Biomarkers and Subclinical Atherosclerosis

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Περίληψη

Οι επιδημιολογικές μελέτες για ισχαιμική καρδιαγγειακή νόσο (ΚΑΝ) χρησιμοποιούν συνήθως κλινικά επεισόδια όπως έμφραγμα του μυοκαρδίου και εγκεφαλικό σαν καταληκτικά σημεία. Όμως, η επίδραση γενετικών πολυμορφισμών κατά τα πρώιμα στάδια της νόσου σε τέτοιου είδους μελέτες είναι δυνατόν να επηρεάζεται και από άλλους παράγοντες κινδύνου καθώς και από ποικοιλομορφίες στην τελική κλινική εικόνα. Επιπρόσθετα, τα ίδια κλινικά καταληκτικά σημεία πιθανώς να οφείλονται σε διαφορετικές βιολογικές διαδικασίες. Έτσι, μελέτες φαινοτύπων των πρώιμων σταδίων της αθηροσκλήρυνσης μπορούν να δώσουν πληροφορίες που δεν θα ήταν διαθέσιμες από μελέτες κλινικών εκδηλώσεων μόνον.

Σκοπός της παρούσας διατριβής ήταν η μελέτη της σχέσης μεταξύ βιοδεικτών και υποκλινικής αθηροσκλήρυνσης όπως αυτή αποκαλύπτεται από υπερηχογραφικές μετρήσεις άλλες από τις μετρήσεις του πάχους του συμπλέγματος έσω-μέσου χιτώνος (ΙΜΤ), όπως η παρουσία αθηρωματικών πλακών, ο αριθμός αρτηριακών διχασμών με πλάκες, το μέγεθος και η ηχοπυκνότητα των πλακών.

Σύνολο 767 εθελοντών, το σύνολο του πληθυσμού του Πεδουλά, οι συγγενείς τους εκτός Πεδουλά και τμήμα της Νήσσου (95% από εκείνους που προσκλήθηκαν), υπεβλήθησαν σε υπερηχογραφική σάρωση των δύο καρωτιδικών και δύο μηριαίων αρτηριακών διχασμών. Ελήφθη λεπτομερές ιατρικό ιστορικό με έμφαση σε καρδιαγγειακούς παράγοντες κινδύνου και έγινε κλινική εξέταση. Επιλέγησαν βιοδείκτες (τόσο βιοχημικοί όσο και γενετικοί) που σχετίζονταν με τις βιολογικές διεργασίες που πιστεύεται ότι λαμβάνουν μέρος στην αθηροσκληρυντική διαδικασία. Αυτοί περιλαμβάνουν δείκτες μεταβολισμού των λιπιδίων, ενδοθηλιακής δυσλειτουργίας, φλεγμονής, θρόμβωσης, μεταβολισμού της ομοκυστεΐνης και αντίστασης στην ινσουλίνη. Επιλέγησαν με βάση αντικρουόμενες αναφορές στη βιβλιογραφία για συσχέτιση με ΚΑΝ και/ή βιολογική πιθανολόγηση για συσχέτιση με την αθηροσκλήρυνση. Συνολικά ταυτοποιήθηκαν 50 δείκτες.

Αριθμός βιοδεικτών έχει δειχθεί να συσχετίζεται με τις υπερηχογραφικές μετρήσεις που μελετήθηκαν. Αυτοί ήταν ο λόγος apoB/apoA1, η ενεργότητα του Lp-PLA_2, οι *apoE* (E2/E3/E4), *CETP* (TaqIB1B2 and I405V), *MGP* (-138C>T) και *MMP-9* (R279Q) γενετικοί πολυμορφισμοί, τα sCD40L, ινοδογόνο, ιστικός παράγοντας, ομοκυστεΐνη, βιταμίνη B12, συστατικά του μεταβολικού συνδρόμου και η αντίσταση στην ινσουλίνη

(δείκτης HOMA). Επιπρόσθετα, έχει δειχτεί για πρώτη φορά η συσχέτιση των επιπέδων του Lp-PLA_2 και του *Lp-PLA_2* A379V πολυμορφισμού στις γυναίκες.

Τα αποτελέσματα της μελέτης υποδεικνύουν πως με τη χρήση επακριβών φαινοτύπων για υποκλινική αθηροσκλήρωση, συνδυασμός βιοχημικών, γενετικών (όπως δοκιμάστηκε) και επιβεβαιωμένων/εδραιωμένων παραγόντων κινδύνου δύναται να εξηγήσει μέχρι και 37% της μεταβλητότητας σε υπερηχογραφικές μετρήσεις του αρτηριακού τοιχώματος. Η συνεισφορά των δεικτών που μελετήθηκαν ήταν της τάξης του 7%.

Ένα σημαντικό έυρημα της μελέτης είναι και η επιβεβαίωση πρόσφατων υποθέσεων ότι διαφορετικοί δείκτες παίζουν διαφορετικό ρόλο στην αθηροσκληρωτική ανάπτυξη και εξέλιξη. Τα αποτελέσματα μας υποδεικνύουν ότι κάποιοι δείκτες όπως η γλυκοζη και ο λόγος apoB/apoA1 συσχετίζονται με πάχυνση του αρτηριακού τοιχώματος, κάποιοι όπως η LDL και ο γονότυπος *apoE* με παρουσία πλακών και άλλοι όπως το sCD40L και ο γονότυπος *CETP* TaqIB1B2 με την ηχοπυκνότητα των πλακών. Επίσης, για πρώτη φορά ο πολυμορφισμός *MGP* (-138T>C) και τα επίπεδα του MCP-1 έχουν δειχτεί να συσχετίζονται με προκλινική αθηροσκλήρωση. Επιπροσθέτως, αυτή είναι η πρώτη αναφορά συσχέτισης μεταξύ επιπέδων και *Lp-PlA_2* A379V γονότυπου σε γενικό πληθυσμό. Δεδομένης της συσχέτισης μεταξύ ενεργότητας Lp-PLA_2 και ΚΑΝ, όπως αυτή έχει δειχθεί σε δύο άλλες μελέτες, τα ευρήματα μας για συσχέτιση μεταξύ ενεργότητας Lp-PLA_2 και A379V γονότυπου στις γυναίκες υποδεικνύει αιτιότητα.

Abstract

Epidemiological studies on ischemic cardiovascular disease have commonly used clinical events such as myocardial infarction and stroke as end-points. However, in such studies the effect of genetic polymorphisms acting on early stages of atherosclerosis can be confounded by other risk factors and variations in clinical presentation. In addition, the same clinical end-point can be the result of different biological pathways. Thus, studies using phenotypes of early stages of atherosclerosis may yield information that cannot be obtained when studying clinical manifestations.

In this thesis, the relationship between biomarkers and subclinical atherosclerosis as assessed by ultrasonic measurements, other than the commonly used intima-media thickness, such as presence of plaques and number of bifurcations with plaque, plaque size and echodensity has been studied.

A cohort of 767 volunteers, total population of Pedoulas, their relatives in another city and a section of Nissou (95% of all invited), underwent duplex scans of both carotid and femoral artery bifurcations. A detailed medical history with emphasis on cardiovascular risk factors was taken and a clinical examination was made. Biomarkers (both biochemical and genetic) related to biological pathways thought to contribute to the atherosclerotic process were selected. They involved markers of lipid metabolism, endothelial dysfunction, inflammation, thrombosis, homocysteine metabolism and insulin resistance.They were selected either because of conflicting reports of association with clinical cardiovascular disease and/or a biological plausibility for association with atherosclerosis. In total, 50 biomarkers were identified.

A number of biomarkers have been shown to be associated with the ultrasonic measurements studied. These were the apoB/apoA1 ratio, Lp-PLA_2 activity, the *apoE* (E2/E3/E4), *CETP* (TaqIB1B2 and I405V), *MGP* (-138C>T) and *MMP-9* (R279Q) genetic polymorphisms, sCD40L, fibrinogen, tissue factor, tHcy, vitamin B12, MetS components and insulin sensitivity (HOMA). In addition, an association between Lp-PLA_2 activity levels and the *Lp-PLA_2* A379V polymorphism, in women only, was shown.
Results indicate that if precise phenotypes for subclinical atherosclerosis are used a combination of genetic, biochemical markers (tested here) and conventional risk factors

can explain up to 37% of the variability in ultrasonic measurements of the arterial wall. The contribution of the biomarkers tested here was of the order of 7%.

An important finding of the study is the confirmation by our data of recent hypotheses that different markers are associated with different stages of atherosclerosis. We have shown that some biomarkers, such as glucose and apoB/apoA1, are associated with thickening of the arterial wall, others such as LDL and *apoE* genotype with plaque presence and others such as sCD40L and *CETP* TaqIB1B2 with plaque echodensity. In addition, this is the first time that the *MGP* (-138T>C) polymorphism and MCP-1 levels heve been shown to be associated with subclinical atherosclerosis. Furthermore, this is the first report of an association between Lp-PLA_2 activity levels and *Lp-PlA_2* A379V genotype in a general population setting; also between Lp-PLA_2 activity and subclinical atherosclerosis. In the presence of an association between Lp-PlA_2 activity and CHD as shown by two other studies, our finding of an association between Lp-PLA_2 activity and A379V genotype in women suggests evidence of causality.

To my family

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Statement of joint work

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DNA exctraction and all the genetic polymorphism determinations were perfomed by the author (A. Panayiotou) as was entry of all genetic data into the database.

Routine biochemical tests (lipid profile, glucose, creatinine, tHcy, folic acid and vitamin B12) were performed at the Nicosia General Hospital by the author with expert assistance by Ms C. Panayiotou and Ms. A. Ilia. Fibrinogen was performed by Ms. C. Panayiotou. The Lp-PLA_2 activity was measured at INSERM, Paris by Dr. E Ninio's group. The remaining biochemical analyses as well as their entry in the data base were done by the author at the pathology laboratory at the Loyola Medical Center, Chicago.

All statistical analyses were performed by the author.

Glossary

ABC-A1	ATP-binding cassette protein A1
ACAS	Asymptomatic Carotid Atherosclerosis Study
ACE	Angiotensinogen Converting Enzyme
ACS	Acute Coronary Syndrome
ADMA	Asymmetric Dimethyl Arginine
AGT	Angiotensinogen
AHA	American Heart Association
AMORIS Study	Apolipoprotein-related Mortality Risk Study
ANOVA	Analysis of Variance
AP-1	Activating protein-1
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARIC Study	Atherosclerosis Risk in Communities Study
BHS	British Heart Study
BMI	Body Mass index
BPB	Black Plaque burden
CAD	Coronary Artery Disease
CARDIA study	Coronary Artery Risk Development in Young Adults
CAMs	Cell Adhesion Molecules
CE	Cholesteryl Ester
CEA	Carotid Endarterectomy
CETP	Cholesteryl Ester Transfer Protein
CCA	Common Carotid Artery
CCR	Chemokine Receptor
CHD	Coronary Heart Disease
CHS	Cardiovascular Health Study
CI (95%)	Confidence Intervals (95%)
CLA Study	Cholesterol Lowering Atherosclerosis Study
COMAC Study	Concerted Multicentre Action Study
CUDAS	Perth Carotid Ultrasound Disease Assessment Study
CVD	Cardiovascular Disease
DASH Study	Dietary Approaches to stop Hypertension
DDAH	Dimethylarginine dimethylaminohydrolase
DM	Diabetes mellitus
ECs	Endothelial Cells
ECG	Electrocardiogram
ECM	Extracellular Matrix
ECST	European Carotid Surgery Trial
ECTIM Study	Etude Cas-Temoin de l'Infarctus Myocarde
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FA	Folic Acid
Fb	Fibrinogen
FHS	Framingham Heart Study
GFR	Glomerular Filtration Rate
GSM	Gray Scale Median
Glu	Glucose
HR	Hazard Ratio
HDL	High Density Lipoprotein

Hcy	Homocysteine
HIFMECH Study	Hypercoagulability and Impaired Fibrinolytic function MECHanisms Study
HL	Hepatic Lipase
HOMA index	Homeostasis Model Assessement index
hsCRP	High sensitivity Cross-Reactive Protein
ICA	Internal Carotid Artery
ICAM	Intracellular Adhesion Molecules
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein
IQR	Interquartile Range
IGT	Impaired Glucose Tolerance
IL-6	InterleU.K.in-6
IMT	Intima-Media Thickness
IMTbif	Intima-Media Thickness bifurcation
IMTcc	Intima-Media Thickness common carotid
IMTmax	Intima-Media Thickness maximum (including plaques)
LCAT	Lecithin Cholesterol Acetyltransferase
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
Lp(a)	Lipoprotein (a)
Lp-PLA_2	Lipoprotein associated Phospholipase A_2
MCP-1	Monocyte Chemoattractant Protein-1
MetS	Metabolic Syndrome
MGP	Matrix Gla Protein
MI	Myocardial Infarct
MMP	Metalloproteinase
MONICA Study	**MONI**toring of trends and determinants in **CA**rdiovascular disease
MP	Microparticles
MPO	Myeloperoxidase
MRFIT	Multiple Risk Factor Intervention Trial
MTHFR	Methyl Hydrofolate Reductase
MT-MMP	Membranous Type Metalloproteinases
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NCEP	National Cholesterol Education Program
NHANES	National Health And Nutrition Examination Survey
NIH	National Institute of Health
NO	Nitric Oxide
NPHS	Northwick Park Heart Study
OR	Odds Ratio
Ox-LDL	Oxidised- LDL
PAF	Platelet Activating Factor
PAF-AH	Platelet Activating Factor Acetyl Hydrolase
PAI-1	Plasminogen Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PCTP	Phosphatidylcholine Transfer Protein
PON	Paraoxonase
PROCAM Study	Prospective Cardiovascular Munster Study
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species

ROC	Reciever Operator Characteristic curve
sCD40L	Soluble CD40 Ligand
SMCs	Smooth Muscle Cells
SNP	Single Nucleotide Polymorphism
SD	Standard Deviation
TChol	Total Cholesterol
TF	Tissue Factor
TG	Triglyceride
TGC	Time Gain Compensation curve
tHcy	total Homocysteine
TIA	Transient Ischemic Attack
TIFPI	Tissue Factor Pathway Inhibitor
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumour Necrosis Factor-α
t-PA	tissue type Plasminogen Activator
TPT	Total Plaque Thickness
TRL	Triglyceride-Rich Lipoprotein
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
UCP	Uncoupling protein
UDACS	UCL Diabetes and Cardiovascular Study
VISP Study	Vitamin Intervention for Stroke Prevention
VLDL	Very Low Density Lipoprotein
VSMCs	Vascular Smooth Muscle Cells
WHO	World Health Organisation
WOSCOPS	West of Scotland Coronary Prevention Study

Contents

PART II: Hypothesis

PART III: Material and Methods

PART V: Discussion and Conclusions

LIST OF FIGURES

Chapter 5

Chapter 7

Chapter 8

Chapter 9

Chapter 12

Chapter 13

Chapter 14

LIST OF TABLES

Chapter 10

Chapter 11

Chapter 12

Chapter 13

Annexe

Part I:

Review of Literature

Chapter 1:

Atherosclerosis

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality globally. An estimated 17.5 million people died from CVD in 2005, representing 30% of all global deaths and half of the deaths in Europe. Over 80% of CVD deaths take place in high- and middle-income countries and occur almost equally in men and women. The World Health Organisation (WHO) estimated that by 2015 almost 20 million people will die from CVD, mainly from coronary heart disease (CHD) and stroke. These are projected to remain the single leading cause of death. The clinical outcomes of coronary heart disease, CVD and cerebrovascular disease (stroke) are largely attributable to the process of atherosclerosis (Loscalzo, 2005) (www.who.int/topics/cardiovascular_diseases/en/-10/2007).

Atherosclerosis is a pathological process which occurs mainly in large and medium-size arteries (i.e. more than 2 mm in diameter) and leads to the aforementioned vascular disorders. It consists of focal thickening of the wall of the arteries, with lipid and connective tissue accumulation that may eventually obstruct blood flow through the lumen (stenosis), predispose the vessel to thrombosis and impair the vessel's ability to respond to hydrodynamic stress (Dean and Kelly, 2000). Regions of low wall shear stress have been shown to be more prone to atherosclerosis (Ku *et al.*, 1985). Altered shear stress induces changes in the endothelial cells, which evoke a cascade of cellular and molecular reactions occurring in the subendothelial tissues leading to the development of atheromatous plaques (Nerem, 1993).

The development of lesions starts at a very young age. Fatty streak formation (the beginnings of a lesion) has been shown to start in human fetal aortas (Dean and Kelly, 2000) but the disease develops silently and clinical evidence is often not seen until after 45 years (Loscalzo, 2005). Although, the clinical symptoms of atherosclerosis are not usually manifested until the later years of adulthood, such studies suggest that primary prevention of atherosclerosis should start at childhood or adolescence (Dean and Kelly, 2000).

1.2 Pathogenesis and development (hypotheses)

Atherosclerosis is generally considered a modern disease. However, mummies from Egypt (Cockburn *et al.*, 1975), North America (Zimmerman and Morilla, 1983) and China and dating from around 3 000 B.C. to 400 A.D. showed extensive macroscopic and microscopic evidence of atherosclerosis of the aorta and of the coronary, carotid and femoral arteries (Ruffer, 1911; Von Eckardstein, 2005). It was probably Leonardo Da Vinci (1452-1519) who first recognised the macroscopic changes of atherosclerosis; when he illustrated the arterial lesions in an elderly man at autopsy, he suggested that the thickening of the vessel wall was due to "excessive nourishment" from the blood. The term "atherosclerosis" was coined around the1860s by Felix J. Marchand (1846-1928) to emphasize the pathological findings of atheroma (Greek for gruel) and sclerosis (Greek for hard) seen in the intimal layer of the arteries (Von Eckardstein, 2005).

Being a multifactorial, complex disease, the mechanisms that govern its initiation and progression are still being debated. It involves numerous cell types and organs and a number of disparate physiological processes. Not surprisingly, the genetic basis of CHD is complex (Lusis *et al.*, 2004a). Suffice to say that up to 27 000 distinct genes are expressed in CVD (Dempsey *et al.*, 2001). Most forms of the disease are the product of many genes with small effects that are modified by the environment and the effects of other genes, rather than of a single highly penetrant gene (Lusis *et al.*, 2004a). Several hypotheses have been formulated through the years in order to explain the genesis of atherosclerosis and the most important ones are discussed in sections 1.2.1-1.2.4.

1.2.1 The Encrustation or Thrombogenic theory

This theory, proposed by Rokitansky in 1852, suggested that atherosclerosis begins in the intima with deposition of small, mural thrombi that are subsequently infiltrated and covered by fibroblasts and secondary lipid deposition. This was based on the histological observations that atheroma is laid down in layers. In recent years extensive studies performed on coronary arteries reported that adhesion of micro thrombi to the vessel intima appeared to be a common event, and the majority of these thrombi undergo dissolution (Duguid, 1946). The observation by Born in 1992 was that although the presence and adherence of thrombi to atherosclerotic plaques is a common

event, it does not necessarily indicate that they are part of the atherosclerotic plaque (Born, 1992).

1.2.2 The lipid theory

In 1858, Virchow proposed that atherosclerosis starts with lipid transudation into the arterial wall and its interaction with cellular and extracellular elements, causing "intimal proliferation." This had followed earlier experiments by Anitschkow on cholesterol fed animals and the later identification of cholesterol as an important constituent of the plaque produced landmark clues in the role of lipids in the pathogenesis of cardiovascular disease (Anitschkow and Chalatow, 1983; Bondjers *et al.*, 1986; Smith, 1974)*;*. Studies by Brown and Goldstein in 1986, demonstrated that single gene mutations in the Low Density Lipoprotein receptor (LDLR) induced atherosclerosis (Brown and Goldstein, 1986). Rapidly transported LDL passes across an intact endothelium and is subsequently trapped in a network of fibres and fibrils secreted by different cells in the arterial wall (Frank and Fogelman, 1989). Endothelium-trapped LDL undergoes modifications by oxidative products and this modified LDL is no longer recognised by the LDL receptors. It is instead, taken up by macrophages thereby resulting in their transformation into foam cells (lipid-laden macrophages) which comprise the fatty streak- the initial lesion of atheroclerosis (Small, 1988).

Initial oxidation of LDL produces molecular changes and secretion by the endothelial cells of cytokines such as monocyte chemotactic protein 1 (MCP-1). As a result, binding of monocytes to the endothelium as well as subendothelial migration takes place (Berliner *et al.*, 1990). It has also been suggested that the effect of mildly oxidised LDL on monocytes is specific, leading to monocyte adherence, migration and conversion to macrophages (De Vries *et al.*, 1999). Interaction between monocyte products such as interleU.K.ins and various growth factors and the cells of the arterial wall result in the initiation and maintenance of complex inflammatory reactions with recruitment of smooth muscle cells (SMCs) in the region of the intima resulting in lesion progression.

1.2.3 The response-to-endothelial injury theory

This more unifying theory was proposed by Ross in 1986. Termed the response-to-injury hypothesis, it postulates that atherosclerosis begins with endothelial injury, making the endothelium barrier susceptible to the accumulation of lipids and the deposition of thrombus (Ross, 1986). The nature of this injury is uncertain, and

multiple factors may interact. Shear stress is a possibility, as plaque tends to develop at branch points where there is increased turbulence (Berceli *et al.*, 1990; Lever, 1994). Infection has also been proposed, since viral elements have been isolated from plaque, although its presence does not prove causality, and therefore some caution should be exercised when interpreting such data (Ridker, 1998). Oxidised LDL is also cytotoxic and may initiate endothelial injury.

After disruption of the endothelial barrier, there is a complex interaction that leads to the propagation of endothelial injury and infiltration of lipids and other blood elements into deeper layers of the arterial wall resulting in the development of atherosclerotic plaques.

1.2.4 The response-to-vascular injury theory

Over the past decade, Fuster and colleagues have proposed that vascular injury starts the atherosclerotic process. The effect of such vascular injury can be classified as follows:

- Type I -Vascular injury involving functional changes in the endothelium with minimal structural changes, (i.e. increased lipoprotein permeability and white blood cell adhesion)
- Type II -Vascular injury involving endothelial disruption with minimal thrombosis
- Type III -Vascular injury involving damage to media, which may stimulate severe thrombosis resulting in unstable coronary syndromes (Fuster *et al.*, 1992)

According to the response-to-vascular injury theory, injury to the endothelium by local disturbances of blood flow at angulated or branch points, along with systemic risk factors (e.g. hyperglycaemia, dyslipidaemia, cigarette smoking, possibly infection) perpetuates a series of events that culminate in the development of atherosclerotic plaque.

1.3 Fluid Mechanical Forces

The risk of developing premature atherosclerosis is dependent on both genetic and environmental risk factors. Despite the fact that the well-established risk factors are

systemic in nature, atherosclerosis tends to develop at branch points and bifurcations in the arterial circulation in both humans and animals (Asakura and Karino, 1990; Gibson *et al.*, 1993; Nakashima *et al.*, 1994). The existence of these "predilected sites" points to the role of localised, nonsystemic factors in the susceptibility to atherosclerosis. It has been long shown that predilected sites are characterised by nonlaminar, pulsatile blood flow with low or oscillating levels of shear stress (Asakura and Karino, 1990). In contrast, regions of the arterial circulation that are more resistant to atherosclerosis are exposed to pulsatile laminar flow with high levels of shear stress. Shear stress represents the frictional force created by the flowing blood and acts almost exclusively on the endothelial cells (ECs) that line the lumen. Within straight sections of vessels the shear stress is essentially laminar, but at curvatures or bifurcations, shear stress becomes turbulent (non laminar) (Brooks *et al.*, 2004).

These observations give rise to the hypothesis that along with systemic factors (e.g. hypercholesterolaemia), local mechanical forces, in particular shear stress, play an important role in the initiation and progression of atherosclerosis (Brooks *et al.*, 2004).

It is well established that fluid mechanical forces regulate the expression of specific genes in endothelial cells (ECs), at least *in vitro*, and are important modulators of vascular ECs functions. Gene expression profiling has revealed that cultured vascular endothelial cells respond to fluid mechanical forces by modulating the mRNA level of a large number of genes. Analysis of recent data indicates that the transcriptional response of cultured ECs to low-shear disturbed flow conditions similar to those at atherosclerosis-prone areas is distinct from that elicited by atheroprotective high shear laminar flow, providing a molecular basis for the focal nature of atherosclerosis. Many of the genes altered by disturbed flow are involved in key biological processes relevant to atherosclerosis such as inflammation, cell cycle control, apoptosis, thrombosis and oxidative stress (Brooks *et al.*, 2004).

1.4 Plaque morphology (Types & classifications):

The walls of the arteries are composed of intima, media and adventitia which are separated by the internal elastic lamina and external elastic lamina.

The *intima* consists of connective tissue, smooth muscle cells (SMCs) and a few isolated mascrophages. It is defined from the luminal surface endothelium to the internal elastic lamina. The arterial intima can be further divided into two layers. The inner layer, called the proteoglycan layer, is composed of abundant proteoglycans, spaced single SMCs and macrophages. The lower layer, called the musculoelastic layer, is composed of abundant SMCs and elastic fibers. Under normal conditions the two layers of the intima are barely visible by light microscopy; however, they are distinct and prominent when adaptive intimal thickening occurs.

The *media* is the muscular part of arterial walls, composed of SMCs, elastin, collagen fibrils and proteoglycans.

The *adventitia* is the outer and highly microvascular layer and contains collagen and elastic fibrils, SMCs and lymphatic channels (Dean and Kelly, 2000). Atherosclerotic lesions are areas of focal thickening of the intima-media.

The main components of the atherosclerotic plaque are: (1) connective tissue extracellular matrix (ECM), including collagen, proteoglycans and fibronectin fibers, (2) crystalline cholesterol, cholesterol esters and phospholipids and (3) cells such as monocyte-derived macrophages, T-lympocytes and smooth muscle cells (Libby, 1995). Various proportions of these components occur in different plaques giving rise to a spectrum of lesions (Falk *et al.*, 1995; Fuster, 1994; Fuster *et al.*, 1999).

Some authors have argued that there may be different types of vulnerable plaques at different sites. Coronary vulnerable plaques are often non-stenotic with large lipid cores, thin fibrous caps and shoulder regions with macrophage and mast cell accumulation (Fayad and Fuster, 2001). Asymptomatic individuals or patients with stable angina have at their majority (~80%) stable, echogenic plaques, rich in collagen content. On the contrary, patients with unstable angina, acute coronary syndromes (ACS), transient ischemic attacks (TIA) and stroke most often have heterogeneous, echolucent plaques (Libby, 1995). In 75-80% of cases, atherosclerotic plaque rupture and superimposed thrombosis is the underlying cause of ACS, the remaining being associated with plaque erosion (see 1.5.3). Thrombosis on atherosclerotic plaque seems to be triggered by tissue factor (TF) present in the boundary layer of the ruptured plaque. Tissue factor is already expressed in the vessel wall of mildly stenotic

coronaries. Upon exposure to circulating blood, TF can trigger the initiation of the coagulation cascade and locally generate thrombin that increases its production by feed-back (Badimon, 2000).

Traditionally atherosclerotic plaques were classified under the following four categories:

- Fatty streaks
- Gelatinous plaques
- Fibrolipid plaques
- Complicated plaques.

However, in the mid 90's the Committee on Vascular Lesions of the Council of Arteriosclerosis of the American Heart Association produced a more precise classification of these lesions based on their morphological and histological characteristics. In all, six types were described. The precursors of advanced lesions were divided into three morphologically distinct groups, types I, II and III. Advanced atherosclerotic lesions were divided into types IV, V, and VI (Stary *et al.*, 1995).

1.4.1 Type I lesions

Type I lesions represent the first microscopically and chemically detectable lipid accumulation in the intima. These lesions have been described most frequently appearing in the first decade of life, but are also found in adults, especially in those individuals with little atherosclerosis. In 1987 and 1989 Stary confirmed the presence of such lesions in the coronary arteries of infants. Such lesions, not always visible to the naked eye, consist of groups of macrophages containing foam cells. It is possible to regress (Griffin, 2004; Stary, 1987).

1.4.2 Type II lesions

Type II lesions are more commonly referred to as fatty streaks appearing as flat, yellowish streaks or patches on the intimal surface of the artery. Type II lesions histologically contain greater numbers of macrophages that are generally stratified in adjacent layers as opposed to isolated groups of cells. Some of the macrophages are not loaded with lipids. T-lymphocytes have also been identified in these lesions (Katsuda *et al.*, 1992; Munro *et al.*, 1987). This particular group of lesions have been subdivided into type IIa (progression prone) and type IIb (progression resistant) based on claims

by Stary and colleagues that at this stage the atherosclerotic lesion may or may not progress. Type IIa differs from type IIb in that the former group contains smooth muscle cells, intercellular matrix, extracellular lipid droplets and particles. Also the foam cells in type IIa lesions are rather deep in the intimal region.

1.4.3 Type III lesions

Type III lesions are characterized by the presence of free lipid droplets among layers of smooth muscle cells. They are often referred to as intermediate lesions and are found from the third decade of life onwards (Griffin, 2004; Stary *et al.*, 1995) .

1.4.4 Type IV lesions

Type IV lesions also known as atheroma, are characterized by further increases in extracellular lipid, known as the lipid core. This results in visible thickening of the arterial wall and a predisposition to ischaemic events. Histologically, these lesions in addition to the lipid core they may contain calcium particles, intimal smooth muscle cells, mast cells, lymphocytes, capillaries, proteoglycans, macrophages and foam cells. Stary and colleagues have shown that there is an abundance of macrophages in areas superficial to the lipid core as well as at the periphery of these lesions. Such sites with abundance of macrophages have been found to be more liable to fissuring and rupture (Griffin, 2004; Stary *et al.*, 1995).

1.4.5 Type V lesion

Beginning around the fourth decade of life, lesions usually have a lipid core with a prominent layer or layers of fibrous connective tissue, mostly collagen. Stary and co-workers in 1994 sub-divided this type of lesion histologically into three groups (Stary *et al.*, 1994):

- Type Va (multi-layered fibroatheroma) characterized by the presence of several cores of lipid material separated by thick fibrous connective tissue.
- Type Vb (calcified fibroatheroma) where the lipid core and other parts of the lesion are calcified.
- Type Vc (fibrotic plaque) characterized by minimal lipid content and an intima which is replaced by fibrous tissue. These lesions are often evident in arteries of the lower limbs (Ross *et al.*, 1984). In this instance the normal intima is

replaced with fibrous connective tissue, while the content of lipid is minimal or even absent.

1.4.6 Type VI lesion

Often referred to as complicated lesions, these may represent any of type IV or V lesions with:

Surface disruption (type VIa)
Haemorrhage/haematoma (type VIb)
Thrombosis (type VIc)
Lesions described as type VI abc often contain all three features listed above.

In 2000, Virmani and colleagues (Virmani *et al.*, 2000) proposed a modification on the AHA classification (Stary, 2000) focusing primarily on type IV, V and VI lesions. For more details on the AHA classification (2000) and the proposed changes see corresponding papers by Stary *et al*. and Virmani *et al*.

1.5 Plaque complications

Every year, more than 1 million people in the U.S.A. and more than 19 million others worldwide experience a sudden cardiac event (acute coronary syndromes and/or sudden cardiac death). A large portion of this population had no prior symptoms (Naghavi *et al.*, 2003a). Responsible for an event is usually a "vulnerable", prone-to-rupture plaque.

1.5.1 Vulnerable plaque

The prone-to-rupture plaque is termed the "vulnerable" plaque and there are some specific criteria that distinguish it. Characteristic histomorphologic features of vulnerable plaques include a high lipid content, increased numbers of inflammatory cells, and extensive adventitial and intimal neovascularity. The fibrous cap of an atherosclerotic plaque may become thin and rupture as a result of the depletion of matrix components through the activation of enzymes, such as matrix-degrading proteinases and cysteine and aspartate proteases, and through the reduction in the number of smooth muscle cells. Activated T cells may also inhibit matrix synthesis through the production of interferon-gamma (IF-γ). A number of triggers of plaque rupture have been identified. Also, some thrombi may occur without rupture of the

fibrous cap. Reducing the lipid component and inflammation in atherosclerotic plaques may help reduce the risk of plaque rupture. This may account for the clinical benefit of risk-factor reduction gained from changes in lifestyle and from drug therapy (Shah, 2003).

The major criteria for a vulnerable plaque are listed below:

1. Active inflammation
2. A thin fibrous cap with a large lipid core (cap thickness <100μm, lipid core >40% of plaque volume)
3. Endothelial denudation with superficial platelet aggregation
4. Fissured/Injured plaque
5. Severe stenosis

On the surface of plaques with severe stenosis, shear stress imposes a significant risk of thrombosis and sudden occlusion. A stenotic plaque may be a vulnerable plaque regardless of ischemia and may indicate the presence of many non-stenotic or less stenotic plaques that can be vulnerable to rupture and thrombosis (Naghavi *et al.*, 2003b; Shah, 2003).

It has been demonstrated that angiographically complex lesions represent vulnerable plaques prone to disruption or truly disrupted plaques (Davies, 1996). Complex lesions are associated with rapid disease progression and a higher restenosis rate after percutaneous transluminal coronary angioplasty as compared with smooth lesions, probably reflecting a tendency toward thrombogenesis or further plaque disruption or both (Yan *et al.*, 2004).

1.5.2. Plaque Rupture

The most common type of plaque complication is plaque rupture, accounting for ~70% of fatal acute myocardial infarctions (MI) and/or sudden coronary deaths. According to Virmani's (2000) definition, plaque rupture is: "an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the lipid core". Ruptured lesions typically have a large necrotic core and a disrupted fibrous cap infiltratated by macrophages and lympocytes. The smooth muscle cell (SMC) content within the fibrous cap at the rupture site may be quite sparse (Virmani *et al.*, 2000). The rupture-prone, vulnerable plaque has been termed a "thin-cap fibroatheroma" (Kolodgie *et al.*, 2004b; Naghavi *et al.*, 2003a).

Rupture of atherosclerotic plaque has been identified as the proximate event in the majority of cases of acute ischemic syndromes. Specifically, plaque ruptures are found in 60% of individuals dying suddenly with luminal thrombi and are the most frequent cause of death in young men (<50 years) and older women (>50 years). Risk factors more predictive for this type of lesion are hypercholesterolaemia, low serum high-density lipoprotein (HDL) and a high TChol/ HDL ratio. In women more than 50 years old, ruptured plaques compose the vast majority of atherosclerotic lesions associated with acute thrombi and similar to men, there is an association with increased total cholesterol (TChol) levels (Virmani *et al.*, 2000).

Plaque disruption and/or rupture usually occurs at the weakest point ("shoulder"), frequently where the cap is thinnest and most heavily infiltrated with inflammatory cells. Once the plaque is disrupted, the highly thrombogenic components of the plaque are exposed to the blood stream, activating the clotting cascade and promoting thrombus formation (Viles-Gonzalez *et al.*, 2004). Future culprit lesions are difficult to identify, however, and angiographic assessment of stenosis severity is prone to underestimation. Compared with plaques that cause severe luminal stenosis, vulnerable plaques may cause relatively minor stenosis, although they account for more cases of rupture and thrombosis. Such unstable, vulnerable plaques may be associated with outward remodeling of the vessel. Because severely stenotic plaques are more likely to stimulate collateral circulation to the post-stenotic segment, plaque rupture and thrombosis at such sites may be clinically silent (Shah, 2003). However, presence of a plaque rupture does not imply a causal association with the thrombus that occluded the lumen. There is amble evidence that non-fatal plaques can contain areas of rupture. For example, Arbustini and collaborators found a 10% incidence of plaque ruptures in lesions of people who died of non-cardiovascular causes (Arbustini *et al.*, 1991). These findings suggest that advanced plaques may undergo many non-fatal ruptures without causing death. Moreover, the existence of non-ruptured but fatal lesions suggests that the equation of rupture with death is overly simplistic (Virmani *et al.*, 2000).

1.5.3. Plaque thrombosis:

Another type of plaque complication is intraplaque haemorrhage (deposition of blood products inside the plaque), which is not necessarily associated with atherosclerotic plaque rupture (Stary, 2000). Sometimes, the thrombus might be superimposed. In all cases that involve a superimposed thrombus, the underlying lesion may be stenotic or

not stenotic. However, non-stenotic lesions are more frequent than stenotic plaques and account for the majority of culprit ruptured plaques (Ambrose *et al.*, 1988; Naghavi *et al.*, 2003b). Virmani et al. have previously shown that in sudden coronary death patients who died of luminal thrombosis, at least 50% of the thrombi occurred at lesion sites with 75% cross-sectional area stenosis by plaque (corresponding to 50% diameter reduction). Therefore, cross-sectional luminal narrowing of more than 75% is not a prerequisite for luminal thrombosis, either acute or healed, or for the development of intraplaque haemorrhage (Virmani *et al.*, 2000).

Another mechanism of coronary thrombosis, plaque erosion, has been identified as an important cause of sudden coronary death. Eroded plaques differ from ruptured plaques in that they have a base rich in proteoglycans and SMCs. These lesions are more often seen in younger individuals and women, have less luminal narrowing and less calcification and less frequently have foci of macrophages and T-cells compared with plaque ruptures. Determinants of plaque erosion are not yet clear (Tedgui and Mallat, 2001b).

1.6 Subclinical atherosclerosis:

Different factors and various pathways may play more or less important roles in different atherosclerotic stages and interact with each other in various ways. Subclinical atherosclerosis as indicated by intima-media thickness (IMT) probably reflects a hypertrophic response of arterial intimal and medial cells to lipid infiltrations (Cheng *et al.*, 2002; Spence, 2002) or hypertension. In contrast, formed arterial plaques probably represent a later stage of atherogenesis related to oxidation, inflammation, endothelial dysfunction and/or smooth muscle cell proliferation (Hegele, 1996). As plaques develop, there may be some compensatory arterial enlargement to maintain a constant shear rate at the interface between the flowing blood and endothelium (Glagov *et al.*, 1987) with minimal haemodynamic compromise. When and if haemodynamically significant stenosis occurs, it probably represents an even later stage of atherogenesis, determined by pathways related to plaque rupture, intraplaque haemorrhage, thrombosis and scarring (Hegele, 1996). All the aforementioned can happen without any clinical signs of disease and represent subclinical atherosclerosis. Clinical events such as stroke or MI possibly reflect determinants of arterial occlusion at the latest stages of atherogenesis, as well as determinants related to thrombosis and thrombolysis (Spence and Hegele, 2004).

Chapter 2:

Screening with Ultrasound

2.1 Introduction

Identification of genes influencing common, complex disorders such as cardiovascular disease has been difficult, partly because of the many interacting factors known to contribute to these traits and the large conceptual, physiological and temporal distance between gene variation and clinical manifestation of adult disease (Manolio *et al.*, 2004; Price *et al.*, 1997). In addition, genetic analysis of a disease end-point is complicated by discrepancies in disease diagnosis. Studies of phenotypes more proximal in the pathway from DNA sequence variation to overt clinical disease, such as ultrasound-defined atherosclerosis, may thus yield valuable information not obtainable by studying clinical conditions alone (Manolio *et al.*, 2004).

"Non-invasive imaging has great clinical potential to stratify patients at intermediate risk and determine whether early intervention is warranted. New technologies enable exploration of the artery wall where the plaque itself resides. With increasing reliability and decreasing costs, the clinical applications are expanding and imaging is being used extensively in clinical trials to speed drug development by providing functional and anatomic information with smaller sample sizes and shorter trial durations than possible with cardiovascular mortality and morbididy trials" (Tardif *et al.*, 2006).

Intimal-medial thickening (IMT) of the carotid artery determined by B-mode ultrasound is a quantitative measure of atherosclerosis that has a graded, predictive relationship to overt clinical disease (O'Leary *et al.*, 1999). Focal carotid wall thickening (plaque) and lumen narrowing (stenosis) can also be imaged and also predict cardiovascular events (Salonen and Salonen, 1991). Ultrasonographic measures of the carotid artery may thus provide a useful intermediate phenotype for the identification of atherosclerosis-related biomarkers.

2.2 Intima-Media Thickness (IMT)

IMT is the most commonly assessed ultrasonographic carotid measurement because of its high measurement precision (O'Leary *et al.*, 1991) and its strong predictve value for subsequent cardiovascular events in large studies (Azen *et al.*, 1996; Bots *et al.*, 1997a; Chambless *et al.*, 1997; O'Leary *et al.*, 1999). Carotid IMT is now an established surrogate marker for atherosclerosis and is recognised as such by the American Heart Association.

IMT is a validated end-point for atherosclerosis. Prospective, epidemiological studies have shown that an increase in IMT due to cardiovascular risk factors is associated with an increase in relative risk for MI and stroke; and that a decrease in IMT due to drug treatment is associated with a decrease in incidence of vascular events (i.e. in the REGRESS trial statins had a favourable impact on coronary lumen, carotid and femoral IMT and clinical CV events) (de Groot *et al.*, 2004).

In addition, at least 5 studies have shown that carotid IMT (as measured by B-mode ultrasound) correlates with presence of coronary atherosclerosis and represents an independent risk factor for CHD and stroke. The ARIC study (Chambless *et al.*, 1997) and the CHS (O'Leary *et al.*, 1999) study which included >15 000 people without CHD at baseline showed that the higher the IMT the greater the risk of MI or stroke. So far, it is established that carotid IMT is an independent predictor of transient cerebral ischemia, stroke and coronary events such as myocardial infarct (MI) (Pearson, 2002).

Measurements are typically performed in the common carotid artery, usually 1 to 3 cm proximal to the origin of the carotid bulb (where the near and far walls cease to be parallel). The carotid bulb, or bifurcation, includes the segment from the initial outward curving of the walls to the proximal tip of the flow divider between the external and internal carotid arteries. The internal carotid artery is more difficult to image as it proceeds beneath the angle of the jaw, and rarely can more than the most proximal centimetres be measured (Manolio *et al.*, 2004). Measurement variability of the internal carotid can be up to 3 times greater than in the common carotid, and missing data are more frequent (O'Leary *et al.*, 1996).

Variability of carotid IMT has been suggested to be higher for the near wall (nearest to the skin and the ultrasound transducer) than the far wall, because of physical characteristics of the transmission and reflection of the ultrasound beam (Wendelhag *et al.*, 1991; Wikstrand and Wiklund, 1992).

Measurements of IMT vary. Some authors obtain IMT measurements in the distal common carotid artery (IMTcc) where plaques rarely occur (Bots *et al.*, 1997a; Kitamura *et al.*, 2004; Salonen and Salonen, 1991). Others measure IMT at several sites of the carotid bifurcation (IMTbif) including both carotid bulb and internal carotid artery obtaining the mean (Chambless *et al.*, 1997; Kablak-Ziembicka *et al.*, 2004; Longstreth *et al.*, 1998) or

maximum thickness at these sites (Longstreth *et al.*, 1998). These IMTbif measurements include the thickness of plaques whenever plaques are present. IMTcc and IMTbif are associated with different risk factors and prevalence of cardiovascular disease (Ebrahim *et al.*, 1999). Irrespective of IMT measurements the presence and number of plaques, however small, are good predictors of future cardiovascular events (Ebrahim *et al.*, 1999) and strokes (Hollander *et al.*, 2002). More recently, other ultrasonic markers of atherosclerotic cardiovascular disease have been identified: the combination of the maximum IMTcc with the maximum IMT in the internal carotid artery provides a better prediction of the risk for stroke than IMTcc on its own (Kitamura *et al.*, 2004); carotid plaque area and plaque volume are associated with increased cardiovascular risk (Brook *et al.*, 2006; Spence *et al.*, 2002; Spence, 2006) and the presence of femoral plaques is a marker of coronary artery disease (Khoury *et al.*, 1997; Schmidt *et al.*, 2005).

Risk factors for subclinical atherosclerosis have been shown to be similar to traditional risk factors for clinical CVD (Kuller *et al.*, 1994). It has been suggested by O'Leary (O'Leary *et al.*, 1999) that thickening of the common carotid artery (CCA) IMT might be more representative of total body atherosclerotic burden (resulting from SMC accumulation and matrix deposition), whereas thickening of the internal carotid artery (ICA) IMT might represent focal atherosclerotic plaques, possibly related to endothelial dysfunction and haemodynamic flow in the ICA (Crouse *et al.*, 1996; Malek *et al.*, 1999). This may explain why ICA IMT has been shown to be somewhat more strongly associated with an increased risk of incidence disease, particularly CHD, than the CCA IMT (Fox *et al.*, 2003; O'Leary *et al.*, 1996; Psaty *et al.*, 1999).

Some authors have previously shown that combined common carotid IMT and internal carotid IMT was more strongly associated with the prevalence of CVD and with traditional risk factors than either variable alone (O'Leary *et al.*, 1996).

Arterial bifurcations are lesion-prone areas that have increased activity of thrombosis, lipid deposition and atherosclerosis (Grabowski and Lam, 1995), though to be a direct result of haemodynamic factors (Arnett *et al.*, 1998; Topper *et al.*, 1996). *In vitro* experimental models have demonstrated that atherosclerotic plaques are most often formed along bifurcations and along the inner wall of curvatures (Cavalli-Sforza and Bodmer, 1971; Levy *et al.*, 2000). In contrast, the CCA is exposed primarily to laminar blood flow and *in vivo* experiments have shown CCA intimal thickness to be inversely

related to wall shear stress, independent of age, blood pressure, body mass index (BMI) and diabetes mellitus (DM) (Carallo *et al.*, 1999; Gnasso *et al.*, 1996; Irace *et al.*, 1999; Jiang *et al.*, 2000). Low wall shear stress is hypothesized to increase the duration of time that blood comes into contact with the endothelial wall, enhancing atherogenic particle delivery and vessel wall adherence (Fox *et al.*, 2003; Gnasso *et al.*, 1996; Malek *et al.*, 1999).

Borderline elevation of multiple risk factors is common in the elderly and the association between risk factors and CVD may weaken in the later years of life (Irace *et al.*, 1999; Schmidt *et al.*, 1998). Therefore, it may be difficult for clinicians to identify older persons with subclinical CVD on the basis of classic risk factors alone. Increased IMT, an indicator of subclinical disease may reflect the consequences of past exposure to risk factors. Addition of IMT to risk equations will help identify asymptomatic individuals who would benefit more from aggressive preventive measures (O'Leary *et al.*, 1999).

2.3 Carotid plaque and stenosis

A carotid plaque is a focal thickening of the carotid wall caused by atherosclerosis. Like definitions of IMT, plaque definitions vary and include focal thickening >50% of the surrounding wall (North *et al.*, 2002; Yi-Heng *et al.*, 2001), focal widening with protrusion into the lumen (Bots *et al.*, 1996), localised IMT more than or equal to the cutpoints in the range of 0.75 mm to 1.5 mm (Fortunato *et al.*, 2003; Hunt *et al.*, 2002; Jartti *et al.*, 2002; Mannami *et al.*, 2001) and focal acceleration of flow as measured by Doppler spectral analysis (O'Leary *et al.*, 1991).

Plaque as a carotid atherosclerosis phenotype is not studied as frequently as carotid IMT. The heritability of plaque determined by localised IMT $\geq$ 1.5 mm was recently estimated at 23% to 28% (Hunt *et al.*, 2002). Carotid plaque has also been reported to be more strongly related to early parental CHD death than is IMT (Zureik *et al.*, 1999). Whereas one could anticipate that genes influencing plaque overlap with those influencing IMT, there are likely to be unique sets of genes related to both (Spence and Hegele, 2004).

When plaques progress they can cause significant stenosis of the lumen which alters shear stress, disturbs blood flow and ultimately increase the risk of an event. Several large randomized multicenter trials, such as the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and the European Carotid Surgery Trial (ECST), concluded that compared with medical therapy, the combination of carotid endarterectomy (CEA) and best medical therapy significantly reduces the risk of stroke only for patients with significant carotid artery stenosis. Similarly, the Asymptomatic Carotid Atherosclerosis Study (ACAS) confirmed the benefits of CEA over medical therapy for patients with >60% asymptomatic carotid stenosis (http://clinicaltrials.gov/ct2/show-4/2008).

However, several trials also noted that most patients with high-grade stenosis (>70%) remained stroke free even with medical therapy alone (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). Factors in addition to the degree of stenosis, such as the histological composition of the plaque, may be responsible for the determination of stroke risk. The composition of plaques from patients with symptoms is significantly different from that of plaques from those without. The former contain more total lipid and cholesterol, and less collagen and calcium. This is further discussed in section 3.6

2.4 Femoral Plaques

The association between femoral artery atherosclerosis and cardiovascular disease (CVD) has received much less attention compared with the carotid artery. Kroger et al. (Kröger *et al.*, 1999) showed that isolated atherosclerosis of the femoral arteries was more common in all examined age groups than isolated atherosclerosis of the carotid arteries or combination of atherosclerosis in both arteries. The femoral arteries seem to be involved earlier in atherosclerosis than the carotid arteries. Cerne and Kranjec (Cerne and Kranjec, 2002) observed that patients with coronary artery disease had a significantly increased IMT in the common femoral artery compared with age- and sex-matched controls. A study performed by Held *et al*., found that plaques in the femoral artery were significantly more common among patients who were revascularized, as compared to event-free patients in a population with stable angina (Held *et al.*, 2001).

In a large perspective study of healthy individuals followed-up for 10 years it was shown that in 51% of subjects the carotid was the worst (most advanced plaque type) artery and in 52.4% the right (carotid or femoral) arteries were more advanced than the left. Therefore combining data from both carotids and femorals has the benefit of a wider sampling, overcoming the problem of not detecting a more advanced lesion in the femoral i.e. when looking at the carotids only. No difference between carotid and femoral arteries in predicting events was observed. It was also demonstrated that carotid and femoral lesions had comparable power in predicting different types of events. Scanning only carotids or only femorals was less predictive (fewer predicted events). On average, only-carotid or only-femoral scans predicted 15% and 13% less events respectively. Scanning four arteries predicted on average 14% more events than one pair of arteries only. This was equally true for coronary (13%), cerebrovascular (15%) or peripheral vascular events/disease (14%). Scanning only one side (1 ipsilateral femoral+carotid) also predicted on average 13% fewer events (Belcaro *et al.*, 2001).

A significant argument against scanning four arteries is the increase in cost. In the same study, it was demonstrated that the cost of scanning both carotids and femorals was only 6% more than scanning only a couple of arteries as the most important component of cost is equipment-time (which increases only by 3–5 min when scanning four instead of two arteries) (Belcaro *et al.*, 2001).

2.5 Other atherosclerotic phenotypes:

Other phenotypes related to carotid atherosclerosis include plaque echodensity (consistent pathologically with more organised and fibrotic plaques) or echolucency (consistent with greater lipid deposition, intraplaque haemorrhage, and vulnerability to rupture) (Diamantopoulos *et al.*, 2002), lumen diameter, distensibility and stiffness (Manolio *et al.*, 2004) and carotid plaque area (Spence and Hegele, 2004).

2.6 Echogenicity

Plaque echogenicity as assessed by B-mode ultrasound has been found to reliably predict the content of soft tissue and the amount of calcification in carotid plaques. Fibrous plaques have a highly echogenic quality and the presence of calcium provides a markedly hyperechoic image with acoustic shadowing formation. As the lipid content

of the plaque increases, the plaque becomes more echolucent on ultrasound (Geroulakos *et al.*, 1993; Tegos *et al.*, 2000b). Gray scale median (GSM) is used to distinguish between hypoechoic (echolucent) and hyperechoic (echogenic) atherosclerotic plaques and it is a measure of plaque echogenicity. It uses 2 reference points in each image: (1) blood which is assigned a value of 0-5 and (2) adventitia which is assigned a value of 185-195. If normalisation of the image is performed, then the gray scale value of all the pixels in the image is adjusted according to the input and output values of the 2 reference points. The GSM of the plaque (the median of the frequency distribution of the gray levels of the pixels in the plaque) in the normalised (or not-normalised) image is used to quantify its echogenicity. Image normalisation though, when performed, results in better reproducibility of plaque echogenicity (Sabetai *et al.*, 2000).

Echodensity has, also, been associated with cardiovascular symptoms. At least 3 studies have reported the association between carotid plaque echogenicity and presence of symptoms in patients with stenosis. By using ultrasound images of plaques and assessing their echogenicity, all studies demonstrated that symptomatic status of patients was significantly associated with plaques with low median GSM (echolucent plaques) (AbuRahma *et al.*, 2002; Grogan *et al.*, 2005; Tegos *et al.*, 2000a; Tegos *et al.*, 2001). A heterogeneous pattern was also shown to be associated with presence of symptoms (as compared to a homogeneous pattern) as well as with the degree of stenosis (AbuRahma *et al.*, 2002; Tegos *et al.*, 2001). Pedro et al. (Pedro *et al.*, 2000) showed that echolucent plaques were associated with a higher neurological risk and Tegos et al. showed that they were associated with retinal symptomatology, whereas intermediate plaque appearance (GSM:16) was associated with cerebrovascular symptoms and presence of ipsilateral CT-demonstrated silent infarct (GSM:14) (Tegos *et al.*, 2000b). Another study, using a three-dimentional reconstruction of parallel two-dimentional gray scale B-mode ultrasound of carotid plaques, demonstrated that progressive plaques were predominantly hypoechoic or had an ulcerated surface in cases of a hyperechoic echogenicity. Traditional risk factors and drug therapy were unrelated to plaque progression (Schminke *et al.*, 2000).

Hence, it has been proposed that echolucent plaques may be more vulnerable and prone to rupture and that it is possible to identify a group of patients at risk of stroke based on

texture features extracted from ultrasonic images of carotid plaques (Christodoulou *et al.*, 2003).

The assessment of plaque echolucency is usually based on the version of classification proposed by Gray-Weale et al. where plaques are graded from 1 to 4 (Gray-Weale *et al.*, 1988):

Type 1: Dominantly echolucent with a thin echogenic cap
Type 2: Substantially echolucent with small areas of echogenicity
Type 3: Dominantly echogenic lesions with small areas of echolucency (<25%)
Type 4: Uniformly echogenic lesions (equivalent to homogenous)

Another commonly used plaque classification system is the one described by Widder (Widder and Pault, 1990). The plaques are graded from 1 to 4 as follows:

Type 1: Uniformely echogenic
Type 2: Predominantly echogenic
Type 3: Predominantly echolucent
Type 4: Uniformely echolucent

The Widder classification was used to assign plaque types in the work presented here with the addition of the calsified plaque which was assigned as type 1 and the rest types 2-5 (for details see chapter 5).

Recent advances in ultrasonography and histological findings have revealed that the majority of clinical events (unstable angina, acute coronary syndrome, transient ischemic attacks) occur to patients with unstable carotid atherosclerotic plaques, whereas patients with stable carotid atherosclerotic plaques usually exibit stable angina or remain asymptomatic throughout their life (see chapter 1.5.1 for vulnerable plaque definitions). Studies comparing the association between different ultrasonic measurements and cardiovascular disease (Ebrahim *et al.*, 1999; Hollander *et al.*, 2002) suggest that the presence of plaques whose thickness is often included in intima-media thickness (IMT) measurements may be a better predictor of cardiovascular events than IMTcc, which was found to add little to the predictive power of conventional easily obtainable risk factors (Iglesias del Sol *et al.*, 2001).

Subclinical cardiovascular disease is an important predictor of subsequent coronary heart disease and stroke even after controlling for conventional risk factors, suggesting that better use of subclinical measurements (such as ultrasonic arterial wall measurements) could improve our ability to stratify patients into those at increased risk of cardiovascular disease (Kuller *et al.*, 2006).

People in developed countries, now face environmental risk factors such as high levels of smoking and alcohol intake that increase an individual's cardiovascular risk. However, even, in the face of these environmental "insults" some individuals maintain cardiovascular health into old age, whereas others –with a different genetic make-up do not. These individuals develop vascular disease including atherosclerosis, myocardial infarction, and stroke. Identification of the genes involved in cardiovascular homoeostasis during environmental challenge, and discovery of whether the effect of environmental risk factors is modulated by an individual's genotype, should lead to progress in understanding the pathophysiology and aetiology of atherosclerosis. Similarly, this will be essential for genetic risk prediction because mutations in such genes are likely to predispose strongly to or protect from atherosclerosis (Humphries and Morgan, 2004).

Recent studies (reviewed in Humphries and Morgan, 2004), have identified several common functional variants in genes coding for proteins likely to be involved in the pathophysiology of carotid-artery thickening, and several association studies examining their effect on IMT have been published. However, studies vary in design, population studied, IMT measurements and end-point selected, thus giving contradictory results for the same genetic polymorphisms. In the present study, more weight was given in selecting genes with contradictory reports, as well as novel ones, in an effort to elucidate their association.

The relative lack of work on biomarkers and subclinical atherosclerosis supports the need for such studies testing the association between biochemical and genetic biomarkers and ultrasonic markers of early, subclinical atherosclerosis.

While the work for this thesis was in progress, publications have come out supporting plaque area and volume as a more predictive marker of atherosclerosis than IMT. In addition, it is becoming increasingly clear that carotid IMT, coronary calcification and

carotid plaque reflect biologically different aspects of the atherosclerotic process and will respond defferentially to therapy. IMT represents mainly hypertensive medial hypertrophy; this measure is more predictive of stroke than of myocardial infarction, and is only weakly associated with traditional coronary risk factors. Carotid plaque area, on the other hand, is more strongly associated with traditional risk factors, and is more predictive of myocardial infarction than of stroke. A quantitative trait, called 'unexplained atherosclerosis', expresses the extent to which an individual has excess carotid plaque not explained by traditional risk factors, or the extent to which an individual is protected from traditional risk factors. Unexplained progression of plaque is an even more powerful tool for genetic research, because age, which accounts for the greatest proportion of baseline plaque, has much less influence on the rate of progression. Compared with IMT, measurement of carotid plaque volume by three-dimensional ultrasound reduces by two orders of magnitude the sample size and duration of treatment needed to evaluate new therapies (Spence, 2006).

Chapter 3:

Atherosclerotic Markers (biochemical and genetic) and Risk Factors

3.1 Introduction

More than 50 years ago, William Kannel (Kannel *et al.*, 1961) first popularised the concept of CVD risk factors for clinicians and clinical epidemiologists (Dzau, 2004). The word 'factor' derives from the Latin (meaning doer) which implies causality; however, from its initial use in 1961, the term 'risk factor' included both causal and predictive factors. Typically, risk factors are surrogates for deeper causes (and better predictors) of CVD (Stampfer *et al.*, 2004).

The NIH Definition Working Group established some working definitions for terms such as biomarker, surrogate marker and surrogate end-point, in order to help end their arbitrary use. According to their definition:

Biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention";

Clinical end-point is "a characteristic or variable that reflects how a patient feels, functions or survives"; and

Surrogate end-point is "a validated biomarker intended to substitute for a clinical end-point. A surrogate end-point should predict clinical benefit (or harm, or lack of benefit or harm) on the basis of epidemiological, therapeutic, pathophysiological or other scientific evidence. Changes in the biomarker that result from therapy are expected to reflect changes in clinically meaningful end-points" (Tardif *et al.*, 2006). Although many potential surrogates correlate well with the true clinical outcome, very few are able to reflect the full therapeutic effect on the clinical outcome (Fleming and DeMets, 1996).

In addition, **Risk Factor** is "an environmental, behavioral, or biologic factor confirmed by temporal sequence, usually in longitudinal studies, which if present directly increases the probability of a disease occurring, and if absent or removed reduces the probability. Risk factors are part of the causal chain, or expose the host to the causal chain. Once disease occurs, removal of a risk factor may not result in a cure" (Beck, 1998).

A number of clinical and biochemical risk factors have been identified thus far, that are associated with the development and progression of atherosclerosis. Most risk factors used in daily practice have demonstrated a consistent graded-response effect and are substantiated by large series of consistent prospective studies in broad populations,

some of which will be discussed in section 3.2; however, new risk factors stand to improve previous predictive capabilities.

Soluble biomarkers and imaging technologies have both advantages and limitations. Imaging technologies can assess disease in human clinical trials, non-invasivly and with a high degree of sensitivity and specificity but may be limited by technical difficulty, availability and cost. Soluble biomarkers, on the other hand, are easily available and at lower cost, but they may not prove as sensitive as imaging modalities in the detection or assesement of disease (Tardif *et al.*, 2006).

Genes that are consistently over-expressed or suppressed in a certain clinical context may be considered as biomarkers. A genetic marker therefore is: variants in the DNA (alleles) that alone or in combination are associated with a specific disease phenotype. Markers whose presence (or absence) confers a high level of probability of disease (high predictive value) would be most useful as diagnostic tools or predictors of prognosis or response to therapy. Even markers with modest effects may provide us with important clues to disease pathophysiology or suggest new ways of therapeutic intervention (Gibbons *et al.*, 2004). Therefore, a combination of all three –soluble biomarkers, imaging techniques and genetic biomarkers- may prove to be the gold standard in the unravelling of complex diseases such as atherosclerosis.

Two approaches are used when pursuing genetic markers: (a) candidate gene studies, which focus on single genes and (b) genomic studies that use the entire genome (Gibbons *et al.*, 2004). The genetic association study design provides greater power to detect common genetic variants conferring susceptibility to complex phenotypes such as atherosclerosis and MI (Kathiresan *et al.*, 2004).

The main problem of association studies is that the results are often not reproducible. This is probably due to inconsistently defined phenotypes (that may differ from study to study), small sample sizes, different ethnic groups, false positive and false negative associations and population stratification (Gibbons *et al.*, 2004). However, a recent examination of 301 published studies of 25 different reported associations found that less than half of all the reported associations had strong evidence of replication (Kathiresan *et al.*, 2004).

3.2 Epidemiological studies

3.2.1 The Framingham Heart Study

The Framingham Heart Study (FHS) is the oldest on-going epidemiological study for cardiovascular disease (CVD) today and it started in 1948 in the town of Framingham in the Boston area (U.S.A.). It was deemed necessary at the time, given that in the 1940s and 1950s, the scourge of the infectious diseases was replaced by a mounting epidemic of CVD with one out of every three men in the United Stated developing CVD before the age of sixty. The study, as it developed, had one main and two subsidiary aims (Dawber and Moore, 1952): First, to secure epidemiological data on arteriosclerotic and hypertensive CVD; second, to secure data on the prevalence of all forms of CVD in a representative population sample and third, to test the efficiency of various diagnostic procedures.

Some 5 000 men and women aged 30-59 years were recruited and its results have provided a wealth of epidemiological information elucidating the relation between total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides and the risk for coronary atherosclerosis (Anderson *et al.*, 1990; Wilson *et al.*, 1999). The major findings of this study established a list of risk factors for CHD, summarised below, that act at all ages and in both sexes:

- Total Cholesterol (TChol) positively related to CHD
- LDL-cholesterol, positively related to CHD
- HDL-cholesterol inversely related to CHD. The ratio TChol: HDL-cholesterol is established as an efficient lipid risk predictor, better than LDL-cholesterol: HDL-cholesterol
- Systolic and diastolic blood pressures are equally good predictors of risk
- Fibrinogen
- Alcohol intake, moderate being protective and excess increasing risk
- Family history – 30% increased risk if a parent died before age 65
- Smoking
- Diabetes
- Lack of exercise

These factors were used to form an equation which can calculate the 10-year risk of an individual for CHD, termed the Framingham Risk Score. The risk score is being used everyday by clinicians to estimate the 10-year risk of their patients.

The Framingham investigators have since extended the study to include the children of the original subjects and their spouses, called the Framingham Offspring Study. This study analysed 2 191 men aged 20 to 54 years and investigated elevated Lp(a), and CHD (Bostom *et al.*, 1996). The subjects had no history of known CHD and were followed for onset of asymptomatic atherosclerosis. Followed for a mean of 15 years, Lp(a) was found to contribute a relative risk of 1.9 for development of cardiovascular disease and therefore was established as an independent risk factor in this sample (http://www.framinghamheartstudy.org-10/2007).

3.2.2 Prospective Cardiovascular Münster Study- PROCAM

The **Pro**spective **Ca**rdiovascular **M**ünster (PROCAM) Study (also known as the Münster Heart Study) was initiated in 1978 by the Institute of Arteriosclerosis Research at the University of Munich. More than 30 000 participants aged between 16 and 65 years were recruited from among the employees of 52 large companies and the public service in Munich and the northern Ruhr area. All participants are still in continuing long term follow-up for heart disease, stroke and mortality. Perhaps the most important result to emerge from PROCAM and other prospective epidemiological studies of coronary heart disease risk factors is the realization that risk factors do not act in isolation, but in synergistic interaction with other risk factors (i.e. total cholesterol and HDL). That is to say, individual risk factors interact in a multiplicative rather than in an additive fashion.

The major findings of this study confirmed a list of risk factors for future coronary events and stroke, summarised below:

- Age
- LDL-cholesterol
- HDL-cholesterol
- Systolic Blood Pressure
- Triglycerides
- Smoking
- Diabetes mellitus
- Family history of MI

These 8 variables listed here, each make an independent contribution to risk for future coronary event and stroke in the PROCAM study. Taken together, they allow a more than 40-fold stratification of risk between the lowest and the highest quintile of risk score.

The PROCAM study resulted in an algorithm which uses the above 8 variables in order to predict an individuals future (10-year) risk for coronary events and stroke, used widely in Europe since (http://www.chd-taskforce.com/risk-english.htm-10/2007).

3.2.3 The Rotterdam Study

The Rotterdam Study is a prospective cohort study that started in 1990 in Ommoord, a suburb of Rotterdam, among 10 994 men and women aged 55 and over. The main objective of the Rotterdam Study is to investigate the prevalence and incidence of and risk factors for chronic diseases in the elderly. The chronic diseases of interest are cardiovascular, neurological, locomotor and ophthalmologic diseases.

Baseline measurements were obtained between 1990 and 1993. All participants were subsequently examined in follow-up examination rounds every 2-3 years. The Rotterdam Study comprises of three cohorts.The initial cohort started in 1990 with 7,983 men and women aged 55 years and over. Follow-up visits were held in 1994-1995, in 1997-1999, and in 2002-2004. In 2000-2001 a second cohort was established. Another 3 011 inhabitants of Ommoord aged 55 years and over agreed to participate. The partakers of this second cohort visited the research center for a follow-up examination in 2004-2005. The third cohort of the Rotterdam Study started in 2006, this time with inhabitants aged 45 years and over. By the end of 2008 about 4 000 participants will have been included in this third cohort.

A wealth of publications has originated from the Rotterdam study, some of which include the association of CRP with coronary atherosclerosis, sodium and potassium intake and risk of cardiovascular events, CETP and CHD mortality and *apoE* genotype and risk of Alzheimer (http://www.epib.nl/ergo.htm-11/2007).

3.2.4 The Cardiovascular Health Study –The CHS

The Cardiovascular Health Study (CHS) is a population-based, longitudinal study of risk factors for the development and progression of CHD and stroke in adults over the age of 65 years. Initially funded for six years, it was renewed for another six-year

period in 1994 and it shifted to morbidity and mortality follow-up (with no further collection of examination data) at the end of the year 11 exam in June 1999.

Within a population of men and women 65 years and older, the objectives of the Cardiovascular Health Study are:

1. To quantify associations of conventional and hypothesized risk factors with CHD and stroke.
2. To assess the associations of non-invasive measures of subclinical disease with the incidence of CHD and stroke.
3. To quantify the associations of risk factors with subclinical disease.
4. To characterize the natural history of CHD and stroke, and identify factors associated with clinical course.
5. To describe the prevalence and distributions of risk factors, non-invasive measures of subclinical disease, and clinical CHD and stroke.

A major emphasis of the study is its focus on subclinical disease, or abnormalities detected noninvasively without signs or symptoms. Subclinical disease measures in CHS include ultrasonography of the carotid artery and abdominal aorta, ankle-brachial index, echocardiography, resting and ambulatory electrocardiography, cerebral magnetic resonance imaging, spirometry, and retinal photography. Some of these measures are conducted three times; at baseline, to assess risk of clinical disease in relationship to subclinical disease; three to four years after entry to assess change in subclinical disease and risk of clinical disease in relationship to change; and toward the end of the study, to assess predictors of subclinical disease itself. Echocardiography, ambulatory ECG, cerebral MRI and aortic ultrasonography are only conducted twice. Preliminary results from the CHS include the absence of correlation between brachial flow-mediated dilation and carotid IMT and the association of CRP and Lp-PLA_2 (PAF-AH) with ischemic stroke and 5-year risk of cardiac death respectively (http://www.clinicaltrials.com-10/2007).

3.2.5 The Tromsø Study

The Tromsø Study was started in 1974 and is a population based prospective study of inhabitants in the municipality of Tromsø, Norway. The aims of the study are to investigate the determinants of chronic diseases in order to assess etiologic significance and to investigate potentially modifiable determinants that may be developed into preventive or therapeutic strategies. The main focus is on cardiovascular disease. The

study design includes repeated population surveys to which total birth cohorts and random samples are invited. So far it has recruited 27 159 participants.

The examination included standardized measurements of height, weight, blood pressure, nonfasting serum lipids, and blood cell counts. A self-administered questionnaire covered information about current and previous cigarette smoking, physical activity in leisure time, currently or previously treated hypertension, and a medical history of angina pectoris, diabetes mellitus, asthma, myocardial infarction, and stroke. A total of 6 892 subjects, were subject to ultrasound measurements of the abdominal aortic diameter (Singh *et al.*, 2001). Results include confirmation of the association between HDL and CHD, more specifically high HDL levels were found to reduce plaque growth in subjects with pre-existing atherosclerosis, and a reported association between hyperhomocysteinemia and CHD risk.

3.2.6 The Bruneck Study

The Bruneck Study is a prospective population-based survey on atherosclerosis and its risk factors carried out in Bruneck, a small town of about 13 500 people in northeastern Italy. Baseline evaluation was carried out between July and November 1990 on subjects aged 40–79 years. Of the 4 793 subjects of the appropriate age range, 125 men and 125 women for each age decade (40–49, 50–59, 60–69, and 70–79 years) were randomly selected and invited to participate in the study; 936 individuals were recruited. Among others, they have so far tested the association between pro-inflammatory variants in *IL-6*, *IL-1* and *CD14* genes and carotid and femoral IMT as well as the risk of subclinical atherosclerosis with LDL and Lp(a) levels.

3.3 Lipid metabolism markers

3.3.1 Introduction

Both genetics and environment have been shown to affect plasma lipid levels and it has been suggested that, overall, the magnitute of their impact is approximately the same. However, it is also true that the metabolism of some lipoproteins such as very low density lipoproteins (VLDL) is strongly influenced by environment (i.e. diet), while that of others, such as Lp(a) is governed almost entirely by inheritance. Lifestyle attributes such as smoking, exercise, obesity and alcohol consumption exert some of

their deleterious or beneficial effects on CAD risk by perturbing lipoprotein transport (Betteridge, 1999).

There are two major pathways for extracellular modification of lipoproteins. The first is the lipolytic cascade which begins with the hydrolysis of triglyceride (TG) from the TG-rich-VLDL by lipoprotein lipase (LPL). Hydrolysis of VLDL yields intermediate density lipoproteins (IDL) which are either catabolised by the liver or further hydrolised in plasma by hepatic lipase (HL) to low density lipoproteins (LDL). Concurrent with these changes are the activities of at least two lipid transfer proteins that transfer single molecules of TG, cholesteryl ester and phospolipid between these lipoproteins and HDL.

The second pathway for lipoprotein modification is the HDL cascade, which involves the co-ordinated actions of lecithin cholesterol acetyltransferase (LCAT), phosphatidylcholine transfer protein (PCTP), cholesteryl ester transfer protein (CETP) and HL. In normolipidaemic subjects, the core of HDL is largely cholesteryl ester, a major fraction of which is exchanged for the triglyceride of VLDL through the action of CETP (Betteridge, 1999).

3.3.2 Total Cholesterol

Total cholesterol is transported in the blood in macromolecular complexes known as lipoproteins. The cholesterol concentration is an aggregate measure of the total amount of cholesterol carried in the various lipoprotein fractions. These lipoproteins are classified in terms of their density on ultracentrifugation: very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL). About two-thirds of the serum total cholesterol is transported in the LDL fraction. In excess it is potentially atherogenic, explaining the relationship between elevated total cholesterol and coronary heart disease (Frohlich *et al.*, 2001; Kuller, 2001). HDL cholesterol accounts for 20-25% of the total serum cholesterol.

Much epidemiological evidence is available to demonstrate that the relationship between CHD and cholesterol levels is continuous, graded and curvilinear and therefore TChol is an established risk factor for atherosclerotic disease. The risk becomes increasingly steep as cholesterol concentration rises. This has been shown in

studies between countries such as The Seven Countries Study (Keys, 1980), and within countries studies that include the Framingham Study (Kannel *et al.*, 1971), and the Multiple Risk Factor Intervention Trial – MRFIT (Martin *et al.*, 1986).

3.3.3 Low Density Lipoprotein cholesterol (LDL)

Low density lipoprotein (LDL) is a plasma lipoprotein produced mostly via catabolism of Very Low Density Lipoproteins (VLDL); there is however evidence that it is also secreted by the liver. It is composed of phospholipids, cholesterol, apoB protein and cholesteryl esters and their lifetime in plasma is linked to the apoB cascade. There is evidence that the core lipid composition of LDL can be significantly effected by the TG-rich apoB-containing proteins via the action of CETP which can result in the generation of small dense LDL particles that have been shown to be more atherogenic.

Epidemiological studies have identified LDL as an independent risk factor that modulates CVD risk (Castelli *et al.*, 1992; Gordon and Rifkind, 1989). LDL measurements are included in both the Framingham and the PROCAM risk scores for identifying individuals at risk for future coronary events. LDL is involved in the early stages of atherosclerotic plaque formation, being taken up by macrophages, after its oxidation, and mediating their transformation into foam cells (discussed further in 2.3.9). LDL has long been considered as a therapeutic target and over the past decade, clinical trials of LDL-lowering drugs have established that reductions in LDL are associated with a 30-45% reduction in clinical events (Downs *et al.*, 1998; Heart Protection Study Collaborative Group, 2002; Sacks *et al.*, 1996; Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995). However, despite lowering LDL many patients continue to have cardiac events. It was therefore, suggested that HDL and its interaction with LDL might represent another significant player in CVD.

3.3.4 High Density Lipoprotein cholesterol (HDL):

HDL is the smallest and most protein rich of the major lipoprotein classes.
Although understanding how HDL protects against CVD is still incomplete, evidence supports three major atheroprotective mechanisms:

1. HDL-mediated efflux of cholesterol-loaded macrophages which can occur by passive diffusion (Yancey *et al.*, 2003), by interaction with the SR-BI receptor (Williams *et al.*, 1999) or by binding to the ABCA1 transporter

(Liu *et al.*, 2003; Oram and Lawn, 2001; Remaley *et al.*, 2001; Takahashi and Smith, 1999).

2. Protection of LDL from oxidation by transferring oxidised lipids from LDL to itself and hydrolysing them with PON and PAF-AH (Lp-PLA_2) (Mackness *et al.*, 1993; Marathe *et al.*, 2002).
3. Selective decrease of endothelial cell adhesion molecule expression *in vivo* (Tedgui and Mallat, 2001a).

Cholesterol in HDL is returned to the liver by two pathways: transfer of cholesteryl esters (CE) by CETP to the VLDL-IDL-LDL lipoproteins with uptake by the liver via the LDL receptor (LDLR) (Goldstein and Brown, 1987) and selective uptake of CE by the hepatic SR-BI receptor (Trigatti *et al.*, 2003).

Epidemiological studies have identified HDL as an independent risk factor that modulates CVD risk (Castelli *et al.*, 1992; Gordon and Rifkind, 1989). A strong association between HDL and risk of CHD has been demonstrated in both the Framingham and the Tromsø, study (Gordon *et al.*, 1977; Miller *et al.*, 1977). An inverse association between HDL and carotid atherosclerosis has also been demonstrated, although not as consistently (Bots *et al.*, 1992; Heiss *et al.*, 1991; Prati *et al.*, 1992; Tell *et al.*, 1998). Low HDL is often present in high-risk patients with CVD and many different studies provide support for the concept that raising HDL may represent an additional therapeutic target for CVD prevention (Brewer, 2004).

3.3.5 Cholesteryl ester transfer protein (CETP)

CETP is a hydrophobic glycoprotein that is secreted mainly from the liver and that circulates in plasma mainly bound to HDL. It promotes the redistribution of cholesteryl esters (CE), triglycerides (TG), and to a lesser extent, phospholipids between plasma lipoproteins. CETP transfers lipids from one lipoprotein particle to another in a process that results in equilibration of lipids between lipoprotein fractions (Barter *et al.*, 2003).

Cholesteryl-ester transport protein (CETP) appears to have both pro-atherogenic and anti-atherogenic effects. CETP-mediated transfer of CE decreases HDL levels and increases CE in VLDL-IDL-LDL. The decreased HDL levels would reduce the atheroprotective functions of HDL and would thus be pro-atherogenic. If the increased CE in LDL is taken up by vessel wall macrophages to increase foam cell formation

then this activity would also be pro-atherogenic. However, if the increased CE transfer to VLDL-IDL-LDL instead results in transport of CE back to the liver via the LDLR, then CETP would facilitate reverse cholesterol transport and would act in an atheroprotective way. Finally, CETP-mediated HDL remodelling during CE transfer results in the production of lipid-poor apoA1, which can be used for ABCA1-mediated cholesterol efflux, an atheroprotective process (Barter *et al.*, 2003; Brewer, 2004). The ultimate outcome of this combination of CETP activities, in terms of atherosclerosis, is not easy or simple to predict and the available clinical data in humans are incomplete and do not allow a definite conclusion about the relation of CETP deficiency to the risk of CHD.

Animal studies of CETP in several rabbit models of atherosclerosis have shown that inhibiting CETP results in a marked reduction in atherosclerosis. A recent study (Okamoto *et al.*, 2000) in cholesterol-fed rabbits, used a chemical inhibitor of CETP that reduced CETP activity by >90%, doubled the levels of HDL cholesterol and decreased the non-HDL cholesterol by ~50%, with an accompanying 70% reduction in atherosclerotic lesions. It was speculated that short-term treatment of humans with the same CETP inhibitor would result in a 40-45% increase in HDL cholesterol and a 15-20% decrease in LDL cholesterol.

Several mutations of the *CETP* gene have been identified as a cause of CETP deficiency and elevated levels of HDL cholesterol. These include a common IsoleU.K.in to Valine aminoacid mutation at position 405 (I405V) and an A to G change in intron 1 (TaqIB1B2). The TaqIB1B2 polymorphism has been associated with plasma CETP activity and concentration, plasma HDL cholesterol and with development of CHD (Freeman *et al.*, 1990; Fumeron *et al.*, 1995; Kakko *et al.*, 2001; Kauma *et al.*, 1996; Kuivenhoven *et al.*, 1998; Ordovas *et al.*, 2000). In the West of Scotland Coronary Prevention Study (WOSCOPS), the TaqIB2B2 homozygous had a 30% reduced risk of a cardiovascular event compared to the B1B1 genotype and a dose-dependent association of risk with the TaqIB genotype was demonstrated for non-smokers (Freeman *et al.*, 2003). The I405V polymorphism has been associated with plasma CETP and HDL choletesterol levels (Bruce *et al.*, 1998; Gudnason *et al.*, 1997; Kakko *et al.*, 2000) and to the degree of carotid atherosclerosis and risk of CHD (Agerholm-Larsen *et al.*, 2000; Bruce *et al.*, 1998; Kakko *et al.*, 2000). The 405VV genotype has been also associated to increased carotid IMT in men who were heavy

drinkers (Kakko *et al.*, 2000). In the men of the Stanislas Cohort, the I405V SNP explained 3.8% of the variability of carotid IMT (Pallaud *et al.*, 2001).

3.3.6 Triglycerides (TG)

Triglyceride-rich lipoproteins (TRLs) include VLDL, IDL and various remnant particles. Elevated TG levels are common in patients with atherosclerotic disease but several factors have made establishing a clear link between elevated TG and atherosclerotic disease difficult. The most important is the lack of an established pathophysiological mechanism linking TRLs to atherosclerosis (Haynes, 2003).

Meta-analyses of observational studies have suggested that 1 mmol/L (89 mg/dL) elevation in TG is associated with a 14-37% higher incidence of CVD, independent of other risk factors; with the highest risk in women (Hokanson and Austin, 1996). Given the variability in plasma TG levels, observational studies may considerably underestimate the cardiovascular consequences of elevated TLRs. An alternative experimental approach is to focus on a plausible, quantitative, intermediate phenotype for atherosclerosis that exhibits changes in a relatively short period of time (Haynes, 2003); such as ultrasonically defined phenotypes.

3.3.7 Apolipoprotein E (*apoE*)

A much studied gene in relation to carotid atherosclerosis is apolipoprotein E (*apoE*). Three common alleles (designated E2, E3 and E4) produce 3 protein isoforms differing at amino acid positions 112 and 158 on the mature polypeptide. The most common allele, E3, produces the apoE E3 isoform with cysteine at position 112 and arginine at position 158, whereas the least common E2 allele, produces the apoE E2 protein with cysteine at both positions and apoE E4 has arginine at both positions. Apolipoprotein levels vary by allele with the E2 allele associated with higher and E4 associated with lower plasma apoE levels (Eichner *et al.*, 2002). *ApoE* E2 is in turn associated with lower LDL levels and E4 with higher levels than is the E3 isoform (Cattin *et al.*, 1997). Carrying an *apoE* E4 allele has generally been associated with a modestly increased risk of coronary disease and carrying an E2 allele has been associated with lower risk, compared with the E3/E3 genotype (Eichner *et al.*, 2002), although the E2 allele has also been related to increased risk of coronary disease (Cattin *et al.*, 1997; Eichner *et al.*, 1993).

Research on *apoE* (E2/E3/E4) and stroke has been inconclusive but many studies were heterogenous and small. In an Italian case-control study for ischemic stroke, the apoE4 allele was found to be more frequent in patients versus controls or healthy subjects. Accordingly, the E3/E3 genotype was less frequent in the stroke group (Margaglione *et al.*, 1998).

In the Rotterdam study, measuring common carotid IMT and presence of plaques in 5401 subjects, the *apoE* E2/E3 genotype was associated with a lower IMT and fewer plaques than E3/E3. The E4 allele was not found to be an important risk factor for carotid atherosclerosis (Slooter *et al.*, 2001). The same was true in another population study of middle-aged men, with the E2 allele being associated with lower carotid IMT (Ilveskoski *et al.*, 2000). In the CUDAS study, the *apoE* polymorphism was found to be associated with carotid plaque formation but not with IMT (Beilby *et al.*, 2003).

Studies on *apoE* and carotid atherosclerosis have been inconsistent (Cattin *et al.*, 1997; De Andrade *et al.*, 1995; Hanon *et al.*, 2000; Kogawa *et al.*, 1997; Sass *et al.*, 1998; Terry *et al.*, 1996). Most studies were not population-based (Hanon *et al.*, 2000; Kogawa *et al.*, 1997; Sass *et al.*, 1998; Terry *et al.*, 1996) and they excluded subjects at high risk of atherosclerosis, thus allowing for selection bias (Slooter *et al.*, 2001). Prospective population studies with intermediate phenotypes such as IMT are needed to further elucidate the relationship of *apoE* and other polymorphisms with atherosclerosis.

3.3.8 Apolipoprotein A1 (apoA1)

ApoA1 is the major protein constituent of plasma HDL and plays a crucial role in lipid transport and metabolism. Its main functions are to act as a structural protein, to mediate transfer of cholesterol from cell surfaces to lipoprotein particles, and to activate the enzyme responsible for cholesterol esterification in the circulation, lecithin:cholesterol acyl transferase (LCAT). It has been shown that apoA1 mediates reverse cholesterol transport (RCT) by acting as a ligand for the ATP-binding cassette protein A1 (ABC-A1), thus promoting cholesterol efflux from peripheral tissues back to the liver. Lipid-bound apoA1 activates the enzyme LCAT which catalyses the esterification of cholesterol in plasma (Loscalzo, 2005).

ApoA1 has been implicated in the inhibition or regression of atherosclerosis in humans and experimental animals. HDL and apoA1 have been reported to have antioxidant and anti-inflammatory properties; they can alter prostacyclin levels and platelet function and modulate nitric oxide (NO) release. ApoA1 may directly or indirectly protect against oxidation of LDL. *In vitro*, apoA1 renders LDL resistant to lipoxygenase-mediated oxidation. An indirect effect rests on the presence on HDL of the antioxidant enzymes paraoxonase (PON) and platelet-activating factor-acetyl hydrolase (PAF-AH or Lp-PLA_2), which prevents the formation of oxidised LDL *in vitro* (Loscalzo, 2005).

3.3.9 Apolipoprotein B100 (apoB100)

Apolipoprotein B100 (apoB), is a large, hydrophobic protein of 4 536 aa which is synthesized in the liver. ApoB comprises 30-40% of the protein content of plasma VLDL and more than 95% of the protein in LDL. It is required for the assembly and secretion of apoB-containing lipoproteins (i.e. LDL) which transport hydrophobic lipids, cholesteryl ester and triglycerides in their cores (Loscalzo, 2005). Thus, apoB serum concentration is a measure of the number of LDL, intermediate density lipoprotein and VLDL atherosclerotic particles (Sniderman *et al.*, 2001) and studies have shown that apoB is a better candidate risk parameter than non-HDL cholesterol for identifying subgroups of individuals with elevated cardiovascular risk as well as predicting carotid intima-media thickness (IMT) (Sniderman *et al.*, 2003).

Large studies have shown an association between apoB/apoA1 ratio and prediction of cardiovascular risk above and beyond TG and cholesterol (Lamarche *et al.*, 1996; Moss *et al.*, 1999; Roeters von lennep *et al.*, 2000; Talmud *et al.*, 2002; Walldius *et al.*, 2001). The apoB/apoA1 ratio has also been associated with all the components of the Metabolic Syndrome (MetS) as well as with a more rapid increase in carotid IMT (Wallenfeldt *et al.*, 2004).

3.3.10 Lipoprotein (a) (Lp(a))

Lp(a) is a cholesteryl-ester rich lipoprotein that resembles LDL, but has distinctive structural, epidemiological and genetic properties. It was discovered in 1963 by Berg (Betteridge *et al.*, 1999). Lp(a) is formed by joining a lipoprotein that is structurally similar to LDL in protein and lipid composition to a carbohydrate-rich, hydrophyllic protein called apo(a). Lp(a) particles contain apo(a) and apoB100 in a 1:1 molar ratio (Fruchart *et al.*, 2004). It is believed to contribute to lipid-induced atherogenesis,

similarly to LDL particles (Kronenberg *et al.*, 1996). Compared to LDL, however, it contains lower amounts of antioxidants and exhibits a high affinity to ECM and fibrinogen (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990; Pillarisetti *et al.*, 1997), which prolongs residence time in the subintima. Both properties of Lp(a) fascilitate its oxidative modification and may enhance its capacity to cause injury. Lp(a) has been suggested to exhibid both atherogenic and thrombogenic properties and may thus promote both early and advanced stages of atherosclerosis (Kronenberg *et al.*, 1999).

Mechanisms of Lp(a)-induced atherosclerosis:

1 Lipid pathway (like LDL).
2 High Lp(a) impairs activation of transforming growth factor-β by downregulation of plasmin generation, thereby contributing to SMCs proliferation (Grainger *et al.*, 1993).
3 Lp(a) enhances expression of ICAM-1. Because Lp(a) accumulates in the subendothelial space of the vessel wall, it may act as a potent chemoattractant for monocytes in human atherosclerosis (Kronenberg *et al.*, 1999).

To date, no clinical trials have shown that lowering Lp(a) decreases CHD risk (Fruchart *et al.*, 2004). However, Lp(a) plasma concentration predicted the risk of early atherogenesis synergistically with high LDL cholesterol in 826 random population individuals with 5-year follow-up on progression of carotid atherosclerosis (Kronenberg *et al.*, 1999).

In the Bruneck Study, the risk of early atherogenesis in the high-LDL group (> 3.3 mmol/L) increased gradually with increasing Lp(a) concentration. A cross-sectional evaluation of the ARIC study, yielded analogous results in that Lp(a) was significantly elevated in subjects with high IMT (Brown *et al.*, 1993).

Therapeutic lowering of LDL by ≥ 10% was found to dilute the predictive value of Lp(a) for coronary artery disease (Maher *et al.*, 1995). A recent study revealed high Lp(a) plasma concentration to increase the risk of familial CAD only if the TChol/HDL ratio was elevated (Hopkins *et al.*, 1997). Apparently, the interaction of Lp(a) with other lipoproteins triggers or enhances its atherosclerotic properties (Kronenberg *et al.*, 1999).

Whether the assessement of Lp(a) adds prognostic information to overall risk in primary prevention remains uncertain, because Lp(a) appears to be an important marker primarily in individuals with markedly elevated risk caused either by diabetes or by hyperlepidaemia (Kronenberg *et al.*, 1999; Luc *et al.*, 2002; von Eckardstein *et al.*, 2001). Other comparative studies have found Lp(a) to predict risk but to have modest magnitute compared with several other novel markers. Some of this effect is caused by the non-linearity of Lp(a) in that risk primarily increases in very high levels (Sharrett *et al.*, 2001).

3.3.11 Lipoprotein-Associated Phospholipase A_2 (Lp-PLA_2 or PAF-AH)

Lp-PLA_2 is a calcium-independent member of the phospholipase A_2 family. It is produced mainly by monocytes, macrophages, T-lymhpocytes, mast and liver cells. Lp-PLA_2 activity occurs in association with macrophages and it has been found to be upregulated in atherosclerotic lesions, especially in complex plaques (Hakkinen *et al.*, 1999), as well as in the fibrous cap of coronary lesions prone to rupture (Kolodgie *et al.*, 2004a). In the bloodstream, two-thirds of the Lp-PLA_2 plasma isoform circulate primarily bound to LDL, the remaining one-third is bounded between HDL and VLDL (Caslake *et al.*, 2000; Tsimihodimos *et al.*, 2002).

LDL provides a circulating reservoir, in which Lp-PLA_2 remains inactive until LDL undergoes oxidatative modification. Lp-PLA_2 acts only on oxidatively modified LDL (OxLDL), and hydrolysis of OxLDL can be performed solely by Lp-PLA_2 (MacPhee *et al.*, 2005 ; Macphee *et al.*, 1999). A product of Ox-LDL hydrolysis is lysophospatidylcholine (LysoPC) which is a potent chemoattractant for T cells and monocytes, promotes endothelial cell dysfunction, stimulates macrophage proliferation and induces apoptosis in SMCs and macrophages (Kume *et al.*, 1992; MacPhee *et al.*, 2005 ; Quinn *et al.*, 1988).

Data however, support both a pro-atherogenic and an anti-atherogenic role for Lp-PLA_2. Recombinant Lp-PLA_2 exhibits anti-inflammatory properties in animal models (Tjoelker *et al.*, 1995), supporting evidence of an anti-atherogenic function. In the same line of evidence, epidemiological data demonstrate that genetic deficiency of Lp-PLA_2 caused by a missense mutation (Val279Phe) in exon 9 of the *Lp-PLA_2* gene (*PLA2G7*) which results in complete loss of catalytic activity is associated with an increased risk

of coronary artery disease (Keiko *et al.*, 2003; Yamada *et al.*, 2000) and stroke (Hiramoto *et al.*, 1997).

Another mutation in the *PLA2G7* gene (A379V) has been reported to have a functional role. Recently, homozygosity for the V allele was found to be associated with a lower risk of MI in the HIFMECH study, a European multicenter case-control study (Abuzeid *et al.*, 2003). In addition, this polymorphism has been found to have an effect, independent of other polymorphisms, on prospective cardiovascular outcome in the AtheroGene study. The V allele was less frequent in CAD patients than in controls and was associated with a lower risk of future cardiovascular events, suggesting that this allele may be protective against the development of CAD (Ninio *et al.*, 2004). A possible interpretation by the authors is that the A379V polymorphisms might modify the enzyme function towards a more anti-atherogenic form. However, the association between A379V genotype and plasma Lp-PLA_2 activity has been inconclusive since in the AtheroGene study VV homozygotes were reported to have increased plasma Lp-PLA_2 activity (Ninio *et al.*, 2004), a finding not replicated by Kruse et al. in functional studies (Kruse *et al.*, 2000). These studies represent different case-control populations and may not be the best setting for testing the association between A379V genotype and plasma Lp-PLA_2 activity.

Data from the ARIC and the MONICA studies confirm that elevated levels of Lp-PLA_2 appear to be predictive of future coronary events and suggest that they may be complementary to CRP levels for identifying individuals at high CHD risk (Ballantyne *et al.*, 2004).

3.4 Endothelial dysfunction markers

3.4.1 Introduction

The endothelium is an active, dynamic tissue that controls many important functions, including regulation of vascular tone and maintenance of blood circulation, fluidity, coagulation and inflammatory responses (Bonetti *et al.*, 2003; Gonzalez and Selwyn, 2003). Cardiovascular risk factors affect many of the normal functions of the endothelium (Gonzalez and Selwyn, 2003). Endothelial dysfunction represents a systemic disorder and a key variable to the pathogenesis of atherosclerosis and its complications. Current evidence suggests that endothelial status is not determined solely by the individual risk factor burden

but rather, may be regarded as an integraded index of all atherogenic and atheroprotective factors present in an individual (Bonetti *et al.*, 2003).

Activated endothelial cells express cell adhesion molecules such as P- and E-selectin, which mediate leucocyte rolling, the first step in the cell-adhesion cascade. Rolling leucocytes become activated by chemokines such as monocyte chemoattracting protein 1 (MCP-1), presented by the activated endothelial surface. The activated leucocytes can bind to other endothelial adhesion molecules, such as ICAM-1 and start transmigrating into the vascular intima (Wagner and Burger, 2003).

The arterial endothelium responds to flow and shear stress via a pathway that leads to phosphorylation of endothelial nitric oxide synthase (eNOS), which produces nitric oxide (NO) and thus leads to vasodilation (Dimmeler *et al.*, 1999; Scotland *et al.*, 2002). This response allows conduit arteries to accomodate increases in flow and control changes in shear stress. In addition, the endothelium limits local thrombosis by producing tissue plasminogen activator, maintaining a negatively charged surface and secreting heparans and thrombomodulin (Behrendt and Ganz, 2002; Gonzalez and Selwyn, 2003). Changes in the endothelium that characterise a procoagulant state include decreased secretion of plasminogen activator inhibitor-1 (PAI-1), activation and increased reactivity of platelets, local production of tissue factor and exposure of collagen (Behrendt and Ganz, 2002).

In the intimal space monocytes and T-lymphocytes modulate the inflammatory response and eventually form foam cells with intimal thickening, plaque formation and vessel narrowing. This local macrophage-mediated inflammatory response includes the secretion of matrix metaloproteinases (MMPs), disruption of type I collagen and plaque disruption (Libby *et al.*, 2002). At the same time, the release of interleU.K.in-6 (IL-6) stimulates the liver to produce increased quantities of Cross-reactive protein (CRP).

Evidence suggests that loss of endothelium-dependent vasodilation by NO is characteristic throughout the development of atherosclerosis and has adverse consequences, specifically vasoconstriction (Gonzalez and Selwyn, 2003).

3.4.2 Endothelial Nitric Oxide Synthetase (eNOS)

Endothelial nitric oxide synthetase (eNOS) plays an important role in the endothelial function, under a wide range of physiological conditions. In the vessels, the endothelial isoform of NO sunthase (eNOS) is responsible for the production of NO. eNOS, is activated by mechanical stress such as blood shear-stress and stimulation with agonists such as bradykinin and acetylcholine (Kawashima and Yokoyama, 2004). NO appears to exert a protective action against different vasculatory disorders such as atherosclerosis and coronary heart disease (CHD) (Albrecht *et al.*, 2003).

A substiantial body of evidence *in vitro* suggests that eNOS-derived NO acts as an anti-atherogenic molecule (De Caterina *et al.*, 1995; Sarkar *et al.*, 1996). NO from eNOS inhibits leucocyte-endothelial adhesion, vascular SMCs (VSMC) migration and proliferation, and platelet aggregation, all of which are important steps in atherogenesis. It is now being widely recognised that eNOS becomes dysfunctional and produces superoxide rather than NO in hyperlipidaemia and atherosclerosis. Dysfunctional eNOS is closely implicated to the endothelial dysfunction in atherosclerotic vessels (Kawashima and Yokoyama, 2004).

The Glu298Asp (894G>T) polymosphism of the *eNOS* gene has been considered to involve a genetic risk of CAD (Colombo *et al.*, 2003), results however remain conflicting. In a population-based case-control study of 600 hypertensives and 600 controls, no association was found between the Glu298Asp polymorphism and blood pressure, left ventricular mass, left ventricular hypertrophy or ultrasonic carotid artery alterations (Karvonen *et al.*, 2002). Similarly, Poirier *et al*. found no association between 10 different polymorphisms of the *eNOS* gene and MI in European subjects in the ECTIM study (Poirier *et al.*, 1999). However, in the French population of the study 298Glu homozygotes were more frequent among patients with MI, a finding that was not replicated in the Irish population.

On the other hand, Hingorani et al. found a strong association between the Glu298Asp polymorphism and CAD in a Caucasian population in the U.K. (Hingorani *et al.*, 1999). The same group reported recently that carriers of the 298Asp allele have enzymatic activity equivalent to that of the 298Glu allele, but selective proteolysis occurs in native cells and tissues such that the steady-state level of active eNOS may be reduced in carriers of this allele (Hingorani, 2003). Lembo et al. also, demonstrated an

association between the Glu298Asp polymorphism and presence of carotid plaques (CCIMT> 1.5mm). In their study, the risk of having carotid plaques increased 3-fold in 298Asp homozygotes compared to 298Glu homozygotes, independently of age, blood pressure or smoking (Lembo *et al.*, 2001).

3.4.3 Oxidised LDL (Ox-LDL)

The hypothesis that Ox-LDL is necessary, if not obligatory, in the development of atherosclerotic lesions was formulated more than 25 years ago with the seminal observation that uptake of native LDL by macrophages did not result in foam cell formation. In contrast, uptake of Ox-LDL via scavenger receptors resulted in the upregulated accumulation of lipids (Steinberg *et al.*, 1989; Tsimikas and Witztum, 2001).

There is substantial evidence that Ox-LDL is present *in vivo* within atherosclerotic but not normal blood vessels. Ox-LDL (*in vivo*) has a wide range of atherogenic properties from early lesion formation to plaque rupture. These include: inducing expression of adhesion molecules on endothelial cells, monocyte chemotaxis and adhesion, cytotoxicity, upregulating inflammatory genes and growth factors, endothelial dysfunction, platelet aggregation and thrombus formation and destabilising plaques through mechanisms including increased expression of MMPs (Aikawa *et al.*, 1998; Berliner *et al.*, 1995; Tsimikas and Witztum, 2001).

LDL undergoes oxidative modifications when trapped in the endothelium. When "fully-Ox-LDL" enters the circulation in minor quantities, it is rapidly cleared by the reticuloendothelial system, particularly in the liver, or it is removed by the pre-existing circulating antibodies against Ox-LDL (Liu *et al.*, 2004). It is generally believed, that "fully-Ox-LDL" does not exist in the circulation (Tsimikas and Witztum, 2001). In contrast, the "minimally-Ox-LDL" in which oxidative modification has not been sufficient to cause changes recognised by scavenger receptors, can be found in the circulation. Circulating minimally-modified-LDL, was described by Avogaro et al. and Sevanian et al. (Avogaro *et al.*, 1988; Sevanian *et al.*, 1997).

High plasma and plaque levels of Ox-LDL have been associated with the vulnerability of plaques (Nishi *et al.*, 2002). The origins of plasma Ox-LDL as well as its determinants are unknown. The characteristics of Ox-LDL isolated from the plasma of

CAD patients are comparable to those of Ox-LDL isolated from lesions (Holvoet and Collen, 1998). The potential origin of circulating Ox-LDL may be a direct release of modified LDL from ruptured or permeable plaques or ischemic injury (Nishi *et al.*, 2002; Tsimikas and Witztum, 2001). Generation of Ox-LDL in the arterial wall is probably affected by susceptibility of Ox-LDL to oxidation, the particle size and the number of LDL in the circulation, the composition of LDL and local oxidative stress in the arterial wall. Thus, circulating Ox-LDL may reflect the combined effect of these factors via additive and synergistic actions (Liu *et al.*, 2004).

Several post-mortem studies have shown that oxidised-cholesterol, cholesteryl esters, phospholipids and their breakdown products are present within the lipid pool of plaques in substantial quantities (Ylä-Herttuala *et al.*, 1994). Presumably, this oxidised lipid material is derived, in part, from necrotic foam cells rich in ox-LDL, that have released their content into the endothelial space (Tsimikas and Witztum, 2001).

Plasma Ox-LDL has been associated with subclinical atherosclerosis in clinically healthy populations. Ox-LDL was univariately correlated with IMT in carotid and femoral arteries and independently associated with subclinical plaque occurrence in carotid and femoral arteries in healthy populations (Hulthe and Fagerberg, 2002).

3.4.4 Paraoxonase (PON)

Serum paraoxonase-1 (PON1) is a 45-kDa glycoprotein which is tightly associated with HDL particles (Araki *et al.*, 2000; Durrington *et al.*, 2001). It is part of a gene cluster on chromosome 7q21.3-22.1 which contains two other members with approximately 65% similarity on the aminoacid level, *PON2* and *PON3*. PON1 was initially identified for its ability to hydrolyse pesticide-derived organophosphates, like paraoxon. Later, it was shown to hydrolyse oxidised phospholipids from LDL (Aviram *et al.*, 1998; Durrington *et al.*, 2001; Heinecke and Lusis, 1998) as well as catalyse the hydrolysis of lipid peroxides and being a potent hydroliser of a number of other substrates. These findings have led to the suggestion that PON1 activity has a role in susceptibility to atherosclerosis (Durrington *et al.*, 2001; Shih *et al.*, 1998).

PON1 and PON3 are associated with HDL and participate in the prevention of LDL oxidation. PON2 also has antioxidant properties but unlike PON1 and PON3, which are expressed primarily in the liver, it is ubiquitously expressed, especially in endothelial

and human aortic SMCs (Primo-Parma *et al.*, 1996). Experimental data suggest that, at these levels, PON2 may play an anti-atherogenic role by reducing the production of intracellular hypoperoxides and/or cell-mediated LDL oxidation (Ng *et al.*, 2001). *In vitro* studies, indicate that PON1 isolated from HDL prevents LDL from excess oxidation, which could partly explain the protective effect of HDL against oxidative stress (Heinecke and Lusis, 1998). Clinical studies support this hypothesis in that serum activity and concentration of PON are lower in patients with CVD than in those without (Araki *et al.*, 2000).

The Q192R mutation in *PON1* gene strongly determines the enzyme activity and influences serum levels of PON1, along with the L55M mutation (Wheeler *et al.*, 2004). The Q to R substitution gives rise to two alloenzymes (Adkins *et al.*, 1993 ; Humbert *et al.*, 1993). It has been shown that the R alloenzyme confers least ability of HDL to prevent oxidation of LDL, mediated through the level of PON activity (Mackness *et al.*, 1997).

Studies of *PON1* and CHD have yielded conflicting results, perhaps because each involved only a small number of cases and controls and because they used different end-points, different populations etc. (Wheeler *et al.*, 2004). In 316 randomly selected individuals in the Austrian Stroke Prevention Study, the 55LL genotype was found to be significantly associated with presence and severity of carotid disease, even after adjusting for age and sex (Schmidt *et al.*, 1998). The authors reported that the L55M polymorphism of the *PON1* gene is a genetic risk factor for carotid atherosclerosis. In addition to this line of evidence, a study of young, healthy, non-diabetic men from families with premature CHD and matched controls demonstrated that the L55M polymorphism was independently associated with the glucose response to an oral glucose tolerance test, with the 55LL homozygotes having a significantly impaired glucose disposal. The L55M –glucose interaction differentiated offsprings of high CHD risk families, suggesting that it may be of particular relevance for vascular disease (Deakin *et al.*, 2002). In an Italian cohort of 310 middle-aged women, the 55L allele was an independent risk factor for increased carotid IMT (plaques at any site) whereas the *PON1* Q192R and *PON2* S311C were not (Fortunato *et al.*, 2003). In contrast, also, in an Italian case-control study of CAD patients, the 55M allele was not associated with increased risk of CAD. Also, no major effect for the L55M alone or in combination with the R192Q on CAD risk was seen (Arca *et al.*, 2002).

Several variants of *PON2* have also been reported, with almost complete allelic association between an A148G change and a C311S change (Boright *et al.*, 1998). The presence of the C allele of S311C variant has been associated with an independent protective effect in FH patients (Leus *et al.*, 2001), and in an Asian–Indian study (Sanghera *et al.*, 1998), but not in a Chinese study (Wang *et al.*, 2003). The *PON2* S311C variant has also been shown to interact with *PON1* Q192R in an additive manner in determining CHD risk (Sanghera *et al.*, 1998).

In the Northwick Park Heart Study II, a large (3 052 subjects), prospective study on healthy middle-aged men looking on both *PON1* L55M and *PON2* S311C polymorphisms, a significant effect on CHD risk was found in men homozygous for the *PON2* C allele who also carried the *PON1* M allele (LM/MM), and showed higher CHD risk compared to SS/LL men (OR = 3.54). In addition, even though genotype frequencies did not differ between cases and controls, the CHD risk associated with smoking was significantly modified by *PON1* L55M genotype with the LL smokers having a hazard ratio of 1.30 compared to LL non-smokers while M-allele carriers had a HR of 1.76. When genotypes were analysed in combination, men with the genotype *PON1* 55 LM/MM+ *PON2* 311 CC, had HR of 3.54 (1.81–6.93) compared to *PON1* LL + *PON2* SS/SC men and these effects were shown to be independent of classical risk factors (Robertson *et al.*, 2003). These data demonstrate the importance of stratifying by environmental factors and the use of multiple SNPs for genetic analysis.

3.4.5 Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a heme enzyme found in phagocytes which transforms LDL into atherogenic particles. During the oxidative burst related to phagocyte activation (as part of the antimicrobial defence system), MPO produces several reactive intermediates on the coronary wall, that are thought to constitute an important pathway of oxidative destruction throughout the process of atherosclerosis (Heinecke, 1999).

MPO is expressed in human atherosclerotic lesions (Daugherty *et al.*, 1994) and it has been shown that in patients with coronary artery disease (CAD) leucocyte- and blood-levels of MPO are significantly higher than in controls (Zhang *et al.*, 2001b). Kutter et al. have also reported a protective effect of MPO deficiency against cardiovascular damage (Kutter *et al.*, 2000).

The promoter region of the *MPO* gene has a single G-to-A base substitution at position -463 inside a strong SP1 transcription factor consensus sequence. This polymorphism was shown to lead to high- (GG) and low-expression (AA/ AG) genotypes in promoter activity and gene expression assays (Mäkelä *et al.*, 2003). Whether this polymorphism is associated with atherosclerosis is not yet clear, however, in patients with angiographically documented CAD, the A allele has been found to be less frequent, but the stage of possible asymptomatic CAD of the contols was not evaluated (Nikpoor *et al.*, 2001). In addition to that line of evidence no direct association was found between the -463G>A polymorphism and MPO activity in the whole population in a study of 243 healthy controls. Thus, the influence of −463G >A was only found in older subjects and opposite effects according to the *MPO* genotype were reported between men and women (Rutgers *et al.*, 2003). The presence of a SP1 binding site in the −463G allele was associated with an increase in MPO expression *in vitro* (Piedrafita *et al.*, 1996), while no effect of this polymorphism on MPO concentration was detected *in vivo* (Hoy *et al.*, 2001).

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Very recently, a new polymorphism in the promoter region of the *MPO* gene was described in position -638 (-638C>A) and the A allele was found to be independently associated with a significant increase in human neutrophil MPO activity, whereas no association was found between the -463G allele and increased MPO activity *in vivo*, even though the -463A allele was mostly associated with the -638A allele (Chevrier *et al.*, 2006).

3.4.6 Angiotensinogen-converting enzyme (ACE)

ACE converts inactive angiotensin I to the vasoconstrictor angiotensin II and it also inactivates the vasodilator vradykinin, leading to increased vascular tone, vascular smooth muscle cell growth, neointimal proliferation and ECM deposition (Jeng, 2000; Sass *et al.*, 1998). Variants associated with higher ACE activity may thus be expected to be related to increased carotid wall thickness and plaque formation.

One of the most studied locus in relation to carotid atherosclerosis and CVD in general, is perhaps the insertion/deletion polymorphisms of the *ACE* gene. Presence (insertion, I) or absence (deletion, D) of a 287-bp alu-repeat sequence in intron 16 of the gene is associated with substantially different levels of plasma ACE activity in a co-dominant fashion, with DD homozygotes having the highest levels (Rigat *et al.*, 1990). Results from association studies for the I/D polymorphisms have proved, however, to be

inconsistent; with the majority of studies showing no association (Dessì-Fulgheri *et al.*, 1995; Diamantopoulos *et al.*, 2002; Mannami *et al.*, 2001; Markus *et al.*, 2001; Zannad *et al.*, 1998), whereas several show higher IMT and plaque frequency in DD homozygotes or D allele carriers. Only 2 studies reported possible interactions of the D allele in relation to IMT, in that IMT increased more steeply with increasing systolic blood pressure and age in D allele carriers than in II homozygotes (Manolio *et al.*, 2004). Another showed an association of the DD genotype with echolucent (high-risk) plaques despite lack of association with IMT or stenosis in diabetic subjects (Diamantopoulos *et al.*, 2002). Of interest are the findings by Balkestein et al. reporting that the number of *ACE* D alleles was associated with IMT of the femorals but not of the carotids and that the effect was confined to carriers of two other polymorphisms in the aldosterone synthase and a-adducin genes (Balkestein *et al.*, 2002).

3.4.7 Angiotensinogen

Angiotensinogen (ang) is the precursor peptide of angiotensin II, a potent vasoconstrictor involved in regulation of blood pressure and fluid and electrolyte balance (Chapman *et al.*, 2001). Angiotensin II stimulates proliferation and migration of VSMCs, causing intimal thickening; it also upregulates monocyte chemoattractant protein 1 (MCP-1) to attract monocytes to the vessel wall and increases oxidation and uptake of LDL by macrophages, thus promoting foam cell formation (Daemen *et al.*, 1991; Hernandez-Presa *et al.*, 1997; Keidar *et al.*, 1996). It has, also, been shown that angiotensin II can stimulate myocardial fibrosis not only by upregulating collagen synthesis but also by down-regulating collagen degradation (Tsuruda *et al.*, 2004).

Polymorphisms in the angiotensinogen gene have been associated with increased CVD risk (Fatini *et al.*, 2000). The -6G>A polymorphism upstream of the transcription initiation site has been shown to lead to increased gene transcription (Corvol and Jeunemaitre, 1997). Few studies have examined this polymorphism with no overall association detected, but women carrying the *ang* -6A and *ang* -20C (a SNP in almost complete linkage) alleles were shown to have higher IMT after adjustement. The same study examined gene-gene interactions, suggesting that the *ACE* I allele is associated with increased IMT in *ang* -6GG homozygotes only (Chapman *et al.*, 2001).

3.5 Inflammatory markers

3.5.1 Introduction

Systemic inflammation is a non-specific term referring to a state in which many pathophysiological processes are involved. It is characterised by a multitude of interactions between leucocytes, endothelial cells and platelets. Irrespective of its aetiology, inflammation causes endothelial activation which in turn promotes leucocyte transmigration into the arterial wall (Wagner and Burger, 2003).

Elevated plasma levels of several markers of the inflammatory cascade have been shown to predict future risk of plaque rupture (Blake and Ridker, 2001). Early atherosclerotic lesion development involves tethering and adherence of monocytes to and subsequent transmigration through the vascular endothelium. Differentiation of monocytes to macrophages and subsequent accumulation of lipid results in foam cell generation and fatty streak formation. Further recruitment of inflammatory cells and proliferation of smooth muscle cells (SMCs) lead to the development of a mature plaque with a fibrous cap separating the prothrombotic lipid pool from luminal blood flow. Fibrous cap thinning may lead to plaque rupture and precipitate the onset of an acute ischemic event (Libby, 2000). Accumulating evidence suggests that inflammatory processes are intimately involved in each of these stages in atherogenesis (Blake and Ridker, 2001).

Atherosclerosis is a typical example of a chronic inflammatory process (Ross, 1999). Various substances have been used as markers of the presence of ongoing inflammation. Some of the most important ones are discussed forthwidth:

3.5.2 Tumor Necrosis Factor α (TNF-α)

Tumor Necrosis Factor-a (TNF-α), is a pro-inflammatory cytokine, with a wide range of proinflammatory activities. It is primarily produced by monocyets/ macrophages, although significant amounts are also secreted by other cell types (Skoog *et al.*, 2002).

TNF-α is a primary proinflammatory cytokine that elicits the expression of the messenger cytokine IL-6, which in turn induces expression of cell adhesion molecules (CAMs) for leucocytes. Adhesion of circulating leucocytes to endothelial cells (ECs) with ensuing transendothelial migration is considered an important step in atherogenesis and increased expression of CAMs may represent one mechanism by

which TNF-α is implicated in atherothrombotic disease (Skoog *et al.*, 2002). Disturbances in the TNF-α metabolism have also been implicated in metabolic disorders such as obesity and insulin resistance (Hotamisligil *et al.*, 1993; Hotamisligil and Spiegelman, 1994), indicating that perturbations of TNF-α metabolism may affect the onset of non-insulin dependent diabetes mellitus (DM) and play a part in the development of cardiovascular disorders.

Increased TNF-α concentrations have been found in patients with premature CAD (Jovinge *et al.*, 1998). However, it remains unclear whether elevated serum TNF-α in patients with atherosclerosis derives from plaques or from non-vascular sources (Skoog *et al.*, 2002).

While many factors can affect TNF-α production (i.e. infection), genetic regulation also plays a significant role (Wang and Oosterhof, 2000). Among many DNA variants in the *TNF-α* gene, a G to A transition at the -308bp position in the promoter was shown to be associated with increased promoter activity (Wilson *et al.*, 1997) and elevated plasma TNF-α levels (Louis *et al.*, 1998). In the ECTIM study, including 641 MI patients and 710 controls that looked at five polymorphisms within the *TNF-α* gene, the only difference was seen for the -308G>A polymorphism; the A allele was found to be more frequent in obese compared to non-obese subjects (Herrmann *et al.*, 1998). In a Mediterranean (Spanish) population of 341 CHD patients (106 with DM), 207 healthy controls and 135 DM patients without CHD, the -308A allele carriers were more frequent in the CHD patients group vs controls independent of other risk factors. CHD patients with DM displayed a greater prevalence of the A allele compared to controls or patients with CHD without DM (Vendrell *et al.*, 2003).

3.5.3 Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 is a chemokine strongly implicated in promoting atherosclerosis in animal models but human genetic evidence is contradictory. For instance, overexpression of MCP-1 in the leucocytes of susceptible mice results in increased plaque size (Aiello *et al.*, 1999). MCP-1 is expressed in human lesions and CCR2 (its receptor) is expressed on leucocytes (Nelken *et al.*, 1991; Takeya *et al.*, 1993; Yla-Herttuala *et al.*, 1991). It induces arrest and transmigration from the circulation of CCR2+ monocytes under conditions of physiological shear stress and promotes monocyte differentiation to lipid-laden macrophages (foam cells) (Gerszten *et al.*, 1999; Tabata *et al.*, 2003). It also

contributes to the proliferation of arterial smooth muscle cells (Viedt *et al.*, 2002), which along with macrophages constitute the key cellular components of plaques. A growing number of human epidemiological studies have suggested links between circulating MCP-1 levels and atherosclerosis. Higher MCP-1 levels have been associated with increased risks of myocardial infarct (MI), sudden death, coronary angioplasty and stent restenosis (Cipollone *et al.*, 2001; de Lemos *et al.*, 2003; Deo *et al.*, 2004; Oshima *et al.*, 2001). However, little is known about the role of MCP-1 levels in CVD in the general population (McDermott *et al.*, 2005).

3.5.4 Cross-Reactive Protein (CRP)

CRP is a 110 kDa protein produced in response to acute injury, infection or other inflammatory stimuli (Ballantyne and Nambi, 2005). It is composed of 5 identical subunits, 22 kDa each. It is synthesized in the liver and its presence in the arterial wall is assumed to result from circulating CRP that filters in the sub-endothelial space in response to or in conjunction with inflammation of the arteries (Fu and Borensztajn, 2002). A recent study, however, has also provided evidence that CRP can be synthesized by macrophages and smooth muscle-like cells present in human atherosclerotic plaques (Yasojima *et al.*, 2001).

While its exact role remains unclear, CRP can stimulate mononuclear cells to release tissue factor (TF), it can activate the complementary pathway and it neutralises platelet activating factor. It is found both in plasma but also in the intima and media layers of human atherosclerotic arteries (Torzewski *et al.*, 2000), as well as on the surface of foam cells (Torzewski *et al.*, 1998). It has been shown that CRP only binds to oxidised LDL but not to native, non-oxidised LDL (Chang *et al.*, 2002). CRP's role in the pathogenesis of atherosclerosis is further supported by experimental evidence suggesting that it may promote foam cell formation by opsonizing native LDL; not only when LDL was in a fluid phase (Zwaka *et al.*, 2001) but also in conditions approximating the ones of the arterial wall (Fu and Borensztajn, 2002). The mechanism, however, of LDL-bound to CRP- uptake by macrophages remains to be elucidated.

Several studies have suggested CRP to be involved in atherosclerosis and development of CVD (Haverkate *et al.*, 1997; Mendall *et al.*, 1996; Rifai and Ridker, 2001). Ridker et al. demonstrated that CRP levels predict the risk of cardiovascular events in

apparently healthy men and women in the Physicians' Health Study (Ridker *et al.*, 1997). Elevated serum levels of CRP were, also, associated with abnormal endothelial vascular reactivity in patients with CAD (Fichtlscherer *et al.*, 2000). The American Heart Association has stated that it is reasonable to measure CRP as an adjunct to the measurement of established risk factors in order to assess the risk of coronary heart disease (Pearson *et al.*, 2003). The report acknowledged, however, that the epidemiologic data to support this view were not entirely consistent and recommended that larger prospective studies be undertaken to improve the reliability of the evidence. Since then at least one large case-control study has been published (Danesh *et al.*, 2004) and their results indicate that CRP is a moderate predictor of CHD. The authors suggest that recommenadations regarding use of CRP in predicting the likelihood of CHD need to be reviewd.

3.5.5 Interleukin-6 (IL-6)

IL-6 is a 26kDa cytokine, produced by many different cells in the body, including lymphocytes, monocytes, fibroblasts and endothelial cells, as well as adipose tissue. Amongst the spectrum of its functions, the most important in the systemic inflammatory response is its action as a regulator of the acute phase response. IL-6 is the only cytokine that can stimulate the synthesis of all the acute phase proteins involved in the inflammatory response (CRP, serum amyloid A, fibrinogen etc). In addition, it can affect platelet function, regulate the accumulation of leucocytes and possibly be the link between obesity and insulin resistance (Woods *et al.*, 2000).

Several studies have demonstrated a link between IL-6 and CVD. IL-6 levels appear to be predictive of future heart disease (Harris *et al.*, 1999) and are elevated in patients with unstable angina compared to those with stable angina (Biasucci *et al.*, 1999). CRP levels correlate strongly with those of IL-6, suggesting that previous studies measuring CRP may also have been indirectly measuring IL-6 levels. Cardiovascular risk factors, such as smoking and strenuous exercise leads to increased IL-6 concentrations (Woods *et al.*, 2000).

There is a relative paucity of direct evidence linking IL-6 and CVD. However, there is a strong association between systemic inflammation and CVD. IL-6 plays such a central part in the inflammatory response that it is extremely likely that there is also a link between IL-6 and CVD. If systemic inflammation is a cause of coronary artery

disease, IL-6 might determine the magnitute of inflammatory response to a particular stimulus and hence also the severity of CVD. Consequently, the discovery of a genetic polymorphism that determines the rate of IL-6 production is even more important (Fishman *et al.*, 1998).

A polymorphism in the promoter region of the gene coding for *IL-6* has been found to be associated with IL-6 levels. It involves a single-base change from guanine to cytosine at position -174 (-174G>C) and the G allele is associated with higher IL-6 production than the C allele. This has been demonstrated both *in vitro* and *in vivo* (Woods *et al.*, 2000). Others, however, have reported higher peak levels of IL-6 after coronary artery bypass surgery for C allele carriers (Brull *et al.*, 2001). In addition to that evidence, a recent study on the effect of the polymorphism on carotid IMT, reported that the risk of elevated IMT was confined to CC homozygotes with no clear heterozygote effect. This suggests that there may be a threshold effect, with only very high levels of IL-6 predisposing to disease.The same study demonstrated that the *IL-6* -174G>C polymorphism may modulate the effect of alcohol on carotid atherosclerosis, with the CC genotype associated with higher IL-6 levels, elevated IMTcc and increased risk of carotid plaque in heavy drinkers (Jerrard-Dunne *et al.*, 2003).

3.5.6 Matrix Gla Protein (MGP)

Matrix Gla protein is a 10kDa, vitamin K-dependent circulating protein that contains Gla residues with strong affinity for calcium phosphate (hydroxyapatite), thus playing an important part in its clearance (Herrmann *et al.*, 2000). It has been shown that MGP is highly expressed in human atheromatous plaques by both VSMCs and macrophages-derived foam cells. The function of MGP in the healthy vessel wall is unknown. In the atheromatous plaque, however, MGP may become trapped in the extracellular matrix, particularly bound to lipid where its affinity for calcium may predispose to tissue calcification (Shanahan *et al.*, 1994). It could be hypothesized that during the development of atherosclerotic plaques, MGP is trapped and inactivated or its expression and function is affected by other atheroma-dependent mechanisms, which could account for the tight correlation existing between plaque evolution and the degree of calcification (Herrmann *et al.*, 2000).

Coronary calcification in asymptomatic patients is known to increase the risk of coronary heart disease and mineral deposits are documented in >90% of patients with

CHD. Recent evidence suggests that calcification in the arteries may be the consequence of a functional deficit leading to an impaired inhibition of calcification rather than the consequence of an active calcification process (Schinke *et al.*, 1999). Morphological studies have shown that VSMCs in atheromatous plaques differ from contractile VSMCs in the media and that plaque VSMCs synthesize matrix proteins which contribute to the composition of the plaque; therefore the proteins they produce may determine the nature of the resulting atherosclerotic lesion (Mosse *et al.*, 1985). Decreased serum MGP levels are correlated with increased severity of coronary arterial calcification as assessed by computerised tomography (Jono *et al.*, 2004). In the ECTIM Study, a change from T to C in position -138 in the promoter of the gene was found to be associated with the promoter activity, with the minor C allele conferring a reduced activity of -20% in rat VSMCs and -50% in human fibroblast cell lines. Data indicate that a nuclear protein binds in the region encompassing the -138T>C polymorphic site and that the extent of this binding is reduced when the minor allele C is present. Despite the identification of a functional effect on *MGP* promoter activity *in vitro*, the -138T>C polymorphism was not related to calcification, femoral artery atherosclerosis or MI in the ECTIM and AXA populations (Cambien *et al.*, 1999). In the CARDIA study, the -138T>C polymorphism was weakly associated with coronary calcification, but this interaction was only observed in the white participants (Taylor *et al.*, 2005).

3.5.7 Metalloproteinases (MMPs)

Integrity of the extracellular matrix (ECM) constitutes a critical determinant in the stability of coronary atheromata. In particular, degradation of fibrillar collagen may decrease the ability of the fibrous cap to withstand mechanical stress. Several members of the metalloproteinase (MMP) family contribute to collagen degradation (Yan *et al.*, 2004).

MMPs are a family of Zinc-containing, Ca^{2+}-dependent endopeptidases that maintain homeostasis of cardiac structure by digesting the ECM. The MMP family consists to date of more than 20 species with different substrates, which include collagenases (such as MMP-1 and MMP-13), gelatinases (such as MMP-2 and MMP-9), stromelysin (MMP-3), and membranous type MMP (such as MT1-MMP) (Tsuruda *et al.*, 2004). Together they can digest all proteins and proteoglycans of the ECM.

Dysregulation of MMPs is thought to play a part in both atherogenesis and the precipitation of acute coronary syndromes by regulating connective tissue remodelling, thus determining the volume expansion of the atherosclerotic plaque, its stability, and the potential for smooth muscle cell proliferation. Indeed, both smooth muscle cells and macrophages synthesize and secrete a range of MMPs, and MMP expression has been demonstrated in human atherosclerotic plaques (Jormsjo *et al.*, 2000) (Table 3.1). MMPs have been shown to be present in rupture-prone areas of plaques (Brown *et al.*, 1995; Galis *et al.*, 1994; Shah *et al.*, 1995). Synthesis of MMPs has also been reported in coronary atherosclerotic lesions in patients with unstable angina and acute myocardial infarction (Kai *et al.*, 1998), which suggests a pathogenic role of MMPs in the development of acute coronary syndromes (ACS) as well (Yan *et al.*, 2004).

The activites of MMPs are controlled on multiple levels: transcription and translation of their inactive precursors (zymogens), post-translational activation of zymogens by proteolysis and interactions with tissue inhibitors of metalloproteinases (TIMPs) and/or tissue factor pathway inhibitor-2 (TFPI-2). Indeed, TIMPs-1, -2, -3 and TIFP-2 are expressed in atherosclerotic lesions (Galis *et al.*, 1994; Herman *et al.*, 2001) and these inhibitors bind to and inactivate most of the MMPs (Takeo *et al.*, 2006).

Induction of MMP-9, as well as MMP-1, expression has been shown in both VSMCs and accumulating macrophages in atherosclerotic plaques, particularly in the shoulder and core of plaques prone to rupture. These observations raised the possibility that these MMPs are strongly associated with the molecular mechanism of the onset and development of acute coronary syndrome (ACS). In addition, the expression of MMP-1, MMP-3 and MMP-9 was shown to be augmented in plaques compared to the adjacent control regions. In the same study, the expression of TFPI-2 was found to be lower in plaques and upregulation of MMPs in plaques was disproportional to that of TIMPs, suggesting that imbalanced degradation and synthesis of ECM persists in advanced lesions, particulary in plaques with disruption (Takeo *et al.*, 2006). In another recent study, measuring mRNA transcript levels of MMP-1, -3, -7, -9 and -12 in carotid atherosclerotic plaques with different histopathological characteristics, it was shown that plaques with a thin fibrous cap (prone to rupture) had significantly higer levels of MMP-1 transcripts compared to plaques with a thick fibrous cap. MMP-3, -7 and -12 levels were also higher but did not reach statistical significance. In ruptured plaques, MMP-12 transcript levels were significantly higher compared to lesions without cap

disruption (Morgan *et al.*, 2004). MMP-3 levels have also been associated with positive arterial remodelling as shown by intravascular ultrasound (IVUS) (Schoenhagen *et al.*, 2002). In a histochemical study looking at presence of MMPs in atherosclerotic plaques both MMP-7 mRNA and protein were expressed primarily at the border between the fibrous cap and the lipid core (Katsuda and Kaji, 2003). Others, have reported similar findings, noting that the degradation by MMP-7 particularly of versican in the border area fasciltates the separation of the fibrous cap from the lipid core (Halpert *et al.*, 1996). MMP-12 mRNA and protein were found in macrophages, particularly at the border between the fibrous cap and the lipid core (Katsuda and Kaji, 2003). These data further support a role of MMPs in determining atherosclerotic plaque stability.

Since the level of expression of these enzymes in the plaque is essentially impossible to measure *in vivo*, genetic polymorphisms of MMPs that predict high or low enzyme levels may add information to measurable plasma risk factors for estimating CAD risk.

MMP1 (1G/2G):

A functional polymorphism is present in the promoter region of the human *MMP-1* gene, with alleles having either one (1G) or two (2G) guanine nucleotides at position -1607 relative to the transcription start site. It has been shown that the 2G allelelic promoter has over 20 fold higher transcriptional activity than the 1G (Rutter *et al.*, 1998).

Nordskog et al. demonstrated recently that in cultured human aortic endothelial cells those homozygous for the 1G allele exhibited low levels of gene expression, whereas those carrying the 2G allele had elevated levels of both mRNA and protein. However, differences in lesion development based on this polymorphisms were not observed in 104 Caucasian males in the Pathobiological Determinants of Atherosclerosis in Youth study (Nordskog *et al.*, 2004).

In a case control study in Japanese patients with MI there was no disease association reported for the *MMP-1* 1G/2G genotype alone; however, there was strong linkage disequilibrium between the *MMP-1* 1G/2G and *MMP-3* 5A/6A polymorphisms and the 5A-1G haplotype was a genetic risk factor for MI in Japanese (Nojiri *et al.*, 2003).

In a British study including 471 indiciduals (204 with evidence of coronary disease), the 2G/2G genotype was associated with lower risk of coronary heart disease compared to 1G/1G homozygous. The heterozygotes exhibited intermediate risk and results remained significant even after adjustement for traditional cardiovascular risk factors (Ye *et al.*, 2003).

MMP-3 (5A/6A):

Another such polymorphism is the 5A/6A in the promoter of the *MMP-3* (stromelysin-1) gene. *In vitro* studies showed that the 5A allele expressed higher activity than the 6A allele and that compared with other genotypes, individuals homozygous for the 6A allele would have lower stromelysis-1 levels in their arterial wall because of reduced gene transcription. In the LOCAT gemfibrozil study, the 6A allele homozygous subjects were associated with rapid progression of angiographically determined CAD, whereas the 5A allele was associated with a beneficial effect on disease progression (Humphries *et al.*, 1998).

In a large Japanese case-control study for MI, the 6A allele was found to be a risk factor for MI in women (Yamada *et al.*, 2002). In addition to these evidence, a study looking at all polymorphisms in the promoter and coding regions of the *MMP-3* gene in 1240 CAD patients, demonstrated that the effect of susceptibility to MI was largely attributed to the 5A/6A polymorphism, with the 5A allele possibly predisposing to unstable plaques and the 6A/6A genotype predisposing to developing more fibrotic, atherosclerotic plaques with significant stenosis (Beyzade *et al.*, 2003).

An association between the 5A/6A genotype and carotid IMT has also been demonstrated in at least three independent studies, which have shown that individuals with the 6A/6A genotype have greater IMT values compared with 5A/6A or 5A/5A individuals (Gnasso *et al.*, 2000; Rauramaa *et al.*, 2000; Rundek *et al.*, 2002). In one of this studies, the 6A/6A genotype was also associated with enlarged arterial lumen and local reduction of wall shear stress, which might predispose them to atherosclerotic plaque localisation (Gnasso *et al.*, 2000).

MMP-9 (R279Q):

As mentioned above, MMP-9 has been found to be highly expressed in the vulnerable regions of atherosclerotic plaques and it has been suggested to be causally involved in

the remodelling processes associated with atherogenesis and plaque rupture. An exonic polymorphism (R279Q) which leads to an amino acid exchange in the catalytic domain of the MMP-9 enzyme has been significantly associated with CV events in patients with stable angina in a large (1 127 patients) prospective study (Blankenberg *et al.*, 2003).

MMP-12 (-82A>G):

A recently described nucleotide change from A to G in the promoter region of the *MMP-12 gene* (-82A>G) influences the binding site of the transcription factor activating protein 1 (AP1) and thus enzyme activity, with the A allele being associated with higher promoter activity *in vitro*. Insulin (a known activator of AP1) was associated with higher affinity for the A allele and higher promoter activity (Jormsjo *et al.*, 2000). In the same study, the A allele was associated with a smaller luminal diameter in 367 diabetic patients with CAD.

Table 3.1: Family of matrix metalloproteinases with known cellular sources in plaques (Katsuda and Kaji, 2003).

MMP name	Substrates	Cellular sources in plaques
MMP-1	Collagens I, II, III, VII, X, gelatine, entactin, proteoglycan, link protein, tenascin.	Macrophage, SMC, endothelial cell.
MMP-2	Gelatin, collagens IV, V, VII, XI, laminin, elastin, fibronectin,proteoglycan, link protein	SMC, macrophage, endothelial cell, T lymphocyte
MMP-3	Proteoglycan, collagens III, IV, IX, X, laminin, fibronectin, gelatin, tenascin, link protein, elastin	Macrophage, SMC
MMP-7	Proteoglycan, gelatine, fibronectin, tenascin, elastin, collagen IV, laminin, link protein	Macrophage
MMP-8	Collagens I, II, III, gelatine, proteoglycan, link protein	Neutrophil, macrophage, SMC, endothelial cell
MMP-9	Gelatin, collagens III, IV, V, elastin, entactin, link protein	Macrophage, SMC, endothelial cell, T-lymhpocyte
MMP-12	Elastin	Macrophage
MMP-13	Collagens I, II, IV, IX, X, XIV, proteoglycan, fibronectin, tenascin	Macrophage, SMC, endothelial cell
MMP-14	Collagens I, II, III, gelatine, proteoglycan, fibronectin, laminin	SMC, endothelial cell, macrophage

3.6 Thrombotic markers

3.6.1 Introduction

The central role of platelets in thrombosis and haemostasis has been known for many years. Recently, however, it has become evident that platelets have relevant functions in inflammation as well. Thrombosis and inflammation share several key molecular mechanisms and are in fact two extrinsically linked processes (Wagner and Burger, 2003).

Among the mechanisms through which cholesterol may facilitate the atherosclerotic complication, the activation of the platelet and clotting system has been suggested to play a pivotal role (Sanguigni *et al.*, 2005). Thrombophilia is basically characterised by platelet hyperactivation and up-regulation of tissue factor (TF) with enhanced thrombin generation (Davi *et al.*, 1992; Ferro *et al.*, 1997). CD40L may represent an important link between platelet activation and TF expression, as well as between inflammation and thrombosis.

3.6.2 CD40-CD40L

CD40 is a 48-kDa phosphorylated glycoprotein, belonging to the TNF receptor superfamily. CD40 is constitutively expressed on platelets and provides a novel mechanism for CD40-mediated platelet activation in thrombosis, inflammation and atherosclerosis (Inwald *et al.*, 2003). CD40-CD40 ligand (CD40L) signalling is critical in the pathogenesis of atherosclerosis. In addition, CD40L is involved in thrombosis; at high shear stress, CD40L binds directly to platelets, enhancing thrombus formation and inducing platelet spreading (Andre *et al.*, 2002).

CD40L is a transmembrane protein expressed on the surface of lymhpocytes as well as on the cells of the vascular system, such as endothelial cells, SMCs and macrophages (Schönbeck and Libby, 2001). It exists in two forms: the 39-kDa, cell-associated form and the soluble, biologically active form (sCD40L) (Schönbeck *et al.*, 2001). The sCD40L is cleaved from the platelet-bound CD40L, which is expressed upon agonist stimulation on platelet surface (Henn *et al.*, 1998). sCD40L in circulation may pass through damaged atherosclerotic endothelium and come into direct contact with cells inside the lesion. More importantly, sCD40L may activate circulating leucocytes or platelets to enhance the release of MMPs and increase the rupture of the plaques. Recently, many studies have demonstrated that the interaction of CD40-CD40L is

associated with the early formation of atherosclerosis and the long-term atherosclerotic process (Ozmen *et al.*, 2001; Phipps *et al.*, 2001).

Patients with unstable angina have higher concentrations of functional sCD40L than do patients with stable angina or healthy volunteers, possibly as a result of release from activated platelets or T-lympocytes (Schönbeck *et al.*, 2000). Platelets express membrane-bound CD40L on activation, which induces pro-inflammatory changes in endothelial cells via endothelial CD40 (Henn *et al.*, 1998; Slupsky *et al.*, 1998). Platelets are also, the major source of sCD40L in the circulation and sCD40L is released from platelets following activation by thrombin, ADP or collagen (Aukrust *et al.*, 1999). The enhancement of platelet-leucocyte adhesion caused by ligation of platelet CD40 and the proinflammatory stimulus caused may be of particular importance when platelets are in proximity to cells expressing CD40L, such as platelets in thrombi and T-lymphocytes, macrophages and endothelial cells in plaques (Phipps, 2000). This could provide a mechanism for directing leucocytes to sites of thrombosis, inflammation or tissue injury in the initiation and progression of atherosclerosis (Inwald *et al.*, 2003).

The binding of CD40L with its receptor (CD40) mediates many inflammatory responses important in atherosclerosis. A wide variety of inflammatory cells express CD40L and stimulation by other pro-inflammatory cytokines increases endothelial cell expression of CD40L (Karmann *et al.*, 1995). Ligation of CD40 induces the expression of leucocyte adhesion molecules and triggers the release of chemoattractants overexpressed in human atheroma (Denger *et al.*, 1999; Kornbluth *et al.*, 1998; Mach *et al.*, 1999) . Furthermore, CD40 ligation potently induces the expression of tissue factor (TF) (Mach *et al.*, 1997; Schonbeck *et al.*, 2000) an important pro-thrombotic component of the intraplaque lipid pool (TF converts factor X to Xa). Thus, the spectrum of functions of CD40L appears to span a wide range from early atherogenesis to late thrombotic complications (Blake *et al.*, 2003).

It remains unknown, whether plasma concentrations of sCD40L have diagnostic or prognostic value among apparently healthy individuals before the onset of acute cardiovascular events. However, findings suggest that CD40L may be a marker of coronary disease activity rather than a measure of the anatomic extent of CAD (Yan *et al.*, 2004).

3.6.3 Fibrinogen (Fb)

Fibrinogen (Fb) is a glycoprotein that circulates largely inactively, in the blood stream. It consists of 6 polypeptide chains held together by disulphide bonds in a molecule with bilateral symmetry (Scott *et al.*, 2004). It is involved in primary haemostasis, platelet aggregation and leucocyte-endothelial cell interactions and is the major determinant of whole blood and plasma viscosity (Woods *et al.*, 2000). Blood viscosity was shown to be positively associated with CHD in men after 8 years of follow-up in the MONICA study (Koenig *et al.*, 1998). There is considerable variation in the fibrin clot structure of different individuals, suggesting that both genetic and environmental factors have a role in determining the balance between stability and susceptibility of the clot to fibrinolysis (Scott *et al.*, 2004).

Elevated Fb levels have consistently been demonstrated to be a major risk factor for thrombosis and CVD (Koenig, 2003) as well as with atherosclerosis, and have been reported in patients with CHD, peripheral vascular disease and carotid stenosis (Ernst and Resch, 1993; Kannel *et al.*, 1997). Fb levels also predict vascular events in population-based studies (Folsom *et al.*, 1997; Lowe *et al.*, 2000; Wilhelmsen *et al.*, 1984). Fibrinogen is as powerful a predictor of future CVD as other classical risk factors such as hypercholesterolaemia, smoking and hypertension. There is a high degree of intra-individual variation in fibrinogen levels, which means that the real association between fibrinogen and coronary disease might be even stronger than reported thus far (Woods *et al.*, 2000).

Fibrinogen is increased in many conditions including advancing age, female sex, smoking, diabetes mellitus (DM), elevated LDL and TG, hypertension, inflammation and infection (Koenig, 2003). It is reduced by exercice/physical fitness, weight loss, and smoking cessation (Scott *et al.*, 2004). Whether Fb is just a marker of the inflammation related to vascular disease, or whether it is involved in actively mediating the vascular disease process is not entirely known, although recent studies have suggested that it is a true modifier of vascular disease, augmenting fibrin deposition in certain organs and regulating fibrin turnover (Kerlin *et al.*, 2004). However, few studies have looked at Fb levels in relation to distinct outcome events (such as stroke) and none has looked at early, subclinical atherosclerosis.

3.6.4 P-selectin

P-selectin is an adhesion molecule present within endothelial cells that is rapidly translocated to the cell membrane upon activation, where it mediates endothelial-leucocyte interactions. P-selectin, released from the cell surface, circulates as a soluble molecule in the plasma. In atherosclerosis, the adhesion of circulating monocytes to the endothelial lining is the earliest detectable event after cholesterol feeding in experimental animal models (Faggioto *et al.*, 1984) and the active stages of atherosclerosis are marked by the extensive infiltration of these blood-derived monocytes through the endothelium into the arterial intima (Hansson *et al.*, 1989). It is now well established that endothelial adhesion molecules play an important part in the emigration of leucocytes from the blood into foci of inflammation, and evidence is accumulating that similar processes are at work in atherosclerosis (Johnson-Tidey *et al.*, 1994).

Immunohistochemical analysis of human atherosclerotic plaques has shown strong expression of P-selectin by the endothelium overlying active atherosclerotic plaques. P-selectin is not, however, detected in normal arterial endothelium or in endothelium overlying inactive fibrous plaques (Johnson-Tidey *et al.*, 1994).

Celi *et al.* showed that P-selectin, either expressed on activated cells or purified, upregulates tissue factor (TF) expression on monocytes *in vitro* (Celi *et al.*, 1994). Soluble P-selectin is often used as a marker of platelet activation, although its origin has not been established with certainty. Higher levels of soluble P-selectin are predictive of future cardiovascular events (Hillis *et al.*, 2002; Ridker *et al.*, 2001). These findings support the concept of P-selectin having procoagulant activity.

In addition, recent findings by Burger et Wagner in apoE and *P-selectin* Knock Out mice sugest that in atherosclerosis the procoagulant sP-selectin reflects endothelial rather than platelet activation; endothelial P-selectin was crucial for the promotion of atherosclerotic lesion growth because in its absence only relatively small lesions developed (Burger and Wagner, 2003)**.**

3.6.5 Tissue Factor (TF)

Tissue Factor (TF) is an integral membrane-bound glycoprotein (47 kDa), that requires the presence of specific phospholipids to function. It serves as both the receptor and essential cofactor(s) for factors VII and VIIa in initiating cell surface procoagulant

activity (extrinsic pathway of coagulation). It also, activates factor X through the intrinsic pathway by activating factor IX which in turn activates factor X (Osterud and Rapaport, 1977) and leads ultimately to thrombin generation and fibrin formation. As a potent initiator of coagulation, TF is believed to have a critical part in haemostasis and thrombogenesis.

Tissue Factor antigen, TF activity, as well as TF mRNA have been found in different cell types within the atheroma, including ECs, vascular SMCs and especially cholesterol-rich macrophages (foam cells) (Toschi *et al.*, 1997). TF antigen has also been detected in the extracellular matrix of atherosclerotic plaque and is though to be derived largely from macrophages present in the plaque with a minor contribution of vascular SNCs and ECs. Several studies have demonstrated that TF is most abundant in the shoulder region and acellular, lipid-rich core of the plaque (Moons *et al.*, 2002) while others have shown the majority of TF expression within human plaques to be in areas of high density of apoptotic cells and microparticles (Tedgui and Mallat, 2001b).

3.6.6 Plasminogen Activator Inhibitor (PAI-1)

PAI-1 is a member of the superfamily of serine protease inhibitors and is derived from several sites, including the liver, the vascular endothelium and adipose. Impaired fibrinolysis can result from an imbalance between clot-dissolving enzymes such as tissue-type plasminogen activator (t-PA) and its endogenous inhibitor plasminogen activator inhibitor (PAI-1) (Fuster *et al.*, 1999). Experimentally, there is strong evidence that PAI-1 directly contributes to arterial thrombosis in specific vascular beds. Transgenic mice that overexpress a stable form of human PAI-1 have age-dependent coronary arterial thrombosis and subendothelial infarction (Eren *et al.*, 2002). Several environmental and genetic factors further modulate PAI-1 and fibrinolytic balance. In particular, a common single 4G/5G polymorphism located upstream from the transcription site of the *PAI-1* gene has been described that influences plasma PAI-1 levels (Dawson *et al.*, 1991). PAI-1 activity is highest in individuals homozygous for the 4G allele and lowest for the 5G allele. This genotype further influences the relationship of insulin resistance and circulating PAI-1 concentrations (Mansfield *et al.*, 1995). In a large case-control study in Japanese subjects looking at 112 polymorphisms in 2819 unrelated patients with MI and 2242 controls, it was demonstrated that only three were significantly associated with the risk of MI. One of those was the 4G/5G polymorphism of the *PAI-1* gene and it was suggested that the 5G allele was a risk

factor for MI in women (Yamada *et al.*, 2002). However, evidence to the contrary also exists. In the Framingham Offspring cohort including 867 men and 911 women no association was shown between carotid IMT and polymorphisms in the haemostatic factor pathway, including the *PAI-1* 4G/5G polymorphism (Fox *et al.*, 2004).

Beyond genetic predisposition, a number of factors are known to directly influence PAI-1 production, including glucose, insulin, type II diabetes and the acute-phase response (Healy and Gelehrter, 1994; Juhan-Vague and Alessi, 1997 ; Kohler and Grant, 2000).

Elevated levels of PAI-1 have been shown to predict cardiovascular risk in middle-aged men (Thogersen *et al.*, 1998) and circulating PAI-1 concentrations are elevated in young men at increased risk for recurrent infarction (Hamsten *et al.*, 1987).

3.6.7 Microparticles (MP)

Microparticles are plasma membrane-derived vesicles shed from stimulated cells (i.e. platelets, T- and B-lymphocytes, endothelial cells etc). Stimulation can happen when they are activated by an agonist, shear stress, or apoptosis. Microparticles harbor cell surface proteins and contain cytoplasmic components of the original cell. They exhibit negatively charged phospholipids, chiefly phosphatidylserine, at their surface, which accounts for their procoagulant character and proinflammatory properties, including alteration of vascular function. Elevated levels of circulating microparticles have been detected in pathological states associated with vascular dysfunction, including attenuation of endothelium dependent vasodilatation and/or alteration of responsiveness of vascular smooth muscle to vasoconstrictor stimuli in conductance and resistance arteries (Martinez *et al.*, 2005). MPs are though to play a part in various normal or pathologic conditions such as antigen-transfer and intercellular cross-talk, vascular function and angiogenesis, haemostasis, thrombosis and inflammation (Hugel *et al.*, 2005). Thus, microparticles can be viewed as a new pathway that can be used by cells to exchange information in addition to the transduction linked to the activation of classical known receptors or transporters. The majority of *in vivo* circulating microparticles are derived from platelets compared with microparticles from other circulating or vascular cells. Under several pathological situations, the number of total microparticles as well as the proportion of their different origins can change. Because of the variety of microparticles, it is plausible that they may exert pleiotropic effects on the vascular wall. Moreover, depending on the microparticle

composition, one can speculate that different subpopulations of microparticles (from platelets, leucocytes, etc.) may communicate different messages in regulating vascular function and dysfunction (Martinez *et al.*, 2005).

3.7 Homocysteine Metabolism

3.7.1 Introduction

Elevated serum homocysteine is considered an independent risk factor for cardiovascular disease (CVD). High levels of homocysteine have been reported through the years in patients with stroke and vascular disease (Boers *et al.*, 1985), whereas Clarke et al. showed that about 30% of patients with premature atherosclerosis had plasma homocysteine levels greater than 14 μmol/L (Clarke *et al.*, 1991).

3.7.2 Homocysteine (Hcy)

Homocysteine is a sulphuric amino acid derived from methionine which is present in dietary proteins. In plasma it exists in two forms; reduced and oxidised. Hcy is primarily found in its oxidised states, homocystine, homocysteine mixed disulfide and protein-bound homocysteine. Measurements of circulating Hcy include both states and represent total homocysteine (tHcy) (Carmel and Jacobsen, 2001).

Hcy is rapidly metabolised during the methylation process back to methionine or catabolised to cysteine. Both pathways are catalysed by group B vitamins- folic acid and B12 or B6 respectively (Skibińska *et al.*, 2004). In vascular cells, homocysteine metabolism is limited to the B12-folate dependent remethylation pathway catalysed by methionine synthase (Carmel and Jacobsen, 2001).

A great number of environmental factors have been found to play a determinant role in Hcy levels, with age and sex being two of the most important. Lussier-Casan et al. showed that the fasting level in a woman is 21% lower than in a man (Lussier-Cacan *et al.*, 1996), a fact that could be partially explained by the observation that the remethylation pathway is more efficient in women than in men (Fukagawa *et al.*, 2000). Hcy levels also increase with age in both sexes. The Hordaland study in Norway found that a rise in Hcy levels was associated with smoking, high arterial pressure, high cholesterol and a sedentary lifestyle (Nygard *et al.*, 1995). Alcoholics have higher tHcy levels, probably due though to malnutrition and malabsorption (Bleich *et al.*, 2000).

3.7.3 Hyperhomocysteinemia treatment

Moderately elevated homocysteine concentrations, which reflect less genetic defects and more a deficiency of nutritional factors (folate/B12/B6) required for homocysteine metabolism are common in the general population (Perry *et al.*, 1995), particularly in elderly people. Vitamin status is the primary determinant in mild and moderate hyperhomocysteinemia, with deficiency in the B complex (folic acid, B12 and B6) being the cause in two thirds of the cases (Naurath *et al.*, 1995). Brattstrom et al. showed that administration of 5 mg of folic acid per day for 14 days was sufficient to diminish Hcy levels in healthy people (Brattström *et al.*, 1988). The main role in diminishing Hcy levels could be explained by the fact that it is consumed during Hcy metabolism for the methylation of Hcy back to methionine in the folate cycle. In this way, folic acid supplementation is the only effective treatment in reducing plasma Hcy levels in healthy subjects (Brattstrom, 1996). Serum folate levels correlate inversely with total homocysteine (tHcy) in virtually all surveys and in all ages in both healthy populations and CVD patients (Aguilar *et al.*, 2004).

There is a limited consensus on the normal reference range for serum folate levels depending on the population studied and the laboratory techniques used. In population studies, the population median is usually used as the cut-off point between normal and subnormal levels.

Folate deficiency could arise from a combination of causes in a single individual, such as dietary inadequacy, malabsorbsion, alcohol or drugs. The most common cause of mild folate deficiency, however, is dietary deficiency. In 1998, fortification of cereal grain foods with 1.4 μg folic acid/g became mandatory in the United States, in order to reduce neural tube defect births. This practise, however, had begun earlier on a voluntary basis (Carmel and Jacobsen, 2001). As a result, median levels of folate rose from 10.4 to 22.5 nmol/L and the prevalence of low levels declined from 22% to 7% , as was shown in the Framingham study (Jacques *et al.*, 1999).

3.7.4 Methylenetetrahydrofolate reductase (MTHFR)

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the re-methylation pathway; it reduces 5,10- methylenetetrahydrofolate to produce 5-methylenetetrahydrofolate, which acts as a carbon donor in the conversion of

homocysteine to methionine (Bova, 1999). Genetic mutations of the *MTHFR* gene have been shown to result in increased levels of homocysteine. One such mutation is the 677C>T which renders the enzyme thermolabile and functionally impaired. Hcy levels tend to be higher in persons homozygous for the thermolabile variant (TT), particularly in the setting of dietary folate deficiency (Jacques *et al.*, 1996). Vascular endothelial cells may be especially vulnerable to the higher levels of circulating and endogenous homocysteine found in patients with hyperhomocysteinemia (Hayden and Tyagi, 2004).

3.7.5 Homocysteine and CVD

The mechanism by which homocysteine induces atherosclerosis is not yet fully understood but homocysteine has been found to: (i) cause cytotoxic effects on the endothelium by impairing the NO synthase pathway (Skibińska *et al.*, 2004 ; Stamler *et al.*, 1993), (ii) stimulate vessel fibrosis (Skibińska *et al.*, 2004), (iii) act in a prothrombotic and proaggregational way (Harpel *et al.*, 1996) and (iv) inhibit anti-oxidant processes (Skibińska *et al.*, 2004 ; Upchurch Jr *et al.*, 1997). *In vitro*, clots formed in the presence of Hcy have thicker, shorter fibers with a more compact structure (Lauricella *et al.*, 2002). Clots formed with Fb from homocysteinemic plasma are more resistant to lysis (Sauls *et al.*, 2003). In addition, Hcy binds to circulating fibronectin and hinders fibrin/fibronectin binding (Majors *et al.*, 2002). This may reduce the amount of fibronectin in the clot, impairing wound healing (Scott *et al.*, 2004).

To date, more than 80 cross-sectional, case-control and cohort studies have linked hyperhomocysteine with CHD risk. In the Framingham study (Bostom *et al.*, 1999), the Trømso study (Arnesen *et al.*, 1995) and in women but not men in the ARIC study (Folsom *et al.*, 1998), homocysteine levels were higher in adults with CHD. In the British Heart Study (BHS), Hcy levels were significantly higher in patients with stroke (Perry *et al.*, 1995). More recently, data from a prospective cohort study (the Hordaland study) in 17 361 people corroborated the finding of increased Hcy in pre-existing CVD (Fruchart *et al.*, 2004; Nurk *et al.*, 2002). In addition, in a large multi-center European study, the COMAC study, it was estimated that the cardiovascular risk associated with hyperhomocysteinemia in fasting conditions (>12 μmol/L) and after methionine load is similar to that of hyperlipidaemia or smoking, although less than that associated to hypertension (Graham *et al.*, 1997). Boushey et al. suggested that a 5

μmol/L Hcy plasma rise could augment coronary disease risk to a similar grade as a 20 mg/dL cholesterol plasma rise (Boushey *et al.*, 1995).

Several studies have analysed the influence of the *MTHFR* 677C>T polymorphism in CAD with ambiguous results. In a meta-analysis done by Brattstrom et al. which included 23 studies, no difference was found between vascular disease patients (CAD) and controls for the T allele frequency (Brattstrom *et al.*, 1998). A recent study which correlated CAD early presentation with Hcy levels and 677C>T genotype, found that hyperhomocysteinemia and TT genotype had a stronger effect in CAD pathogenesis when they were combined and that a marked Hcy raise (>15 μmol/L) in TT homozygous patients was a risk factor for the early beginning of CAD, not being the case for homozygotes with normal Hcy levels (Mager *et al.*, 2002). In concordance with these results, a recent meta-analysis that included 11 162 patients with CAD and 12 758 controls concluded that under good folate ingestion conditions, and therefore low Hcy levels, it does not have any clinical value to look for 677C>T genotype in order to predict CAD (Klerk *et al.*, 2002).

3.7.6 Assymetric Dimethyl Arginine (ADMA)

NO is formed from L-arginin in a reaction catalysed by NO synthase (NOS), with L-citrullin being the second reaction product. The most important endogenous inhibitor of NOS is ADMA. ADMA is derived from the catabolism of proteins containing methylated arginine residues. When these proteins undergo hydrolysis their methylated arginine residues are released and excreted in the urine. This explains the increase in plasma ADMA levels in patients with renal insufficiency. The major metabolic pathway for ADMA is regulated by dimethylarginine dimethylaminohydrolase (DDAH). Both isoforms of DDAH have been found in every cell type examined. ADMA is constantly being produced in the course of normal protein turnover and its production is balanced by its metabolism by DDAH. Accordingly, inhibition of DDAH activity will cause a gradual accumulation of ADMA sufficient to induce vasoconstriction (Cooke, 2000).

Plasma levels of ADMA are normally in the range of ~1μmol/L but they can increase two-fold in subjects with risk factors for vascular disease and increase even further (up to 10-fold) in patients with with clinical atherosclerosis.

The hypothesis that endogenous ADMA accelerates atherosclerosis is supported by the observation that supplemental dietary arginine enhances NO synthesis in the rabbit aorta (Tsao *et al.*, 1994). There are similar data for human; in 120 Japanese individuals with varying levels of risk factors, carotid IMT was correlated with blood pressure, lipid profile, smoking history, blood sugar and ADMA. A multivariate analysis revealed that ADMA and age were the only independent predictors (Miyazaki *et al.*, 1999).

3.8 Metabolic Syndrom (MetS)

3.8.1 Introduction

The metabolic syndrome (MetS) is defined as a clustering of risk factors for cardiovascular disease (CVD), including abdominal obesity, dyslipidaemia, hypertension and insulin resistance. Insulin resistance can lead to metabolic changes, endothelial dysfunction, intima-media thickening (IMT) and later to macro- and micro-vascular complications (Anand *et al.*, 2000; Feinstein, 2006; Jashnani *et al.*, 2005; Kablak-Ziembicka *et al.*, 2004; Kablak Ziembicka *et al.*, 2003; Tenenbaum *et al.*, 2004; Wassink *et al.*, 2007). Individuals with MetS have significantly greater IMT values compared to individuals without MetS (Pollex *et al.*, 2006; Wallenfeldt *et al.*, 2005), and the IMT increases with each additional component of MetS (Pollex *et al.*, 2006).

There are currently three common definitions of the MetS, the World Health Organization (WHO) (Alberti and Zimmet, 1998), the National Cholesterol Education Program Expert Panel (NCEP) (Adult Treatment Panel III, 2001) and the International Diabetes Federation (IDF) (International Diabetes Federation, 2005). These definitions are in general agreement on the essential components of MetS, but differ in their cut-offs and methods of combining the individual components. A study done by Paras et al. on the relationship of these 3 definitions with subclinical atherosclerosis demonstrated that the WHO definition was the only one associated with all three measures of atherosclerosis (intima-media thickening, total plaque area and prevalence of focal lesions); thus the WHO definition appears to better discriminate those individuals at risk (Paras *et al.*, 2007).

3.8.2 Insulin resistance –Homeostasis Model Assesement index (HOMA)

Glucose metabolism is critical to the well-being of mammals and is maintained, in large part, by pancreatic β-cells which secrete insulin in proportion to increasing concentration of glucose. Insulin lowers blood glucose by stimulating glucose uptake into skeletal muscle and adipose tissue and by decreasing glucose production by the liver. Diabetes, defined as hyperglycaemia of sufficient magnitute to produce adverse effects, results when insulin is extremely low or absent due to absolute deficiency of β-cell mass (T1DM) or, more commonly, when resistance to insulin's blood glucose-lowering effects occurs in association with relative β-cell dysfunction, as in T2DM (Kahn and Flier, 2000). The mechanism responsible for β-cell dysfunction in common forms of T2DM, which is usually associated with obesity, is largely unknown. It has been proposed that uncoupling protein-2 (UCP-2) limits production of ROS by decreasing the mitochondrial membrane potential.

The Homeostasis Model Assessement of insulin resistance (HOMA) equation can be used in order to assess insulin resistance. It is used instead of the gold standard of the oral euglycemic clump which is time consuming and invasive. For the HOMA index calculation one needs to have measurements of fasting insulin and fasting glucose as shown in the equation that follows:

HOMA= (fasting Insulin x fasting Glucose)/ 22.5

Units: insulin in μU/ml and fasting plasma glucose in mmol/L

3.8.3 Diabetes mellitus

It has been estimated that the number of diabetic patients will more than double within 15 years (Amos *et al.*, 1997) and an emerging issue is the recent increase in diagnoses of type 2 Diabetes mellitus (T2DM) and pre-diabetic conditions in children (Rosenbloom *et al.*, 1999).

Diabetes is associated with significantly accelerated rates of cardiovascular complications such as atherosclerosis and hypertension (Natarajan and Nadler, 2004). In particular, type 2 diabetes (T2DM) is associated with 2- to 4-fold increase in coronary artery disease (Beckman *et al.*, 2002). This has been attributed to the

clustering of several risk factors, including insulin resistance, hypertension, obesity and dyslipidaemia (Libby and Plutzky, 2002; Mokdad *et al.*, 2001).

The close relationship between DM and CVD has led to the "common soil" hypothesis (Stern, 1995), postulating that DM and CVD share common genetic and environmental antecedents. One of the most important of these possible antecedents is considered insulin resistance. Insulin resistance, impaired glucose tolerance (IGT) and overt diabetes appear to be associated, although to a variable degree with an increased risk of CVD (Balkau *et al.*, 1999; Lakka *et al.*, 2002).

In the first 20 years of Framingham, the incidence of CVD among men with DM was twice that of men without. In women with DM the incidence was 3 times higher vs women without (Kannel and McGee, 1979). A meta-analysis of 17 prospective trials found hypertriglyceraemia to be an independent risk factor for CVD (Hokanson and Austin, 1996). Data from the PROCAM study showed a significant relationship between hypertriglyceraemia and CHD risk independent of LDL and/or HDL cholesterol (Assmann *et al.*, 1996; Fruchart *et al.*, 2004).

3.8.4 Uncoupling proteins-2 and -3 (UCP-2, UCP-3)

Uncoupling proteins -1, -2 and -3 are members of the mitochondrial transporter superfamily that uncouples proton entry in the mitochondrial matrix from ATP synthesis. UCP-1 was the first to be discovered but it is only expressed in brown adipose tissue, which humans have very little of. UCP-2 and UCP-3 were discovered later, in 1997, and present a much more widespread expression pattern. UCP-3 expression is largely restricted to skeletal muscle (Zhang *et al.*, 2001a) whereas UCP-2 is widely expressed, including in adipose tissue, the immune system and the pancreatic islets (Fleury *et al.*, 1997; Zhang *et al.*, 2001a). Their physiological role remains to be established; they are considered, however, candidate genes for association with energy metabolism and obesity (Le Fur *et al.*, 2004; O'Rahilly, 2001).

Esterbauer et al. recently reported that a functional G/A polymorphism in the promoter of the *UCP-2* gene is associated with obesity in middle-aged European adults. The A allele was associated with enhanced transcription of the gene and with a decreased risk of obesity, whereas the AA genotype was found to be associated with slightly higher

fasting insulin levels (Esterbauer *et al.*, 2001). Their results indicate that enhancing UCP-2 expression might help reduce and/or prevent obesity. Studies with *UCP-2* Knock Out obese mice have in turn demonstrated that UCP-2 is a negative regulator of insulin secretion (Zhang *et al.*, 2001a) and could thus be a link between obesity and β-cell dysfunction in obesity-induced type 2 diabetes. The authors suggest that inhibiting *UCP-2* might be a worthwhile approach to tackle the diabetes that results from obesity. Several studies have shown that expression of *UCP-2* gene can be increased by exposing cells to free fatty acids. The increased levels of circulating fatty acids seen in obesity provide a possible link between obesity and increased *UCP-2* expression (O'Rahilly, 2001).

Another study by Pedersen et al. showed that first degree relatives of patients with T2DM exhibit reduced expression of *UCP-2* mRNS in subcutaneous adipose tissue compared to age- and BMI- matched controls. After multiple regression analysis, though, only amount of adipose tissue was associated with *UCP-2* expression. *UCP-3* expression was similar in both groups (Pedersen *et al.*, 2005).

PART II:

Hypothesis

Chapter 4:

Hypothesis and Specific Aims of Thesis

4.1 Hypothesis

Multiple studies demonstrate that 20% to 25% of all future cardiovascular events occur in individuals with only 1 of the traditional risk factors (Khot *et al.*, 2003). Moreover, the prevalence of traditional risk factors is almost as high in those without disease as in affected individuals (Greenland *et al.*, 2001). This indicates that other, unknown, factors also play a part in the development of atherosclerosis as well as to its progression to clinical symptoms or not.

It has also been indicated in the literature review that ultrasound can be used to detect subclinical atherosclerosis many years before symptoms develop and that ultrasonic subclinical findings are the combined result of many risk factors (known, unknown, environmental, biochemical and genetic). Work on biomarkers and subclinical atherosclerosis has been confined mostly to IMT (carotid IMT in the vast majority of cases). It is now believed that IMT is not the best measure of subclinical atherosclerosis in comparison to plaque thickness, presence and echolucency as indicated in chapter 2.

What is currently known is that the *CETP* (I405V), *apoE* (E2/E3/E4), the apoB/apoA1 ratio, ox-LDL, the *PON1* (L55M), the *IL-6* (-174C>G), the *MMP-3* (5A/6A), the *ACE* (I/D), components of the MetS and ADMA levels have been shown to be associated with carotid IMT in various populations. The *apoE* (E2/E3/E4), *eNOS* (-894G>T), Ox-LDL, *IL-6* (-174C>G) and the *ACE* (I/D) have been associated in at least one study with presence of plaques in different populations (chapter 3). Studies in general populations which will use specific intermediate phenotypes such as plaques, plaque thickness and plaque echolucency, better fitted to assess subclinical atherosclerosis should have greater power in detecting possible associations between biochemical and genetic markers and subclinical atherosclerosis.

The aim of the work presented in this thesis was to determine the association between subclinical atherosclerosis as indicated by ultrasonic measurements of the arterial wall and markers of atherosclerosis, either protective or harmful. In addition we tested the hypothesis that different markers are associated with different ultrasonic measurements of subclinical atherosclerosis and that therefore there is a different genetic backround and different biological mechanisms acting at different stages of atherosclerosis.

4.2 Specific aims

1. Lipid markers. The lipid markers studied were: TChol, HDL, LDL, TG, apoA1, apoB, Lp(a), Lp-PLA_2 activity, *apoB* (-516C>T), *apoE* (E2/E3/E4), *CETP* (TaqIB1B2B2 and I405V) and *Lp-PLA_2* (A379V). Both routine established markers (TChol, HDL, LDL, TG) as well as novel ones (apoA1, apoB, Lp(a), Lp-PLA_2 activity) were chosen after thorough review of reports in the literature. Genetic polymorphisms were chosen on the basis of conflicting reports for association with clinical CHD and/or IMT in the literature (*apoE* E2/E3/E3, *apoB* -516C>T, *CETP* TaqIB1B2B2 and I405V) (pages: 76-86) as well as new reports on their biological properties and lack of data on atherosclerosis (*Lp-PLA_2* A379V).
2. Endothelial dysfunction markers. The markers tested were: soluble NO, MPO, serum creatinine levels, *eNOS* (894G>T), *PON1* (L55M), *PON2* (S311C), *MPO* (-638C>A), *ACE* (I/D) and *ang* (-6G>A). Conflicting findings have been reported for *eNOS* and *PON* genes and MPO is a novel marker with no reports of its association with subclinical atherosclerosis (pages: 86-94).
3. Inflammation markers. The inflammatory markers tested were: CRP, IL-6, MCP-1, *IL-6* (-174C>T), *TNF-α* (-308G>A), *MGP* (-138C>T), *MMP-1* (1G/2G), *MMP-3* (5A/6A), *MMP-7* (-181A>G), *MMP-9* (R279Q) and *MMP-12* (-82A>G). A lot of interest has focused on CRP in the last ten years with early reports stating that it is a risk factor strongly associated with CVD (pages: 97-98). However, more recent reports indicate that it might not be a causal risk factor. Increasing evidence indicates that MMPs are associated with atherosclerosis and plaque rupture but are inconclusive (pages: 100-105). MGP is a novel marker associated with artery calcification.
4. Thrombotic markers. The thrombotic markers tested were: soluble CD40L, Fb, P-selectin, tissue factor, microparticles and *PAI-1* (4G/5G). These markers are also implicated in inflammatory cascades and were chosen with that in mind as well as lack of reports for their association with subclinical atherosclerosis. sCD40L and MP are novel markers only now being tested for association, based on reports of their biological properties.

5. Homocysteine metabolism markers. The homocysteine metabolism markers tested were: total serum Hcy, folic acid, vitamin B12, ADMA and the *MTHFR* (677C>T). Homocysteine has long been considered to be associated with CVD; however reports from folic acid implementation trials have yielded inconsistent results (pages: 111-115). In addition, it is not known if homocysteine exerts its effects at the early stages of atherosclerosis formation or at plaque progression. Also the importance of other markers in the Hcy metabolism cycle is unknown.
6. Metabolic syndrome components. Presence of the MetS and number of its components were tested. In addition, the HOMA index for insulin resistance, presence of DM and the *UCP-2* (-866G>A) and *UCP-3* (-55C>T) polymorphisms were also tested (pages: 115-118). Uncoupling proteins are relatively new genes that are implicated in energy expenditure and metabolism and whose relationship with atherosclerosis is not yet known.

The above markers (biochemical and genetic) were chosen because of available and validated measurements, sufficient and/or conflicting reports for association with clinical CHD and/or IMT in the literature and/or a biological plausibility for association with atherosclerosis.

PART III:

Material and Methods

Chapter 5:

Collection of Demographic, Clinical and Biochemical Data from the Population studied

This chapter describes material and methods for the population selection, study design, blood sampling, clinical examination, ECG, medical history and ultrasonic examination undertaken for each participating volunteer. All data collected were entered in an SPSS electronic data base with an ID number for each volunteer. The statistical analysis performed is described in section 7.4.

5.1 Sample population

The Cyprus Study is a prospective follow-up study on cardiovascular disease in 2 000 subjects, aged 40 or more. Part I consists of a pilot study of 500 subjects, part II currently in progress aims to extend the pilot study to 2 000 subjects and part III to provide a minimum of 5 year follow-up. The material presented here is based on the first 767 subjects recruited. (Subject number included in each analysis may vary depending on the number of people with complete data for each biomarker.) Baseline data have been collected from Pedoulas, a village in the Troodos Mountains of Cyprus (n=271), their relatives who live in any one of the main towns (n=250) and from a section of Nissou (first 250 people on the population list) a village in the Mesaoria plain ten kilometres south of the capital, Nicosia (n=246). Both villages were selected randomly by throwing darts on a map of Cyprus. All inhabitants were identified through the population-list held at the Mayor's office in both villages and all those over the age of 40 were invited to participate. Under the age of 40 the resolution of ultrasound techniques is not able to distinquish IMT thickening. The overall participation rate of those invited was 95%. The Ethics Committee of the Cyprus Institute of Neurology and Genetics approved the study. All participants provided written informed consent. A table with the general characteristics of the population can be found in Annexe 1 (Table A1).

5.2 Clinical examination

A vascular internist obtained a full medical history and physical examination with emphasis on conventional cardiovascular risk factors and cardiovascular symptoms including angina, myocardial infarction, ischemic hemispheric neurological events and intermittent claudication. All past and current medications were recorded. The physical examination included height and weight measurements, a sitting blood pressure, measured three times with the first measurement being discarded.

5.3 ECG

A resting 12-lead ECG was obtained. The diagnosis of angina was based on the history and confirmatory hospital investigations including ECG or Thallium stress test; that of myocardial infarction was based on the history and hospital documentation including information on coronary artery intervention (angioplasty or coronary artery bypass). Myocardial ischemia was diagnosed in the presence of ST-segment depression or symmetrical T-wave inversion. ST-segment depression was considered present if it was horizontal or down-sloping and at least 0.05 mV. Inverted T-wave was considered present if it was isoelectric, negative or biphasic in leads V3-V6, aVL (if R > 0.5 mV), I and II. At least 0.1 mV T-wave inversion was required in leads V2 and aVF (positive QRS also required). An old myocardial infarct was diagnosed in the presence of a pathological Q wave: amplitude greater than 0.1 mV with Q/R ratio greater than 0.33 and duration greater than 0.04 sec or when QS patterns were present in precordial leads.

5.4 Medical History and risk factor questionnaire

A detailed questionnaire on risk factors for CVD was filled in for each participant by the examining doctor (questionnaire available on request).

The diagnosis of ischemic stroke, TIA or transient monocular blindness was based on the patient's history, hospital records, and reports from neurologists who saw the patient at the time of the event. Intermittent claudication was diagnosed from the history of recurrent pain on walking at a fixed distance, the presence of weak or absent pulses and an ankle/brachial index less than 0.9. Presence of diabetes mellitus was assumed if subjects were on treatment or had fasting glucose greater than 110 mg/dL Hyperlipidaemia was considered present if subjects were on treatment or had fasting total cholesterol greater than 235 mg/dL. Hypertention was considered present if subjects were on treatment or had a systolic BP greater than 160 mm Hg or a diastolic BP greater than 90 mm Hg.

5.5 Blood Sampling

A fasting (6-12 hours) blood sample was taken for the biochemical analyses (described in chapter 6). Blood was drawn, in the sitting position, into sodium citrate and EDTA containing tubes, as well as plain tubes and samples were left at room remperature for 15-30 min before centrifugion. They were then centrifuged at 3 000 rpm for 15 min and

serum and plasma were separated and aliquoted immediately. Samples were stored at -80 ^{o}C. A separate sample in EDTA antigoagulant was taken for DNA extraction and the genetic analyses (as described in chapter 7), which was also stored at -80 ^{o}C.

5.6.1 Ultrasonic scan methodology

All scans were performed using a Philips (ATL) HDI 5000 duplex scanner (Philips Corp., Seattle, USA). A high-resolution broadband linear array transducer 10-5 MHz was used. Technical ultrasound parameters were preset and constituted a special programme known as Intima-Media Thickness, so that the settings, 2-D map (map 1), post-processing curve (linear), dynamic range (170 db), persistence (low) and frame rate (high) remained constant. The magnification and depth (3-7 cm) were also preset but adjustments of those could be made depending upon patient anatomy and size. Regular use of an RMI (model 415) ultrasound phantom ensured the system's continual accuracy in sensitivity, measurements, axial and lateral resolution.

Each carotid bifurcation was examined transversely first, and then longitudinally with all subjects supine and a slight neck extension. Careful circumferential scanning and adjustment of the position of the probe ensured the optimal demonstration of the intima-media complex of the far wall of the common carotid artery 1.5 to 2.0 cm proximal to the bifurcation. Each measurement was performed on a frozen on-screen image, using the automated measuring callipers. The IMT complex of the far wall of the artery was measured at its thickest part (mean of three readings) on both transverse and longitudinal sections. The mean of the measurements from both carotid arteries was used in the analysis (IMTcc).

An arterial bifurcation (internal carotid or common femoral bifurcation) was classified as being affected by plaque if there was a localised thickening of greater than 1.2 mm that did not uniformly involve the whole arterial wall. Careful transverse scanning in addition to longitudinal scanning and the use of colour flow allowed for a better appreciation of the geometry and borders of the plaque, thereby allowing a more accurate assessment and measurement of plaque thickness. Once again on-screen callipers were used to measure maximum plaque thickness (IMTmax) in both longitudinal and transverse sections. In the absence of plaques the IMTcc measurement was used. The mean of the measurements from both carotid arteries was used in the analysis.

Next, the common femoral artery bifurcations were visualised and plaques were localised if present using both transverse and longitudinal scanning. On-screen callipers were used to measure maximum plaque thickness in both longitudinal and transverse sections. Total plaque thickness (TPT) was defined as the sum of the maximum plaque measurements made in each of the four bifurcations scanned.

For images of plaques in longitudinal section the ultrasound beam was kept at 90 degrees to the arterial wall and plaque under investigation. The gain was adjusted so that at least some areas of blood appeared without noise and the time gain compensation curve (TGC) was positioned vertically through the lumen of the vessel, as there was little attenuation of the beam through blood. This ensured that the returning echoes of the structures of the posterior vessel wall would be of equal strength to those of the near wall, and therefore of equal brightness. A section of adventitia had to be clearly visualised on either side of the plaque. Images were stored in B-mode, then with the plaque outlined using on-screen callipers and finally where considered appropriate (e.g. in the presence of hypoechoic plaques) a colour flow image.

A strict scanning protocol was observed with each patient, i.e., the left carotid bifurcation followed by the right carotid bifurcation, right common femoral artery and finally the left common femoral artery. The whole examination was recorded on VHS videotape. Frozen images with the measurements were also recorded on magneto-optical disc (230 Mbyte rewritable). Images from normal carotid IMT measurements, as well as thickened IMT and types of plaques follow (Figs. 5.1-9).

5.6.2 Reproducibility of ultrasound measurements

The two ultrasonographers who performed the ultrasonic scans were not aware of the clinical or biochemical risk factors of the subjects. In a reproducibility study in which measurements in 35 subjects were repeated by both ultrasonographers the inter-observer mean difference between repeat measurements of IMTcc was -0.03 mm, the within-subject standard deviation was 0.12 mm and the intra-class correlation coefficient was 0.79. For IMTmax which included plaque thickness the corresponding values were 0.02 cm, 0.26 cm and 0.94. These results are within the accepted range of reproducibility published by other groups.

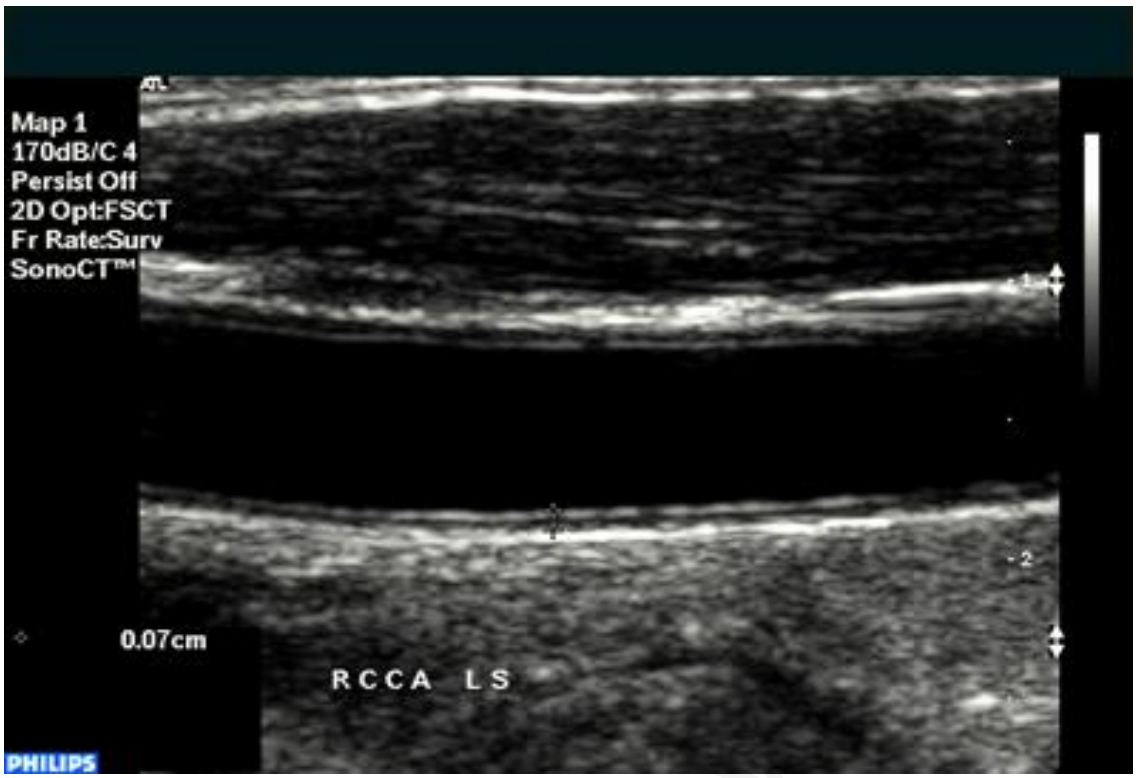

Figure 5.1: Normal IMT in the common carotid artery. IMT (0.07 cm) measurement is shown by the points on the arterial wall

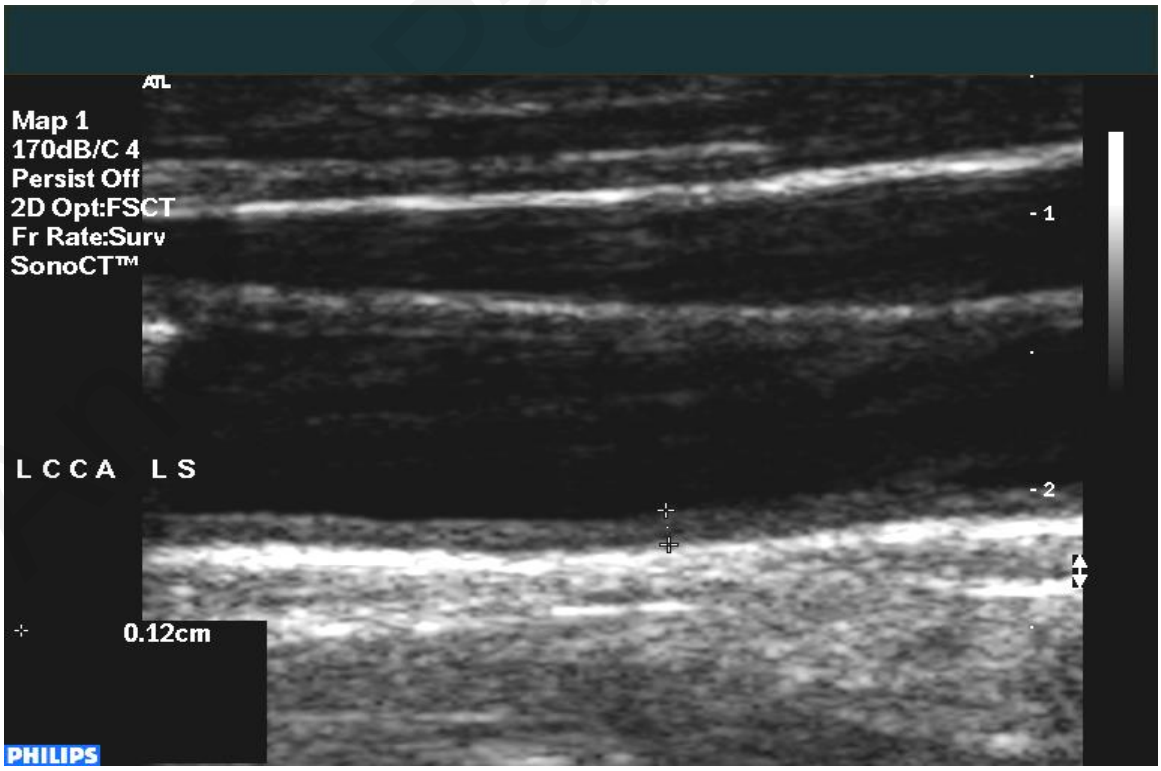

Figure 5.2: Thickenned IMT in the common carotid artery. IMT measurement (0.12 cm) is shown by the points on the arterial wall

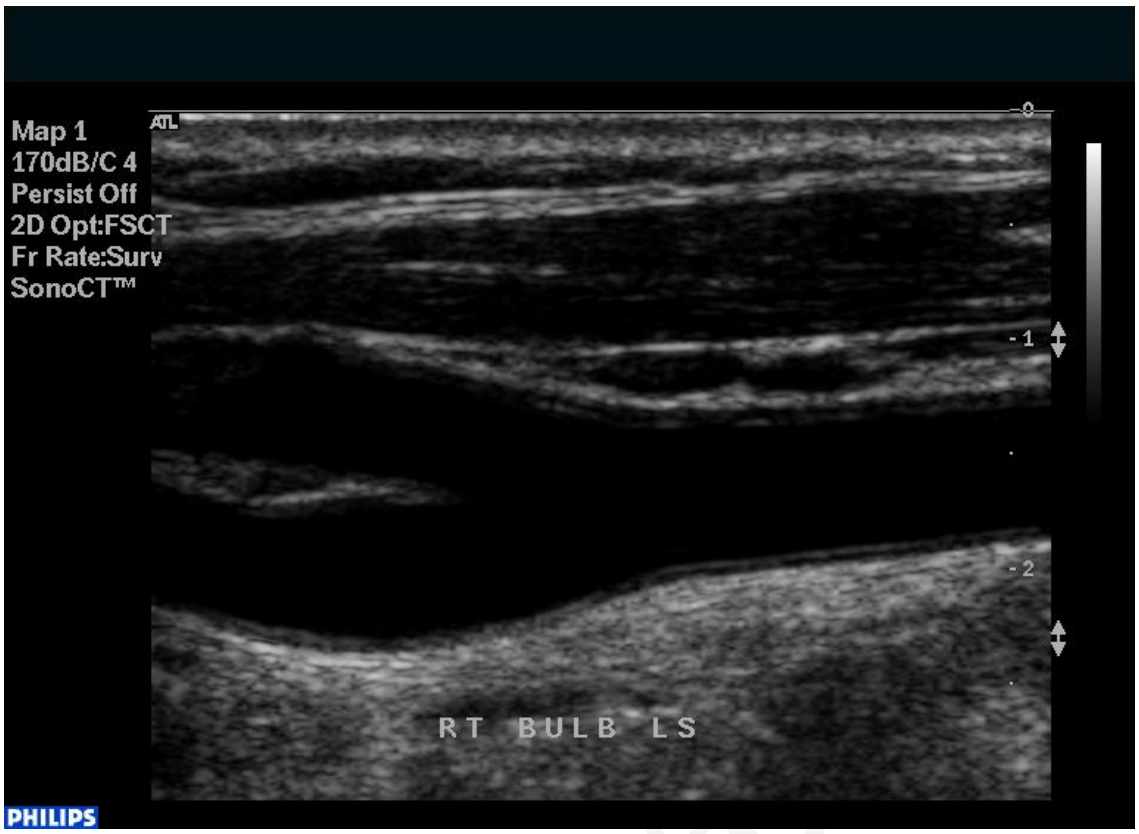

Figure 5.3: Normal carotid bifurcation

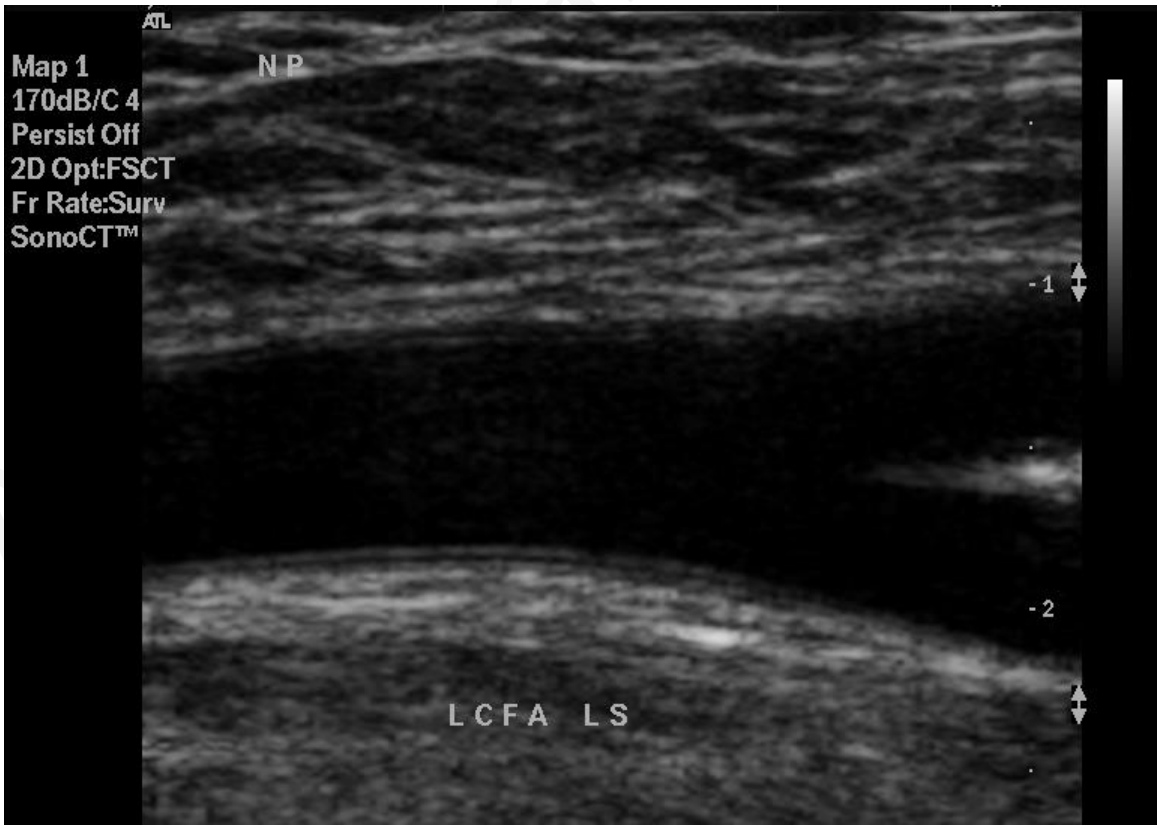

Figure 5.4: Normal femoral bifurcation

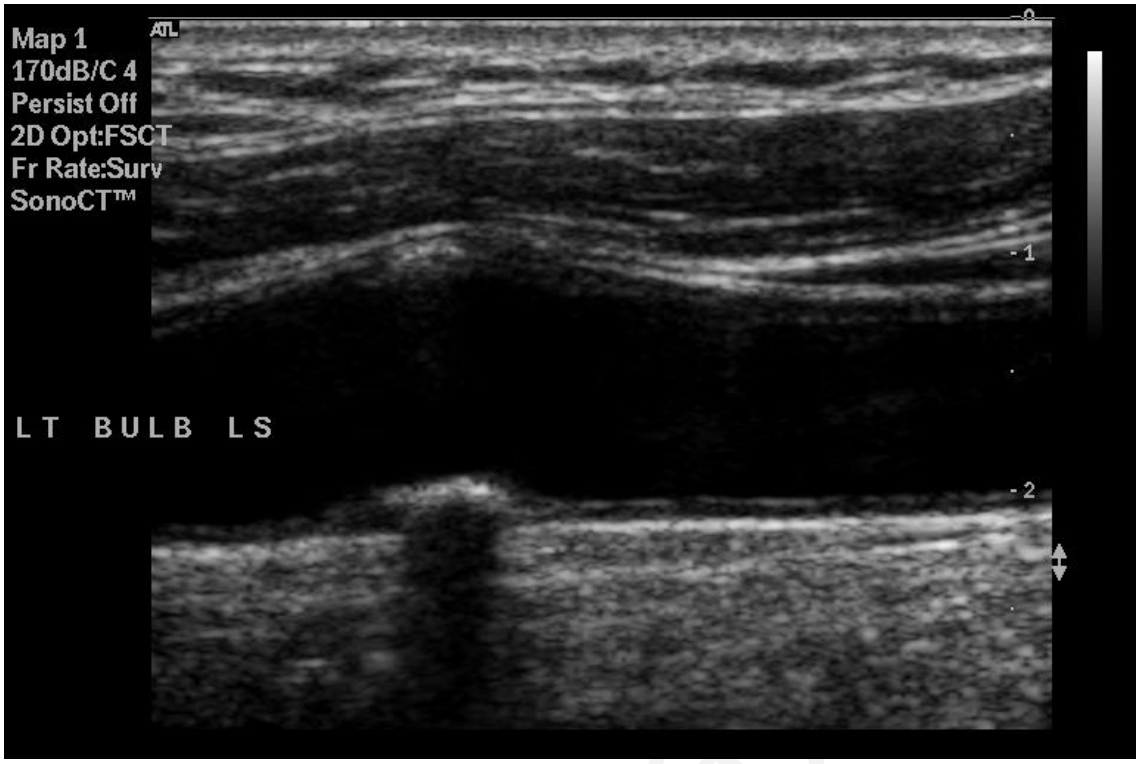

Figure 5.5: Type 1 atherosclerotic plaque

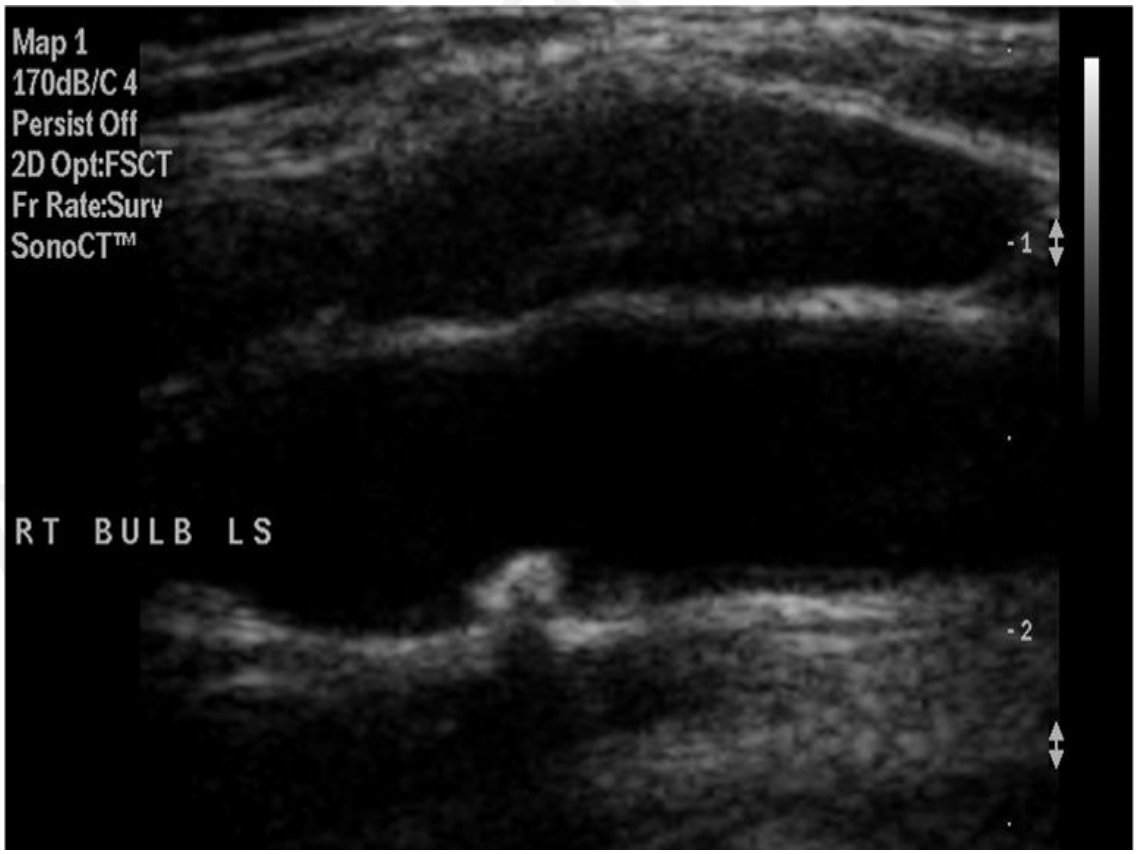

Figure 5.6: Type 2 atherosclerotic plaque

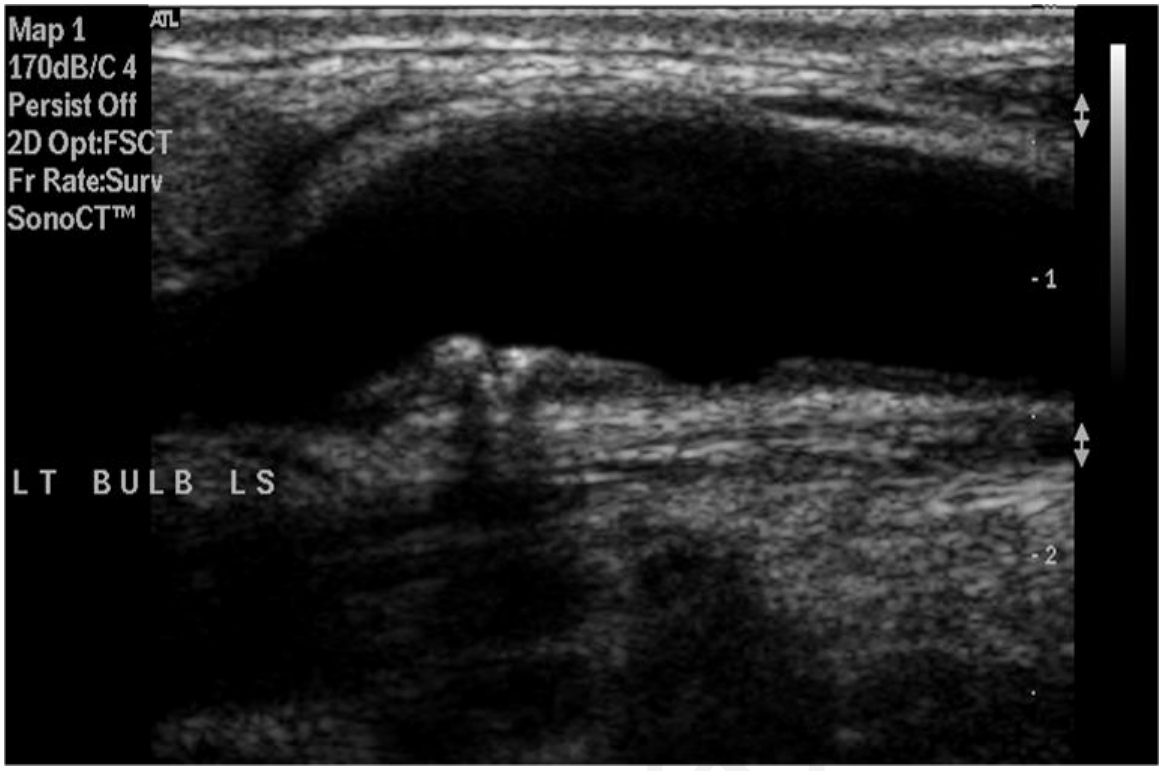

Figure 5.7: Type 3 atherosclerotic plaque

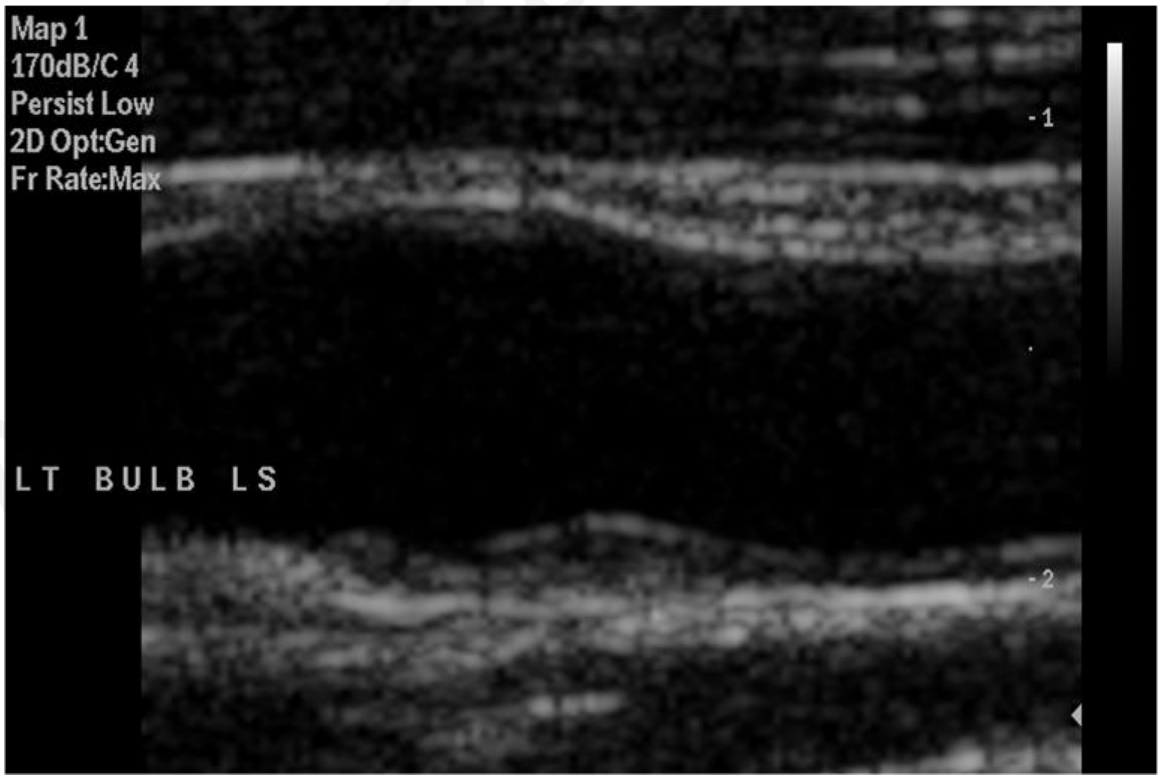

Figure 5.8: Type 4 atherosclerotic plaque.

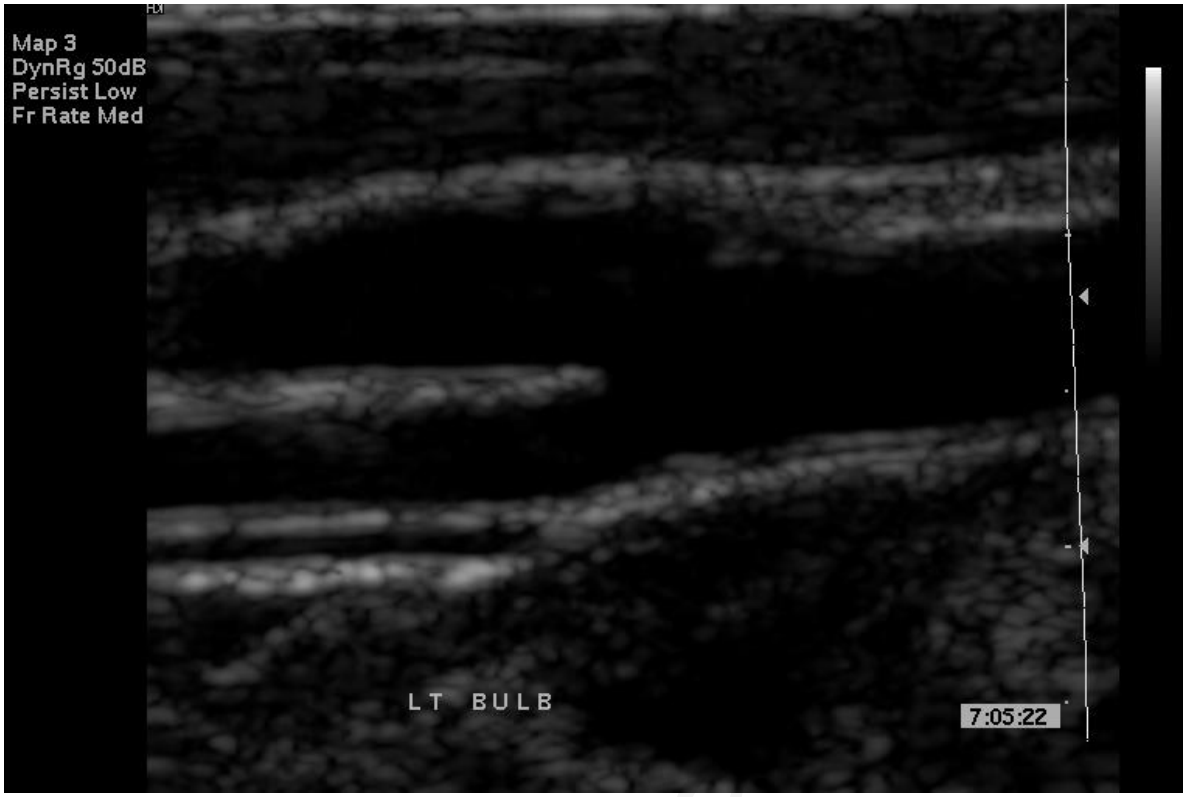

Figure 5.9a: Type 5 atherosclerotic plaque

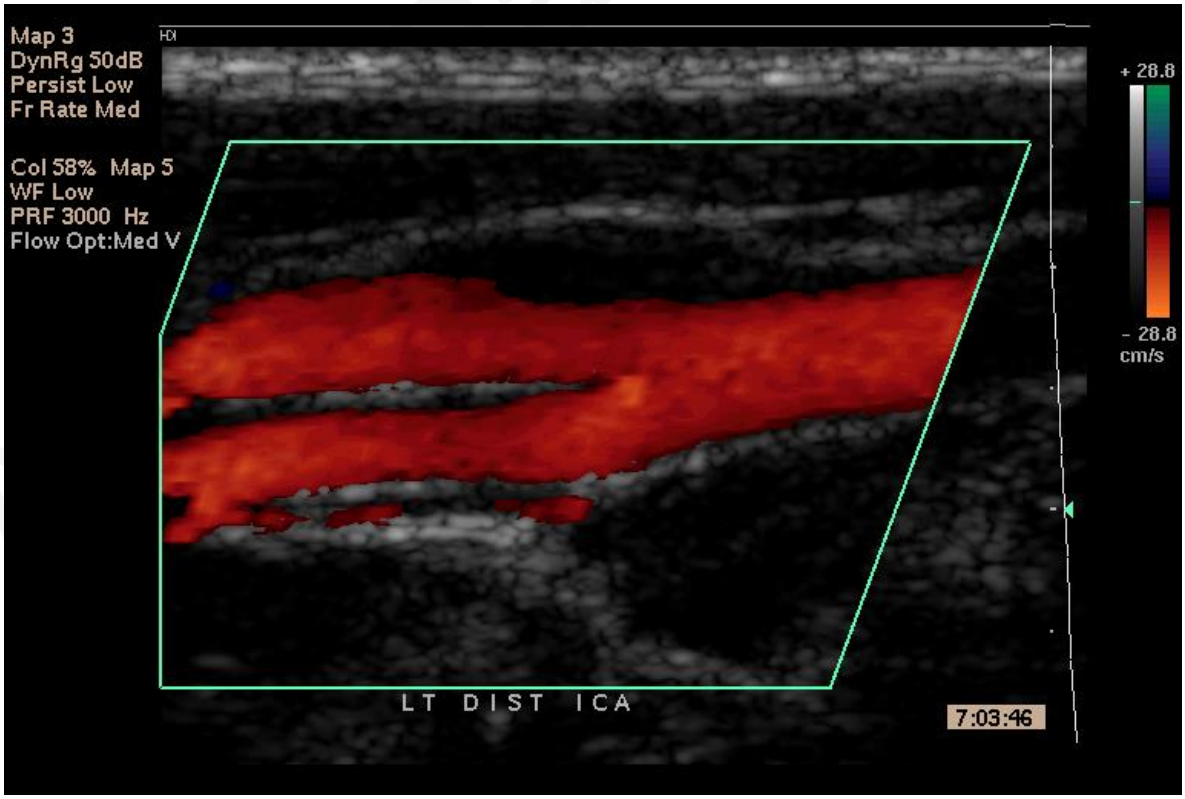

Figure 5.9b: Type 5 atherosclerotic plaque shown as a black filling defect when colour is swithed on

Chapter 6:

Methodology for biochemical analyses

This chapter describes the methods for the determination of serum and plasma levels of the selected biochemical markers. Common and automated methods were mostly used and are described below.

6.1 ELISA methods

Commercially available ELISA kits from various suppliers were used for the quantification of the chosen markers. Following review of the literature we chose the following biochemical markers for analysis with ELISA: C-Reactive Protein (CRP), sCD40 Ligand (sCD40L), P-selectin, Nitric Oxide (NO), Monocyte Chemoattractant Protein 1 (MCP-1), Interleukine-6 (IL-6), Tissue Factor (TF), ADMA, Myeloperoxidase (MPO), Microparticles (MP) and insulin. Standard sandwich ELISA was performed for all samples according to the manufacturers' instructions. A brief description of the principle follows:

6.1.1 Principle of ELISA

The assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonic (or monoclonic) antibody with specific affinity for the marker (antigen) in question is pre-coated onto a microplate. Standards and samples are added into the wells and any marker present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the marker in question is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of marker bound in the initial step. The colour development is stopped and the intensity of the colour is measured in the appropriate wavelength with the use of a plate spectrophotometer. A standard curve is created by plotting the mean absorbance for each standard on the Y axis against the concentration on the X axis and drawing a best fit curve through the points. If samples have been diluted, the concentration read from the standard curve is multiplied by the dilution factor. The standard curve and sample concentrations for all markers (except insulin) were done with use of Excel (Microsoft Corp. Redmond, WA). Insulin calculations were done with the use of the spectophotometer software.

All ELISA analyses –except insulin measurements- were performed in the Pathology Department Laboratory at the Loyola Medical Center, Chicago, IL, U.S.A. The insulin ELISA measurements took place in the Molecular Genetics Laboratory of the Department of Biological Sciences, University of Cyprus. For all assays sodium citrated plasma was used. A list of manufacturers follows:

Table 6.1: List of kit manufacturers for ELISA

Marker	**Kit Manufacturer**
hsCRP	American Diagnostica Inc
sCD40L	R & D Systems
P-selectin	R & D Systems
Total NO	R & D Systems
MCP-1	R & D Systems
IL-6	R & D Systems
TF	American Diagnostics Inc
ADMA	Cardiovasics
MPO	Assay Designs Inc
Insulin	DaKoCytomation Ltd
MP	Hyphen BioMed

6.1.2 Quality control of ELISA measurements

. In each plate, standard graded concentrations of the marker (provided by the manufacturer) were run in duplicate and the mean of each measurement was used for plotting the standard curve. In addition, commercial high and low controls for each individual marker as well as in-house normal and pathological pools were used in each plate to ensure quality control of the assays

6.2 Automated Methods

Lipid profile (apoA1, apoB, total Cholesterol, HDL, LDL, Lp(a), triglycerides,) glucose, creatinine and homocysteine measurements of serum were performed on the Olympus A320 automated analyser. Fibrinogen was measured in citrated plasma by the clotting time method and serum folic acid and vitamin B12 were measured on the Immulite 2000 automatic analyser. All analyses were performed at the clinical laboratory of the Nicosia General Hospital. Basic principle of each assay follows:

Total Cholesterol was determined by an enzymatic colour test. Cholesteryl esters in the sample are hydrolysed by cholesterol esterase. The free cholesterol produced is oxidised by cholesterol oxidase to cholestene-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromophore. The red quinoneimine dye formed can be measured spectrophotometrically at 540/600 nm as an increase in absorbance.

HDL cholesterol (Olympus Diagnostics) was measured with an immunoinhibition method. Anti human-b-lipoprotein antibody binds to lipoproteins other than HDL (LDL, VLDL and chylomicrons). The antigen-antibody complexes form block enzyme reactions when a second reagent is added. HDL cholesterol is quantified by the presence of an enzyme chromogen system.

LDL cholesterol (Olympus Diagnostics) was measured with an enzymatic colour test. By adding cholesterol esterase and cholesterol oxidase to the sample, all the lipoproteins (HDL, VLDL, CM) are oxidised, except LDL which is protected against oxidation by addition of a protective agent. Then it is determined by further adding cholesterol oxidase which produces hydrogen peroxide, quantified by the production of blue dye.

Triglycerides (Olympus Diagnostics) were measured with an enzymatic colour test. Triglycerides are enzymatically hydrolysed by lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminoantipyrine and 4-chlirophenol under the catalytic influence of peroxidase.

Glucose (Olumpus Diagnostics) was determined with an enzymatic UV test (hexokinase method). Glucose in the sample is phosphorylated by hexokinase in the presence of adenosine triphosphate and magnesium ions to produce glucose-6-phosphate and adenosine diphosphate. Glucose-6-phosphate dehydrogenase specifically oxidises glucose-6-phosphate to gluconate-6-phosphate with the concurrent reduction of NAD^+ to NADH. The increase in absorbance at 340 nm is proportional to the glucose concentration in the sample.

ApoA1 and ApoB (Olympus Diagnostics) were measured with an immuno-turbimetric test. ApoA1/apoB in the sample reacts specifically with anti-human apoA1/apoB antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the apoA1/apoB concentration in the sample.

Lp(a) (Dako Diagnostics) was determined with an immunoturbimetric test. Lp(a) in the sample reacts specifically with anti-human Lp(a) antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the Lp(a) concentration in the sample.

Creatinine (Olumpus Diagnostics) was determined with a colorimetric test (Jaffe method). Creatinine forms a yellow-orange colour compound with picric acid in an alkaline medium. The rate of change in absorbance at 520/800 nm is proportional to the creatininee concentration in the sample.

Fibrinogen (Dade Behring) was measured with the clotting method. The sample is brought to coagulation with a large excess of thrombin. The coagulation time depends on the fibrinogen content of the specimen which is determined from a standard values table provided.

Homocysteine (Carolina Liquid Chemistries) was determined by use of Hcy metabolism reactions. The method utilises a reaction of homcosysteine and L-serine to

form cystathionine b-synthase followed by the cystathionine b-lyase catalysed conversion of cystathionine to homocysteine, pyruvate and ammonia. The rate of pyruvate production can be measured by inclusion of LD and NADH in the reaction mixture and is directly proportional to the concentration of homocysteine.

Folic acid (Immulite) was measured with a competitive immunoassay. In a two-cycle reaction, folic acid from binding proteins in the sample competes with ligand-labeled folic acid for binding to folate binding protein. Anti-ligand is added and it binds to the ligand-labeled folate. Unbound enzyme conjugate is removed, substrate is added and colour develops in proportion.

Vitamin B12 (Immulite) was measured with a solid-phase, competitive chemiluminescent enzyme immunoassay involving an automated alkaline denaturation procedure. Vitamin B12 released from endogenous binding proteins in the treated sample competes with immobilised B12 for binding with hog intrinsic factor (HIF). Labeled anti-hog intrinsic factor is introduced and binds to any HIF immobilised on the beads. Substrate is added and colour develops in proportion.

Lp-PLA$_2$ (PAF-AH) activity was measured by standard radioimmuno methods at INSERM, Paris, France. It was measured by the trichloroacetic acid precipitation procedure in 96-well plates. Briefly, plasma was diluted with assay buffer and [^{3}H]acetyl PAF with specific activity and samples in duplicate were incubated. After precipitation, the radioactivity was measured in the supernatant. The activity of PAF-AH is expressed in nmol PAF hydrolysed/min/ml of plasma. A pool of control plasma (n=10) served as an internal standard for all measurements.

6.3 Reproducibility of clinical laboratory measurements

Each time samples were run, the automatic analyser (Olympus A320 and Immunolite 2000) was calibrated with standards and checked with high and low controls for each parameter. All the tests were performed in 3 separate runs within the interval of a month, using the same controls to ensure minimum inter-assay variability due to day-to-day variation.

For the lipid profile, creatinine and fibrinogen, intra-assay variability was < 5%. For homocysteine measurements intra-assay variability was <10%. For biochemical measurements of lipids a variability of < 5% is considered quite good. Homocysteine is a more sensitive molecule to measure so a higher variability (up to 10%) is accepted. Bland-Altman plots were used to calculate variability. The Bland & Altman plot (Bland and Altman, 1986) is a statistical method that may be used to assess the repeatability of a technique by comparing repeated measurements using one single method on a series of subjects. In this graphical method the differences (or alternatively the ratios) between the two techniques or measurements are plotted against the averages of the two techniques. The graph is used to check whether the variability or precision of a method is related to the size of the characteristic being measured.

The Clinical Laboratory of the Nicosia General Hospital, where all the automated tests were performed, is subject to the NEQAS and REGAS European and American programs of external control for clinical laboratories which further validates our results.

For Lp-PLA_2 activity measurements all samples were run in duplicate and the mean of the two measurements was used.

Chapter 7:

Methodology for genetic analyses (SNPs)

This chapter describes material and methods for identification of all the genetic polymorphism tested in the Cyprus Study. Both RFLP and TaqMan techniques were used and are described below.

7.1 DNA extraction

For DNA extraction, whole blood in EDTA anticoagulant was used and extraction was performed with the QIAmp DNA Blood Maxi kit (Qiagen group) according to the manufacturer's instructions.

All samples yielded approximately 20 ng/μl of DNA in a total volume of 1.7-1.9 ml. An aliquote stored in -20 ^{o}C was used for all gene analyses and the rest (stock DNA) was stored at -80 ^{o}C.

7.2 Restriction Fragment Length Polymorphism (RFLP)

A number of genetic polymorphisms (*ACE* I/D, *MTHFR* 677C>T, *MMP3* 5A/6A) were analysed with the classic RFLP method.

7.2.1 Principle of RFLP

Specific amplification of the sequence around the genetic polymorphism in question is performed with a standard polymerace chain reaction (PCR). A pair of primers (forward and reverse) covering the sequence around the polymorphism is designed and used in each case. The PCR product is then digested with a suitable restriction enzyme and run in an agarose gel which separates bands of different length according to their molecular weight. The gel is read and a genotype is assigned to each sample according to its bands pattern (Figs. 7.1-2).

Primers and PCR conditions for each SNP are shown in 7.2.2 and 7.2.3.

7.2.2 Primers

***ACE* (I/D)**

For better identification of the insertion/deletion, two sets of primers were used (A covering the sequence outside the insertion & B inside the insertion)

(A) F: GCCCTGCAGGTGTCTGCAGCATGT

R: GGATGGCTCTCCCCGCCTTGTCTC

(B) F: TGGGACCACAGCGCCCGCCACTAC

R: TCGCCAGCCCTCCCATGCCCATAA

***MTHFR* (C677T)**

F: TGAAGGAGAAGGTGTCTGCGGGA

R: AGGACGGTGCGGTGAGAGTG

***MMP3* (5A/6A)**

F: GGTTCTCCATTCCTTTGATGGGGGGAAAGA

R: CTTCCTGGAATTCACATCACTGCCACCACT

7.2.3 PCR reagents and conditions

Polymerace Chain Reaction (PCR) reagents for *ACE* (I/D):

DNA → 2 μl

dNTPs → 3 μl

Buffer → 3 μl

Primer forward → 0.5 μl

Primer reverse → 0.5 μl

AmpliTaq polymerase → 0.1 μl

dH_2O → 20.9μl

Total volume = 30 μl per reaction

PCR conditions for *ACE* I/D:

94 ^{O}C for 5 min

94 ^{O}C for 30 sec
64 ^{O}C for 40 sec
72 ^{O}C for 2 min
} **X 34**

72 ^{O}C for 8 min

Pause at 4 ^{O}C

Analysis of the PCR product in an agarose gel (1.5%) separated the bands as seen in figure 7.1, according to their length. The *ACE* I/D genotype of each volunteer was identified according to their band pattern as follows. Subjects homozygous for the I allele carry only the higher band, subjects homozygous for the D allele carry only the lower band and heterozygots have both bands (Figs. 7.1)

PCR reagents for *MTHFR* (677C>T):

DNA → 2 μl

dNTPs →2 μl

Buffer → 2 μl

$MgCl_2$ → 1,2 μl

Primer forward → 1 μl

Primer reverse →1 μl

TaqExpress polymerase → 0,2 μl

dH_2O → 10.6 μl

Total volume = 20 μl per reaction

PCR conditions for the *MTHFR* (677C>T):

96 ^{O}C for 5 min

94 ^{O}C for 50 sec

58 ^{O}C for 50 sec } **X 36**

68 ^{O}C for 30 sec

68 ^{O}C for 5 min

Pause at 4 ^{O}C

PCR product digestion with Hinf1 restriction enzyme:

PCR product → 5 μl

Buffer Hinf1 →1 μl

Hinf1 (5 units per reaction) → 0.5 μl

dH_2O → 3.5 μl

Digestion takes place at 37 ^{O}C for 6 hours.

Same for the *MTHFR* 677C>T polymorphism, analysis of the restriction product in an agarose gel (Nuisive 3%) gives a band pattern indicative of the *MTHFR* 677C>T

genotype. The presence of the T allele creates a restriction point for the Hinf1 restriction enzyme which results in the cleavage of a second smaller band (175-bp) in T allele carriers. Carriers of the C allele have a longer (higher) band (198-bp), as seen in figure 7.2.

PCR reagents for *MMP3* (5A/6A):

DNA → 2 μl

dNTPs →2 μl

Buffer → 2 μl

$MgCl_2$ → 2 μl

Primer forward → 1 μl

Primer reverse →1 μl

TaqExpress polymerase → 2 μl

dH_2O → 8 μl

Total volume = 20 μl per reaction

PCR conditions for *MMP3* (5A/6A):

95 ^{O}C for 5 min

94 ^{O}C for 5o sec

64 ^{O}C for 50 sec } **X 39**

72 ^{O}C for 35 sec

72 ^{O}C for 5 min

Pause at 4 ^{O}C

PCR product digestion with Tthl11I restriction enzyme

PCR product → 5 μl

Buffer Hinf1 →1 μl

Tth111I (5 units per reaction) → 0.375 μl

dH_2O → 3.725 μl

Digestion takes place at 65 ^{O}C for 15 minutes.

Analysis of the restriction product in agarose gels gives a band pattern as seen in figure 7.3.

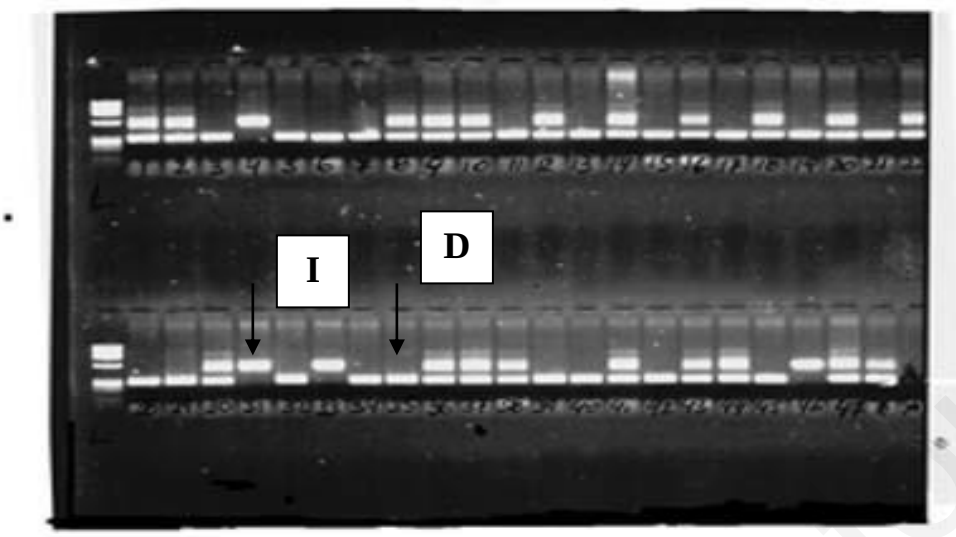

Figure 7.1: Agarose gel showing the band pattern for *ACE* (I/D). The higher band indicates the presence of the 287-bp insertion (~600-bp fragment), whereas the lower indicates its absence, deletion (~300-bp fragment). Presence of both bands indicated heterozygotes

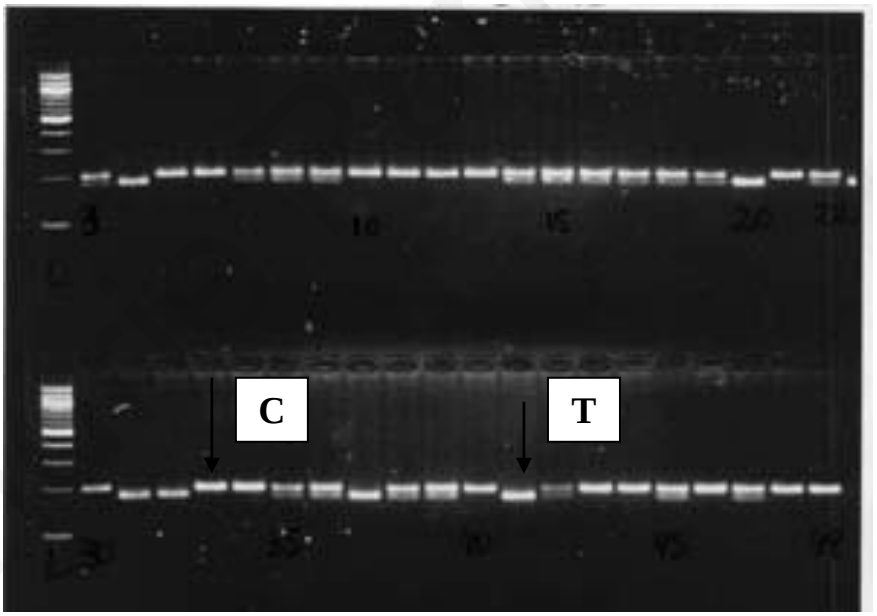

Figure 7.2: Agarose gel showing the band pattern for *MTHFR* (677C>T). Presence of the T allele results in a second shorter band (175bp). Presence of the C allele results in the upper longer band (198 bp)

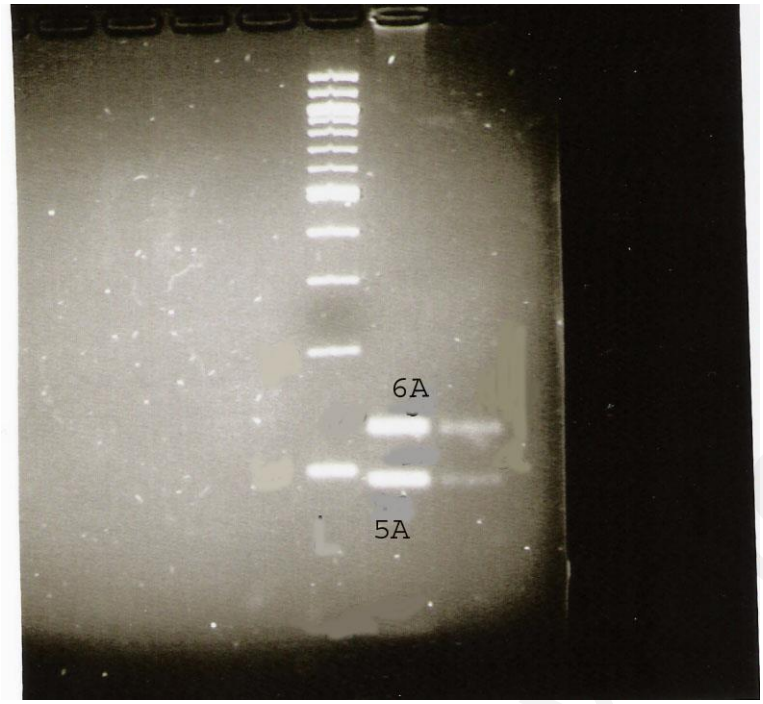

Figure 7.3: Agarose gel showing the band pattern for *MMP-3* (5A/6A). Presence of 6A results in the upper band and 5A to the lower

7.2.4 Reproducibility and quality control

Each time samples were analysed, a negative control was included in the run and known samples for each polymorphism were run next to the unknown samples. A reproducibility assessement performed by analysing 100 samples in duplicate demonstrated an inter-assay reproducibility of 99% for *ACE* (I/D) and an intra-assay reproducibility of 98% for *MTHFR* (677C>T) (blinded assignement of genotype in duplicate by the same researcher). For the *MMP3* (5A/6A) polymorphism, results from the RFLP method were compared with results from the TaqMan method in 618 samples. Of these 618 samples 27 had different results for each method, which is 4.4% deviation between methods or 95.6% agreement. An aggrement of 95.6% between the two methods is deemed as quite high and within the acceptable error rates. It is worth noting that 13 out of the 27 misstyped samples were found in two clusters (two particulate PCR and gel runs), which indicates that the disaggrement between methods was primarily due to poor gel quality in two occasions and the real deviation is actually smaller.

7.3 TaqMan method

7.3.1 Principle of TaqMan

The TaqMan method uses the standard PCR reaction with the difference lying in a set of flyorescent probes used in addition of the primer set. Each TaqMan MGB probe contains:

1. A reporter dye at the 5 end of each probe:
   A VIC dye is linked to the 5 end of the Allele 1 probe and a 6-FAM dye is linked to the 5 end of the Allele 2 probe.
2. A minor groove binder (MGB).
   *This modification (MGB) increases the melting temperature ™ without increasing probe length (Afonina *et al.,* 1997),which allows for the design of shorter probes. This results in greater differences in Tm values between matched and mismatched probes, which produce more accurate allelic discrimination.
3. A non-fluorescent quencher (NFQ) at the 3 end of the probe.

During the PCR reaction:

1. Each TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites.
   When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence (Lakowicz and Keating-Nakamoto, 1984)
2. AmpliTaq Gold DNA polymerase only cleaves probes that are hybridised to the target.
3. Cleavage separates the reporter dye from the quencher dye which results in increased fluorescence emission from the reporter.

The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.

Mismatches between a probe and a target reduce the efficiency of probe hybridization. Furthermore, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye.

Each SNP assay contains two primers for amplifying the sequence of interest and two TaqMan MGB probes for distinguishing between two alleles.

7.3.2 Primers and probes for the genetic polymorphisms analysed with the TaqMan method (alphabetically)

***ApoB* (-516C>T)**

F: TCACCAGACCTCCCTGCAT
R: CCACACCCTAATCCTGATCAGAATC
VIC: CCTTCTCTCTCCTCCCC NFQ
FAM: CTTCTCTCTTCTCCCC NFQ

***ApoE* (112C>T)**

F: GCGGGCACGGCTGT
R: GCTTGCGCAGGTGGGA
VIC CATGGAGGACGTGTGC NFQ
FAM ATGGAGGACGTGCGC NFQ

***ApoE* (158C>T)**

F: TCCGCGATGCCGATGAC

R: CCCCGGCCTGGTACAC

VIC CAGGCGCTTCTGC NFQ

FAM CAGGCACTTCTGC NFQ

Angiotensinogen (-6G>A)

F: CCCACCCCTCAGCTATAAATAGG

R: CGGCTTACCTTCTGCTGTAGTAC

VIC: TTCTTCCCCCGGCCGG NFQ

FAM: CTTCTTCCCCTGGCCGG NFQ

***CETP* (I405V)**

F: CTCACCATGGGCATTTGATTGG

R: CGGTGATCATTGACTGCAGGAA

VIC: CTCCGAGTCCGTCCAGA NFQ

FAM: TCCGAGTCCATCCAGA NFQ

***CETP* (TaqIB1B2B2_G>A)**

F: GCCAGGTATAGGGATTTGTGTTTGT

R: CCCCTAACCTGGCTCAGATC

VIC: CCCTAACTCGAACCC NFQ

FAM: CCCTAACTTGAACCC NFQ

***eNOS* (894G>T)**

F: GGCTGGACCCCAGGAAA

R: CACCCAGTCAATCCCTTTGGT

VIC: CCCAGATGATCCCCCA NFQ

FAM: CCAGATGAGCCCCCA NFQ

***IL-6* (-174C>G)**

F: CATCAGGCTTTTGGGCTTTCAA

R: AGTCTACTGTTAATGGGACTACAGGAA

VIC: TCTCTACATTAAGAAATAC NFQ

FAM: TCTCTACAATAAGAAATAC NFQ

***MMP1* (1G/2G)**

F: ACATGTTATGCCACTTAGATGAGGAAA

R: GCGTCAAGACTGATATCTTACTCATAAACAATA

VIC: TTGAGATAAGTCATATCCTTTCTA NFQ

FAM: TTGAGATAAGTCATATCTTTCTA NFQ

***MMP7* (-181A>G)**

F: GGAGTCAATTTATGCAGCAGACAGA

R: AGTGTTTTCTTTCTTTTTAGAGTCTACAGAACT

VIC: CTTTGAAAGACAAATACA NFQ

FAM: CTTTGAAAGACGAATACA NFQ

***MMP9* (R279Q)**

F: TCCCCCTTTCCCACATCCT

R: CAGGGTTTCCCATCAGCATTG

VIC: CTCTACACCCAGGACGG NFQ

FAM: TCTACACCCGGGACGG NFQ

***MMP12* (-82A>G)**

F: TGCTTTTGTTTGCATGTTTTTGAGATAGA

R: CCGGGTTCTGTGAATATGAATCCT

VIC: ATGATATCAACTATGAGTCACT NFQ

FAM: ATATCAACTGTGAGTCACT NFQ

***MGP* (138C>T)**

F: GAAAACTGCCCACTCAGAGTAGATA

R: GCACTGAACTAGCATTGGAACTTT

VIC: TCCCAAACAGTCATTC NFQ

FAM: CCAAACGGTCATTC NFQ

MPO (-638C>A)

F: CCAGTTCCTGACTTTTGTTCCTTTC

R: GATACACACAACTAACCCCTCTGAA

VIC: ACCCTGGATAACCAGTGTA NFQ

FAM: ACCCTGGATAAACAGTGTA NFQ

***PAI-1* (4G/5G)**

F: AGCCAGACAAGGTTGTTGACA

R: GCCGCCTCCGATGATACAC

VIC: CTGACTCCCCCACGTGT NFQ

FAM: CTGACTCCCCACGTGT NFQ

***PAF-AH* (A379V)**

F: GCTTTTGTAAGAATGCTAATGAAGCTTTGT

R: ACACATGCTCAAATTAAAGGGAGACA

VIC: TAAGATCAATAGCTACATTTG NFQ

FAM: ATCAATAGCTGCATTTG NFQ

***PON1* (L55M)**

F: ACAACCTGTACTTTCTGTTCTCTTTTCTG

R: CAGAGCTAATGAAAGCCAGTCCAT

VIC: AGTATCTCCAAGTCTTC NFQ

FAM: CAGTATCTCCATGTCTTC8 NFQ

***PON2* (S311C)**

F: TGTGGAAAACAGGGCTTATTGATGA

R: CCCATTGTTGGCATAAACTGTAGTC

VIC: TAGGCTTCTCAGATAGAA NFQ

FAM: AGGCTTCTCACATAGAA NFQ

***TNF-α* (-308G>A)**

F: CCAAAAGAAATGGAGGCAATAGGTT

R: GGACCCTGGAGGCTGAAC

VIC: CCCGTCCCCATGCC NFQ

FAM: CCCGTCCTCATGCC NFQ

***UCP-2* (-866G>A)**

F: GCCAGAGGGCCCAATTGTT

R: GGGCCTGGTTCGCCTTTAATT

VIC: CACGCGTCAGTTAC NFQ

FAM: TTCACGCATCAGTTAC NFQ

***UCP-3* (-55C>T)**

F: GCTGTCAACCAACTTCTCTAGGATA

R: ACTGTTGTCTCTGCTGCTTCTG

VIC: TCTTATACACACGGGCTGA NFQ

FAM: TCTTATACACACAGGCTGA NFQ

7.3.3 PCR reagents and conditions

Amplification was performed in a 5 µl volume with 4.25 ng of genomic DNA, 2.5 µl of TaqMan® Universal PCR Master Mix, 0.125 µl of 40X Assay Mix and 2.375 µl of DNase-free dH_2O according to the ABI protocol for dry template dispersion (plates left overnight for DNA to dry). The PCR conditions were also according to the ABI TaqMan protocol and 384-well plates were used. After amplification, the plates were read with the ABI Prism 7900HT Sequence Detection System for allelic discrimination and the results were analyzed using the SD 2.1 software. The PCR conditions are shown in table 7.1 and an example of the result sheet is shown in figure 7.4 (the oligonucleotides were synthesized by Applied Biosystems, U.K.).

Table 7.1: PCR conditions for the TaqMan method (same conditions for all the polymorphisms)

Times and Temperatures [5]		
Initial Steps	Each of 40 Cycles	
	Denature	Anneal/Extend
HOLD	CYCLE	
10 min 95°C	15 sec 92°C	1 min 60°C

7.3.4 Quality control

DNA was measured and diluted to a concentration of 1.25 ng/μl after being placed in 96-well plates. A random number generator was used to decide on sample duplicates (4 duplicates per 96-well plate) and the last 4 wells in each plate were left blank and assigned a no template (negative) control identity (NTCs), in order to ensure quality control. The 96-well plates were then merged into 384-well plates thus resulting in 16 duplicates and 16 NTCs in each 384-well plate. The duplicates and the NTCs were checked after every run to ensure the run was succesfull and there was no contamination.

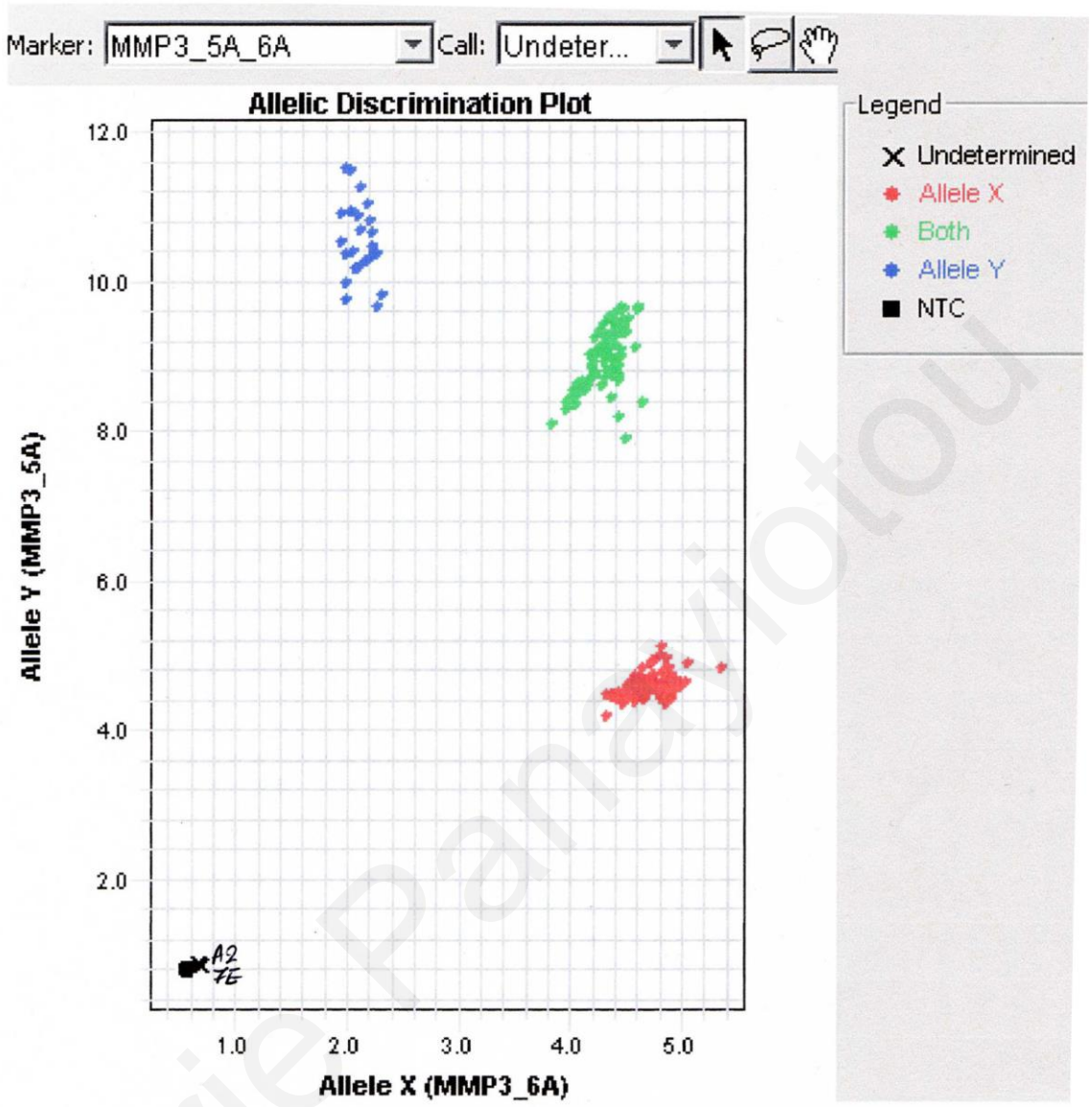

Figure 7.4: Results sheet from the TaqMan method for *MMP-3* (5A/6A). Red dots show 6A/6A homozygotes, blue dots 5A/5A homozyhotes and green dots 5A/6A heterozygotes

7.4 Statistical analysis

Descriptive statistics were performed first for all the measurements (biochemical and genetic) that were included in the study. Genetic polymorphisms were tested for deviation from the Hardy-Weinberg equilibrium with a chi^2 test. Histograms were drawn for all the biochemical measurements as well as a Q-Q plots in order to establish their normality (data not shown). Normally distributed data are presented as means and standard deviation (±SD), whereas skewed data are presented as median and interquartile range (iqr) as shown in Annexe 1. Biochemical and genetic markers were tested for association with ultrasonic markers of early atherosclerosic (IMTcc, IMTmax, TPT, BPB) in groups according to function.

Parametric methods were used for analysis of normal data and non-parametric for skewed data. Indepentent samples t-test and one-way ANOVA were used to compare between two or more independent groups of normally distributed data and Mann-Whitney U test and Kruskal-Wallis test for comparing two or more independent groups of skewed data. Cut-off significance level for P value was set to 0.05. The assumption that samples were independent was satisfied by checking that the ratio of standard deviations was between 0.5 and 2.

Analysis of categorical data was done with 2X2 frequency tables for analysis of binary data and larger frequency tables for analysis of categorical data with more than two categories. P values reported are from a chi^2 test.

Analysis of continuous outcomes with continuous factors was done with correlation and linear regression analysis (skewed data were logtransformed to fit the assumption of normality before linear regression was performed). Linear regression was also used to adjust for traditional risk factors (covariates) when the dependent variable was continuous. Analysis of binary dependent variables and adjustement for covariates was done with multiple, binary logistic regression analysis. R^2 refers to the adjusted R^2.

The means and SD (for normally distributed data), or median (iqr) (for not-normally distributed data) are presented alphabetically for the biochemical measurements. Table A2 shows means (±SD) for symmetrical data and Table A3 median (iqr) for skewed data (Annexe).

Categorical data, such as genetic polymorphisms, are described with the use of relative frequencies (RF) (percentage). Percentages of all the studied polymorphisms are listed in Table A4 alphabetically (Annexe).

All analyses were performed with the SPSS 13.0 as well as MedCalc statistical packages.

PART VI:

RESULTS

Chapter 8:

Results of association between ultrasonic measurements and disease prevalence

As indicated in the review of the literature (chapter 2), carotid intima-media thickness (IMT) and the presence and number of carotid plaques are surrogate markers of atherosclerosis. They are associated with the extent and severity of coronary atherosclerosis and future episodes of myocardial infarction and stroke.

The aim of this chapter was to present the results of the association between ultrasonic markers of subclinical atherosclerosis and clinical CVD in the population studied. Demonstration of the strength of the association between these measurements and actual disease prevalence would form the basis for the subsequent investigation on biomarkers.

8.1 Ultrasonic measurements and disease prevalence

Quartiles of ultrasonic measurements were analysed for association with clinical cardiovascular disease. Odds ratios before and after adjustement for known factors are shown in table 8.1. After correcting for traditional risk factors (age, sex, smoking in pack-years, systolic blood pressure, total cholesterol, diabetes, administration of cholesterol lowering therapy and antihypertensive therapy), IMTcc was no longer associated with CVD. In contrast, IMTmax, TPT, the number of bifurcations with plaques and plaque echodensity (MPT and BPB) were strongly associated wth prevalence of clinical CVD even after adjustement. Figure 8.1 shows the increase in odds ratios for presence of clinical CVD with increasing quartiles of ultrasonic measurements. The odds ratios of the novel ultrasonic measurements increase most steeply in the 4^{th} quartile.

8.1.2 TPT and disease prevalence

A cut-off point of total plaque thickness greater than 0.52 cm (maximum combined sensitivity (75.2%) and specificity (75.2%) determined from an ROC curve) identified 247 (32%) of the population that contained 85 (75%) of the 113 individuals with clinical cardiovascular disease (Table 8.3). The positive predictive value (PPV) was 34.4%, negative predictive value (NPV) 94.6% and the likelihood ratio of a positive test result 3.03. The PPV represents the probability of a positive test result indicating the true present of disease and the NPV the probability of a negative test result indicating that the disease is truly absent. A Receiver Operating Characteristic (ROC) curve is a graph that plots the true positive rate in function of the false positive rate at different cut-off points.

8.1.3 MPT and disease prevalence

Plaques were assigned a plaque type from 1 to 5 according to the Widder classification modification (with type 1 being the most echogenic and type 5 the most echolucent as described in chapter 2). Mean plaque type (MPT) was the mean type in the four arteries tested. The association between MPT and clinical disease was tested (Table 8.1). Even after adjustements for other known risk factors, MPT remained significantly associated with clinical CVD. It is apparent that the second quartile is associated with the highest prevalence of CVD, so analyses with biomarkers in following chapters were done by using the median (2.75) as a cut-off point, in those with plaques, instead of using MPT as a continuous measurement.

8.1.4 BPB and disease prevalence

Plaques were classified 1 to 5 according to Widder classification (with type 1 being the most echogenic and type 5 the most echolucent as described in chapter 2) (Figs.2.1-7) and plaque type was assigned by the two ultrasonographers. The ultrasonic features were defined as follows: Total plaque thickness (TPT) was the sum of the thickest plaque in each of the four arteries and mean plaque type (MPT) the mean plaque type of the arteries with plaque present. BPB is the product of TPT * MPT. The association between BPB and clinical disease was tested (Table 8.1). Figure 8.2 shows the relationship between TPT in mm per year (i.e. an average measure of progression of TPT in time) and BPB. The scattergram suggests that rapidly growing plaques are more echolucent (high BPB) and produce most of the clinical events (CVD).

8.2 Presence of plaques and disease prevalence

The association between carotid bifurcation plaques, femoral bifurcation plaques, number of bifurcations with plaque and clinical CVD was also tested (Table 8.2). Both carotid and femoral plaques were associated with CVD even after adjustement for each other and traditional risk factors. The number of bifurcations with plaque (which includes all four bifurcations measured) is a stronger predictor of CVD than both carotid and femoral bifurcations if used individually.

Table 8.1: Association between ultrasonic measurements and clinical CVD

Ultrasonic Measurements	**Range of measurements**	**Subjects studied** n	**CVD present** n (%)	**Crude Odds ratio (95% CI)**	**Adjusted odds ratio***
IMTcc (mm)					
Quartiles					
1_{st}	0.04-0.06	174	10 (5.4%)	1	1
2_{nd}	0.06-0.07	216	26 (10.7%)	2.09 (0.98 to 4.46)	1.10 (0.48 to 2.56)
3_{d}	0.07-0.08	123	29 (19.1%)	4.10 (1.93 to 8.73)	1.32 (0.55 to 3.12)
4_{th}	≥ 0.080	141	48 (25.4%)	5.92 (2.89 to 12.13)	1.31 (0.56 to 3.06)
IMTmax (mm)					
Quartiles					
1_{st}	0.04-0.07	219	8 (3.5%)	1	1
2_{nd}	0.07-0.12	146	16 (9.9%)	3.00 (1.25 to 7.19)	1.72 (0.67 to 4.38)
3_{d}	0.12-0.20	159	27 (14.5%)	4.65 (2.06 to 10.50)	1.77 (0.73 to 4.31)
4_{th}	≥ 0.20	130	62 (32.3%)	13.05 (6.06 to 28.13)	2.87 (1.19 to 6.91)
Total plaque thickness (cm)					
Quartiles					
1_{st}	No plaques	209	5 (2.3%)	1	1
2_{nd}	0.12-0.33	162	9 (5.3%)	2.37 (0.78 to 7.20)	1.41 (0.45 to 4.45)
3_{rd}	0.33-0.64	168	26 (13.4%)	6.59 (2.48 to 17.54)	2.67 (0.94 to 7.56)
4_{th}	≥ 0.64	113	73 (39.2%)	27.52 (10.81 to 70.05)	6.34 (2.22 to 18.06)
Mean Plaque Type (mm)					
Quartiles					
1_{st}	No plaques	215	5 (2.3%)	1	1
2_{nd}	0.0-2.75	169	52 (30.8%)	18.67 (7.25 to 48.03)	4.24 (1.49 to 12.06)
3_{d}	2.75-3.50	197	30 (15.2%)	7.55 (2.87 to 19.87)	2.26 (0.78 to 6.53)
4_{th}	≥ 3.50	184	26 (14.1%)	6.91 (2.60 to 18.40)	3.26 (1.14 to 9.33)
Black Plaque Burden (mm)					
Quartiles					
1_{st}	No plaques	218	6 (2.8%)	1	1
2_{nd}	0.13-1.02	165	12 (7.3%)	2.77 (1.02 to 7.55)	1.46 (0.50 to 4.23)
3_{d}	1.03-2.49	268	53 (19.8%)	8.71 (3.66 to 10.69)	2.86 (1.11 to 7.34)
4_{th}	≥ 2.50	113	42 (37.2%)	20.90 (8.53 to 51.24)	4.49 (1.64 to 12.30)

* Adjusted for age, sex, pack-years, systolic blood pressure, total cholesterol, diabetes; also administration of cholesterol lowering therapy and antihypertensive therapy

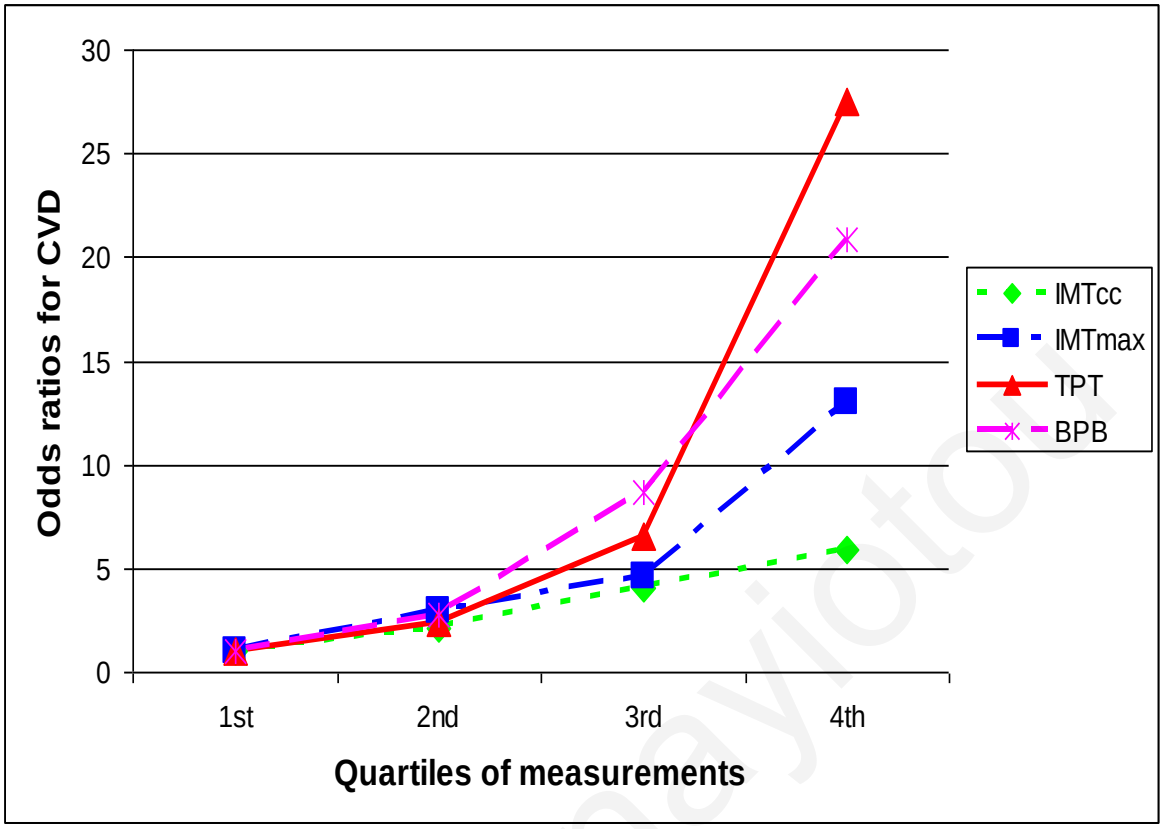

Figure 8.1: Association between quartiles of ultrasonic measurements and Odds Ratios for clinical CVD

Table 8.2: Association between carotid bifurcation plaques, femoral bifurcation plaques, number of bifurcations with plaque and cardiovascular disease (CVD)

Carotid plaques n	Subjects studied n	CVD present n (%)	Crude Odds ratio (95% CI)	OR adjusted for femoral plaques	OR adjusted for femoral plaques and risk factors*
0	310	15 (4.8%)	1	1	1
1	196	22 (11.2%)	2.49 (1.26 to 4.92)	1.86 (0.92 to 3.77)	1.37 (0.65 to 2.89)
2	261	76 (29.1%)	8.08 (4.51 to 14.48)	4.35 (2.35 to 8.07)	2.21 (1.12 to 4.33)

Femoral plaques n	Subjects studied n	CVD present n (%)	Crude Odds ratio (95% CI)	OR adjusted for carotid plaques	OR adjusted for carotid plaques and risk factors*
0	394	16 (4.1%)	1	1	1
1	151	24 (15.9%)	4.46 (2.30 to 8.67)	3.75 (1.90 to 7.39)	2.68 (1.31 to 5.50)
2	222	73 (32.9%)	11.57 (6.52 to 20.53)	7.37 (4.05 to 13.39)	3.70 (1.87 to 7.32)

Bifurcations with plaques n	Subjects studied n	CVD present n (%)	Crude Odds ratio (95% CI)	OR adjusted for risk factors*
0	214	5 (2.3%)	1	1
1	145	10 (6.9%)	3.10 (1.04 TO 9.26)	2.51 (0.70 to 8.86)
2	174	16 (9.2%)	4.23 (1.52 to 11.80)	2.53 (0.79 to 8.11)
3	96	24 (25.0%)	13.93 (5.12 to 37.88)	6.48 (2.03 to 20.74)
4	138	58 (42.0%)	31.08 (12.02 to 80.36)	9.07 (2.84 to 28.94)

* Adjusted for age, sex, pack-years, systolic blood pressure, total cholesterol, diabetes; also administration of cholesterol lowering therapy and antihypertensive therapy

Table 8.3: Association of total plaque thickness (TPT) greater than 0.52 cm (value with highest sensitivity 75.2% and specificity 75.2% in an ROC curve) with prevalence of CVD: TPT >0.52 cm identifies 347 (32%) of the population that contains 85/113 (75%) of the events (OR: odds ratio; PPV: positive predictive value; NPV: negative predictive value; LR: likelihood ratio).

TPT cm	No disease	Disease	n
<0.52	492 (96.4%)	28 (5.4%)	520
>0.52	162 (65.6%)	85 (34.4%)	247
Total	654 (85.3%)	113 (14.7%)	767

OR 9.20 (95% CI 5.79 to 14.61)
PPV 34.4%; NPV 94.6%; LR 3.03

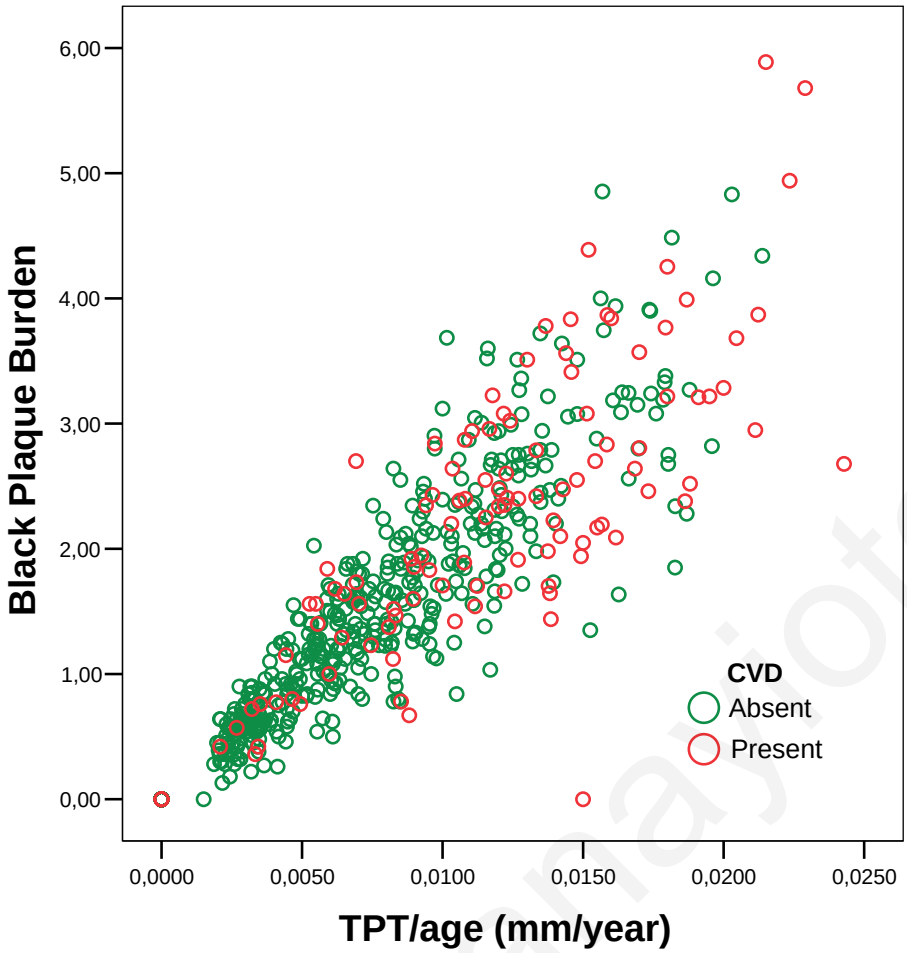

Figure 8.2: Association between progress of plaque thickness in mm per year (TPT/age) and black plaque burden

8.3 Framingham risk score and ultrasonic measurements

The widely used Framingham 10-year risk score was calculated for each subject with complete data in the study (n=767). The predictive ability of the Framingham risk score for presence of clinical CVD was 0.76 (area covered by an ROC curve). If IMTcc was added to the model the area under the ROC curve did not improve. However, if IMTmax or TPT were added to the model the area improved to 0.81 and 0.83 respectively. This makes little difference to the prediction of absolute numbers of individuals with cardiovascular disease. The upper quartile of the Framingham 10 year risk, though, could determine a high-risk group, which contained 59 (37.8%) of the 93 individuals with cardiovascular disease in the population studied. The combination of Framingham 10 year risk with total plaque thickness (value with highest sensitivity 75.2% and specificity 75.2% in ROC curve) could reclassify individuals into low and high groups in the upper 3 quartiles (Figs.8.3-4). If BPB (population median cut-off point 1.02) was added to the Framingham risk, individuals were reclassified into low and high groups in the upper 3 quartiles. Combining MPT with the Framingham risk could identify 49.2% of clinical events (upper quartile). This suggests that inclusion of ultrasonic measurements to the Framingham 10 year risk score may improve its predictive ability as shown in figures 8.3-4.

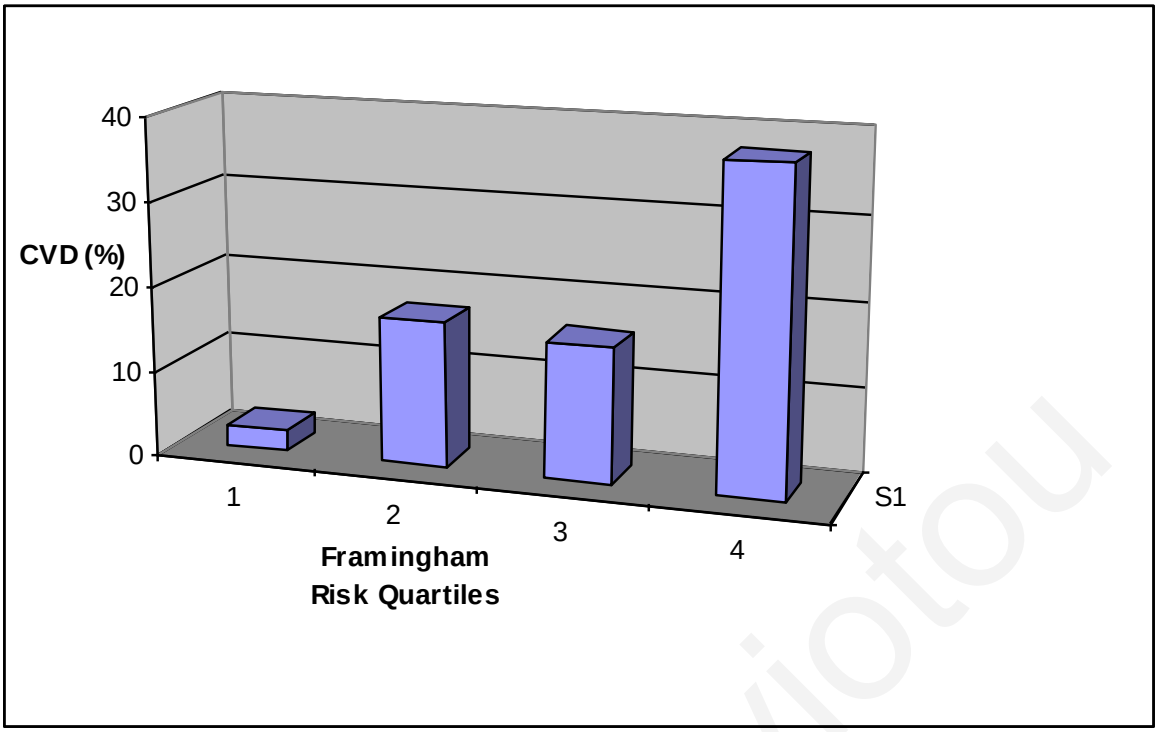

Figure 8.3: Association between percentage of clinical CVD and quartiles of Framingham 10-year risk score

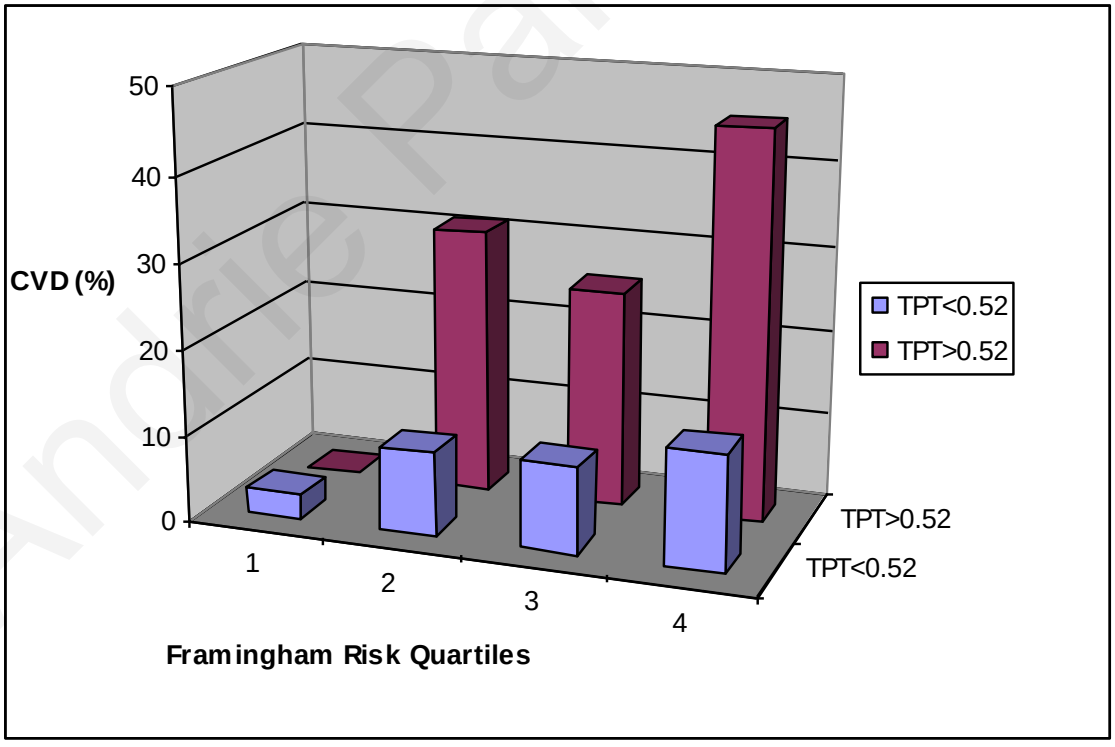

Figure 8.4: Association between percentage of clinical CVD and quartiles of Framingham 10-year risk score if TPT is added to the model. Value with highest sensitivity 75.2% and specificity 75.2% in ROC curve is used as cut-off point

Summary of results

In the Cyprus study population examined, the presence of plaque at three or four bifurcations (indicating the presence of both carotid and femoral atherosclerosis), TPT, MPT and BPB (TPT*mean plaque type) appear to have a stronger association with the prevalence of cardiovascular disease than IMTcc or IMTmax measurements (Tables 8.1-2).

As far as we know this is the first time that the association between cardiovascular disease prevalence and different ultrasonic measurements that include IMTcc, IMTmax, presence of plaque at the common femoral bifurcation, number of vessels with plaque and total plaque thickness has been compared in a single population study (only exlusion criterion was age). The results of this study indicate that total plaque thickness when four vessel bifurcations are scanned has a stronger association with cardiovascular disease than IMT measurements (IMTcc or IMTmax). The findings of this study suggest that the presence, number, size and type of plaques have a stronger association with clinical cardiovascular disease than any IMT measurements and warrant further investigation in large prospective studies to determine whether they are associated with the future development of clinical events.

Chapter 9: *Results of association between lipids and subclinical atherosclerosis*

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and lipid markers (both biochemical and genetic). The lipid markers chosen were: serum levels of TChol, HDL, LDL, TG, apoA1, apoB and Lp(a), plasma levels of Lp-PLA$_2$ activity and genetic polymorphisms of *apoB* (-516C>T), *apoE* (E2/E3/E4), *CETP* (TaqIB1B2B2 and I405V) and *Lp-PLA$_2$* (A379V). The reasons for the choise of the above markers are stated at the hypothesis (chapter 4).

Association between blood levels of markers and relative genetic polymorphisms

The association between plasma Lp-PLA$_2$ activity and A379V genotype has only been studied in two case-control studies thus far -the AtheroGene and the UDAC study populations- with inconclusive results as indicated in the literature review (chapter 3). Although this was not a primary aim, it was decided that the association between A379V genotype and Lp-PLA$_2$ activity had to be elucidated before analysing the results, especially given the lack of data in the literature. Exploratory associations between *apoB* (516C>T) and *apoE* (E2/E3/E4) genotypes and lipid levels were also undertaken.

Plasma Lp-PLA$_2$ activity and A379V polymorphism

Lp-PLA$_2$ 379VV genotype (n=48) was associated with higher plasma Lp-PLA$_2$ activity levels compared to 379VA and 379AA subjects (n=697), but the results were not statistically significant in the population as a whole (63.88 mmol/min/ml (±20.70) vs 58.51 mmol/min/ml (±17.79); P = 0.086). However, in a sex specific analysis, it became obvious that the relationship was driven by women (65.43 (±18.51) vs 54.9 (±16.87); P=0.016) (Fig.9.1) and the association was not significant in men (P=0.98). After adjusting for factors known to affect Lp-PLA$_2$ levels (age, smoking in packyears and BMI) the association was even stronger (P=0.003) and could explain 1.9% of its variability. This is the first time such an association is shown in a general population study and especially in women (further discussed in chapter 16).

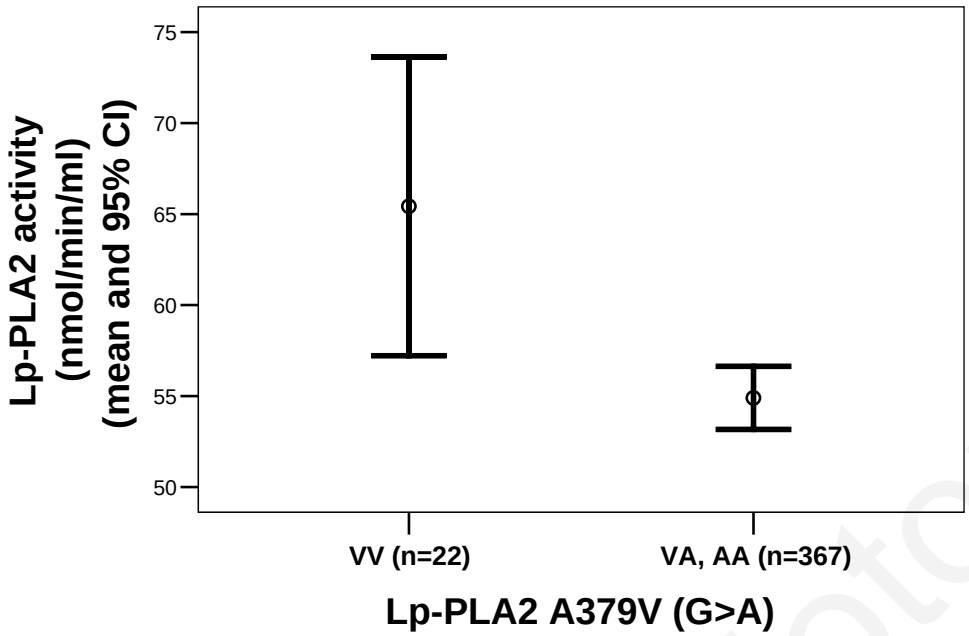

Figure 9.1: Association between plasma Lp-PLA$_2$ activity and *Lp-PLA$_2$* A379V genotype in women only (Independent t-test; P=0.016)

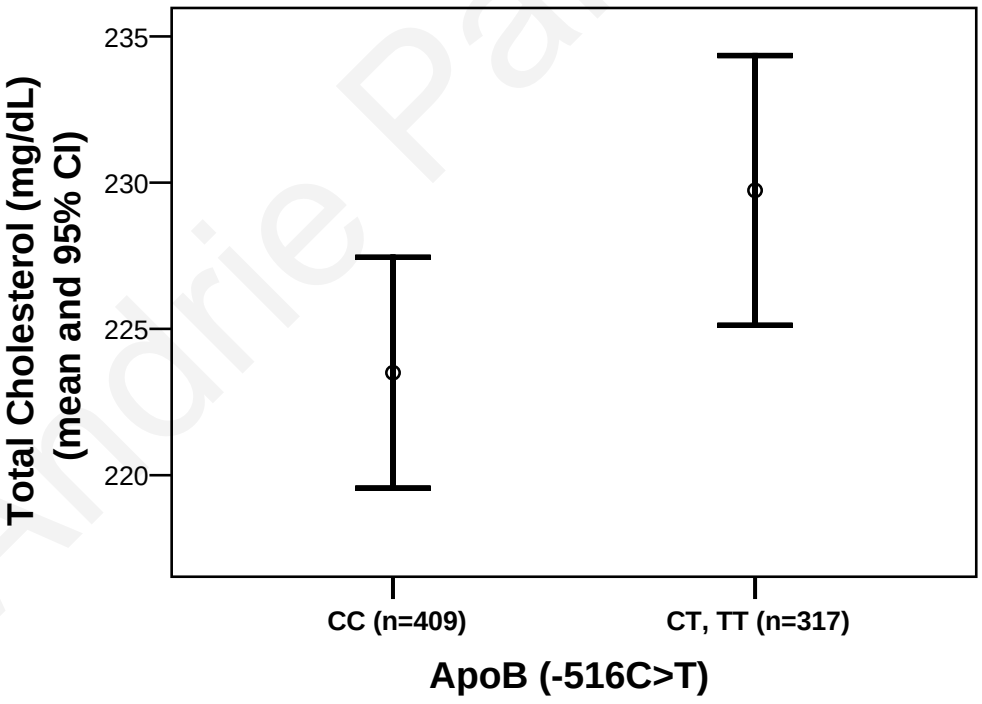

Figure 9.2: Association between total cholesterol levels and *apoB* (-516C>T) genotype (Independent t-test; P=0.044)

Serum apoB and cholesterol levels and apoB (-516C>T) polymorphism

Serum levels of apoB were not significantly associated with *apoB* (-516C>T) genotype. However, the *apoB* -516CC genotype was significantly associated with higher circulating total cholesterol levels and LDL levels compared to -516CT and -516TT genotypes. Significant results are shown in figures 9.2-3 and p values are tabulated in table 9.1.

Serum cholesterol levels and apoE (E2/E3/E4) polymorphism

Serum levels of total cholesterol, TChol/HDL ratio and LDL were all significantly associated with the *apoE* (E2/E3/E4) polymorphism with the E3/E3, E3/E4 genotypes being associated with higher levels of total cholesterol, TChol/HDL and LDL compared to the E2/E2 and E2/E3 genotypes. (E4/E4 genotype was too rare in our population and was left out of the analysis). Results are shown in figures 9.4-6 and p values are tabulated in table 9.1.

Table 9.1: Results of association between plasma levels markers and their corresponding genetic polymorphisms (Independent t-test used; P value is 2-tailed for equal variances not assumed)

Genetic polymorphism and corresponding biochemical measurements:

Genetic polymorphism	**Biochemical Measurement**	**P value**	**95% CI for P**
Lp-PLA$_2$(379VVvsAA)	Lp-PLA$_2$ activity	P=0.016	2.54 to 19.49
ApoB(-516CCvsCT,TT)	Tchol levels	P=0.044	0.17 to 12.30
	HDL levels	P=0.036	0.13 to 3.79
ApoE (E2/E2, E2/E3vs E3/E3, E3/E4)	Tchol levels	P=0.001	8.49 to 29.65
	Tchol/HDL	P=0.004	0.11 to 0.91
	LDL levels	P<0.001	13.59 to 27.46

* Lp-PLA$_2$ analysis is for women only

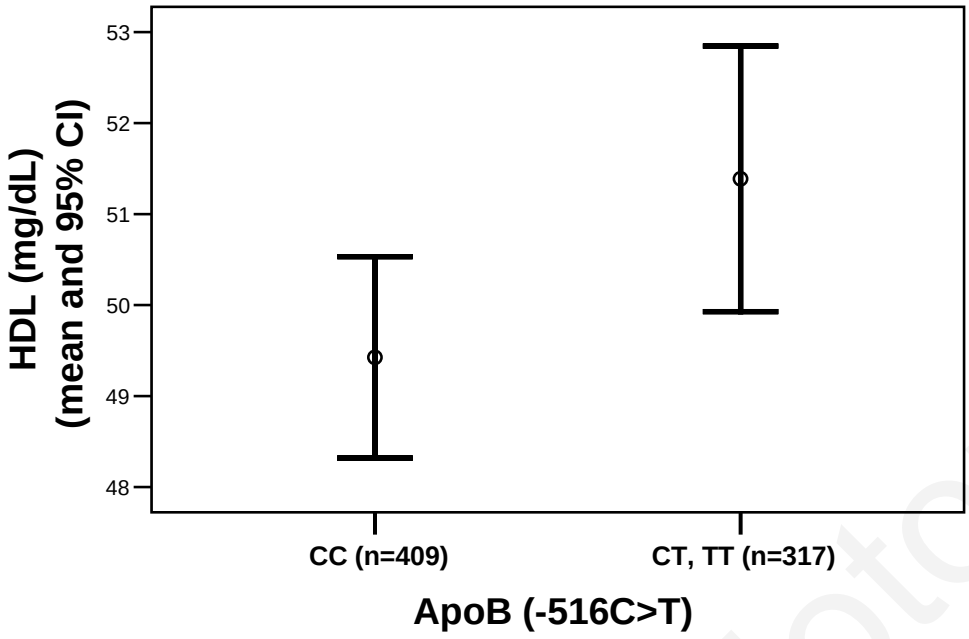

Figure 9.3: Association between HDL levels and *apoB* (-516C>T) genotype (Independent t-test; P=0.036)

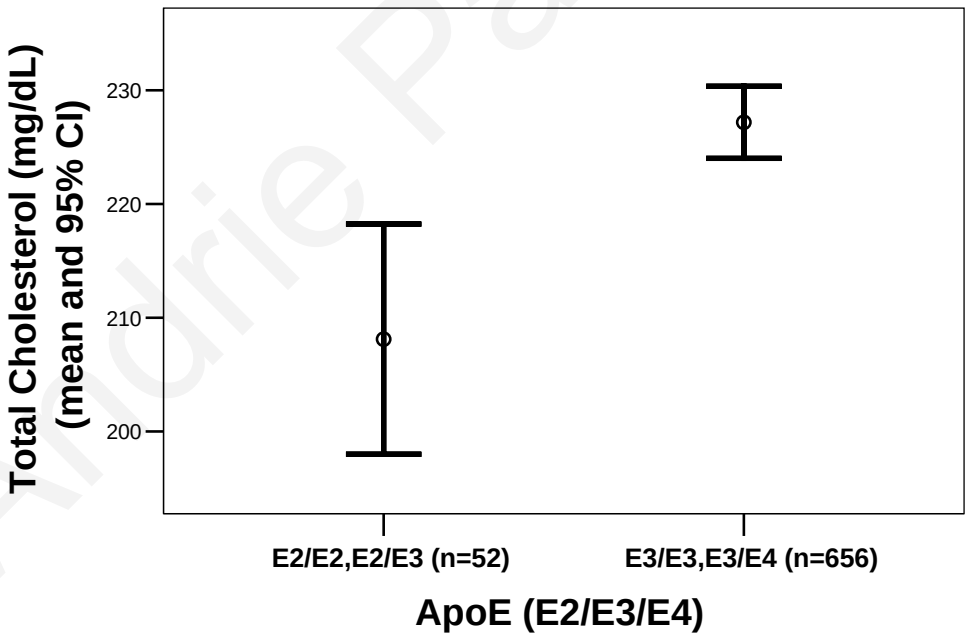

Figure 9.4: Association between total cholesterol levels and *apoE* (E2/E3/E4) genotype (Independent t-test; P=0.001)

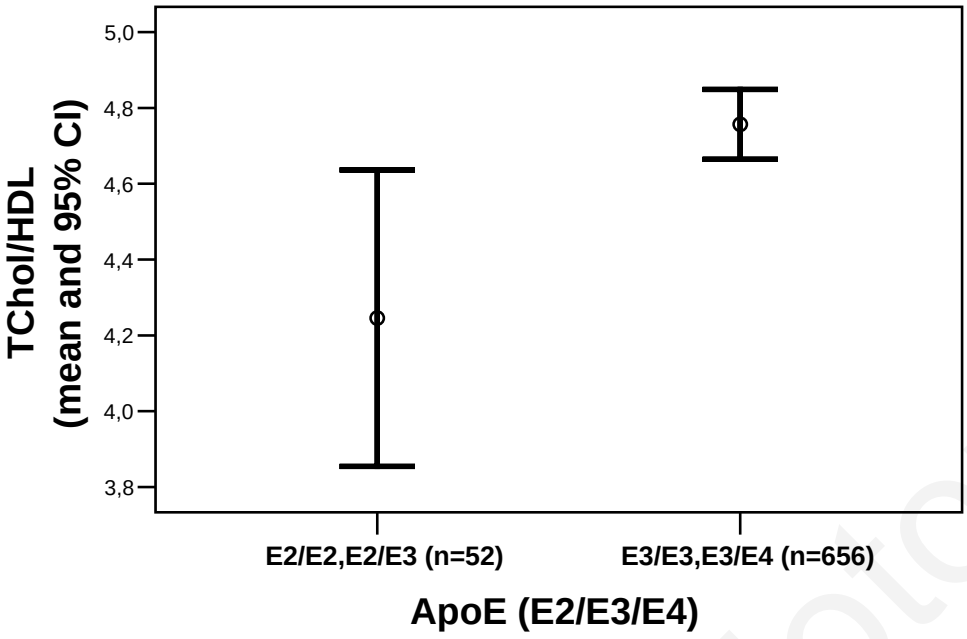

Figure 9.5: Association between total cholesterol/ HDL ratio and *apoE* (E2/E3/E4) genotype (Independent t-test; P=0.004)

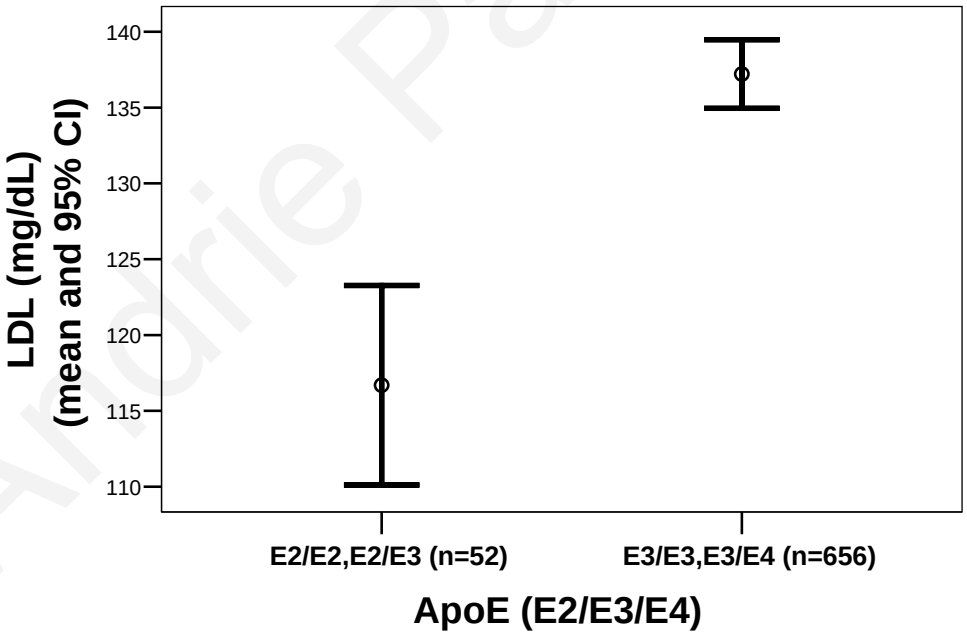

Figure 9.6: Association between LDL levels and *apoE* (E2/E3/E4) genotype (Independent t-test; P<0.001)

Association between lipid markers and IMTcc

The association between lipid markers and IMTcc was tested. In univariate linear regression analyses HDL, apoA1, the ratio Tchol/HDL, the ratio of apoB/apoA1 and Lp-PLA_2 activity were significantly associated with IMTcc. None of the genetic polymorphisms affecting lipid metabolism was associated with IMTcc in linear regression. Results are shown in figures 9.7-11 and table 9.2.

In multivariate linear regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes and hypertension) apoA1 levels, the ratio of apoB/apoA1 and the ratio of TChol/HDl remained significantly associated with IMTcc. In a multivariate analysis including all the independent predictors: age, sex, smoking in packyears, diabetes, hypertention and the apoB/apoA1 ratio the model could explain 29.5% of the variability in IMTcc. Results are shown in table 9.3. Adding the ratio of apoB/apoA1 to the model improved its predictive ability by 1.1% whereas adding the ratio of TChol/HDL only improved the model by 0.3%. Results indicate that the ratio of apoB/apoA1 is a risk factor significantly associated with IMTcc over and above traditional risk factors and seems to contribute more than the commonly used TChol/HDL ratio to the development of arterial wall thickening

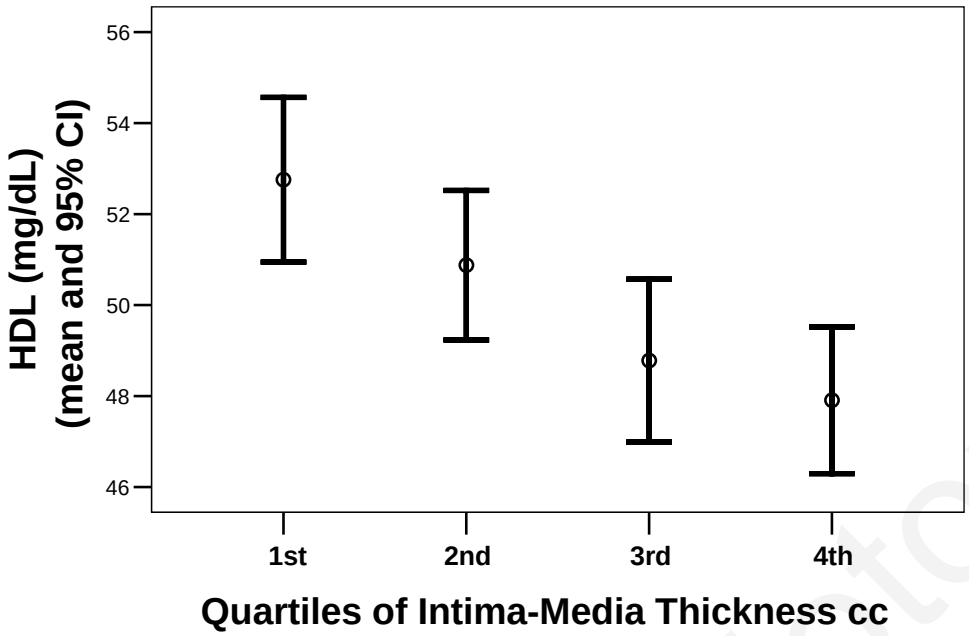

Figure 9.7: Association between HDL levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.001)

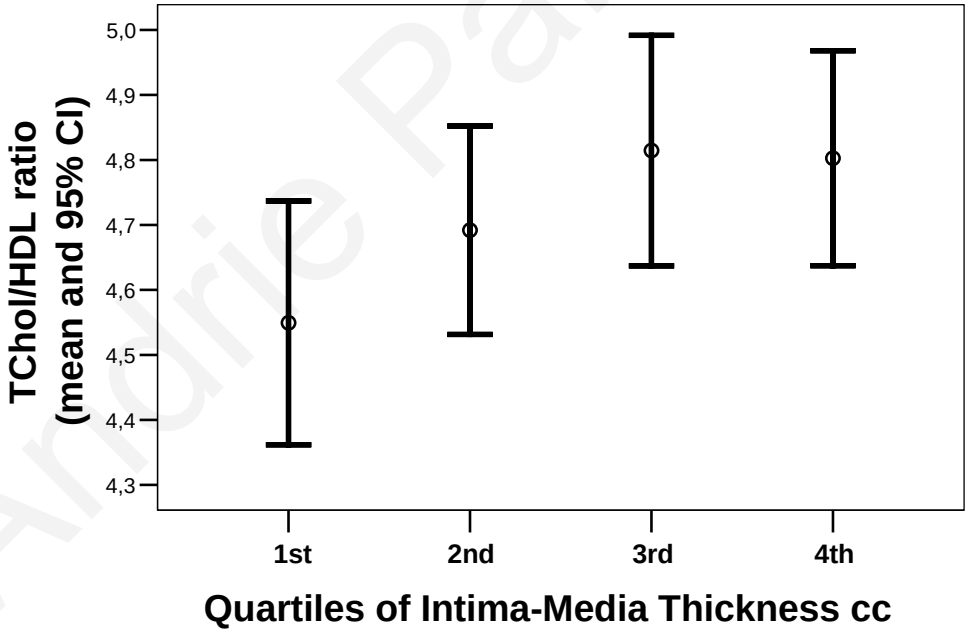

Figure 9.8: Association between TChol/HDL ratio and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.018)

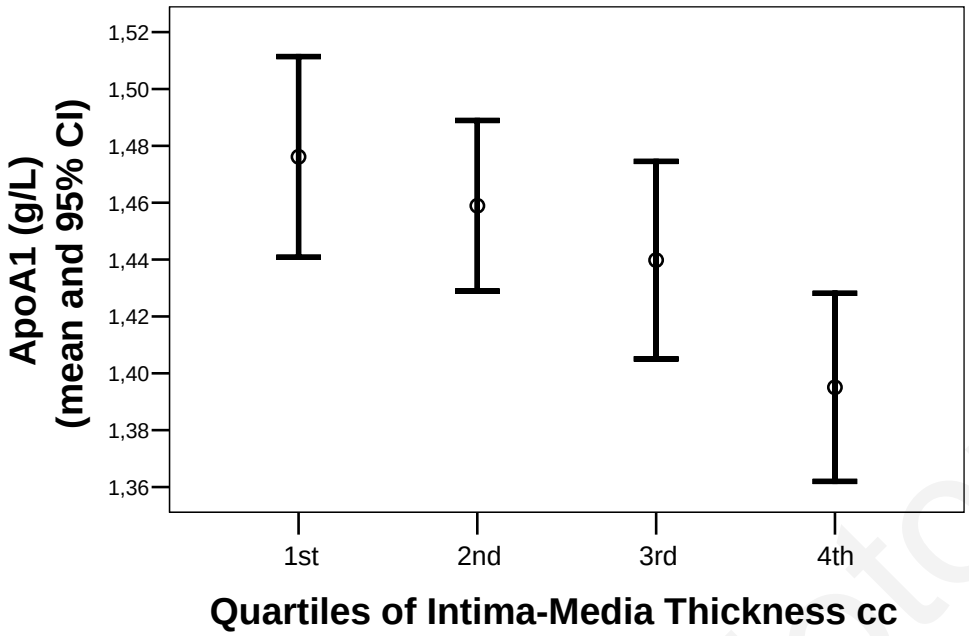

Figure 9.9: Association between apoA1 levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.007)

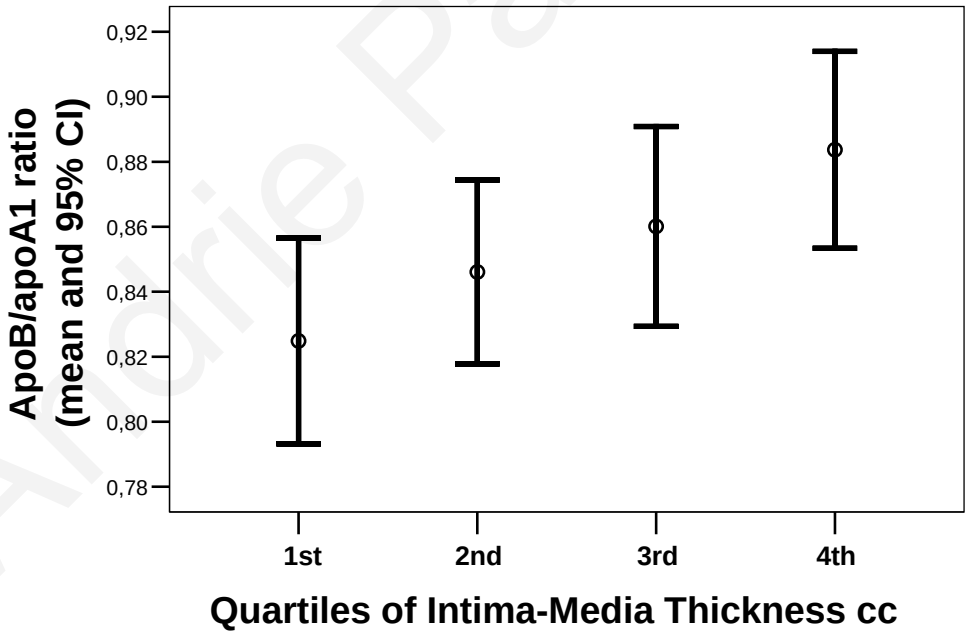

Figure 9.10: Association between apoB/apoA1 ratio and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.033)

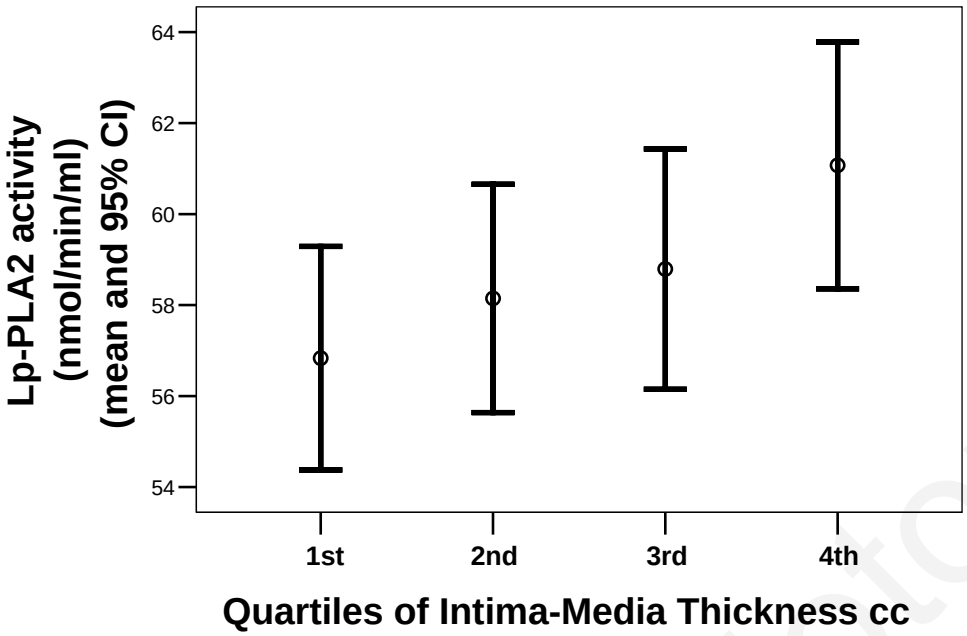

Figure 9.11: Association between Lp-PLA_2 activity and quartiles of IMTcc (Kruskal-Wallis test; P=0.115)

Table 9.2: Results of univariate linear regressions of associations between lipid markers and IMTcc

Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for Beta	R^2
TChol	770	-2.0E-005	P=0.165	-0.05	0.00 to 0.00	0.001
HDL	770	0.00	**P<0.001**	-0.143	0.00 to 0.00	0.019
TChol/HDL	770	0.001	**P=0.02**	0.084	0.00 to 0.002	0.006
LDL	770	1.62E-005	P=0.42	0.029	0.00 to 0.00	0.000
TG	770	1.15E-005	P=0.09	0.06	0.00 to 0.002	0.002
ApoA1	770	-0.009	**P<0.001**	-0.127	-0.014 to -0.004	0.015
ApoB	770	0.003	P=0.24	0.042	-0.002 to 0.008	0.000
ApoB/ApoA1	770	0.009	**P=0.002**	0.113	0.003 to 0.014	0.012
Lp(a)	743	0.001	P=0.41	0.030	-0.001 to 0.002	0.001
Lp-PLA$_2$ activity	752	8.08E-005	**P=0.017**	0.087	0.00 to 0.00	0.006
***Lp-PLA$_2$* (A379V)**	743	0.001	P=0.31	0.037	-0.001 to 0.003	0.000
***ApoB* (-516C>T)**	724	0.001	P=0.30	0.039	-0.001 to 0.003	0.000
***ApoE* (E2/E3/E4)**	706	-0.003	P=0.74	-0.039	-0.007 to 0.002	0.000
***CETP* (TaqIB1B2B2)**	725	0.002	P=0.058	0.070	0.00 to 0.003	0.004
***CETP* (I405V)**	708	-0.001	P=0.10	-0.062	-0.003 to 0.000	0.002

Table 9.3: Results of association between lipid markers and IMTcc using multivariate linear regression analyses (a-e) in which each variable was adjusted for age, sex, smoking (packyears), diabetes and hypertention (Baseline R^2=0.284)

Model	Marker	Estimate (B)	P value	Exponential (Beta)	95% CI for Beta	R^2
(a)	**HDL**	-5.7E-005	P=0.21	-0.042	0.00 to 0.00	0.285
(b)	**TChol/HDL**	0.001	**P=0.049**	0.063	0.00 to 0.002	0.287
(c)	**ApoA1**	-0.005	**P=0.031**	-0.071	-0.10 to 0.00	0.288
(d)	**ApoB/ApoA1**	0.009	**P<0.001**	0.114	0.004 to 0.014	0.295
(e)	**Lp-PLA$_2$ activity**	5.67E-005	P=0.054	0.061	0.00 to 0.00	0.282

Association between lipid markers and IMTmax

The association between lipid markers and IMTmax was tested. In univariate linear regression analyses HDL, the ratio TChol/HDL, apoA1, the ratio apoA1/apoB, Lp-PLA_2 activity and the *CETP* TaqIB1B2B2 polymorphism (B2B2 vs B1B2, B1B1) were significantly associated with IMTmax (logtransformed to fit the assumption of normality). Significant results are shown in figures 9.12-17 and table 9.4.

In multivariate linear regression analyses including traditional risk factors (age, sex, smoking in packyears, diabetes and hypertension) and each of the significant variables, HDL, the ratio of TChol/HDL, LDL, apoA1, apoB, the ratio of apoB/apoA1, Lp-PLA_2 activity and *CETP* TaqIB1B2B2 (A>G) polymorphism all emerged to be independently associated with IMTmax over and above traditional risk factors. Results are shown in table 9.5. A multivariate model including all the above variables and correcting for traditional risk factors could explain 36% of the variability in IMTmax and improved the model by 3.4%; HDL, TChol/HDL, Lp-PLA_2 activity and *CETP* (TaqIB1B2B2) polymorphism remained independently associated with IMTmax and LDL had a borderline p value (P=0.025; P=0.009; P=0.003; P=0.024; P=0.069 respectively).

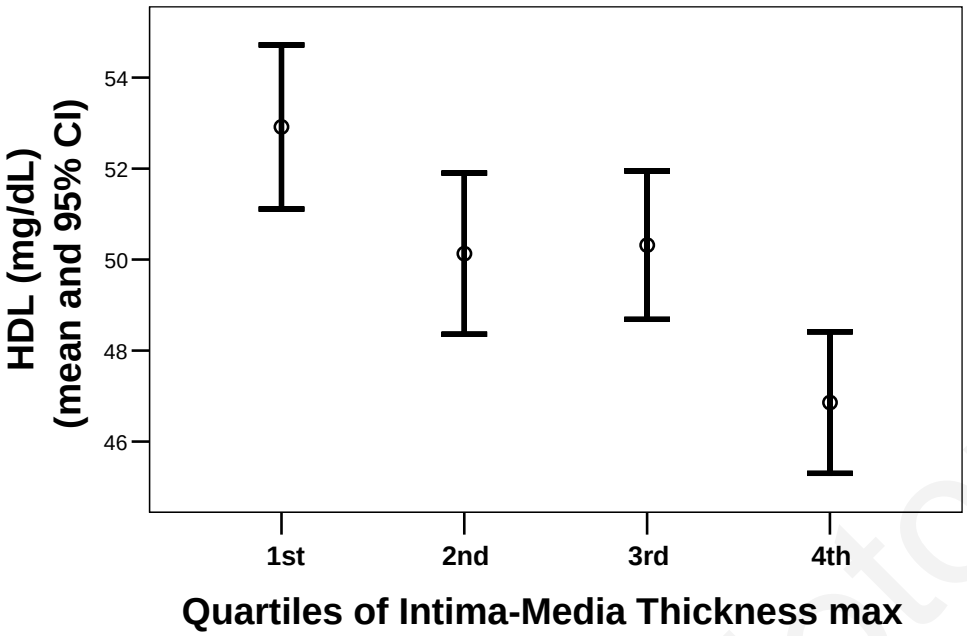

Figure 9.12: Association between HDL levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend<0.001)

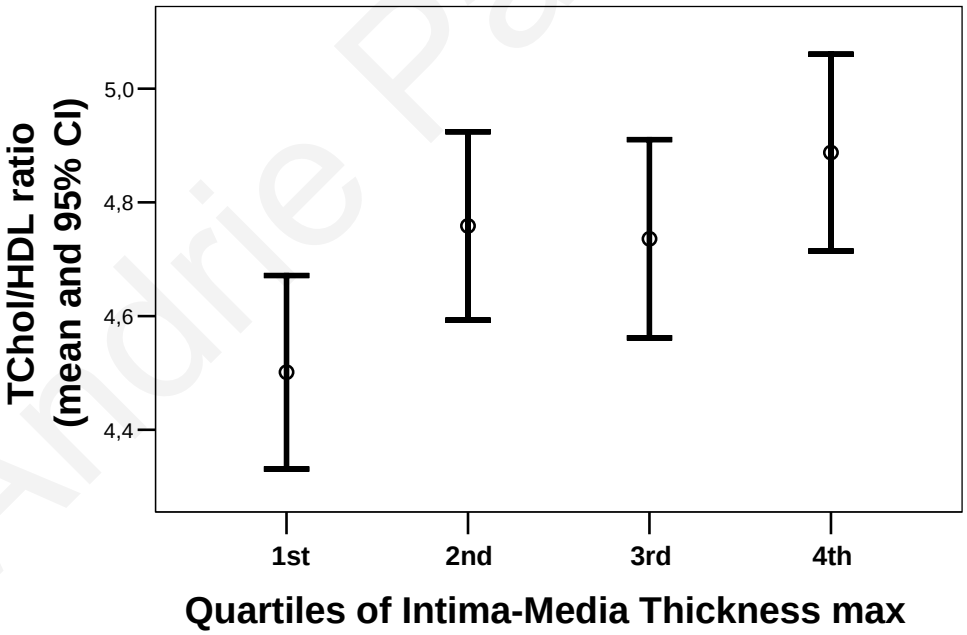

Figure 9.13: Association between TChol/HDL ratio and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.001)

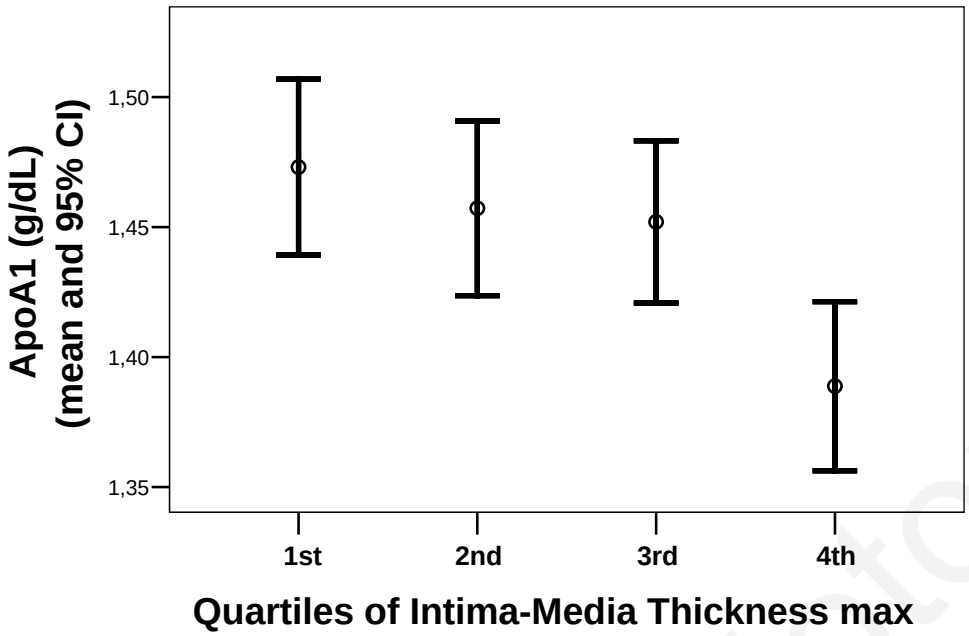

Figure 9.14: Association between apoA1 levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.017)

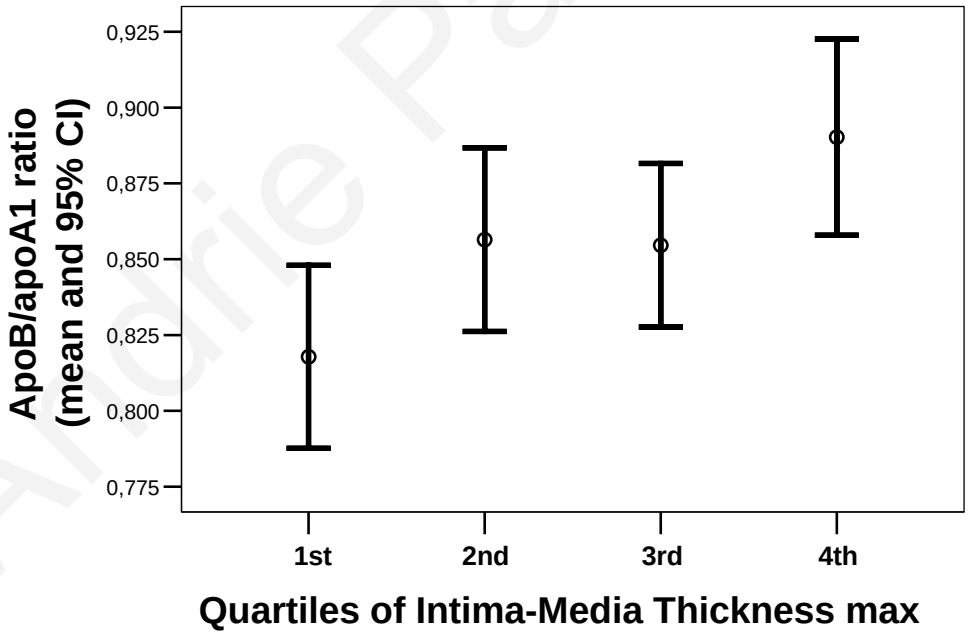

Figure 9.15: Association between apoB/apoA1 ratio and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.006)

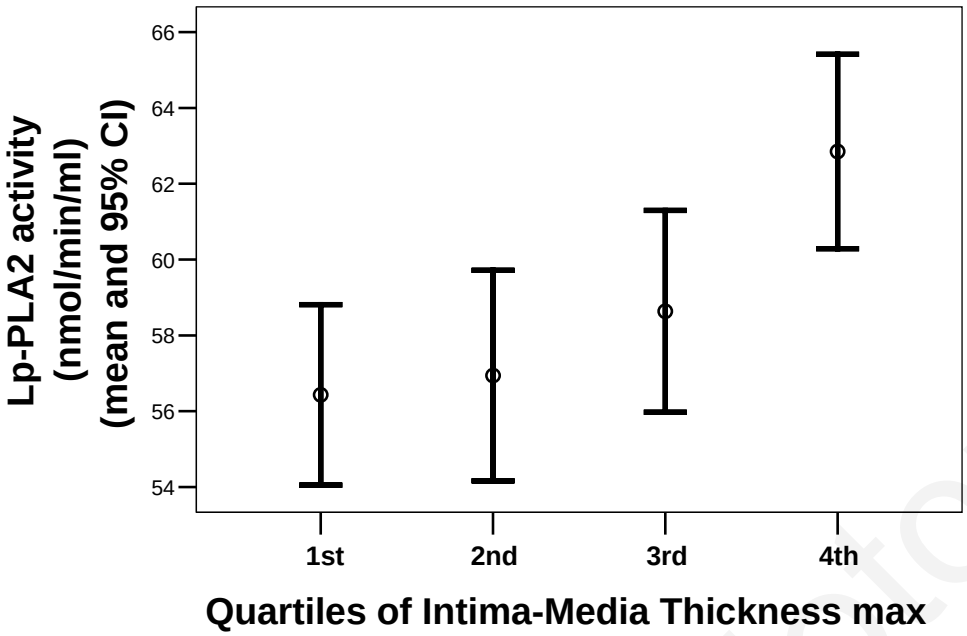

Figure 9.16: Association between Lp-PLA_2 activity levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend<0.001)

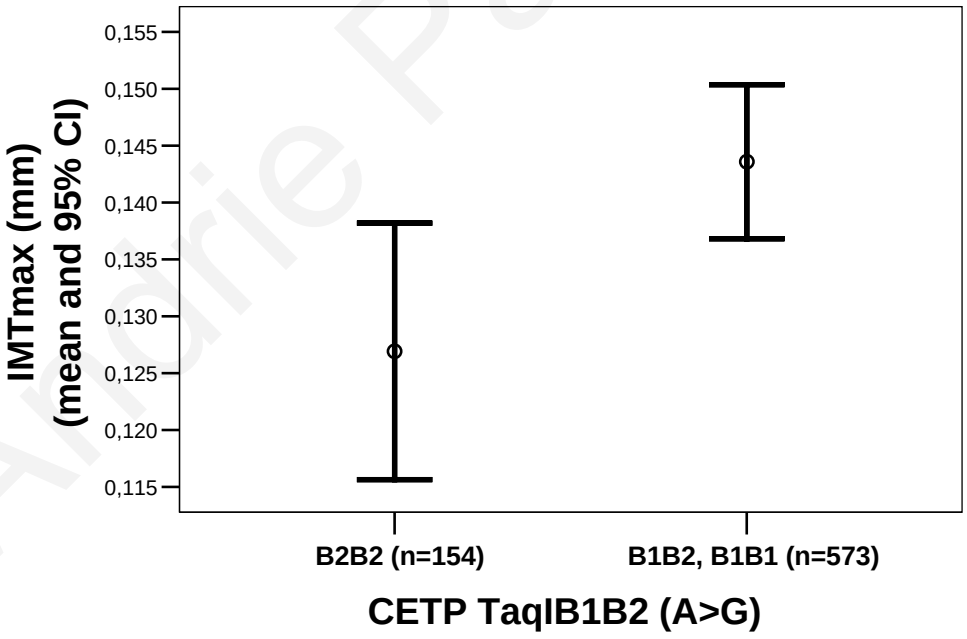

Figure 9.17: Association between IMTmax and *CETP* TaqIB1B2B2 (A>G) polymorphism (Mann-Whitney test; P=0.03)

Table 9.4: Results of univariate linear regressions of associations between lipid markers and IMTmax (logtransformed)

Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
HDL	770	-0.008	**P<0.001**	-0.172	-0.011 to -0.005	0.028
TChol/HDL	770	0.045	**P=0.009**	0.095	0.011 to 0.078	0.009
LDL	770	0.00	P=0.72	0.013	-0.001 to 0.002	0.000
ApoA1	770	-0.310	**P<0.001**	-0.126	-0.483 to -0.137	0.015
ApoB	770	0.106	P=0.23	0.043	-0.069 to 0.280	0.002
ApoB/ApoA1	770	0.282	**P=0.004**	0.104	0.092 to 0.473	0.010
Lp-PLA$_2$ activity	752	0.004	**P<0.001**	0.132	0.002 to 0.006	0.016
***CETP* (TaqIB1B2B2)**	725	0.110	**P=0.033**	0.079	0.009 to 0.212	0.005

Table 9.5: Results of association between lipid markers and IMTmax (logtransformed) using multivariate linear regression analyses (a-h) in which each variable was adjusted for age, sex, smoking (packyears), diabetes and hypertention (Baseline R^2=0.326)

Model	Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
(a)	**HDL**	770	-0.004	**P=0.018**	-0.077	-0.007 to -0.001	0.330
(b)	**TChol/HDL**	770	0.038	**P=0.010**	0.079	0.009 to 0.066	0.331
(c)	**LDL**	770	0.002	**P=0.004**	0.085	0.001 to 0.003	0.333
(d)	**ApoA1**	770	-0.184	**P=0.019**	-0.075	-0.338 to -0.030	0.336
(e)	**ApoB**	770	0.208	**P=0.005**	0.084	0.064 to 0.351	0.333
(f)	**ApoB/ApoA1**	770	0.306	**P<0.001**	0.113	0.143 to 0.468	0.337
(g)	**Lp-PLA_2 activity**	752	0.003	**P<0.001**	0.110	0.002 to 0.005	0.333
(h)	***CETP* (TaqIB1B2B2)**	725	0.113	**P=0.008**	0.081	0.029 to 0.196	0.337

Association between lipid markers and TPT

The association between lipid markers and TPT was tested. In univariate linear regression analyses HDL, the ratio TChol/HDL, TG, apoA1, the ratio apoA1/apoB, Lp-PLA_2 activity and the *CETP* I405V polymorphism were significantly associated with TPT (logtransformed to fit the assumption of normality). Significant results are shown in figures 9.18-24 and table 9.6.

In multivariate linear regression analyses including traditional risk factors (age, sex, smoking in packyears, diabetes and hypertension) and each variable HDL, the ratio of TChol/HDL, LDL, TG, apoA1, apoB, the ratio of apoB/apoA1, Lp-PLA_2 activity and *CETP* I405V (VV vs VI vs II) polymorphism all remained significantly associated with TPT over and above traditional risk factors. Results are shown in table 9.7. A multivariate model including all the above variables (except apoB and apoA1) and adjusting for traditional risk factors, could explain 32.6% of the variability in TPT compared to 29% if the model included only the traditional risk factors (age, sex, smoking in packyears, diabetes and hypertention).

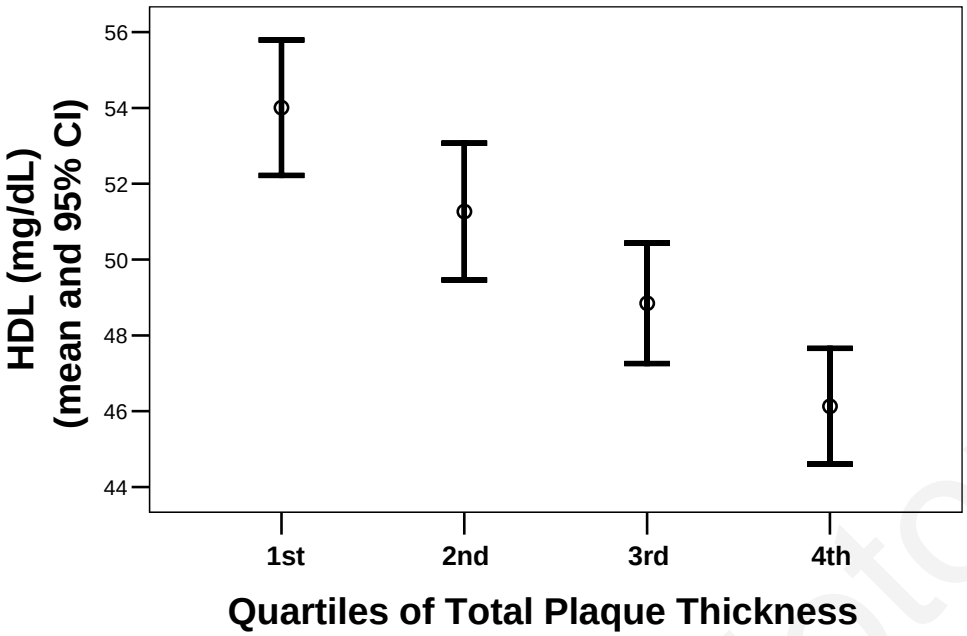

Figure 9.18: Association between HDL levels and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

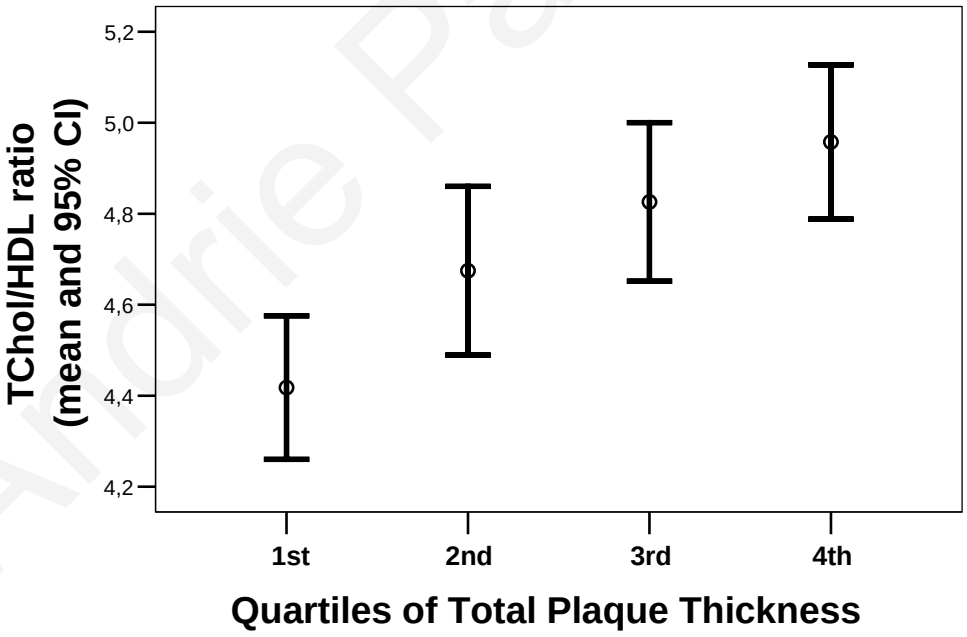

Figure 9.19: Association between TChol/HDL ratio and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

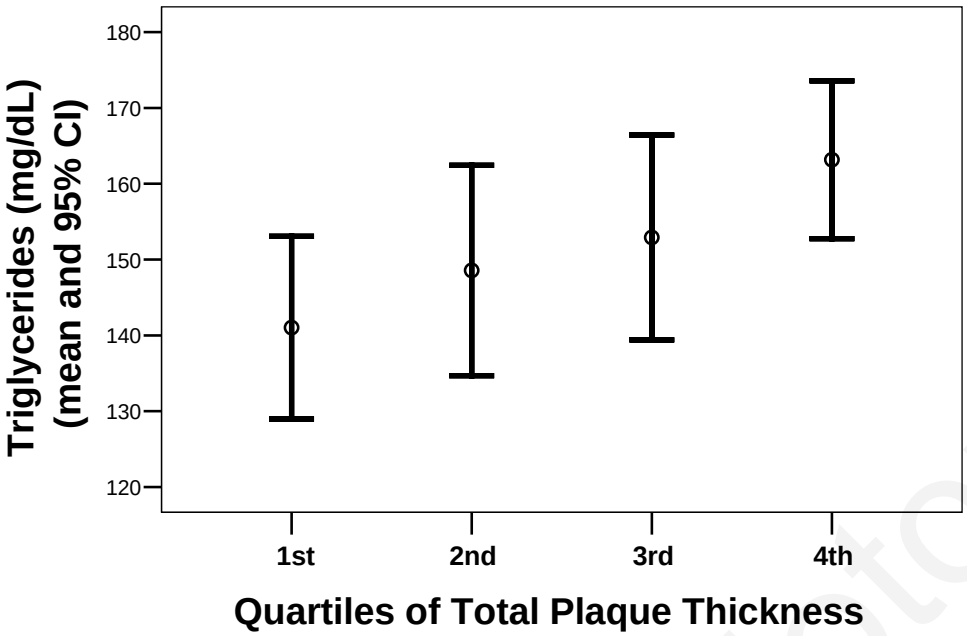

Figure 9.20: Association between triglyceride levels and quartiles of TPT. (Kruskal-Wallis test; P for trend<0.001)

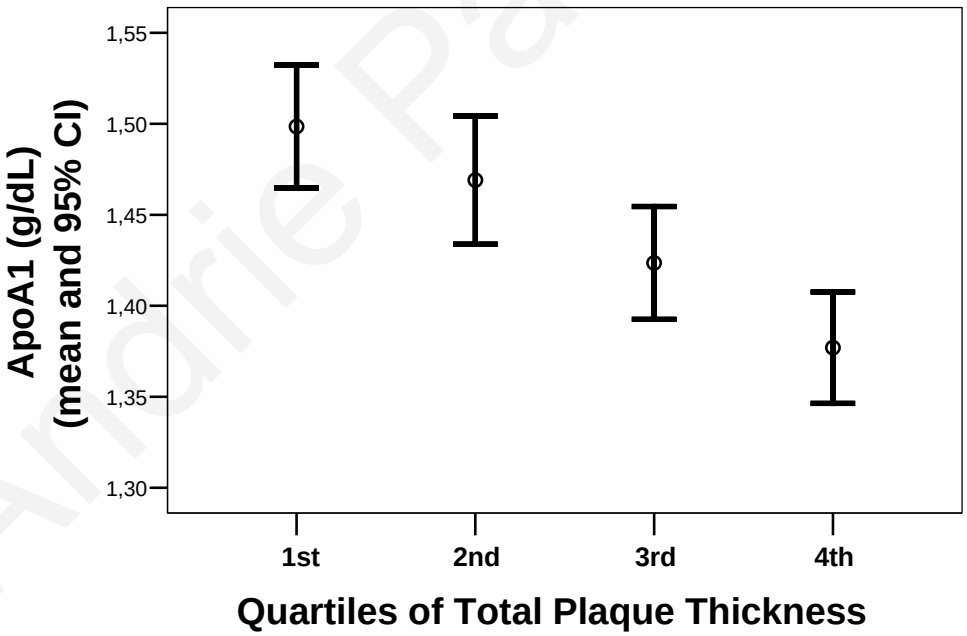

Figure 9.21: Association between apoA1 levels and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

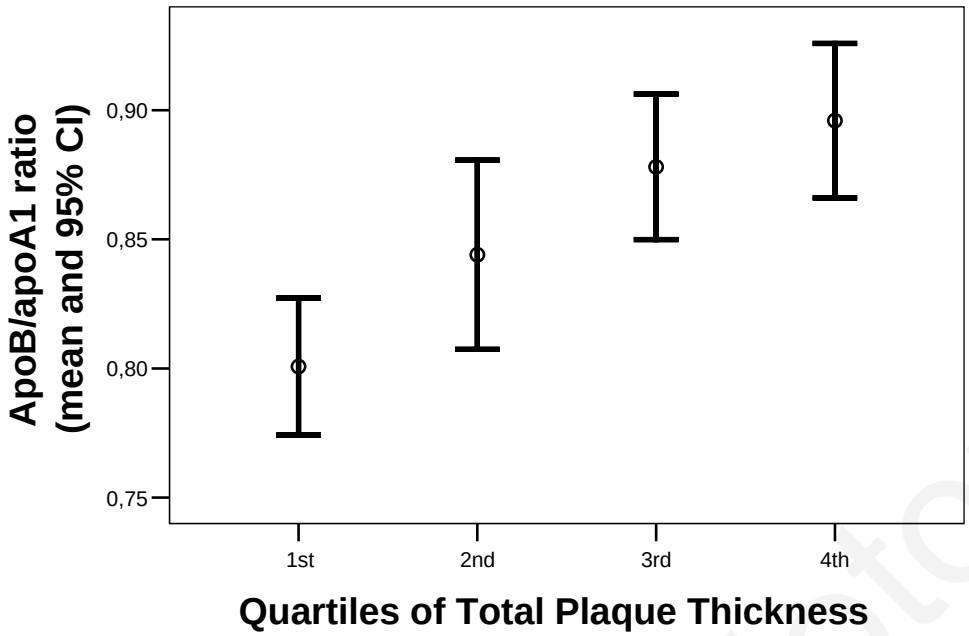

Figure 9.22: Association between apoB/apoA1 ratio and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

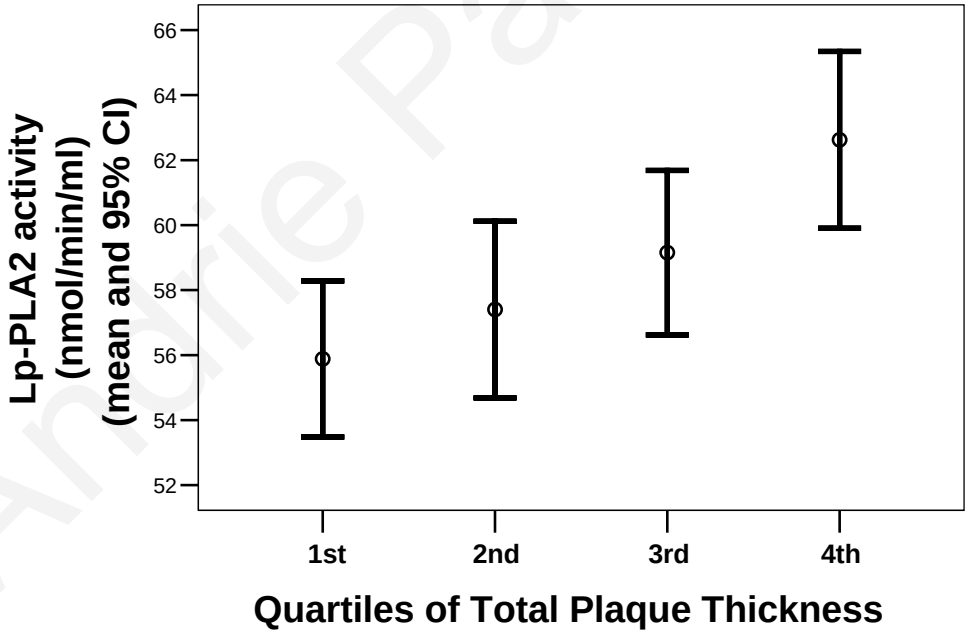

Figure 9.23: Association between Lp-PLA$_2$ activity and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

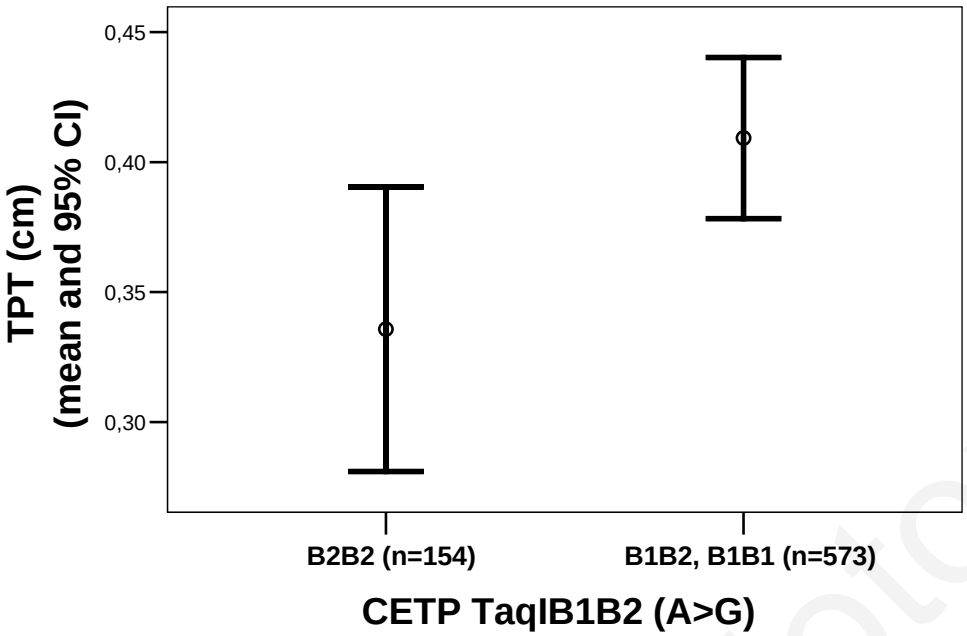

Figure 9.24: Association between TPT and *CETP* I405V genotype (Kruskal-Wallis test; P for trend=0.37)

Table 9.6: Results of univariate linear regressions of association between lipid markers and TPT (logtransformed)

Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
HDL	770	-0.011	**P<0.001**	-0.192	-0.016 to -0.006	0.035
TChol/HDL	770	0.062	**P=0.007**	0.115	0.017 to 0.106	0.011
LDL	770	-3,2E-005	P=0.97	-0.001	-0.002 to 0.002	-0.002
TG	770	0.001	**P=0.020**	0.099	0.00 to 0.001	0.008
ApoA1	770	-0.528	**P<0.001**	-0.181	-0.768 to -0.287	0.031
ApoB	770	0.090	P=0.45	0.033	-0.142 to 0.322	-0.001
ApoB/ApoA1	770	0.377	**P=0.003**	0.127	0.126 to 0.627	0.014
Lp-PLA$_2$ activity	752	0.005	**P=0.003**	0.130	0.002 to 0.008	0.015
***CETP* (I405V)**	708	-0.081	P=0.057	-0.084	-0.163 to 0.002	0.005

Table 9.7: Results of multivariate linear regression analyses of association between lipid markers and TPT (logtransformed) (a-i) in which each variable was adjusted for age, sex, smoking (packyears), diabetes and hypertention (Baseline R^2=0.29)

Model	Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
(a)	**HDL**	770	-0.005	**P=0.026**	-0.087	-0.009 to -0.001	0.295
(b)	**TChol/HDL**	770	0.064	**P=0.002**	0.119	0.024 to 0.103	0.301
(c)	**LDL**	770	0.002	**P=0.003**	0.108	0.001 to 0.004	0.300
(d)	**TG**	770	0.001	**P=0.016**	0.087	0.00 to 0.001	0.296
(e)	**ApoA1**	770	-0.312	**P=0.006**	-0.107	-0.534 to -0.09	0.298
(f)	**ApoB**	770	0.312	**P=0.002**	0.113	0.114 to 0.509	0.301
(g)	**ApoB/ApoA1**	770	0.446	**P<0.001**	0.148	0.227 to 0.666	0.309
(h)	**Lp-PLA_2 activity**	752	0.004	**P=0.006**	0.103	0.001 to 0.006	0.303
(i)	***CETP* (I405V)**	708	-0.076	**P=0.032**	-0.080	-0.145 to 0.004	0.306

Association between lipid markers and presence of plaques

The association between lipid markers and presence of plaques was tested. Independent samples t-test was used to compare means of continuous (biochemical) lipid markers for presence against absence of plaques. The ratio TChol/HDl, TG, apoB, the ratio of apoB/apoA1 and Lp-PLA_2 activity were significantly associated with presence of plaques. HDL and apoA1 levels were associated with absence of plaques. Significant results are shown in figures 9.25-31 and p values are tabulated in table 9.8. Crosstabulation was used to check for association between the genetic polymorphisms and presence of plaques. *Lp-PLA_2* (A379V), *apoB* (-516C>T) or *CETP* (TaqIB1B2B2 and I405V) were not associated with presence of plaques. The *apoE* (E2/E3/E4) polymorphism was significantly associated with presence of plaques (P=0.036 for E2/E3 vs E3/E3, E3/E4) (Fig.9.37).

In binary logistic regression analyses testing for association with presence of plaques and adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes and hypertention) the ratio of TChol/HDL, LDL, apoB, the ratio of apoB/apoA1 and the *apoE* (E2/E3/E4) polymorphism emerged as independent predictors of the presence of plaques over and above the traditional risk factors. In a multivariate model including all the significant variables and correcting for traditional risk factors, only the *apoE* (E2/E3/E4) polymorphism remained significantly associated with presence of plaques (P=0.02 for E2/E2, E2/E3 vs E3/E3, E3/E4). This model could explain 33.8% of the variability in presence of plaques compared to 32.3% if the model included only the traditional risk factors. P values and OR are tabulated in table 9.9.

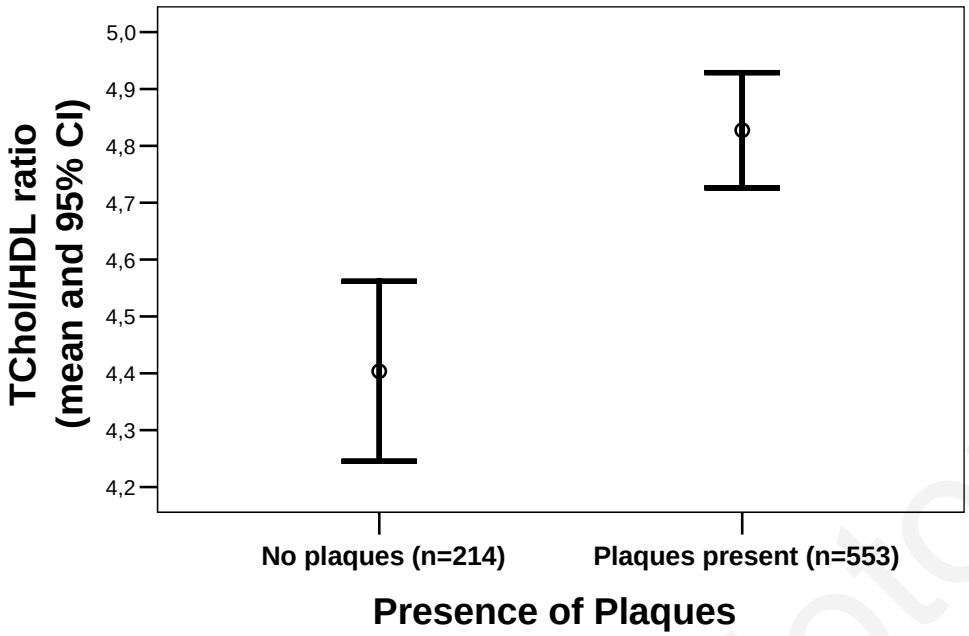

Figure 9.25: Association between TChol/HDL ratio and presence of plaques (T-test; P<0.001)

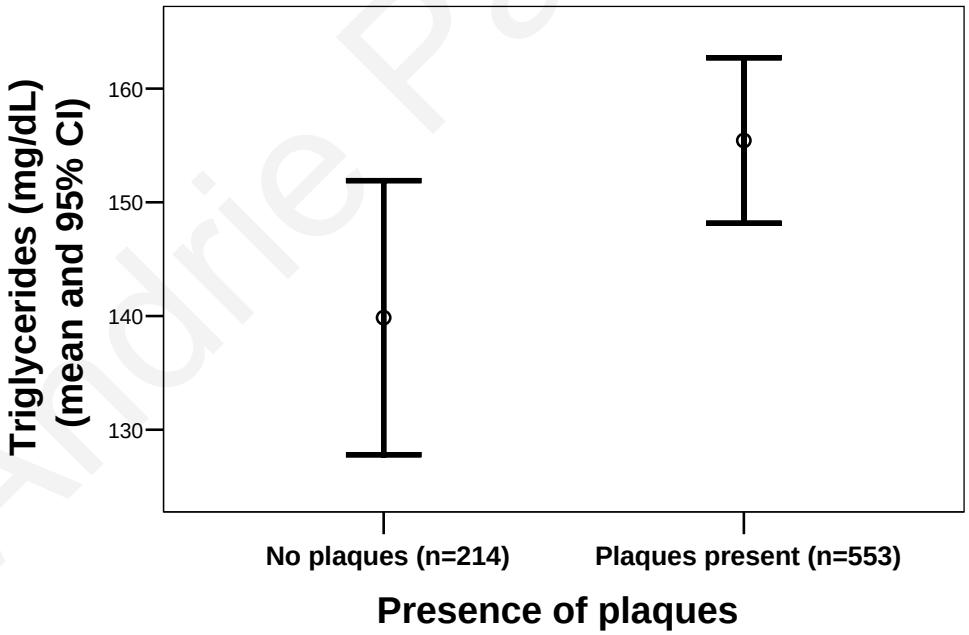

Figure 9.26: Association between triglyceride levels and presence of plaques (T-test; P=0.03)

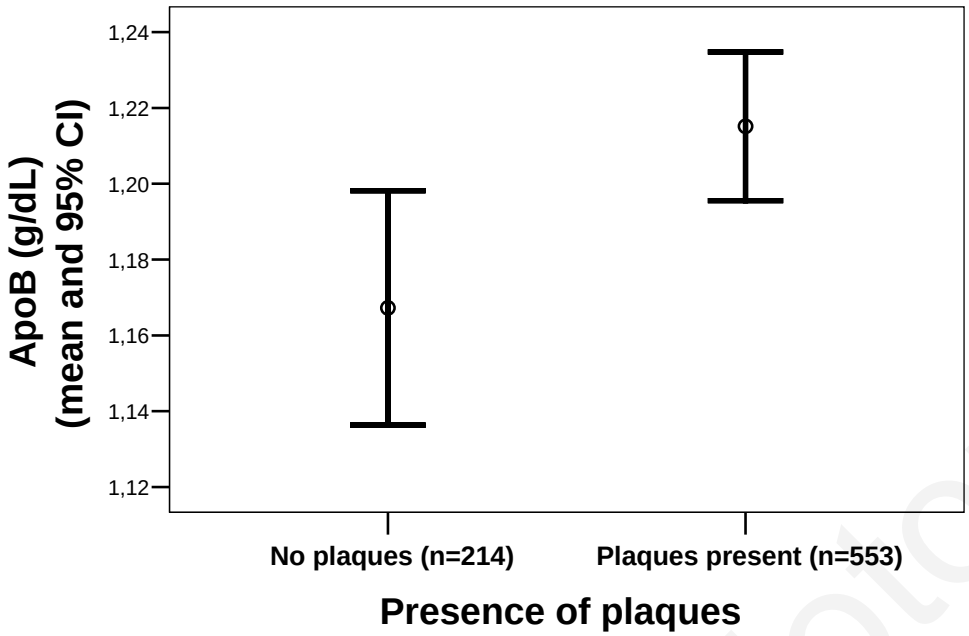

Figure 9.27: Association between apoB levels and presence of plaques (T-test; P=0.01)

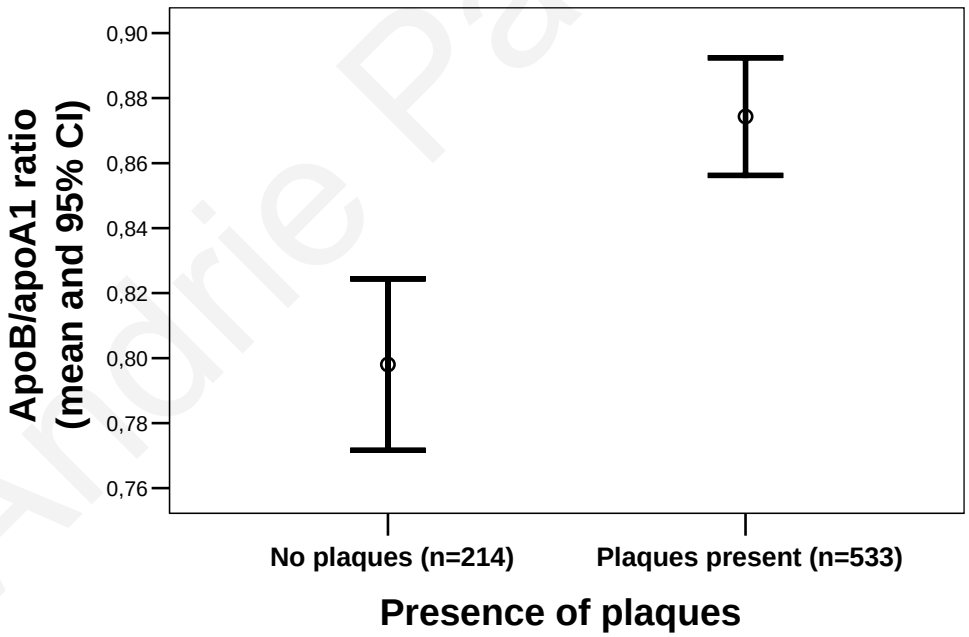

Figure 9.28: Association between apoB/apoA1 ratio and presence of plaques (T-test; P<0.001)

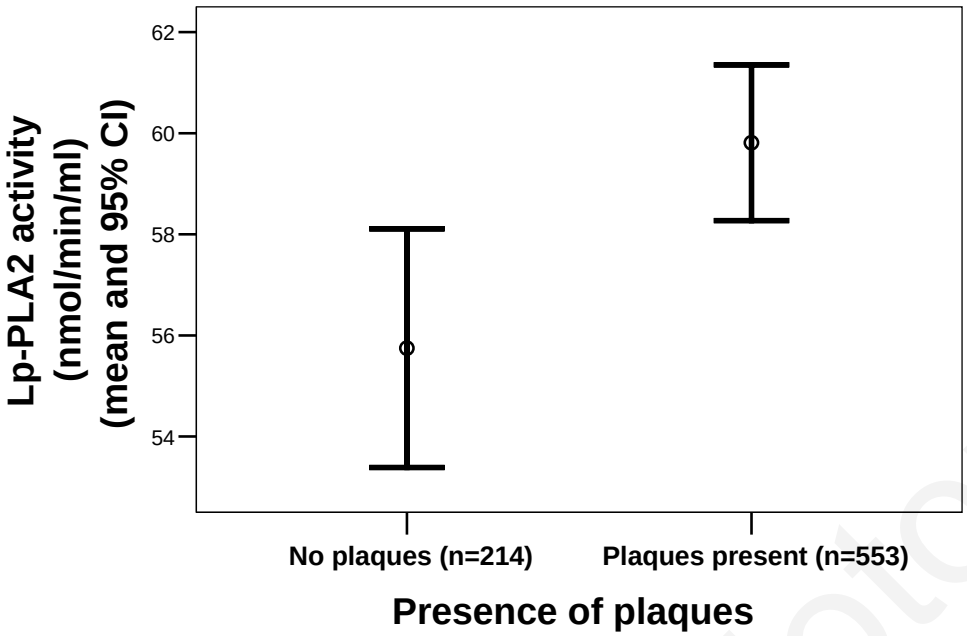

Figure 9.29: Association between Lp-PLA$_2$ activity and presence of plaques (T-test: P=0.005)

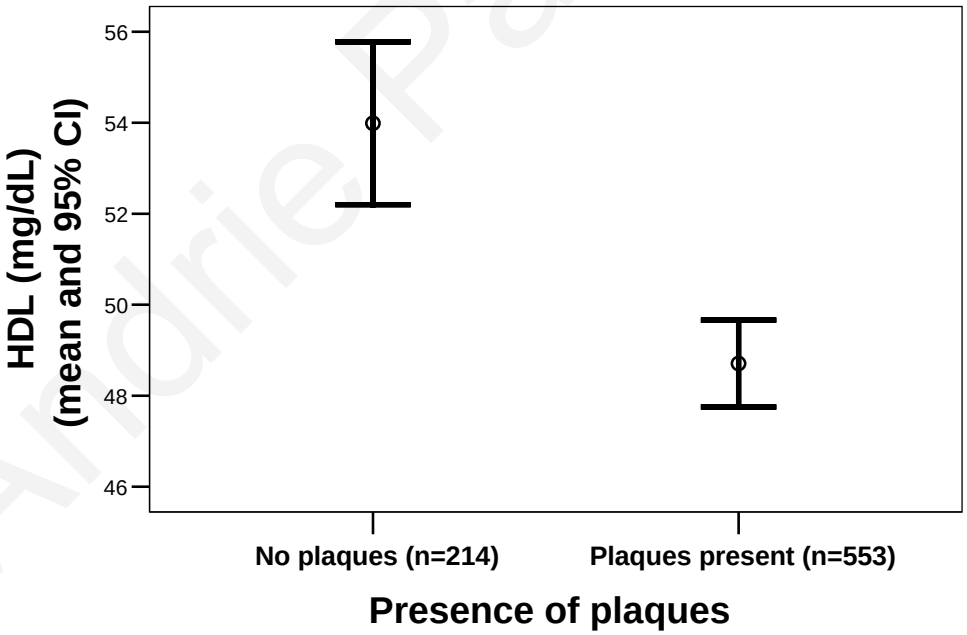

Figure 9.30: Association between HDL levels and absence of plaques (T-test; P<0.001)

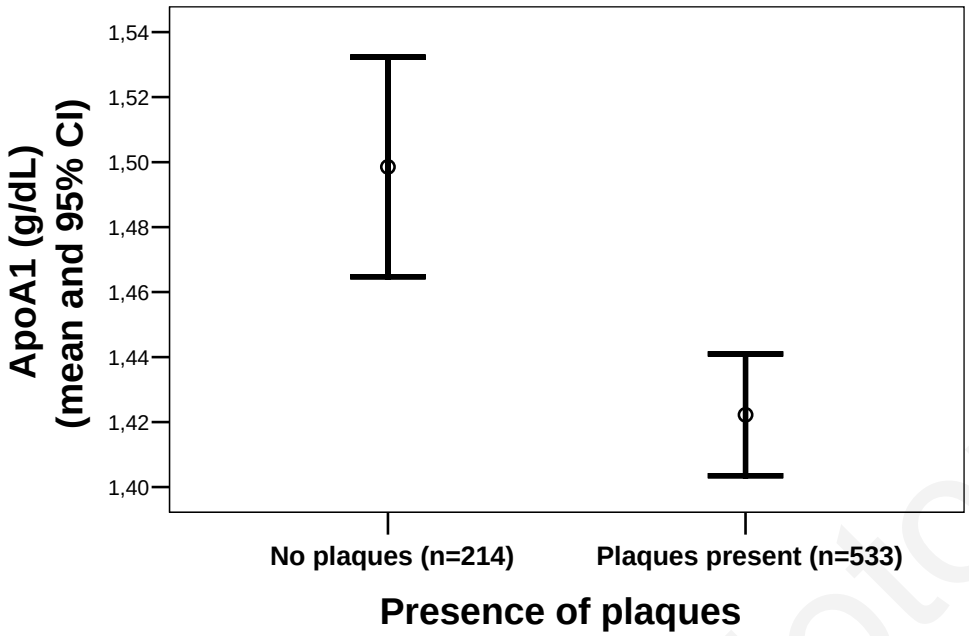

Figure 9.31: Association between apoA1 levels and absence of plaques (T-test; P<0.001)

Table 9.8: Results of Indepentend t-test (univariate) of association between lipid markers (continuous) and presence of plaques

Marker	**P value Univariate**	**95% CI for P Univariate**
TChol	P=0.77	-7.34 to 5.42
HDl	**P<0.001**	-7.31 to -3.26
TChol/HDL	**P<0.001**	0.24 to 0.61
LDL	P=0.10	-0.78 to 8.27
TG	**P=0.03**	1.52 to 29.63
ApoA1	**P<0.001**	-0.12 to 0.38
ApoB	**P=0.01**	0.011 to 0.085
ApoB/ApoA1	**P<0.001**	0.04 to 0.11
*Lp(a)	P=0.34	
Lp-PLA_2 activity	**P=0.005**	1.25 to 6.88
ApoE (E2/E3/E4)	**P=0.036**	

* Lp(a) was tested with a Mann-Whitney test for skewd data

Table 9.9: Results of binary logistic regressions (a-k) of association between lipid markers (continuous) and presence of plaques (ORs are adjusted for age, sex, smoking in packyears, DM and hypertension)

Model	Marker	P value for multivariate	OR (95% CI)
(a)	TChol	P=0.08	1.0 (1.0 to 1.009)
(b)	HDL	P=0.12	0.99 (0.97 to 1.003)
(c)	TChol/HDL	**P=0.035**	1.2 (1.01 to 1.41)
(d)	LDL	**P=0.004**	1.01 (1.003 to 1.02)
(e)	TG	P=0.83	1.0 (1.0 to 1.002)
(f)	ApoA1	P=0.15	0.55 (0.24 to 1.24)
(g)	ApoB	**P=0.006**	3.10 (1.39 to 6.89)
(h)	ApoB/ApoA1	**P=0.002**	4.34 (1.68 to 11.20)
(i)	Lp(a)	P=0.10	1.0 (1.0 to 1.001)
(j)	Lp-PLA_2 activity	P=0.25	1.0 (1.0 to 1.02)
(k)	*ApoE* (E2/E3/E4)	**P=0.005**	2.76 (1.37 to 5.57)

Association between lipid markers and number of bifurcations with plaques

The association between lipid markers and number of bifurcations with plaques was also tested. HDL, the ratio of TChol/HDL, apoA1, the ratio of apoB/apoA1 and Lp-PLA_2 activity were significantly associated with number of bifurcations with plaques (Figs.9.32-36).

By using 2 bifurcations with plaque as a cut-off point, with more than 2 bifurcations with plaque indicating generalised atherosclerosis (presence of plaques in both carotid and femoral arteries), TChol, HDL and apoA1 levels were associated with less than 2 bifurcations with plaque and the ratio of TChol/HDL, TG, the ratio apoB/apoA1 and Lp-PLA_2 activity were all significantly associated with more than 2 bifurcations with plaques (Table 9.10).

In binary logistic regression analyses, testing for association with more than 2 bifurcations with plaques and adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes and hypertention), HDL, the ratio of TChol/HDL, LDL, apoA1, apoB, the ratio apoB/apoA1, Lp(a) and Lp-PLA_2 activity were all independently associated with number of bifurcations with plaques. P values and OR are tabulated in table 9.11

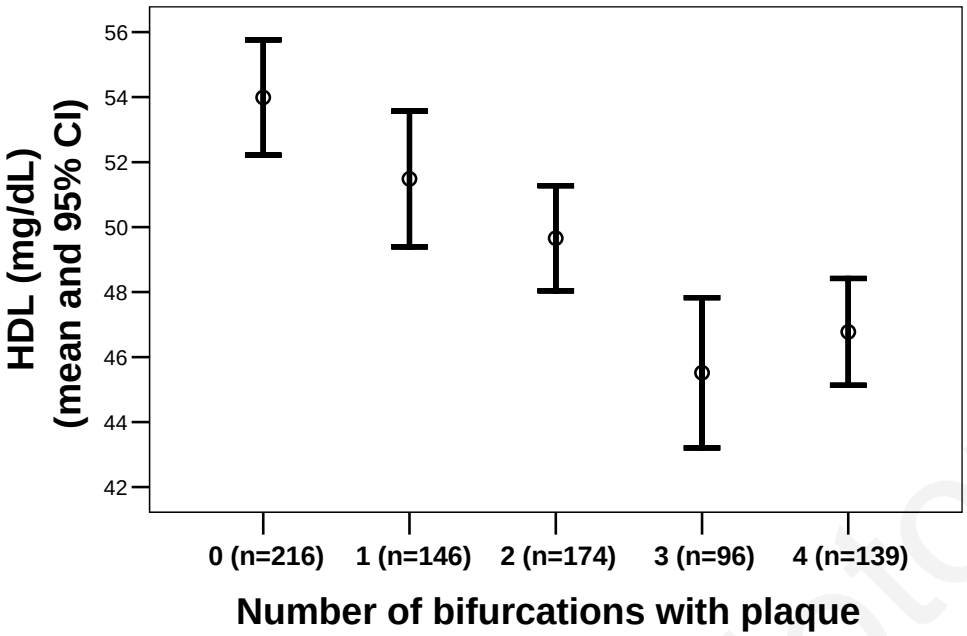

Figure 9.32: Association between HDL levels and number of bifurcations with plaque (ANOVA; P for trend<0.001)

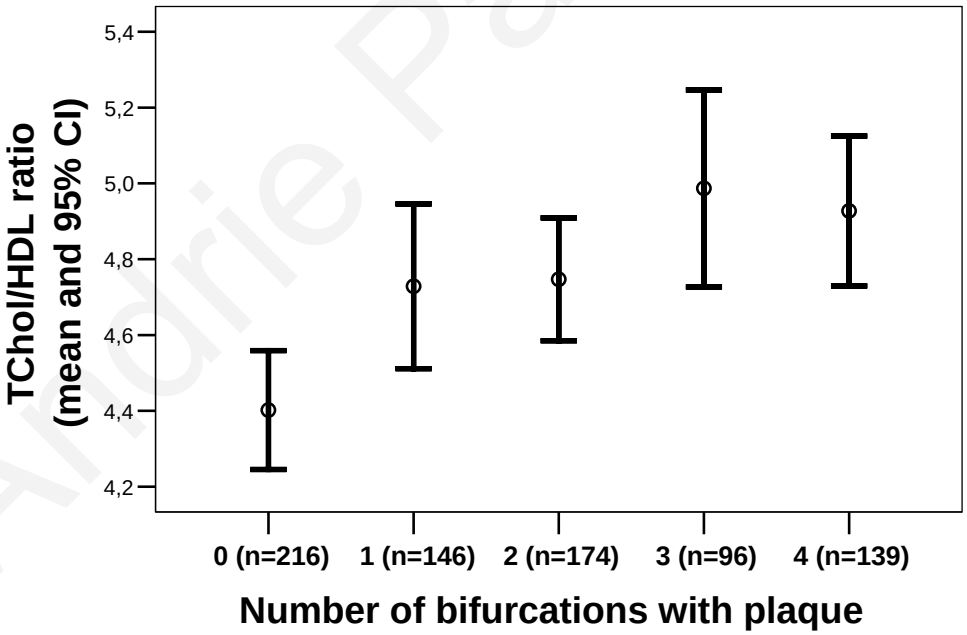

Figure 9.33: Association between TChol/HDl ratio and number of bifurcations with plaques (ANOVA; P for trend<0.001)

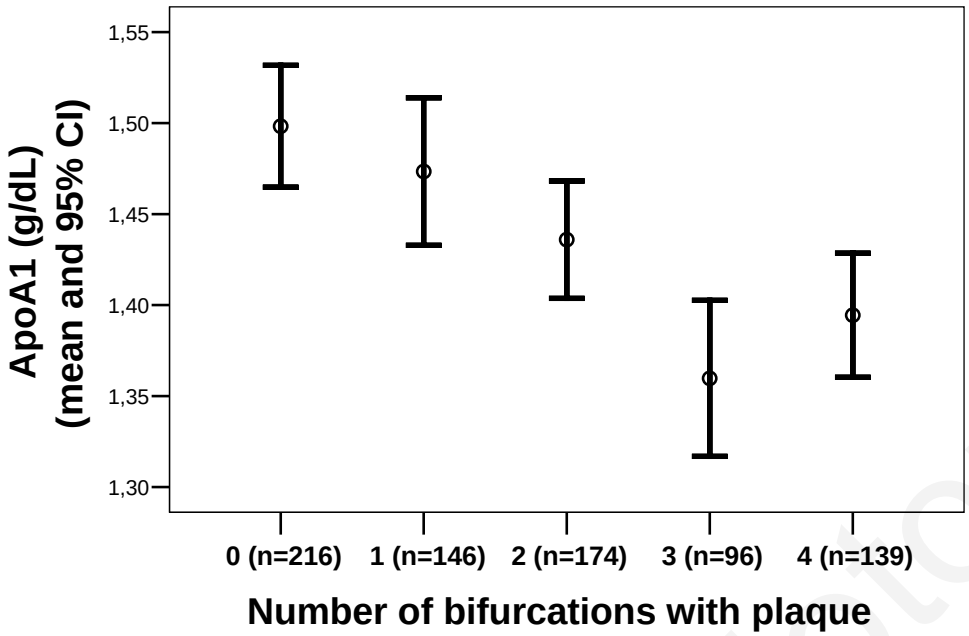

Figure 9.34: Association between apoA1 levels and number of bifurcations with plaque (ANOVA; P for trend<0.001)

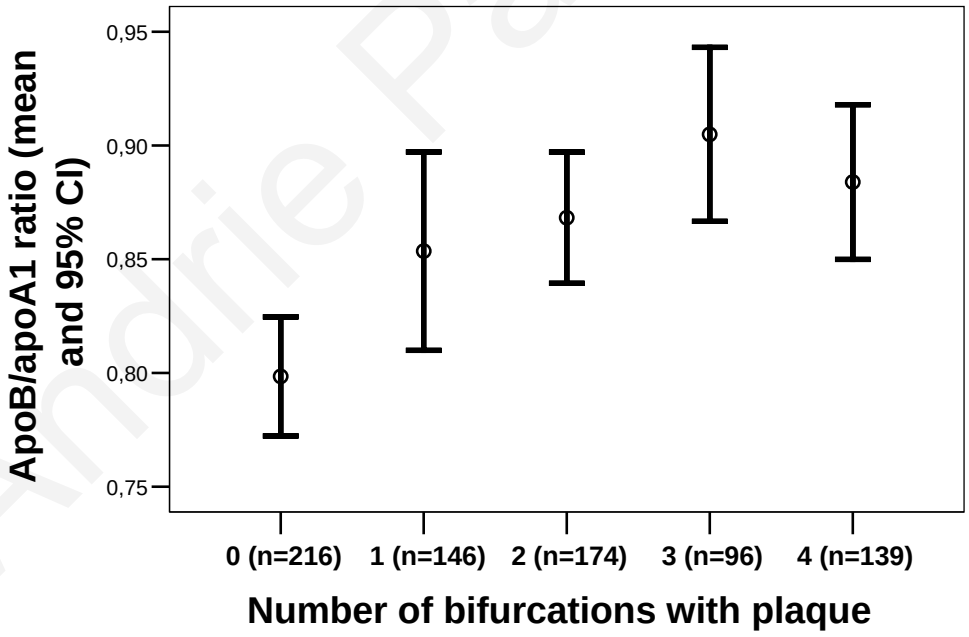

Figure 9.35: Association between apoB/apoA1 ratio and number of bifurcations with plaques (ANOVA; P for trend<0.001)

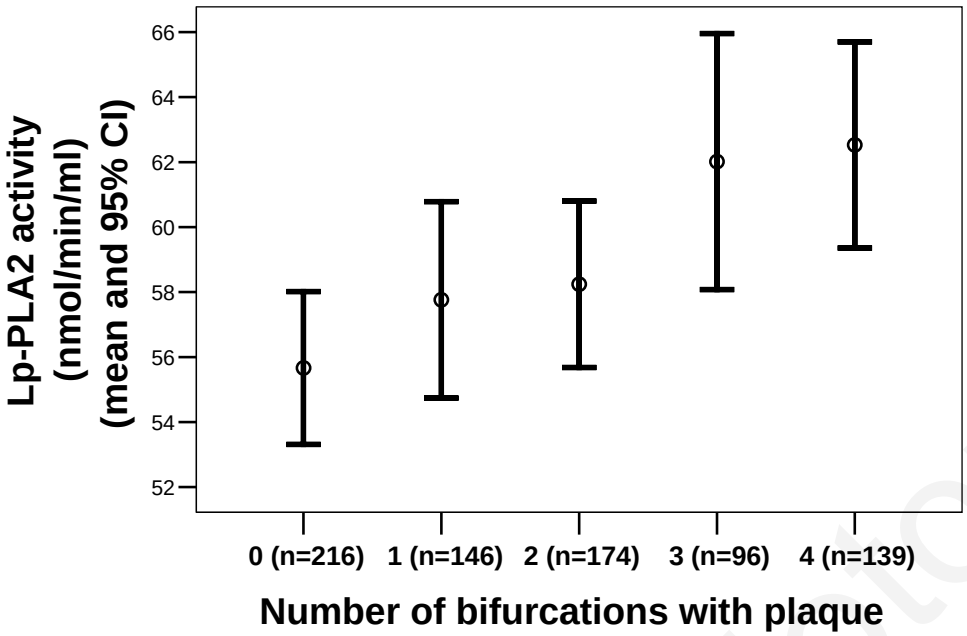

Figure 9.36: Association between Lp-PLA$_2$ activity levels and number of bifurcations with plaques (ANOVA; P for trend=0.003)

Table 9.10: Results of indepentend t-tests (univariate) of association between lipid markers (continuous) and more than 2 bifurcations with plaques

Marker	P value	95% CI for P
TChol	P=0.014	1.59 to 14.27
HDl	**P<0.001**	3.29 to 7.38
TChol/HDL	**P<0.001**	-0.54 to -0.16
LDL	P=0.66	-3.52 to 5.53
TG	**P=0.04**	-25.49 to -0.059
ApoA1	**P<0.001**	0.058 to 0.125
ApoB	P=0.55	-0.046 to 0.025
ApoB/ApoA1	**P<0.001**	-0.088 to -0.025
*Lp(a)	P=0.26	
Lp-PLA_2	**P<0.001**	-8.10 to -2.33

*Lp(a) was skewed so a Mann-Whitney test was used instead.

Table 9.11: Results of binary logistic regressions (a-j) of association between lipid markers (continuous) and more than 2 bifurcations with plaque (ORs are adjusted for age, sex, smoking in packyears, DM and hypertension)

Model	Marker	P value for multivariate	OR (95% CI)
(a)	TChol	P=0.39	1.0 (1.0 to 1.007)
(b)	HDl	**P=0.005**	0.98 (0.96 to 0.99)
(c)	TChol/HDL	**P=0.002**	1.3 (1.09 to 1.48)
(d)	LDL	**P=0.038**	1.01 (1.0 to 1.013)
(e)	TG	P=0.19	1.0 (0.99 to 1.003)
(f)	ApoA1	**P=0.008**	0.3 (0.12 to 1.73)
(g)	ApoB	**P=0.027**	2.5 (1.11 to 5.59)
(h)	ApoB/ApoA1	**P=0.001**	4.23 (1.76 to 10.18)
(i)	Lp(a)	**P=0.035**	1.0 (1.0 to 1.001)
(j)	Lp-PLA_2 activity	**P=0.007**	1.0 1 (1.004 to 1.024)

Association between lipid markers and MPT

The association between lipid markers and mean plaque type was tested. As described in chapter 8, for analyses of association between MPT and lipid markers the MPT median was used as a cut-off point. In univariate analyses, HDL levels, the TChol/HDL ratio, apoA1, apoB levels, the apoB/apoA1 ratio and Lp-PLA_2 activity were significantly associated with MPT. Triglycerides had a borderline P value (P=0.058). From the genetic polymorphisms tested, the *apoE* (E2/E3/E4) and the *CETP* (TaqIB1B2B2B2) (B2B2 vs B1B2, B1B1) were also associated with MPT <2.75 (P=0.042 and P=0.04 respectively). Significant results are shown in figures 9.37-45.

In multivariate binary logistic analyses, in those with plaques, adjusting for age, sex, smoking (packyears), DM and hypertension only the *CETP* (TaqIB1B2B2) polymorphisms remained significantly associated with MPT (P=0.017; OR: 0.516; 95% CI: 0.299 to 0.89). Adding *CETP* to the model improved its predictive ability by 1.9% and could explain 16.5% of the variability in MPT. In all the other multivariate models tested only age was significantly associated with MPT over 2.75.

Association between lipid markers and BPB

In univariate analyses HDL, the ratio TChol/HDL, TG, apoA1, apoB, the ratio apoA1/apoB, Lp(a) and Lp-PLA_2 activity were significantly associated with BPB. None of the genetic polymorphisms tested was associated with BPB. Significant results are shown in table 9.12 and figures 9.45-52.

In multivariate analyses adjusting for age, sex, smoking (packyears), DM and hypertension HDL, the ratio TChol/HDL, LDL, TG, apoA1, apoB, the ratio apoA1/apoB, Lp(a) and Lp-PLA_2 activity all remained significantly associated with BPB over and above traditional risk factors. In the multivariate analyses, the *CETP* TaqIB1B2 (B2B2 vs B1B2, B1B1) genetic polymorphism was also associated with BPB. Results are shown in table 9.13. In a multivariate model including all the significant markers and adjusting for traditional risk factors, HDL (P=0.047), the TChol/HDL ratio (P=0.014), LDL (P=0.009), TG (P=0.016), Lp(a) (P=0.015) and Lp-PLA_2 activity (P=0.014) were all significant predictors of BPB and the model could predict 32.2% of the variability in BPB compared with 30.3% if only the traditional risk factors were included in the model. Adding only the ratio of apoB/apoA1 improved the model by 1.7%.

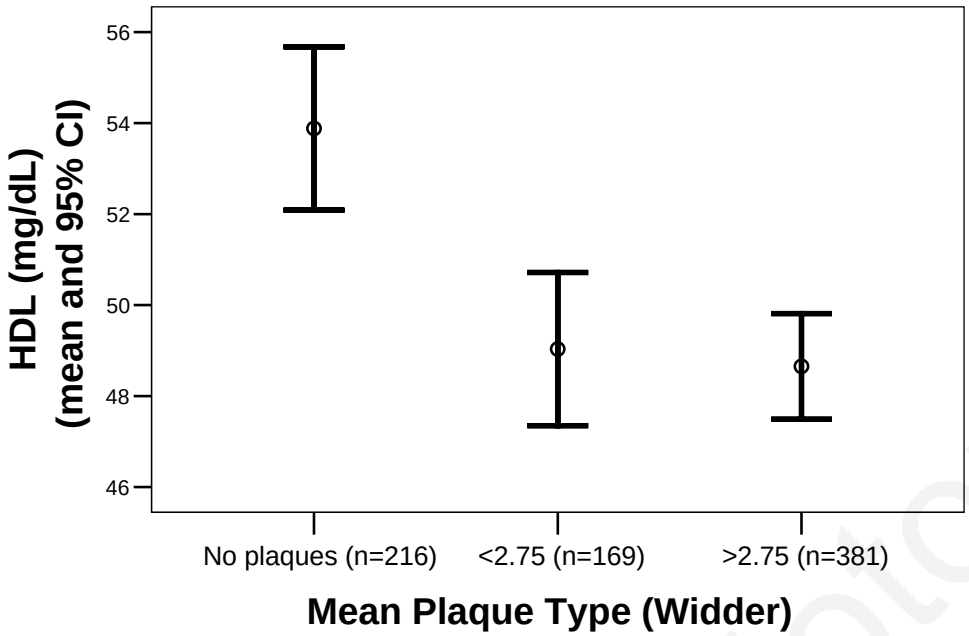

Figure 9.37: Association between HDL levels and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

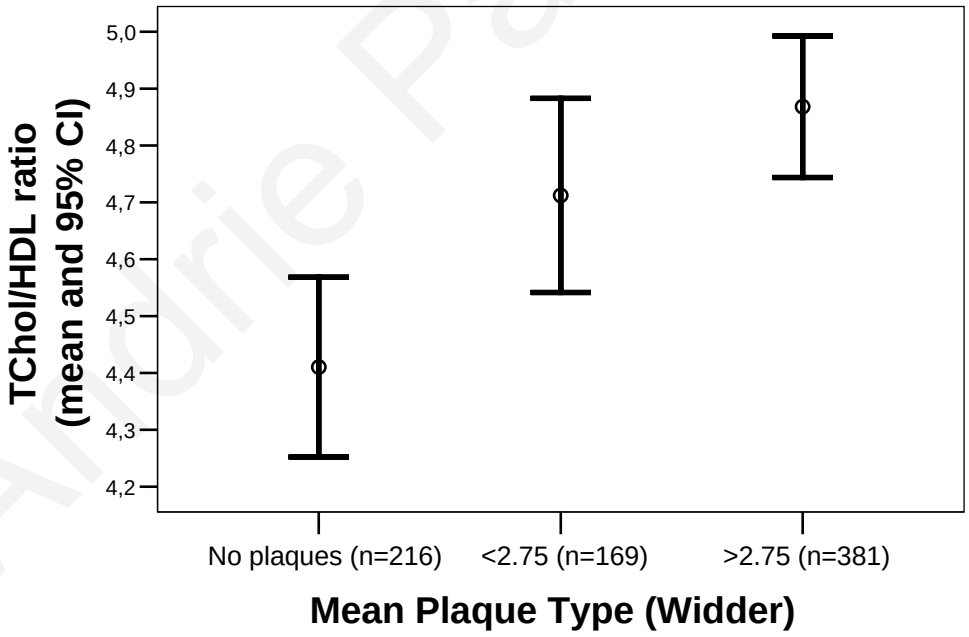

Figure 9.38: Association between TChol/HDL ratio and and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

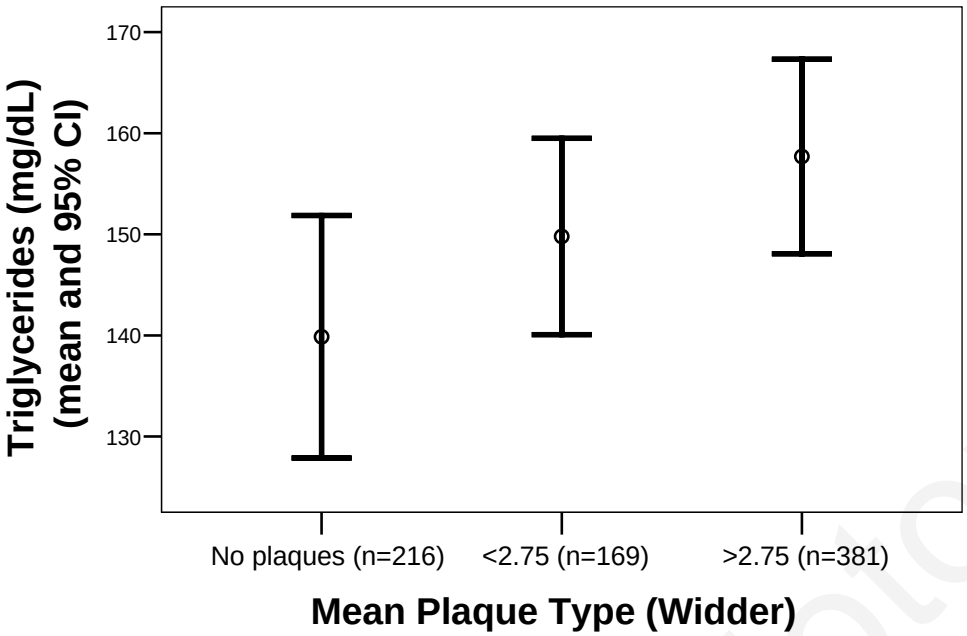

Figure 9.39: Association between triglyceride levels and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

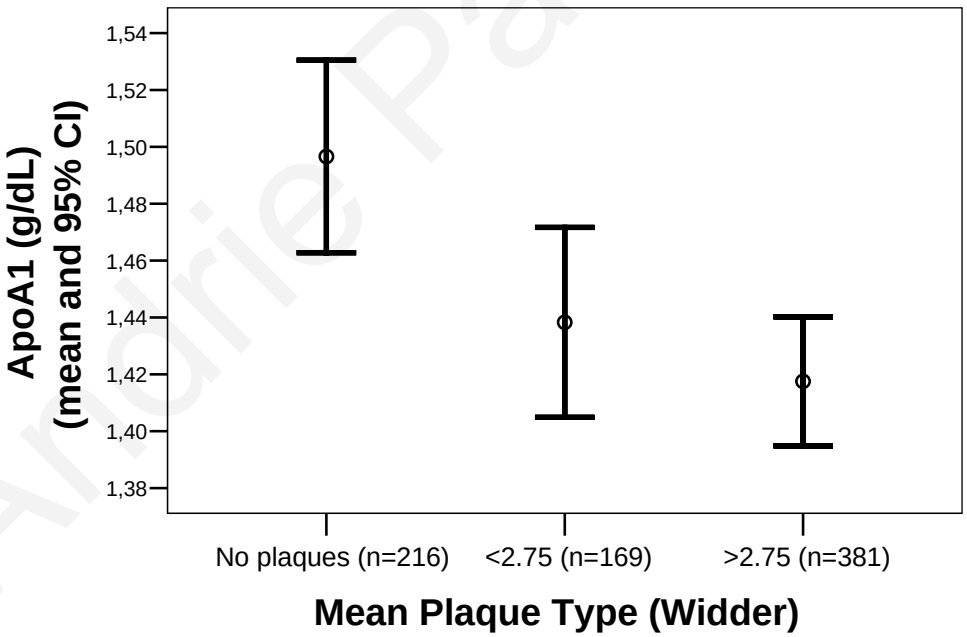

Figure 9.40: Association between apoA1 levels and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

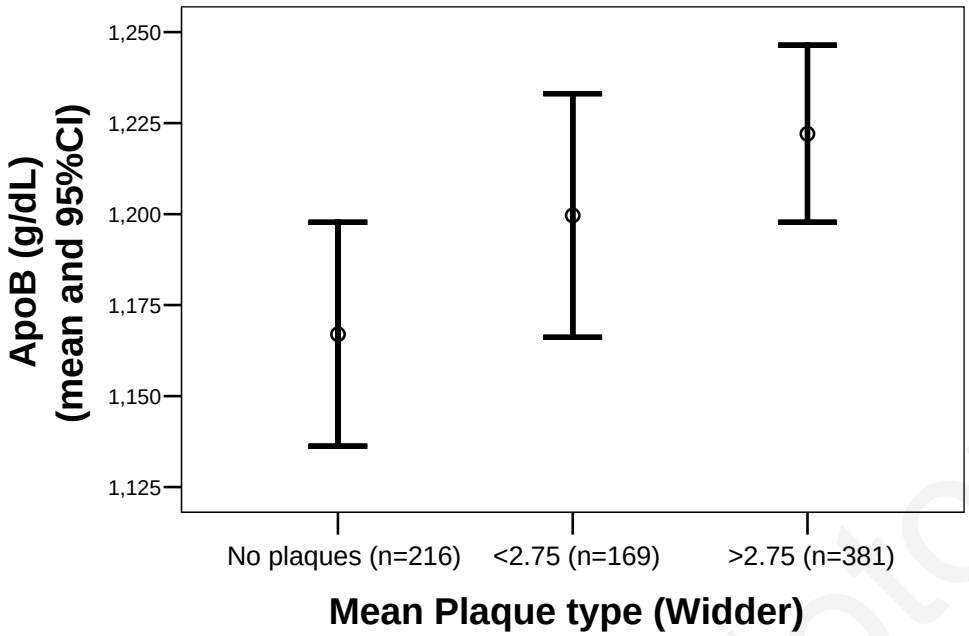

Figure 9.41: Association between apoB levels and MPT (Widder) below and over the median (ANOVA; P for trend=0.022)

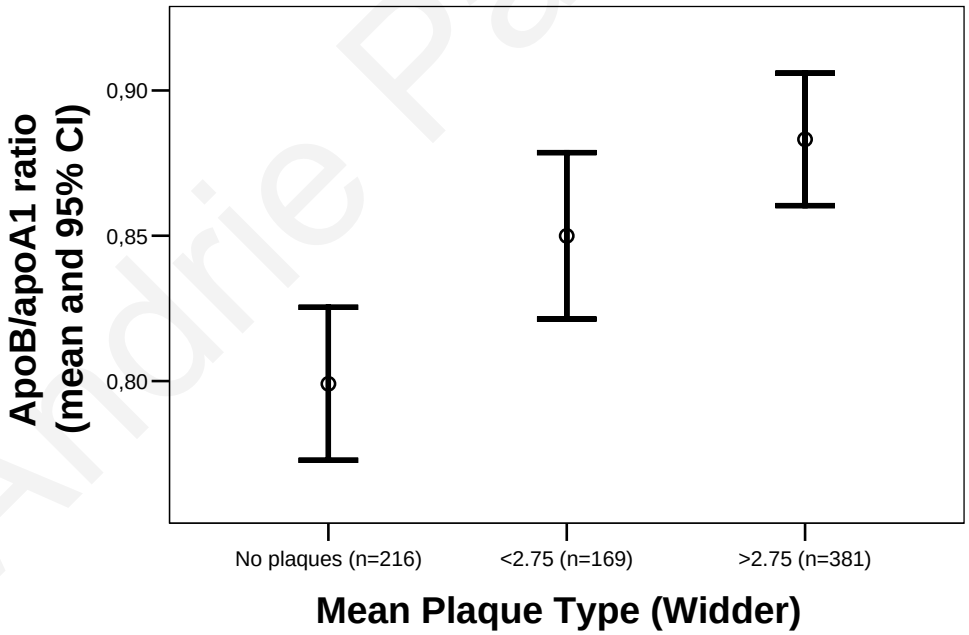

Figure 9.42: Association between apoB/apoA1 ratio and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

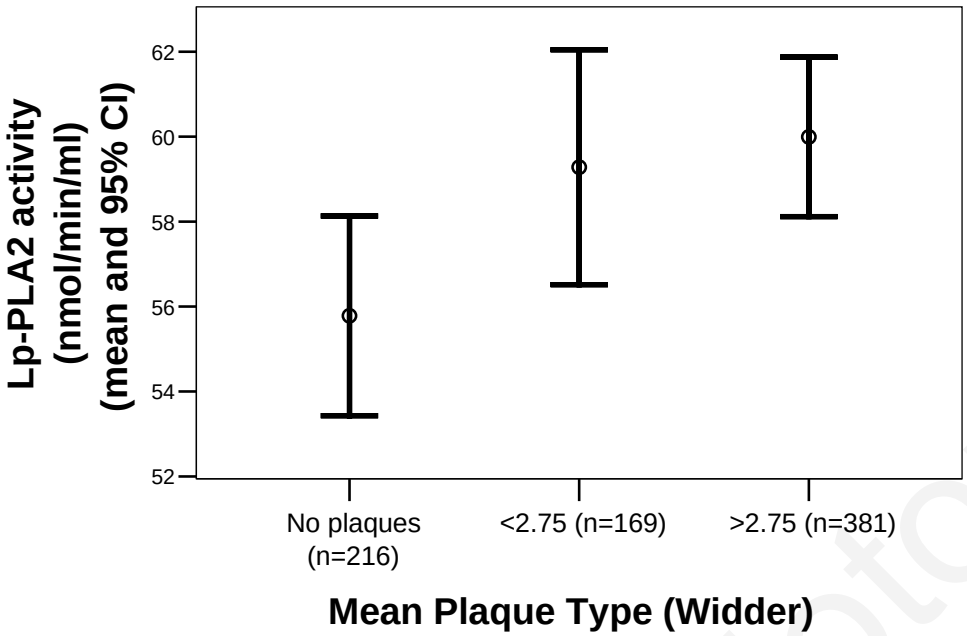

Figure 9.43: Association between Lp-PLA$_2$ activity levels and MPT (Widder) below and over the median (ANOVA; P for trend<0.001)

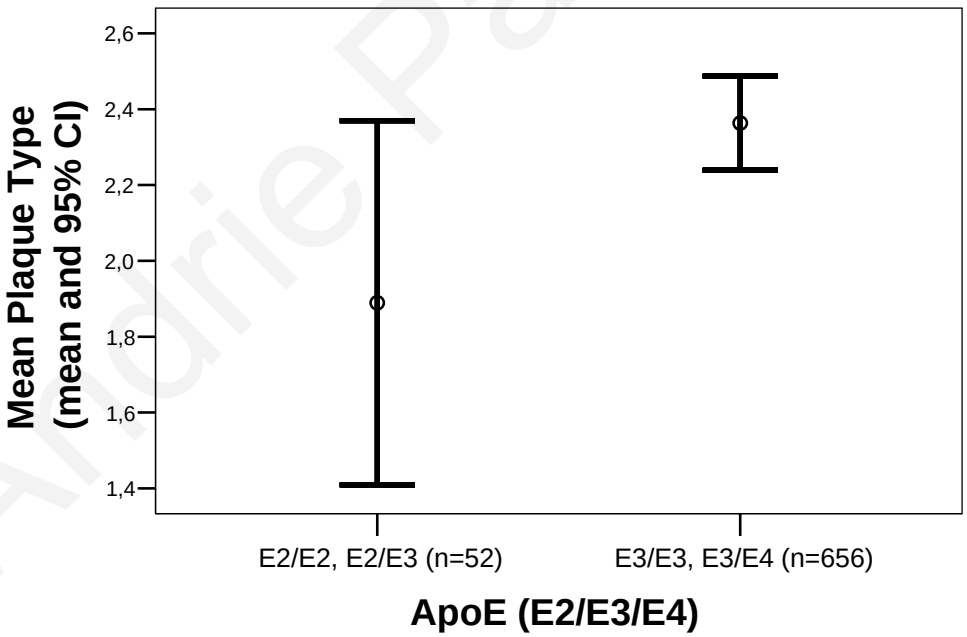

Figure 9.44: Association between MPT (Widder) and *apoE* (E2/E3/E4) polymorphism (Mann-Whitney test; P=0.042)

Table 9.12: Results of association between lipid markers and BPB using univariate linear regression analyses

Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
HDL	763	-0.024	**P<0.001**	-0.252	-0.03 to -0.017	0.062
TChol/HDL	763	0.172	**P<0.001**	0.182	0.106 to 0.238	0.032
LDL	763	0.001	P=0.406	0.030	-0.002 to 0.004	0.000
TG	763	0.002	**P=0.001**	0.124	0.001 to 0.003	0.014
ApoA1	763	-1.06	**P<0.001**	-0.217	-1.40 to 0.721	0.046
ApoB	763	0.344	P=0.052	0.070	-0.003 to 0.69	0.004
ApoB/ApoA1	763	1.001	**P<0.001**	0.186	0.626 to 1.377	0.033
Lp(a)	763	0.00	**P=0.047**	0.0072	0.0 to 0.001	0.004
Lp-PLA$_2$ activity	745	0.011	**P<0.001**	0.169	0.006 to 0.015	0.027

Table 9.13: Results of association between lipid markers and BPB using multivariate linear regression analyses (a-j) in which each variable was adjusted for age, sex, smoking (packyears), diabetes and hypertention (Baseline R^2=0.303)

Model	Marker	N	Estimate (B)	P value	Exponential (Beta)	95% CI for B	R^2
(a)	**HDL**	762	-0.009	**P=0.003**	-0.099	-0.015 to -0.003	0.310
(b)	**TChol/HDL**	762	0.107	**P<0.001**	0.113	0.049 to 0.165	0.314
(c)	**LDL**	762	0.004	**P=0.001**	0.100	0.002 to 0.006	0.312
(d)	**TG**	762	0.001	**P=0.017**	0.073	0.00 to 0.002	0.307
(e)	**ApoA1**	762	-0.494	**P=0.002**	-0.101	-0.806 to -0.182	0.311
(f)	**ApoB**	762	0.473	**P=0.001**	0.097	0.183 to 0.764	0.311
(g)	**ApoB/ApoA1**	762	0.748	**P<0.001**	0.139	0.419 to 1.076	0.320
(h)	**Lp(a)**	762	0.00	**P=0.009**	0.08	0.0 to 0.001	0.308
(i)	**Lp-PLA_2 activity**	744	0.007	**P=0.001**	0.108	0.003 to 0.011	0.301
(j)	***CETP* (TaqIB1B2)**	720	0.178	**P=0.04**	0.064	0.008 to 0.349	0.298

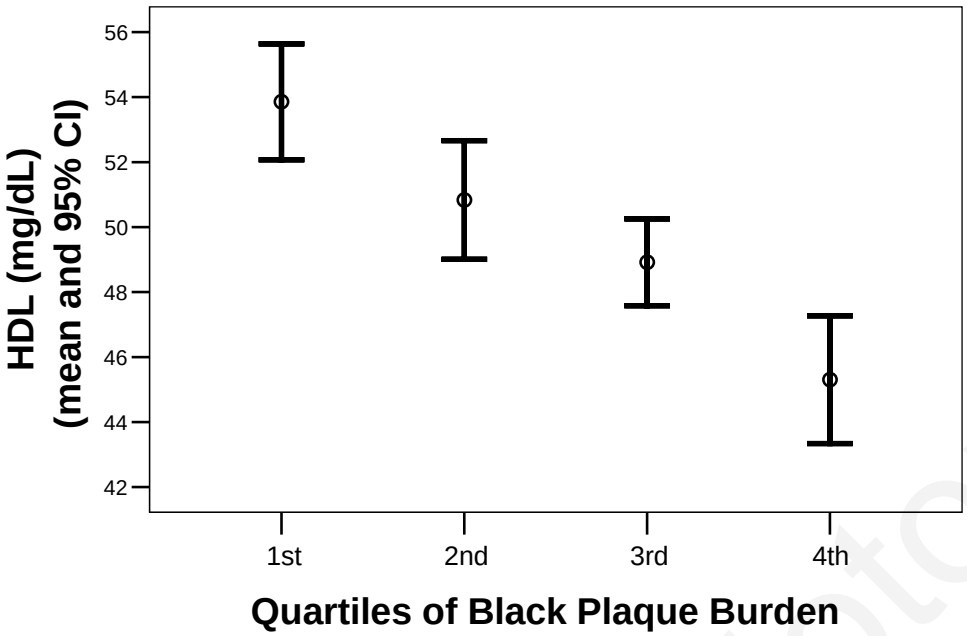

Figure 9.45: Association between HDL levels and quartiles of BPB (ANOVA; P for trend <0.0001)

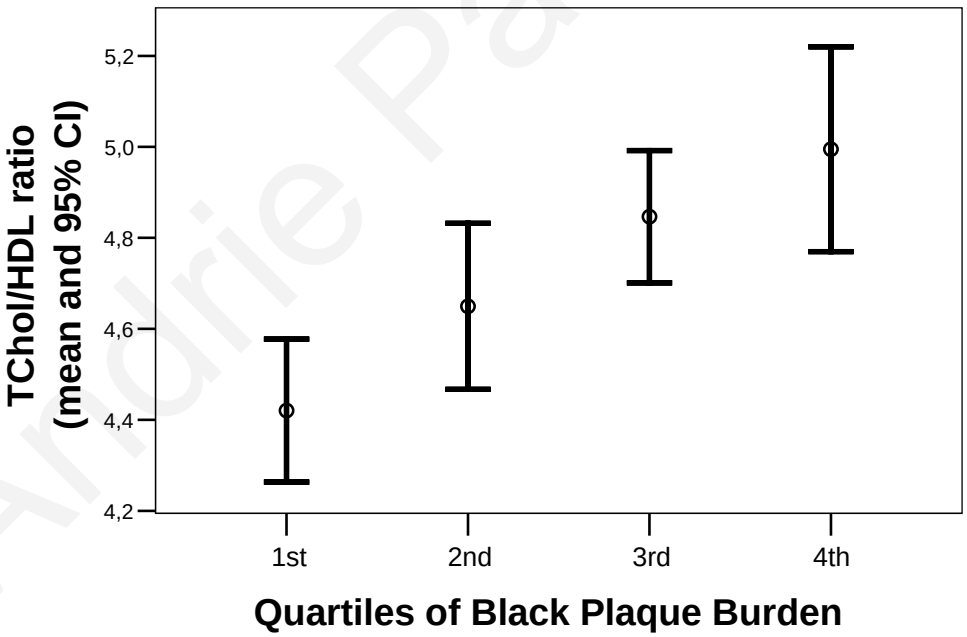

Figure 9.46: Association between TChol/HDL ratio and quartiles of BPB (ANOVA; P for trend <0.0001)

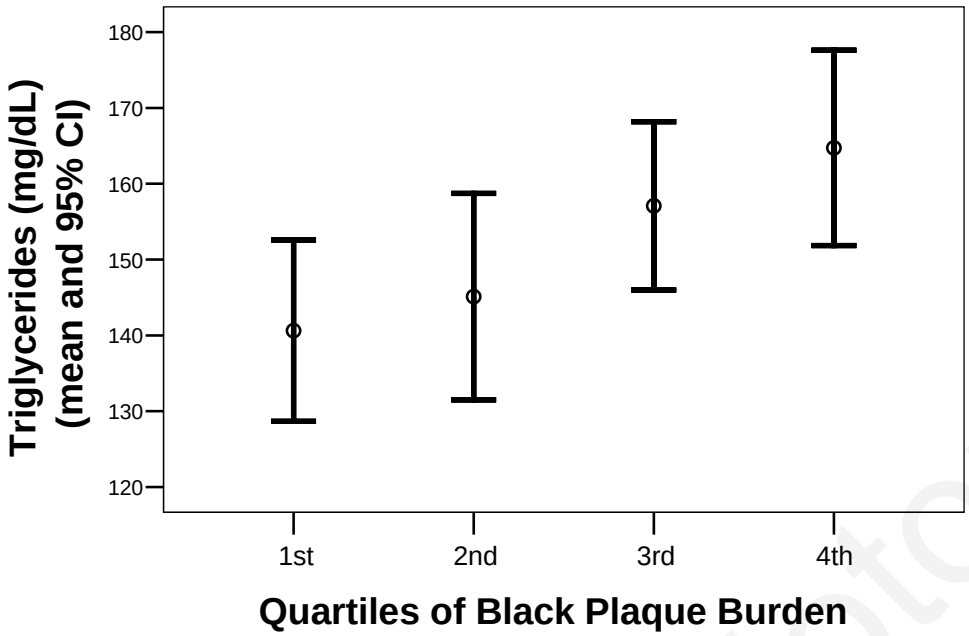

Figure 9.47: Association between triglyceride levels and quartiles of BPB (ANOVA; P for trend <0.05)

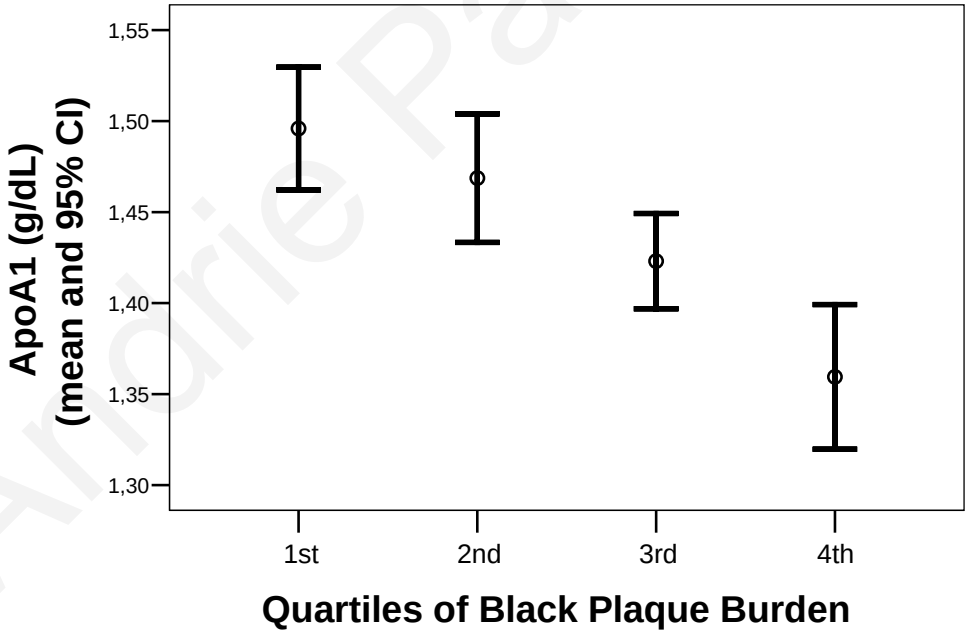

Figure 9.48: Association between apoA1 levels and quartiles of BPB (ANOVA; P for trend <0.0001)

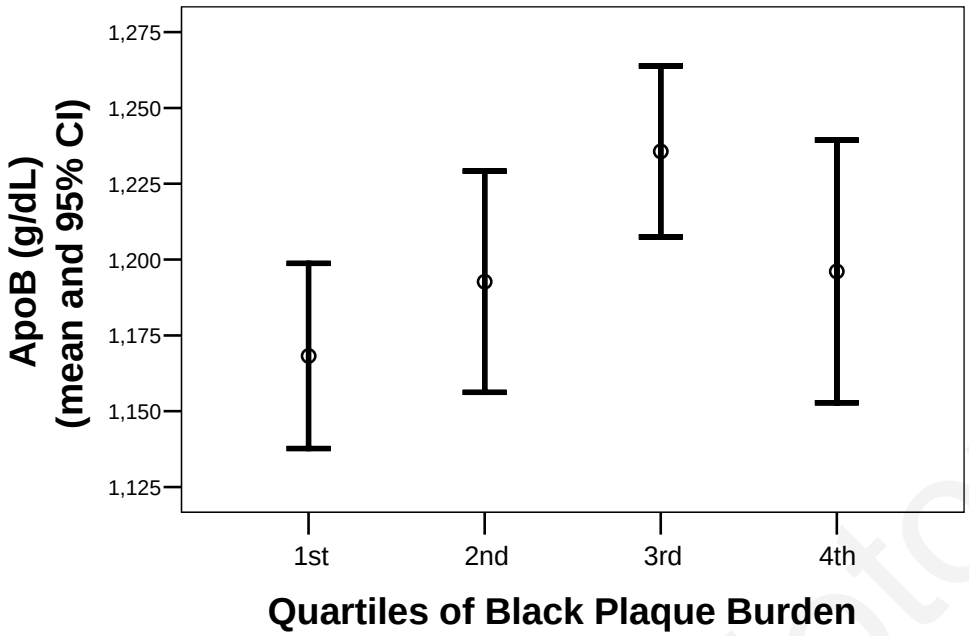

Figure 9.49: Association between apoB levels and quartiles of BPB (ANOVA; P for trend=0.015)

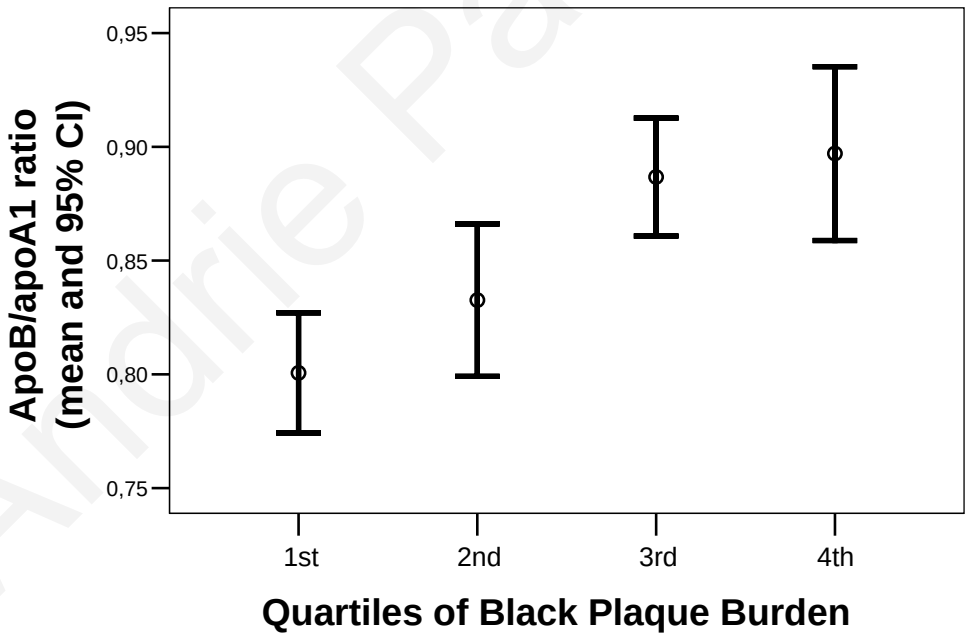

Figure 9.50: Association between apoB/apoA1 ratio and quartiles of BPB (ANOVA; P for trend <0.0001)

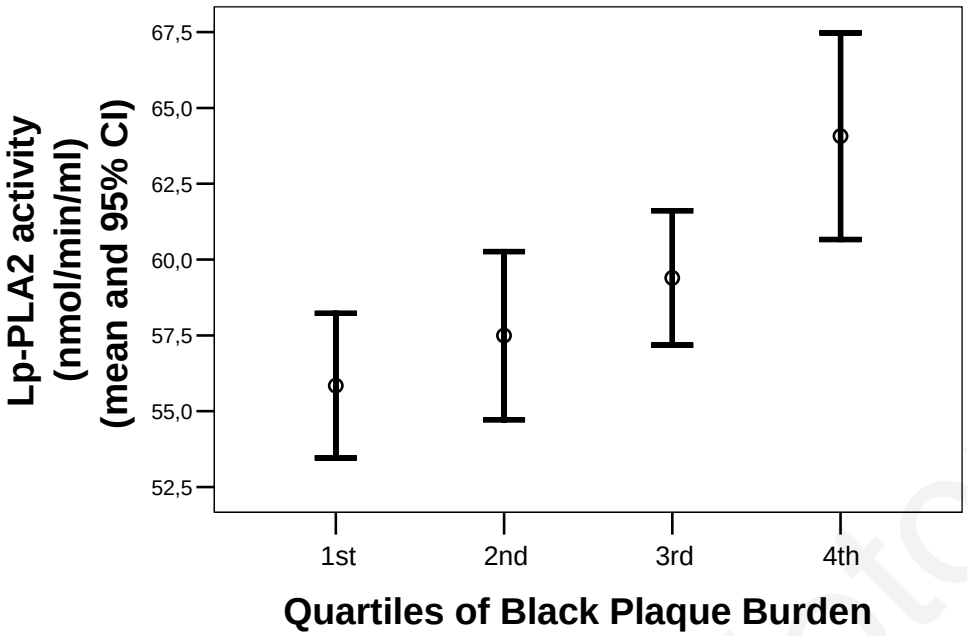

Figure 9.51: Association between Lp-PLA_2 activity levels and quartiles of BPB (ANOVA; P for trend=0.001)

Table 9.14: Compiled associations between lipid markers and ultrasonic measurements in univariate analyses

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
TChol	-	-	-	-	-	-
HDL	+	+	+	+	+	+
TChol/HDL	+	+	+	+	+	+
LDL	-	-	-	-	-	-
TG	-	-	+	+	+	*+
ApoA1	+	+	+	+	+	+
ApoB	-	-	-	+	+	+
ApoB/apoA1	+	+	+	+	+	+
Lp(a)	-	-	-	-	+	-
Lp-PLA_2	+	+	+	+	+	+
ApoB (-516C>T)	-	-	-	-	-	-
ApoE	-	-	-	+	-	+
CETP TaqIB1B2	-	+	-	-	-	+
CETP (I405V)	-	-	-	-	-	-
Lp-PLA_2 (A379V)	-	-	-	-	-	-

*Borderline P value for TG.

Table 9.15: Compiled table for association between lipid markers and ultrasonic measurements in multivariate analyses adjusting for age, sex, smoking in packyears, diabetes and hypertention

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
TChol	-	-	-	-	-	-
HDL	-	+	+	-	+	-
TChol/HDL	+	+	+	+	+	-
LDL	-	+	+	+	+	-
TG	-	-	+	-	+	-
ApoA1	+	+	+	-	+	-
ApoB	-	+	+	+	+	-
ApoB/apoA1	+	+	+	+	+	-
Lp(a)	-	-	-	-	+	-
Lp-PLA_2	-	+	+	-	+	-
ApoB (-516C>T)	-	-	-	-	-	-
ApoE	-	-	-	+	-	-
CETP TaqIB1B2	-	+	-	-	+	+
CETP (I405V)	-	-	+	-	-	-
Lp-PLA_2 (A379V)	-	-	-	-	-	-

Summary of results

Results from this chapter confirm the important role of certain lipids in early stages of atherosclerosis development and bring new lipid markers into the spotlight for association with subclinical atherosclerosis as discussed further in chapter 16.

1. This is the first time, as far as we know that an association between plasma levels of Lp-PLA_2 (PAF-AH) and the *Lp-PLA_2* A379V genotype has been demonstrated in a general population, especially in a sex specific analysis. In our study population, the VV genotype was significantly associated with higher plasma levels of Lp-PLA_2 and the association was apparent only in women.
2. The *apoB* (-516C>T) polymorphism was significantly associated with TChol and HDL cholesterol levels and the *apoE* (E2/E3/E4) polymorphism was significantly associated with TChol and LDL cholesterol levels as well as with the ratio of TChol/HDL.
3. Determinants of IMTcc, plaque presence, TPT and BPB are different. Practically all the biochemical lipid markers associated with TPT were also associated with IMTcc as well. However, TG and genetic markers were associated with presence and/or thickness of plaque but not IMTcc (Tables 9.14 and 9.15).

Chapter 10:

Results of association between Endothelial Dysfunction markers and subclinical atherosclerosis

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and endothelial dysfunction markers (both biochemical and genetic). The endothelial dysfunction markers chosen were: soluble NO, MPO, serum creatinine levels and the *eNOS* (894G>T), *PON1* (L55M), *PON2* (S311C), *MPO* (-638C>A), *ACE* (I/D) and *ang* (-6G>A) genetic polymorphisms. The reasons for the choise of the above markers are stated at the hypothesis (chapter 4).

Association between endothelial dysfundtion markers and IMTcc

The association between levels of endothelial dysfunction markers and IMTcc was tested using linear regression. Only creatinine levels were associated with IMTcc (Fig.10.1). Neither plasma nitric oxide (NO) nor myeloperoxidase (MPO) were significantly associated with IMTcc (Table 10.1). From the genetic polymorphisms tested no one was significantly associated with IMTcc.

In multivariate analyses adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with IMTcc.

Association between endothelial dysfunction markers and IMTmax

The association between levels of endothelial dysfunction markers and IMTmax was tested in linear regression analysis. Creatinine levels were significantly associated with IMTmax (Fig.10.2). Neither plasma NO or MPO were significantly associated with IMTmax (logtransformed to fit the assumption of normality) (Table 10.1). From the genetic polymorphisms tested none was significantly associated with IMTcc.

In multivariate analyses adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with IMTmax.

Table 10.1: Univariate linear regression for association between ultrasonic measurements and plasma levels of oxidation markers (IMTmax, TPT, NO and MPO were logtransformed to achieve normality)

	IMTcc		**IMTmax**		**TPT**		**BPB**	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
NO	0.002 (-0.001 to 0.004)	P=0.211	0.066 (-0.018to0.15)	P=0.123	0.091 (-0.02 to 0.21)	P=0.118	0.09 (-0.08 to 0.26)	P=0.299
MPO	0.001 (-0.002 to0.003)	P=0.579	-0.043 (-0.13 to 0.04)	P=0.310	0.061 (-0.064 to0.19)	P=0.335	-0.024 (-0.207 to0.158)	P=0.793
Creatinine	0.016 (0.01 to 0.022)	**P<0.001**	0.062 (0.42 to 0.082)	**P<0.001**	0 (0.108 to 0.24)	**P<0.001**	0.255 (0.134 to 0.377)	**P<0.001**

Association between endothelial dysfunction markers and TPT

The association between levels of endothelial dysfunction markers and TPT was tested with linear regression analysis. Only creatinine levels were associated with TPT (Fig.10.3). Neither plasma NO or MPO were significantly associated with TPT (logtransformed to fit the assumption of normality) (Table 10.1). From the genetic polymorphisms tested none was significantly associated with TPT.

In multivariate analyses adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with TPT.

Association between endothelial dysfunction markers, presence of plaques and number of bifurcations with plaque

The association between levels of endothelial dysfunction markers, presence of plaques and number of bifurcations with plaques was tested using Mann-Whitney and Kruskal-Wallis tests. In addition to number of bifurcations with plaques ranging from 0 to 4 we also used a cut-off point of 2 bifurcations with plaque (0-2) or more (3-4) to indicate generalised atherosclerosis. Again only creatinine levels were significantly associated with both presence of plaque and number of bifurcations with plaque (Figs.10.4-5). Neither NO levels nor MPO levels were significantly associated with presence of plaques or number of bifurcations with plaques (Table 10.2). From the genetic polymorphisms tested none was significantly associated with plaques.

In a multivariate analysis adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with presence of plaque or number of bifurcations with plaque.

Association between endothelial dysfunction markers and MPT

The association between levels of endothelial dysfunction markers and MPT was tested with Kruskal-Wallis test. Serum creatinine levels were significantly associated with MPT over the median ($P<0.001$) whereas plasma NO or MPO levels were not. From the genetic polymorphisms tested none was significantly associated with BPB. In a multivariate analysis adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with IMTcc.

Association between endothelial dysfunction markers and BPB

The association between levels of endothelial dysfunction markers and BPB was tested with linear regression. Serum creatinine levels were significantly associated with BPB (Fig.10.6) whereas plasma NO or MPO levels were not (Table 10.1). From the genetic polymorphisms tested none was significantly associated with BPB.

In a multivariate analysis adjusting for age, sex, smoking (packyears), diabetes and hyperlipidaemia, creatinine levels were no longer associated with IMTcc.

Table 10.2: Association between presence of plaques and number of bifurcations with plaque and plasma levels of endothelial dysfunctio markers (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaques as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque)

Marker	**N**	**Presence of plaques**	**Number of bifurcations With plaques (0-4)**	**0-2 bifurcations with plaque vs 3-4**
NO	266	P=0.618	P=0.738	P=0.253
MPO	201	P=0.186	P=0.220	P=0.777
Creatinine	765	**P<0.001**	**P<0.001**	**P<0.001**

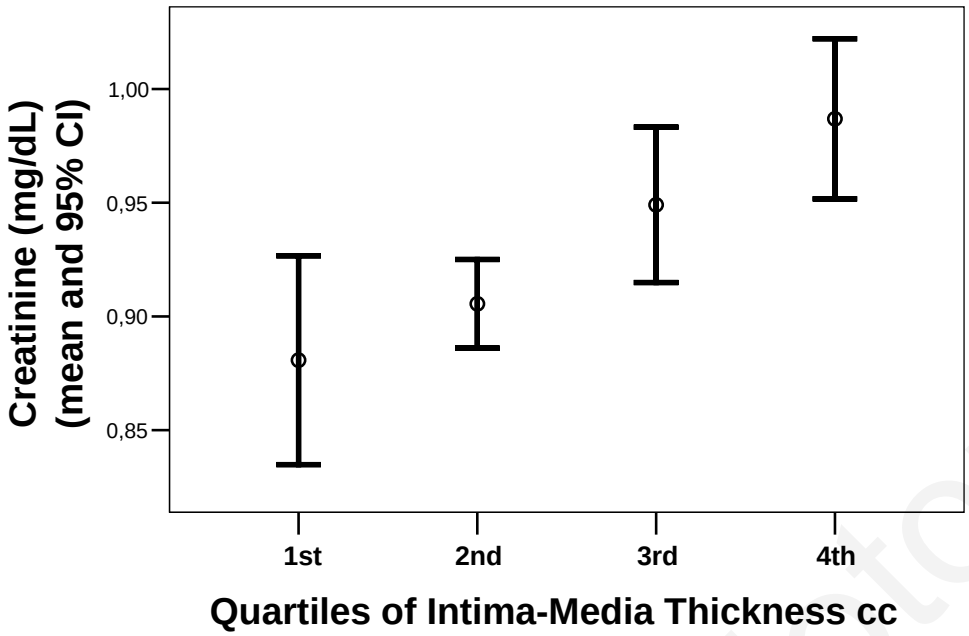

Figure 10.1: Association between creatinine levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend <0.0001)

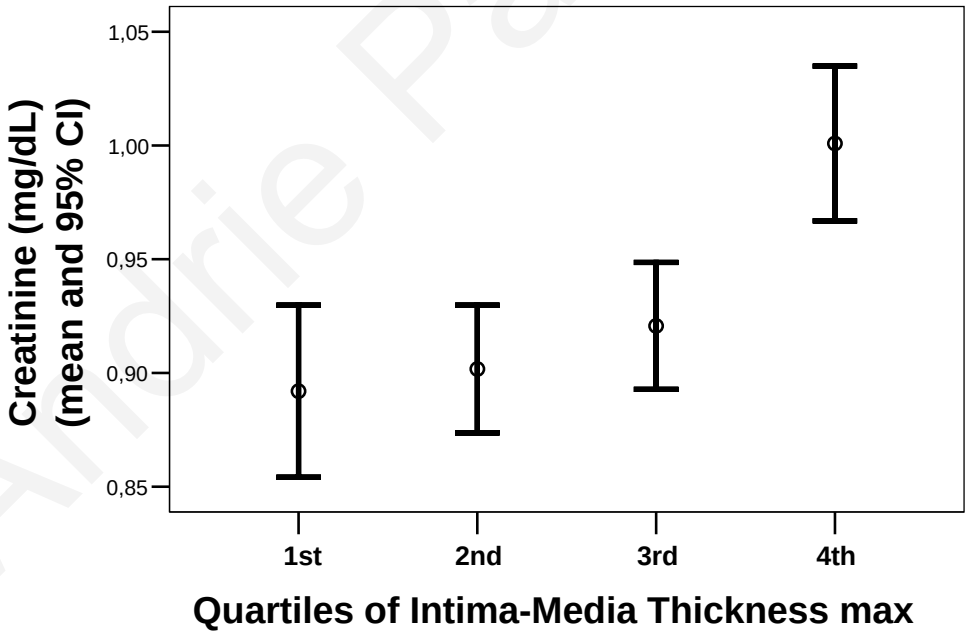

Figure 10.2: Association between creatinine levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend <0.0001)

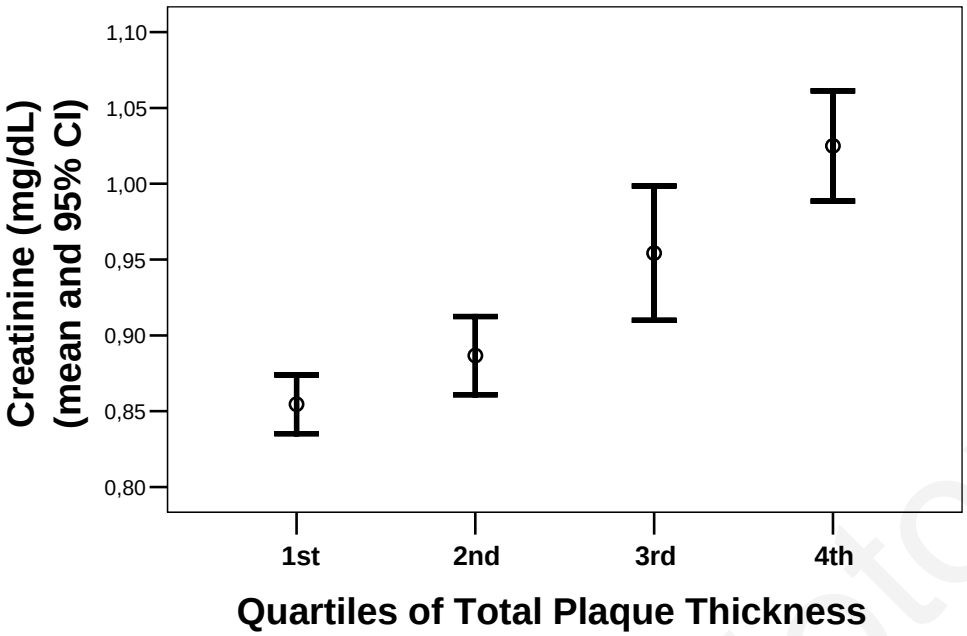

Figure 10.3: Association between creatinine levels and quartiles of TPT (Kruskal-Wallis test; P for trend <0.0001)

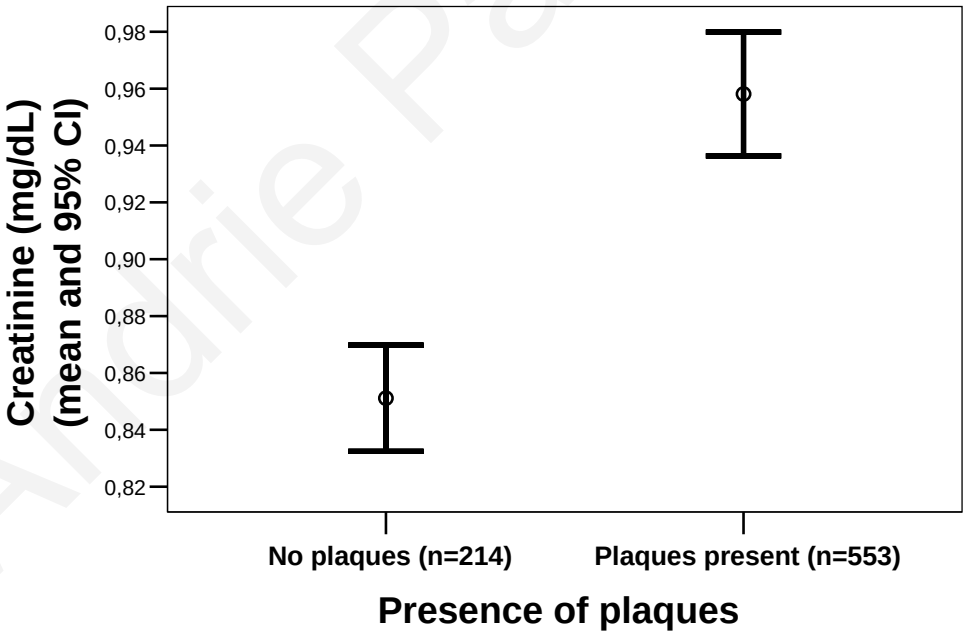

Figure 10.4: Association between creatinine levels and presence of plaques (Mann-Whitney test; P for trend <0.0001)

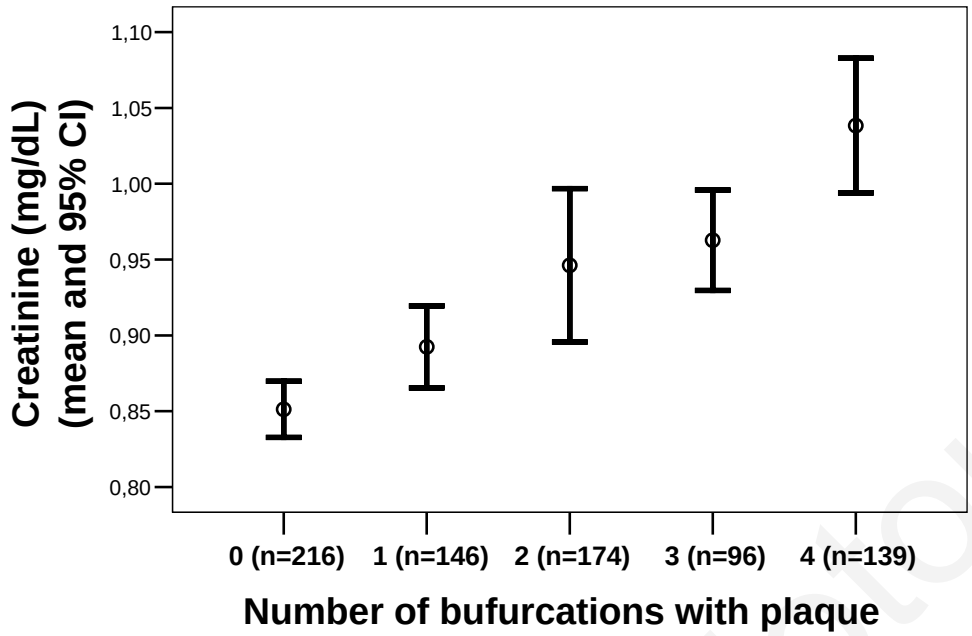

Figure 10.5: Association between creatinine levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend <0.0001)

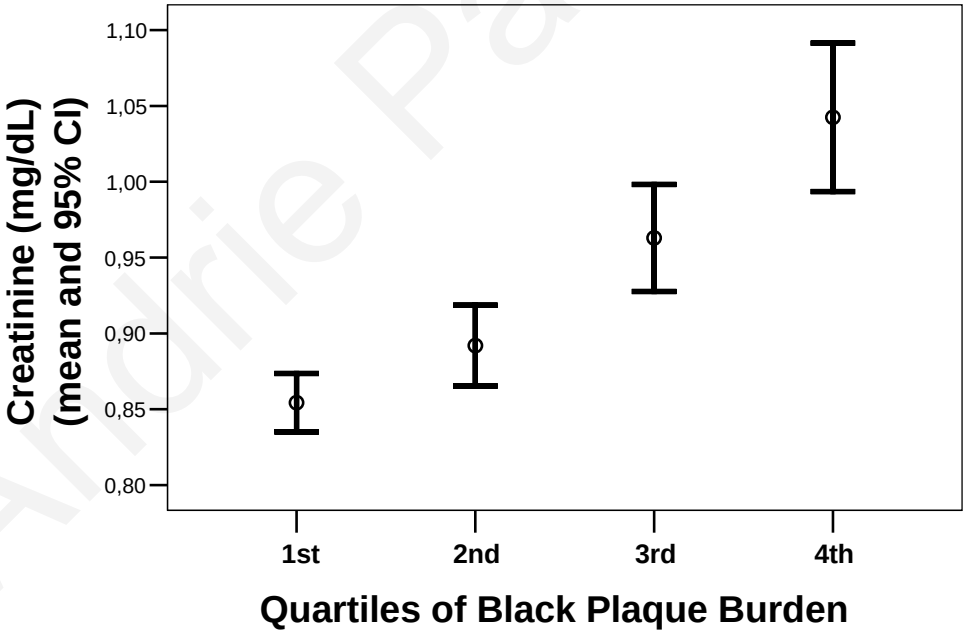

Figure 10.6: Association between creatinine levels and quartiles of BPB (Kruskal-Wallis test; P for trend <0.0001)

Summary of results:

The results do not seem to support a role of endothelial dysfunction in the early stages of atherosclerotic process and development. However, more markers need to be tested in a prospective study before definite conclusions can be drawn.

1. From the endothelial dysfunction markers tested only creatinine levels were significantly associated with all the ultrasonic markers tested. The association, however, did not remain significant after adjusting for age, sex, amoking, diabetes and hyperlipidaemia.
2. None of the genetic polymorphisms tested was associated with any of the ultrasonic markers tested.

Chapter 11:

Results of association for inflammation markers and subclinical atherosclerosis

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and inflammatory markers (both biochemical and genetic). The inflammatory markers chosen were: CRP, IL-6, MCP-1, *IL-6* (-174C>T), *TNF-α* (-308G>A), *MGP* (-138C>T), *MMP-1* (1G/2G), *MMP-3* (5A/6A), *MMP-7* (-181A>G), *MMP-9* (R279Q) and *MMP-12* (-82A>G).The reasons for the choise of the above markers are stated at the hypothesis (chapter 4).

Association between inflammatory markers and IMTcc

The association between plasma levels of inflammatory markers and IMTcc was tested. IL-6 and MCP-1 were significantly associated with increasing IMTcc. Estimate (B) and p values for univariate linear regression are shown in table 11.1. CRP was not associated with IMTcc in linear regression but there was a significant trend with increasing IMTcc quartiles. From the genetic polymorphisms tested only *TNF-α* (-308A>G) was associated with IMTcc. Significant results are shown in figures 11.1-4 and p values of all associations are tabulated in table 11.2.

In multivariate, binary logistic regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia), *TNF-α* (-308A>G) was no longer a significant predictor of IMTcc (population median used as a cut-off point) and neither were plasma levels of CRP, IL-6 or MCP-1.

Association between inflammatory markers and IMTmax

The association between levels of plasma inflammatory markers and IMTmax was tested. Plasma levels of CRP were not associated with IMTmax but IL-6 and MCP-1 levels were significantly associated with increasing IMTmax. Estimate (B) and p values for univariate linear regression are shown in table 11.1. From the genetic polymorphisms tested *MGP* (-138C>T) and *MMP-9* (R279Q) were both significantly associated with IMTmax. Significant results are shown in figures 11.5-8 and p values of all associations are tabulated in table 11.2.

In multivariate binary logistic regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia), *MGP* (-138C>T) polymorphism remained significantly associated with IMTmax (OR=2.4;

95% CI=1.07 to 5.37 for TC compared to CC, OR=1.7; 95% CI=0.78 to 3.70 for TT compared to CC; P=0.043). After adjustement, *MMP-9* (R279Q) was no longer a significant predictor of IMTmax (population median used as a cut-off point) but with a borderline p value (OR=1.8; 95% CI=0.89 to 3.61 for GG compared to AA, OR=1.2; 95% CI=0.61 to 2.43 for GA compared to AA; P=0.053). Plasma levels of IL-6 or MCP-1 were no longer significantly associated with IMTmax after adjustement for traditional risk factors.

Association between inflammatory markers and TPT

The association between levels of plasma inflammatory markers and TPT was tested. Plasma levels of CRP and IL-6 were not associated with IMTmax but MCP-1 levels were significantly associated with increasing TPT. Estimate (B) and p values for univariate linear regression are shown in table 11.1. From the genetic polymorphisms tested *TNF-α* (-308G>A), *MMP-7* (-181A>G) and *MMP-9* (R279Q) were significantly associated with TPT. Significant results are shown in figures 11.9-13 and p values of all associations are tabulated in table 11.2. The *MMP-3* (5A/6A) polymorphism was not associated with TPT in a Kruskal-Wallis test, however, if a cut-off point was taken for TPT, the 5A/6A and 6A/6A genotypes were associated with TPT <0.52cm compared to the 5A/5A genotype (OR=0.62; 95% CI= 0.41 to 0.96; P=0.032 for 5A/6A and OR=0.72; 95% CI= 0.43 to 1.05; P=0.078 for 6A/6A).

In multivariate binary logistic regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia), *TNF-α* (-308G>A), *MMP-3* (5A/6A) and *MMP-7* (-181A>G) polymorphisms were no longer significantly associated with TPT. In contrary, the *MMP-9* (R279Q) polymorphism remained a strong, significant predictor of TPT (best cut-off point from ROC curve used), even after adjustement for traditional risk factors (OR=7.5; 95% CI=2.30 to 24.27 for GG compared to AA, OR=5.6; 95% CI=1.74 to 17.99 for GA compared to AA; P=0.003). Plasma levels of MCP-1 were no longer significantly associated with TPT after adjustement for traditional risk factors.

Table 11.1: Results of univariate linear regressions for association between ultrasonic measurements and plasma levels of inflammatory markers (IMTmax, TPT, CRP and IL-6 were logtransformed to achieve normality)

	IMTcc		**IMTmax**		**TPT**		**BPB**	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
CRP	0.002 (0.0 to 0.004)	P=0.078	0.037 (-0.038to0.11)	P=0.337	-0.017 (-0.12 to 0.08)	P=0.73	-0.002 (-0.15 to 0.15)	P=0.977
IL-6	0.002 (0.0 to0.004)	P=0.110	0.104 (0.039 to 0.17)	**P=0.002**	0.067 (-0.025 to0.16)	P=0.15	0.186 (0.05 to0.323)	**P=0.008**
MCP1	3,34E-005 (0.0 to 0.0)	**P=0.03**	0.001 (0.0 to 0.003)	**P=0.008**	0.188	**P=0.03**	0.003 (0.001 to 0.005)	**P=0.008**

Table 11.2: Results of univariate associations between genotypes for inflammatory genetic polymorphisms and ultrasonic measurements used as continuous variables; each genotype was used as a different category except when homozygotes for one allele were too few (Kruskal-Wallis- test)

Gene	Variant	Allele	N	IMTcc	IMTmax	TPT	BPB	MPT
IL-6	-174 C>G	CC, CG, GG,	736	P=0.302	P=0.256	P=0.416	P=0.516	P=0.65
TNF-α	-308 G>A	GG vs AA, AG	490	**P=0.028**	P=0.245	***P=0.034**	***P=0.043**	P=0.41
MGP	-138 C>T	CC, CT, TT	734	P=0.096	**P=0.035**	P=0.284	P=0.248	P=0.89
MMP-1	1G/2G	1G/1G,1G/2G,2G/2G	734	P=0.551	P=0.823	P=0.967	P=0.720	P=0.83
MMP-3	5A/6A	5A/5A,5A/6A,6A/6A	762	P=0.313	P=0.625	P=0.300	P=0.305	P=0.64
MMP-7	-181A>G	AA, AG, GG	725	P=0.436	P=0.413	**P=0.037**	P=0.087	P=0.53
MMP-9	R279Q	GG, AG, AA	745	P=0.175	**P=0.009**	**P=0.013**	**P=0.011**	*P=0.067
MMP-12	-82A>G	AA, AG, GG	738	P=0.300	P=0.192	P=0.224	P=0.111	P=0.45

* If *MMP-9* AA, GG vs AG was tested P=0.033

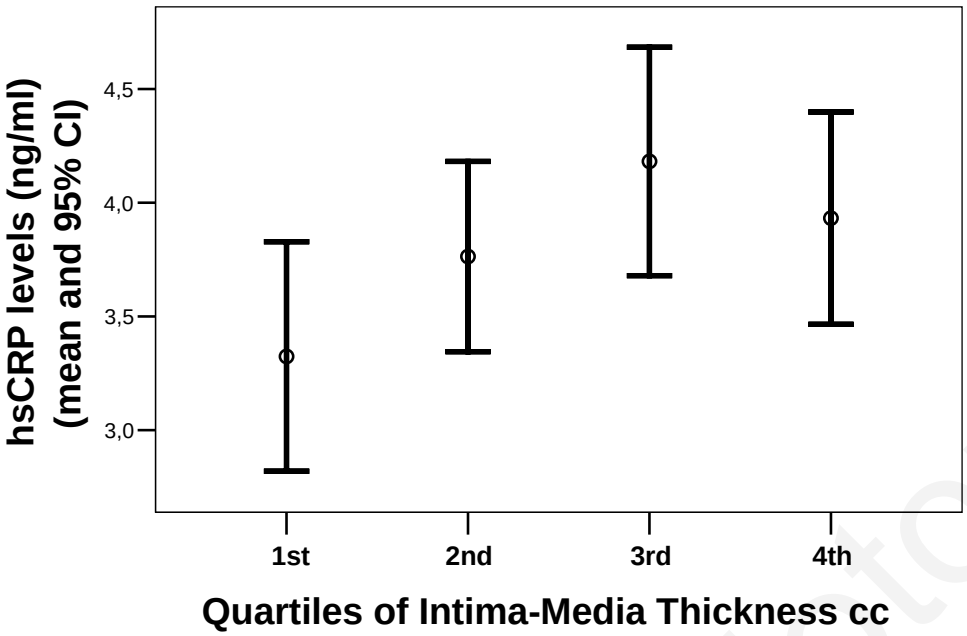

Figure 11.1: Association between CRP levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.009)

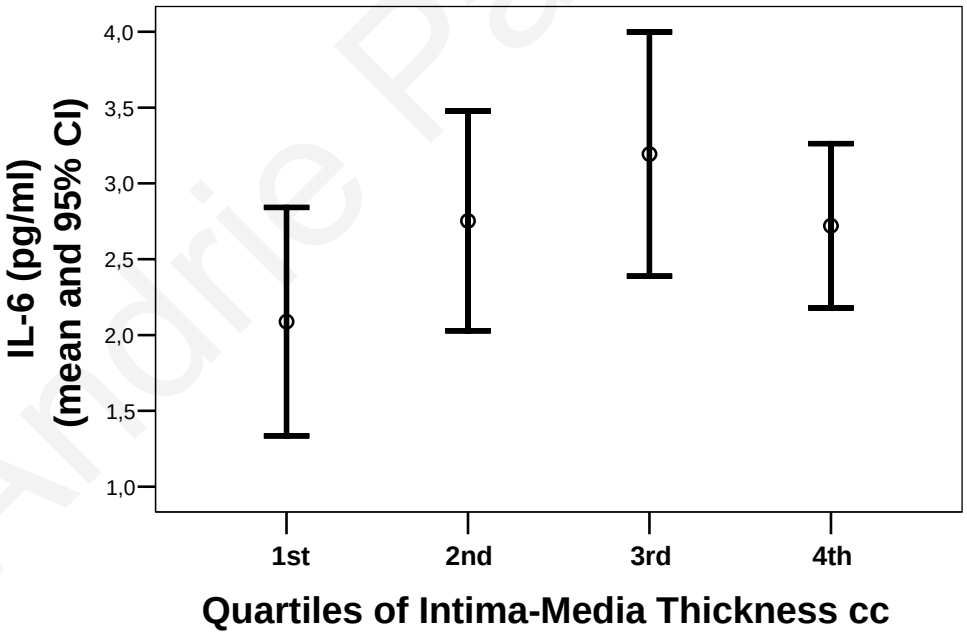

Figure 11.2: Association between IL-6 levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend =0.015)

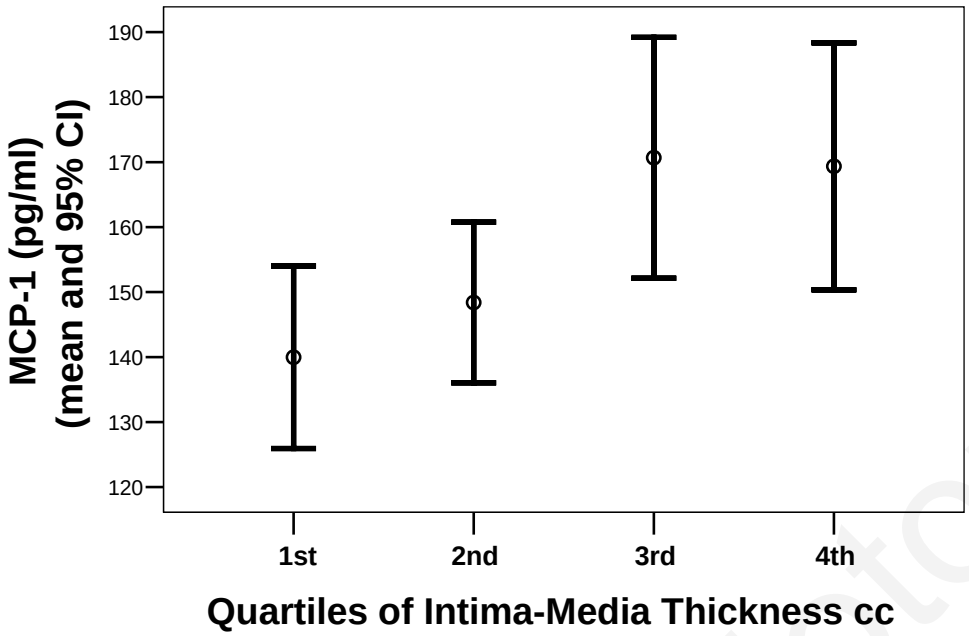

Figure 11.3: Association between MCP-1 levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.002)

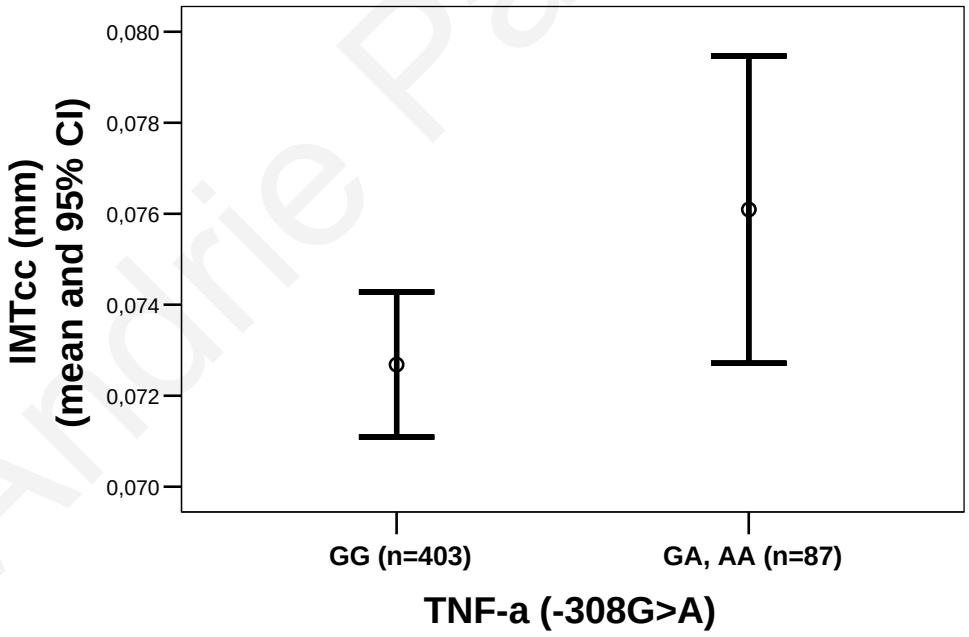

Figure 11.4: Association between IMTcc and *TNF-α* (-308A>G) polymorphism (Kruskal-Wallis test; P for trend =0.028)

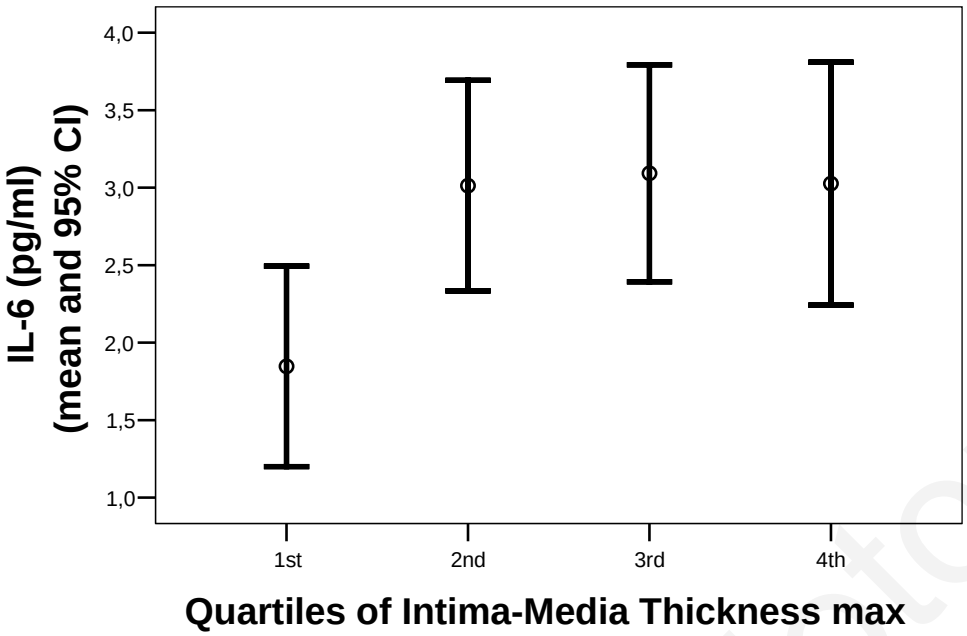

Figure 11.5: Association between IL-6 levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend<0.001)

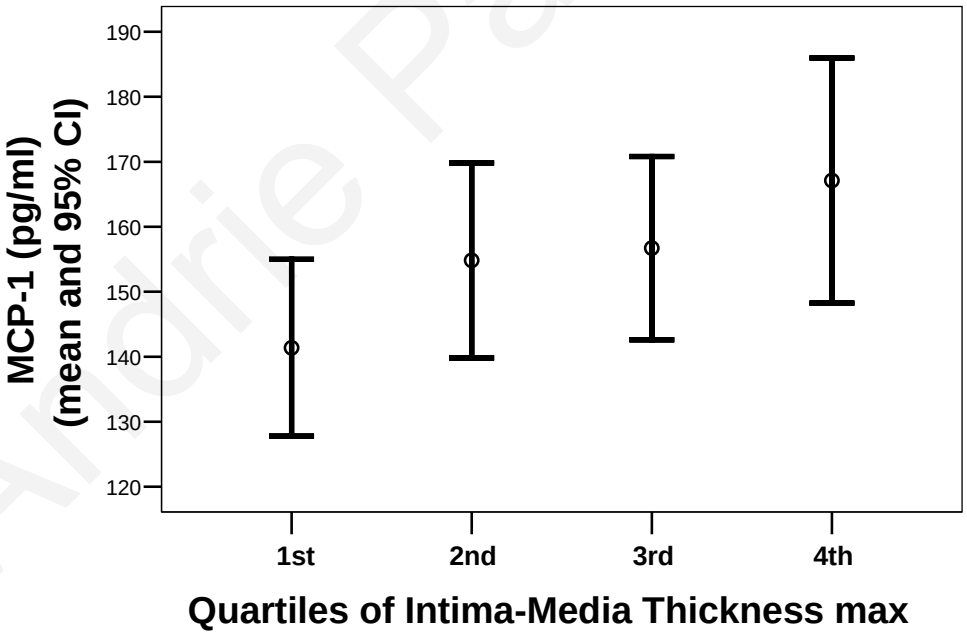

Figure 11.6: Association between MCP-1 levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.021)

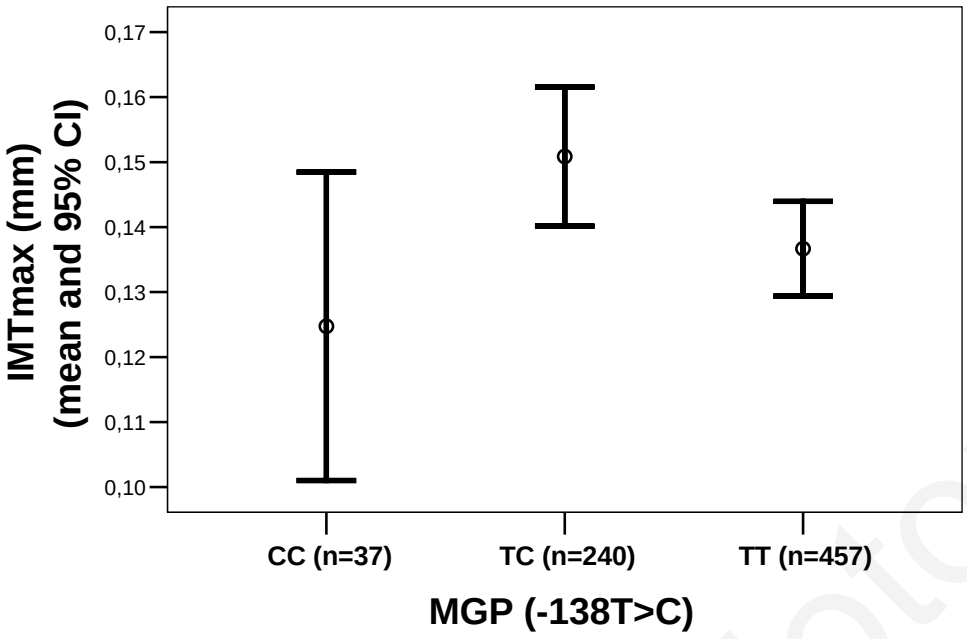

Figure 11.7: Association between IMTmax and *MGP* (-138C>T) genotype. (Kruskal-Wallis test; P for trend=0.035)

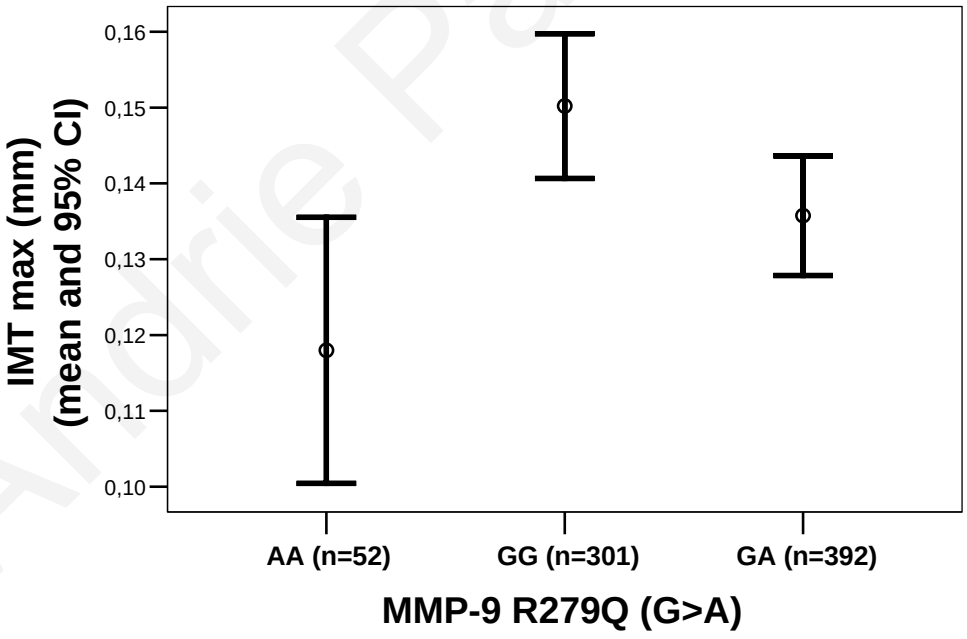

Figure 11.8: Association between IMTmax and *MMP-9* (R279Q) genotype (Kruskal-Wallis test; P for trend =0.009)

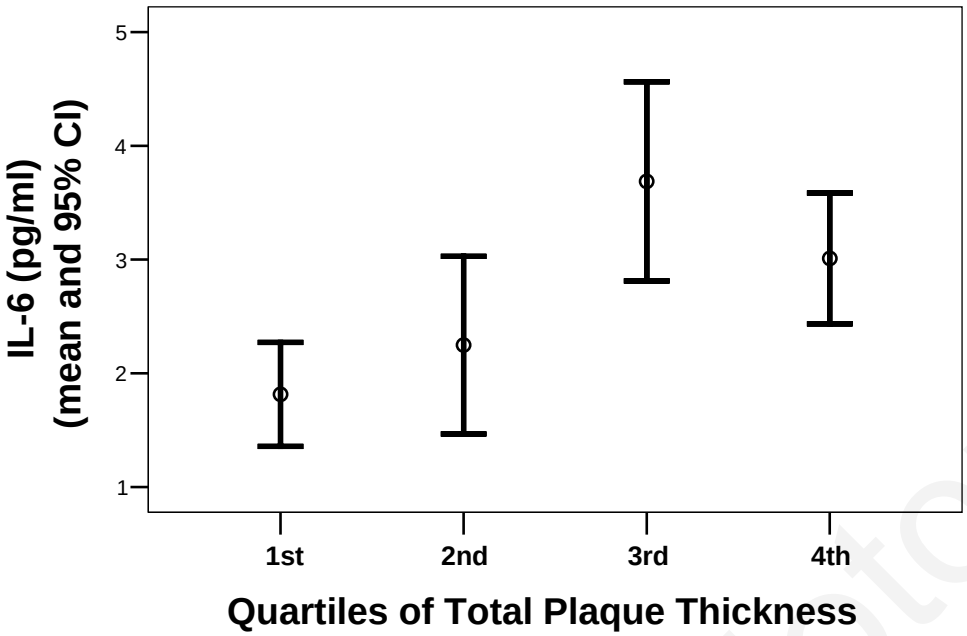

Figure 11.9: Association between IL-6 levels and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

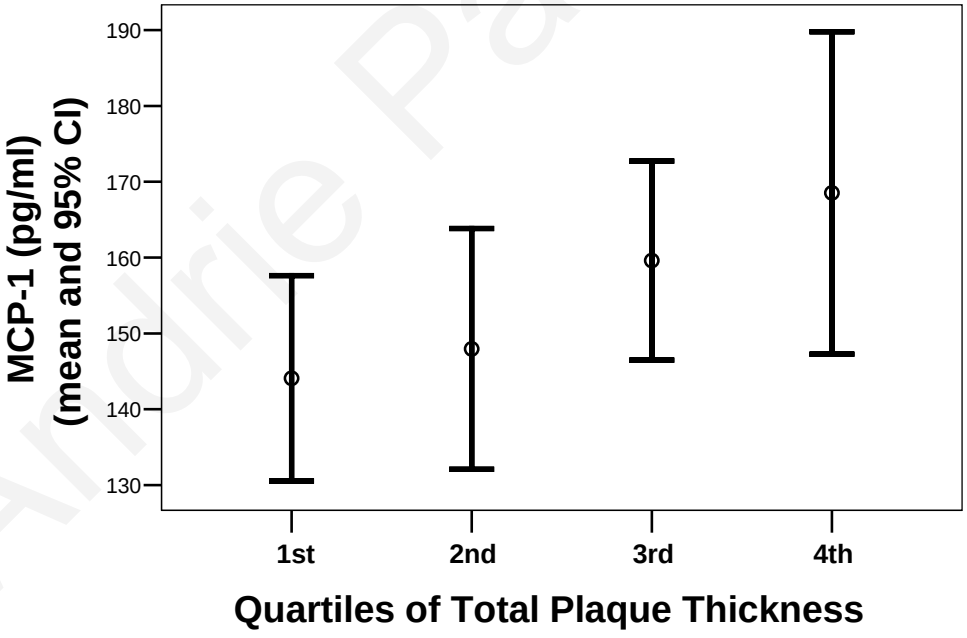

Figure 11.10: Association between MCP-1 levels and quartiles of TPT (Kruskal-Wallis test; P for trend=0.0042)

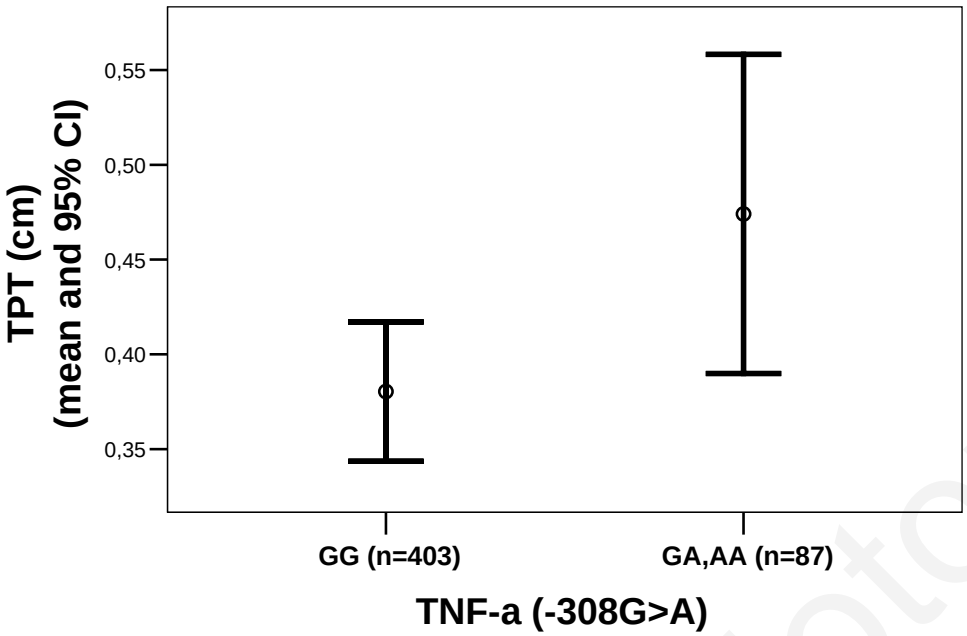

Figure 11.11: Association between TPT and *TNF-α* (-308A>G) genotype. (Kruskal-Wallis; P for trend= 0.034)

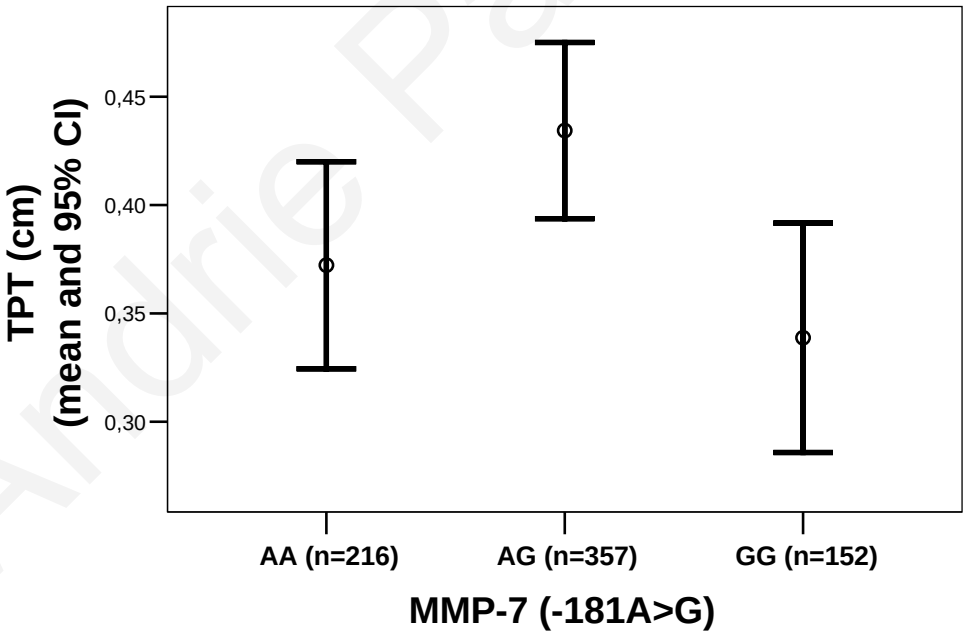

Figure 11.12: Association between TPT and *MMP-7* (-181A>G) genotypes. (Kruskal-Wallis test; P for trend=0.037)

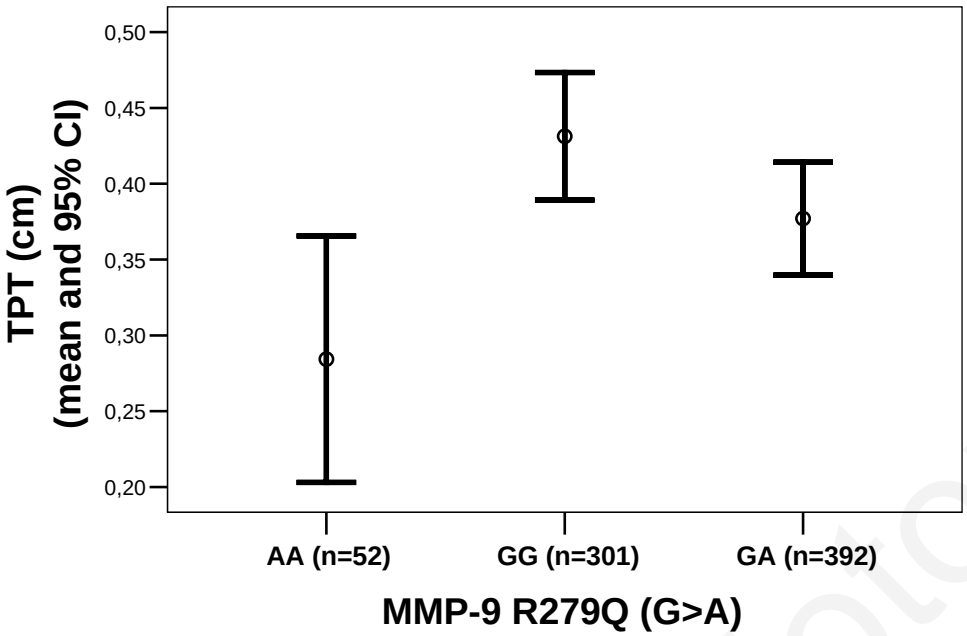

Figure 11.13: Association between TPT and *MMP-9* (R279Q) genotypes. (Kruskal-Wallis test; P for trend=0.013)

Association between inflammatory markers, presence of plaques and number of bifurcations with plaque

The association between plasma levels of inflammatory markers and presence of plaques and number of bifurcations with plaque was tested. In addition to number of bifurcations with plaque ranging from 0 to 4 we also used a cut-off point of 2 bifurcations with plaque (0-2) or more (3-4) to indicate generalised atherosclerosis. CRP plasma levels were not associated with either variable tested but IL-6 and MCP-1 levels were significantly associated with both presence of plaques and number of bifurcations with plaque. Significant results are shown in figures 11.14-23 and p values are tabulated in table 11.3. From the genetic polymorphisms tested only *MMP-9* (R279Q) was consistently associated with all the variables tested. *MMP-7* (-181A>G) was also significantly associated with presence of plaque in more than 2 bifurcations (3-4). Significant results are shown in figures 11.14-19 and p values of all associations are tabulated in table 11.4

In multivariate binary logistic regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia), neither *MMP-7* (-181A>G) or *MMP-9* (R279Q) polymorphisms remained independently associated with the number of bifurcations with plaques (0-2 vs 3-4). However, the *MMP-9* (R279Q) polymorphism was still strongly associated with both the presence of plaques (OR=1.33; 95% CI=0.64 to 2.80 for GG compared to AA, OR=0.78; 95% CI=0.38 to 1.60 for GA compared to AA; P=0.029) and more than two bifurcations with plaque (OR=4.4; 95% CI=1.56 to 12.43 for GG compared to AA, OR=3.12; 95% CI=1.14 to 8.98 for GA compared to AA; P=0.011), even after adjustement for traditional risk factors. Plasma levels of MCP-1 were no longer significantly associated with presence of plaques or number of bifurcations with plaques after adjustement for traditional risk factors but levels of IL-6 were (P=0.038).

Table 11.3: Results of association between presence of plaques and number of bifurcations with plaque and plasma levels of inflammatory markers (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaque as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque)

Marker	**N**	**Presence of plaques**	**Number of bifurcations With plaques (0-4)**	**0-2 bifurcations with plaque vs 3-4**
CRP	728	P=0.093	P=0.265	P=0.584
IL-6	252	**P<0.001**	**P=0.001**	**P=0.003**
MCP-1	262	**P=0.009**	**P=0.033**	**P=0.022**

Table 11.4: Results of association between genotypes for inflammatory genetic polymorphisms and presence of plaques, number of bifurcations with plaques and presence of more than 2 bifurcations with plaques (Kruskal-Wallis test for association)

Gene	Variant	Allele	N	Presence Of Plaques	Number of bifurcations with plaques	0- 2 bifurcations with plaques vs 3-4
IL-6	-174 C>G	CC, CG, GG,	734	P=0.399	P=0.148	P=0.141
TNF-α	-308 G>A	GG vs AA, AG	490	P=0.293	P=0.444	P=0.094
MGP	138 C>T	CC, CT, TT	732	P=0.546	P=0.944	P=0.869
MMP-1	1G/2G	1G/1G,1G/2G,2G/2G	732	P=0.748	P=0.980	P=0.991
MMP-3	5A/6A	5A/5A,5A/6A,6A/6A	756	P=0.871	P=0.699	P=0.249
MMP-7	-181A>G	AA, AG, GG	723	P=0.262	P=0.281	**P=0.028**
MMP-9	R279Q	GG, AG, AA	743	**P=0.016**	**P=0.016**	**P=0.004**
MMP-12	-82A>G	AA, AG, GG	736	P=0.374	P=0.733	P=0.480

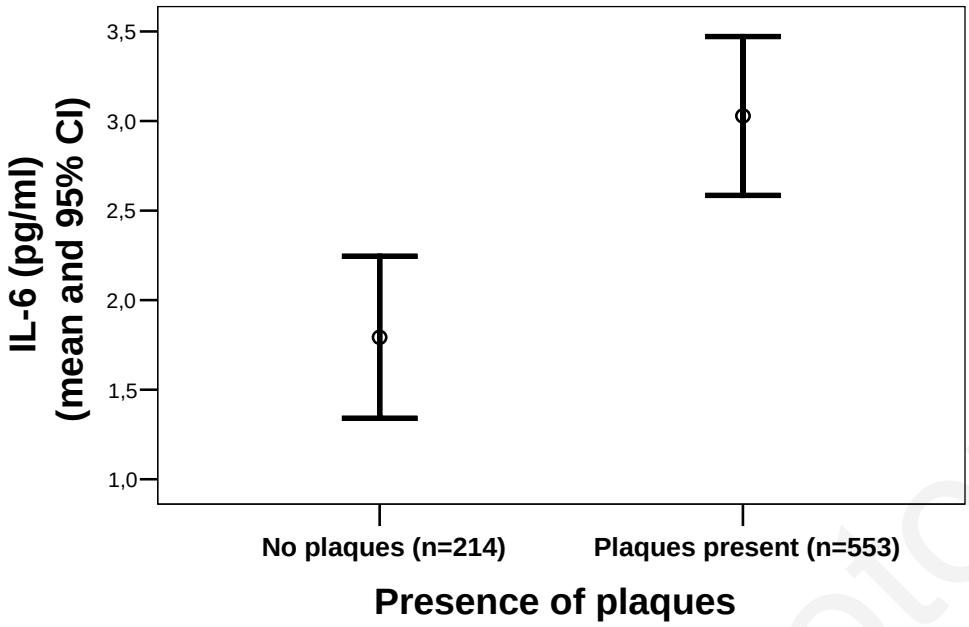

Figure 11.14: Association between IL-6 levels and presence of plaques (Mann-Whitney test; P <0.0001)

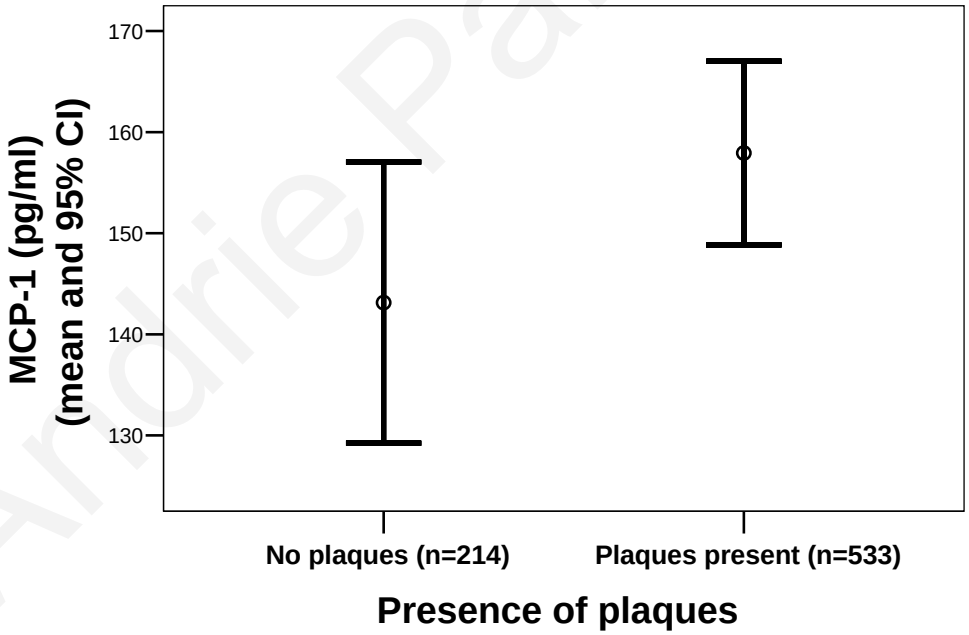

Figure 11.15: Association between MCP-1 levels and presence of plaques (Mann-Whitney test; P=0.009)

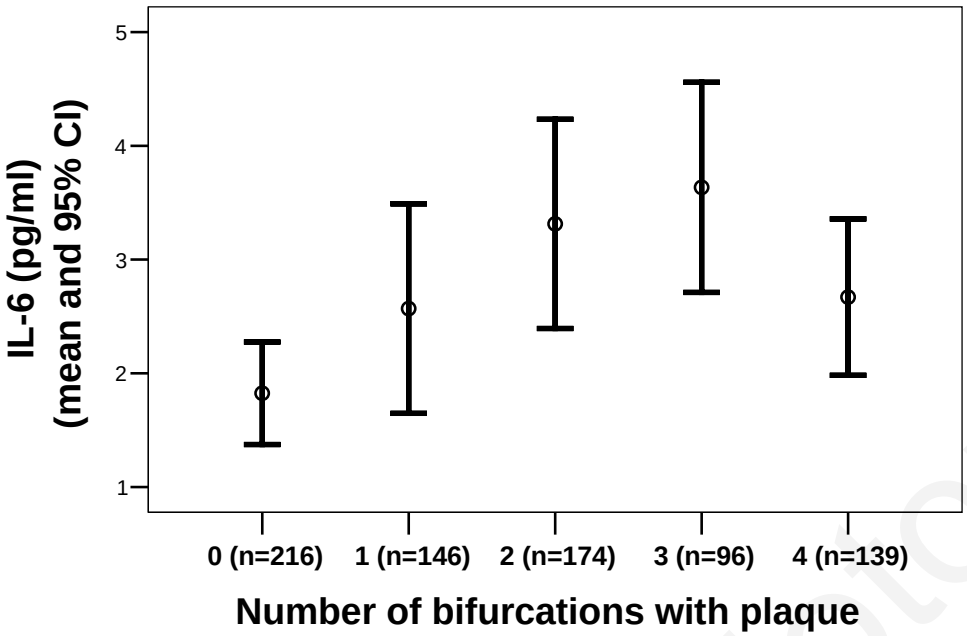

Figure 11.16: Association between IL-6 levels and number of bifurcations with plaque (Kruskal-Wallis test; P=0.001)

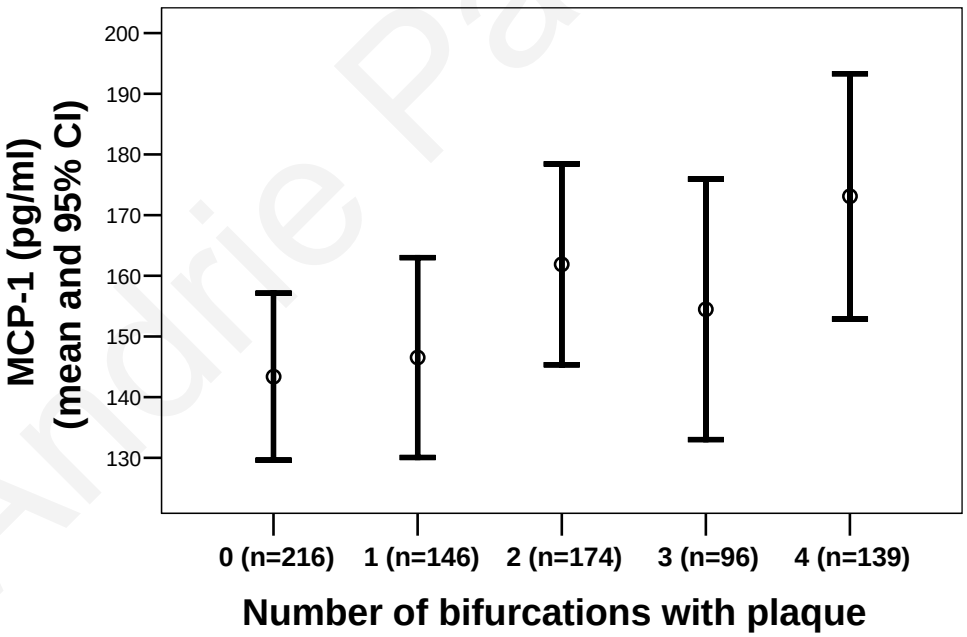

Figure 11.17: Association between MCP-1 levels and number of bifurcations with plaque (Kruskal-Wallis tets; P for trend=0.033)

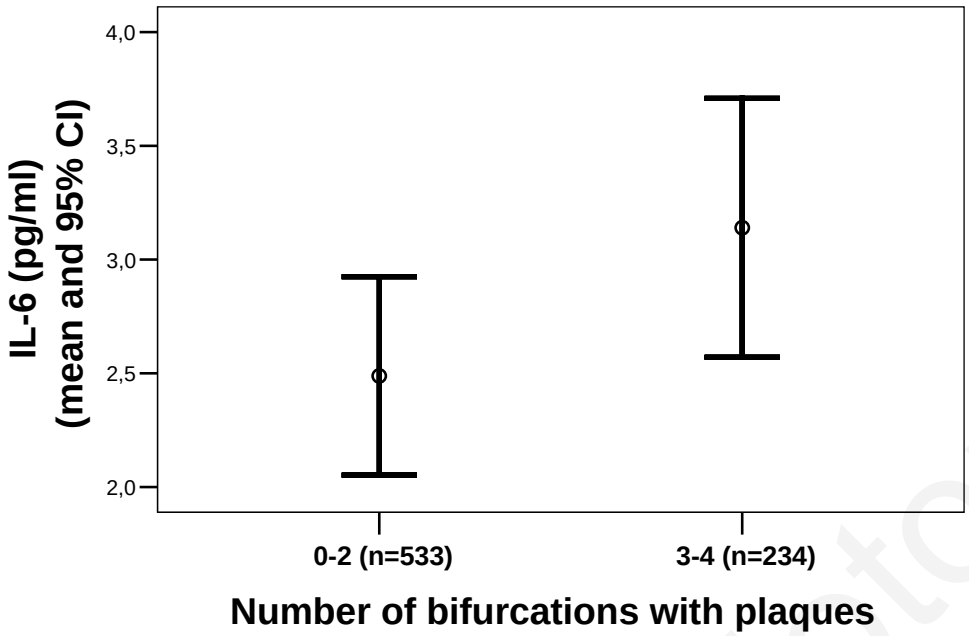

Figure 11.18: Association between IL-6 levels and more than 2 bifurcations with plaque (Mann-Whitney test; P=0.003)

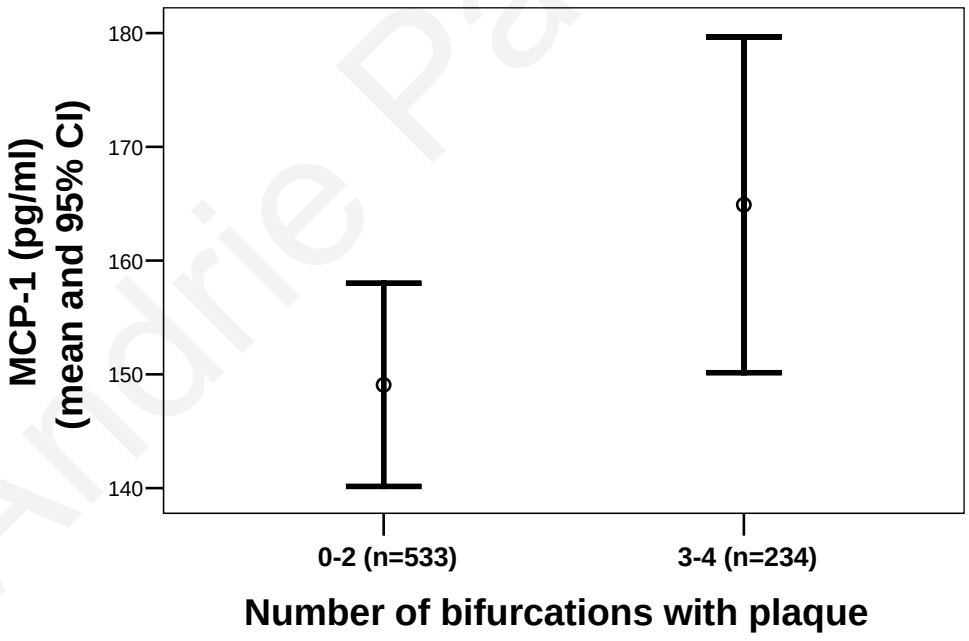

Figure 11.19: Association between MCP-1 levels and more than 2 bifurcations with plaque (Mann-Whitney test; P=0.022)

Association between inflammatory markers and MPT

The association between inflammatory markers and mean plaque type was tested with the use of Kruskal-Wallis tests and crosstabulation. Plasma levels of CRP were not associated with MPT but IL-6 and MCP-1 levels were significantly associated with MPT over the median (P=0.001 and P=0.023 respectively). From the genetic polymorphisms tested only the *MMP-9* (R279Q) was associated with MPT (P=0.033 for AA vs AG, GG). Significant results are shown in figures 11.20-21.

In multivariate binary regression analyses, in those with plaques, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) none of the markers remained associated with MPT. Only age was significantly associated with MPT in the multivariabe model.

Association between inflammatory markers and BPB

The association between levels of plasma inflammatory markers and BPB was tested. Plasma levels of CRP were not associated with IMTmax but IL-6 and MCP-1 levels were significantly associated with increasing BPB. Estimate (B) and p values for univariate linear regression are shown in table 11.1. From the genetic polymorphisms tested *TNF-α* (-308G>A) and *MMP-9* (R279Q) were significantly associated with BPB. Significant results are shown in figures 11.22-25 and p values of all associations are tabulated in table 11.2.

In multivariate binary logistic regressions adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia), neither *TNF-α* (-308G>A) or *MMP-9* (R279Q) polymorphisms remained significantly associated with BPB (population median used as cut-off point) although *MMP-9* had a borderline p value (OR=1.66; 95% CI=0.83 to 3.34 for GG compared to AA, OR=1.12; 95% CI=0.57 to 2.22 for GA compared to AA; P=0.058) and should be taken under consideration. Plasma levels of IL-6 or MCP-1 were no longer significantly associated with BPB after adjustement for traditional risk factors.

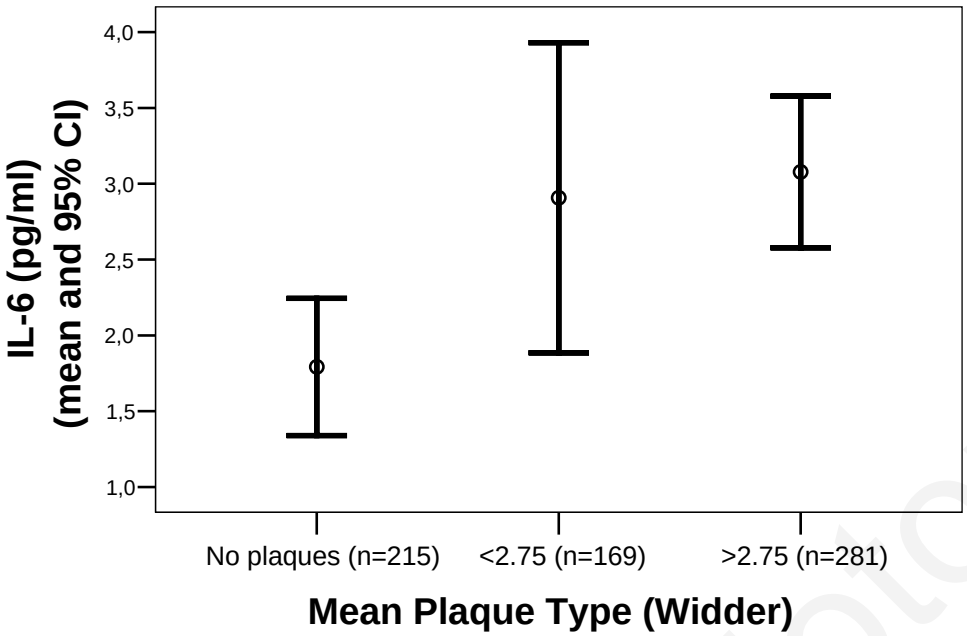

Figure 11.20: Association between IL-6 levels and MPT below and over the median (Kruskal-Wallis test; P for trend=0.001)

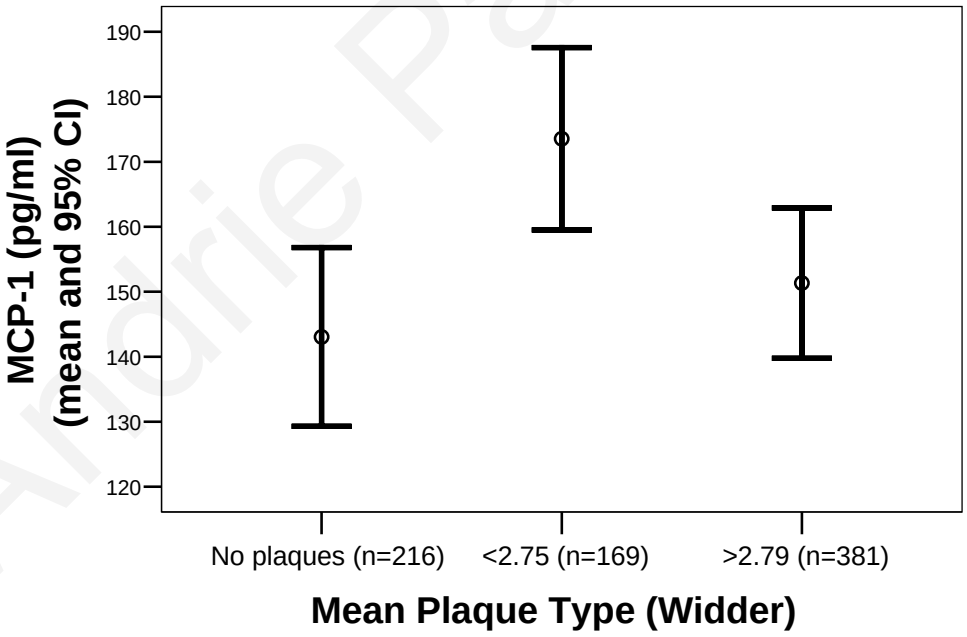

Figure 11.21: Association between MCP-1 levels and MPT below and over the median (ANOVA; P for trend=0.023)

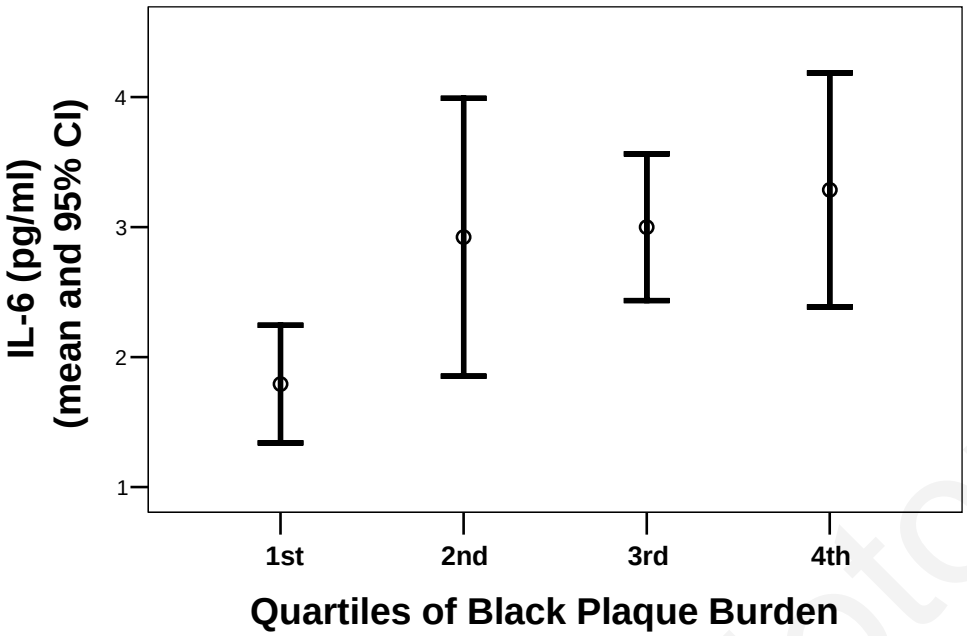

Figure 11.22: Association between IL-6 levels and quartiles of BPB (Kruskal-Wallis test; P for trend=0.001)

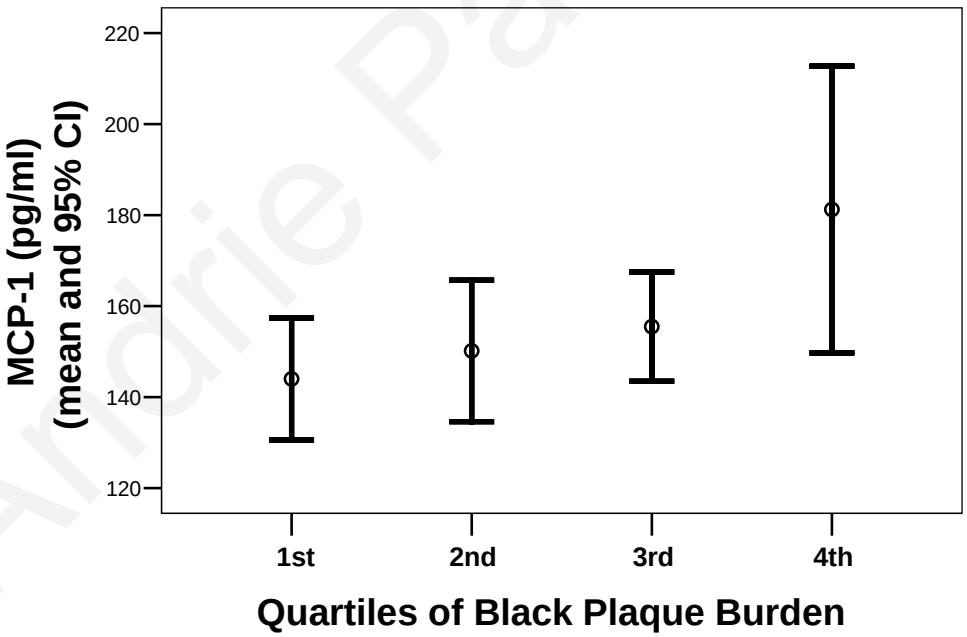

Figure 11.23: Association between MCP-1 levels and quartiles of BPB (Kruskal-Wallis test; P for trend=0.037)

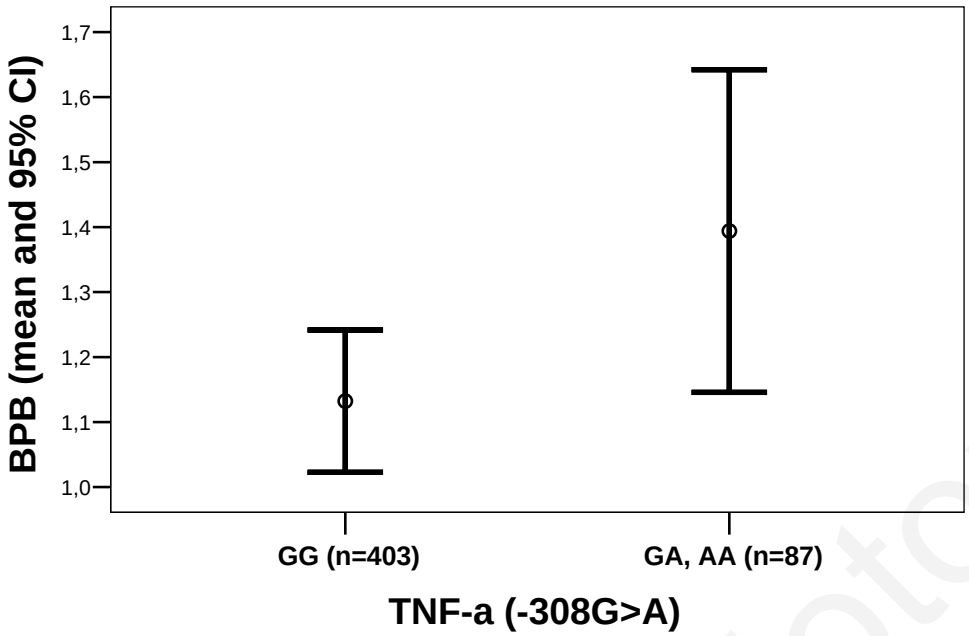

Figure 11.24: Association between BPB and *TNF-α* (-308A>G) genotype (Kruskal-Wallis test; P for trend=0.011)

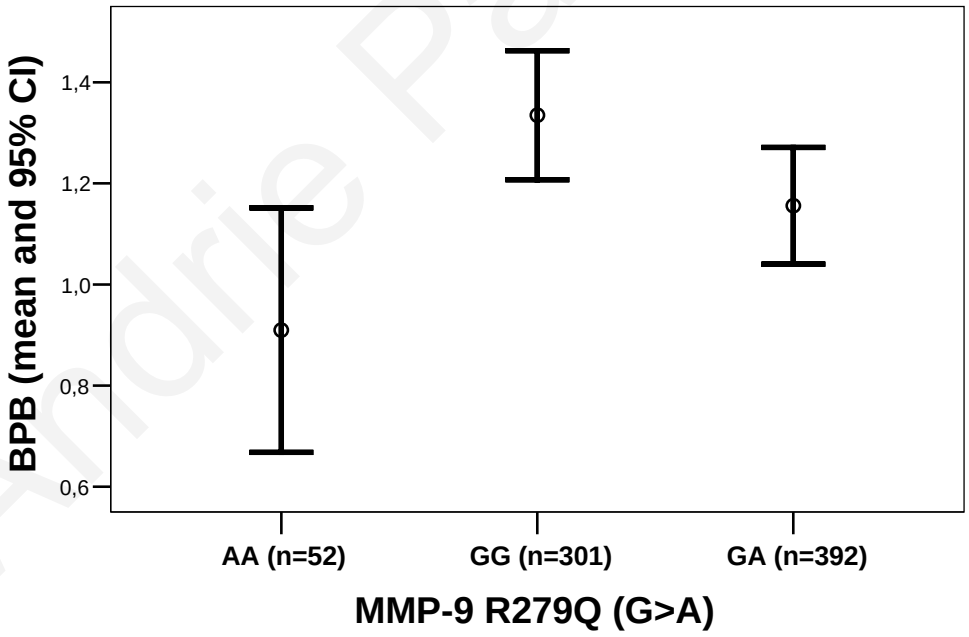

Figure 11.25: Association between BPB and *MMP-9* (R279Q) genotype (Kruskal-Wallis test; P for trend=0.011)

CRP sex specific analysis

In a sex specific analysis for CRP, plasma levels were significantly associated with IMTcc, IMTmax and BPB as well as with presence of plaques but not with TPT, MPT or number of bifurcations with plaque in women (no age difference between sexes). Results are shown in tables 11.5 and figure 11.26.

In a multivariabe binary logistic regression analysis adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) CRP levels were no longer independently associated with any of the ultrasonic measurements.

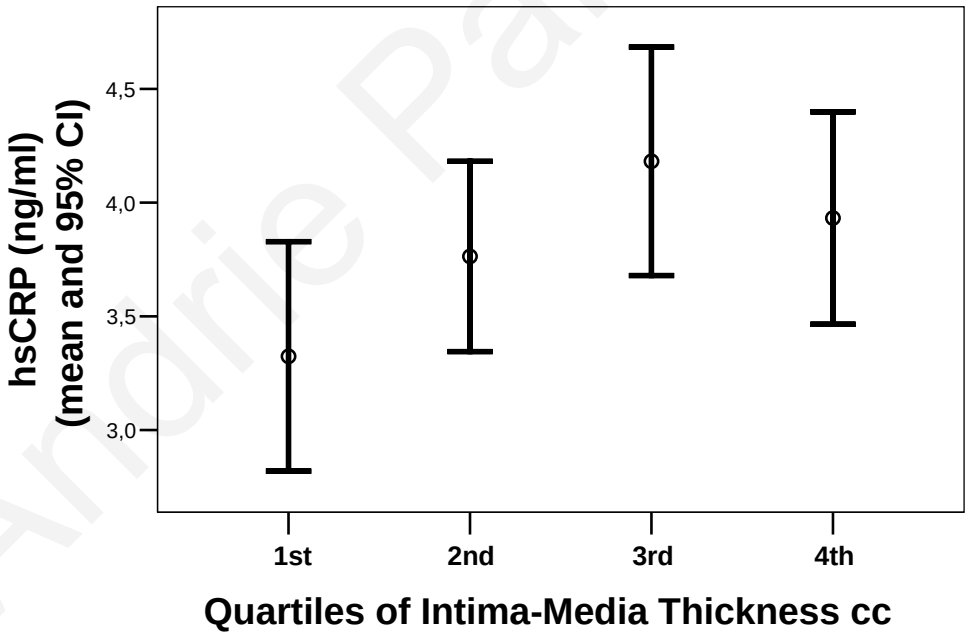

Figure 11.26: Association between CRP levels and quartiles of IMTcc in women only (Kruskal-Wallis test; P for trend=0.074)

Table 11.5: Results of association between CRP and ultrasonic measurements in women only; linear regression is used and CRP, IMTmax and TPT were logtransformed to fit the assumption of normality

	IMTcc		IMTmax		TPT		BPB	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
CRP	0.005 (0.002 to 0.007)	**P=0.003**	0.153 (0.043 to 0.27)	**P=0.007**	0.060 (-0.11 to 0.24)	P=0.50	0.208 (0.015 to 0.40)	**P=0.034**

Table 11.6: Results of association between presence of plaques and number of bifurcations with plaque, CRP in women (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaques as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque)

Marker	**N**	**MPT<2.75>**	**Presence of plaques**	**Number of bifurcations With plaques (0-4)**	**0-2 bifurcations with plaque vs 3-4**
CRP	728	P=0.052	**P=0.093**	P=0.265	P=0.584

Table 11.7: Compiled results of associations between inflammatory markers and ultrasonic measurements in univariate analysis

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
hsCRP	-	-	-	-	-	-
IL-6	-	+	-	+	+	+
MCP-1	+	+	+	+	+	+
IL-6 (-174C>G)	-	-	-	-	-	-
TNF-α (-308A>G)	+	-	+	-	+	-
MGP (138C>T)	-	+	-	-	-	-
MMP-1 (1G/2G)	-	-	-	-	-	-
MMP-3 (5A/6A)	-	-	-	-	-	-
MMP-7 (-181A>G)	-	-	+	-	-	-
MMP-9 (R279Q)	-	+	+	+	+	+
MMP-12 (-82A>G)	-	-	-	-	-	-

Table 11.8: Compiled results of association between inflammatory markers and ultrasonic measurements in multivariate analyses adjusting for age, sex, smoking in packyears, diabetes and hypertention

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
hsCRP	-	-	-	-	-	-
IL-6	-	-	-	+	-	-
MCP-1	-	-	-	-	-	-
IL-6 (-174C>G)	-	-	-	-	-	-
TNF-α (-308A>G)	-	-	-	-	-	-
MGP (138C>T)	-	+	-	-	-	-
MMP-1 (1G/2G)	-	-	-	-	-	-
MMP-3 (5A/6A)	-	-	-	-	-	-
MMP-7 (-181A>G)	-	-	-	-	-	-
MMP-9 (R279Q)	-	-	+	+	*+	-
MMP-12 (-82A>G)	-	-	-	-	-	-

* Borderline P value (P=0.058)

Summary of results

The results support the hypothesis that inflammatory factors and markers are associated with early, subclinical atherosclerosis and that different markers are associated with IMT or with plaques.

1. Plasma levels of CRP, which has received much attention these past years as a novel marker for atherosclerosis and CVD, wer only significantly associated with increasing IMTcc quartiles in our whole population and not with any other ultrasonic measurement tested. In a sex specific analysis, CRP levels were also significantly associated with IMTcc, IMTmax and BPB in women. They were also significantly associated with presence of plaque before adjustement.
2. Both plasma levels of IL-6 and MCP-1 (a novel marker) were significantly associated with all the ultrasonic markers tested (IMTcc, IMTmax, TPT, presence of plaques, number of bifurcations with plaque, BPB and MPT), which implies that these (and therefore inflammation) act at early stages of both IMT thickening and plaque formation and progression, possibly through macrophage recruitment.
3. *TNF-α* (308A>G) genetic polymorphism was significantly associated with IMTcc, TPT and BPB but not with IMTmax.
4. *MGP* (-138C>T) was significantly associated with IMTmax.
5. *MMP-7* (-181A>G) polymorphism was significantly associated with TPT and with more than 2 bifurcations with plaques.
6. *MMP-9* (R279Q) was significantly associated with presence and plaque size (IMTmax, TPT), plaque type (BPB, MPT), extent of atherosclerosis (number of bifurcations with plaques) but not with IMTcc and emerged as an important factor contributing to a high degree to the development and progression of subclinical atherosclerosis.

Chapter 12:

Results of association between thrombotic markers and subclinical atherosclerosis

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and thrombotic markers (both biochemical and genetic). The thrombotic markers tested were: soluble CD40L, Fb, P-selectin, tissue factor, microparticles and *PAI-1* (4G/5G). The reasons for the choise of the above markers are stated at the hypothesis (chapter 4).

Association between thrombotic markers and IMTcc

The associaton between levels of plasma thrombotic markers and IMTcc was tested. Only fibrinogen levels were significantly associated with IMTcc (Table 12.1) (Fig.12.1). *PAI-1* (4G/5G) polymorphism was not significantly associated with IMTcc (P for trend=0.82).

In multivariate linar regression analyses adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertension and hyperlipidaemia) none of the markers tested was significantly associated with IMTcc (*TF was 0.045 if adjusted only for age and sex).

Association between thrombotic markers and IMTmax

The association between levels of plasma thrombotic markers and IMTmax was tested. Plasma levels of sCD40L and fibrinogen were significantly associated with IMTmax; P-selectin, tissue factor and microparticles were not. Significant results are shown in figures 12.2-3 and p values are tabulated in table 12.1.

In multivariate linear regression analyses, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) only sCD40L (P=0.008) remained significantly associated with IMTmax (logtransformed to fit the assumption of normality) although tissue factor had a borderline value (P=0.066). All the factors in the model (age, sex, smoking, diabetes, hypertention and hyperlipidaemia) could explain 33.4% of the variability in IMTmax. Adding sCD40L (logtransformed for normality) to the model improved its predictive ability by 1.8% reaching 34.9%.

Table 12.1: Results of univariate linear regressions for association between ultrasonic measurements and plasma levels of thrombosis markers (IMTmax, TPT, sCD40L, T.F and MP were logtransformed to achieve normality; for Fb the square root was used)

	IMTcc		**IMTmax**		**TPT**		**BPB**	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
sCD40L	-0.001 (-0.002 to 0.001)	P=0.32	-0.048 (-0.085to-0.12)	**P=0.010**	-0.057 (-0.105 to -0.01)	**P=0.019**	-0.106 (-0.18 to -0.036)	**P=0.003**
Fb	4.55E-008 (0.0 to0.0)	**P=0.017**	1.94E-005 (0.0 to 0.0)	**P=0.003**	1.20E-006 (0.0 to 0.0)	P=0.14	3.53E-006 (0.0 to 0.0)	**P=0.007**
P-S	-1.6E-005 (0.0 to 0.0)	P=0.46	0.00 (-0.001to0.002)	P=0.83	-0.001 (-0.003to0.001)	P=0.45	-0.001 (-0.004 to 0.002)	P=0.60
T.F	-0.001 (-0.004 to 0.003)	P=0.74	-0.016 (-0.124 to 0.91)	P=0.76	-0.205 (-0.35 to -0.06)	**P=0.007**	-0.116 (-0.33 to 0.99)	P=0.29
MP	0.00 (-0.001 to 0.002)	P=0.77	-0.024 (-0.07 to 0.26)	P=0.35	-0.19 (-0.091 to-0.052)	P=0.60	-0.037 (-0.131 to 0.057)	P=0.44

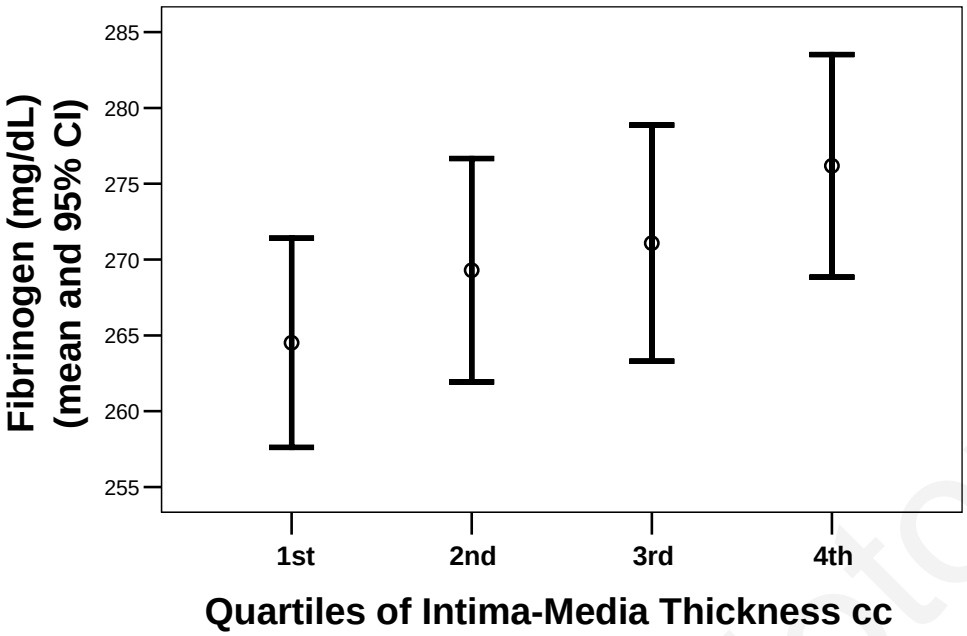

Figure 12.1: Association between fibrinogen levels and quartiles of IMTcc (Kruskal-Wallis test; P=0.003)

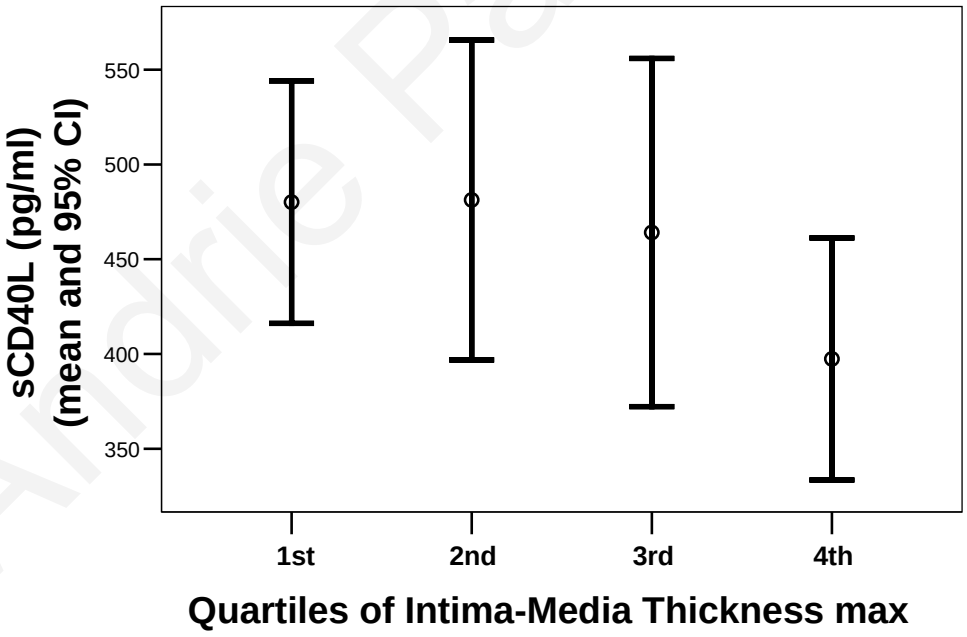

Figure 12.2: Association between sCD40L levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.071)

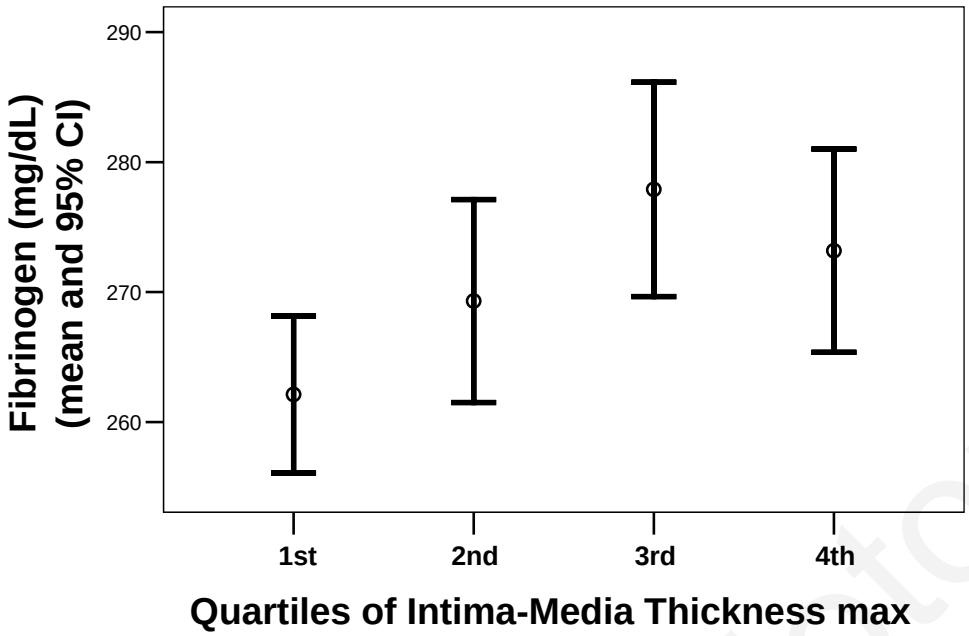

Figure 12.3: Association between fibrinogen levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.001)

Association between thrombotic markers and TPT

The association between plasma levels of thrombotic markers and TPT was tested. Plasma levels of sCD40L and tissue factor were significantly associated with TPT. Estimate (B) and p values for univariate linear regression are shown in table 12.1. Fibrinogen, P-selectin, microparticles and the *PAI-1* (4G/5G) polymorphism were not associated with TPT. Significant results are shown in figures 12.4-5.

In multivariate linear regression analyses, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) both sCD40L and tissue factor remained significantly associated with TPT over and above traditional risk factors (P=0.007 and P=0.001 respectively). Adding sCD40L or tissue factor to the model improved its predictive ability (31.5% and 31.1% respectively compared to 30.7%). A multivariate model including both sCD40L and tissue factor and adjusting for traditional risk factors could predict 33.1% of the variability in TPT and both markers remained significant (P=0.002 and P=0.001 respectively).

Figure 12.4: Association between sCD40L levels and quartiles of TPT (Kruskal-Wallis test; P for trend=0.023)

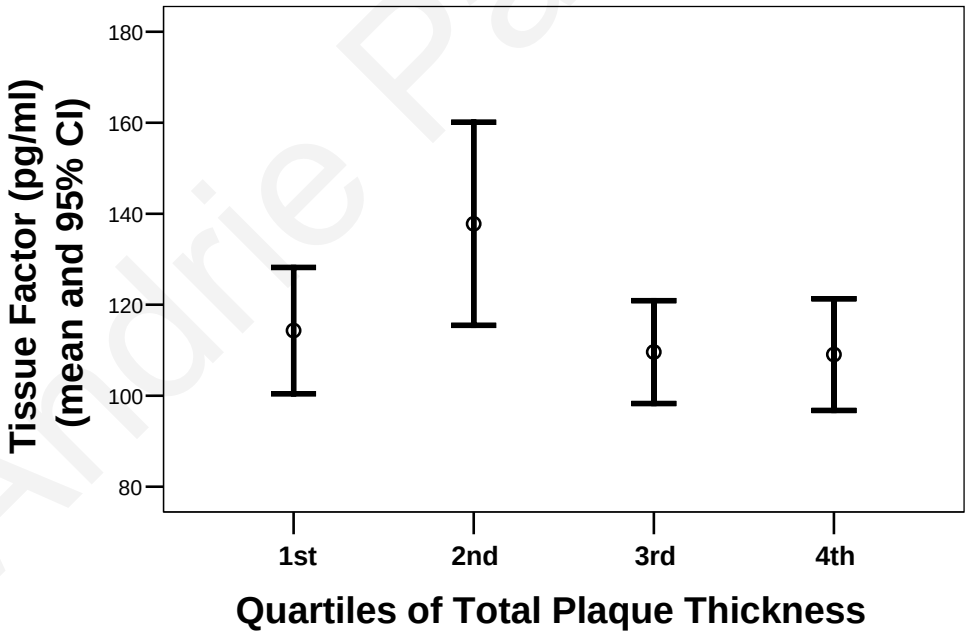

Figure 12.5: Association between tissue factor levels and quartiles of TPT (Kruskal-Wallis test; P=0.28)

Association between thrombotic markers, presence of plaques and number of bifurcations with plaque

The association between plasma levels of thrombotic markers and presence of plaques and number of bifurcations with plaque was tested. In addition to number of bifurcations with plaque ranging from 0 to 4 we also used a cut-off point of 2 bifurations with plaque (0-2) or more (3-4) to indicate generalised atherosclerosis (plaque in both carotid and femoral bifurcations). Fibrinogen levels were significantly associated with both presence and number of bifurcations with plaque and sCD40L and tissue factor levels was associated with only the number of bifurcations with plaque (Table 12.2). Significant results are shown in figures 12.6-10. *PAI-1* (4G/5G) polymorphism was not associated with either the presence or the number of bifurcations with plaque.

In multivariate binary logistic regression analyses adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) fibrinogen levels remained significantly associated with presence of plaques (P=0.043). In a multivariate binary regression, sCD40L levels remained significanty associated with number of bifurcations with plaque (0-2 vs 3-4) over and above traditional risk factors (P<0.001).

Table 12.2: Results of association between presence of plaques and number of bifurcations with plaque and plasma levels of thrombosis markers (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaques as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque)

Marker	N	Presence of plaques	Number of bifurcations With plaques (0-4)	0-2 bifurcations with plaque vs 3-4
sCD40L	759	P=0.23	**P=0.001**	**P<0.001**
Fibrinogen	736	**P=0.001**	**P=0.006**	**P=0.004**
P-Selectin	532	P=0.98	P=0.78	P=0.87
Tissue Factor	755	P=0.35	**P=0.037**	P=0.62
Microparticles	413	P=0.69	P=0.70	P=0.35

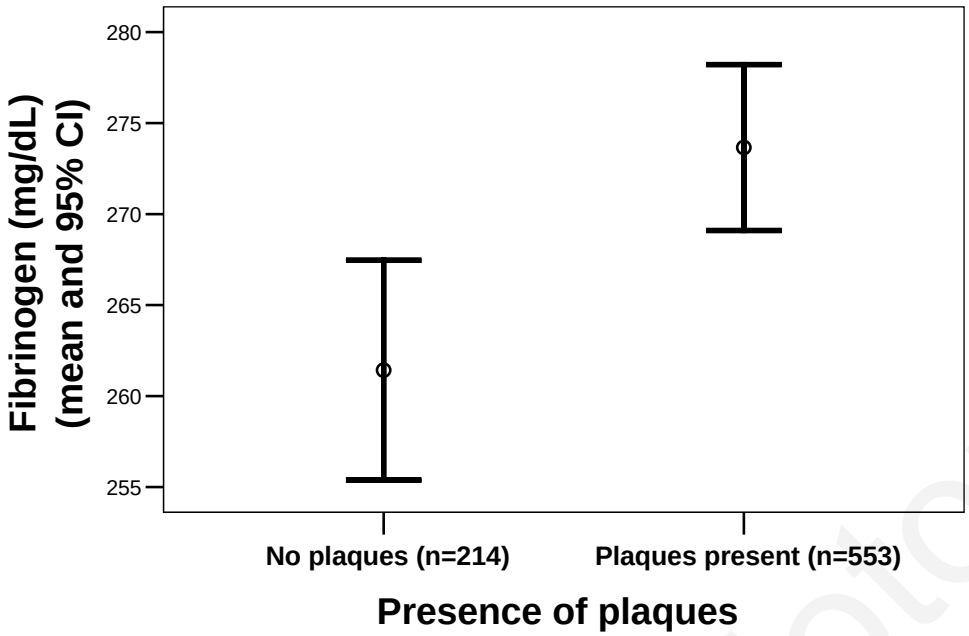

Figure 12.6: Association between fibrinogen levels and presence of plaques (Mann-Whitney test; P=0.001)

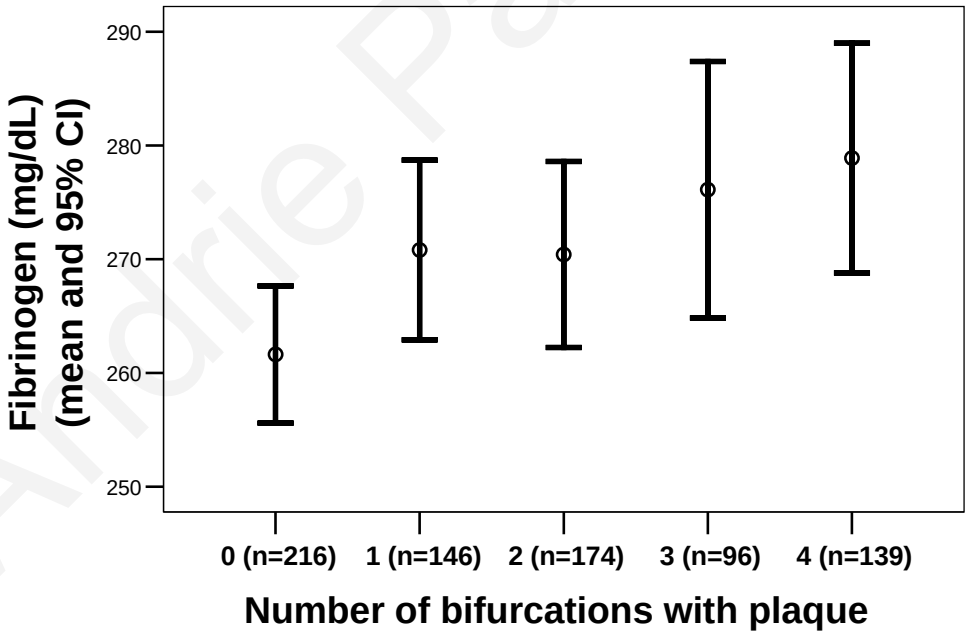

Figure 12.7: Association between fibrinogen levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend=0.006)

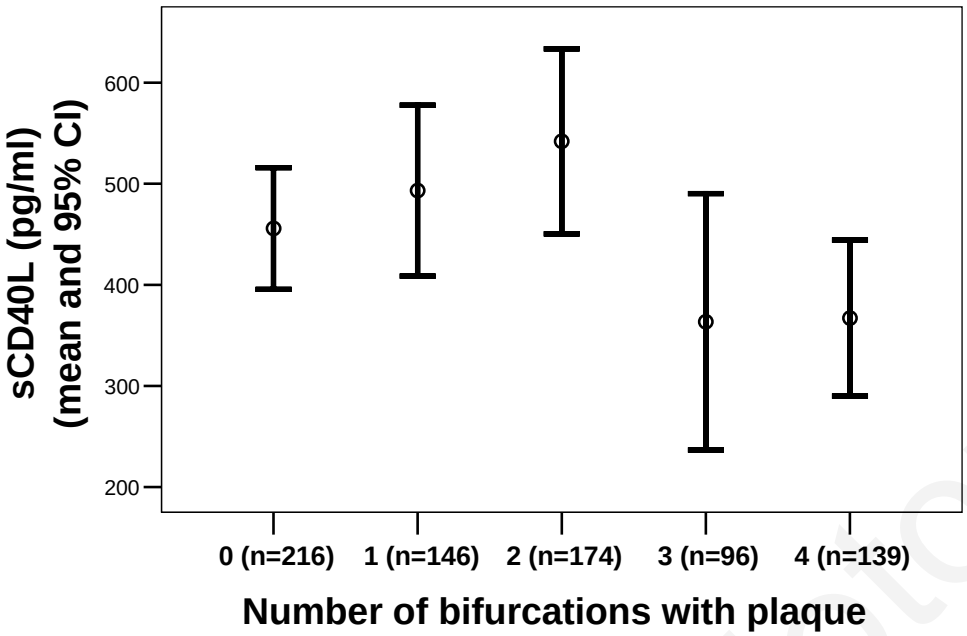

Figure 12.8: Association between sCD40L levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend=0.001)

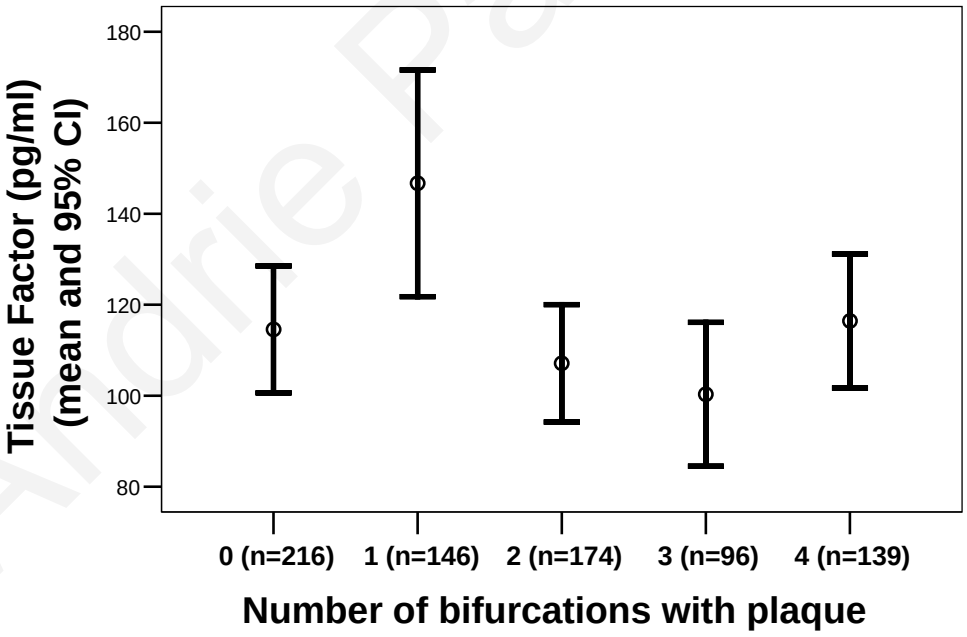

Figure 12.9: Association between tissue factor levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend=0.037)

Association between thrombotic markers and MPT

The association between thrombotic markers and mean plaque type was tested with the use of Kruskal-Wallis tests and crosstabulation. Plasma levels of sCD40L and fibrinogen were significantly associated with MPT (P=0.014 and P<0.001 respectively). Tissue factors, MP and P-selectin were not associated with MPT and neither was the *PAI-1* (4G/5G) polymorphism. Significant results are shown in figures 12-10-11.

In multivariate binary regression analyses adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) only sCD40L remained associated with MPT (P=0.01).

Association between thrombotic markers and BPB

The association between plasma levels of thrombotic markers and BPB was tested. Fibrinogen and sCD40L levels were significantly associated with BPB in univariate linear regression analyses (P=0.007 and P=0.003) respectively). P-selectin, tissue factor, microparticles and *PAI-1* (4G/5G) polymorpshism were not associated with BPB. Significant results are shown in figures 12.12-13.

In multivariate linear regression analyses adjusting for traditional risk factors both fibrinogen and sCD40L remained significantly associated with BPB over and above traditional risk factors (P=0.043 and P=0.002 respectively). Tissue factor levels also emerged to be independently associated with BPB (P=0.016). Adding sCD40L to the model could improve its predictive ability by 0.9% and it reached 32.4%.

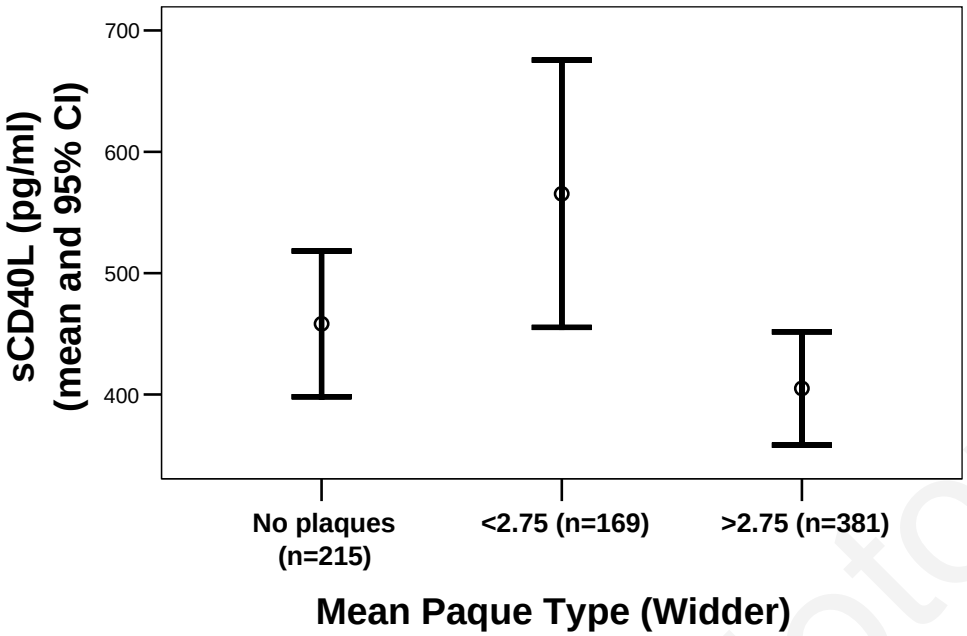

Figure 12.10: Association between sCD40L levels and MPT below and over the median (Kruskal-Wallis test; P for trend=0.014)

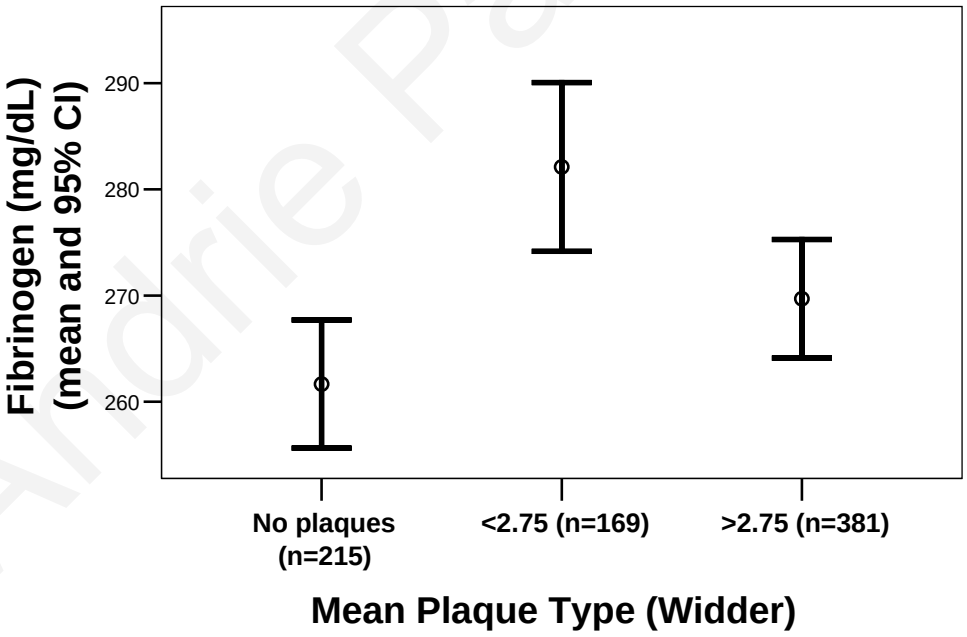

Figure 12.11: Association between fibrinogen levels and MPT below and over the median (Kruskal-Wallis test; P for trend <0.0001)

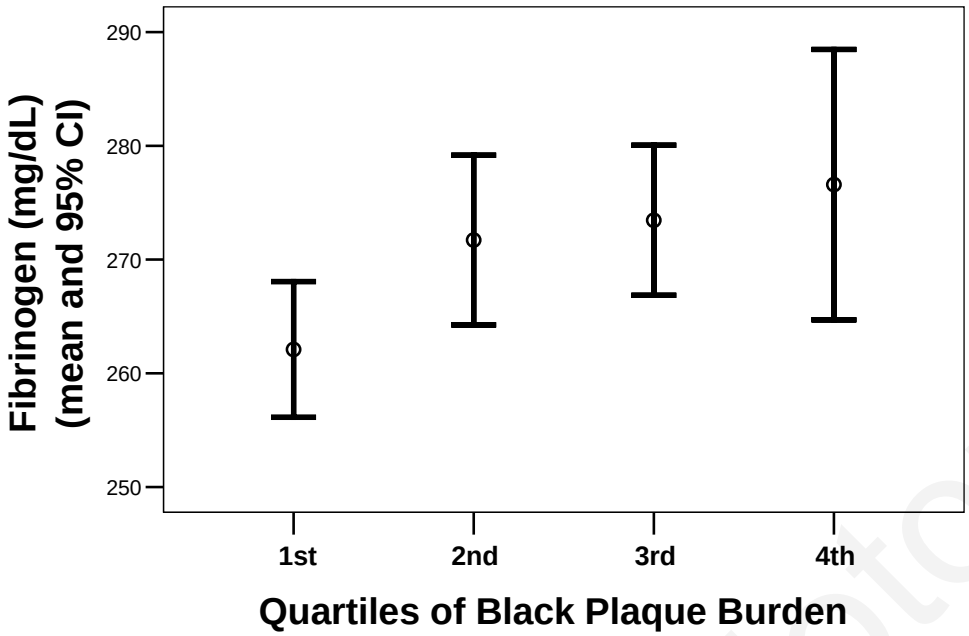

Figure 12.12: Association between fibrinogen levels and quartiles of BPB (Kruskal-Wallis test; P for trend=0.011)

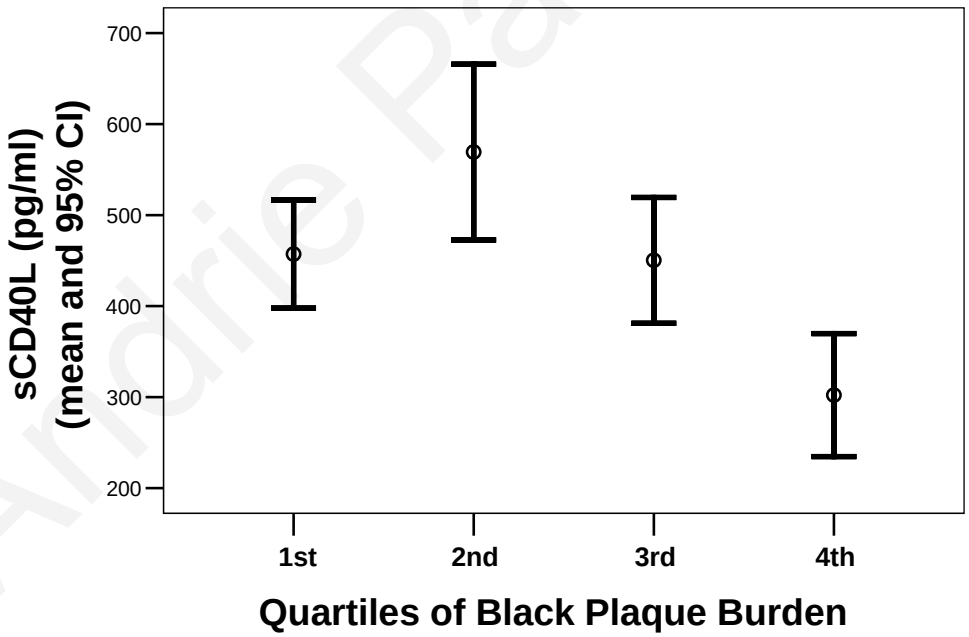

Figure 12.13: Association between sCD40L levels and quartiles of BPB (Kruskal-Wallis test; P for trend<0.001)

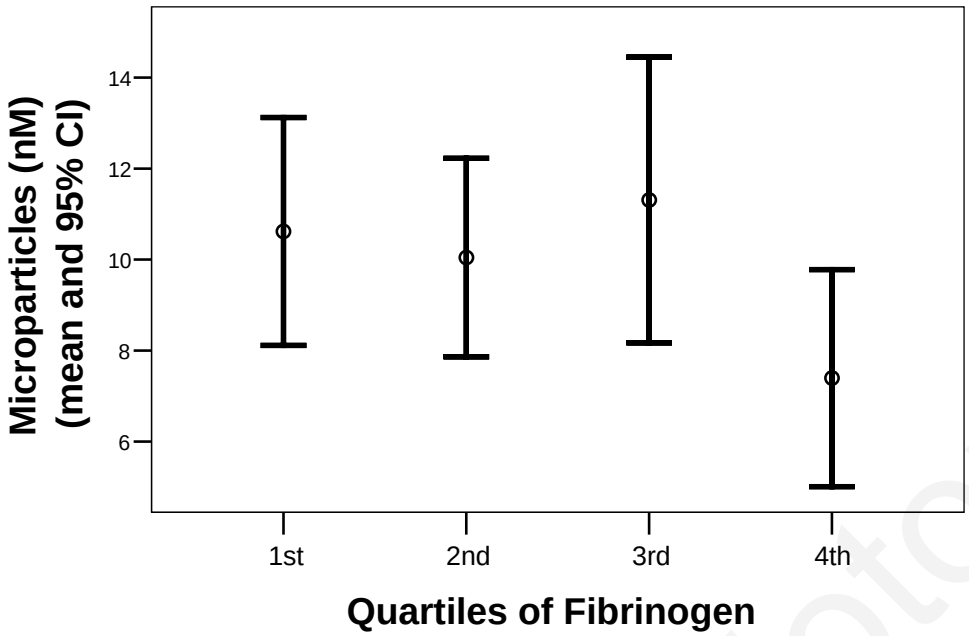

Figure 12.14: Association between microparticle levels and quartiles of fibrinogen measurements (Kruskal-Wallis test; P for trend=0.016)

Table 12.3: Compiled results of association between thrombosis markers and ultrasonic measurements in univariate analyses

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
sCD40L	-	+	+	-	+	+
Fb	+	+	-	+	+	+
TF	-	-	+	-	-	-
MP	-	-	-	-	-	-
P-selectin	-	-	-	-	-	-
PAI-1 (4G/5G)	-	-	-	-	-	-

Table 12.4: Compiled results of association between thrombosis markers and ultrasonic measurements in multivariate analyses adjusting for age, sex, smoking in packyears, diabetes and hypertention

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
sCD40L	-	+	+	-	+	+
Fb	-	-	-	+	+	-
TF	-	-	+	-	+	-
MP	-	-	-	-	-	-
P-selectin	-	-	-	-	-	-
PAI-1 (4G/5G)	-	-	-	-	-	-

Summary of results

The results support the hypothesis that certain thrombotic markers that also participate in inflammatory cascades are associated with early, subclinical atherosclerosis as assessed by ultrasound.

1. Plasma sCD40L levels were significantly associated with IMTmax, TPT, number of bifurcations with plaque, MPT and BPB but not IMTcc or presence of plaques in univariate analyses. After adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hpertention and hyperlipidaemia) sCD40L remained associated with IMTmax, TPT, number of bifurcations with plaque, MPT and BPB over and above established risk factors.
2. Plasma fibrinogen levels were significantly associated with IMTcc, IMTmax, presence of plaques and number of bifurcations with plaque, MPT and BPB but not TPT in univariate analyses. After adjusting for traditional risk factors, Fb remained significantly associated with presence of plaques, number of bifircations with plaque and BPB. These results indicate that Fb is mostly associated with plaques and more specifically with echolucent, vulnerable plaques.
3. Plasma P-selectin levels were not associated with any of the ultrasonic markers tested. It is possible that P-selectin plays its part mostly during late (clinical) disease and not at its early stages.
4. Plasma tissue factor levels were associated univariatly with TPT and number of bifurcations with plaque. After adjustement for traditional risk factors, tissue factor levels remained significantly associated with TPT as well as with BPB.
5. Plasma microparticle levels, a new and not very well studied marker, have not been associated with any of the ultrasonic markers tested in our population. However, they were significantly associated with fibrinogen quartiles.
6. The *PAI-1* (4G/5G) polymorphism was not associated with any of the ultrasonic markers tested in our population.

Chapter 13:

Results of association between homocysteine metabolism markers and subclinical atherosclerosis

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and homocysteine metabolism markers (both biochemical and genetic). The homocysteine metabolism markers tested were: total serum Hcy, folic acid, vitamin B12, ADMA and the *MTHFR* (677C>T) polymorphism. The reasons for the choise of the above markers are stated at the hypothesis (chapter 4).

The association between folic acid, vitamin B12 levels and tHcy levels has been previously established, however, given the absence of folic acid fortification in our population and the relatively high population median for tHcy (12 μmol/L) it was decided that the association be verified before procedding with further analysis of the data. The association between *MTHFR* (677C>T) genotype and serum tHcy levels was also tested, given the controversy in the literature, with most studies not supporting an association between the *MTHFR* (677C>T) polymorphism and homocysteine levels (see chapter 3.8).

Association between serum folic acid, vitamin B12 levels and serum tHcy levels

In linear regression analyses, both folic acid and vitamin B12 were significanlt associated with tHcy levels (P<0.001 for both) (Figs.13.1-2). Folic acid could explain 5.4% of the variability in tHcy levels and B12 could explain 6.3%.

Association between *MTHFR* (677C>T) genotype and serum levels of tHcy

In a univariate linear regression analysis, the *MTHFR* 677C>T polymorphism (TT compared to CC, CT) had a borderline p value for association with higher levels of tHcy (P=0.055) that merits further investigation.

In a multivariate linear regression model adjusting for age, sex, folic acid and B12 (all factors affecting tHcy levels), the *MTHFR* 677C>T genotype was significantly associated with tHcy levels (P=0.003) and the model could predict 33.8% of the variability in tHcy levels. Sex-specific univariate linear analysis showed that the relationship was driven solely my men (P=0.004) and it was not significant in women (P=0.64) (Fig.13.3).

Since Hcy levels are known to be affected by age and this was also true in our population, we examined the association of *MTHFR* (677C>T) genotype and tHcy levels in subjects below and over the population mean age (60 years). In a univariate linear regression analysis, the genotype-Hcy association was confined to men below (≤) 60 years of age (P=0.005); in people over 60 years the association was not significant (P=0.947). In people below 60 years of age adjusting for sex, folate and vitamin B12 levels in a multivariate model further increased the significance of the *MTHFR* (677C>T) genotype (P=0.001 for 677TT compared to CT, CC; P<0.001 for sex, F.A. and B12).

If only men below (≤) 60 yrs of age were included in the analysis, the *MTHFR* (677C>T) genotype was the most significant predictor of tHcy blood levels (P<0.001), followed by folic acid (P=0.001). Vitamin B12 levels were no longer significant.

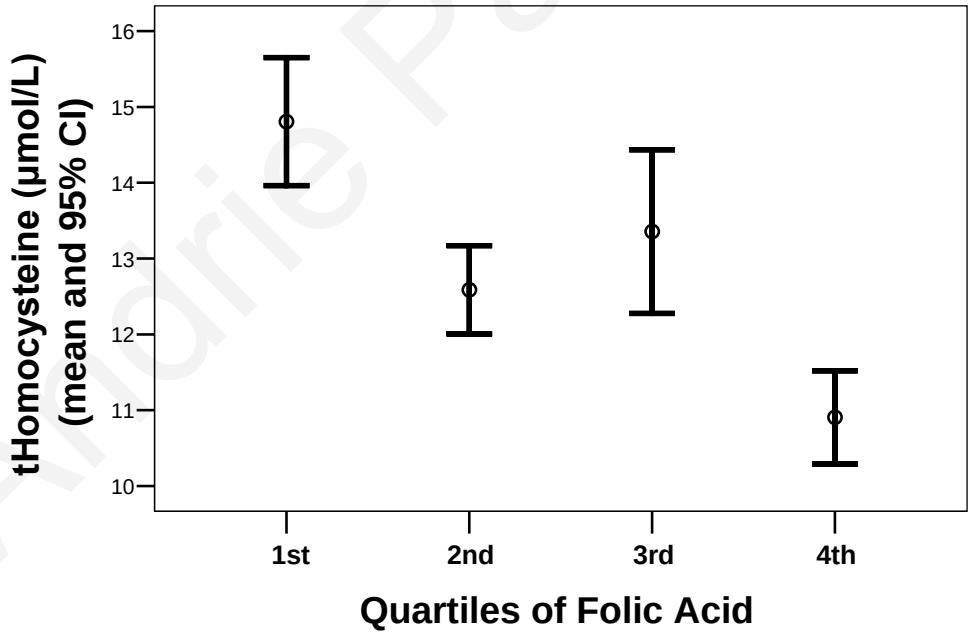

Figure 13.1: Association between tHcy levels and quartiles of folic acid measurements (Kruskal-Wallis test; P<0.001)

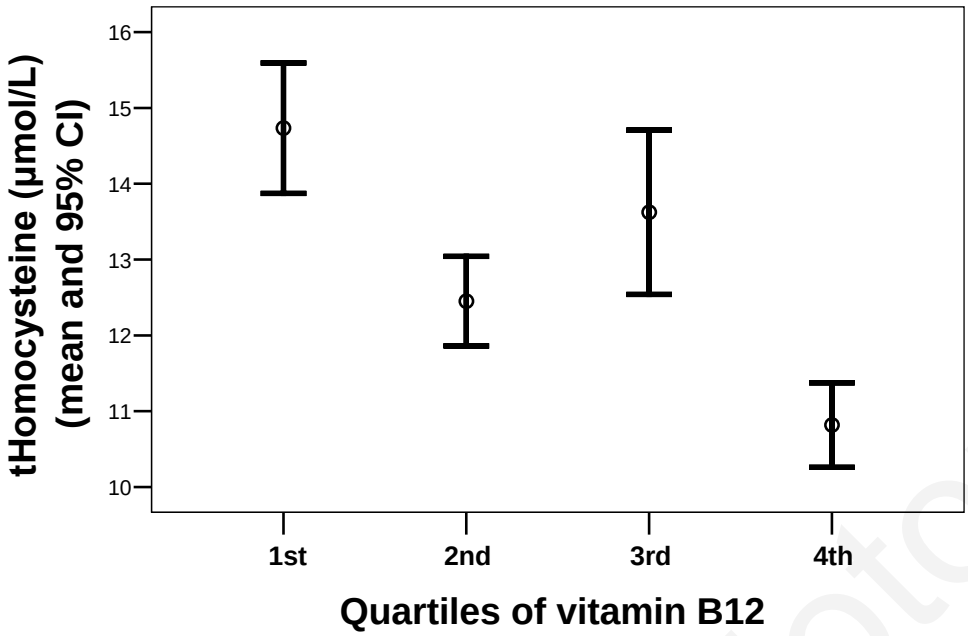

Figure 13.2: Association between tHcy levels and quartiles of vitamin B12 measurements (Kruskal-Wallis test; P for trend<0.001)

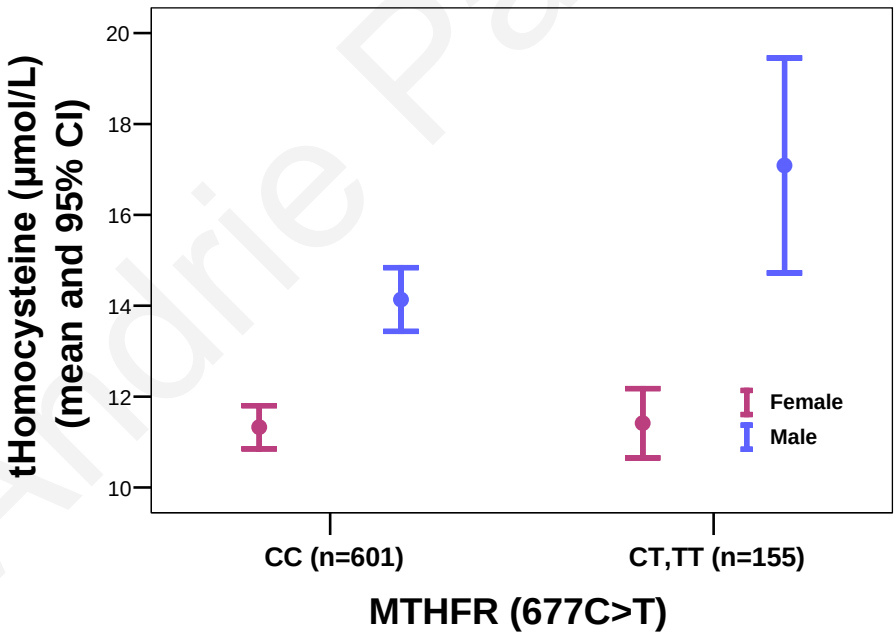

Figure 13.3: Association between tHcy levels and *MTHFR* 677C>T genotype (Mann-Whitney test; P for men=0.017; P for women=0.53)

Association between homocysteine metabolism markers and IMTcc

The association between homocysteine metabolism markers and IMTcc was tested. Serum tHcy, folic acid and vitamin B12 levels were all significantly associated with IMTcc. Plasma levels of ADMA and the *MTHFR* (677C>T) genotype were not. Estimate (B) and p values for univariate linear regression are shown in table 13.1. Significant results are shown in figures 13.4-6.

In multivariate linear regression analyses, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) and each of the variables (by using the population median as a cut-off point), tHcy (>12 μmol/L) and vitamin B12 levels (>400 pg/ml) remained significantly associated with IMTcc (P=0.022 and P=0.009 respectively) over and above traditional risk factors. When both tHcy and B12 were added to the model, they still remained significant and increased its predictive ability from 28.6% to 29.4%.

Association between homocysteine metabolism markers and IMTmax

The association between homocysteine metabolism markers and IMTmax was tested. Serum tHcy and vitamin B12 levels were significantly associated with IMTmax. Estimate and p values are shown in table 13.1. Serum levels of folic acid, plasma levels of ADMA and the *MTHFR* (677C>T) genotype were not associated with IMTmax. Significant results are shown in figures 13.7-8.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) and using the population media as a cut-off point for tHcy and B12, none of the variables remained significanlty associated with IMTmax, although tHcy had a borderline p value (P=0.052). If both tHcy and B12 were added to the model, tHcy levels were significantly associated with IMTmax over and above traditional risk factors (P=0.034) and improved the predictive ability of the model by 0.5%.

Table 13.1: Results of univariate linear regressions for association between ultrasonic measurements and serum levels of homocysteine metabolism markers (IMTmax, TPT, tHcy, F.A, B12 and ADMA were logtransformed to achieve normality)

	IMTcc		**IMTmax**		**TPT**		**BPB**	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
tHcy	0.031 (0.024 to 0.038)	**P<0.001**	1.076 (0.836 to 1.316)	**P<0.001**	1.263 (0.939 to 1.558)	**P<0.001**	2.344 (1.871 to 2.818)	**P<0.001**
F.A.	-0.003 (-0.006to 0.00)	**P=0.041**	-0.098 (-0.20 to 0.005)	P=0.061	-0.198 (-0.33 to -0.064)	**P=0.004**	-0.366 (-0.057 to- 0.16)	**P<0.001**
B12	-0.006 (-0.008 to -0.003)	**P<0.001**	-0.128 (-0.219to-0.037)	**P=0.006**	-0.146 (-0.265to-0.027)	**P=0.016**	-0.239 (-0.42 to -0.058)	**P=0.01**
ADMA	0.0 (-0.003 to 0.002)	P=0.72	-0.057 (-0.146 to 0.033)	P=0.21	0.106 (-0.023 to 0.234)	P=0.107	-0.018 (-0.22 to 0.184)	P=0.86

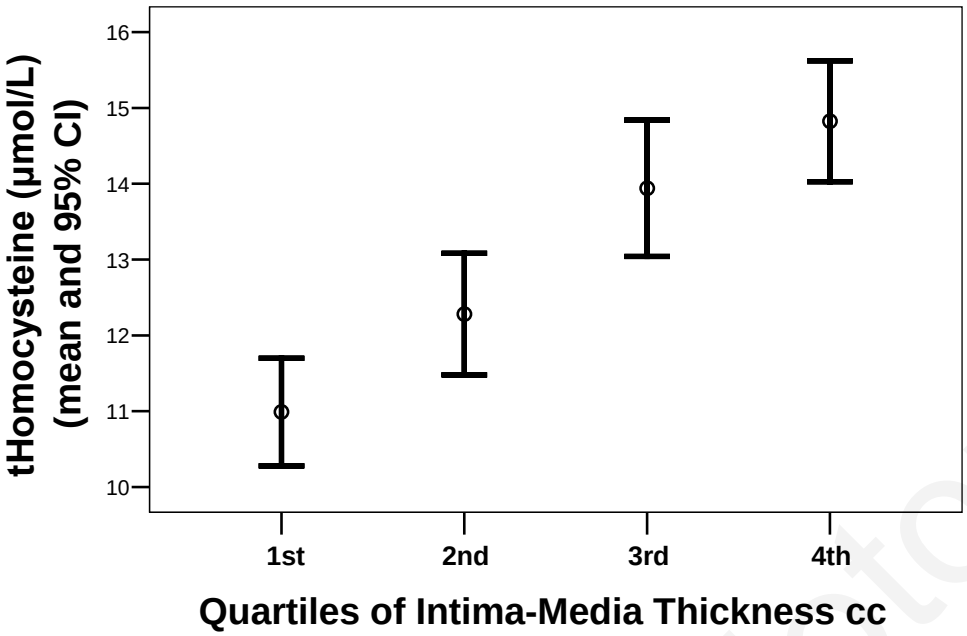

Figure 13.4: Association between tHcy levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend<0.001)

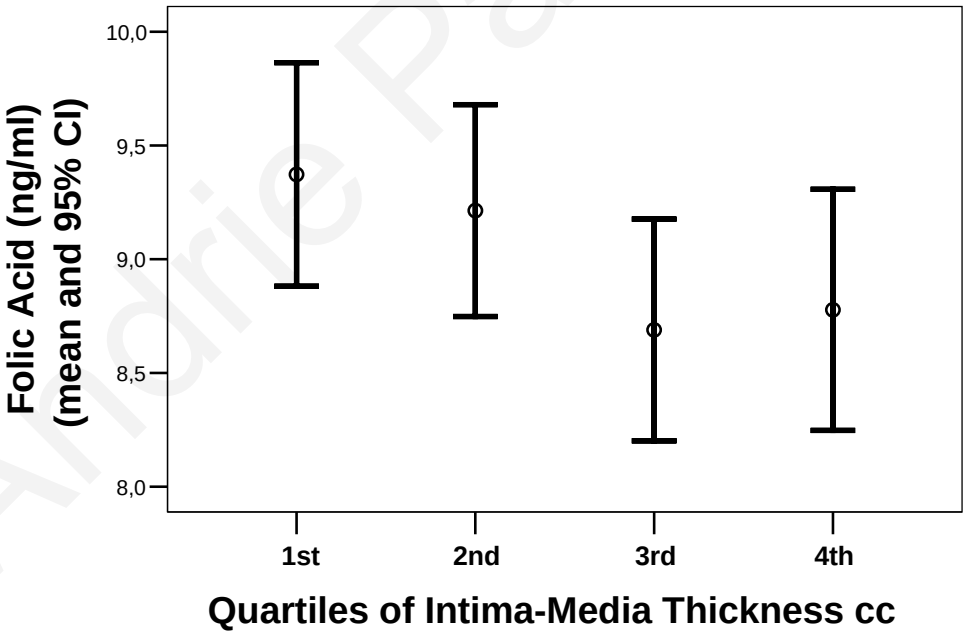

Figure 13.5: Association between folic acid levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend=0.082)

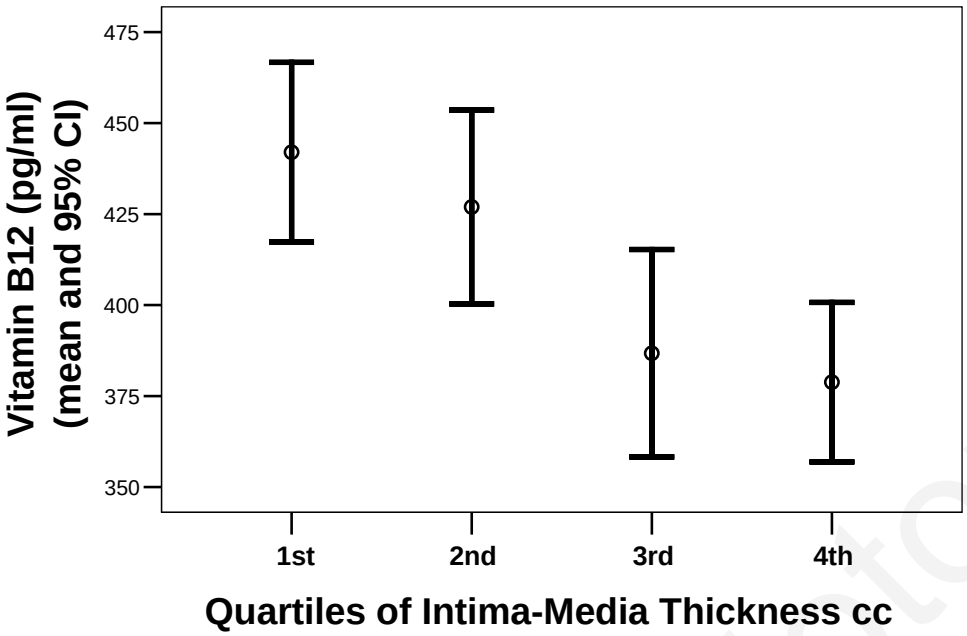

Figure 13.6: Association between vitamin B12 levels and quartiles of IMTcc (Kruskal-Wallis test; P for trend<0.001)

Association between homocysteine metabolism markers and TPT

The association between homocysteine metabolism markers and TPT was tested. Serum tHcy, folic acid and vitamin B12 levels were significantly associated with TPT. Estimate and p values are shown in table 13.1. Plasma levels of ADMA and the *MTHFR* (677C>T) genotype were not associated with TPT. Significant results are shown in figures 13.9-11.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) and using the population media as a cut-off point for tHcy, F.A. and B12, only tHcy levels remained significanlty associated with TPT above traditional risk factors (P=0.014). Adding tHcy to the model improved its predictive ability by 0.6% and could predict 31.3% of the variability in TPT in individual level.

Association between homocysteine metabolism markers, presence of plaques and number of bifurcations with plaque

The association between markers of homocysteine metabolism, presence of plaques and number of bifurcations with plaque was tested. In addition to number of bifurcations with plaque, ranging from 0 to 4, a cut-off point of 2 bifurcations with plaque (0-2) was used with more (3-4) indicating generalised atherosclerosis (plaque present in carotid and femoral bifurcations). Serum levels of tHcy and plasma levels of ADMA were significantly associated with both presence of plaque and number of bifurcations with plaque. Folic acid levels were only associated with number of bifurcations with plaque; vitamin B12 levels and the *MTHFR* (677C>T) genotype were not associated with neither presence of plaques nor number of bifurcations with plaque Significant results are shown in figures 13.12-16 and p values are tabulated in table 13.2.

In multivariate binary logistic regression analyses adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes and hyperlipidaemia) only tHcy levels remained significantly associated with more than 2 bifurcations with plaque (P=0.004).

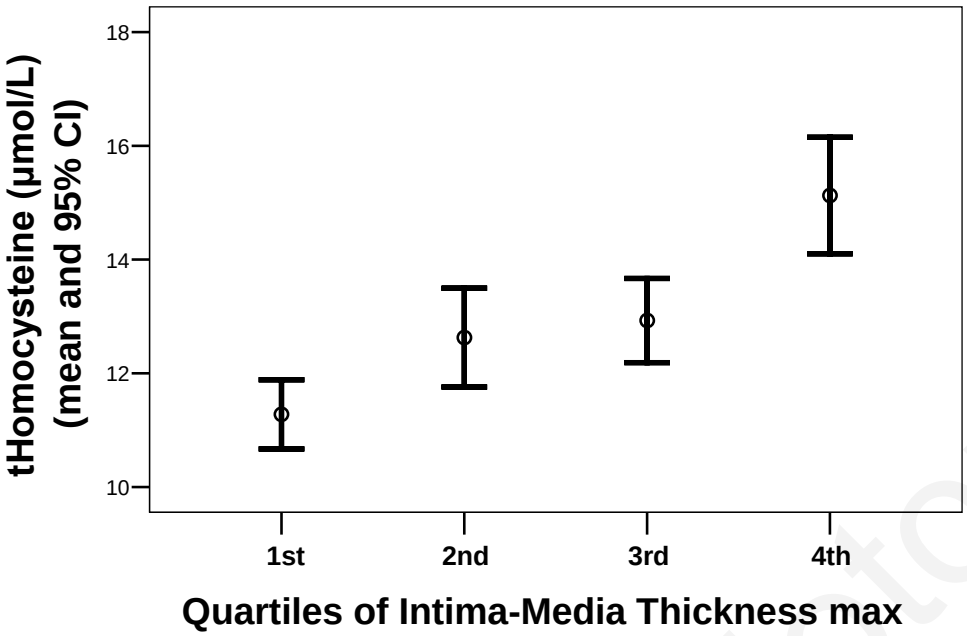

Figure 13.7: Association between tHcy levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend<0.001)

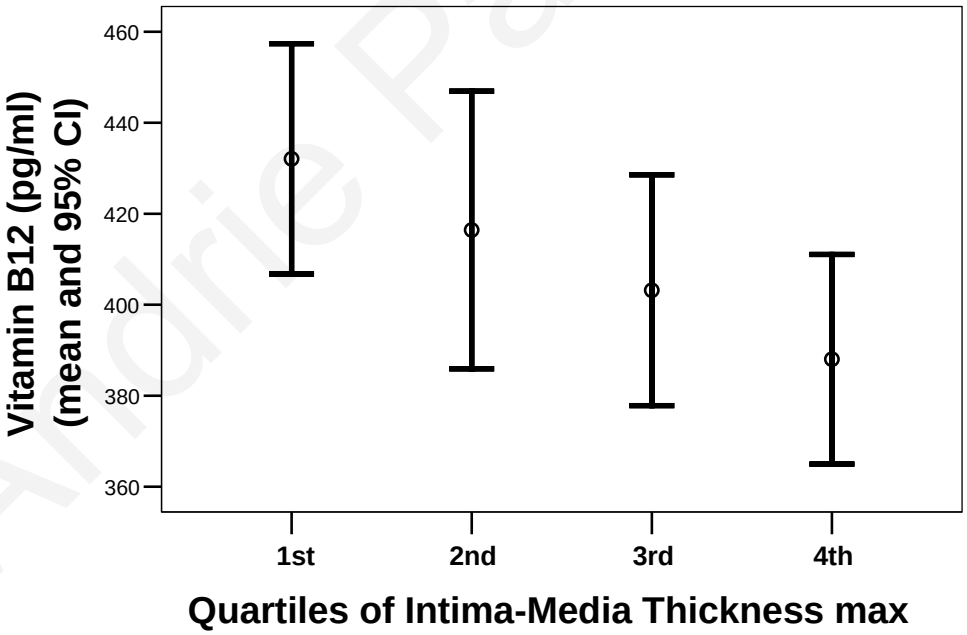

Figure 13.8: Association between vitamin B12 levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.35)

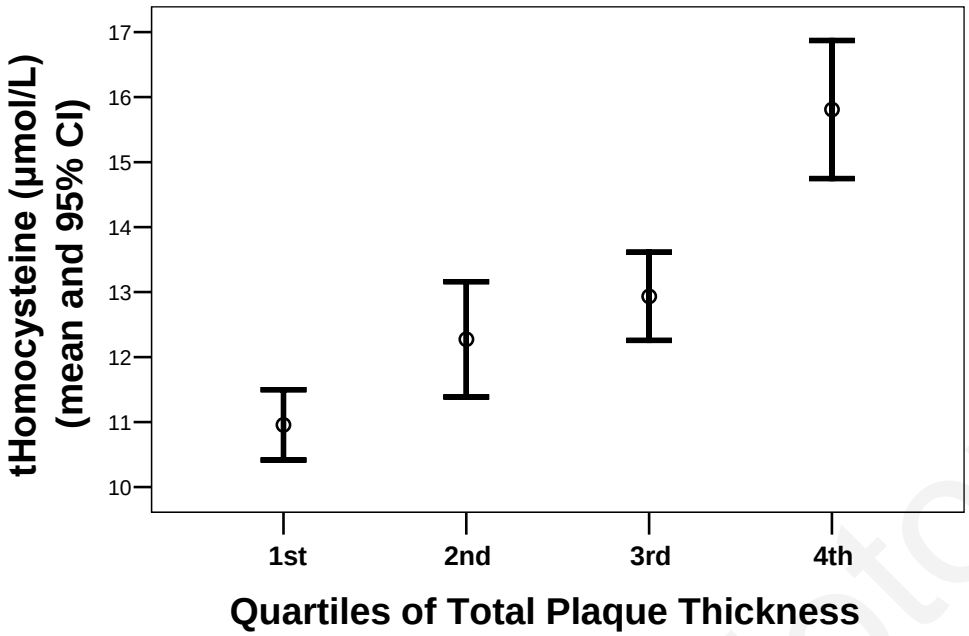

Figure 13.9: Association between tHcy levels and quartiles of TPT (Kruskal-Wallis test; P for trend<0.001)

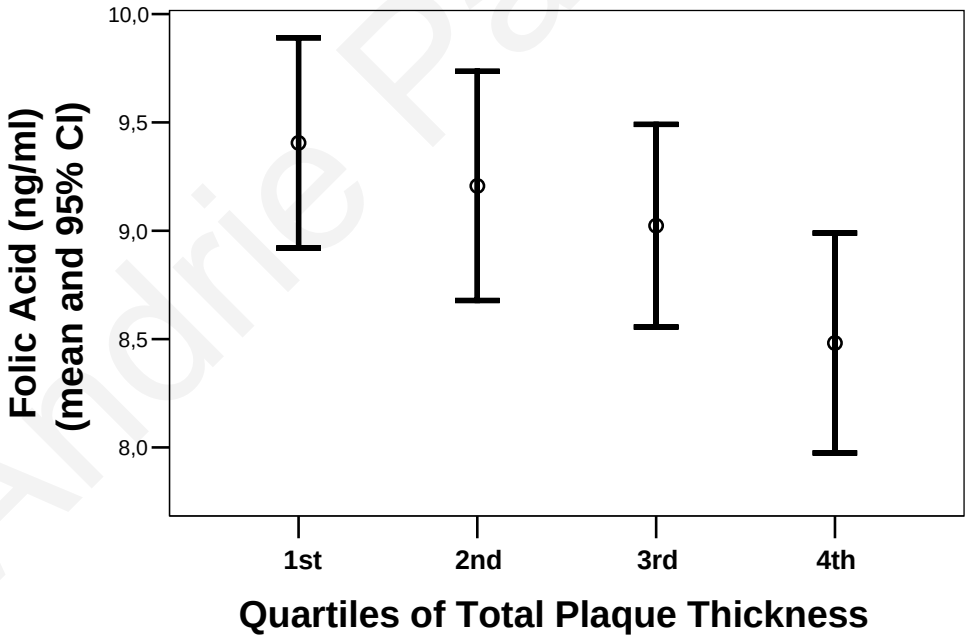

Figure 13.10: Association between folic acid levels and quartiles of TPT (Kruskal-Wallis test; P for trend=0.026)

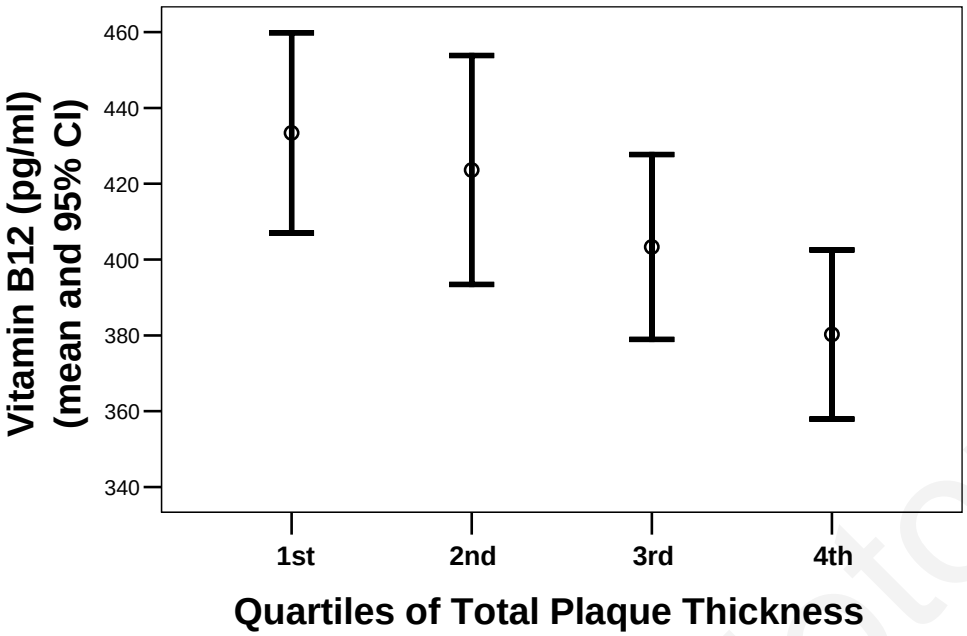

Figure 13.11: Association between vitamin B12 levels and quartiles of TPT (Kruskal-Wallis test; P for trend=0.1)

Table 13.2: Results of association between presence of plaques and number of bifurcations with plaque and serum levels of homocysteine metabolism markers (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaques as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque)

Marker	**N**	**Presence of plaques**	**Number of bifurcations With plaques (0-4)**	**0-2 bifurcations with plaque vs 3-4**
tHcy	767	**P<0.001**	**P<0.001**	**P<0.001**
Folic acid	767	P=0.220	**P=0.024**	**P=0.011**
Vitamin B12	767	P=0.079	P=0.222	P=0.064
ADMA	188	**P=0.029**	**P=0.018**	P=0.27
MTHFR	754	P=0.687	P=0.967	P=0.697

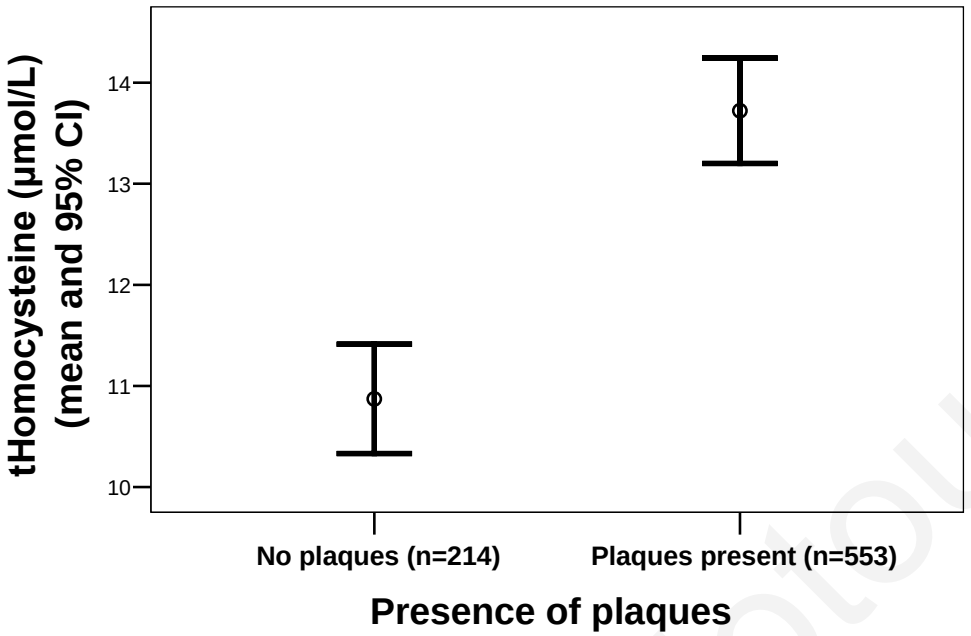

Figure 13.12: Association between tHcy levels and presence of plaques (Mann-Whitney test; P<0.001)

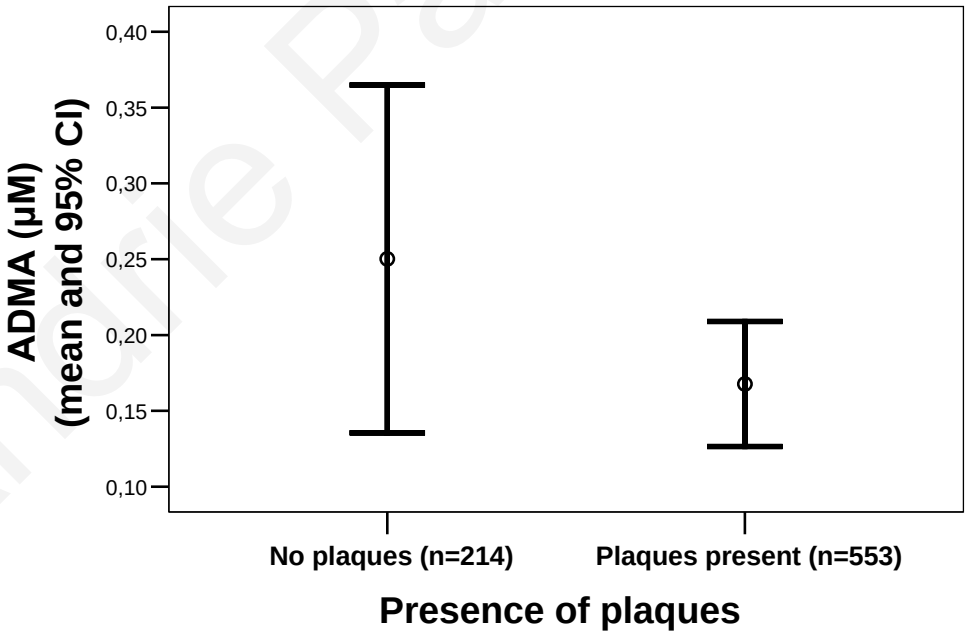

Figure 13.13: Association between ADMA levels and presence of plaques (Mann-Whitney test; P=0.029)

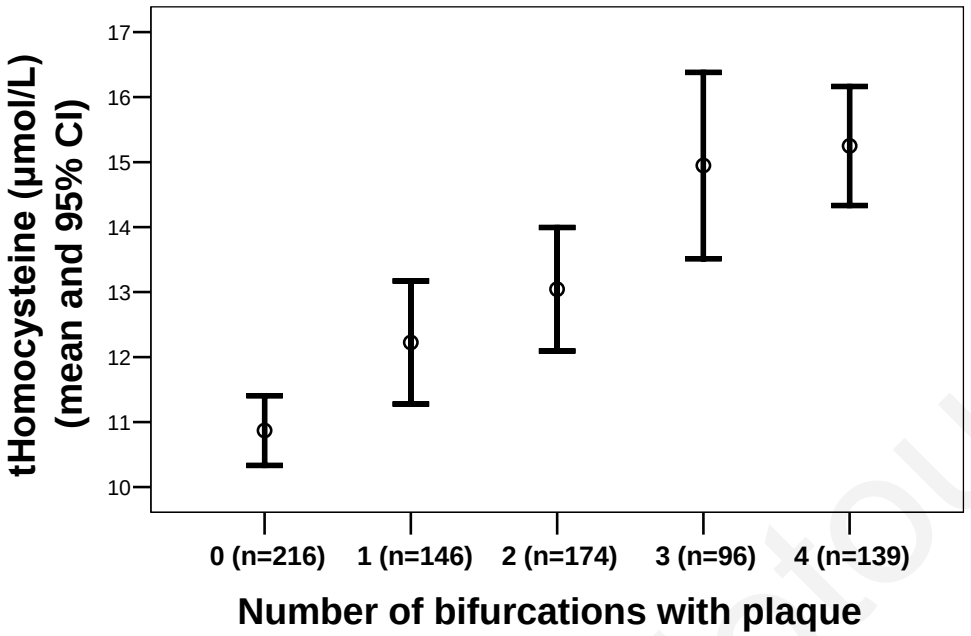

Figure 13.14: Association between tHcy levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend<0.001)

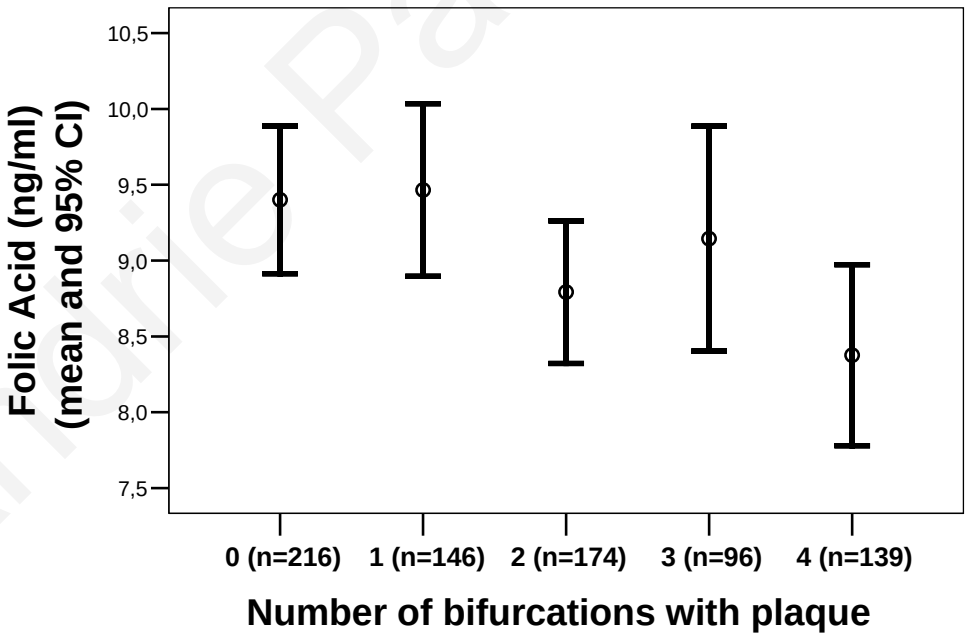

Figure 13.15: Association between folic acid levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend=0.024)

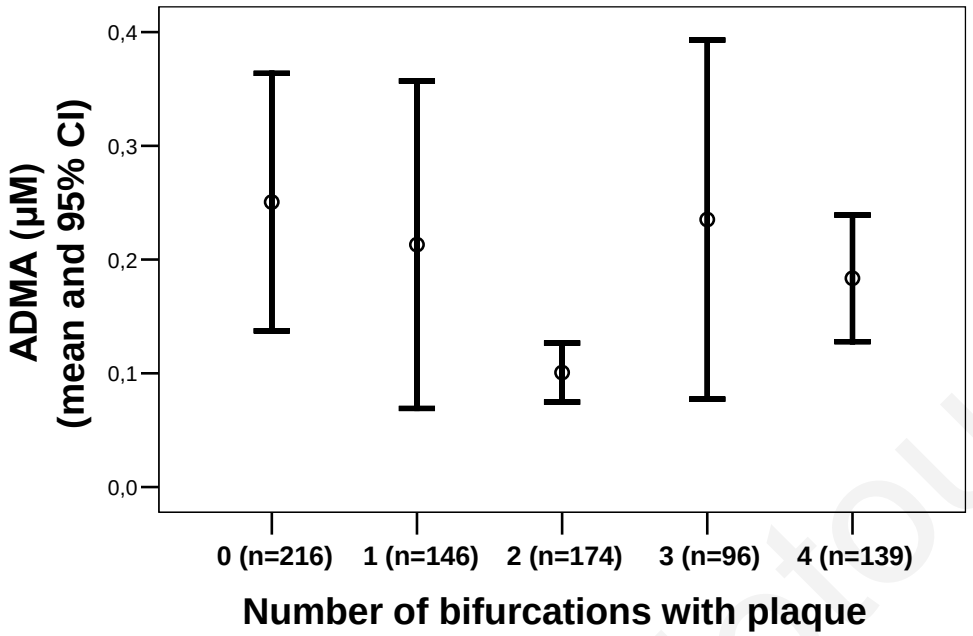

Figure 13.16: Association between ADMA levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend=0.018)

Association between homocysteine metabolism markers and MPT

The association between homocysteine metabolism markers and mean plaque type was tested with the use of Kruskal-Wallis tests and crosstabulation. Only serum tHcy levels were significantly associated with MPT ($P<0.001$). Folic acid, vitamin B12 and ADMA levels were not associated with MPT and neither was the *MTHFR* (677C>T) polymorphism. Significant results are shown in figure 13.17.

In multivariate binary regression analyses adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) tHcy was no longer associated with MPT. Only age was associated with MPT from the traditional risk factors in the model.

Association between homocysteine metabolism markers and BPB

The association between homocysteine metabolism markers and BPB was tested. Serum levels of tHcy, folic acid and vitamin B12 were significantly associated with BPB. Estimate (B) and p values are tabulated in table 13.1 and significant results are shown in figures 13.18-20. ADMA levels and the *MTHFR* (677C>T) genotype were not associated with BPB.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex, smoking in packyears, diabetes, hypertention and hyperlipidaemia) and using the population media as a cut-off point for tHcy, F.A. and B12, only tHcy levels remained significanlty associated with BPB above traditional risk factors ($P=0.001$). Adding tHcy to the model improved its predictive ability by 0.8% and could predict 32.3% of the variability in BPB in individual level.

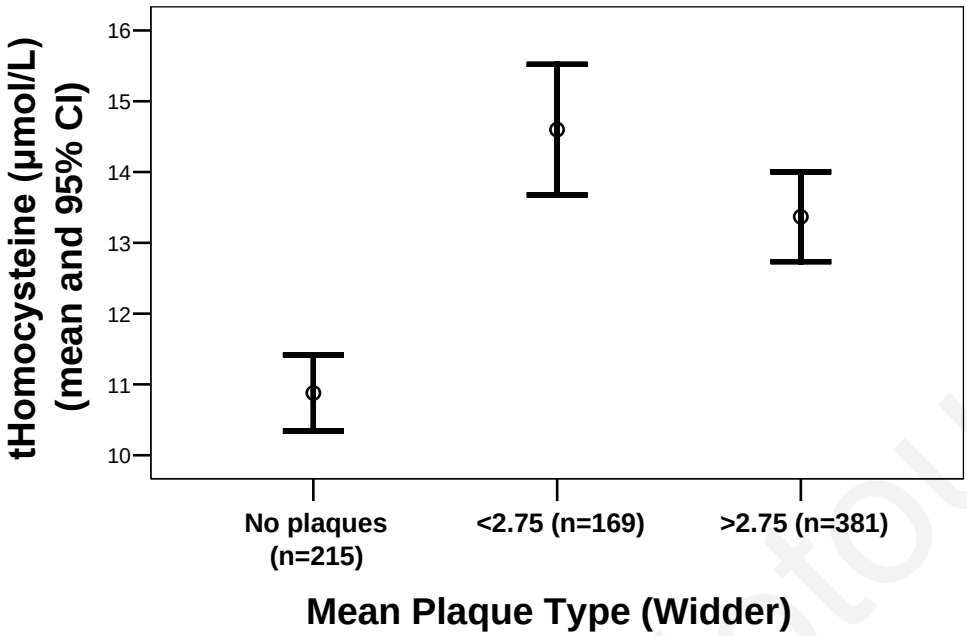

Figure 13.17: Association between tHcy levels and MPT below and over the median (Kruskal-Wallis test; P for trend<0.001)

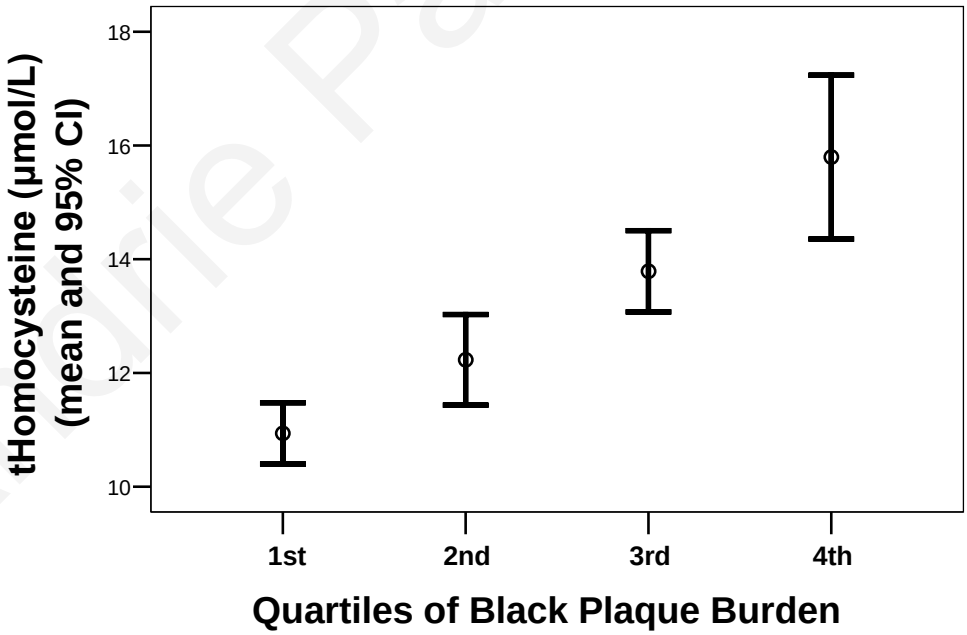

Figure 13.18: Association between tHcy levels and quartiles of BPB (Kruskal-Wallis test; P for trend<0.001)

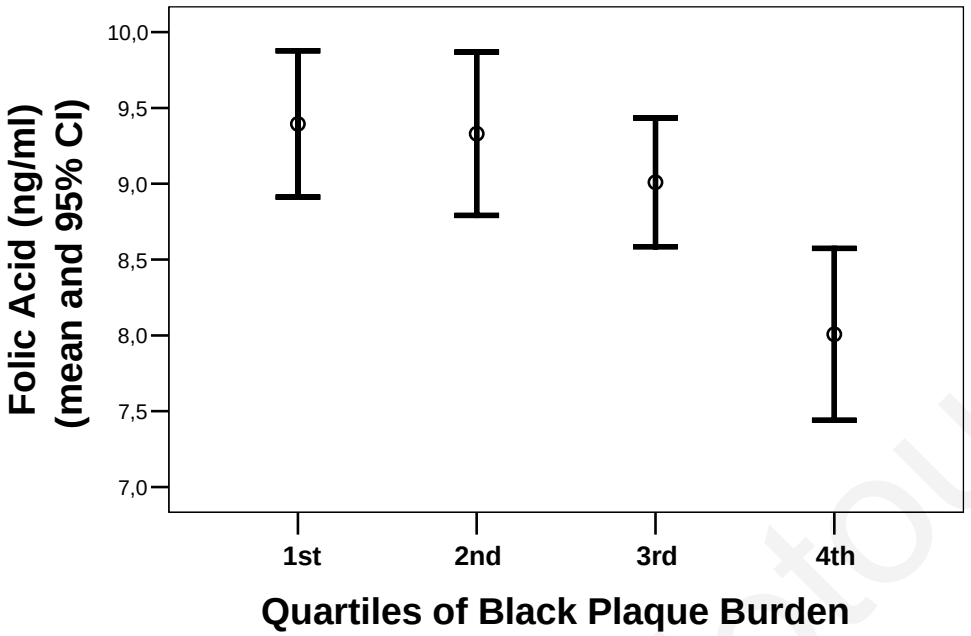

Figure 13.19: Association between folic acid levels and quartiles of BPB (Kruskal-Wallis test; P for trend=0.004)

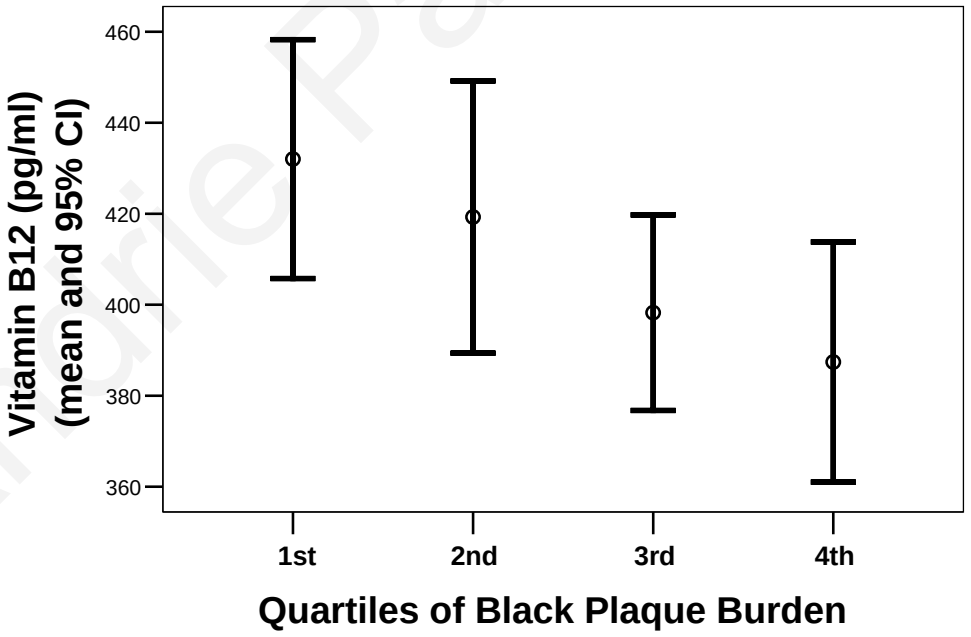

Figure 13.20: Association between vitamin B12 levels and quartiles of BPB (Kruskal-Wallis test; P for trend=0.312)

Table 13.3: Compiled results of association between homocysteine metabolism markers and ultrasonic measurements in univariate analysis

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
tHcy	+	+	+	+	+	+
Folic Acid	+	-	+	-	+	-
Vit B12	+	+	+	-	+	-
ADMA	-	-	-	+	-	-
MTHFR (677C>T)	-	-	-	-	-	-

Table 13.4: Compiled results of association between homocysteine metabolism markers and ultrasonic measurements in multivariate analyses adjusting for age, sex, smoking in packyears, diabetes and hypertention

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
tHcy	**+**	**-**	**+**	**+**	**+**	**-**
Folic Acid	**-**	**-**	**-**	**-**	**-**	**-**
Vit B12	**+**	**-**	**-**	**-**	**-**	**-**
ADMA	**-**	**-**	**-**	**-**	**-**	**-**
MTHFR (677C>T)	**-**	**-**	**-**	**-**	**-**	**-**

Summary of results

Homocysteine metabolism markers have been the source of much controvercy in atherosclerosis for the past decades. Although, the association of high homocysteine blood levels and atherosclerosis has been shown several times, its causative role has been questioned especially in randomised trials of folic acid fortification. The association between the *MTHFR* 677C>T polymorphism and Hcy blood levels has also been inconclusive with some studies showing association and some not. In this chapter we have tested both tHcy levels as well as markers in its metabolic pathway and their association with novel ultrasonic measurements.

1. The *MTHFR* (677C>T) polymorphism is the most significant predictor of blood tHcy levels in men under 60 years of age in our population. Age, gender, folic acid levels and vitamin B12 levels significantly affect the association between *MTHFR* 677C>T genotype and tHcy levels.
2. Serum levels of tHcy were significantly associated with all the ultrasonic markers tested (IMTcc, IMTmax, TPT, presence of plaques and number of bifurcations with plaque), which indicates that homocysteine metabolism plays a part in atherosclerosis initiation and progression.
3. Serum levels of folic acid were significantly associated with IMTcc, TPT, number of bifurcations with plaques and BPB.
4. Serum levels of vitamin B12 were significantly associated with IMTcc, IMTmax, TPT and BPB but not with presence of plaques and number of bifurcations with plaque.
5. Plasma levels of ADMA, a novel marker for atherosclerosis participating in Hcy metabolism, were significantly associated with presence of plaques and number of bifurcations with plaque. They were not associated with IMTcc, IMTmax, TPT or BPB. However, adding ADMA to multivariate models for prediction of IMTcc, IMTmax, TPT and BPB significantly improved their predictive ability (R^2), even though its p value did not reach statistical significance. This could be due to the small number of subjects included in the analysis (n=188).

Chapter 14:

Results of association between metabolic syndrome and subclinical atherosclerosis

This chapter reports on the results of the association between early, subclinical, atherosclerosis as assessed by ultrasound and components of the metabolic syndrome. In addition, we have tested the association between subclinical atherosclerosis and the HOMA index of insulin sensitivity and two novel genetic polymorphisms associated with energy expenditure and metabolism (*UCP-2* -866G>A and *UCP-3* -55C>T). The definition for the MetS used was presence of 3 or more of the following components:

1. HDL <50 mg/dL for men and <40 mg/ldL for women
2. BMI >30 or waist circumference >102 cm for men and >88 cm for women
3. Fasting glucose > 110 mg/dL
4. Hypertension: DBP >85 mm Hg and SBP >130 mm Hg
5. Fasting triglycerides >150 mg/dL

Association between *UCP-2* and *UCP-3* polymorphisms and components of the MetS

Although not a specific aim of the thesis, given the lack of data in the literature and relative personal communications from the UDACS study (UCL Diabetes and Cardiovascular study), it was decided to test the association between the *UCP-2* and *UCP-3* polymorphisms and relative metabolic markers. The *UCP-3* (-55C>T) polymorphism was found to be significantly associated with BMI in diabetic subjects (P=0.001 for CC vs CT, TT genotypes) (Fig. 14.1).

Association between MetS markers and IMTcc

The association between MetS markers and IMTcc was tested. Presence of MetS, number of components, presence of diabetes, fasting glucose and the HOMA index of insulin sensitivity were all significantly associated with IMTcc. Estimate (B) and p values for univariate linear regression are shown in table 14.1. The *UCP-2* (-866G>A) and *UCP-3* (-55C>T) polymorphisms were not associated with IMTcc, although the *UCP-2* (-866AA vs AG,GG) genotype had a borderline value (P=0.05) which shouldn't be discarded without furter analysis. Significant results are shown in figures 14.2-6.

In multivariate linear regression analyses, adjusting for traditional risk factors (age, sex and smoking in packyears) and each of the variables, number of components, presence of diabetes and the HOMA index remained significantly associated with IMTcc (P<0.001, P=0.026, P=0.002 respectively) over and above traditional risk factors. Adding number of components of MetS to the model increased its predictive ability from 27.6% to 28.6%. Presence of MetS had a borderline P value (P=0.051).

Association between MetS markers and IMTmax

The association between MetS markers and IMTmax was tested. Presence of MetS, number of components, presence of diabetes, fasting glucose levels and the HOMA index were all significantly associated with IMTmax. Estimate and p values are shown in table 14.1. The *UCP-2* (-866G>A) and *UCP-3* (-55C>T) polymorphisms were not associated with IMTmax. Significant results are shown in figures 14.7-11.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex and smoking in packyears), MetS, number of components, diabetes, glucose levels and the HOMA index all remained significantly associated with IMTmax over and above traditional risk factors (P=0.005, P<0.001, P=0.004, P=0.004 and P=0.005 respectively). Adding number of components of MetS in the model improved its predictive ability by 1.3% and reached 32.1%.

Association between MetS markers and TPT

The association between MetS markers and TPT was tested. MetS, number of components and presence of diabetes mellitus were significantly associated with TPT (Figs.14.12-14). Estimate (B) and p values are shown in table 14.1. Glucose levels, the HOMA index and the *UCP-2* (-866G>A) and *UCP-3* (-55C>T) polymorphisms were not associated with TPT.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex and smoking in packyears) only number of components of MetS remained significanlty associated with TPT (P=0.017). Adding number of components to the model improved its predictive ability from 27.4% to 28.1%.

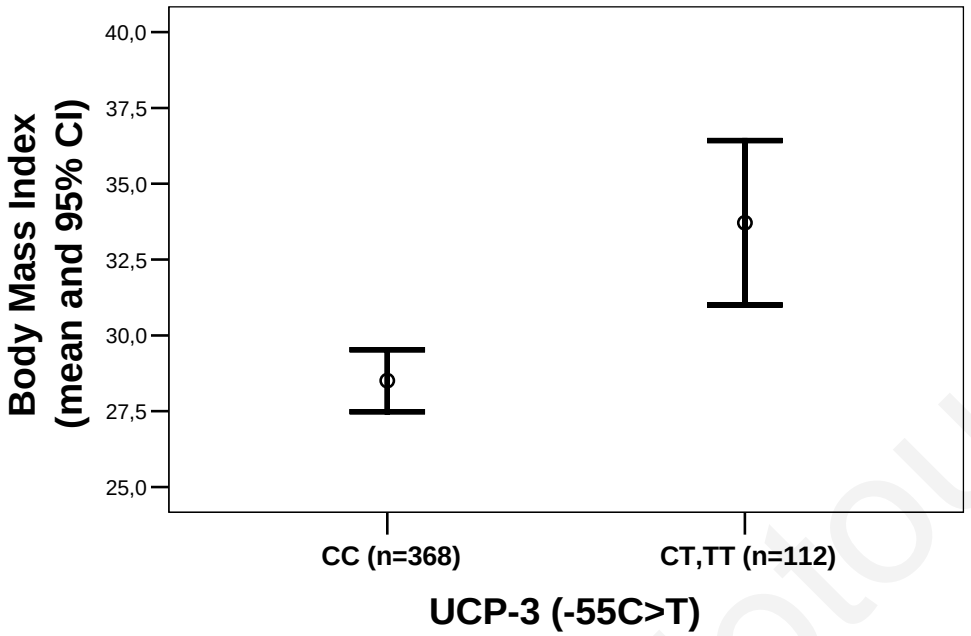

Figure 14.1: Association between body mass index and UCP-3 (-55C>T) polymorphism in diabetics (Independent t-test; P=0.001)

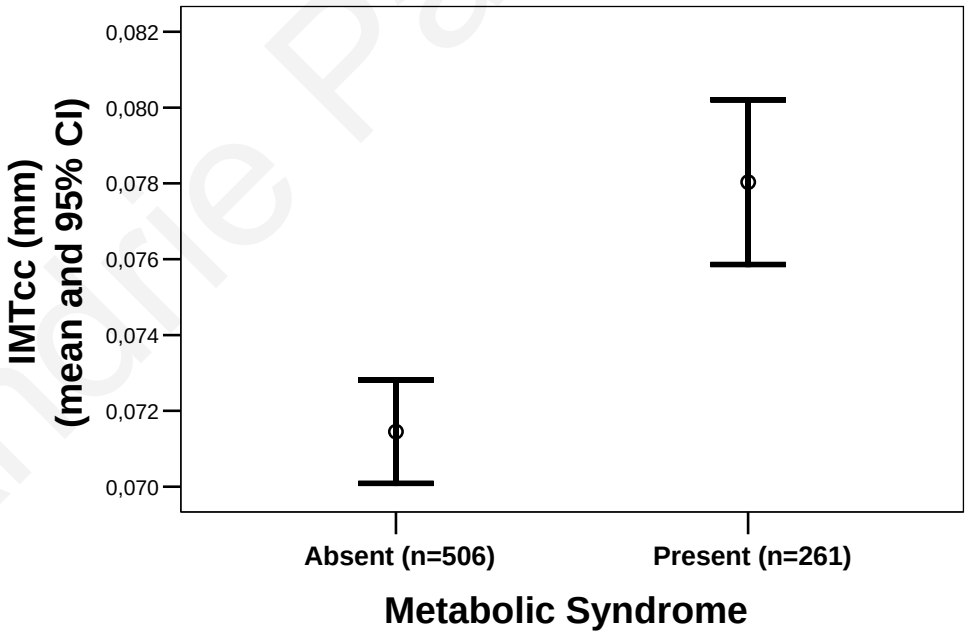

Figure 14.2: Association between IMTcc and presence of MetS (t-test; P<0.001)

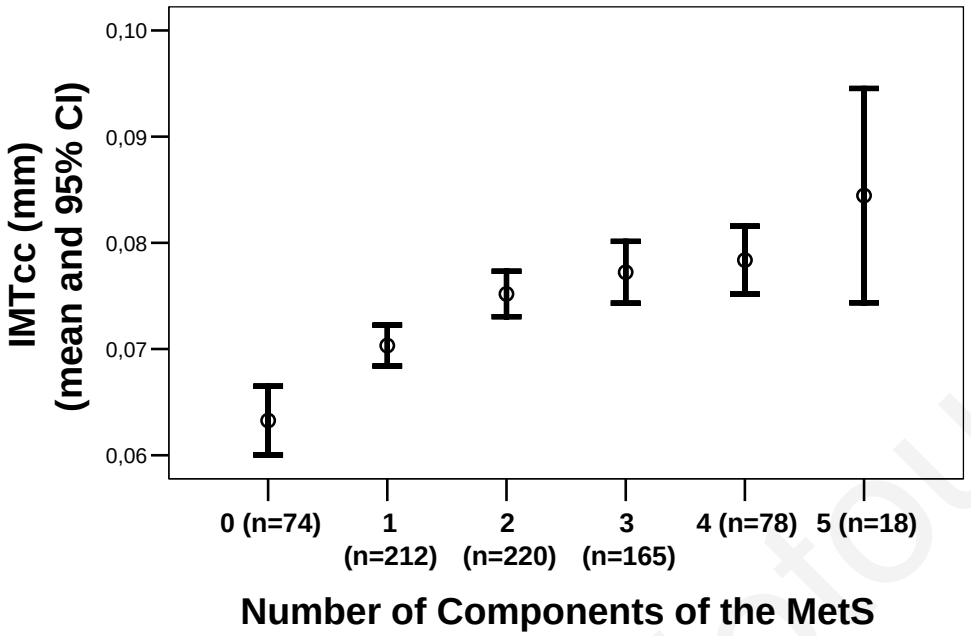

Figure 14.3: Association between IMTcc and number of components of MetS (ANOVA; P for trend<0.001)

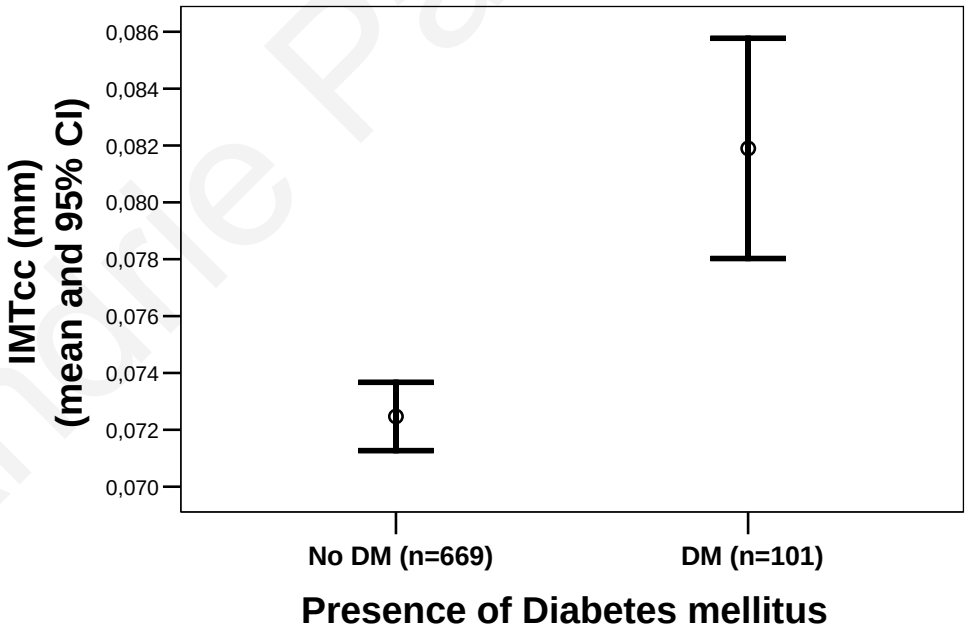

Figure 14.4: Association between IMTcc and presence of diabetes mellitus (t-test; P <0.0001)

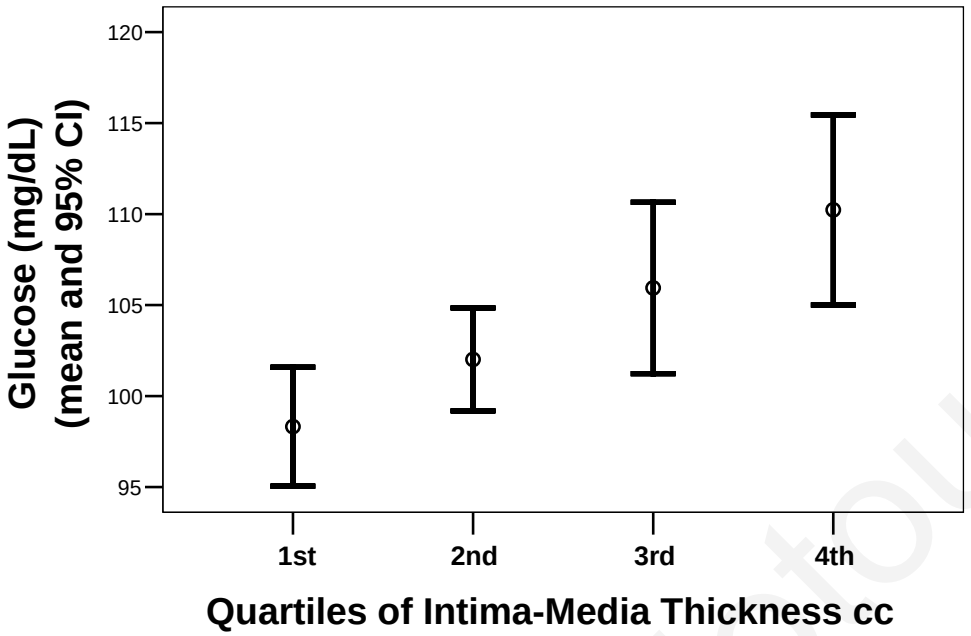

Figure 14.5: Association between glucose levels and quartiles of IMTcc (ANOVA; P for trend<0.001)

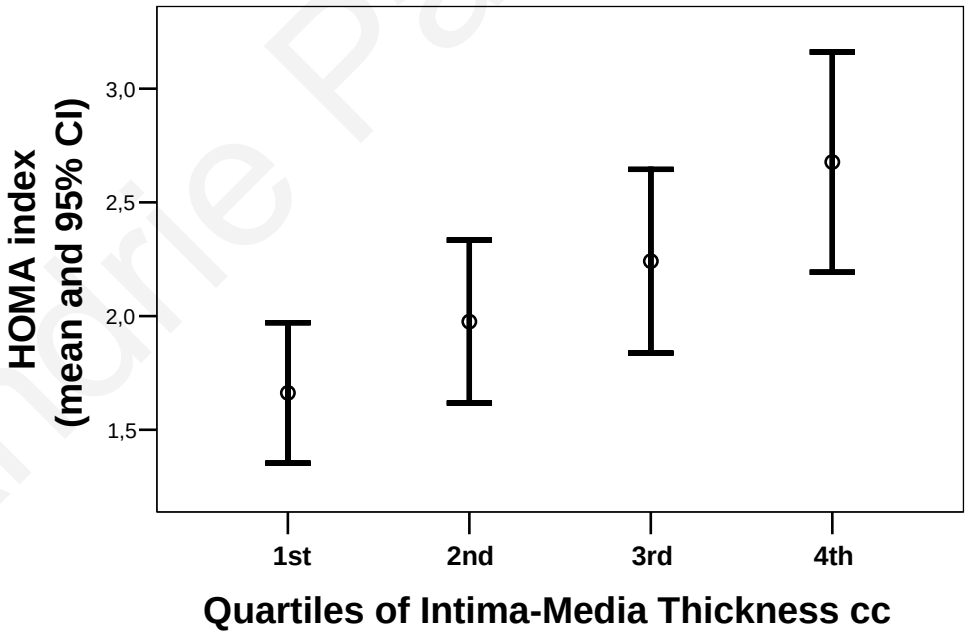

Figure 14.6: Association between the HOMA index and quartiles of IMTcc (Kruskal-Wallis tes; P for trend<.0001)

Table 14.1: Results of univariate linear regressions for association between ultrasonic measurements and metabolic syndrome markers (IMTmax, TPT and HOMA were logtransformed to achieve normality)

	IMTcc		**IMTmax**		**TPT**		**BPB**	
	Estimate (95% CI)	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**	**Estimate (95% CI)**	**P value**
MetS	0.007 (0.004 to 0.009)	**P<0.001**	0.257 (0.173 to 0.341)	**P<0.001**	0.216 (0.106 to 0.326)	**P<0.001**	0.565 (0.398 to 0.731)	**P<0.001**
MetS compon	0.004 (0.003 to 0.005)	**P<0.001**	0.129 (0.097 to 0.161)	**P<0.001**	0.109 (0.064 to0.154)	**P<0.001**	0.275 (0.211 to 0.339)	**P<0.001**
DM	0.009 (0.006 to 0.013)	**P<0.001**	0.369 (0.251 to 0.487)	**P<0.001**	0.286 (0.142 to 0.429)	**P<0.001**	0.73 (0.494 to 0.965)	**P<0.001**
Glucose	8.68E-005 (0.0 to 0.0)	**P<0.001**	0.004 (0.003 to 0.005)	**P<0.001**	0.001 (0.00 to 0.003)	P=0.108	0.006 (0.003 to 0.009)	**P<0.001**
HOMA	0.003 (0.001 to 0.004)	**P<0.001**	0.087 (0.043 to 0.131)	**P<0.001**	0.031 (-0.027 to0.089)	P=0.30	0.091 (0.004 to 0.179)	P=0.041

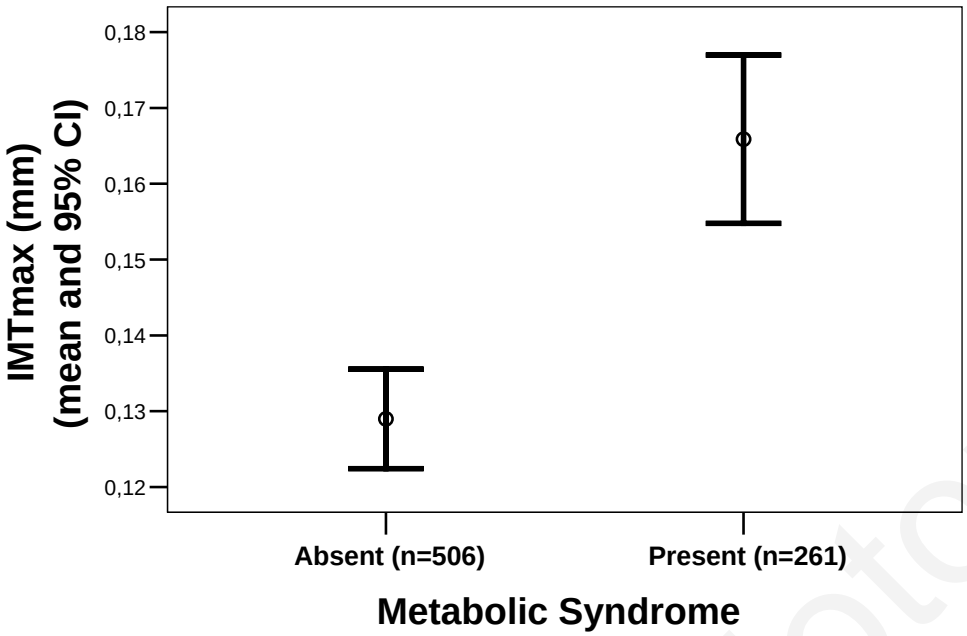

Figure 14.7: Association between IMTmax and presence of MetS (Mann-Whitney test; P<0.001)

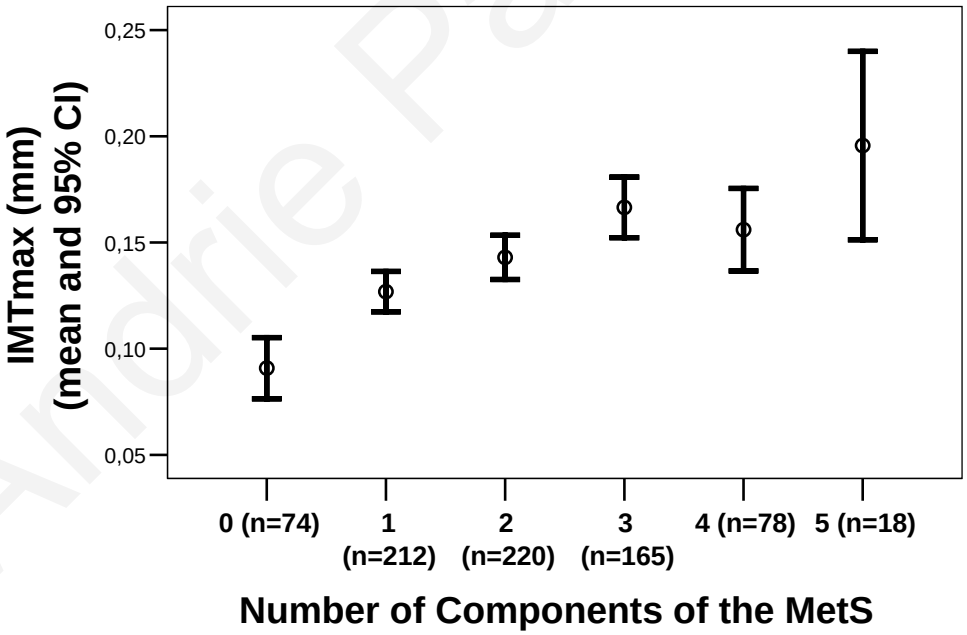

Figure 14.8: Association between IMTmax and number of components of the MetS (Kruskal-Wallis test; P for trend<0.001)

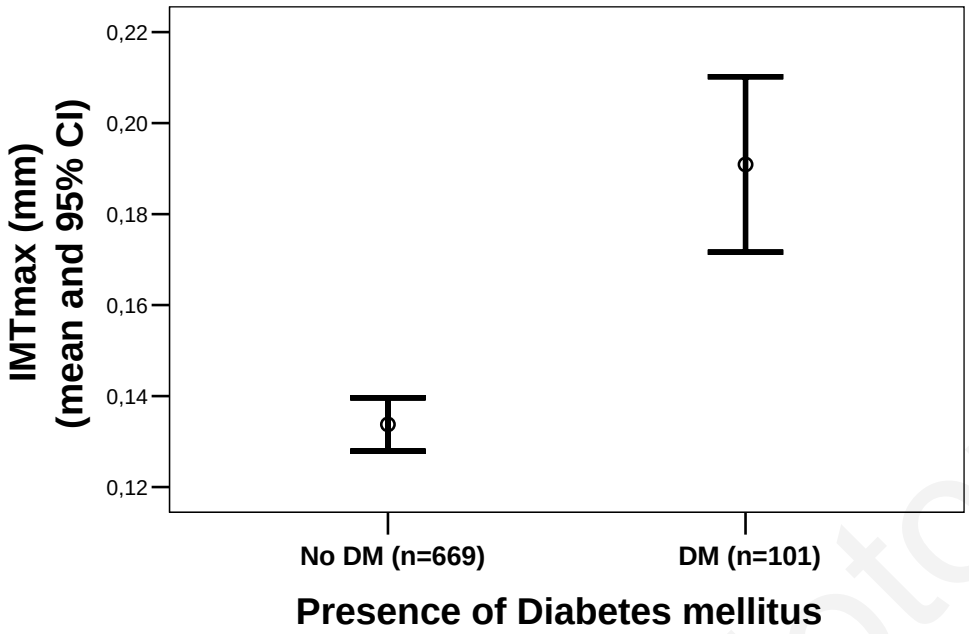

Figure 14.9: Association between IMTmax and presence of diabetes mellitus (Mann-Whitney test; P<0.001)

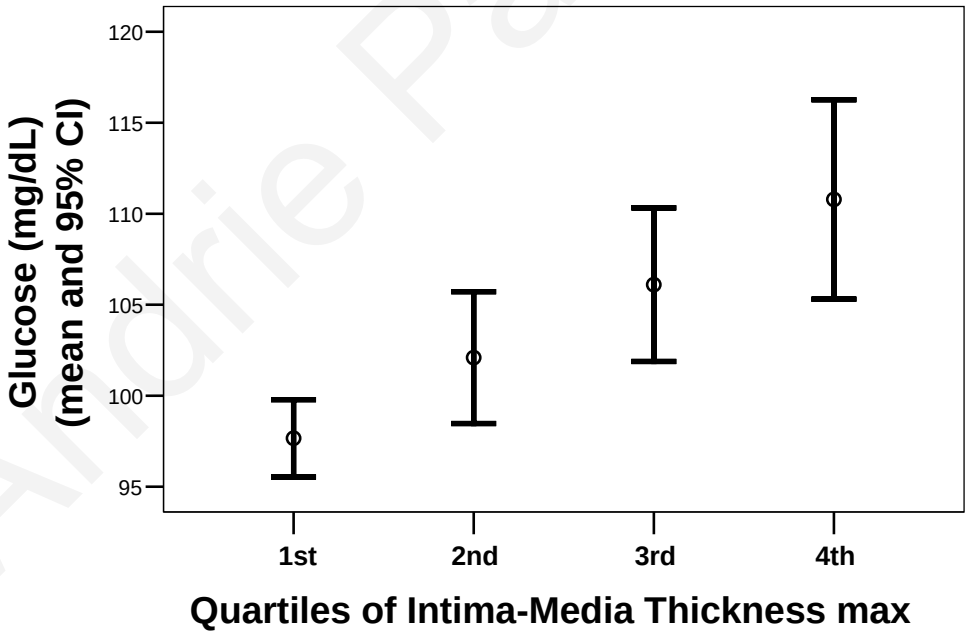

Figure 14.10: Association between glucose levels and quartiles of IMTmax (Kruskal-Wallis test; P for trend<0.001)

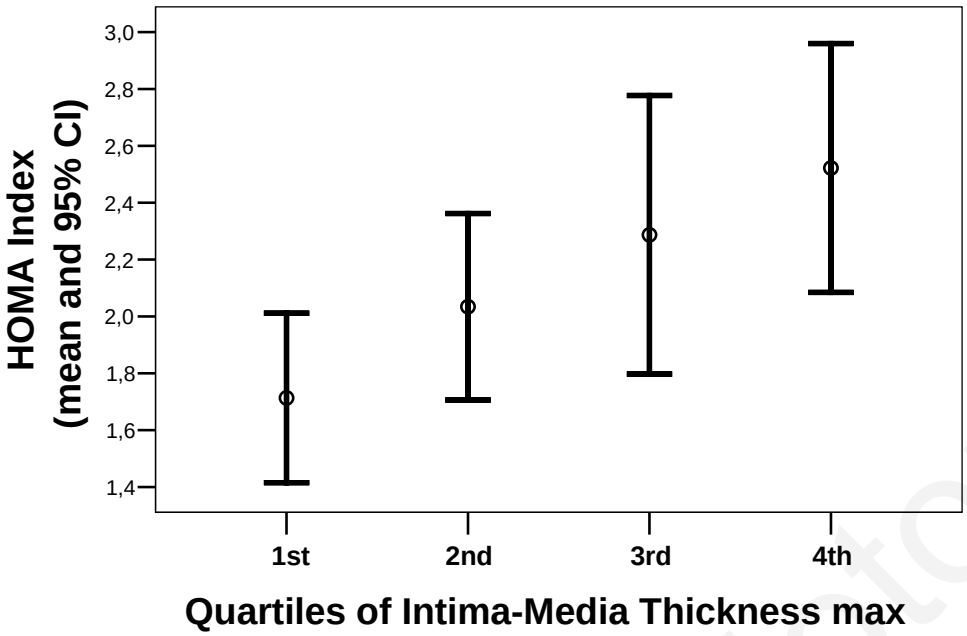

Figure 14.11: Association between the HOMA index and quartiles of IMTmax (Kruskal-Wallis test; P for trend=0.001)

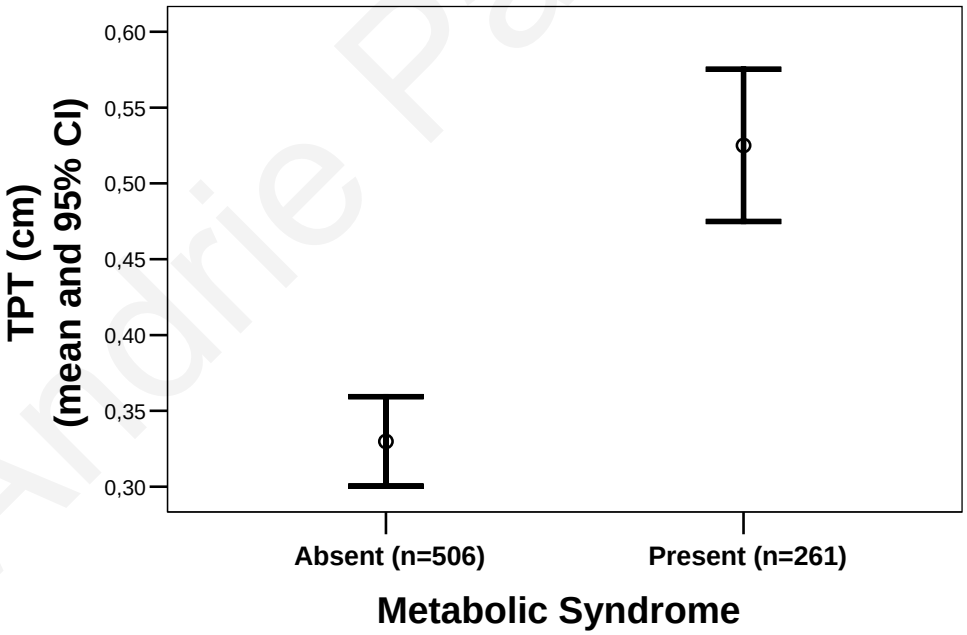

Figure 14.12: Association between TPT and presence of MetS (Mann-Whitney U-test; P<0.001)

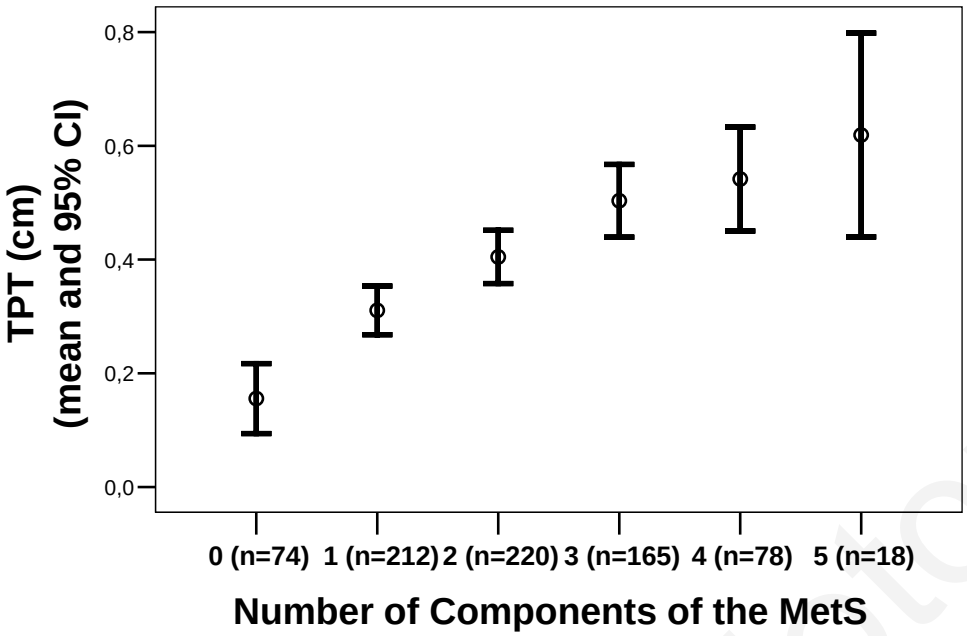

Figure 14.13: Association between TPT and number of components of MetS (Kruskal-Wallis test; P for trend<0.001)

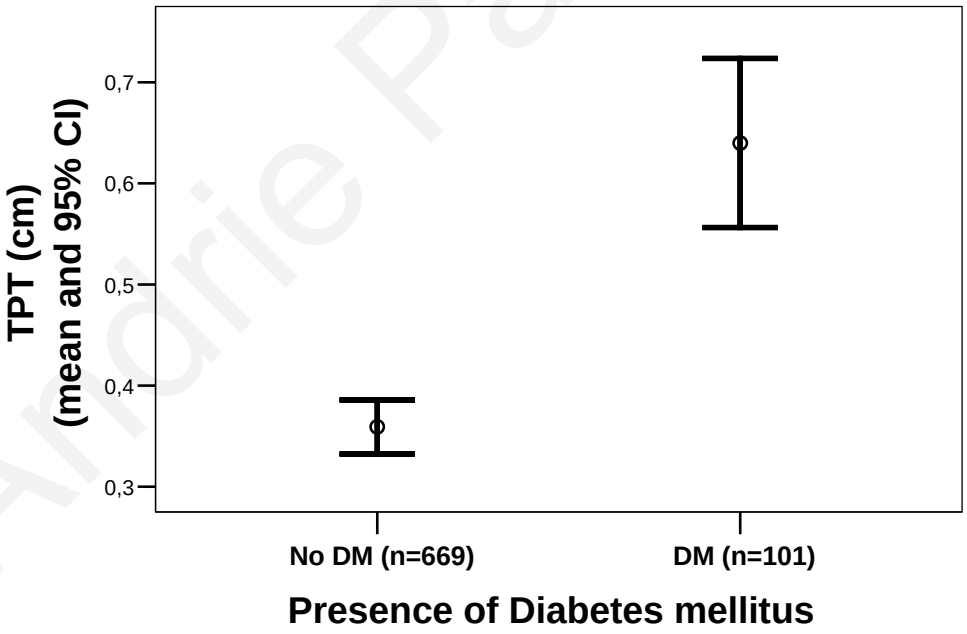

Figure 14.14: Association between TPT and presence of diabetes (Mann-Whitney test; P<0.001)

Association between MetS markers, presence of plaques and number of bifurcations with plaque

The association between markers of the metabolic syndrome, presence of plaques and number of bifurcations with plaque was tested. In addition to number of bifurcations with plaque, ranging from 0 to 4, a cut-off point of 2 bifurcations with plaque (0-2) was used with more (3-4) indicating generalised atherosclerosis (plaques present in carotid and femoral bifurcations). MetS, number of components, presence of diabetes and glucose levels were significantly associated with both presence of plaques and number of bifurcations with plaque. The HOMA index was not associated with the number of bifurcations with plaque but it had a borderline p value for association with presence of plaques (p=0.051). Significant results are shown in figures 14.15-16 and P values are tabulated in table 14.2. None of the genetic polymorphisms tested was significantly associated with presence of plaque or number of bifurcations with plaque, although there was a trend for the *UCP-2* -866AA genotype to be associated with presence of plaques (P=0.084; only 66 subjects AA homozygotes).

In multivariate binary logistic regression analyses adjusting for traditional risk factors (age, sex and smoking in packyears) only number of components and presence of diabetes remained significantly associated with presence of plaques (P=0.039 and P=0.013).

Table 14.2: Results of association between presence of plaques and number of bifurcations with plaque and MetS markers (Mann-Whitney test used for presence or absence of plaques and for 2 bifurcations with plaques as a cut-off point and Kruskal-Wallis test for number of bifurcations with plaque; crosstabulation for MetS and SNPs)

Marker	**N**	**Presence of plaques**	**Number of bifurcations With plaques (0-4)**	**0-2 bifurcations with plaque vs 3-4**
MetS	767	**P<0.001**	**P<0.001**	**P<0.001**
MetS Compon.	767	**P<0.001**	**P<0.001**	**P<0.001**
Diabetes	768	**P<0.001**	**P<0.001**	**P<0.001**
Glucose	768	**P<0.001**	**P=0.011**	**P<0.001**
HOMA	730	P=0.051	P=0.167	P=0.35
***UCP-2* (AA vs AG, GG)**	732	P=0.084	P=0.094	P=0.176
***UCP-3* (CC vs CT, TT)**	480	P=0.905	P=0.296	P=0.342

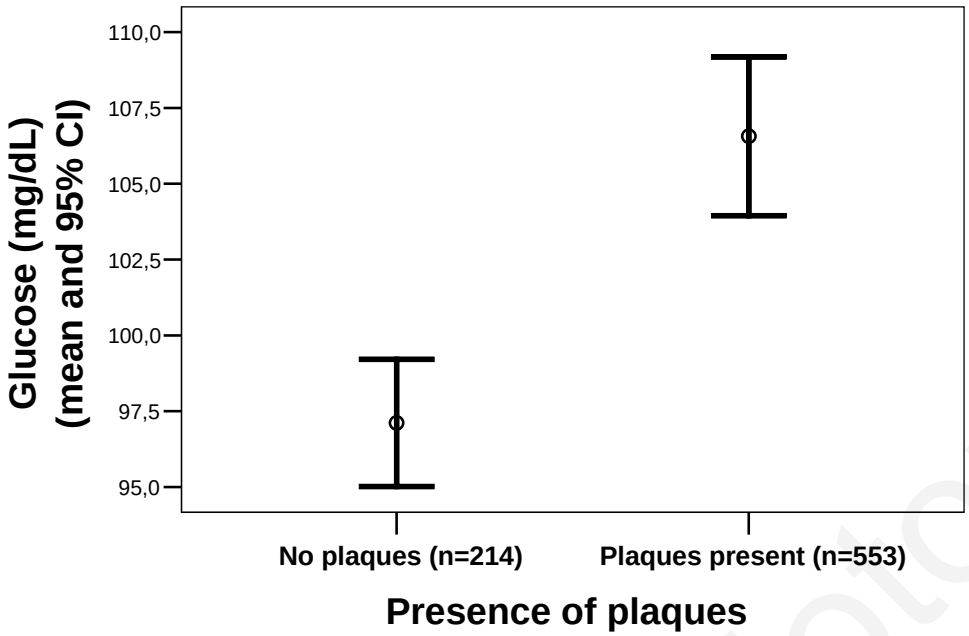

Figure 14.15: Association between glucose levels and presence of plaques (Mann-Whitney test; P<0.001)

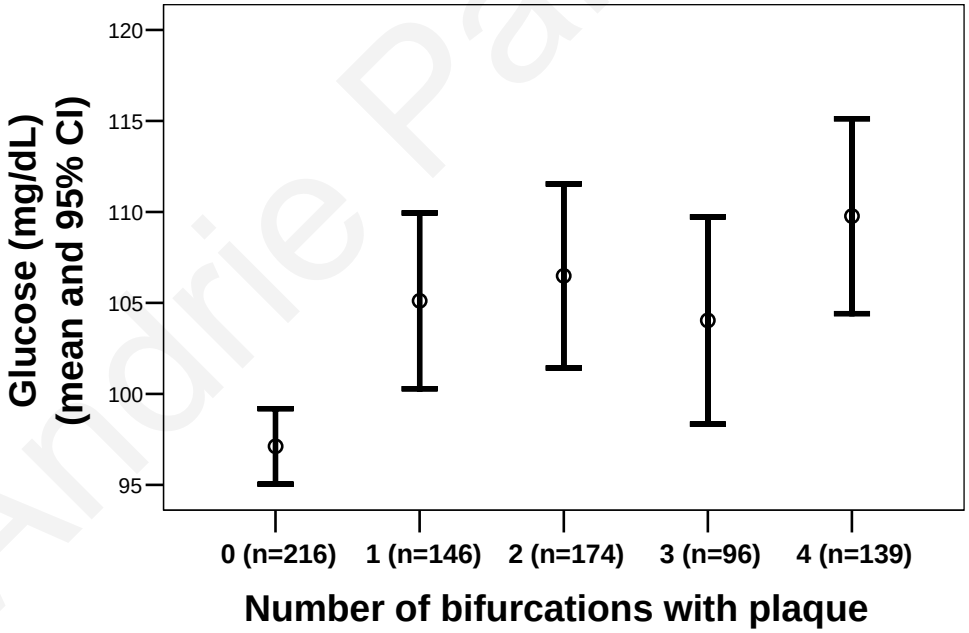

Figure 14.16: Association between glucose levels and number of bifurcations with plaque (Kruskal-Wallis test; P for trend<0.001)

Association between MetS markers and MPT

The association between MetS markers and mean plaque type was tested with the use of Kruskal-Wallis tests and crosstabulation. MetS, number of components, presence of diabetes and fasting glucose were significantly associated with MPT below and over the median ($P<0.001$ for all). The HOMA index and the genetic polymorphisms tested were not associated with MPT. Significant results are shown in figure 14.17.

In multivariate binary regression analyses adjusting for traditional risk factors (age, sex and smoking in packyears) none of the variables remained associated with MPT.

Association between MetS markers and BPB

The association between metabolic syndrome markers and BPB was tested. MetS, number of components, presence of diabetes, glucose levels and the HOMA index were all significantly associated with BPB. Estimate (B) and p values are tabulated in table 14.1 and significant results are shown in figures 14.18-22. The genetic polymorphisms tested were not associated with BPB.

In multivariate linear regressions, adjusting for traditional risk factors (age, sex and smoking in packyears), MetS, number of components and presence of diabetes remained significantly associated with BPB above traditional risk factors ($P=0.003$, $P<0.001$ and $P=0.004$ respectively). Adding MetS or diabetes to the model improved its predictive ability by 0.7% and could predict 30.0% of the variability in BPB in individual level. Adding number of components could predict 30.5% of the variability.

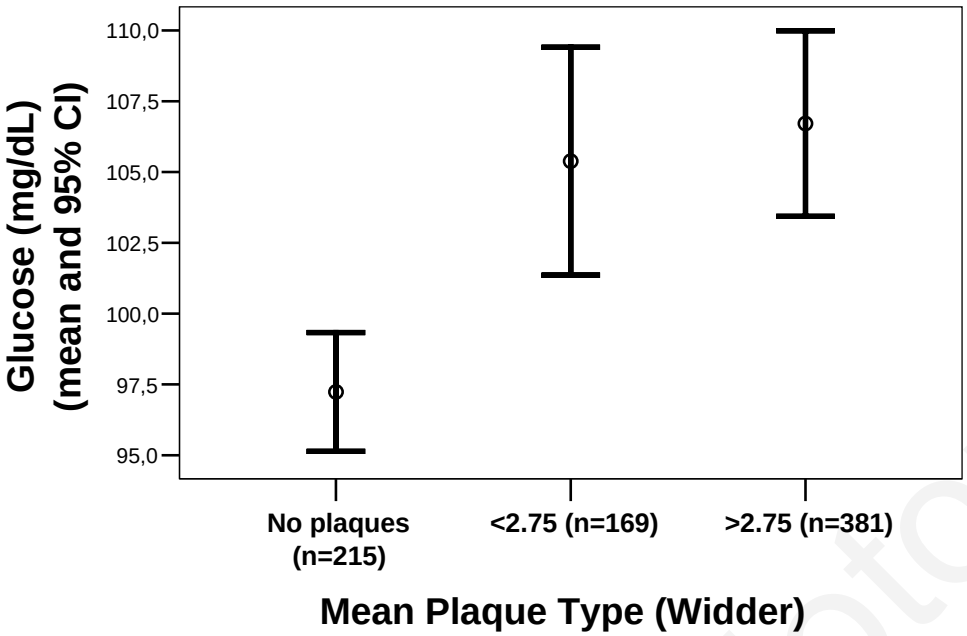

Figure 14.17: Association between glucose levels and MPT below and over the median (Kruskal-Wallis: P for trend<0.001)

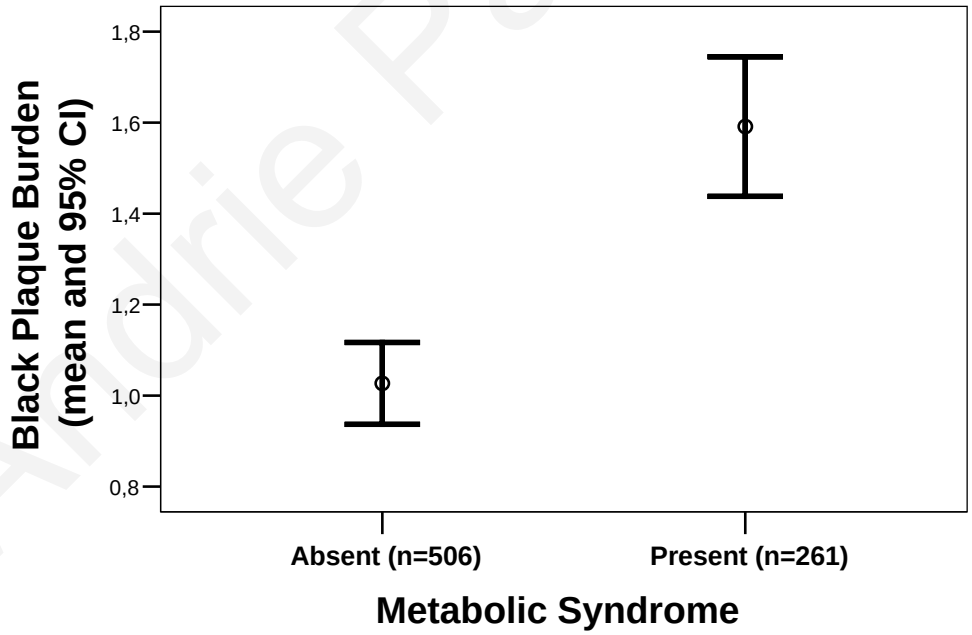

Figure 14.18: Association between BPB and presence of MetS (t-test; P<0.001)

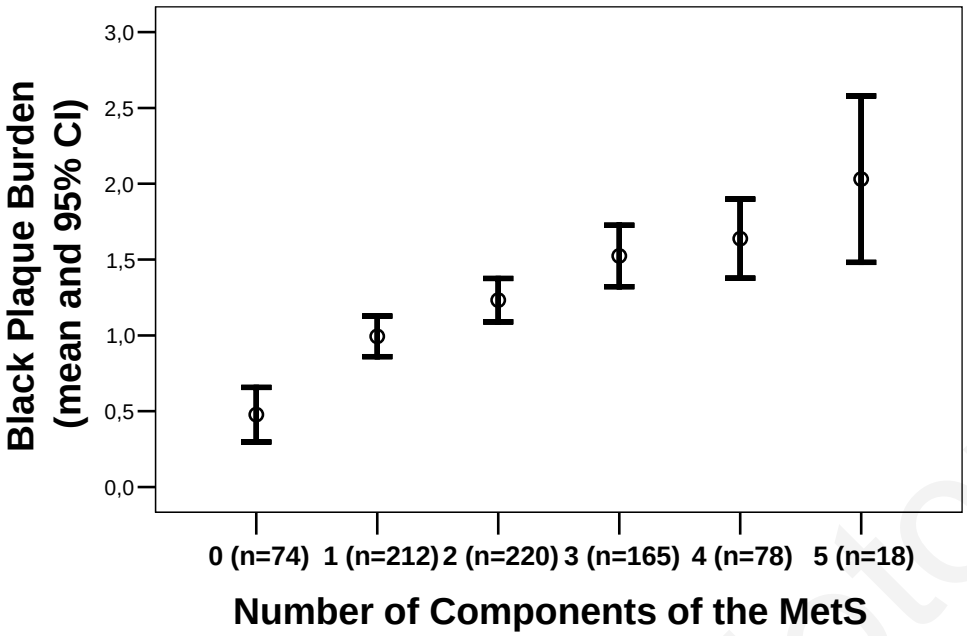

Figure 14.19: Association between BPB and number of components of MetS (ANOVA; P for trend<0.001)

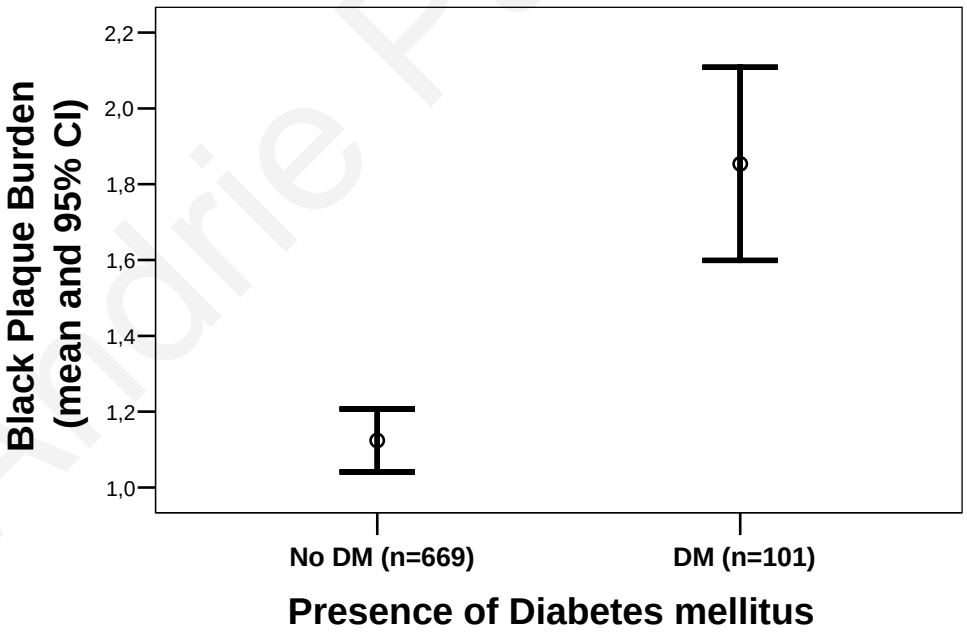

Figure 14.20: Association between BPB and presence of diabetes (t-test; P<0.001)

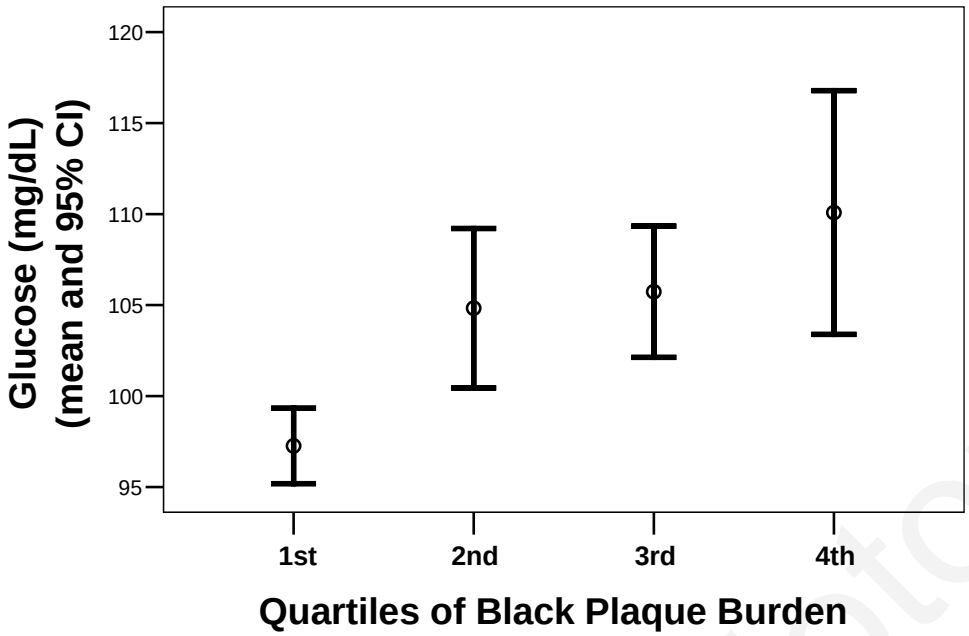

Figure 14.21: Association between glucose levels and quartiles of BPB (ANOVA: P for trend<0.001)

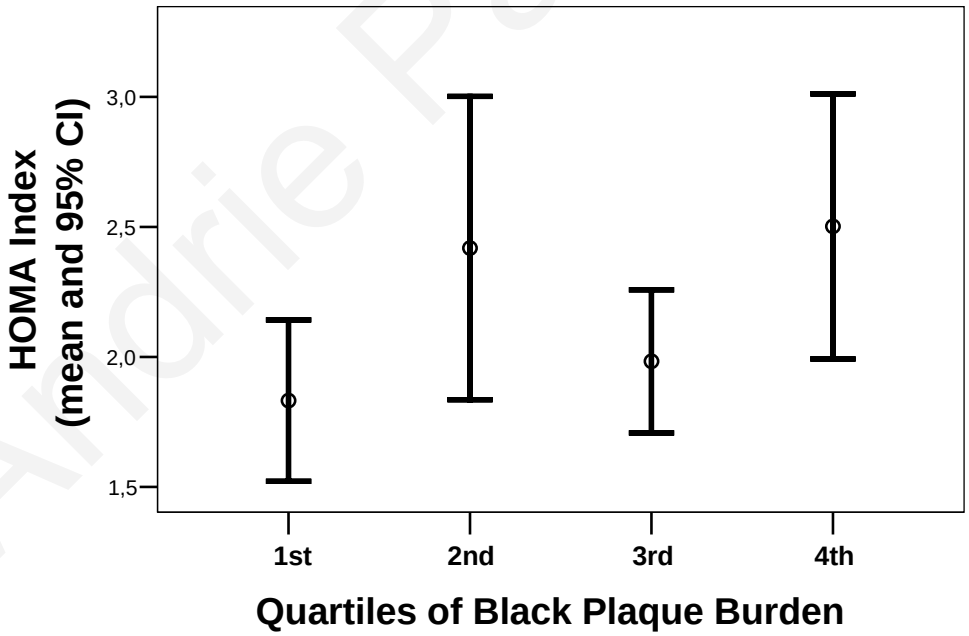

Figure 14.22: Association between the HOMA index and quartiles of BPB (Kruskal-Wallis test; P for trend=0.115)

Table 14.3: Compiled results of association between MetSmarkers and ultrasonic measurements in univariate analysis

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
MetS	+	+	+	+	+	+
MetS Compon.	+	+	+	+	+	+
DM	+	+	+	+	+	+
Glucose	+	+	-	+	+	+
HOMA	+	+	-	*+	+	-
UCP-2 (-866G>A)	**+	-	-	-	-	-
UCP-3 (-55C>T)	-	-	-	-	-	-

*Borderline p value (p=0.051)

**Borderline p value (p=0.05)

Table 14.4: Compiled results of association between MetS markers and ultrasonic measurements in multivariate analyses adjusting for age, sex, smoking in packyears, diabetes and hypertention

Marker	**IMT cc**	**IMT max**	**TPT**	**Presence of plaques**	**BPB**	**MPT**
MetS	-	+	-	-	+	-
MetS Compon.	+	+	+	+	+	-
DM	+	+	-	+	+	-
Glucose	-	+	-	-	-	-
HOMA	+	+	-	-	-	-
UCP-2 (-866G>A)	-	-	-	-	-	-
UCP-3 (-55C>T)	-	-	-	-	-	-

Summary of results

As discussed in chapter 3.10, the metabolic syndrome is considered to significantly increase the risk for CHD and people with the MetS (3 or more components) are at high risk for CHD.

1. Presence of MetS was significantly associated with all the ultrasonic markers tested. After adjustement for age, sex and smoking it remained significantly associated with IMTmax and BPB.
2. Adding components of the MetS were significantly associated with all the ultrasonic markers tested. The association was gradual and risk increased when even one component of the MetS was present. After adjustement for age, sex and smoking, adding components of the MetS remained significantly associated with IMTcc, IMTmax, TPT, presence of plaques and BPB.
3. Presence of diabetes was significantly associated with all the ultrasonic markers tested (IMTcc, IMTmax, TPT, presence of plaque, number of bifurcations with plaque and BPB) ($P<0.001$). After correction for age, sex and smoking (packyears), it remained associated with IMTcc, IMTmax, presence of plaques, number of bifurcations with plaque and BPB. Our results indicate that diabetes plays a significant part in all stages of atherosclerosis from early development to clinical disease, which corruborates the "common soil" hypothesis described in chapter 2.10.
4. Fasting serum levels of glucose were significantly associated with all the ultrasonic measurements tested exept TPT. If corrected for age, sex and smoking (packyears) glucose remained associated only with IMTmax.
5. The HOMA insulin resistance index was significantly associated with IMTcc, IMTmax and BPB but not with TPT and presence of plaques or number of bifurcations with plaque. After correcting for age, sex and smoking (packyears), it remained associated with IMTcc and IMTmax.
6. From the genetic polymorphisms (*UCP-2, UCP-3*) tested only *UCP-2* (-866AA vs GA, GG) showed a borderline association with IMTcc ($P=0.05$) and a trend for association with presence of plaques ($p=0.084$). The small number of AA homozygotes could be the reason for lack of statistical significance. The *UCP-3* (-55C>T) polymorphism was associated with BMI in diabetic subjects with the -55CC genotype associated with lower BMI.

Chapter 15:

Multivariate Predictive Models

This chapter reports on the results of the best predictive and explanatory multivariate models for each of the ultrasonic measurements used. Guided by the analysis reported in chapters 9-14, the best predictive models for IMTcc, IMTmax, TPT, MPT, presence of plaques, number of bidurcations with plaque and BPB were devised.

Best predictive model for IMTcc

In a linear multivariate regression analysis with IMTcc as a dependent variable the best fitted model included the independent variables: age, sex, smoking in packyears, hypertension, Tchol/HDL ratio, apoB/apoA1 ratio, creatinine, tHcy and number of MetS components. Results are tabulated in table 15.1. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 2.9% and could explain 30.5% of the variability in IMTcc in individual level.

Best predictive model for IMTmax

In a linear multivariate regression analysis with IMTmax (logtransformed) as a dependent variable the best fitted model included the independent variables: age, sex, smoking in packyears, hypertension, Tchol/HDL ratio, apoB/apoA1 ratio, Lp-PLA_2 activity, the *CETP* (TaqIB1B2) polymorphism, sCD40L, creatinine, glucose and number of MetS components. Results are tabulated in table 15.2. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 6.2% and could explain 37.0% of the variability in IMTmax in individual level.

Best predictive model for TPT

In a linear multivariate regression analysis with TPT (logtransformed) as a dependent variable the best fitted model included the independent variables: age, sex, smoking in packyears, hypertension, apoB/apoA1 ratio, Lp-PLA_2 activity, the *CETP* (I405V) and *MMP-9* (R279Q) polymorphisms, sCD40L and TF. Results are tabulated in table 15.3. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 6.9% and could explain 34.3% of the variability in TPT in individual level.

Best predictive model for presence of plaques

In a binary logistic multivariate regression analysis with presence of plaques as a dependent variable, the best fitted model included the independent variables: age, sex, smoking in packyeasrs, HDL, LDL, the *apoE* (E2/E3/E4) polymorphism, fibrinogen, IL-6, DM and number of MetS components. The goodness-of-fit of the model was tested with a Hosmer-Lemeshow test and the estimated likelihood was close to the observed one, indicating that the model accounts well for the outcome. Results are tabulated in table 15.4. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 10.2% and could explain 31.7% of the variability in presence of plaques in individual level.

Best predictive model for number of bifurcations with plaque

In a binary logistic multivariate regression analysis with number of bifurcations with plaque (0-2 vs 3-4) as a dependent variable, the best fitted model included the independent variables: age, sex, smoking in packyears, HDL, LDL, fibrinogen, the *MMP-9* (R279Q) polymorphism, glucose, DM and MetS. The goodness-of-fit of the model was tested with a Hosmer-Lemeshow test and the estimated likelihood was close to the observed one, indicating that the model accounts well for the outcome. Results are tabulated in table 15.5. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 4.2% and could explain 28.4% of the variability in more than 2 bifurcations with plaque in individual level.

Best predictive model for MPT

In a binary logistic multivariate regression analysis with MPT (below and over the median 2.75) as a dependent variable in individuals with plaques, the best fitted model included the independent variables: age, smoking in packyears, the *CETP* (TaqIB1B2) polymorphism, sCD40L, fibrinogen, creatinine, DM and MetS. The goodness-of-fit of the model was tested with a Hosmer-Lemeshow test and the estimated likelihood was close to the observed one, indicating that the model accounts well for the outcome Results are tabulated in table 15.6. Adding the markers tested here to the baseline model (age and smoking in packyears) improved its predictive ability by 4.5% and could explain 14.0% of the variability in MPT in those with plaques.

Best predictive model for BPB

In a linear multivariate regression analysis with BPB as a dependent variable the best fitted model included the independent variables: age, sex, smoking in packyears, LDL, Lp-PLA_2 activity, the *CETP* (TaqIB1B2) and *MMP-9* (R279Q) polymorphisms, sCD40L, DM and number of MetS components.. Results are tabulated in table 15.7. Adding the markers tested here to the baseline model (age, sex and smoking in packyears) improved its predictive ability by 4.2% and could explain 33.5% of the variability in BPB in individual level.

Table 15.1: Best predictive model for IMTcc in multivariate linear regression analysis (R^2=0.304) (n=766)

Variables in the model	Estimate (B)	95% CI for B	Beta	P value
Age	0.001	0.001 to 0.001	0.425	<0.0001
Sex	0.004	0.001 to 0.007	0.117	0.005
Smoking in packyrears	5.7E-005	0.000 to 0.00	0.097	0.005
TChol/HDL	-0.002	-0.003 to 0.00	-0.138	0.017
ApoB/apoA1	0.015	0.007 to 0.023	0.190	<0.0001
Creatinine	-0.007	-0.013 to 0.00	-0.084	0.041
tHcy	0.007	-0.001 to 0.015	0.068	0.071
MetS components	0.002	0.000 to 0.003	0.111	0.005
Hypertension	0.002	0.000 to 0.005	0.065	0.057

Table 15.2: Best predictive model for IMTmax in multivariate linear regression analysis (R^2=0.370) (n=637)

Variables in the model	Estimate (B)	95% CI for B	Beta	P value
Age	0.026	0.022 to 0.030	0.467	<0.0001
Sex	0.139	0.040 to 0.238	0.120	0.006
Smoking in packyears	0.001	0.000 to 0.03	0.069	0.069
ApoB/apoA1	0.326	0.024 to 0.628	0.119	0.035
Lp-PLA$_2$ activity	0.004	0.002 to 0.006	0.121	0.001
***CETP* (TaqIB1B2- AA vs AG,GG)**	0.110	0.021 to 0.199	0.078	0.015
sCD40L	-0.045	-0.075 to -0.015	-0.093	0.004
MetS components	0.042	0.001 to 0.84	0.091	0.043
Hypertension	0.117	0.034 to 0.201	0.098	0.006
TChol/HDL	-0.058	-0.115 to -0.002	-0.126	0.043
Glucose	0.001	0.000 to 0.003	0.072	0.041
Creatinine	-0.159	-0.379 to 0.061	-0.057	0.155

Table 15.3: Best predictive model for TPT in multivariate linear regression analysis (R^2=0.343) (n=438)

Variables in the model	Estimate (B)	95% CI for B	Beta	P value
Age	0.026	0.021 to 0.031	0.394	<0.0001
Sex	0.260	0.145 to 0.376	0.201	<0.0001
Smoking in packyears	0.003	0.001 to 0.005	0.149	0.001
ApoB/apoA1	0.331	0.054 to 0.608	0.108	0.019
MMP-9 **(R279Q- GG vs AA,GA)**	0.217	0.028 to 0.407	0.088	0.025
sCD40L	-0.074	-0.115 to -0.032	-0.136	0.001
Hypertension	0.189	0.082 to 0.295	0.144	0.001
Lp-PLA2 activity	0.002	-0.001 to 0.005	0.055	0.217
Tissue Factor	-0.140	-0.275 to -0.005	-0.080	0.043
CETP **(I405V-VV vs VI,II)**	0.070	-0.035 to 0.174	0.052	0.191

Table 15.4: Best predictive model for presence of plaque in multivariate logistic regression analysis (chi^2=6.623, P=0.58) (n=217)

Variables in the model	P value	Odds Ratio	95% CI for OR
Age	<0.0001	1.083	1.039 to 1.129
Sex	0.001	4.712	1.836 to 12.091
Smoking in packyears	0.140	1.023	0.992 to 1.055
HDL	0.367	1.021	0.975 to 1.069
***ApoE* (E2/E3 vs E3/E3, E3/E4)**	0.575	1.620	0.300 to 8.747
MetS components	0.040	1.505	1.019 to 2.223
IL-6	0.040	1.178	1.007 to 1.377
Diabetes mellitus	0.998	2E+0.008	0.000 to 0.00
Fibrinogen	0.811	0.999	0.991 to 1.007
LDL	0.397	0.993	0.979 to 1.009

Table 15.5: Best predictive model for number of bifurcations with plaque in multivariate logistic regression analysis (chi^2=4.594, P=0.80) (n=712)

Variables in the model	P value	Odds Ratio	95% CI for OR
Age	<0.0001	1.115	1.090 to 1.142
Sex	<0.0001	2.872	1.769 to 4.664
Smoking in packyears	0.002	1.015	1.006 to 1.024
Fibrinogen	0.157	1.003	0.999 to 1.006
Diabetes mellitus	0.002	3.076	1.498 to 6.318
HDL	<0.0001	0.959	0.939 to 0.980
LDL	0.022	1.008	1.001 to 1.015
Glucose	0.015	0.989	0.980 to 0.998
***MMP-9* (R279Q-GG vs AA,AG)**	0.010	3.793	1.384 to 10.389
MetS	0.197	0.727	0.448 to 1.180

Table 15.6: Best predictive model for MPT below and over the median (0.275) in multivariate logistic regression analysis (chi^2=6.96, P=0.54) (n=491)

Variables in the model	P value	Odds Ratio	95% CI for OR
Age	<0.0001	0.916	0.893 to 0.940
sCD40L	0.026	1.000	0.999 to 1.000
***CETP* (TaqIB1B2- AA vs AG,GG)**	0.037	0.546	0.309 to 0.963
Fibrinogen	0.232	0.998	0.993 to 1.002
MetS	0.304	1.284	0.797 to 2.068
Creatinine	0.153	2.024	0.769 to 5.324
Diabetes mellitus	0.343	0.752	0.417 to 1.356
Smoking in packyears	0.190	0.995	0.988 to 1.002

Table 15.7: Best predictive model for BPB in multivariate linear regression analysis (R^2=0.335) (n=630)

Variables in the model	Estimate (B)	95% CI for B	Beta	P value
Age	0.035	0.028 to 0.043	0.329	<0.0001
Sex	0.492	0.324 to 0.661	0.222	<0.0001
Smoking in packyears	0.006	0.003 to 0.009	0.144	<0.0001
Lp-PLA$_2$ activity	0.007	0.003 to 0.011	0.114	0.001
sCD40L	-0.114	-0.174 to -0.055	-0.124	<0.0001
Diabetes mellitus	0.208	-0.026 to 0.441	0.064	0.081
MetS components	0.083	0.015 to 0.151	0.092	0.017
***CETP* (TaqIB1B2-AA vs AG,GG)**	0.121	-0.054 to 0.295	0.044	0.175
***MMP-9* (R279Q-GG vs AA,GA)**	0.330	0.062 to 0.598	0.079	0.016
LDL	0.003	0.000 to 0.005	0.072	0.041

PART V:

Discussion and Conclusions

Chapter 16:

Discussion

In this chapter the findings of the thesis are discussed for their significance in relation not only to what was available in the literature when the work began but also, to studies published while the work of the thesis was in progress.

Epidemiological studies have identified individuals and groups at increased risk for cardiovascular events using established risk factors (e.g. hyperlipidaemia, diabetes, hypertension, smoking and family history). However, high risk groups at best contain 50-60% of subsequent cardiovascular events and the remaining events occur in the low or medium risk population which is quite large. It is now well established that 50% of individuals with symptomatic cardiovascular disease do not have any of the conventional risk factors (Chockalingham and Balaguer-Vinto, 1999). This suggests that there should be many unknown factors.

Several risk assessment models (Framingham, Canadian, British, European, Munster and MONICA) have been developed; however, all of them are based on traditional risk factors known to contribute to the chronic development of atherosclerosis. Addition of emerging risk factors particularly those indicative of the activity of the disease (i.e. plaque inflammation) may allow individualized risk assessments to be made. Those who have no indication of coronary stenosis or myocardial ischemia and who may even lack traditional risk factors may benefit from the techniques now under development that evaluate plaque biology and inflammation (Naghavi *et al.*, 2003b) as well as non-invasive methods of visualising plaques in vivo. One such established method is B-mode ultrasound.

With the use of ultrasound, arterial wall thickening, presence or absence of plaques and plaque type can be studied, in an attempt to improve on epidemiological studies and advance basic knowledge on atherosclerosis. It can be argued that arterial wall changes will be the end result of all, exogenous and endogenous (genetic) factors known, and unknown. In addition to that, because atherosclerosis progresses over decades, epidemiological studies and intervention trials with clinical end-points require long-term follow-up, participation of large populations or both. Use of surrogate markers, such as ultrasonic measurements of the arterial wall, has been proposed (Wittes *et al.*, 1989) in order to overcome such difficulties (de Groot *et al.*, 2004).

Measurements of arterial wall IMT with B-mode ultrasound was first presented as a means of assessing atherosclerotic changes in the aorta in 1986. The rate of IMT change has been found to approximate 0.01 to 0.03 mm/year and the Cholesterol-Lowering Atherosclerosis Study (CLAS) showed that for each 0.03 mm carotid IMT increase/year the relative risk for any coronary event increased 3.1 times (O'Leary and Polak, 2002). As discussed further in chapter 2, only carotid IMT is currently recognised by the American Heart Association (AHA) and included in the evaluation of risk. Effective therapeutic intervention has advanced the concept of primary prevention, which focuses on identifying asymptomatic individuals with no prior history of CHD who are at sufficiently high risk for a future event to justify gradually more intensive risk reduction efforts.

This notion was set forth recently, given the fact that in a significant number of patients, sudden cardiac death is the first manifestation of underlying heart disease and it is still responsible for > 450 000 deaths annually in the U.S.A. Despite extensive studies and development of several risk prediction models, traditional CHD risk factors fail to predict development of CHD in a large group of cases (25% to 50%) (Khot *et al.*, 2003; Naghavi *et al.*, 2003a). For example, the Framingham Coronary Risk Score (with family history) can predict at best, only 50-60% of cardiovascular events (Naghavi *et al.*, 2003a). This implies that other factors play a significant role in the development of the disease and there is a void in current understanding of its pathogenesis.

The aim of the work presented in this thesis was to determine the association between subclinical atherosclerosis as indicated by novel ultrasonic measurements of the arterial wall and selected biomarkers of lipi d metabolism, endothelial dysfunction, inflammation, thrombosis, homocysteine metabolism and insulin resistance.

Identification of genetic differences influencing the pathways of measurable risk factors or novel pathways may allow for the determination of risk that is additive to the measurement of conventional risk factors. More over, some genetic polymorphisms (such as *apoE* E2/E3/E4, *IL-6* -174G>C) show evidence of specific environmental interactions (e.g. smoking and alcohol) in which the overall risk is more than additive. It has been argued that because an individual's personal characteristic and plasma risk-trait values reflect both genotype and exposure, they may be a superior predictor of

clinical outcome (Stephens and Humphries, 2003). However, (a) a number of recently identified genes are likely to be independent of measured traits, (b) they may guide therapy and (c) they may reveal highly penetrant disorders that could influence risk substiantially for a patient and/or his/her family (Lusis *et al.*, 2004a). In addition, studying genetic polymorphisms -even if alone do not improve risk assessement- may give further insight into the pathophysiology of a complex disease leading into new pathways of innovation.

Ultrasonic measurements and clinical disease

In the Cyprus study population included in the analysis, we tested 7 ultrasonic measurements as presented with detail in chapter 8. IMTcc, IMTmax (including plaques), TPT, presence of plaques and number of bifurcations with plaque and mean plaque type; also BPB (TPT*MPT). In our study population, the presence of plaque at three or four bifurcations (indicating the presence of both carotid and femoral atherosclerosis), TPT, MPT and BPB were more strongly associated with prevalence of clinical CVD than IMTcc or IMTmax measurements; indicating that the presence of plaques in the common femoral artery as well as the type of plaques (MPT-based on Widder classification) could be used as ultrasonic markers associated with CVD (Table 8.2).

While this thesis was being written, a study published by Kablak-Ziembicka *et al.* demonstrated that carotid IMT has a diagnostic value in indicating multi-level atherosclerosis. They included 415 patients with measurements of carotid IMT (common, bulb and internal carotid), coronary and renal angiography, and supraaortic, iliac/femoral arteries ultrasound evaluation. It was shown that aggregate IMT (mean value of common, bulb and internal carotid measurement i.e. including plaques) increased with increasing number of involved territories (1 to 4) being thus an independent predictor of significant multi-level atherosclerosis, showing high sensitivity and specificity for indicating more advanced territorial atherosclerotic involvement (Kablak-Ziembicka *et al.*, 2007).

A meta-analysis including data from 37 197 subjects who were followed up for a mean of 5.5 years, provided robust age and sex-adjusted risk estimates of common carotid IMT prediction for MI and stroke in the general population. For an absolute carotid IMT difference of 0.1 mm, the future risk of MI was shown to increase by 10% to 15%,

and the stroke risk increased by 13% to 18%. The risk for both end points decreased with the number of adjustments for risk factors (Lorenz *et al.*, 2007). Such large meta-analyses also, highlight the heterogeneity that exists between studies in IMT measurement protocols.

Another recent study by Schmidt et al. (2005) has showed that subjects with a plaque present in the femoral artery at baseline are at increased risk for having a cardiovascular event during a 6.6 year follow-up and to a large extent the risk was confined to those with an echolucent plaque at baseline (Schmidt *et al.*, 2005). These data further support the notion that femoral plaques and plaque type increase the risk of developing clinical CVD. One of the shortcomings of the above study was that only one femoral artery was scanned.

This is the first time, to the best of our knowledge that four bifurcations (two carotid and two femoral) have been scanned in one study and that the associations between CVD prevalence and different ultrasonic measurements that include IMTcc, IMTmax, presence of femoral plaques, number of vessels with plaque and total plaque thickness have been compared in a single population study. Our data, also, indicate that "rapidly growing" plaques are mostly echolucent (as shown in fig.9.2) and as discussed in detail in chapter 2, there are at least 3 studies supporting that echolucent plaques are associated with an increased risk of stroke (Grønholdt *et al.*, 2001; Mathiesen *et al.*, 2001; Polak *et al.*, 1998). Recent publications by the Trømso group (Johnsen *et al.*, 2005; Johnsen *et al.*, 2007) have demonstrated that slowly growing plaques are echogenic, a fact which further supports our findings.

National guidelines from the American Heart Association (AHA) and the National Heart, Lung and Blood Institutes NECP recommend an approach to initial global risk assessment of the asymptomatic patient to obtain an estimate of absolute cardiovascular risk. On the basis of standard risk factors and related risk correlates, the concept was set forth that asymptomatic patients can be placed into one of three risk categories: low, intermediate and high. The approach to therapy is guided by the principle that the intensity of risk-factor management should be adjusted by the severity of risks. Low risk patients should adhere to healthy lifestyle habits; high risk patients are advised to start directly on an aggressive risk reduction regiment (non-drug and drug regimens). Patients at intermediate risk become candidates for further risk stratification through new markers and non-invasive methods for presence of MI or

atherosclerosis (Lusis *et al.*, 2004b). The NHANES III panel has estimated in 2000 that in the U.S.A., intermediate risk subjects account for approximately 40% of the general population (Greenland *et al.*, 2001). All of the above highlight the need for better discrimination and risk stratification in indermediate risk groups.

The most widely used risk scoring system today (especially in the U.S.A.) is the Framingham risk score, so in order to compare its predictive ability (though cross-sectionally) in our study population we calculated the 10-year Framingham risk for each individual and then added TPT, MPT and BPB –using the best ROC point and the population median as cut-off points- in the equation. The addition of total plaque thickness to the Framingham 10 year risk increased the positive predictive value from 56% to 66%, the negative predictive value for presence of clinical CVD from 92.3% to 94% and the likelihood ratio from 3.00 to 3.52. If IMTcc was added to the model the area under the ROC curve did not improve. However, if IMTmax or TPT were added to the model the area improved to 0.81 and 0.83 respectively. The combination of Framingham 10 year risk with total plaque thickness could determine a high-risk group (upper quartile), which contained 45.4% of cardiovascular events. If BPB (cut-off point 1.02) was added to the Framingham risk, the upper quartile could identify a group that contained 40.1% of the CVD events. Combining MPT with the Framingham risk could identify a group that contained 49.2% of clinical events (upper quartile). This suggests that inclusion of ultrasonic measurements to the Framingham 10 year risk score may improve its predictive ability. However, this needs to be validated in prospective studies.

Lipid markers and subclinical atherosclerosis

Hypercholesterolaemia is considered one of the most important factors associated with CVD and it has even been linked to plaque rupture leading to acute ischemic syndromes (Virmani *et al.*, 2000). The most widely used markers for hypercholesterolaemia are total cholesterol (TChol), HDL and LDL cholesterol and triglycerides (TG). In this thesis, we have tested the association between blood levels of both commonly used lipid markers (TChol, HDL, LDL, the ratio of TChol/HDL and TG) as well as those not used routinely (apoA1, apoB, the ratio of apoB/A1, Lp(a) and Lp-PLA_2) and the novel ultrasonic measurements of subclinical atherosclerosis described in chapter 8. In addition, we tested five common genetic polymorphisms (*apoB* -516C>T, *apoE* E2/E3/E4, *CETP* TaqIB1B2B2 and I405V and *Lp-PLA_2*

A379V) influencing lipoprotein metabolism and function. Compiled results are shown in tables 9.14 and 9.15. In brief, of the biochemical markers studied the ratio of apoB/apoA1 and Lp-PLA_2 activity were significantly associated with most of the ultrasonic markers of subclinical atherosclerosis, including plaque echolucency. The ratio of TChol/HDL was also significantly associated with the ultrasonic markers but the association was even stronger for the ratio of apoB/apoA1.

Evidence is now accumulating that the apoB/apoA1 ratio is superior to the TChol/HDL ratio for prediction of cardiovascular risk in both sexes and in all ages as shown by Walldius group (Lamarche *et al.*, 1996; Walldius and Jungner, 2006; Walldius *et al.*, 2001). Given that the apoB/apoA1 ratio is a measurement of the number of apoB atherogenic particles over the number of apoA1 anti-atherogenic particles, there is also a biological plausibility that it is a more important factor than the amount of lipids carried per particle. So far, studies have correlated apoB/apoA1 ratio with late atherosclerosis, mainly clinical events. Our data suggest that the apoB/apoA1 ratio is associated with subclinical atherosclerosis and may be a key-factor in the early formation of stable and unstable plaques. In our study apoB/apoA1 ratio was associated among others with MPT (measure of echolucency) and BPB and by inference with rapidly growing plaques. However, prospective studies are required in order to determine causality.

Another significant finding of our study is the association between plasma Lp-PLA_2 activity and all the ultrasonic measurements tested. Lp-PLA_2 is a relatively new "player" in atherosclerosis and it has not been studied –to the best of our knowledge- in association with subclinical atherosclerosis. This is also the first time that the association between Lp-PLA_2 activity and A379V genotype has been demonstrated in a general population cohort. We have shown that the 379VV genotype is associated with higher mean Lp-PLA_2 activity and that this association is driven exclusively by women. Only two case-control studies have looked at this association so far, the AtheroGene and the UDACS. The first showed a consistent association between 379VV genotype and higher Lp-PLA_2 activity in both men and women and cases and controls. However, after adjustement for age, smoking and BMI the genotype effect on activity levels was weak (<1%) (Ninio *et al.*, 2004). The UDAC study which excluded women from the analysis due to lack of activity data resulted in not significant results (Wootton *et al.*, 2006). The authors argue that if a causal relationship between A379V genotype and activity was clearly established, then an association between A379V genotype and CHD risk (as indicated in the AtheroGene study) would provide

indirect evidence for the causality of the association between Lp-PLA_2 activity and CHD risk. Our data provide evidence for an association between *Lp-PLA_2* A379V polymorphism and Lp-PLA_2 activity in women and explain why such an association has not been demonstrated in the UDAC study. Furthermore, after adjustement for age, smoking and BMI the genotype effect could explain 1.9% of the variability in activity levels (P=0.003) in women.

Our results, indicating an association between higher Lp-PLA_2 activity levels and greater IMTcc, IMTmax, TPT, presence of plaques and BPB are in accordance with previously published data from the ARIC (Ballantyne *et al.*, 2004) and AtheroGene (Ninio *et al.*, 2004) studies showing that higher Lp-PLA_2 activity was an independent predictor of CVD. Data seem to support a causal role for Lp-PLA_2 starting from the early stages of atherosclerosis development and support the notion that Lp-PLA_2 may represent an important "missing link" between the oxidative modification of LDL in the intimal layer of the arterial wall and local inflammatory processes within the atherosclerotic plaque that may be specific for atherosclerosis (MacPhee *et al.*, 2005). Experimental studies in Watanabe hyperlipidemic rabbits have shown that inhibition of Lp-PLA_2 leads to reduction of atherosclerotic lesion formation. In addition, a recent study in patients with CHD demonstrated that increased Lp-PLA_2 plasma concentrations predict future cardiovascular events independently of a variety of potential risk factors, including markers of inflammation, renal function and haemodynamic stress (Koenig *et al.*, 2006).

An association between the 379VV genotype and arterial wall measurements has not been shown in our study, a fact which could be due to the small number of VV homozygotes (n=48).

As far as the other genetic polymorphisms are concerned, the *apoE* (E2/E3/E4) polymorphism was associated with presence of plaques (E3/E3, E3/E4 genotypes compared to E2/E2, E2/E3); the *CETP* TaqIB1B2B2 polymorphism was associated with IMTmax (B2B2 compared to B1B2, B1B1) and the *CETP* I405V polymorphism was associated with TPT as presented in chapter 9. A very recent meta-analysis of 22 studies on *apoE* genotype and carotid IMT presents positive evidence of association and suggest a possible specific association with large artery ischemic stroke (Paternoster *et al.*, 2008). Our results provide strong evidence for a positive association

between apoE genotype and presence of plaque over and above other traditional risk factors.

Endothelial dysfunction markers and subclinical atherosclerosis

The observation that certain individuals do not develop atherosclerotic manifestations despite the presence of several cardiovascular risk factors suggests the existence of a "threashold switch" that, only when activated translates the risk factor into an unfavourable vascular effect. The endothelial cell layer that represents a mechanical and biological barrier between the blood and the vascular wall is likely to serve as the "missing link" between any given risk factor and its detrimental vascular effect (Bonetti *et al.*, 2003). In this thesis, we have tested the association between certain oxidation markers (sNO, MPO, *eNOS* 894G>T, *MPO* -638C>A, *PON1* L55M and *PON2* S311C) and novel ultrasonic markers of subclinical atherosclerosis.

Independently, none of the oxidation markers tested was associated with any of the ultrasonic measurements.

As far as the *eNOS* (894G>T) polymorphisms is concerned, our data are in contrast with those from Hingorani's group who found a striking over-representation of the 894T allele and TT homozygotes among cases of CAD compared to controls in a U.K. population (Hingorani, 2000). The same was also true for an Italian population of 375 hypertensives where the 894TT homozygotes were found to be associated with presence of atherosclerotic plaques (Lembo *et al.*, 2001). However, an association between the 894G>T polymorphism and blood pressure, left ventricular mass and carotid IMT was not demonstrated in the OPERA population study, including 1024 subjects giving it greater power to detect a possible relationship (Karvonen *et al.*, 2002). The authors of the Finnish study argue that although the *eNOS* 894G>T polymorphism is likely to alter the function of the eNOS protein, this altered function does not affect endothelium-dependent vasodilatation in Caucasian subjects. The NO production catalysed by *eNOS* Glu298Asp protein may be sufficient to relax the endothelial vascular smooth muscle cells, or other mechanisms regulating the blood pressure might compensate for the shortness of the action of NO. It also seems likely that this polymorphism has a greater effect on Japanese than Caucasian, e.g. Finnish subjects. This is in accordance with our data that the *eNOS* 894G>T polymorphism is not a major risk factor for subclinical atherosclerosis in a

Cypriot general population cohort. In addition, the Finnish as well as the Cypriot populations are likely to be genetically more homogeneous than the U.K. population (Karvonen *et al.*, 2002).

The *MPO* (-638C>A) polymorphism has not been studied extensively. However, a recent study has shown that the A allele is correlated with higher human neutrophil MPO activity and it has been suggested that it may be a genetic determinant of cardiovascular risk (Chevrier *et al.*, 2006). There are no data concerning the association between plasma MPO levels and the -638C>A polymorphism and as far as we know this is the first time that such an association is tested. In our Cypriot population cohort there is no apparent association or trend between plasma MPO levels and the -638C>A genotype. It is worth noting that haplotypes were used in the aforementioned study by Chevrier et al. which increases the power for detection of multiple associations. Our study included only one polymorphism for the *MPO* gene which allows for the possibility that haplotypes and not single polymorphisms change the risk for complex traits such as atherosclerosis.

A relationship between the *PON1* L55M polymorphism and carotid atherosclerosis measured by plaque size was shown in an Austrian general population cohort (Schmidt *et al.*, 1998). Our data do not support such findings since in our study population no association was found between the L55M genotype and any of the ultrasonic measurements of subclinical atherosclerosis tested. This could be explained by the findings of Jarvik et al. who demonstrated that *PON1* L55M genotype did not predict case-control status unless the activity phenotype was also included as a predictor (Jarvik *et al.*, 2000). The authors concluded that examining *PON1* Q192R and/or *PON1* L55M genotypes alone may mistakenly lead to the conclusion that there is no role of *PON1* in carotid artery disease. Such results support the benefit of a "level crossing" approach that includes intervening phenotypes in the study of complexly inherited disease.

PON2 S311C polymorphism and CHD has been described previously in an Asian Indian population (Sanghera *et al.*, 1998). One of the largest studies looking at *PON1* and *PON2* polymorphisms and CHD risk was the prospective Northwick Park Heart study II including 3052 subjects which demonstrated that genotype frequencies did not differ between cases and controls but when considered together the *PON1* L55M and *PON2* S311C did influence the risk of CHD and the *PON1* L55M modified the CHD

risk associated with smoking (Robertson *et al.*, 2003). Multiple associations combining two or more polymorphisms were not performed here.

In our population cohort, creatinine levels were significantly associated with all the ultrasonic markers studied. Our data are in accordance with previous reports indicating an association between slightly raised creatinine concentrations (within the normal range) and subsequent mortality from CAD (reviewd in 3.7.4). The mechanism underlying the link between minor impairement of renal function and CVD is still unclear. However, it can be ventured that whatever causes endothelial dysfunction in the kidneys –causing a rise in creatinine levels- would commonly cause endothelial dysfunction in the arterial wall as well (Solomon *et al.*, 2007). One such mechanism could be systemic inflammation causing a continuous activation of oxidative factors and endothelial dysfunction throughout the body, giving rise to slightly impaired function in the kidneys and atherosclerosis in the arterial wall.

The *ACE* (I/D) polymorphism was not significantly associated with any of the ultrasonic markers of subclinical atherosclerosis in our population. There are previous reports both supporting and refuting an association between the *ACE* I/D polymorphism and CVD with most studies, however, refuting it (see 3.7.2 for details). In our cohort the II genotype was associated with diastolic but not systolic blood pressure.

The angiotensinogen (-6G>A) polymorphism was not associated with any of the ultrasonic markers of subclinical atherosclerosis in our population. In a sex-specific analysis there was a trend towards higher TPT, BPB and more than 2 bifurcations with plaque for the GG homozygotes in women and for presence of plaques in men but none reached statistical significance. This is in accordance with previous reports not supporting an association with the -6G>A genotype but in disagreement with one study reporting an association between the -6A allele and higher IMT in women (Chapman *et al.*, 2001). Interactions between diet and the *ang* (-6G>A) genotype resulting in increased blood pressure have been reported previously (DASH study), however, such an association could not be tested here due to lack of diet data.

Inflammation markers and subclinical atherosclerosis

Over the past few years, it has become increasingly clear that inflammation is at the root of atherosclerosis and its complications. As the mechanisms underlying this process are deciphered, new markers may emerge to assist the clinician in the determination of patient risk for CVD.

In this thesis, we have tried to elucidate the association between genetic (*IL-6* -174C>G, *TNF-α* 308A>G, *MGP* -138C>T, *MMP-1* 1G/2G, *MMP-3* 5A/6A, *MMP-7* -181A>G, *MMP-9* R279Q and *MMP-12* -82A>G) and biochemical (CRP, IL-6 and MCP-1) inflammatory markers and novel ultrasonic markers of subclinical atherosclerosis. In our population, both plasma levels of IL-6 and MCP-1 were significantly associated with all the ultrasonic markers tested (IMTcc, IMTmax, TPT, presence of plaques, number of bifurcations with plaque MPT and BPB). CRP levels were associated with IMTcc, IMTmax, presence of plaques and BPB in women only.

CRP has received much attention over the past decade as a new marker for CHD and several studies have demonstrated an association between CRP levels and risk of CVD (as discussed further in chapter 3.5.4). In 2003, the AHA stated that: "it is reasonable to measure CRP as an adjunct to the measurement of established risk factors in order to assess the risk of CHD" (Pearson *et al.*, 2003). Our data are not in complete accordance with these since in our population CRP levels were only moderately associated with increasing IMTcc and not associated with any other ultrasonic marker tested. However, in a sex-specific analysis CRP levels were significantly associated with IMTcc, IMTmax, presence of plaques, BPB and borderline for MPT in women only. This is the first time that CRP levels are associated with subclinical atherosclerosis in women only. A global risk prediction model that includes hsCRP improved cardiovascular risk classification in women, particularly among those with a 10-year risk of 5% to 20%, as was shown in the prospective Womens Health Study (Cook *et al.*, 2006). However, a recent review on published studies concluded that there is no definitive evidence that, for most individuals, CRP adds substantial predictive value above that provided by risk estimation using traditional risk factors for CVD. Also, women have a higher incidence of rheumatoid diseases which increase inflammatory markers such as CRP, a fact which may influence results. Use of CRP may add to risk estimation in a limited subset of individuals who are at intermediate predicted risk according to the Framingham risk score (Lloyd-Jones *et al.*, 2006). Other findings also support a more moderate role for CRP (Danesh *et al.*, 2004).

MCP-1 is a chemokine implicated in atherosclerosis mostly in animal models while data from humans are contradictory. Higher levels of MCP-1 have been associated with increased risk of MI, sudden death and restenosis after stending (discussed further in 3.5.3). However, data is lacking about the role of MCP-1 in the general population and especially in subclinical atherosclerosis. As far as we know, this is the first time that an association between MCP-1 blood levels and ultrasonic markers of subclinical atherosclerosis is shown in a large general population cohort. Our findings support a significant role for MCP-1 in the first stages of atherosclerosis since higher levels of MCP-1 were significantly associated with both presence and plaque size (IMTcc, IMTmax, TPT) as well as with extend of atherosclerosis (number of bifurcations with plaque) and plaque echolucency (BPB and MPT).

From the genetic polymorphisms studied, the *MGP* (-138C>T) and *MMP-7* (-181A>G) were significantly associated with some but not all the ultrasonic markers, suggesting that different genes play part in different stages of atherosclerotic disease progression. The TNF-α (-308G>A) polymorphism was significantly associated with IMTcc, TPT and BPB but not IMTmax or presence of plaques. The *MMP-9* (R279Q) polymorphism was significantly associated with presence and plaque size (IMTmax and TPT), extend of atherosclerosis (number of bifurcations with plaque) and plaque type (MPT) but not with IMTcc or BPB.

To the best of our knowledge, this is the first report of an association between the *MGP* (-138C>T) polymorphism and subclinical atherosclerosis as measured by ultrasound. In our population the CC genotype was associated with lower IMTmax. The -138C allele was previously shown to confer reduced promoter activity (Cambien *et al.*, 1999).

The *TNF-α* (-308G>A) functional polymorphism has been associated with greater promoter activity and increased plasma TNF-α levels. In a U.K. study of 641 subjects reffered to for coronary angiography, the -308A allele was associated with homocysteine but not with number of significantly diseased vessels (Wang and Oosterhof, 2000). TNF-α plasma levels have been associated with common carotid IMT in healthy middle-aged men (Skoog *et al.*, 2002) as well as with higher MCP-1 levels in the French Stanislas Cohort (Berrahmoune *et al.*, 2007). On the other hand, findings from the Helsinki Sudden Death study in 700 autopsy cases do not support an

association between the *TNF-α* -308G>A genotype and coronary atherosclerosis (Keso *et al.*, 2001).

Our data support a role for *TNF-α* in subclinical atherosclerosis. The -308A allele (AA, GA vs GG) was associated with higher IMTcc, TPT and BPB which indicates that higher TNF-α activity is associated mainly with the initial thickening of the arterial wall and the type of plaques formed (echolucent).

A variety of MMPs have been found to be expressed in atherosclerotic lesions and all the *MMPs* studied in this thesis have been implicated in some way in atherosclerosis (discussed further in 3.5.7). Our results support a role for *MMP-7* (-181A>G) and *MMP-9* (R279Q) in subclinical atherosclerosis as assessed by ultrasound. The *MMP-1* (1G/2G) and the *MMP-12* (-82A>G) polymorphisms were not associated with the ultrasonic markers tested. The *MMP-3* (5A/6A) was associated with TPT greater than 0.52 cm only in univariate analysis. Our results are not in complete accordance with the Northern Manhattan Prospective Cohort study, which reported an association between the *MMP3* 6A/6A and *IL-6* -174GG genotypes and higher IMTcc; however, this was so in only 87 subjects of mixed race (Rundek *et al.*, 2002). Our results suggest a weak association between *MMP-3* 5A/5A genotype and higher TPT. A study looking at MMPs expression in plaques, using tissue from carotid endarterectomies, found augmented expression of MMP-1, MMP-3 and MMP-9 in plaques compared with control regions. MMP-9 expression, in particular was significantly upregulated in plaques with disrupted fibrous cap (Takeo *et al.*, 2006), while MMP-9 and MMP-8 activities were, also, found to be higher in macrophage-rich lesions in another recent study (Sluijter *et al.*, 2006). Combined elevated plasma levels of MMP-9 and plaque echolucency were associated with a 4-fold risk for ipsilateral stroke or cardiovascular death and a 3-fold risk for ipsilateral stroke in patients with carotid stenosis (Eldrup *et al.*, 2006). Matrix metalloproteinase-9 has been shown to degenerate structural components of plaque matrix such as connective tissue collagens IV, V, VII, X and XII and elastin (Lijnen, 2001). Increased production of MMP-9 is thought to contribute to the progressive deterioration of the elastic lamellae associated with vessel remodeling, which could be closely related to the occurrence of plaque disruption.

Considerable evidence suggests inflammation is a key process in the pathogenesis of atherosclerosis. However, the question remains: does this represent a truly causal

relationship or do elevated levels of inflammatory markers simply reflect subclinical disease already present many years before symptomatic events (reverse causality). By studying associations with functional candidate gene polymorphisms and applying the principle of "Mendelian randomization" we can examine causality in such situations (Davey Smith and Ebrahim, 2003). When it comes to inflammation, if it indeed plays a causal role, individuals with genetic variants that predispose them to an increased inflammatory response would be expected to develop more atherosclerosis. This hypothesis was also tested in the Bruneck study which tested the association between proinflammatory variants in three genes (IL-6, IL-1 and CD14) and common carotid and femoral IMT. They demonstrated a significant relationship between gene-variant score and carotid IMT as well as synergistic effects of gene-variant score and smoking on IMT measurements. Interactive effects of gene-variant score, a risk factor score composed of the acquired inflammatory conditions were highly significant and results were similar for femoral artery IMT (Markus *et al.*, 2006). These findings, in addition to providing support for a causal role of inflammation in atherosclerosis, they also emphasize the importance of gene-gene and gene-environment interactions in this and possibly other pathogenic pathways and are in accordance with findings from our study indicating the importance of inflammation in the early stages of atherosclerosis.

Thrombotic markers and subclincal atherosclerosis

Overlying thrombus constitutes one of the characteristics of vulnerable plaque and is usually associated with coronary events. In this thesis we tested the association between thrombotic markers (sCD40L, fibrinogen, P-selesctin, tissue factor, microparticles and the *PAI-1* 4G/5G polymorphism) and novel ultrasonic markers of subclinical atherosclerosis as described in chapter 12.

Plasma sCD40L and fibrinogen levels were signifficantly associated with several ultrasonic markers of subclinical atherosclerosis in our general population cohort and may contribute significantly to atherosclerosis initiation and progression. In addition, tissue factor levels were associated with TPT and BPB.

It is not yet clear whether plasma concentrations of sCD40L have diagnostic or prognostic value among apparently healthy subjects. We found sCD40L levels to be associated with IMTmax, TPT, number of bifurcations with plaque, MPT and BPB

even after adjustement for age, sex, smoking, diabetes, hypertention and hyperlipidaemia. Our findings suggest that sCD40L plays a major part in subclinical atherosclerosis formation; whether or not though it is actively mediating disease or is just a marker of inflammation and thrombogenicity is not clear yet. Of interest is the fact that lower levels of sCD40L were associated with higher IMTmax, TPT, number of bifurcations with plaque, MPT and BPB. A possible mechanism of action has been recently proposed by Sanguigni et al, who showed for the first time that CD40L promotes clotting activation by enhancing oxidative stress. They argue that, at the lesion site, CD40L-induced oxidative stress could fascilitate accumulation of Ox-LDL within macrophages and enhance thrombogenicity of the plaque (Sanguigni *et al.*, 2005). It is possible that in complicated plaques, CD40L is consumed within the thrombi, thus depleting the circulation of sCD40L.

Fibrinogen levels have been consistently associated with thrombosis and CVD and have been found to be a good predictor of future CVD, comparable to hypercholesterolaemia, hypertention and smoking (see 3.6.3 for details). Our findings are in accordance with fibrinogen being a risk factor for atherosclerosis. In our population study, fibrinogen levels were significantly associated with presence of plaques, number of bifurcations with plaque and BPB even after adjusting for age, sex, smoking, diabetes, hypertention and hypercholesterolaemia. This data support an association between fibrinogen and presence of plaques and more specifically echolucent, vulnerable plaques.

Plasma levels of tissue factor were associated with TPT and BPB even after adjustement for traditional risk factors and improved the predictive ability of the models. Thus, TF appears to be associated with plaques and plaque echolucency which would agree with previous reports that TF is most abundant in the shoulder-region and acellular, lipid-rich core of plaques (Moons *et al.*, 2002). This suggests that TF exerts its action in the formation of a plaque and may mediate thrombus formation, resulting in the development of a complex, vulnerable plaque.

The *PAI-1* (4G/5G) polymorphism was not associated with any of the ultrasonic measurements tested in our study. Data on *PAI-1* (4G/5G) have been controvercial though an association between the polymorphism and carotid IMT has been shown in the Framingham Offspring cohort. Our data do not support any association between the *PAI-1*

(4G/5G) polymorphism and ultrasonic measurements of subclinical atherosclerosis including IMTcc and IMTmax.

Microparticles have only recently been implicated in disease and are thought to contribute to various normal or pathological conditions such as antigen-transfer and intercellular cross-talk, vascular function and angiogenesis, haemostasis, thrombosis and inflammation (Hugel *et al.*, 2005). Nevertheless, little is still known about the potential participation of circulating MP to early stages of arterial disease before complication has occurred. A recent study by Mitsios et al. demonstrated that human platelets secrete the plasma type of Lp-PLA_2 (PAF-AH) that is primarily associated with MP. Platelet activation either by shear stress or by the combination of thrombin plus collagen leads to the secretion of PAF, which is mainly associated with MP (Mitsios *et al.*, 2006).

A recent study in 216 subjects without CVD, showed that leucocyte-derived MP level was higher in the presence than in the absence of moderate to high Framingham risk, metabolic syndrome, high C-reactive protein (CRP), or 2- to 3-sites disease, and correlated positively with number of metabolic syndrome components, tertiles of fibrinogen, and number of diseased sites. In multivariate analysis, 2- to 3-sites disease was independently associated only with leucocyte-derived MP level, Framingham risk and metabolic syndrome (Chironi *et al.*, 2006). In our study population, MP levels were only significantly associated with fibrinogen quartiles (lower MPs in the upper Fb quartile) and not with any of the other ultrasonic markers tested. One possible explanation is that depending on their composition (i.e. their cellular origin), MP exert different effects on the vascular wall. The method used here was specific for the thrombotic activity of MP. Different methods that cannot distinguish between different subpopulations have been used in various studies and could thus explain different findings. Nevertheless, more studies are needed in order to elucidate their role in atherogenesis.

Homocysteine metabolism markers and subclinical atherosclerosis

Homocysteine is considered an independent risk factor for CVD and insight into its metabolism and biology is likely to reveal new pathways involved in atherosclerosis. In the population study presented here, we tested the association between homocysteine metabolism markers (tHcy, folic acid, vitamin B12, ADMA and the *MTHFR* 677C>T

polymorphism) and novel ultrasonic markers of subclinical atherosclerosis as described in chapter 13.

Serum total homocysteine (tHcy) levels were significantly associated with all the ultrasonic markers tested. If the population median was taken as a cut-off point, tHcy remained predictive of all the ultrasonic markers even after adjusting for traditional risk factors (except MPT). These findings indicate that Hcy exerts its role in both atherosclerosis initiation and progression and the possible existence of a threshold value over which Hcy levels become deleterious or affect other pathways such as inflammatory ones.

In our study population, the *MTHFR* 677C>T genotype was the most significant predictor of tHcy blood levels in men under 60 years of age. Age, sex, folic acid and vitamin B12 levels significantly affected the association between *MTHFR* 677C>T genotype and tHcy levels. Serum folic acid levels were associated with IMTcc, TPT, number of bifurcations with plaque and BPB. Serum vitamin B12 levels were associated with IMTcc, IMTmax, TPT and BPB.

Several studies have suggested that elevated plasma homocysteine levels have both atherogenic and thrombogenic effects. Hyperhomocysteinemia causes endothelial dysfunction by increasing oxidant stress (Kanani *et al.*, 1999) and decreases the release of NO, impairing vasodilation (Stuhlinger *et al.*, 2001). Excess of homocysteine stimulates smooth muscle cell proliferation and collagen synthesis, promoting intima-media thickening (Majors *et al.*, 1997; Voutilainen *et al.*, 1998). It also causes increased platelet aggregation and coagulation abnormalities (Khajuria and Houston, 2000) and high homocysteine is associated with increased lipid peroxidation (Soinio *et al.*, 2004; Voutilainen *et al.*, 1999).

Most studies performed so far, involving subclinical atherosclerosis, have investigated the role of homocysteine and carotid IMT in different patient groups. Eight studies (Adachi *et al.*, 2002; Bots *et al.*, 1997b; de Bree *et al.*, 2003; Durga *et al.*, 2003; Inamoto *et al.*, 2003; Malinow *et al.*, 1993; Markus *et al.*, 2001; McQuillan *et al.*, 1999; Vermeer *et al.*, 2002) have done so in the general population and have found a weak or often absent association between homocysteine and carotid IMT (Durga *et al.*, 2004). Apart from the ARIC study (Malinow *et al.*, 1993) that used a mean of several measurements, some of which included

plaques, and the study by Vermeer et al. (Vermeer *et al.*, 2002) where the site of measurement is not specified the remaining six studies measured IMTcc.

In a meta-analysis of *MTHFR* 677C>T polymorphism and coronary heart disease covering 80 studies, a wide regional variation was observed and only studies from the Middle East and Asia (Japan) supported a positive relationship (OR 2.61) (Lewis *et al.*, 2005). Geographical differences may be related to nutritional habits and it has been shown that differences in homocysteine levels according to *MTHFR* genotype are greater at lower levels of folate intake (Ashfield-Watt *et al.*, 2002). Cyprus falls well within the region of Middle East and our data are similar with those from other regional studies (Lewis *et al.*, 2005; Papoutsakis *et al.*, 2005). We have found that homocysteine is strongly associated with subclinical atherosclerosis, as indicated by the presence and size of plaques (TPT) as well as with plaque echolucency (BPB) using B-mode ultrasound, in a general population and in a wide age range (40-90 years). Our data are also in accord with studies by Spence who showed that homocysteine levels and not the *MTHFR* 677C>T genotype are associated with subclinical atherosclerosis as measured by carotid plaque area even if *MTHFR* genotype was associated with homocysteine levels (Spence, 1999).
Most studies performed so far investigated the association between homocysteine levels and *MTHFR* 677C>T with a clinical end-point (CVD, CHD, stroke etc) often in populations such as European and American with high folate intake. We have attempted to associate homocysteine, folate levels and the *MTHFR* 677C>T polymorphism with subclinical atherosclerotic plaque formation. In the Cypriot population studied homocysteine is strongly and significantly associated with both clinical CVD and subclinical atherosclerosis. This represents a total population, a fact which eliminates CVD selection bias. In addition, our population does not take folate supplementation which could mask the effect of *MTHFR* polymorphisms on homocysteine levels. On the basis of our results, it can be concluded that homocysteine is independently associated with subclinical atherosclerosis and that *MTHFR* 677TT homozygote men, less than 60 years have a significantly higher serum homocysteine. Our results are in accord with recently published results from the Framingham Offspring study (Russo *et al.*, 2003), which also reported that the homocysteine levels were higher in 677TT homozygote compared to CT/CC but only in those with folate levels below their population median. They also, showed that the association between genotype and homocysteine was confined to men younger than 55 years and that age and sex modify the contribution of the 677C>T polymorphism to fasting homocysteine concentration. The inconsistency regarding the

influence of age and sex on the relationship between 677C>T and homocysteine concentration may be partly explained by the findings of Kauwell et al. who did not observe any cross-sectional relationship between *MTHFR* genotype and homocysteine concentration in a sample of women aged 60-85 years (Kauwell *et al.*, 2000). However, after 7 weeks of folate depletion the relationship was apparent. This suggests that the relationship is present in women and persists into older ages. Cross-sectional studies, such as the Framingham and the present one, may fail to find true relationships between genotype and homocysteine concentrations in the elderly and women, because of the increased influence in those population subgroups of age- and sex-related risk factors for hyperhomocysteinemia.

Randomized controlled trials, now in progress, are attempting to demonstrate the effects of folate supplementation on lowering the number of clinical events in patients with pre-existing cardiovascular events often at advanced stage and preliminary data appear not to be encouraging. Multivitamin treatment to lower plasma homocysteines does not seem to prevent recurrent stroke in the VISP study (Toole *et al.*, 2004). However, new data on the quantities or the combinations of vitamins used indicate that they may not have been sufficient to control for folic acid fortification practiced in N. American and European countries. In the Framingham Offspring Study the proportion with folate deficiency declined from 22% before grain fortification to 7% after (Jacques *et al.*, 1999). In an efficacy analysis performed by the authors of the VISP study excluding patients with very low and very high levels of vitamin B12 or malabsortion of B12 they reported a 21% reduction in the risk of events in the high-dose vs the low-dose group, highlighting the role of B12 in vitamin therapy for hyperhomocysteinemia (Spence *et al.*, 2005). Our results indicate that B12 plays an equally important –if not more important- part as folic acid in Hcy metabolism and that high homocysteine is associated with subclinical atherosclerosis (i.e. plaque development and progression). Thus, in addition to the studies already performed in patients with clinical CVD randomized control trials for subclinical atherosclerosis in populations with high homocysteine and low folate are needed. Because homocysteine exerts its effects mostly on the endothelium and is associated with the development of subclinical atherosclerosis, such studies will test the hypothesis that folate (and B12) supplementation (for lowering homocysteine) is effective in the prevention or in reducing progression of early atherosclerotic disease in asymptomatic individuals.

Plasma levels of ADMA, a new marker for atherosclerosis which participates in Hcy and NO metabolism, were found to be associated with presence of plaques and number of bifurcations with plaque in our population. Of interest is the fact that adding ADMA to multivariate models for prediction of IMTcc, IMTmax, TPT and BPB significantly improved their predictive ability, although p value did not reach the significance level. This could be explained by the small number of subjects included in the analysis (n=188) which influenced the p value. In a Japanese population cohort without overt CVD (n=712), plasma levels of ADMA were shown to be an independent determinant of IMT of the carotid artery (Kumiko *et al.*, 2007). ADMA levels were also independently associated with cerebral small vessel disease in a small case-control study (Khan *et al.*, 2007).

Metabolic syndrome and subclincal atherosclerosis

Individuals with the MetS are more likely to develop subclinical atherosclerosis, CVD and type 2 diabetes (DM) and have greater CVD mortality rates than individuals without MetS. Central obesity and insulin resistance are considered to be the principal underlying components in the development of the MetS (Paras *et al.*, 2007). In the present study, we tested the association between components of the MetS, diabetes, glucose levels and two polymorphisms in genes involved in energy metabolism (*UCP-2* -866G>A and *UCP-3* -55C>T) and novel ultrasonic markers of subclinical atherosclerosis as described in chapter 14.

In our study cohort, fasting serum levels of glucose were univariatly significantly associated with all the ultrasonic markers tested except TPT. After adjustement for traditional risk factors glucose levels remained associated with IMTmax. The HOMA insulin resistance index also remained associated with IMTcc and IMTmax after adjustement. When diabetes mellitus was tested, its presence was significantly associated with all the ultrasonic markers tested (except TPT) even after adjustement for traditional risk factors. Our results indicate that insulin resistance (pre-diabetes) participates in the early stages of intima-media thickening. If it progresses into clinical diabetes over time, then it influences atherosclerosis (over time) from subclinical development to clinical disease, which corroborates the “common soil” hypothesis as explained hither.

Because evidence suggests that overnutrition, insulin resistance, impaired glucose tolerance, diabetes mellitus and CVD share in common the presence of oxidative stress, oxidative stress generation has been proposed as the common, persistent pathogenic factor mediating the appearance of insulin resistance. It also mediates the passage from insulin resistance to overt diabetes via impaired glucose tolerance, while producing the increased cardiovascular risk condition typical of pre-diabetic and diabetic subjects, by favouring atherosclerotic complications. When caloric intake exceeds the energy expenditure, an increase in reactive oxygen species (ROS) follows, which eventually leads to insulin resistance. Thus, insulin resistance may be considered as a compensatory mechanism that protects the cells against further insulin-stimulated glucose and fatty acid uptake and therefore oxidative damage (Ceriello and Motz, 2004).

Even though, convincing evidence is now available supporting the hypothesis that oxidative stress plays a key part in the development of both DM and CVD, clinical trials with anti-oxidants (in particular vitamin E) have failed to demonstrate any beneficial effect . It has been suggested that antioxidant therapy with vitamin E or other antioxidants, is limited to scavenging already formed oxidants and may, therefore, be considered a more "symptomatic" rather than a causal treatment for oxidative stress (reviewed in (Ceriello and Motz, 2004)).

A study by Pollex et al. (2006), demonstrated that mean IMT was elevated in subjects with the MetS as was total plaque volume, another measurement of subclinical atherosclerosis. However, after adjustement for age and sex the difference remained significant only for IMT. A significant trend was also observed towards increased IMT with increasing numbers of MetS components. Their results suggest that standard IMT measurements show a more consistent and stronger association with the MetS than plaque volume (Pollex *et al.*, 2006). This might indicate that components of the MetS are more strongly associated with the initiation of atherosclerosis rather than with its progression or even support the hypothesis that the risk factors influencing IMT thickening are different from those influencing plaque formation and progression. Our results suggest that components of the MetS are signifiantly associated with both presence and plaque size (IMTcc, IMTmax, TPT) as well as with plaque echolucency (MPT and BPB). Adding to this evidence, a recent Japanese study trying to associate IMTcc with DM and the MetS, showed that IMTcc was higher in the groups with any metabolic abnormalities compared to the group

without abnormalities. Even after taking into account each individual component of the MetS clustering of visceral obesity with at least 2 of 3 components and diabetes was independnently associated with increased IMTcc. This suggests that the components of MetS and type 2 diabetes interact synergistically to affect vascular thickness in Japanese subjects (Kawamoto *et al.*, 2007).

As far as the two new genetic polymorphisms in the uncoupling proteins are concerned, only *UCP-2* (-866G>A) showed a borderline association (P=0.05) with IMTcc and a trend for presence of plaques (AA genotype compared to GA, GG) (p=0.084). The small number of AA homozygotes could account for the insignificant p value. However, the *UCP-3* (-55C>T) polymorphism was significantly associated with BMI in diabetic subjects with the -55CC genotype being associated with lower BMI.

The exact physiological role of uncoupling proteins remains to be established. However, they are considered to be good candidates for energy metabolism and obesity. Our results are in accord with a role of the UCP-3 protein in body mass regulation and this is the first time that an association between the *UCP-3* (-55C>T) polymorphism and BMI is demonstrated in diabetics and not in non-diabetic subjects of a general population cohort.

Chapter 17:

Conclusions and Suggestions for Future Work

Conclusions

The 2007 AHA scientific statement acknowledged the importance of identifying specific intermediate phenotypes (i.e. phenotypes that mediate disease as opposed to phenotypes that represent the ultimate manifestation of a disease) that may prove more amenable to genetic analysis. With respect to phenotype measurements, many authors have stated that the quality of linkage and association studies is only as good as our ability to measure phenotypes (Arnett *et al.*, 2007). Ultrasonic measurements of the arterial wall, like the ones described in this thesis, provide a novel and presice phenotype of subclinical atherosclerosis which is strongly associated with clinical CVD, as shown in chapter 8.

Although all commonly used risk assessement models can identify a high risk group, if followed up only 40-50% of the cardiovascular events will occur in the high risk group. The remaining events will occur in the low and intermediate risk groups which are very large. This emphasizes the need for additional risk factor evaluation in subclinical atherosclerosis and a more specific classification of risk, especially for the intermediate-risk group. Those who do not have clinical indication of coronary disease and who may even lack traditional risk factors may benefit from the techniques now under development that evaluate plaque biology and inflammation (Naghavi *et al.*, 2003b) as well as non-invasive imaging methods such as ultrasound.

When assessing individual atherosclerotic burden, it is essential to take into account total vulnerability burden and not just search for a single, unstable coronary plaque. A composite risk score (e.g. vulnerability index), that comprises the total burden of atherosclerosis and vulnerable plaque in the coronaries (and aorta and carotid, femoral, etc arteries) and that includes blood and myocardial vulnerability factors, should be a more accurate method of risk stratification (Naghavi *et al.*, 2003b).

On the other side of the argument, there is evidence supporting the fact that the 4 major "conventional" risk factors (i.e. cigarette smoking, diabetes, hyperlipidaemia and hypertension) actually account for 80-90% of the presence of CHD in men and women. This data came from a meta-analysis of 14 international randomized clinical trials of CHD including 122 458 patients. They showed that among patients with CHD, at least 1 of the 4 conventional risk factors was present in 84.6% of women and 80.6% in men. In younger patients (men ≤ 55 years and women ≤65 years) and most patients

presenting with either unstable angina or for percutaneous coronary intervention, only 10-15% of patients lacked any of the 4 conventional risk factor and this pattern was largely independent of sex, geographic region, trial entry criteria or prior CHD (Khot *et al.*, 2003). However, hyperlipidaemia, diabetes and hypertension are themselves multifactorial and smoking affects a variety of markers (both environmental and genetic).

Genetic polymorphisms, despite 20 years of excessive research, are not currently being used in risk assessment for CHD. This could be attributed to the fact that it is unlikely for any one polymorphism to significantly add to CHD risk. However, combinations of various polymorphisms coupled with environmental risk factors (such as smoking) may change risk assessement models especially in younger ages where hyperlipidaemia, hypertension and diabetes have not yet developed.

It has been argued that an individual's personal characteristics and plasma risk-trait levels (which reflect both genotype and exposure) at present are the best predictors of clinical outcome (Humphries *et al.*, 2004). On the other hand, genetic markers with even modest effects may provide us with important clues to disease pathophysiology or suggest new ways of therapeutic intervention (Gibbons *et al.*, 2004). As age of risk stratification decreases, genetic risk factors that are unchangeable by age might gain further importance and add predictive value to risk scores applied to younger people (i.e. before blood levels of risk factors have risen or before the age that ultrasound can be used to visualise changes in the arterial wall).

In the work presented here, we have combined intermediate phenotypes for subclinical atherosclerosis (shown to be associated with prevalence of clinical disease) with blood levels of various markers as well as with common genetic markers for atherosclerosis. Frequent polymorphisms may not confer an important risk of disease in mutation carriers but exactly because they are frequent they may have a population impact that is far from negligible, despite a weak effect at the individual level. The strength of the work lies mainly in the specific phenotypes used. This is the first time that ultrasonic measurements in four arteries (both common carotids and both common femorals) have been tested in a single population study and a variety of biochemical and genetic markers tested for association with early, subclinical atherosclerosis. Some weaknesses of the study must be acknoweledged; mainly that the results presented here come from

a cross-sectional study with no follow-up data yet and should therefore be interpreted with caution. The predictive ability of the ultrasonic measures will be tested prospectively in the long-term follow-up of the study. Also, many of the volunteers as indicated in table A1 (Annexe 1) are on antihypertensive, cholesterol lowering therapy and antidiabetic drugs that have a potent influence on both carotid atherosclerosis and the occurrence of cardiovascular events. Thus, the effect of the classical risk factors on the development of clinical cardiovascular disease may be underestimated as compared to the ultrasonic measurements. However, when we adjusted for diabetes, antihypertensive and cholesterol lowering therapy we found that IMTmax, presence of common carotid and femoral plaques, total number of bifurcations with plaque present, total plaque thickness, mean plaque type and black plaque burden, were still strongly associated with the presence of clinical cardiovascular disease. The large number of people with hypertention, diabetes and/or hyperlipidaemia in our population cohort is worth noting and should be further validated with the addition of more participants from other regions of Cyprus before we can say with certainty that the prevalence of these risk factors is as high as shown here in the entire Cypriot population over 40 (Table A1).

A number of biomarkers, both biochemical and genetic, have been shown to be associated with the ultrasonic measurements studied over and above traditional risk factors (age, sex, smoking, diabetes, hypertension and hyperlipidaemia accordingly) and to add on the predictive ability of these factors. These were the apoB/apoA1 ratio, Lp-PLA_2 activity, the *apoE* (E2/E3/E4), *CETP* (TaqIB1B2 and I405V), *MGP* (-138C>T) and *MMP-9* (R279Q) genetic polymorphisms, sCD40L, fibrinogen, tissue factor, tHcy, vitamin B12, MetS components and the HOMA index of insulin sensitivity.

It has been argued that p values should not be dichotomised as “significant” and “non-significant”, but should be interpreted in terms of strength of the evidence against the null hypothesis (Sterne *et al.*, 2001). In light of that, other markers with a p value little over 0.05 should not be dismissed without further analyses and thinking and markers with a p value little less 0.05 should be interpreted with care. However, most of the biomarkers tested here that were assigned no association with the ultrasonic biomarkers according to p values, had a p value of over 0.1. For the purposes of this thesis the cut-off point of 0.05 for significance was used. P values > 0.05 and < 0.06 were assigned a

borderline significance, whereas p values > 0.06 and <0.01 were described as a trend.

Based on the biomarkers studied in this thesis and traditionally used risk factors, multiple predictive models for all the ultrasonic measurements were devised as described in chapter 15. From these models it is apparent that new biochemical and genetic markers tested here, can improve the percentage of variability on the individual level explained by the model. More specifically, adding apoB/apoA1 ratio to the models improved its predictive ability for IMTcc, IMTmax and TPT. Adding Lp-PLA_2 improved the predictive ability of the models for IMTmax, TPT and BPB. Creatinine improved the models for IMTcc, IMTmax and MPT and sCD40L for IMTmax, TPT, MPT and BPB. The *CETP* TaqIB1B2 polymorphism improved the models for IMTmax, MPT and BPB; whereas the *CETP* I405V polymorphism improved the model for TPT. The *MMP-9* (R279Q) polymorphism improved the models for TPT, more than 2 bifurcations with plaque and BPB. Tissue factor, tHcy and the *apoE* (E2/E3/E4) polymorphism improved the models for TPT, IMTcc and presence of plaque respectively. (For detailed results see chapter 15).

Despite certain scepticism on wheather individual genetic polymorphisms would add to risk prediction for CHD, our results indicate that if more precise phenotypes for subclinical atherosclerosis are used (i.e. IMTmax, TPT, MPT, BPB), a combination of genetic, biochemical markers (tested here) and traditional risk factors can explain up to 37% of the variability in ultrasonic measurements of the arterial wall, previously shown to be associated with prevalence of clinical disease. The variability attributed to the biomarkers is of the order of about 7%.

Ultrasound is a non-invasive, relatively inexpensive technique that allows for the visualisation of arterial wall thickening and plaque, being also able to distinguish between types of plaques. Association between ultrasonic measurements of plaque and more importantly vulnerable plaques which account for the majority of clinical events, with easily measured biomarkers (biochemical and genetic) may allow identification of at risk individuals before the age of 40-45 when ultrasound can be used. Thus, a more precise and early model for prediction of risk, including a combination of markers, might aid in a better and more individualised prevention.

Genome-wide association studies, that are now appearing, have greater power in revealing novel genetic factors that contribute to disease risk. Wheather these variants will be clinically usefull remains to be seen. However, as time passes, the interest for genetic research on common CVD moves progressively from the direct expectation of risk stratification to the more fundamental understanding of disease origin and pathophysiology and their indirect diagnostic and therapeutic implications (Cambien and Tiret, 2007). Thus, confirmatory data from our study for recent hypotheses that different markers play a part in different stages of atherosclerosis development and progression is another important finding. It has been proposed that IMT may reflect a hypertrophic response of arterial intimal and medial cells to lipid infiltration or hypertension; whereas formed arterial plaques probably represent a later stage of atherosclerosis related to oxidation, inflammation, endothelial dysfunction and/or SMC proliferation. Thrombosis and thrombolysis probably act at even later stages. We have shown that some biomarkers are associated with thickening of the arterial wall (i.e. IMTcc), some with plaque presence (IMTmax, TPT), and others with plaque echolucency (MPT, BPB).

Association studies, such as the present one, though downplayed at a certain degree by some academicians and medical research funding institutions as compared to clinical trials, are nonetheless a fundamental part of the advancement of medical knowledge. A number of practical and theoretical issues in medicine and biology can only be answered by nonexperimental epidemiological studies. Results from our population study will help advance knowledge in the field of early, subclinical atherosclerosis atherosclerosis and merit further investigation in prospective studies. Association between ultrasonic measurements of plaque and more importantly vulnerable plaques which account for the majority of clinical events, with easily measured biomarkers (biochemical and genetic) in such prosepective studies may allow identification of at risk individuals before the age of 40 when, because of absence of plaques, ultrasound can not be used. Thus, a more precise and early model for prediction of risk leading to individualised and targeted prevention may be developed.

Future Work

As described in section 5.1, the Cyprus Study is a prospective follow-up study aiming to include 2 000 subjects aged 40 and over from several regions in Cyprus. In addition, a minimum 5-year follow-up of the population is another of its basic aims. From the present study, a number of markers –both novel and established ones- have emerged to be significantly associated with subclinical atherosclerosis and most of them can be measured relatively easily. Follow-up of the study population for at least 5 years and addition of more participants from different areas in Cyprus will provide important prospective data on atherosclerosis progression and may validate the use of the ultrasonic markers in assessing subclinical atherosclerosis long before clinical symptoms occur. Inclusion of other areas, such as villages from the Limassol and Paphos districts will help generalise our findings to the whole of Cyprus and exctract important information on the prevalence of cardiovascular risk factors and their significance in our population.

Furthermore, the inclusion of family members of the participants, such as parents, siblings and children, will lead to the creation of another complementary family cohort, giving us the possibility of studying the heritability of all the ultrasonic measurements studied here, a project now under way. As genome-wide microarrays are now becoming more accessible in matters of reproducibility and cost, having a complete population and offspring cohort with available DNA and ultrasonic measurements can lead to identification of causal genome loci with the use of linkage analysis. We are already in the middle of a project aiming at identifying genetic loci associated with the QT interval of the ECG in the Cyrpus population. In addition, mRNA expression analysis from different types of plaques (from endarterectomy samples from surgery patients) is near completion, in an attempt to study possible diferential gene expression in various plaque types.

It is now obvious that clinical end-points can be the result of different pathways so use of more specific and early phenotypes in combination with several biochemical and genetic analyses can help decipher even further the mechanisms that govern atherosclerosis initiation and progression.

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Annexe

Table A1: Overall baseline characteristics of the population (n = 767)

Age, y, mean ± sd	60.5 ± 10.2
Male sex	46.1 %
Body mass index, kg/m^2, mean ± sd	28 ± 4.5
Ever Smokers	39%
Current Smokers	19%
Pack-years (iqr)	0 (0, 15.6)
Systolic BP, mm Hg	140.8 ± 16.9
Diastolic BP, mm Hg	84.0 ± 9.6
Antihypertensive therapy	37.5%
Total Cholesterol, mmol/L, mean ± sd	5.75 ± 1.06
HDL Cholesterol ± sd	1.30 ± 0.32
Cholesterol lowering	
Therapy	18.9%
Triglycerides mmol/L, mean ± sd	1.70 ± 0.89
Diabetes mellitus present	13%
IMTcc, mm mean ± sd	0.074 ± 0.017
IMTmax mm median (iqr)	0.120 (0.07, 0.20)
Vessel Bifurcations with plaques	
None	214 (27.9%)
One	145 (18.9%)
Two	174 (22.7%)
Three	96 (12.5%)
All four	138 (18.0%)
TPT, cm, median (iqr)	0.330 (0, 0.64)

Table A2: Means, SD and SE for normally distributed biochemical measurements in the entire population

Biochemical Measurement	**Mean**	**Standard Deviation (SD)**	**Standard Error of Mean (SE)**	**N**
ApoA1	1.44	0.24	0.008	744
ApoB	1.20	0.24	0.008	743
Creatinine	0.93	0.24	0.008	744
Fibrinogen (Fb)	270.21	51.11	1.88	738
Glucose	103.89	28.57	1.05	745
Homocysteine (Hcy)	12.91	5.96	0.22	741
HDL chol	50.18	12.40	0.46	744
LDL chol	135.95	30.19	1.10	744
Lp-PLA_2 activity	58.70	18.04	0.66	755
P-selectin	60.94	33.83	1.47	532
Total Cholesterol	226.19	41.94	1.54	744
Triglycerides (TG)	150.97	89.45	3.28	744

Table A3: Median, iqr for skewed biological measurements for the entire population

Biological Measurement	**Median**	**Inter-Quartile Range (25^{th}-75^{th} percentile)**	**N**
ADMA	0.12	0.0608 – 0.198	190
B12	358.50	276.75 – 502.25	646
C-Reactive Protein (CRP)	2.95	1.13 – 5.66	734
Folic Acid (FA)	8.39	6.19 – 11.30	674
Interleukin-6 (IL-6)	1.99	0.68 – 3.91	254
Insulin	5.65	3.15 – 9.32	734
Lp(a)	192.00	112.00 – 383.00	745
Microparticles (MP)	6.19	3.16 – 12.38	417
Myeloperoxidase (MPO)	4.26	2.10 – 7.66	206
Nitric Oxide (NO)	8.41	4.92 – 17.04	270
sCD40L	292.33	105.00 – 637.71	761
Tissue-Factor (TF)	93.63	59.34 – 144.64	761

Table A4: Relative frequencies (percentage) of studied polymorphisms in Cyprus. Polymorphisms are shown alphabetically

Polymorphism Name	**Frequent allele**	**Rare allele**	**Hardy-Weinberg**
***ACE* (I/D)**	D (0.686)	I (0.314)	In equilibrium
***Angiotensinogen* (ang -6G>A)**	A (0.507)	G (0.493)	In equilibrium
***ApoB* (-516 T >C)**	C (0.769)	T (0.231)	In equilibrium
***ApoE* (E2/E3/E4)**	E3 (0.893)	E2/E4 (0.062 /0.045)	In equilibrium
***CETP* (int1A>G ή TaqIB1B2/B2)**	G (0.526)	A (0.474)	In equilibrium
***CETP* (I405V)**	V (0.564)	I (0.436)	In equilibrium
***eNOS* (894A>G)**	G (0.634)	T (0.366)	In equilibrium
***IL-6* (-174 G >C)**	G (0.775)	C (0.225)	In equilibrium
***MMP-1* (1G/2G)**	2G (0.54)	1G (0.46)	In equilibrium
***MMP--3* (5A/6A)**	6A(0.615)	5A(0.385)	In equilibrium
***MMP-7* (-181A>G)**	A (0.545)	G (0.455)	In equilibrium
***MMP-9* (R279Q)**	A (0.729)	G (0.271)	In equilibrium
***MMP-12* (-82A>G)**	A (0.811)	G (0.189)	In equilibrium
***MPO* (-638C>A)**	C (0.863)	A (0.137)	In equilibrium
***MGP* (-138 T >C)**	T (0.740)	C (0.260)	In equilibrium
***MTHFR* (677C>T)**	C (0.556)	T (0.444)	In equilibrium
***Lp-PLA$_2$* (A379V)**	G (0.755)	A (0.245)	In equilibrium
***PAI-1* (4G/5G)**	5G (0.611)	4G (0.389)	In equilibrium
***PON1* (L55M)**	T (0.575)	A (0.425)	In equilibrium
***PON2* (S311C)**	C (0.792)	G (0.208)	In equilibrium
***UCP-2* (-866A>G)**	G (0.76)	A (0.33)	In equilibrium
***UCP-3* (-55T>C)**	C (0.877)	T (0.123)	In equilibrium

Table A5: Compiled results of positive associations between ultrasonic measurements and all the biomarkers tested in univariate analyses

Ultrasonic Measurement	**Biomarkers associated in univariate analyses**
IMTcc	HDL, TChol/HDL ratio, apoA1, apoB/apoA1 ratio, Lp-PLA$_2$ activity, creatinine, MCP-1, *TNF-α* (-308 A>G), Fb, tHcy, FA, vit B12, MetS, MetS components, DM, HOMA
IMTmax	HDL, TChol/HDL ratio, apoA1, apoB/apoA1 ratio, Lp-PLA$_2$ activity, creatinine, IL-6, MCP-1, *MGP* (-138C>T), *MMP-9* (R279Q), sCD40L, Fb, tHcy, vit B12, MetS, MetS components, DM, HOMA
TPT	HDL, TChol/HDL ratio, TG, apoA1, apoB/A1 ratio, Lp-PLA$_2$ activity, creatinine, MCP-1, *TNF-α* (-308A>G), *MMP-7* (-181A>G), *MMP-9* (R279Q), sCD40L, TF, tHcy, FA, vit B12, MetS, MetS components, DM
Presence of plaques	HDL, TChol/HDL ratio, TG, apoA1, apoB, apoB/apoA1 ratio, Lp-PLA$_2$ activity, *apoE* (E2/E3/E4), creatinine, IL-6, MCP-1, *MMP-9* (R279Q), Fb, tHcy, ADMA, MetS, MetS components, DM
MPT	HDL, TChol/HDL ratio, TG, apoA1, apoB, apoB/apoA1 ratio, Lp-PLA$_2$ activity, *apoE* (E2/E3/E4), *CETP* (TaqIB1B2), creatinine, IL-6, MCP-1, *MMP-9* (R279Q), sCD40L, Fb, tHcy, MetS, MetS components, DM
BPB	HDL, TChol/HDL ratio, TG, apoA1, apoB, apoB/apoA1 ratio, Lp(a), Lp-PLA$_2$ activity, creatinine, IL-6, MCP-1, *TNF-α* (-308A>G), *MMP-9* (R279Q), sCD40L, Fb, tHcy, FA, vit B12, MetS, MetS components, DM, HOMA

Table A6: Compiled results of positive associations between ultrasonic measurements and all the biomarkers tested in multivariate analyses (adjusting for age, sex, smoking in packyears and DM, hypertension and hyperlipidaemia accordingly)

Ultrasonic Measurement	**Biomarkers associated in multivariate analyses**
IMTcc	TChol/HDL ratio, apoA1, apoB/apoA1 ratio, tHcy, FA, vit B12, MetS, MetS components, DM, HOMA
IMTmax	HDL, TChol/HDL ratio, LDL, apoA1, apoB, apoB/apoA1 ratio, Lp-PLA_2 activity, *CETP* (TaqIB1B2), *MGP* (-138C>T), sCD40L, MetS, MetS components, DM, HOMA
TPT	HDL, TChol/HDL ratio, LDL, TG, apoA1, apoB/A1 ratio, Lp-PLA_2 activity, *CETP* (I405V), *MMP-9* (R279Q), sCD40L, TF, tHcy, MetS components
Presence of plaques	TChol/HDL ratio, LDL, apoB, *apoE* (E2/E3/E4), *MMP-9* (R279Q), Fb, tHcy, MetS components, DM
MPT	*CETP* (TaqIB1B2), sCD40L
BPB	HDL, TChol/HDL ratio, LDL, TG, apoA1, apoB, apoB/apoA1 ratio, Lp(a), Lp-PLA_2 activity, *CETP* (TaqIB1B2), *MMP-9* (R279Q), sCD40L, Fb, TF, tHcy, MetS, MetS components, DM

PAPERS and ABSTRACTS

Panayiotou A, Griffin M, Georgiou N, Bond D, Tyllis T, Tziakouri Ch, Fessas Ch, Nicolaides A. ApoB/ApoA1 ratio and Subclinical Atherosclerosis. *Int Angiol.* 2008; 27(1):74-80.

Panayiotou A, Nicolaides A,MS, Georgiou N, Tyllis T, Griffin M, Martin RM, Bond D, Tziakouri Ch, Fessas Ch, Deltas C. Serum Total Homocysteine, Serum Folate, MTHFR 677C>T Genotype and Subclinical Atherosclerosis. Submited.

Panayiotou A, Georgiou N, Griffin M, Bond D, Tyllis T, Fessas C, Panayiotou C, Ilia A, Chakouri C, Nicolaides AN. ApoB/ApoA1 ratio, atherosclerotic plaque burden and plaque stability. Atherosclerosis Supplements 2005; Vol 6(1):125. Oral presentation at the International Congress of the European Atherosclerotic society, Praque, 23-26 April 2005.

Panayiotou A, Georgiou N, Griffin M, Bond D, Tyllis T , Humphries S.E, Nicolaides AN. Genotypes at the matrix metalloproteinase (MMP) loci for *MMP9* and *MMP12* are associated with carotid IMT measures of plaque stability. Atherosclerosis Supplements 2006; Vol 7(3):145. Poster at the International Congress of the European Atherosclerotic society. Rome, June 19-22, 2006.

Panayiotou A, Tyllis T, Georgiou N, Griffin M, Bond B, Tziakouri CH, Fessas CH, Ilia A, Panayiotou C, Nicolaides A. Genotypes at the Matrix Metalloproteinase (MMP) loci for MMP9 and MMP12 are associated with carotid IMT measures of plaque stability. Oral presentation at the World Congress of the International Union of Angiology. Lisboa, June 24-28, 2006.

Panayiotou A, Georgiou N, Nicolaides A, Tyllis T, Griffin M, Martin R, Bond D, Tziakouri Ch, Fessas Ch, Panayiotou C, Doré CJ**.** Association of serum homocysteine, folate and MTHFR 677C>T genotypes with early atheroslerosis. Results from the Cyprus Study. Atherosclerosis Supplements 2007; Vol 8(1):16-17. Oral presentation at the International Congress of the European Atherosclerotic society, Helsinki, 10-13 June, 2007.

Award-Winning Presentations

1st IUA prize from the International Union of Angiology for best original presentation. World Congress of the International Union of Angiology. Lisboa, June 24-28, 2006. **Panayiotou A**, Tyllis T, Georgiou N, Griffin M, Bond B, Tziakouri CH, Fessas CH, Ilia A, Panayiotou C,Nicolaides A. ApoB/ApoA1 Ratio Atherosclerotic Plaque Burden and Plaque Stability

1st prize from the Nicosia-Keryneia Medical Association for best original presentation. 19th annual congress, Nicosia, November 4-5, 2006. **Panayiotou A**, Tyllis T, Georgiou N, Griffin M, Bond B, Tziakouri CH, Fessas CH, Ilia A, Panayiotou C, Nicolaides A. Association of serum Homocysteine, Folate and MTHFR C677T genotype with early atherosclerosis. Results from the Cyprus Study.

1st prize from the Limassol Medical Association for best original paper. 17th annual contest for research papers during the 27th annual congress, Limassol, June 17-18, 2006. Nicolaides A, tyllis T, Griffin M, Martin R, Bond D, Chakouri Ch, Fessas Ch, **Panayiotou A**, Ilia A, Panayiotou C, Ioannidou E, Dore CJ. Ultrasonic arterial wall measurements and prevalence of cardiovascular disease: The Cyprus Study.