Vascular characterisation using multimodal imaging and relationship to functional lipid indices in blood

Mohammad Alkhalil

BSc M.D MSc MRCP





Linacre College

Division of Cardiovascular Medicine

Radcliffe Department of Medicine

University of Oxford

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Doctoral Supervisor: Professor Robin P. Choudhury

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Abstract

Reduction in LDL cholesterol (LDL-c) has been shown to be effective in decreasing cardiovascular events. However, 1 in 5 patients return with a second event within 5 years despite achieving very low LDL-c. New lipid-lowering drugs have been shown to be efficacious in reducing LDL-c, however, in unselected populations the numbers needed to treat with these agents were unmanageably high. Therefore, attention should be sharply focused on developing stratification tools that not only identify high risk patients but also incorporate a mechanistic understanding when applying targeted therapies. Matching a prominent disease feature (inflammation, lipid accumulation etc.) with a drug mechanism of action may offer an opportunity to achieve more precise intervention. Remarkably, however, it is still unclear *how* reducing LDL-c translates into improved cardiovascular outcomes. One prevalent theory is that LDL-c reduction leads to evacuation of lipid from atherosclerotic plaque, rendering plaque less prone to rupture. Tools to measure plaque lipid content in humans *in vivo* have been fundamentally insensitive and incapable of systematically quantify lipid in every individual. Thus limiting the applicability of using plaque lipid quantification as an approach to select individuals for new lipid-lowering drugs.

Recently, a new quantitative magnetic resonance imaging (MRI) T2 mapping technique has been shown to be capable of accurate, reproducible quantification of carotid plaque lipid on a voxel-by-voxel basis. Accordingly, this thesis hypothesized that using this newly validated technique, plaque lipid content and distribution can be systematically evaluated in all patients with clinical evidence of atherosclerosis. In addition, lipid content quantified on T2 mapping is reduced following intensive LDL-c reduction which can be assessed on subjects with no pre-defined carotid disease. Furthermore, blood lipid biomarkers are dissociated from plaque lipid content and its change on therapy. Finally, plaque lipid content is not influenced by perivascular adipose tissue, marker of inflammation, via *paracrine local* effect.

T2 mapping MRI technique was able to identify patients with propensity to develop lipid-rich plaques at multi-sites vascular territories in patients scheduled for carotid endarterectomy (CEA) or presenting with acute coronary syndrome (ACS). Plaque lipid content and distribution quantified on T2 mapping MRI technique were related to the symptomatic status of plaques in patients scheduled for CEA. Intensive LDL-c reduction using high intensity statin treatment was associated with significant changes in plaque composition which was detected on T2 mapping as early as three months. There was no significant predictor of changes in carotid plaque lipid content using *baseline*, *attained* or *change* in blood lipid biomarkers following statin treatment. There was no spatial relationship between carotid plaque lipid content and adjacent perivascular adipose tissue.

These findings support the use of plaque imaging as a potential tool to identify suitable patients for intensive lipid-lowering treatments and to track patients' response to these therapies.

Table of Contents

List of Figures 9

List of abbreviations 13

1 Chapter 1: General Introduction 17

1.1 The burden of cardiovascular disease in the post-statin era 18

1.1.1 Epidemiology of cardiovascular disease and recent changes 18

1.1.2 Statin and reduction in cardiovascular events 19

1.1.3 LDL hypothesis: “lower is better” 20

1.2 Residual cardiovascular risk despite optimal therapies 26

1.2.1 Residual risk with low LDL-c 26

1.2.2 Targeting HDL-c to reduce residual risk 28

1.2.3 Failure of targeting non-LDL-c, rationale and the need for stratification tools. 33

1.3 Mechanistic staging and the role of imaging in risk stratification 36

1.3.1 Non-imaging biomarkers to identify high risk group 36

1.3.2 Role of imaging in the management of the residual risk 37

1.3.3 Impact of lipid-lowering therapies at the level of atherosclerotic plaque 40

1.3.4 Changes in plaque composition in response to LDL-c reduction 43

1.3.5 Plaque lipid content and future cardiovascular risk, the role of mechanistic staging 46

1.3.6 Plaque lipid quantification and quantitative T2 map 48

1.4 Summary 51

1.5 Hypotheses 51

1.6 Experimental aims 52

2 Chapter 2: General Methodology 53

2.1 Ethics 54

2.2 Eligibility Criteria 54

2.3 Study Population 55

2.4 MRI protocol 58

2.4.1 Carotid atherosclerosis 58

2.4.2 Plaque characterisation on T2 mapping 60

2.4.3 Perivascular adipose tissue 62

2.4.4 Follow up MRI 62

2.5 Image analysis 65

2.5.1 Carotid atherosclerosis 65

2.5.2 T2 mapping image analysis 68

2.5.3 PVAT image analysis 70

2.6 Blood analyses 70

2.6.1 Standard lipid and lipoprotein pleotropic function analyses 74

2.6.2 Lipoprotein (a) analysis 74

2.6.3 Apolipoprotein A1 analysis 77

2.6.4 High sensitivity CRP (hsCRP) analysis 79

2.6.5 PCSK9 analysis 81

2.6.6 Cholesterol efflux capacity analysis 83

2.6.7 Cholesteryl ester transfer protein activity analysis 86

3 Chapter 3: Quantification of carotid plaque lipid content with magnetic resonance T2 mapping in patients undergoing carotid endarterectomy 88

3.1 Abstract 89

3.2 Introduction 91

3.3 Hypotheses and aims 93

3.4 Methods 93

3.4.1 Study population 93

3.4.2 MRI protocol 94

3.4.3 Data analysis 95

3.4.4 Statistical analysis 98

3.5 Results 98

3.5.1 Difference in plaque burden and lipid content between symptomatic and asymptomatic patients 100

3.5.2 Difference in lipid distribution between symptomatic and asymptomatic patients 104

3.5.3 Relationship between T2 mapping MRI outputs and luminal degree of stenosis on ultrasound duplex 107

3.5.4 Relationship between left and right carotid arteries 109

3.6 Discussion 112

3.7 Conclusion 116

4 Chapter 4: The extent of carotid plaque lipid in patients with acute coronary syndrome and the impact of high intensity statin initiation as determined by T2 mapping 117

4.1 Abstract 118

4.2 Introduction 120

4.3 Hypotheses and aims 121

4.4 Methods 122

4.4.1 Study population 122

4.4.2 MRI protocol 123

4.4.3 MRI data analysis 124

4.4.4 Coronary angiogram analysis 124

4.4.5 Statistical analysis 126

4.5 Results 126

4.5.1 Extent of carotid plaque burden and lipid volumes in ACS patients 129

4.5.2 Change in plaque composition with high intensity statin 132

4.5.3 Changes in carotid atherosclerosis burden following three months high intensity statin 136

4.5.4 Relationship between changes in carotid plaque burden and lipid volume 138

4.5.5 Changes in lipid distribution with statin treatment 140

4.6 Discussion 143

4.7 Conclusion 148

5 Chapter 5: Relationship of plaque lipid content quantified using T2 mapping MRI technique and functional lipid indices in blood 150

5.1 Abstract 151
5.2 Introduction 153
5.3 Hypotheses and aims 155
5.4 Methods 156
5.4.1 Study population 156
5.4.2 MRI protocol and data analysis 156
5.4.3 Blood lipid biomarkers assays analyses 157
5.4.4 Statistical analysis 157
5.5 Results 158
5.5.1 Changes in blood lipid indices with statin 158
5.5.2 Relationship between carotid plaque lipid content and blood lipid indices 161
5.5.3 Predictors of lipid percentage reduction 166
5.5.4 Blood lipid biomarkers between patient groups stratified according to the magnitude of lipid percentage reduction 166
5.5.5 Change in plaque lipid content according to the attained plasma LDL-c 173
5.6 Discussion 174
5.7 Conclusion 177
6 Chapter 6: The relationship between perivascular adipose tissue and plaque lipid content in the carotid arteries, as determined by magnetic resonance imaging 178
6.1 Abstract 179
6.2 Introduction 181
6.3 Hypotheses and aims 183

6.4 Methods 184
6.4.1 Study population 184
6.4.2 MRI protocol 184
6.4.3 MRI data analysis 185
6.5 Results 189
6.5.1 Spatial distribution of PVAT and its relationship with the symptomatic status of the carotid artery 191
6.5.2 Spatial relationship between PVAT and vessel wall lipid content 196
6.6 Discussion 197
6.7 Conclusion 200
7 Chapter 7: General discussion 201
7.1 Original aims and hypotheses 202
7.2 Summary of key findings 203
7.3 Future directions 205
8 Chapter 8: Appendices 208
8.1 Attributions 209
8.2 Published work from this period of research 209
8.2.1 Review article 209
8.2.2 Original Research 209
8.2.3 Abstracts 211
8.3 Acknowledgments 211
9 Chapter 8: References 213

List of Figures

Figure 1.1 Scatter-plot of reduction in LDL-c and major vascular events.....24

Figure 2.1 Schematic flow chart of study population.....57

Figure 2.2 Assessment of atherosclerosis burden.....59

Figure 2.3 T2 mapping sequence acquisition using different echo times.....61

Figure 2.4 Raw image of carotid perivascular adipose tissue.....64

Figure 2.5 Carotid atherosclerosis assessment.....67

Figure 2.6 Inner and outer boundaries segmentation of carotid vessel.....69

Figure 2.7 Lp (a) measurements from serum human.....76

Figure 2.8 ApoA1 measurements from human plasma.....78

Figure 2.9 hsCRP measurements from human plasma.....80

Figure 2.10 PCSK9 measurements from human plasma.....82

Figure 2.11 Cholesterol efflux capacity schematic diagram.....85

Figure 2.12 CETP activity measurement in human plasma.....87

Figure 3.1 Calculation of lipid aggregation index.....97

Figure 3.2 Difference in plaque burden between symptomatic and asymptomatic patients.....101

Figure 3.3 Difference in lipid percentage between symptomatic and asymptomatic patients.....102

Figure 3.4 Difference in lipid content between symptomatic and asymptomatic patients.....103

Figure 3.5 Difference in lipid distribution between symptomatic and asymptomatic patients.....105

Figure 3.6 Assessment of lipid distribution using two imaging indices.....106

Figure 3.7 Luminal degree of stenosis and its relationship with both plaque burden and lipid volume 108

Figure 3.8 Relationship between left and right carotid arteries in patients scheduled for carotid endarterectomy 110

Figure 3.9 Relationship between carotid plaque volumes and their corresponding lipid volumes in patients scheduled for carotid endarterectomy 111

Figure 4.1 Study flow chart 127

Figure 4.2 Relationship between left and right carotid arteries in patients admitted with ACS 130

Figure 4.3 Relationship between carotid vessel wall volumes and their correspondent lipid volumes in patients admitted with ACS 131

Figure 4.4 Reduction in lipid percentage as quantified on T2 mapping in two different patients 133

Figure 4.5 Carotid lipid percentage change over study period 134

Figure 4.6 Carotid fibrous percentage change over study period 135

Figure 4.7 Changes in carotid atherosclerosis burden following three months of intensive statin treatment 137

Figure 4.8 Changes in carotid lipid content and vessel wall volume stratified according to AHA classification 139

Figure 4.9 Changes in lipid distribution as assessed by LLD % and LAI 141

Figure 4.10 Changes of lipid distribution following statin treatment 142

Figure 4.11 Relationship between carotid vessel wall and lipid volumes 145

Figure 6.1 Carotid PVAT segmentation and circumferential measurement 188

Figure 6.2 Circumferential distribution of PVAT and vessel wall lipid thickness of the carotid arteries 192

Figure 6.3 Individual distribution of PVAT thickness of the carotid arteries 193

Figure 6.4 Difference in PVAT volume between symptomatic and asymptomatic patients 194

Figure 6.5 Difference in PVAT volume between symptomatic and asymptomatic carotid vessels 195

List of Tables

Table 2.1 Summary of acquired magnetic resonance sequences 63

Table 2.2 Carotid vessel wall Classification using conventional and modified approach 66

Table 2.3 Summary of analysed blood lipid indices with their corresponding technical methodology 71

Table 2.4 Role of functional blood lipid biomarkers in lipid metabolism and future cardiovascular events 72

Table 3.1 Clinical characteristics of recruited patients 99

Table 4.1 Clinical characteristics of recruited patients 128

Table 5.1 Baseline blood lipid characteristics and their changes over time 160

Table 5.2 Relationship between baseline carotid plaque lipid content and baseline blood lipid indices 162

Table 5.3 Relationship between baseline blood lipid indices and change in carotid lipid content with statin treatment 163

Table 5.4 Relationship between follow up blood lipid indices and change in carotid plaque lipid content with statin treatment 164

Table 5.5 Relationship between change in both blood lipid indices and carotid plaque lipid content with statin treatment 165

Table 5.6 Predictors of change in lipid percentage using baseline blood lipid biomarkers 167

Table 5.7 Predictors of change in lipid percentage using follow up blood lipid biomarkers 168

Table 5.8 Predictors of change in lipid percentage using changes in blood lipid biomarkers with statin 169

Table 5.9 Baseline and changes in standard and functional blood lipid biomarkers between responders and no-responders groups 170

Table 5.10 Baseline and changes in carotid vessel wall volume and lipid content between patients with LDL-c of more and less 70 mg/dL following statin treatment 173

Table 6.1 Clinical characteristics of recruited patients 190

Table 6.2 Spatial relationship between PVAT and vessel wall lipid content in both symptomatic and asymptomatic patients 196

List of abbreviations

AAR, absolute risk reduction

ACS, acute coronary syndrome

AHA, American Heart Association

ApoA1, Apolipoprotein A1

AVIC, Acute Vascular Imaging Centre

BP, blood pressure

CCA, common carotid artery

CEA, carotid endarterectomy

CETP, Cholesteryl ester transfer protein

CT, computed tomography

CTT, Cholesterol Treatment Trialists

CVA, cerebrovascular event

CVD, cardiovascular disease

DANTE, Delay Alternating with Nutation for Tailored Excitation

DMSO, dimethyl sulfoxide

DS%, degree of luminal stenosis

DTT, dithiothreitol

eNOS, nitric oxide synthase

FBS, fetal bovine serum

ECG, electrocardiogram

EEM, external elastic membrane

ELISA, enzyme-linked immunosorbent assay

FLASH, Fast Low Angle Shot

FMD, flow-mediated dilatation

FOV, field of view

HDL-c, high-density lipoprotein cholesterol

HDL-p, high-density lipoprotein particle concentration

HDL-s, high-density lipoprotein size

HMG Co- A, 3-hydroxy-methylglutaryl coenzyme A

HR, hazard ratio

hsCRP, high sensitive C reactive protein

IHD, ischaemic heart disease

ICA, internal carotid artery

IL, interleukin

IPH, intra-plaque haemorrhage

IQR, interquartile range

IVUS-VH, intravascular ultrasound coupled with virtual histology

LAI, lipid aggregation index

LDL-c, low-density lipoprotein cholesterol

LDL-p, low-density lipoprotein particle concentration

LDL-s, low-density lipoprotein size

LLD%, percentage of largest lipid deposit

LP(a), lipoprotein (a)

Lp-PLA2, lipoprotein-associated phospholipase A_2

LRNC, lipid-rich necrotic core

MC, multi contrast

MCP-1, monocyte chemoattractant protein-1

MESE, Multiecho Spin-Echo

MRI, magnetic resonance imaging

NA, nicotinic acid

NIRs, near-infra red spectroscopy

NMR, nuclear magnetic resonance

NNT, number needed to treat

NPC1L1, Niemann-Pick C1-Like 1

NRES, National Research Ethics Services

OR, odds ratio

PAV, percent atheroma volume

PCSK9, Proprotein convertase subtilisin-kexin type 9

PD, proton density

PVAT, perivascular adipose tissue

RLD%, percentage of remaining lipid deposit

ROI, region of interest

SD, standard deviation

STEMI, ST-segment elevation myocardial infarction

TC, total cholesterol

TCFA, Thin cap fibro atheroma

TIA, transient ischaemic attack

TNF-α, tumour necrosis factor

TMB, 3,3',5,5'- tetramethylbenzidine

TOF, time of flight

TSE, turbo spin echo

USS, ultrasound scan

VLDL-p, very low-density lipoprotein particle concentration

VLDL-s, very low-density lipoprotein size

vWA, vessel wall area

vWT, vessel wall thickness

Chapter 1: General Introduction

1.1 The burden of cardiovascular disease in the post-statin era

1.1.1 Epidemiology of cardiovascular disease and recent changes

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for almost one third of deaths.[1, 2] Atherosclerosis-related disorders, such as ischaemic heart disease (IHD) and cerebrovascular events (CVA), continue to be the most common CVD deaths across the globe.[1] The total number of CVD-related deaths has increased from 12.3 million in 1990 to 17.3 million deaths, accounting for 31.5% of total deaths in 2013.[1, 3] Although developing countries account for the majority of the global burden of CVD with four out of five patients coming from low and middle income nations,[4] CVD in advanced economies is still a significant cause of death.[3, 5] CVD caused more than 4 million deaths leading to almost half of deaths (45%) in Europe and more than 750 thousands deaths, almost one third of deaths in the United States.[3] Moreover, the case fatality for acute myocardial infarction and stroke continued to be significant and was reported between 7.6% and 10.6% in 2013 in the UK.[2, 3] The increase in populations alongside aging, and among other factors, are contributing to this rise in number of CVD deaths.[1] Additionally, CVD has posed a large health and economic burden. Nearly 1 in 10 admissions to hospitals is related to CVD with an average in-hospital stay of 7.1-days costing more than £8 billion for the National Health Service.[2, 6]

Despite the rise in expenditure and number of deaths of CVD, its mortality rate has decreased over the last decade.[1] Indeed, age-standardised morality rate of CVD has decreased by 22%, with countries like UK the mortality rate of IHD was falling more rapidly by 65%.[1-3] More importantly, in 12 European countries including the UK, CVD is no longer the leading cause of death.[2, 3] In 2012, cancer took over CVD as the most common aetiology of death in men while CVD remained the foremost cause in women, accounting for 28% of deaths in females in the UK.[2] This reduction in the mortality rate of CVD was multi-factorial and reflected a

better control over risk factors coupled with an improvement in case fatality and available treatments.[7, 8] Moreover, medical therapy of established atherosclerosis has significantly evolved and more patients are currently on antiplatelet, antihypertensive and lipid-lowering drugs.[9] The Health Survey for England reported that nearly 1 in 7 of the population have been prescribed lipid-lowering treatment.[2] The recognition of low-density lipoprotein cholesterol (LDL-c) as a significant and modifiable cardiovascular risk has led to a major improvement in patients' care and contributed to the wide spread use of statin therapy.[2, 4, 10-12] Therefore, it is essential to review the available evidence of statin treatment on cardiovascular events.

1.1.2 Statin and reduction in cardiovascular events

3-hydroxy-methylglutaryl coenzyme A (HMG Co- A) reductase inhibitors, statins, have been shown to be effective in decreasing LDL-c and reducing cardiovascular events.[13, 14] The magnitude of benefits was proportional to the level of attained LDL-c and these benefits were noted in both primary and secondary prevention settings.[15-17] The Scandinavian Simvastatin Survival Study (4S) was the first trial to demonstrate survival benefits of statins in 4444 patients with coronary artery disease (CAD).[15] The probability of survival was 91.3% in the simvastatin group compared to 87.6% in the placebo one, yielding an absolute risk reduction (AAR) of 3.7% over a median follow-up of 5.4 years. Moreover, high intensity statin (atorvastatin 80 mg) was shown to be superior to moderate dose (pravastatin 40 mg) in reducing major cardiovascular events (defined as composite of all-cause mortality, myocardial infarction, unstable angina requiring hospitalisation and coronary revascularisation).[16] Early administration of high dose atorvastatin in high risk cohort (i.e. following acute coronary syndrome, ACS) has resulted in 16% relative risk reduction of major cardiovascular events.[16] These benefits were attributed to the reduction in LDL-c to nearly 60 mg/dL in the atorvastatin group compared to 95 mg/dL in the pravastatin one.[16]

Equally, statins showed clinical benefits in patients with no previous documented history of myocardial infraction.[18, 19] The West of Scotland Coronary Prevention Study Group (WOSCOPS) recruited patients with elevated LDL-c (5 ± 0.5 mmol/L) and demonstrated that pravastatin was effective in reducing the relative risk of major coronary events by 31% over 4.9 years follow up.[18] The corresponding reduction in LDL-c of 1.4 mmol/L was proposed to be the driver of these clinical benefits.[18] Moreover, in a large scale meta-analysis, including 170,000 patients from 26 randomised trials conducted by the Cholesterol Treatment Trialists' (CTT) collaboration, statins were shown to reduce the relative risk of major vascular events (defined as major coronary event, coronary revascularisation and ischaemic stroke) by over a fifth for every 1.0 mmol/L reduction in LDL-c.[14] Similarly, the advantages of statins were also reported in patients with low vascular risk (defined as 5-year major vascular event risk of less than 10%) in a large patient-level meta-analysis from 27 randomised trials.[20] However the AAR was relatively small of 1.1% over 5 years.[20] The remarkable evidence of the beneficial effects of statins in reducing future cardiovascular events combined with a favourable side-effect profile, have led to the wide spread use of statin in CVD.[21] Nonetheless, it has been argued that the benefits of statins on cardiovascular outcomes were not adequately explained by their impact on LDL-c. Rather it was debated that these benefits were driven from the 'pleotropic' effects of statin on inflammation, endothelial dysfunction, and anti-oxidant properties.[22] This debate will be discussed in more details in the next section to illustrate the growing evidence that LDL-c reduction is the main driver of statins benefits, in what is referred to as the *LDL hypothesis*.

1.1.3 LDL hypothesis: "lower is better"

The significance role of LDL-c in atherosclerosis has been established decades ago.[23, 24] Anitschkow was the first to demonstrate that cholesterol is responsible for developing

atherosclerosis.[23, 24] In 1913, he showed that the formation of atherosclerotic lesions within the rabbit arterial intima was a consequence of a high cholesterol diet.[23, 24] Moreover, when standardising the cholesterol diet he recognised that the development of atherosclerosis was proportional to the cholesterol uptake irrespective of the stage of atherosclerosis.[23, 24] This was subsequently established using state-of-art ultrastructural techniques confirming that LDL particles of cholesterol-fed rabbits were trapped within the arterial intima among entwined collagen fibres.[25, 26] Moreover, the "response-to-retention" hypothesis has also supported the causal role of LDL-c in the development of atherosclerosis.[27] Lipoproteins retention within the arterial sub-endothelium emerged as an absolute requirement for early athrogenesis.[27] Other processes such as endothelial dysfunction, shear stress or macrophage chemotaxis were not sufficient as a sole stimulus to induce plaque formation in an otherwise normal aretry.[27]

Evidence from large epidemiological studies established a strong relationship between serum cholesterol level and vascular events.[10, 28] The Framingham Heart Study showed that LDL-c is directly related to the risk of CVD.[10, 28] In addition, evidence from Mendelian randomisation revealed that alleles associated with LDL-c were associated with an increased risk of CVD.[29] Furthermore, lowering LDL-c has produced a corresponding reduction in cardiovascular events and this reduction was not exclusive to statin therapy.[13, 14, 30] Nicotinic acid (NA) when compared to placebo in the Coronary Drug Project treatment produced 11% relative reduction in mortality after 15 years follow up.[30] NA in the same study reduced cholesterol level by 0.6 mmol/L potentially accounting for the reduction in cardiovascular events.[30] These advantages of NA were further confirmed in a meta-analysis including 6500 patients.[31] Despite this evidence, other hypotheses were put forward to explain the reduction in cardiovascular events using lipid-lowering drugs.[22, 32] The *Statin hypothesis* suggested that the favourable outcomes of atherosclerosis after using statin cannot be merely explained by

the reduction in LDL-c. The reasons behind this argument were threefold. Firstly, statins were shown to have anti-inflammatory properties by reducing pro-inflammatory cytokines,[33] improving endothelial function,[34] and increasing the bioavailability of nitric oxide.[35] Secondly, adding a non-statin drug in previous clinical trials has failed to demonstrate any incremental benefits in reducing cardiovascular events.[36, 37] The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) trial and The Action to Control Cardiovascular Risk in Diabetes (ACCORD)-Lipid trial were examples of this lack of success.[36, 37] Thirdly, the Justification for the Use of Statins in Prevention: an intervention Trial Evaluating Rosuvastatin (JUPITER) trial demonstrated that the attained high sensitive CRP (hsCRP) was a significant predictor of cardiovascular event independent of the achieved lipoprotein levels.[38] However, recent evidence from non-statin drugs, namely ezetimibe and evolocumab, have halted this long-standing argument.

Ezetimibe

The Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) was a landmark study that showed an incremental cardiovascular benefits when adding a non-statin drug.[39] Combining ezetimibe and simvastatin has resulted in an absolute reduction of 2% in composite endpoints of cardiovascular death, non-fatal myocardial infarction, stroke, hospitalisation for unstable angina and coronary revascularization.[39] The LDL-c in the combination group was 1.4 mmol/L compared to 1.8 mmol/L in the simvastatin group after a median follow up of 6 years. The significance of this study was not only related to the fact that ezetimibe was the first non-statin treatment to show additional cardiovascular benefits when added to statin, but also in reinforcing the LDL hypothesis. Ezetimibe has a distinct mechanism in decreasing LDL-c by stopping the absorption of cholesterol from the intestines.[40] This unique mechanism of blocking Niemann-Pick C1-Like 1 (NPC1L1) protein

provides an excellent opportunity to study the LDL lowering effect in isolation from statin mode of action.[41] Remarkably, the decrease in cardiovascular events using ezetimibe followed a similar slope of LDL-c reduction as seen using statins. This implied that improvement in cardiovascular events in response to LDL-c lowering drugs is independent of the mechanism by which a certain treatment, statin or non-statin, can exert this effect (see Figure 1.1).

Proprotein convertase subtilisin-kexin type 9 inhibitors

Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a serine protease that is secreted from the liver and has an important role in regulating LDL-c.[42, 43] The catalytic subunit of PCSK9 binds to the LDL-R (receptor) promoting its degradation in lysosomes and preventing it from circulating back to the hepatocytes cell membrane.[43, 44] In 2003, Abifadel *et al*. were the first to describe a gain-of-function mutation of PCSK9 as a cause of familial hypercholesteraemia.[45] This was quickly followed by reports of low LDL-c secondary to a loss-of-function mutation of PCSK9.[46] Cohen *et al.* showed that this lifelong reduction in LDL-c, albeit small (15-28%), conferred a significant reduction in the risk of CVD to half (hazard ratio (HR) 0.50; 95% confidence interval, 0.32 to 0.79; P=0.003).[47] Therefore, development of treatments that target PCSK9 was a promising approach to reduce LDL-c and to potentially modify the risk of CVD.

Evolocumab and alirocumab were the first two human monoclonal antibodies to be studied, followed by the humanised monoclonal antibodies (bococizumab and LY3015014).[44] These drugs were all effective in reducing LDL-c (up to 70% when added to statin).[43, 44] This reduction was dose-dependent and following the administration of these drugs there is an interval of 5 to 6 weeks for LDL-c to return to its baseline.[43, 44]

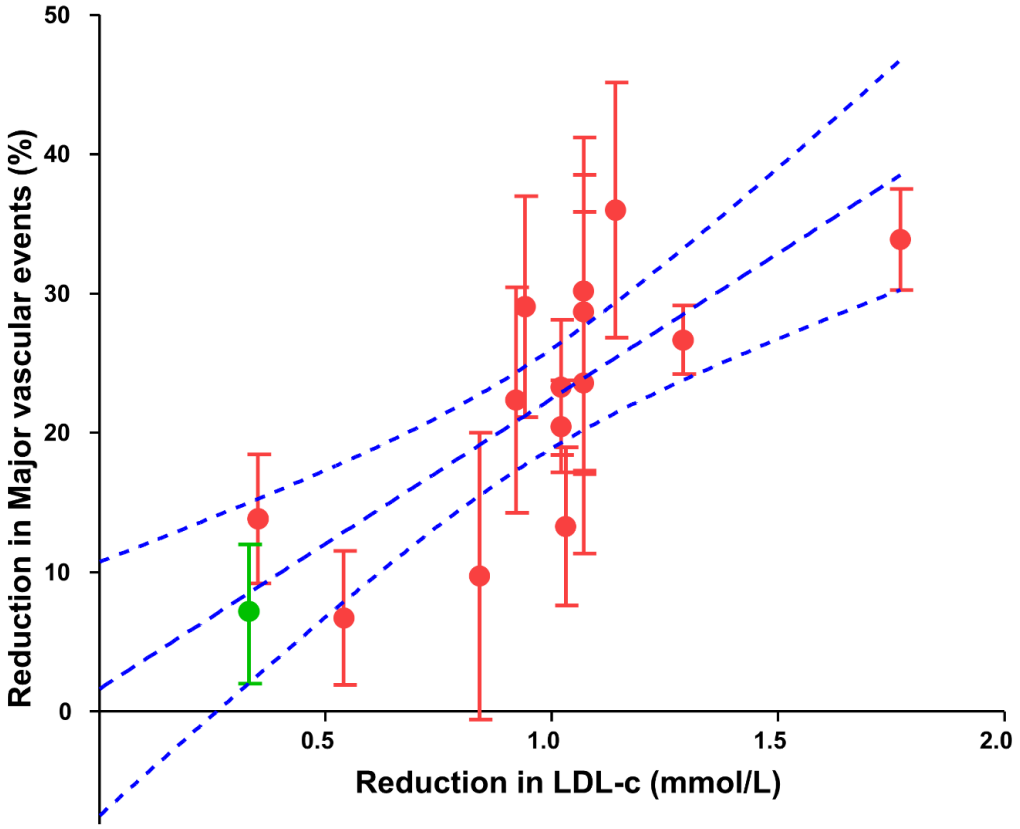


Figure 1.1 Scatter-plot of reduction in LDL-c and major vascular events

The IMPROVE-IT trial is depicted in green compared to the rest of the statin studies which are exhibited in red. The error bars are representative of the 95% confidence intervals. The dotted blue line represents the linear relationship of all the studies combined and evidently demonstrated that LDL-c reduction using ezetimibe was within the regression line. These studies are (from left to right): Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI Prevenzione);[48] Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial–Lipid Lowering Trial (ALLHAT-LLT);[49] Assessment of Lescol in Renal Transplantation (ALERT);[50] Lescol Intervention Prevention Study (LIPS);[51] Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS);[19] Cholesterol and Recurrent Events (CARE);[52] Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID);[53] Prospective Study of Pravastatin in the Elderly at Risk (PROSPER);[54] Anglo-Scandinavian Cardiac Outcomes Trial–Lipid Lowering Arm (ASCOT-LLA);[55] West of Scotland Coronary Prevention Study (WOSCOPS);[18] Post–Coronary Artery Bypass Graft (Post CABG);[56] Collaborative Atorvastatin Diabetes Study (CARDS);[57] Heart Protection Study (HPS);[58] and Scandinavian Simvastatin Survival Study (4S).[15]

The magnitude of LDL-c reduction using PCSK9 inhibitors was superior to any other existing therapies, including ezetimibe.[59] In the GAUSS-3 (Goal Achievement After Utilizing an Anti-PCSK9 Antibody in Statin Intolerant Subjects 3) randomised clinical trial, ezetimibe caused a modest mean percent reduction of 16.7% from a baseline LDL-c of 220 mg/dL compared to 52.8% reduction using evolocumab ($P < 0.01$) after 24 weeks.[59] Initial exploratory analyses suggested possible cardiovascular benefits from reducing LDL-c using PCSK9 inhibitors, such conclusions were criticized given the small number of events (less than 100).[60-62] The FOURIER study (Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk) was designed to address this aspect and recruited 27,564 number of patients with almost three thousands events.[63] Over a median follow up duration of 26 months, subcutaneously administered evolocumab (either 140 mg every 2 weeks or 420 mg every month) has resulted in an absolute reduction in LDL-c of almost 40 mg/dL reaching a median LDL-c of 30 mg/dL.[63] These changes in LDL-c were translated into 1.5% AAR in the evolocumab arm with an event rate of 9.8% compared to 11.3% in the placebo group. Importantly, this reduction in the event rate was in combined primary composite endpoints of cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization.[63] The FOURIER study has, therefore, reinforced the LDL hypothesis and added a second non-statin drug, beside ezetimibe, that modified future cardiovascular events.

Despite these encouraging results of statin and non-statin drugs in reducing cardiovascular events, trials have consistently revealed a significant residual risk that was recognised across all stain and non-statin treatments.[39, 63, 64] This risk endured despite achieving a very low LDL-c and highlighted the need to think beyond LDL-c to tackle the remaining risk. Different pharmacological therapies were used to target the remaining or the "residual" risk

and an overview of these drugs is discussed next to understand the rationale and challenges to modify the residual risk.

1.2 Residual cardiovascular risk despite optimal therapies

1.2.1 Residual risk with low LDL-c

The pathology of the residual risk is widely accepted to be heterogeneous and is recognised to include factors such as diabetes, smoking, and hypertension, in addition to cholesterol-related risks.[64, 65] Therefore, a multifaceted approach was promoted to address the individual risk, including lifestyle modification.[64-66] Certain lifestyle behaviours that include smoking, physical inactivity or poor-quality diet are well-established risk factors for CVD.[66-68] Data from the EPIC-Norfolk population study suggested that combining these behaviour risks has additive effect on total morality.[69] Individuals with four unhealthy behaviours (smoking, physical inactivity, small intake of fruits and vegetables, and more than 14 units a week of alcohol) had four times more likelihood of death compared to those with no risks.[69] On the other hand, adherence to healthy lifestyle was associated with low risk of coronary heart disease.[69, 70] Moreover, applying healthy modifications in early adulthood (age < 30 years) were linked to reduction in vascular risk and subclinical atherosclerosis.[66] Dietary elements such as fibre, plant sterol and olive oil have been associated with favourable lipid profile including lower LDL-c and improvement in HDL function.[71, 72] More importantly this was translated into reduction in future cardiovascular events.[71, 73] In the Prospective Urban Rural Epidemiology (PURE) study, there was reduction in all-cause mortality associated with lower carbohydrate intake.[73] Following 7 years of follow up of more than 135,000 individuals, there was 28% relative increase in total morality for each quantile increase in carbohydrate intake.[73] In addition, there is a strong evidence supporting the role of regular exercise on reducing

LDL-c.[74] There was a negative correlation between physical activity and improvement in both LDL-c and total cholesterol.[74] Therefore, guidelines have constantly reinforced the importance of lifestyle modifications as a first measure before any pharmacological intervention.[75, 76] Statins are considered the standard therapy for atherosclerosis, and tackling lipoprotein-related residual risk, after statin therapy, has employed two main strategies. Firstly further reduction in LDL-c using non-statin drugs,[39] and secondly targeting other non-LDL-c parameters such as high density lipoprotein cholesterol (HDL-c), other pleotropic properties of lipoprotein particles,[77] or even inflammation biomarkers, such as CRP.[38] All these parameters have been targeted as they are intricately related to the development and progression of atherosclerotic plaque.

Two landmark studies, Pravastatin or Atorvastatin Evaluation and Infection Therapy Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI) and Treating to New Targets (TNT),[16, 78] demonstrated incremental benefits from reducing LDL-c using intensive statin treatments and outlined that the residual risk post pedestrian statin use was potentially modifiable. Further reduction in LDL-c in both the IMPROVE-IT and FOURIER trials was translated into better cardiovascular outcomes.[39, 63] However, the residual risk was still reported as 32.7% at 7 years and 9.8% at 2.2 years, respectively despite achieving very low LDL-c.[39, 63] Whether further reduction in LDL-c is still feasible and, more importantly, whether achieving that level of LDL-c is going to have any impact on clinical outcomes is still to be determined. In fact there are signals to suggest that there may be diminishing return upon attaining very low LDL-c.[79]

The second approach of targeting non-LDL-c parameters was proposed not only as therapeutic targets but also as a tools to identify high risk patients beyond LDL-c.[77, 80] Most treatments were mainly focused on raising HDL-c and more recently some therapies targeted inflammation.[80, 81]

1.2.2 Targeting HDL-c to reduce residual risk

Evidence from epidemiological studies have consistently demonstrated an inverse association between HDL-c and cardiovascular risk.[82, 83] A combined analysis from four large studies suggested that for every 0.03 mmol/L increase in HDL-c there was 2-3% decrease in future cardiovascular risk.[82] A post-hoc analysis from the TNT trial has demonstrated the significant role of HDL-c in predicting major cardiovascular risks in statin-treated patients.[84] This showed that in patients with low LDL-c (<70 mg/dL) the 5-year future relative cardiovascular risk was reduced by 25% in the highest quintile compared to the lowest quintile of HDL-c.[84] Such association suggested that the residual risk post statin treatment may be related to the low level of HDL-c and, subsequently, HDL-c- raising treatments were proposed as additional therapy. Moreover, the well-established anti-athrogenic properties of HDL were also supportive of the approach to target HDL in reducing the residual risk.[85] The fundamental involvement of HDL particles in the reverse cholesterol transport, measured as cholesterol efflux capacity would also back up this approach.[86, 87] In fact, cholesterol efflux capacity has subsequently been shown to be a significant predictor of future cardiovascular events, highlighting the potential gain of targeting HDL to further reduce the residual risk.[85-87]

Nicotinic acid (NA)/ niacin

NA is very effective drug in raising HDL-c.[30] In addition, it has been shown to have favourable impacts on other lipoproteins, including LDL-c, which were translated into benefits in reducing cardiovascular outcomes when tested against placebo.[30, 88] However, the AIM HIGH (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes) trial did not demonstrate any incremental benefits of adding niacin to intensive simvastatin treatment.[36] After 2 years, niacin treatment

had significantly increased HDL-c by 25% and decreased LDL-c by 12% but its impact on clinical outcomes was matched to placebo (HR 1.02; 95% confidence interval, 0.87 to 1.21; P=0.79).[36] The relatively low-risk population of the AIM HIGH study that excluded acute events, with a relatively short follow up duration (36 months) coupled with well-managed patients (baseline LDL-c of 74 mg/dL with 94% previously statin-treated) may explain these results. In addition, the use of ezetimibe within the study across the two groups was not similar and as a result almost 10% of the NA group received ezetimibe compared to nearly 20% in the placebo group.[36, 43] Such difference may have offset any potential benefits from NA and contributed to its neutral outcome when taking into account the results from the IMPROVE-IT trial.[39] Nonetheless, the HPS2-THRIVE (The Heart Protection Study 2–Treatment of HDL to Reduce the Incidence of Vascular Events) trial, was also unsuccessful in reducing cardiovascular events when adding NA to optimally-treated patients with baseline LDL-c of 64 mg/dL.[89] Patients in the NA group had suffered more adverse side effects when compared to placebo and per 1000 treated patients there were 13 cases of newly diagnosed diabetes, 37 patients requiring hospitalization for diabetes-control disturbance and 14 cases of serious infections.[89] These effects, among others, have led to a non-significant proportional increase of 9% in all-cause mortality in the NA compared to placebo (HR 1.09; 95% confidence interval, 0.99 to 1.21; P=0.08).[89]

Cholesteryl ester transfer protein (CETP) inhibitors

CETP transfers cholesteryl esters from HDL to apo-B lipoproteins in exchange for triglycerides in a bidirectional fashion.[90] The inhibition of CETP will halt this interchange leading to an increase in HDL-c and a decrease in LDL-c producing a favourable lipid profile that may potentially reduce cardiovascular events.[91, 92] Four molecules targeting CETP activity have been studied and demonstrated safety and tolerability in humans trials, namely torcetrapib,[91] dalcetrapib,[93] evacetrapib,[92] and anacetrpib.[94] The inhibition of CETP activity

using these molecules has consistently led to an increase in HDL-c which ranged between 31% using dalcetrapib to 138% with anacetrapib.[93, 95] On the other hand, the impact on LDL-c was less consistent with more that 35% reduction in LDL-c using evacetrapib and anacetrapib compared to no change with dalcetrapib.[92, 93, 95] Torcetrapib was the first CETP inhibitor to be studied in a randomised clinical trial.[77] In the ILLUMINATE (the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events) trial, torcetrapib resulted in an excess of adverse events, including mortality, after 12 months of treatment and the study was prematurely terminated despite increasing HDL-c by 70% and decreasing LDL-c by 25% with torcetrapib.[77] The unexpected outcomes from the ILLUMINATE study were attributed to 'off-target' effects related to the torcetrapib molecule rather than the mechanism of inhibiting CETP activity.[96-99] These effects included raising blood pressure (BP), increasing aldosterone level and worsening endothelial function.[96-99] Subsequently, dalcetrapib was investigated after having established no 'off-target' effects on both vascular function, measured as the change from baseline of flow-mediated dilatation (%FMD) of the right brachial artery, and blood pressure using 24 hours ambulatory blood pressure in the dal-VESSEL study.[93] Despite this dalcetrapib did not reveal any difference in future risk of cardiovascular events (HR 1.04; 95% confidence interval, 0.93 to 1.16; P=0.52).[100] Yet genotyping the dal-OUTCOMES cohort suggests that subjects with certain gene, accounting for 20% of the dal-OUTCOMES population, showed significant benefits from dalcetrapib compared to placebo (HR 0.67, 95% confidence interval 0.47 to 0.95).[101] These findings are currently investigated in the dal-GENE study to confirm whether a subgroup of patients may demonstrate benefits from CETP inhibition.[102] More recently, evacetrapib was studied in the Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition with Evacetrapib in Patients at a High Risk for Vascular Outcomes (ACCELERATE) trial.[103] The addition of evacetrapib to standard medical therapy, yet again,

did not result in reduction of cardiovascular events after 26 months follow up, although there was more than 35% decrease in LDL-c combined with more than 130% increase in HDL-c (HR 1.01; 95% confidence interval, 0.91 to 1.11; P=0.91).[103] Notably, there was a marginal increase in BP and CRP which may reflect a more severe vasomotor function and a higher inflammation response to CETP inhibition, in contrary to statins' effect.[103] The last member of CETP family inhibitors was anacetrapib which was studied in the REVEAL (Randomized EValuation of the Effects of Anacetrapib Through Lipid-modification) trial.[104, 105] Anacetrapib was tested among patients with atherosclerotic vascular disease who were already on statin treatment and a significant reduction in combined clinical outcomes was demonstrated after 4.1 years follow up.[105] While these benefits were statistically significant, their clinical impacts were considered to be very marginal since the REVEAL trial included more than 30,000 patients with an AAR of 1% compared to placebo (10.8% *vs*. 11.8%, HR 0.91; 95% confidence interval, 0.85 to 0.97; P=0.004) yielding a very large number-needed-to-treat (NNT) of 100.[105]

Anti-inflammatory drugs

In addition to being a disease of lipoprotein accumulation, atherosclerosis is acknowledged to be a disease with inflammatory components.[106] The strong association between pro-inflammatory cytokines and future cardiovascular events has provided an attractive method to reduce the residual risk by targeting inflammatory signalling pathways.[107] Two decades ago, aspirin was shown to have incremental benefits on reducing myocardial infarction across increasing quartile levels of CRP.[108] These benefits were independent of the aspirin antiplatelet properties which did not change across the CRP quartile groups.[108] Similarly data from the JUPITER trial demonstrating that using statin in patients with high level of inflammation led to a reduction in cardiovascular events, independent of LDL-c reduction.[17, 38] However, anti-inflammatory drugs have failed to show significant clinical impacts in

humans over the last decade.[80, 81] Darapladib is a lipoprotein-associated phospholipase A_2 (Lp-PLA_2) inhibitor which was shown to impede the progression of coronary atherosclerotic plaques in swine model and to halt the increase of human coronary plaque's necrotic core volume measured using intravascular ultrasound virtual histology (IVUS-VH).[109, 110] Nonetheless, darapladib in the Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy (STABILITY) trial failed to show any difference in cardiovascular deaths, MI or stroke after 3.7 years follow up in patients with stable CAD (HR 0.94; 95% confidence interval, 0.85 to 1.03; P=0.20).[80] The lack of significant impact of phospholipase A_2 inhibitors on the central pathway of IL-1, IL-6, tumour necrosis factor (TNF-α), and CRP may explain the lack of success with darapladip.[109, 111] This pathway is a critical mechanism related to the progress and development of atherosclerosis.[109, 111] Canakinumab, on the other hand, is a human monoclonal antibody against IL-1ß which was studied in the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial. When compared to placebo, canakinumab produced a significantly lower rate of cardiovascular events in patients with previous myocardial infarction (HR 0.85, 95% confidence interval 0.74 to 0.98, P= 0.021).[112, 113] However, this was associated with significant increase in the rate of fatal infection (0.31 *vs.* 0.18 events per 100 person-years, P=0.02).[113] Moreover, the NNT was, yet again, significantly large (1 in 51), questioning the applicability of this drug in the real world.

This recurrent drawback of anti-atherosclerotic drugs in producing large NNT requires urgent attention to the current, unselective approach of adding a second drug to statin treatment to tackle the residual risk. Notably, the common theme among these drugs was the small margin of benefits questioning their clinical suitability and cost-effectiveness.[62, 114, 115] In addition, it highlighted an important issue of unnecessary exposing patients to potential side-effects with the unselective approach of using these drugs.[62, 114, 115] If the potential clinical benefits of these drugs are to be realised attention should be sharply focused on developing stratification

tools to identify patients who may benefit maximally. In other words, patients who stand to benefit greatly may miss an important therapeutic opportunity and, therefore, there is an urgent need to identify this group of patients. The current challenges facing trials of new anti-atherosclerotic drugs and the rationale for stratification tools will be discussed in more detail in the next section.

1.2.3 Failure of targeting non-LDL-c, rationale and the need for stratification tools

As displayed from the previous overview, the residual risk following statin treatment has been the focus of pharmacological developments over the last decade. Despite this extensive effort, the high annual residual risk continued to pose a challenge in patients with previous CAD and low LDL-c.[64] Although most of these therapies have shown promise in pre-clinical stages or when using surrogate biomarkers, they have failed to produce any significant impact on cardiovascular events in large phase III clinical trials.[43] The basis behind this lack of success is complex and heterogeneous. Firstly, while some of these lipid-modifying and anti-atherosclerotic drugs may potentially show a degree of clinical benefit when assessed against placebo as a monotherapy, they have to demonstrate incremental benefits when added to statin treatment.[43] The robust evidence of statins in reducing cardiovascular events have made it ethically unacceptable and clinically irrelevant to conduct a study without using statin in patients at risk of future cardiovascular events. Secondly, advances in antiplatelet therapies and increase prescriptions of beta-blockers and angiotensin converting enzyme inhibitors combined with intensive statin treatment have aggregated the benefits of optimal medical therapy leading into further reduction of future cardiovascular events.[43] Indeed, both the European Society of Cardiology and American Heart Association have emphasised on the importance of optimal medical therapy in their guidelines, thus, detecting any potential benefits related to any new drug is becoming more challenging.[116, 117] Thirdly, there has been

lack of mechanistic understanding of how targeting certain biomarker is translated into clinical benefits. For example, raising HDL-c does not necessitate any change in the anti-atherogenic properties of HDL particles.[118] In fact, inhibition of CETP activity may produce dysfunctional HDL with impaired reverse cholesterol transfer from cultured monocytes.[118, 119] Finally, the studied cohorts in these trials were very heterogeneous and were recruited based on the patient's category of disease (i.e. previous stroke or post ACS) rather than the disease or patient's characteristics.[43] These characteristics of the atherosclerotic disease have significant variations among patients, for example, in lipid accumulation, inflammation status, thrombosis risk, and arrhythmia burden [120-122] Such features should be distinguish from the 'traditional' risk factors which are useful markers to estimate the future risk of a cohort but not on an individual basis.[43, 123, 124] Therefore, characterising the disease process and recognising the prominent features of the atherosclerotic process may be informative in identifying high risk individual of future adverse cardiovascular events.[17, 43, 121, 125] In the European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis (ATHEROREMO) and the Integrated Biomarker Imaging Study 3 (IBIS-3) studies, coronary lipid content, quantified using near infra-red spectroscopy (NIRs), of the non-culprit artery was inversely related to the incidence of major adverse cardiovascular events (MACE) in ACS patients.[126] Remarkably, the individual response to similar treatments is also variable, and probably reflects differences in both the disease characteristics and the subject susceptibility contributing to the individual future risk. In the CANTOS trial, almost 20% of the screened ACS subjects were excluded compared to 58% included patients using hsCRP cut-off of more than 2 mg/L.[113] This suggests that the inflammatory pathway may still be "switched on" in the included group highlighting the individuals with poor response to medical therapy.[113] Therefore, the heterogeneity of the recruited patients in the trials investigating lipid-lowering drugs may also contribute to the failure of these drugs.

Moreover, these lipid-lowering drugs were proposed to target lipoprotein-related risks. However, the recorded events in these trials were again heterogeneous and included other disease processes such as thrombosis and arrhythmias, perhaps, diluting any potential benefits from these drugs.[43] Therefore, refinement of these clinical trials is increasingly needed in order, firstly, to detect any signal of benefits above noise and to identify patients who may benefit maximally. Secondly, it would minimise any harmful side-effects where new therapies would be tailored toward specific high risk cohort rather than applying them to "all-comers". Thirdly, the cost to plan and conduct such clinical trials is anticipated to be less, in particularly with smaller sample size and probably shorter follow up period to reveal any benefits. These arguments would support the need for stratification tools to identify high risk groups where studying new drugs would maximise the intended benefits. Importantly, even in trials where the studied drugs were considered 'successful', such as ezetimibe and evolocumab, the AAR was only modest.[39, 63, 113] This is very important given the large number needed to treat (NNT) to yield benefits in a combination of composite endpoints as highlighted before.[39, 63, 113] Moreover, these anti-atherosclerotic drugs are 'biological', expensive, and their application on "all-comers" patients is probably not practical. Cost-effective analyses of evolocumab, for example, have highlighted a major limitation of this drugs and the need for risk stratification.[114, 127, 128] It was estimated that adding PCSK9 inhibitors to the current treatment of patients with atherosclerotic disease would increase health care costs by $414,000 per QALY, a substantial increase which would probably limit their use.[114] Therefore, the indiscriminate use of new therapies in unselected vascular populations is undesirable, unaffordable and potentially harmful. Yet, without a clear means of stratification, patients who stand to benefit greatly will miss an important therapeutic opportunity. Current candidates to stratify patients, based on imaging and non-imaging biomarkers are discussed in the next section.

1.3 Mechanistic staging and the role of imaging in risk stratification

1.3.1 Non-imaging biomarkers to identify high risk group

In order to identify patients at a higher risk of future cardiovascular events, numerous blood biomarkers, including lipoprotein-related markers, were proposed. These biomarkers were identified not only as means of stratifying patients but some were also therapeutic targets to reduce residual risk. These biomarkers ranged from pleotropic properties of LDL such as LDL particle size and number,[129, 130] other lipoprotein functions such HDL-c and cholesterol efflux capacity,[86, 87] to inflammatory markers like serum amyloid A and CRP.[17, 131] Low HDL-c is a well-established risk factor, however, treatments raising HDL-c have been largely unsuccessful.[103] Recent evidence has suggested the lack of causal role of HDL-c in atherosclerosis. Mendelian randomisation studies have demonstrated that alleles associated with HDL-c are not associated with future cardiovascular events after adjusting for triglycerides, LDL-c and statin use.[29] Moreover, a genetic variant of scavenger receptor B1 was showed to cause profound increase in HDL-c but it did not decrease the risk of CVD.[132] To the contrary, carriers of P376L variant had significantly increased risk of future events (odds ratio (OR) 1.79, P=0.018).[132] While the lack of success in HDL-c raising therapies may be related to absence of causality role of HDL-c in atherosclerosis, it also may be related to the studied molecule or the drug mode of action in raising HLD-c. It is plausible that the benefits of raising HDL-c are not merely related to achieve high concentration of HDL-c but to the mechanism to raise HDL-c. These findings reinforce the importance of identifying a stratification tool that is based on clear understanding of the disease process.[43] In addition, the complex processes and interactions that lead to lipoprotein retention within the intima of the arterial wall would render a single blood marker imprecise in predicting future cardiovascular events.[27, 43] For example, small dense LDL particles were found to be a significant predictor of atherosclerosis progression.[133] Using this solitary biomarker as a surrogate of

cardiovascular risk to elucidate the impact of ezetimibe on atherosclerosis may have produced perplexing results.[134] In a randomised three-group open-label study sole ezetimibe treatment was associated with increased small dense LDL particles, producing a more pro-atherogenic LDL profile.[134] Moreover, the marginal athero-protective profile induced by simvastatin was offset by ezetimibe treatment in the combination arm.[134] Nonetheless, ezetimibe was shown to have incremental benefits of cardiovascular outcomes when combined with simvastatin in the IMPROVE-IT trial.[39] Therefore, new tools that can probe the relationship with future cardiovascular risk, beyond blood biomarkers, are urgently needed.

1.3.2 Role of imaging in the management of the residual risk

Imaging of the atherosclerotic plaque has been used as a surrogate of clinical outcomes when studying new pharmacotherapies added to statin treatment.[81, 135, 136] However, results from imaging and clinical outcomes studies have not always been consistent.[81, 137] Discordant results were reported from NA and canakinumab.[81, 137] In the Oxford Niaspan Study, NA significantly reduced carotid atherosclerosis in statin-treated patients, quantified as mean change in carotid wall area on magnetic resonance imaging (MRI), compared to placebo [-1.64, 95% confidence interval (-3.12 to -0.16), P= 0.03].[137] Yet, the results from AIM HIGH and HPS2-THRIVE studies did not demonstrate incremental benefits from adding niacin to statin treatment.[36, 89] To the contrary, canakinumab did not produce any significant effect on vascular structure or function measured as mean carotid wall area and aortic distensibility.[81] Despite these neutral effects, adding canakinumab in the CANTOS trial led to a significantly lower MACE when compared to placebo.[113]

On the other hand, other drugs had concordant results from imaging and clinical outcomes studies.[135, 136] Non-invasively quantified carotid atherosclerosis was not significantly different from placebo with either dalcetrapib or torcetrapib.[135, 136] In the Rating Atherosclerotic Disease Change by Imaging with a New CETP Inhibitor (RADIANCE 1) trial, the increase in maximum carotid intima-media thickness, measured on B-mode ultrasonography, was 0.0047 ± 0.0028 mm per year in the combination of atorvastatin and torcetrapib group compared to 0.0053 ± 0.0028 mm per year in the atorvastatin-only group (P=0.87).[135] Similarly, dalcetrapib did not instigate any change in the carotid wall area [absolute change -2.2 mm^2, 90% confidence interval -4.54 to 0.13, P= 0.12], wall thickness [absolute change −0·03 mm, 90% confidence interval (−0·11 to 0·04), P=0.45] or normalised wall index [absolute change 0·60%, 90% confidence interval (−1·20 to 2·50), P=0.57] measured on MRI after 24 months of treatment.[136] Subsequently, both the ILLUMINATE and dal-OUTCOMES studies did not show any reduction in MACE with torcetrapib and dalcetrapib, respectively.[77, 100] On the other hand, anacetrapib showed reduction in the burden of atherosclerosis within the aortic root which was translated into clinical benefits. Evidence from experimental mice model demonstrated that anacetrapib, dose-dependent, reduced aortic plaque volume and improved plaque stability.[138] Consequently, anacetrapib in the REVEAL trial resulted in lower MACE compared to placebo in patients with atherosclerotic vascular disease.[105]

These results highlight the lack of mechanistic insights into the effects of studied drugs on atherosclerosis. It may also question the validity of using total plaque imaging as a surrogate for outcomes when studying new anti-atherosclerosis drugs. Recent advances in technology have led to rapid improvements of *in-vivo* imaging techniques that enable us, with high degree of accuracy, to characterise atherosclerosis processes within the arterial wall.[43] Moreover, improvements in technology allowed us to study new imaging biomarker that may be related to the initiation and progression of atherosclerosis, such as perivascular adipose

tissue.[139, 140] These new techniques allow serial measurements at the target lesion level and can provide useful metrics to predict future cardiovascular outcomes beyond plaque burden and blood biomarkers.[43, 121, 126] Numerous imaging biomarkers were proposed as tools to identify high risk atherosclerotic patients.[139, 141-145] Coronary calcifications detected on computed tomography (CT) can potentially categorise patients into high and low risk groups.[141, 142] Surprisingly, there is still a degree of uncertainty as to what calcifications reflect within the atherosclerotic process making calcification less attractive as a mechanistic tool for risk stratification.[146] One prevalent theory is that calcifications are a surrogate of elevated coronary plaque burden. However, statins have been shown to increase major calcifications despite their well-documented role in inducing atherosclerosis regression.[147] Therefore, these calcifications would not serve as a good imaging biomarker to track effects of lipid-lowering drugs. In fact, imaging calcifications could be referred to as a non-mechanistic staging where current therapies do not specifically target the disease process that categorises the individual at an increased risk, nor does it track the response of the targeted process to the used treatment. More recently, new imaging biomarkers have been promising in adding insights to the development of atherosclerosis.[139, 145] Perivascular adipose tissue (PVAT) has recently been shown to have a bidirectional communication with the vascular wall.[139, 140] Quantifying and characterising PVAT have provided important information on future cardiovascular risk, independent from the classical risk factors.[139, 143, 144] Importantly, understanding the association between PVAT and atherosclerotic plaque burden and/ or composition is pivotal to exploit its potential role for risk stratification. Identifying a relationship between PVAT and features of high risk plaque adds further insights into the future role of PVAT as an imaging biomarker. Features of advanced atherosclerosis, such as large lipid content, may or may not be related to the presence of PVAT. Such a relationship should be evaluated if plaque lipid content could be accurately and systematically quantified

for each individual. Notably, whether PVAT would be able to track response to anti-atherosclerotic drugs is yet to be determined. Overall, the utility of an imaging tool as a risk stratification method would be better valued if this tool is capable of tracking response to the tested drug. The ability to stratify patients based on a disease characteristic (calcification, lipid accumulation, inflammation etc.) and to apply a drug that *targets* this particular feature and, equally, *tracks* the response of the disease characteristic to the applied drug has been referred to as '*mechanistic staging*'. [43] This concept is distinct from the approach of conducting a risk assessment of the atherosclerotic plaque by pursuing features suggestive of plaque instability.[43] Thin cap fibro atheroma (TCFA) reflects plaque's vulnerability with increased likelihood to rupture, however, it does not indicate a single disease process but rather an assembly of different features within the atherosclerotic plaque.[43] In fact, pursuing vulnerable plaque has failed to have any major impact on clinical events.[146] Therefore, the proposed matching between a drug-related mechanism of action and the basis for an individual amplified risk may offer the opportunity to achieve more precise intervention and greater efficacy generating more cost-effective prescribing.[43] This approach targets a specific process of the atherosclerotic disease rather than merely a category of diagnosis (e.g. post ACS). Herein a comprehensive review on the impacts of lipid-lowering drugs at the level of the atherosclerotic plaque using currently available vascular imaging tools.

1.3.3 Impact of lipid-lowering therapies at the level of atherosclerotic plaque

Remarkably, the precise mechanism behind the efficacy of lowering LDL-c in reducing cardiovascular events is still unclear and, in particular, at the level of atherosclerotic plaque. In order to understand the impact of LDL-c reduction on atherosclerosis progression and regression, both invasive and non-invasive imaging modalities have been used.[148, 149] The earliest evidence on the influence of cholesterol modification on atherosclerosis regression

came from rhesus monkeys in 1970.[150] Armstrong *et al*. demonstrated 80% increase in the average cross-sectional area of the lumen of coronary arteries following 40 months of cholesterol-free diet.[150] These data initiated the concept that cholesterol adjustments instigate reduction in plaque volume with concurrent increase in lumen area. Twenty-five years later the Multicentre Anti-Atheroma Study (MAAS) replicated these finding in humans.[151] In this randomised double-blinded trial, simvastatin (20 mg) slowed progression and induced regression of diffuse and focal atherosclerosis measured as minimum and mean luminal area using quantitative coronary angiography.[151] Over 4 years follow up, the investigators reported a significant reduction in degree of stenosis (-2.6%) compared to placebo (+3.6%) with 0.06 and 0.08 mm increase in mean and minimum luminal area, respectively (combined P=0.006).[151] However, measuring the luminal area and degree of luminal stenosis virtually disregard the pathological process of atherosclerosis, which reside in the wall of the artery. This process has variable incursion into the lumen with a certain lag in the arterial wall before encroachment is detectable by coronary angiogram.[43] This is important as a clinical outcomes study in the same era (4S) demonstrated 42% relative risk reduction in coronary deaths.[15] This mismatch between clinical outcomes and the studied mechanism of statins using invasive coronary angiogram had several knock-on effects. It posed a challenge to the proposed mechanism of statin in reducing the degree of luminal stenosis and also highlighted the relative insensitivity of coronary angiography as a tool to study effects of a drug on atherosclerotic plaque. This motivated the search for better tools to quantify changes of atherosclerosis within the arterial wall in response to LDL lowering treatments. MRI, on the other hand, can identify the atherosclerotic disease within the arterial wall and may serve as alternative imaging modality to study the effects of LDL-c reduction.[152] Corti *et al*. demonstrated that simvastatin produced a reduction of 8% and 15% in vessel wall area in aorta and carotid arteries, respectively.[153] This decrease was coupled with significant

reduction in LDL-c of 38% following 12 months treatment.[153] Subsequently, Lee *et al*. revealed that similar changes in aorta and carotid arteries can occur as early as three months.[154] With advancing technologies, Nissen and his colleagues demonstrated a significant decrease in atherosclerosis burden using the IVUS-driven parameter of percent atheroma volume (PAV).[155] This parameter was calculated as the difference between external elastic membrane (EEM) area and lumen area divided by the EEM area, and its absolute change over 18 months follow up was significantly lower in the intensive statin treatment compared to the moderate group (-0.9% *vs.* 4.4%, P=0.02).[155] This was significantly related to the magnitude of LDL-c reduction, with patients attaining LDL-c of less than 100 mg/dL, having slower changes in atheroma volume (-0.9% *vs.* 1.9%, P=0.07).[155] These findings were borne out in the PRECISE-IVUS study (Plaque Regression With Cholesterol Absorption Inhibitor or Synthesis Inhibitor Evaluated by Intravascular Ultrasound).[156] Adding ezetimibe to atorvastatin resulted in a greater coronary atherosclerosis regression (absolute change in PAV -1.4% *vs.* -0.3%, P= 0.001) and a high percentage of patients showing atherosclerosis regression (78% *vs.* 58%, P=0.004).[156] These effects were mirrored with the reduction in LDL-c in the combination group compared to the atorvastatin monotherapy (63.2 ± 16.3 mg/dl *vs.* 73.3 ± 20.3 mg/dl; $p < 0.001$).[156] Similarly, in the Global Assessment of Plaque Regression With a PCSK9 Antibody as Measured by Intravascular Ultrasound (GLAGOV) trial, evolocumab caused a significant reduction in the absolute PAV compared to placebo (difference -1.0%, 95% confidence interval -1.8% to -0.64%, P<0.001) with a greater percentage of patients showing plaque regression (64.3% *vs.* 47.3%, P<0.001).[157] A large meta-analysis, including more than 4000 patients, demonstrated that the reduction in atherosclerosis burden was associated with adverse clinical cardiovascular events.[158] However, the modest change in plaque burden did not match and might not fully explain the

decrease in the clinical risk seen with LDL-c reduction and, therefore, other hypotheses related to change in plaque composition were proposed.[158, 159]

1.3.4 Changes in plaque composition in response to LDL-c reduction

Original imaging studies have focused on changes in plaque burden in response to LDL-c reduction.[158, 159] Since atherosclerotic plaques are heterogeneous with significant variations in lipid content,[120, 126] the next phase of studies attempted to investigate effects of LDL-c reduction on plaque composition. In a preclinical study, Libby *et al*. demonstrated that low-cholesterol diet in rabbits did not only reduce plaque lipid content within aortic plaque but also produced a number of features suggestive of plaque stabilisation (an increase in interstitial collagen and smooth muscle cell maturation whilst a decrease in macrophage number and MMP, matrix metalloproteinase expression).[159] In human *ex-vivo* study, Crisby *et al.* showed that three months of pravastatin treatment was associated with a decrease in lipid content measured as percentage oil red O staining of the total plaque area of excised endarterectomy samples compared to placebo (8.2 ± 8.4% *vs.* 23.9 ± 21.1%, $P<0.05$).[160] They also demonstrated a higher collagen content measured similarly but using Sirius red staining (12.4 ±3.1% *vs.* 7.5 ±3.5%, $P<0.005$).[160] These changes support the lipid depletion hypothesis, where reduction in LDL-c leads to a net depletion of cholesterol from atherosclerotic plaque with replacement of fibrous tissue.[43] This in turn has a 'stabilising effect' on plaques and subsequently less likely to precipitate acute events. To demonstrate these effects *in-vivo* in humans, Schartl *et al.* illustrated that intensive statin treatment resulted in changes in plaque echogenicity based on a grey-level intensity analysis using IVUS.[161] They speculated that these changes reflected a reduction in lipid-rich plaque.[161] Concurrently, advances in non-invasive imaging modalities facilitated delineation of plaque components and precise characterisation of atherosclerotic plaque.[162] Combining four *in-vivo*

MRI sequences of T1-weighted, T2-weighted, proton-density (PD), and time-of-flight (TOF) allowed identification of key components of carotid plaques, namely lipid-rich necrotic core (LRNC), fibrous components, intra-plaque haemorrhage (IPH), and calcium.[163] The initial MRI-based evidence of change in plaque composition in response to lipid-lowering therapy, was derived from a case-control post-hoc study from the Familial Atherosclerosis Treatment Study (FATS).[164] Eight CAD patients who received 10-years combination therapy of lovastatin, niacin and colestipol where compared to age- and lipid profile-matched coronary patients who never received lipid-lowering treatments.[164] In this study, lipid core area was reported to be smaller in the treatment group compared to placebo (0.7 *vs.* 10.2 mm^2, P=0.01) despite having similar luminal and plaque areas.[164] In the Outcome of Rosuvastatin treatment on carotid artery atheroma: a magnetic resonance Imaging ObservatioN (ORION) trial, 24-months treatment of rosuvastatin was associated with 41% reduction in the percentage of lipid-rich necrotic core out of the carotid wall area (%LRNC). Importantly, this reduction was quantified on MRI in a *pre-screened* cohort for carotid atherosclerosis using duplex ultrasound and only patients with identifiable LRNC were included in the study.[165] Similarly, in the CPC Study (Carotid Plaque Composition by MRI During Lipid-Lowering), lipid-lowering treatment was associated with 48% reduction in %LRNC over three years follow up another cohort that was pre-screened for carotid atherosclerosis.[148] In this study, Zhao *et al*. showed a reduction rate of approximately 3% per year in LRNC% in the first two years with no change during the third year.[148] On the other hand, the percentage of fibrous tissue was significantly increased (83.4% *to* 90.1%, P<0.001) while its volume was significantly decreased (571 mm^3 *to* 547 mm^3, p = 0.007).[148] Notably, in both the ORION and CPC studies changes in plaque composition were reported in a minority of recruited patients (only 55% in the ORION and 27% in the CPC study) given the limitations with the used MRI technqiue.[148, 165, 166] The original MRI technology relied on patients with sufficiently large areas of

coalesced lipid to be quantified non-invasively. This means that only a minority of patients, who have sufficient lipid to be quantified serially, were included in these studies.[43, 167]

Progress in invasive imaging modalities has overcome the limitations of the originally-used grey-scale IVUS and allowed a degree of characterisation of the atherosclerotic plaque.[168] Although not consistent, IVUS-VH provided evidence on changes in plaque composition in response to LDL-c reduction.[168, 169] One year of treatment with fluvastatin caused a significant reduction in both necrotic core volume and fibro-fatty volume compared to placebo (-2.6 ± 10.3 mm^3 *vs.* 8.9 ± 17.7 mm^3, P= 0.004 and -48.4 ± 47.2 mm^3 *vs.* 23.6 ± 16.7 mm^3, P<0.001, respectively) coupled with an increase in fibrous volume (16.8 ± 15.0 mm^3 *vs.* 4.4 ± 5.3 mm^3, P=0.03).[168] In addition, the magnitude of reduction in fibro-fatty volume was positively correlated with changes in LDL-c (rho= 0.70, P<0.001).[168] However, calcification was noted to be a major drawback of IVUS measurement and became a considerable limitation when using ultrasound-based technologies.[170] NIRs is a promising intra-coronary imaging tool that detects lipid core plaques based on their spectral signals and has been validated in autopsy specimens.[171] The YELLOW (Reduction in YEllow Plaque by Aggressive Lipid LOWering Therapy) trial, displayed a significant reduction in lipid core, measured as lipid-core burden index, after short intensive statin therapy (*only 7 weeks*) (-149 (-211 to -43) *vs.* 2.4 (-36 to 45) P=0.01).[172] Nonetheless, the invasive nature of the intra-coronary imaging devices combined with the large dropout rate in follow up (up to 28%), are potential drawbacks when attempting to use these invasive imaging tools.[173] Similarly, the utility of MRI-based imaging tools have been confined to patients with predefined carotid atherosclerosis.[43, 167] The limited sensitivity to study changes in plaque composition in patients with no coalesced lipid-core has affected the validity and generalisability of the observations of net lipid depletion in response to LDL-c reduction.[43, 167] In addition, the relatively long duration of follow-up to detect response in atherosclerotic plaque lipid

impedes the potential utility of plaque lipid imaging as a risk stratification tool in unselected vascular populations. Despite the limitations of the current imaging techniques to reliably demonstrate the impact of LDL-c reduction on plaque lipid content, net lipid depletion appears to be the most plausible hypothesis behind cardiovascular benefits in response to LDL-c reduction. Therefore, matching lipid-lowering drugs to patients with propensity to accumulate lipid across the arterial tree may serve as a refined and more cost-effective platform to test and apply these drugs. Yet, it is important to establish whether plaque lipid content has any significant role in identifying high risk patients. This is essential since the proposed approach of using mechanistic staging dictates matching the mechanism of lipid-lowering drugs via net lipid depletion with a corresponding disease characteristic (lipid accumulation) that differentiates high from low risk group. The evidence to support the prognostic role of plaque lipid content and to back up its utility to identify high risk individuals will be reviewed.

1.3.5 Plaque lipid content and future cardiovascular risk, the role of mechanistic staging

The association between plaque lipid content, among other key components of the atherosclerotic plaque, and adverse cardiovascular events was initially inferred from histological studies.[174-176] In fact, plaques with a large lipid core were four times more likely to be associated with ruptured plaques (OR 4.03, 95% confidence interval 2.35 to 6.09, P<0.001).[174, 175] It was one of the best discriminators, alongside macrophage infiltration and thickness of fibrous cap, to determine the vulnerability of atherosclerotic plaques.[174, 175] Moreover, lipid volume within the vascular wall was related to the ischaemic events in the subtended vascular territory.[177, 178] In asymptomatic patients with 50% to 70% stenosis of their carotid arteries there was 60% increase in the relative risk of ipsilateral stroke for every

10% increase in %LRNC (P=0.008).[178] These results were borne out in a meta-analysis of almost 800 patients.[179] Similarly, data from patients with ST-segment elevation myocardial infarction (STEMI) demonstrated that lipid volume, quantified using NIRs, was 5.8-fold greater in the culprit compared to the non-culprit segments.[120] More recently, studies using both invasive and non-invasive imaging tools have presented a strong association between plaque lipid content and systemic cardiovascular outcomes. The European Collaborative Project on Inflammation and Vascular Wall Remodelling in Atherosclerosis - Intravascular Ultrasound Study (AtheroRemo-NIRS) study, displayed the prognostic value of high plaque lipid content, detected using NIRs, on major cardiovascular outcomes at both 1 and 4 years follow up.[126, 177] Likewise, in the MRI sub-study of the AIM-HIGH, there was an almost 60% relative increase in MACE for every 1 standard deviation increase in percent lipid volume quantified on carotid MRI.[121] Crucially, plaque lipid content has also been shown to track response to LDL-c reduction in relatively small but pre-selected patient samples (16 patients in FATS, 18 in ORION and 33 in CPC studies).[148, 164, 165]

These studies underline the importance of quantifying plaque lipid content to (a) identify a subgroup of high risk patients (patients with large plaque lipid content) and to (b) track patients' response to lipid-lowering therapy (identify those with persistent large plaque lipid content that might benefit from new targeted therapies). However, these studies have been fundamentally limited by the inherent insensitivity of the imaging tools, their invasive nature, and long follow-up duration to probe changes in plaque lipid content.[43, 166] Needed is an imaging tool that is capable of *systematically* measuring lipid content, and tracking lipid response to LDL-c reduction that can be employed to study new lipid-lowering drugs. Such tool would potentially establish the influence and relationship between pro- and anti-atherosclerotic blood biomarkers by imaging the end results of these markers on the atherosclerotic plaque. This tool may also allow us to study the relationship of emerging

imaging biomarkers, such as PVAT, with lipid content to add a mechanistic understanding on the progression of atherosclerosis. Overall, such tool will enhance our understanding on how LDL-c reduction is translated into improved cardiovascular outcomes.[148] Novel quantitative mapping techniques may provide solutions and overcome the limitations associated with available vascular imaging tools.

1.3.6 Plaque lipid quantification and quantitative T2 map

Recently Chai *et al*. validated a new non-invasive technique that quantifies lipid at high resolution on a voxel by voxel level based on the physical properties of the tissue in that voxel (T2-mapping).[180] In forty patients with established carotid artery disease, lipid content of explanted carotid artery specimens was directly compared with lipid area derived from T2 mapping technique. Using leave-1-out cross-validation method, a threshold of T2< 42 ms for lipid produced maximal correlation coefficient of R= 0.85 (P< 0.001) against percentage of lipid area on histology. This correlation was similar when using slice or the whole plaque as unit of analysis. More importantly, the association between lipid area on T2 map and histology was identical irrespective of the symptomatic status of the plaque.[180] In addition, T2 mapping demonstrated excellent scan-rescan reproducibility when tested in ten unselected patients. With range of lipid percentage between 5.7% and 36.6%, the intra-class correlation coefficient was 0.89 (95% CI 0.59 to 0.98) with coefficient of variation of less than 10%.[180] This quantitative technique appears to be promising in overcoming the current limitations with the established multi contrast (MC) MRI technique.[43, 180] The need for extensive post-acquisition interpretation and the non-quantitative nature for plaque lipid measurement has limited the widespread application of *in vivo* MC-MRI. Moreover, MC-MRI relies on sufficiently large areas of coalesced lipid to be discernible, such that only a minority of patients can be quantified serially. This was reflected by the large number of excluded

patients using this technique in the ORION and CPC studies.[148, 165] Using new T2 mapping techniques may enable plaque lipid quantification without the need to pre-screen patients for established carotid atherosclerosis. This technique would allow to stage atherosclerotic disease, an approach that is analogous to using imaging to stage cancers. T2 mapping would facilitate identification of plaques with large lipid content at multi vascular territories and patients having these plaques throughout the arterial tree may be candidates for intensive lipid interventions. In line with the concept of staging atherosclerosis, there is increasing evidence highlighting the prognostic role of incorporating risk assessment of non-culprit sites even at the stage of subclinical atherosclerosis. [181-184] The PROSPECT (A Prospective Natural-History Study of Coronary Atherosclerosis) trial demonstrated that non-culprit lesions were unexpectedly responsible for relatively high events (11.6%).[181] Plaque burden of more than 70% was more than five-fold associated with events, highlighting the need to evaluate the pathological process of atherosclerosis across both culprit and non-culprit sites.[181] Similarly data from the PESA (Progression of Early Subclinical Atherosclerosis) study showed that subclinical atherosclerosis, assessed using various non-invasive imaging modalities, was detected in 58% of what was considered to be low risk patients according to the Framingham Heart Study.[184] In 36% of these patients atherosclerotic plaques were observed in at least two different vascular sites (carotid, aorta, ilio-femoral and coronary arteries).[184] Moreover, results from the BioImage (Clinical Study of Burden of Atherosclerotic Disease in an At-Risk Population) study have suggested that asymptomatic patients (average Framingham 10-years risk of 9.2%) with large carotid plaque burden had 2.36-fold increase risk of vascular events over a median follow up of 2.7 years.[183] Therefore, staging atherosclerosis by identifying subclinical plaques improves risk prediction and potentially offers a better tool to distinguish high risk patients.[183] While these studies focused on total plaque burden, plaque composition of non-culprit sites was also shown to have an

important prognostic role.[182] Data from the COLOR (Observational Study of Cholesterol in Coronary Arteries) study demonstrated that almost 60% of the determined future adverse events were related to NIRs-quantified plaque lipid content in the non-culprit coronary vessel with less than 50% degree of stenosis.[182] More importantly plaque lipid content continued to show its significance role of future events even after adjusting for plaque burden reinforcing the significance role of quantifying plaque lipid content.[121, 126]

The ability to quantify plaque lipid accurately and reproducibly with high sensitivity opens several new opportunities. Firstly, it will be possible to determine the extent and distribution of atherosclerotic plaque lipid and to establish whether some patients have a propensity to develop lipid-rich plaques throughout the arterial tree. Such patients may be candidates for new lipid-lowering drugs, such as PCSK9 inhibitors. Secondly, direct quantification of lipid will allow the effects of intensive LDL reduction to be monitored serially with much greater precision than has been possible previously. This would allow to establish those patients with poor response to high intensity statin therapy and offer an opportunity to add a second lipid-lowering drug. Thirdly, the ability to quantify both baseline plaque lipid content and its change over time will allow the functional significance of potential blood biomarkers to be evaluated for the first time. These measures are of current and real importance since drugs targeting some of these molecules and pathways are already in advanced stages of clinical development. Finally, accurate measurements of plaque lipid content will enable investigation of the relationship with new imaging biomarkers like PVAT. The proposed role of PVAT in promoting atherosclerosis maybe better recognised in vascular segments adjacent to areas of PVAT reflecting an accelerated atherosclerotic process. T2 mapping MRI technique would be a useful technique in identifying these segments by quantifying lipid contents as surrogate of advanced atherosclerosis.

1.4 Summary

Statins have been shown repeatedly to be effective in reducing cardiovascular events. That their benefits are extracted through LDL-c reduction has been reinforced by recent trials of ezetimibe and evolocumab, in which additional LDL-c reduction was associated with a further and proportionate reduction in cardiovascular events. Remarkably, however, it is still unclear precisely *how* reducing LDL-c translates into improved cardiovascular outcomes. One prevalent theory is that LDL-c reduction leads to evacuation of cholesterol from atherosclerotic plaque and that this in turn has a 'stabilising effect' on plaques, which are then less likely to precipitate acute events.

However, until recently, tools have not been available to test this in humans *in vivo*. As new biological agents targeting LDL-c are already in late phase clinical trials, it will be important to establish (a) the individual variation in plaque lipid content, and whether this might be a useful metric in guiding patient selection; (b) whether LDL-c reduction really does affect plaque lipid content; (c) whether contemporary blood biomarkers do or do not reflect both plaque lipid content and propensity to change on treatment and (d) to evaluate the relationship between lipid accumulation as the cornerstone of atherosclerosis and PVAT as a new determinant of promoting atherosclerosis. The development and validation of a new quantitative T2 mapping technique allows accurate, reproducible, systematic volumetric quantification of plaque lipid on a voxel-by-voxel basis. Accordingly, I seek to apply this new technique to resolve these important questions.

1.5 Hypotheses

Using newly validated T2 mapping MRI technique the tested hypotheses are: (1) plaque lipid volume and distribution can be systematically evaluated in patients with clinical evidence of

atherosclerosis in order to identify those with propensity to develop lipid-rich plaques in multi-sites vascular territories; (2) lipid content quantified on T2 mapping is reduced following intensive LDL-c reduction; (3) blood lipid biomarkers are dissociated from plaque lipid content and its propensity to change on therapy; and (4) plaque lipid content is influenced by perivascular adipose tissue and there is a spatial relationship between PVAT and plaque lipid content.

1.6 Experimental aims

Chapter 3: To systematically study the volume and distribution of carotid plaque lipid content in patients scheduled for carotid endarterectomy and to compare lipid content between symptomatic and asymptomatic carotid patients.

Chapter 4: To compare the effects of intensive LDL-c reduction on carotid plaque composition after three months of high intensity statin treatment by quantifying plaque lipid and fibrous contents at both baseline and follow up time points.

Chapter 5: To study the relationship between carotid plaque lipid content and blood lipid biomarkers and to establish if changes in carotid lipid content with statin treatment is related to baseline blood biomarkers or their tendency to change following treatment.

Chapter 6: To establish if PVAT had any local impact on plaque lipid content by evaluating the spatial relationship between PVAT and vessel wall lipid content.

Chapter 2: General Methodology

2.1 Ethics

All research was carried out according to the principle of the Declaration of Helsinki. Ethical approval was obtained from National Research Ethics Services (NRES) (16/SC/0102/ 178653) and local R&D committee within the Oxford University Hospitals prior to commencement of the study. All participants gave their written informed consent.

Prospective patients were identified by their direct clinical care team, who have been appraised of the study target population and the study aims. Potentially interested patients were approached by the author. Patients with acute coronary syndromes were approached during their inpatient stay while those scheduled for endarterectomy were contacted once they have been listed for their procedure.

The participants were allowed as much time as they wished to consider participation in the study, and the opportunity to question the author, their GP or other independent parties to decide whether they want to be recruited to the study. It was clearly stated that the participant was free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

2.2 Eligibility Criteria

The following generic eligibility criteria were applied to the recruited participants in all of the presented studies:

Inclusion Criteria

Participants must satisfy the following conditions:

1. Participant is willing and able to give informed consent for participation in the study.
2. Male or female, aged 18 to 90 years.

3. Scheduled for coronary angiogram to investigate an elevated cardiac biomarkers (Troponin T > 0.03 ng/ mL as defined nationally and by the local biochemistry department within the John Radcliffe Hospital) .
4. Not on statin treatment before study participation (statin was introduced as part of clinical care as atorvastatin 80 mg daily)

   OR

1. Participant is willing and able to give informed consent for participation in the study.
2. Male or female, aged 18 to 90 years.
3. Scheduled for carotid endarterectomy irrespective of the symptomatic status of the participant.

Exclusion Criteria

The participant may not enter the study if ANY of the following are known to apply:

1. Patients in whom safety or clinical concerns preclude participation.
2. Pregnant or breast feeding female.
3. Claustrophobia.
4. Standard contra-indications to MRI such as pacemaker implantation or retained ocular metallic fragments.
5. Participants who cannot lie flat on the scan table.

2.3 Study Population

The total number of study population was one hundred and nine, comprising three different studies. This population was a combination of 27 (25%) ACS patients and 82 (75%) carotid endarterectomy scheduled (CEA) patients. ACS was defined according to the third universal definition of myocardial infarction.[185] In details, subjects presenting with chest discomfort

associated with electrocardiogram (ECG) changes and/ or rise in blood biomarker i.e. troponin as defined in the inclusion criteria were recruited. CEA patients were either asymptomatic with no previous documented clinical symptoms or symptomatic of CVA or transient ischaemic attack (TIA). Carotid stenosis was considered significant if the degree of stenosis on carotid duplex ultrasound scan (USS) was 50-99% according to NASCET (North American Symptomatic Carotid Endarterectomy Trial),[186] or 70-99% according to ESCT (European Carotid Surgery Trial) criteria.[187]

Patients' demographics and medical history, including past medical history, list of medications and clinical procedures were obtained from patient's interview and the relevant sections of the medical notes or the local electronic databases.

Patients underwent at least one carotid MRI scan at the Acute Vascular Imaging Centre (AVIC). Based on the study aim, a second follow up carotid MRI scan was also planned.

In one study blood samples were collected at the two respective time point (baseline and follow-up) and were prepared under standard laboratory protocols. The average volume of blood samples for research analyses taken at each sampling point was 30- 35 ml. Blood and its derived components were stored in aliquots in appropriate conditions for further analyses.

Below is a schematic graph demonstrating the different studies with various time points, including imaging and blood sampling procedures.

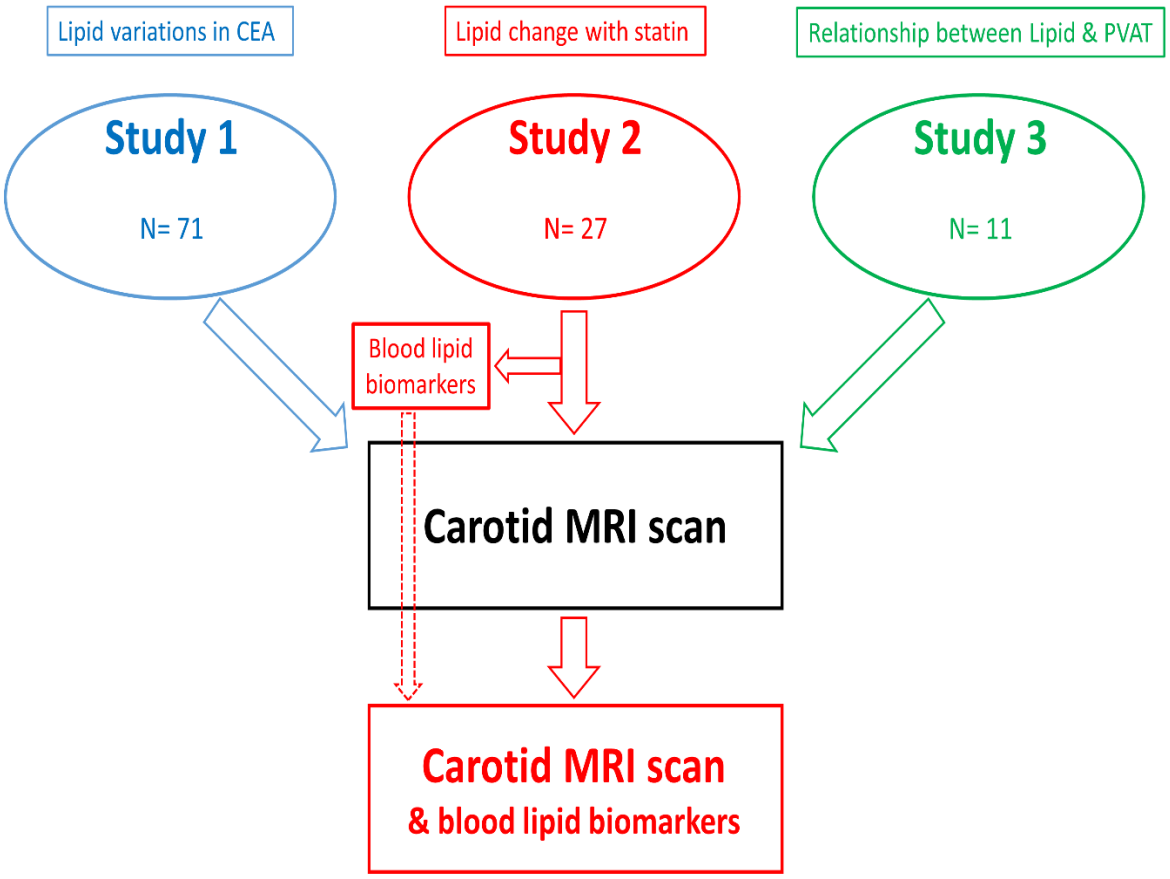


Figure 2.1 Schematic flow chart of study population

Three studies, colour coded, to summarise patients' flow chart in each individual study. The first study investigated the lipid variations of right and left carotid arteries in 71 CEA patients. The second study evaluated changes in carotid lipid content in response to LDL-c reduction via statin treatment in 27 ACS patients. The third study assessed the relationship between PVAT and carotid lipid volume in 11 CEA patients. Only participants in Study 2 underwent two MRI scans coupled with two blood samplings at the same time points, while the other two studies (1 & 3) involved a single MRI scan for each participant.

2.4 MRI protocol

Patients were imaged on a Verio 3T scanner (Siemens Healthcare, Erlangen, Germany) with either a 4-channel phased-array carotid coil (Machnet, Roden, the Netherlands) or a 10-channel phased-array carotid coil (Pulseteq, Surrey, UK). Bright-blood time-of-flight (TOF) angiography of the carotid arteries was acquired using 3D Fast Low Angle Shot (FLASH) with flip angle equal to 25º, TR = 20 ms, TE = 3.3 ms, Field of view (FOV) = 200 X 150 mm^2, matrix size = 384 X 288 and slice thickness = 1mm. Consecutive slices of TOF were obtained to localize carotid bifurcation and atherosclerotic plaque.

2.4.1 Carotid atherosclerosis

Assessment of atherosclerosis was performed using T1-weighted turbo spin echo (TSE), cross-sectional images of both carotid arteries (sequence parameters: TR 1280 ms, TE 13 ms, resolution in plane 0.29 X 0.29 mm, Field of view (FOV) 150 X 150 mm, slice thickness 2 mm, non-breath-hold imaging) and T2-weighted TSE cross sectional images (sequence parameters: TR 2000 ms, TE 59 ms, resolution in plane 0.29 X 0.29 mm, FOV 150 X 150 mm, slice thickness 2 mm, non-breath-hold imaging).

Evaluation of the atherosclerotic disease along the acquired cross-sectional images of the carotid arteries included the common carotid, the bifurcation, and the internal carotid arteries (Figure 2.2).

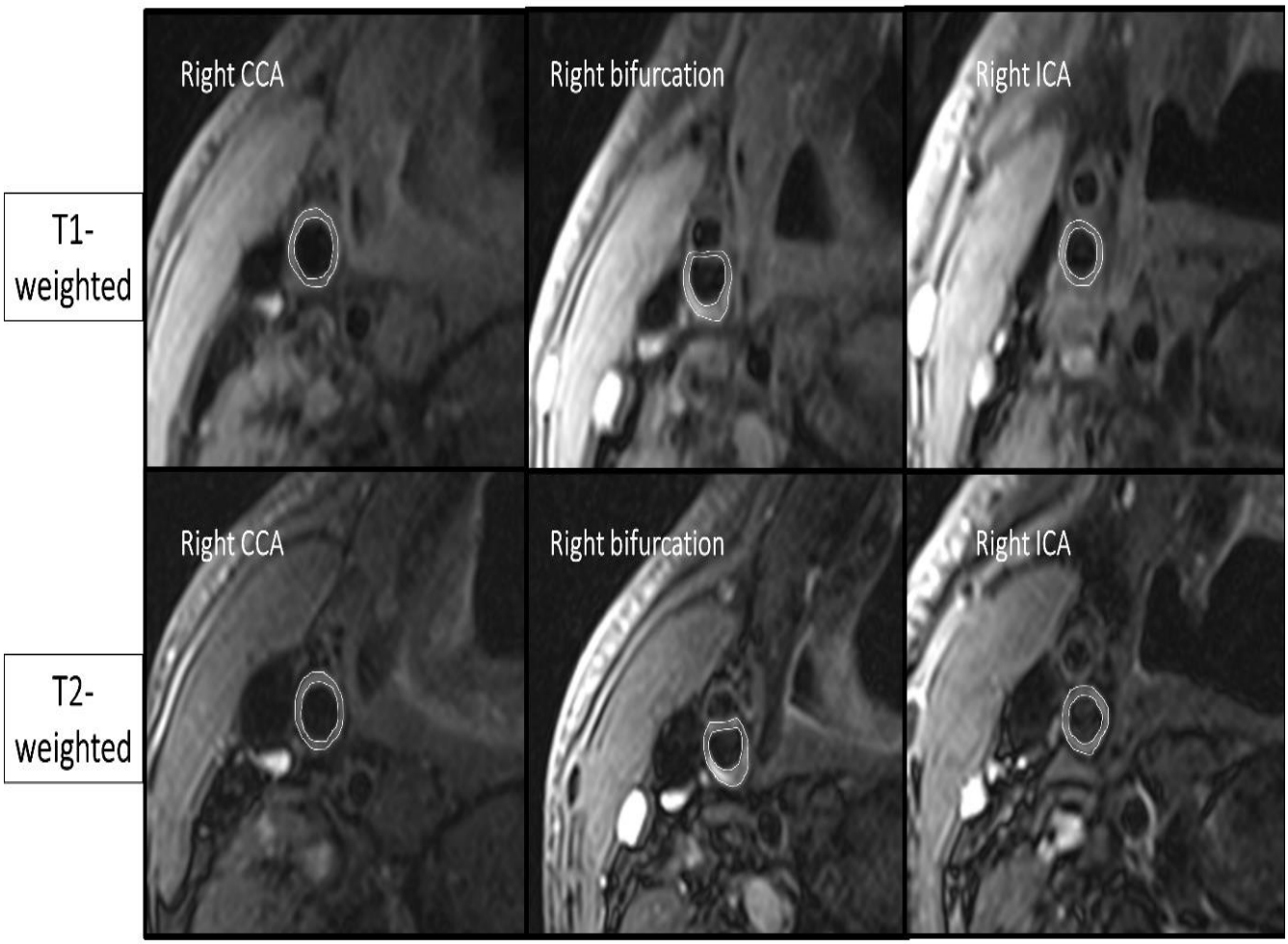


Figure 2.2 Assessment of atherosclerosis burden

Both T1-weighted (upper panel) and T2-weighted (lower panel) sequences where used to assess vessel wall thickness (vWT) as marker of atherosclerosis burden. This was evaluated across all slices and demonstrated in this figure for CCA (common carotid artery), carotid bifurcation, and ICA (internal carotid artery).

2.4.2 Plaque characterisation on T2 mapping

Multi-slice carotid T2 maps were generated from 14 images with incremental echo times TE = 9-127 ms, acquired using a DANTE-MESE sequence that combined black-blood preparation based on non-selective Delay Alternating with Nutation for Tailored Excitation (DANTE) pulse trains with chemical-shift-selective fat saturated Multiecho Spin-Echo (MESE) (sequence parameters: TR 2000, TE 9-127 ms, resolution in plane 0.33 X 0.33 mm, FOV 128 X 128 mm, slice thickness 2 mm, slice gap = 2 mm, non-gated ECG and non-breath-hold imaging).[167, 188]

Initially five slices of DANTE-MESE were acquired and subsequently the sequence was repeated with 2 mm off-set yielding a total of 10 interleaved slices, each 2 mm thickness spanning the carotid artery with total coverage of 2 cm.[167, 188] Slices were centralised on the maximum plaque volume identified on TOF and T1 weighted images and the off-set level was adjusted up or down so full plaque length was covered within 10 slices. If no plaque was identified, slices were centralised on the carotid bifurcation.

Each DANTE-MESE sequence was acquired in almost 4 minutes, with total acquisition time of 8 minutes to obtain the 10 interleaved slices from two different consecutive sequences. During the 4 minutes scanning time, subjects were asked to avoid any neck movements, including swallowing, given the susceptibility of DANTE-MESE sequence for movement artefacts. Patients had a standardised position with shoulders pulled-down and full neck extension. In addition, a customised neck brace was applied to aid patients and minimise neck movement.

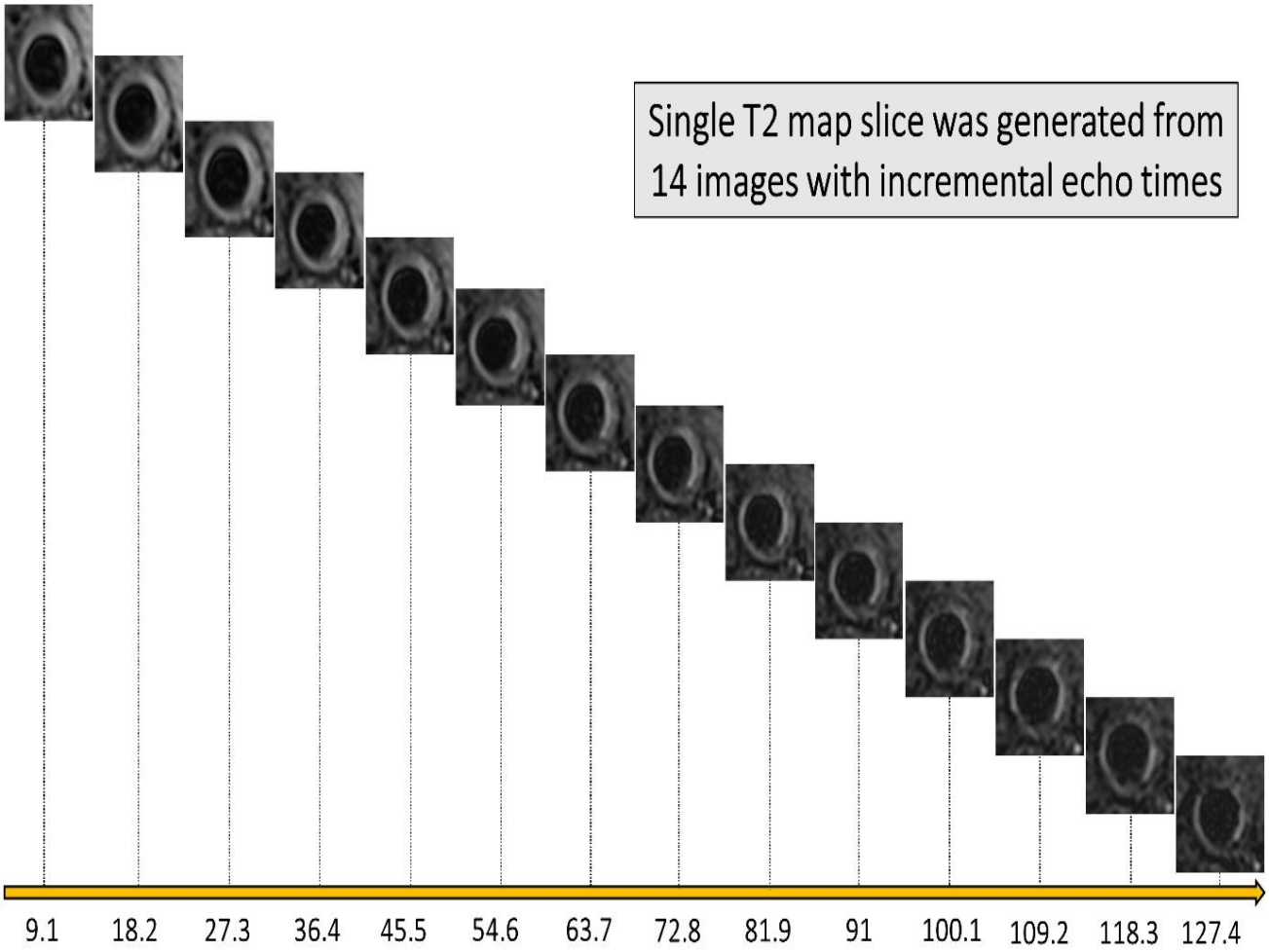


Figure 2.3 T2 mapping sequence acquisition using different echo times

An individual slice was generated from 14 images with incremental echo times. For each echo time, starting from 9 ms, the signal intensity of each voxel in these images was fitted to a mono-exponential decay curve ($SI = \beta \cdot e^{-TE/T2}$) to derive T2 constant of that individual voxel, where SI is the signal intensity, β includes the effect of proton density, T1 weighting, coil sensitivity and signal amplification.[167] The process was repeated for different echo time until reaching 127 ms.

2.4.3 Perivascular adipose tissue

Perivascular adipose tissue was detected using a dedicated water-suppressed MRI sequence to saturate fat. Given the close proximity of PVAT and vessel wall, such sequence will be useful in delineating adipose tissue from vessel adventitia. PVAT slices were generated using TSE black blood fat saturated (sequence parameters: TR 750, TE 12, FOV 150 X 150, in plane resolution 0.39 X 0.39 mm, slice thickness 3 mm). This sequence yielded 13 cross-sectional images of the carotid arteries and spanned more than 2.5 cm of the carotid artery. In order to match PVAT slices with DANTE-MESE ones, slice thickness was dropped to 2 mm but with an increase in oversampling of 60% yielding a very good signal to noise ratio. PVAT slices were centred on the first generated slices of DANTE-MESA sequence so slices of T2 mapping and PVAT sequences were matched up. Fat saturated sequence yields raw images which were subsequently processed to identify perivascular adiposity using newly devised methodology (Figure 2.4) (see Chapter 6 for further details).

Acquired magnetic resonance sequences are summarised in Table 2.1. T2 weighted sequence was not routinely obtained since it could be reconstructed from T2 mapping sequence if necessary. Similarly, proton density was not acquired as it had no role in the tested hypotheses and it would add relatively significant scanning time in patients presenting with acute vascular syndromes.

2.4.4 Follow up MRI

In Study 2, subjects were scheduled for follow up MRI scan. The protocol of this scan was similar to the baseline MRI. In summary, bright blood TOF angiography sequence of the carotid arteries was acquired and followed by T1-weighted sequence to localize carotid bifurcation and atherosclerotic plaque. Combing cervical vertebras with carotid bifurcation as

internal landmarks, baseline and follow-up T1-weighted slices were matched and used as a framework to generate T2 map slices. Left and right carotid arteries were matched separately to account for any subtle difference in patient's neck position between baselines and follow up.

Table 2.1 Summary of acquired magnetic resonance sequences

Acquired sequence	Magnetic resonance parameters
Time-of-flights (bright blood)	Flip angle equal to 25º, TR = 20 ms, TE = 3.3 ms, Field of view (FOV) = 200 X 150 mm^2, matrix size = 384 X 288 and slice thickness 1mm
T1 weighted (turbo spin echo)	TR 1280 ms, TE 13 ms, resolution in plane 0.29 X 0.29 mm, Field of view (FOV) 150 X 150 mm, slice thickness 2 mm.
T2 mapping (Delay Alternating with Nutation for Tailored Excitation (DANTE) pulse trains with chemical-shift-selective fat saturated Multiecho Spin-Echo (MESE)	TR 2000, TE 9-127 ms, resolution in plane 0.33 X 0.33 mm, FOV 128 X 128 mm, slice thickness 2 mm, slice gap = 2 mm. The sequence was repeated to cover these gaps in an interleaved fashion.
Fat saturated (turbo spin echo black blood fat saturated)	TR 750, TE 12, FOV 150 X 150, in plane resolution 0.39 X 0.39 mm, slice thickness 3 mm

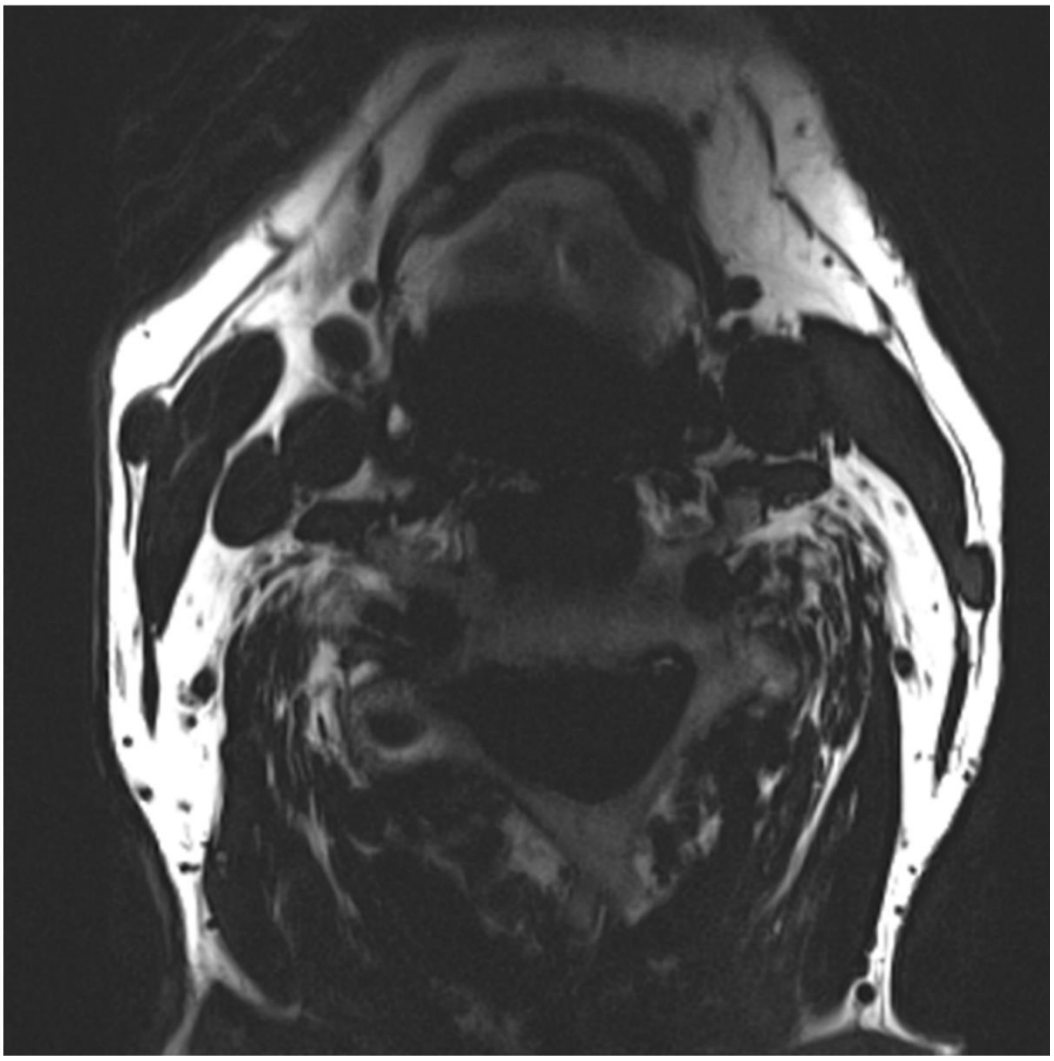


Figure 2.4 Raw image of carotid perivascular adipose tissue

Dedicated water suppression sequence was used to detect adipose tissue in the perivascular and subcutaneous locations. The increase signal intensity within the raw image represents perivascular and subcutaneous adipose tissue. Structures with significant water content, such vessel wall, are suppressed and appear black with low signal intensity. Further processing was required to quantify PVAT from this raw image (see Chapter 6).

2.5 Image analysis

Analyses of Carotid atherosclerosis and plaque composition were implemented in Matlab (MathWorks, Natick, USA). Lipid distribution within the atherosclerotic plaque was executed using ImagePro Plus software (Media Cybernetics, Rockville, USA).

2.5.1 Carotid atherosclerosis

Cross-sectional images of the carotid artery were computed to produce a binary image matrix of the vessel wall area and the background. Inner and outer boundaries were extracted and the total number of pixel were calculated to yield vessel wall area (vWA). Radii-based method was applied to compute distance between vessel centre and the outer boundary. Similarly the distances between vessel centre and the inner boundary were calculated using same number of radii with the difference in measured distance to determine vWT. Using a modified American Heart Association (AHA) classification of carotid vessel wall,[152] the obtained slices were categorised into '*normal*' corresponding to type I and II and '*abnormal*' for type III onward of AHA classification (Table 2.2).

The conventional AHA classification comprised eight types of the carotid plaque and is based on the histopathological classification described by Stary *et al*. in 1995.[189] The modifiable approach by Cai *et al*.[152] was proposed to overcome challenges related to MRI spatial resolution to differentiate type I from type II and to account for the limitation of MRI in discriminating proteoglycan (type IV) from dense collagen (type V) fibrous cap (Table 2.2). The adopted dichotomised classification of normal *versus* abnormal vessel wall was reliant on the structural abnormalities of the vessel wall rather than the compositional one. This negated the need to obtain other sequences, such as proton density.

To ensure objective analysis, vessel wall thickness of each slice was plotted against the lumen perimeter for every individual radii to identify regions of eccentric thickening (Figure 2.5). This process was repeated for every slice of both right and left carotid arteries, yielding an average of 20 slices per patient.

Table 2.2 Carotid vessel wall Classification using conventional and modified approach

<table>
<tr><th>Conventional AHA Classification</th><th>Modified AHA Classification for MRI</th><th>Adopted classification</th></tr>
<tr><td>Type I Discrete foam cells</td><td rowspan="2">Type I-II Near- normal wall thickness, no calcification</td><td rowspan="2">Normal</td></tr>
<tr><td>Type II Fatty streak</td></tr>
<tr><td>Type III pre-atheroma with extracellular lipid pools</td><td>Type III Diffuse intimal thickening or small eccentric plaque</td><td rowspan="6">Abnormal</td></tr>
<tr><td>Type IV atheroma with a confluent extracellular lipid core</td><td rowspan="2">Type IV-V Lipid rich necrotic core plaque</td></tr>
<tr><td>Type V Fibro atheroma</td></tr>
<tr><td>Type VI Complex plaque with possible surface defect, haemorrhage or thrombus</td><td>Type VI Complex plaque with possible surface defect, haemorrhage or thrombus</td></tr>
<tr><td>Type VII Calcified plaque</td><td>Type VII Calcified plaque</td></tr>
<tr><td>Type VIII Fibrotic plaque</td><td>Type VIII Fibrotic plaque</td></tr>
</table>

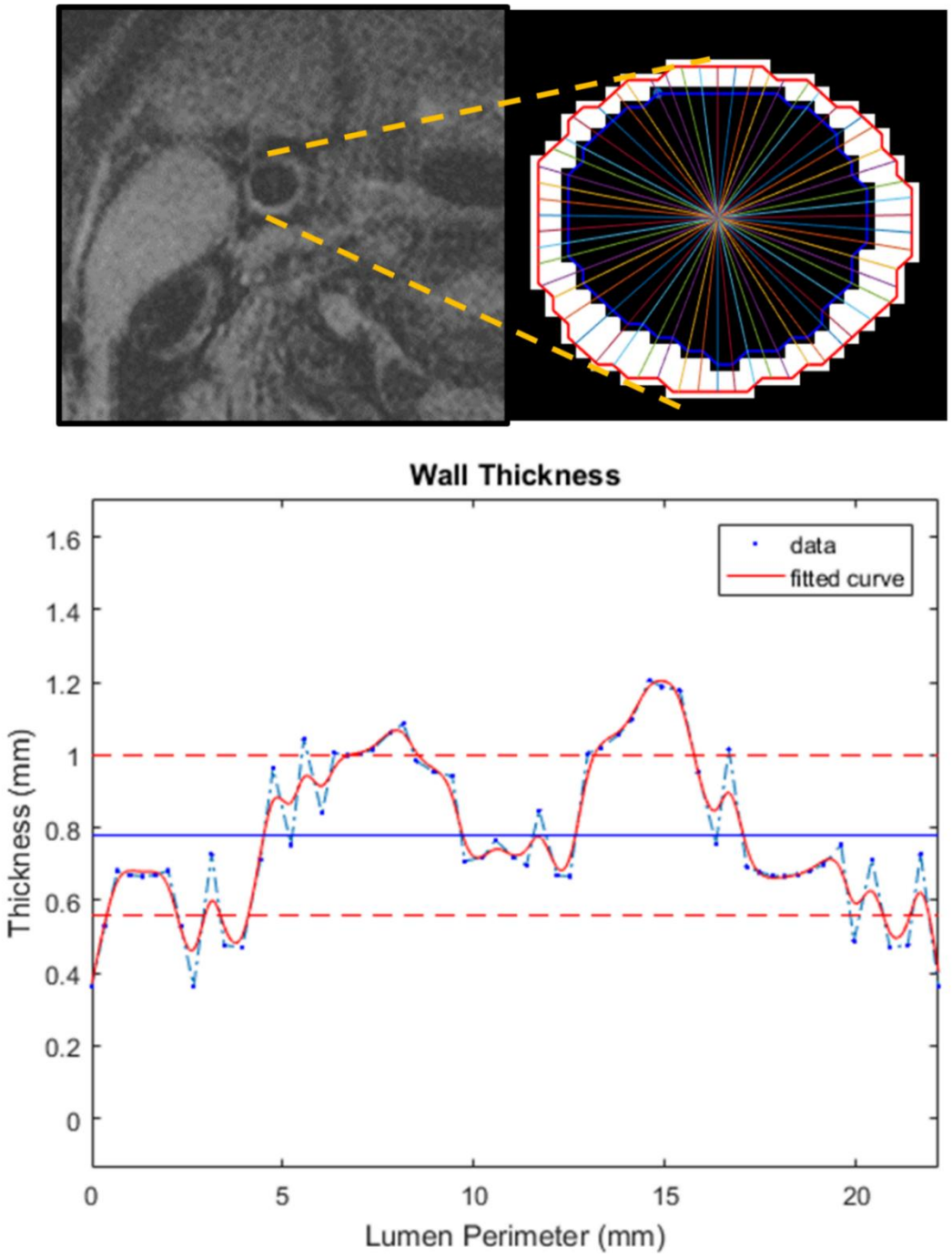


Figure 2.5 Carotid atherosclerosis assessment

Carotid vessel wall was binary segmented and vWT was subsequently calculated as the average distance difference between inner and outer boundaries of the vessel. The lumen perimeter was plotted against the vessel thickness highlighting the near-normal classification (type I-II) of the carotid slice.

2.5.2 T2 mapping image analysis

DANTE-MESE sequence yields 10 interleaved slices for each carotid vessel with total of 20 slices per patients, which were processed individually in Matlab. A region of interest (ROI) was selected to include the vessel wall and the adjacent sternocleidomastoid muscle. T2 relaxation times were calculated for every voxel within ROI and producing a colour map image of the raw T2 map. This map was generated on voxel-by-voxel using mono-exponential nonlinear fitting decay curve (Figure 2.3).[167] The signal intensities of an individual voxel was derived from the corresponding 14 echo times which were used to calculate the T2 time constant of that particular voxel. This T2 time constant reflects the decay curve of the protons within this voxel at a given field strength and is affected by the physical property of the tissue occupying that voxel, thus, mapping the vessel wall according to its components.

The mapped circular vessel structure was subsequently 'unwrapped' to segment both lumen and external vessel boundaries using a validated semi-automated procedure.[190] Using Black blood technique to suppress luminal signal aided differentiating lumen from vessel wall and subsequently tracking lumen edge. Similarly external boundaries were strictly contoured using raw mapping images to ensure accurate inclusion of the vessel wall. Voxels between external and luminal border were finally segmented using a validated segmentation method to identify lipid. This segmentation method used T2 threshold of less than 42 ms (Figure 2.6).[180]

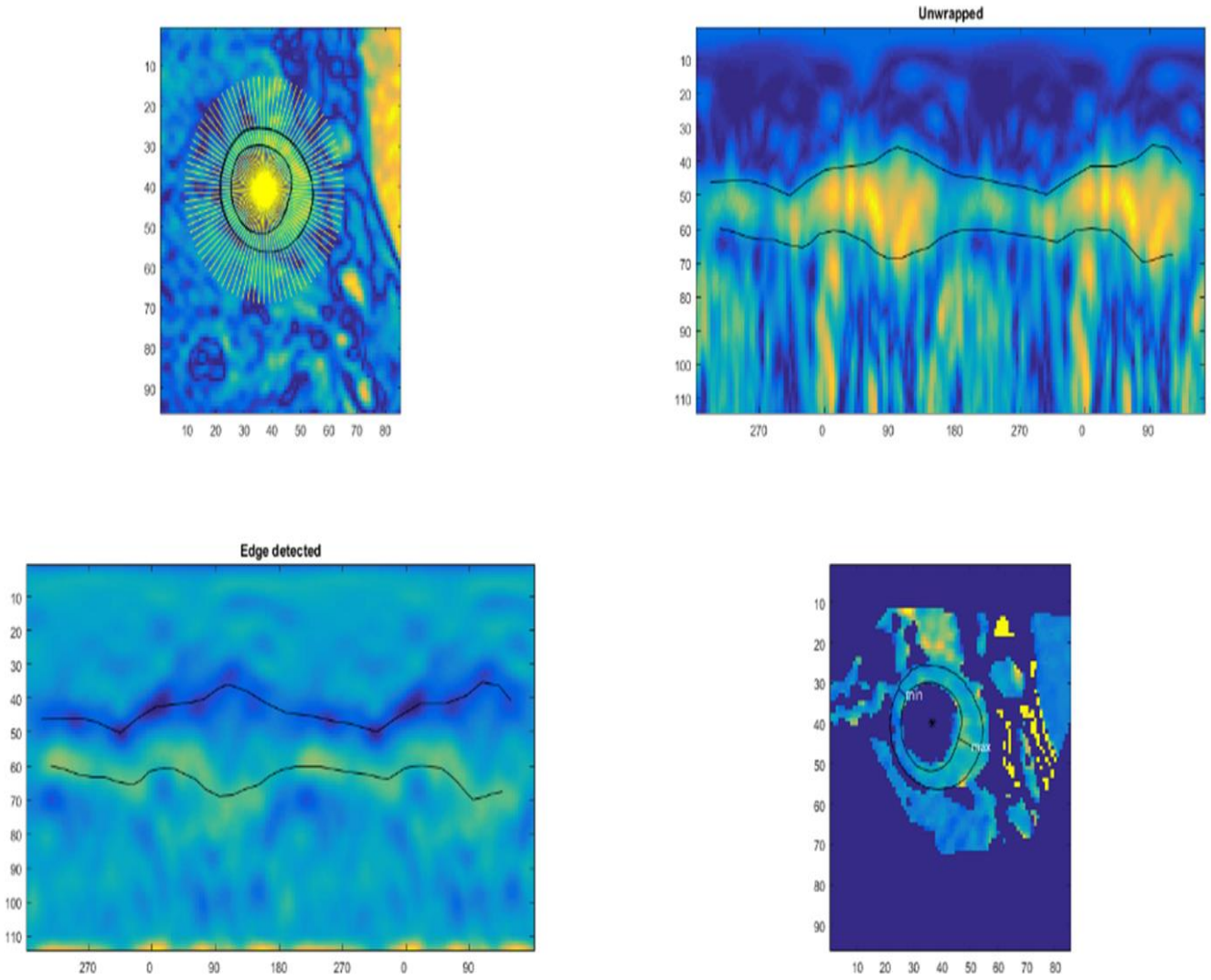


Figure 2.6 Inner and outer boundaries segmentation of carotid vessel

Top left top panel shows the selected ROI including the sternocleidomastoid muscle and the vessel wall. Each voxel within the ROI had its T2 relaxation time calculated using a mono-exponential non-linear fitting curve of the 14 incremental echo times. Accordingly, a colour map was produced based on the T2 relaxation time of each voxel. The top right panel demonstrates the process of 'unwrapping' the vessel, where both external and luminal boundaries were segmented to include all voxels of the vessel wall. The bottom right panel displays the included area of ROI for final lipid segmentation.

Lipid content and plaque volume were quantified using a single voxel as one unit (0.33 x 0.33 x 2 mm) where the thickness of the slice was 2mm. Voxels less than 42 ms threshold were grouped together to quantify lipid volume, according to the validated segmentation method.[180] Lipid and plaque volumes of the studied vessel were calculated using the average lipid and plaque volume in all the 10 studied slices, respectively, and irrespective of the degree of stenosis (unit of analysis) as previously described.[81] Lipid percentage per vessel was calculated using this formula, as previously described,[148]

Lipid percentage = (Lipid volume / vessel wall volume) 100*

For analysis per patients, an average of right and left carotid values was calculated as previously described.[154]

2.5.3 PVAT image analysis

PVAT analysis was performed using image intensity threshold method where segmentation of PVAT was dependent on the mean signal intensity of the acquired raw image (see Chapter 6 for further details).

2.6 Blood analyses

Blood samples were obtained via venepuncture using winged butterfly system (vacuum extraction) and standard laboratory tubes. Serum and EDTA-plasma were centrifuged for 15 minutes at 3,300 x g at 4^{o} C within one hour of blood collection and stored in aliquots at −80^{o} for future analyses. These analyses were performed according to standard laboratory protocols and are summarised in Table 2.3. Some of these blood lipid biomarkers demonstrated to have a significant role in future cardiovascular events (Table 2.4).

All blood lipid analyses, except for NMR spectroscopy, were performed by the author of this thesis with support from Dr Akbar (post doc within the group).

Table 2.3 Summary of analysed blood lipid indices with their corresponding technical methodology

Name of laboratory test	Methodology
Total cholesterol	Nuclear magnetic resonance spectroscopy
LDL-c	Nuclear magnetic resonance spectroscopy
HDL-c	Nuclear magnetic resonance spectroscopy
Triglycerides	Nuclear magnetic resonance spectroscopy
Lipoprotein (a)	Enzyme-linked immunosorbent assay
Apolipoprotein A1	Enzyme-linked immunosorbent assay
Lipoprotein particle size	Nuclear magnetic resonance spectroscopy
Lipoprotein particles concentration	Nuclear magnetic resonance spectroscopy
High sensitive CRP	Enzyme-linked immunosorbent assay
PCSK9	Enzyme-linked immunosorbent assay
Cholesterol efflux capacity	Fluorescence-labelled cholesterol
Cholesterol ester transfer protein activity	Fluorometric method

Table 2.4 Role of functional blood lipid biomarkers in lipid metabolism and future cardiovascular events

Functional lipid marker	Role in lipid metabolism	Biomarker prognostic role
LDL particles	Number of particles that are carrying mass of cholesterol by low-density lipoprotein fraction.	There was a direct relationship between LDL particles and incidence of cardiovascular events HR 1.32, 95% CI (1.19, 1.47).[191]
LDL size	The average size of LDL particles related to the compositional variability of transported cholesterol and triglycerides.	Small LDL particles were 3.6-fold increase in the risk of IHD (95% CI, 1.5 to 8.8) compared with those with large LDL particles.[192, 193]
HDL particles	Number of particles that are carrying mass of cholesterol by high-density lipoprotein fraction.	There was an inverse association between HDL particles and cardiovascular events HR 0.75; (95% CI: 0.61 to 0.93).[194]
HDL size	The average size of HDL particles related to the compositional variability of transported cholesterol and triglycerides.	Top quartile HDL size had lower risk of future cardiovascular events than low quartile OR 0.51 (95% CI, 0.39 to 0.67).[195] This risk was abolished when adjusting to apolipoprotein B and triglycerides.
Lp(a)	Lp(a) contains apolipoprotein B-100, linked by a disulfide bond to	Elevated level of Lp(a) was associated with 2.3-fold (95% CI 1.7 to 3.2) of

	apo(a). has enhanced atherothrombotic properties, potentially related to the presence of plasminogen-like kringle structure.[196]	severe coronary stenosis and greater rate of major cardiovascular events (41.8 *vs.* 35.8%, $P = 0.005$).[196]
PCSK9	PCSK9 can bind the hepatocyte LDL receptor leading its degradation in the lysosome and, thus, preventing re-circulation of LDL receptor onto the hepatocyte surface.[197]	Serum PCSK9 level was associated with risk of future cardiovascular events HR 1.48 (95% CI 1.12–1.95).[197]
CETP activity	CETP is a plasma glycoprotein that facilitate bidirectional transfer of cholesteryl esters from HDL to LDL and VLDL in exchange for triglycerides.[198]	CETP activity was inversely related to the incidence of cardiovascular events HR 0.74 (95% confidence interval, 0.57 to 0.90).[198]
HDL efflux capacity	HDL functionality assessment by measuring macrophage-specific reverse cholesterol transport back to the liver.[87]	There was an inverse association between HDL efflux capacity and reduction in cardiovascular events comparing highest versus lowest quartiles HR 0.33; 95% CI, 0.19 to 0.55.[86]

2.6.1 Standard lipid and lipoprotein pleotropic function analyses

Standard lipid measurements (i.e. LDL-c, total cholesterol, HDL-c and triglyceride) in addition to lipoproteins particle size and concentrations were analysed using nuclear magnetic resonance (NMR) spectroscopy.

This was performed using Axinon lipoFIT-S100 system (Numares AG, Regensburg, Germany). 630 µL of serum were used and gently mixed with 70 µL of an internal standard (with reference substances, NaN3 and D2O). From this solution 600 µL were transferred into 5 mm NMR tubes with barcode labelled caps. All NMR spectra were recorded at a temperature of 310 K on a shielded 600 MHz Bruker Avance III HD spectrometer with a 5 mm triple resonance TXI probe head including deuterium lock channel and a z-gradient coil.

2.6.2 Lipoprotein (a) analysis

Lipoprotein (a), Lp(a), was measured in serum using enzyme-linked immunosorbent assay (ELISA) kit from Abcam (Cambridge, UK) and the instructions from manufacturer were strictly followed.

The 10X diluent M concentrate was diluted 1:10 with reagent grade water and, subsequently, 990 µL of the produced 1X diluent M was added to 10 µL of human serum. The diluted serum was then added to 790 µL of 1X diluent M to achieve 1:8,000 concentration of human serum.

50 µL (per well) of the diluted serum and Lp(a) standards were added to the 96 wells which were pre-coated with Lp(a) specific antibody.

Standards were prepared by reconstituting from the provided Lp(a) standard vial to prepare 50 ng/mL and then adding 120 µL of 1X diluent M to achieve decremental concentrations of standard Lp(a) in Eppendorf tubes with the diluent acting as the zero standard (0 ng/ mL).

Following an incubation period of two hours, the samples were mechanically washed (6 times) using 300 µL of 1X Wash Buffer. 50 µL of 1X Biotinylated Lp(a) antibody (detection antibody) was then added to each well and incubated for one hour. The microplate was again mechanically washed using the same protocol.

50 µL of 1X Streptavidin-Perioxidase Conjugate was added to each well and incubated for 30 minutes. Subsequently the unbound conjugates were mechanically washed using the same protocol.

To visualise the Streptavidin-Peroxidase enzymatic reaction, 50 µL of TMB (3,3',5,5'-tetramethylbenzidine) Chromogen Substrate was added to each well and incubated for 10 minutes. TMB was catalysed by Streptavidin-Peroxidase producing a blue colour that changed into yellow after adding 50 µL of acidic stop solution. The density of the yellow colouration was directly proportional to the concentration of Lipoprotein A in each well (Figure 2.7).

This was measured on microplate reader at 450 nm. Further readings were made at 570 nm and were subtracted from those at 450 nm to correct for optical imperfections in the plate. For statistical reasons, all samples and standards were performed in duplicates.

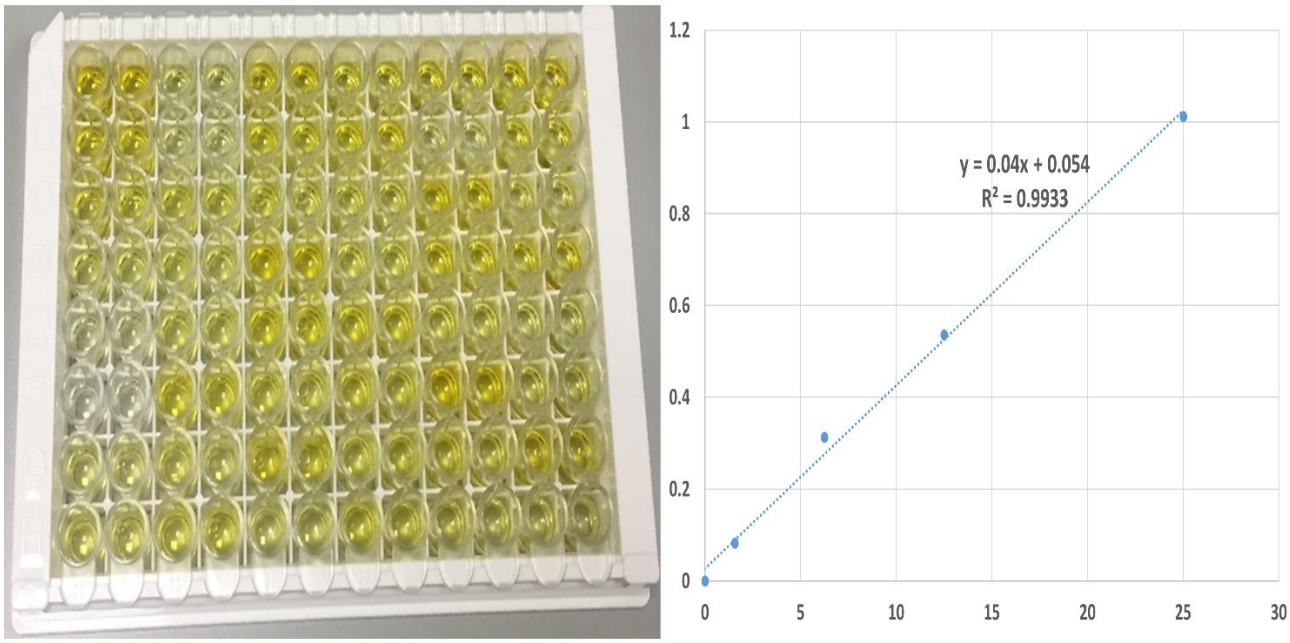


Figure 2.7 Lp (a) measurements from serum human

A: 96 microplate following the addition of the acidic stop solution to visualise Streptavidin-Peroxidase enzymatic reaction changing from blue to yellow. The density of yellow colouration was related to the concentration of Lp (a). Standards were initially prepared and used to construct the standard curve to determine the concentrations of Lp (a) in patients samples performed in duplicates.

B: Standard curve for Lp (a) concentration with best fit line added to the chart to establish the concentration of Lp (a) based on the absorbance reads on the microplate reader. Using standards concentrations with their corresponding absorbance reads, an equation was derived with R^2 value of 0.993.

2.6.3 Apolipoprotein A1 analysis

Apolipoprotein A1 (ApoA1) was determined in plasma by ELISA using kits from R&D systems Ltd (Abingdon, UK).

Samples were diluted to 20,000-fold by adding 20 µL of human plasma to 480 µL of Calibrator Diluent RD5P Concentrate and, subsequently, 20 µL of the diluted plasma were added to 480 µL of the diluent, and, finally, 20 µL of the latest dilute were added to 620 µL of the diluent achieving 20,000-fold dilution.

Human ApoA1 standards were obtained by reconstitute recombinant human ApoA1 with distilled water to produce stock solution pf 1000 ng/mL. Dilution series were achieved by adding 200 µL of Calibrator Diluent RD5P Concentrate to each tube and transferring the mixture to another Eppendorf tube with the diluent acting as the zero standard (0 ng/ mL).

100 µL of buffered protein base with preservative (Assay Diluent RD1W) were added to either 50 µL of diluted plasma or 50 µL of standards on 96 wells plate with pre-coated monoclonal antibody specific for human ApoA1. The mixture was incubated for one hour. Following four mechanical washes with 400 µL Wash Buffer, 200 µL of Human ApoA1 polyclonal antibody conjugated to horseradish perioxdase were added to each well and incubated for 2 hours. Following mechanical washes to remove any unbound antibody-enzyme reagent, 200 µL of stabilised hydrogen peroxide mixed with stabilised chromogen (tetramethylbenzidine) were added and incubated for 30 minutes and protected from light.

50 µL of N sulfuric acid were added to each well and colour density was measured using a microplate reader set at 450 nm. Further readings were made at 570 nm and were subtracted from those at 450 nm to correct for optical imperfections in the plate. All samples and standards were performed in duplicates for statistical reasons (Figure 2.8).

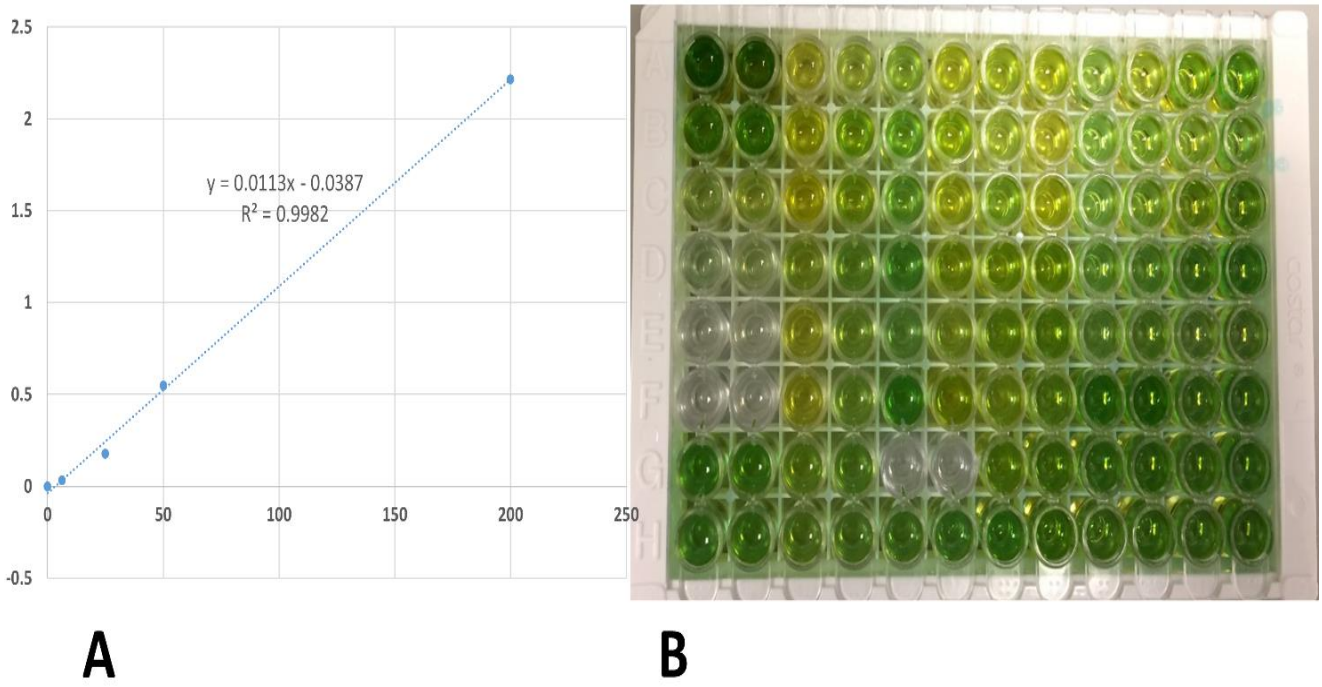


Figure 2.8 ApoA1 measurements from human plasma

A: Standard curve for ApoA1 concentration with best fit line added to the chart to establish the concentration of ApoA1 based on the absorbance reads on the microplate reader. Using standards concentrations with their corresponding absorbance reads, an equation was derived with R^2 value of 0.998.

B: 96 microplate following the addition of sulfuric acid stop solution to visualise substrate solution producing optical density which is proportionate to the concertation of ApoA1. Standards were initially prepared and used to construct the standard curve to determine the concentrations of ApoA1 in patients samples performed in duplicates. Notably, in two wells (one sample) the colour density was less that standard zero and this subject was excluded from any subsequent analyses.

2.6.4 High sensitivity CRP (hsCRP) analysis

hsCRP was measured in plasma by quantitative sandwich ELISA technique using a kit from R&D systems Ltd (Abingdon, UK). Samples were diluted to 100-fold dilution by adding 10 µL of human plasma to 990 µL of 1X Calibrator Diluent RD5P.

The CRP standards were prepared by adding 200 µL of the 1X Calibrator Diluent RD5P to 200 µL of standard recombinant CRP in a buffered protein base with preservatives to produce a stock solution of 25 ng/ mL. Serial dilutions were performed by transferring 200 µL of the mixture to another Eppendorf tube with 1X Calibrator Diluent RD5P serving as the zero standard (0 ng/ mL).

100 µL of Assay Diluent RD1F was added to each well of the 96 microplate with pre-coated monoclonal antibody specific for CRP. Subsequently, 50 µL of either standards or diluted samples were added to each well and incubated for 2 hours at room temperature. At the end of the incubation period, four mechanical washes were performed using 400 µL of Wash Buffer after aspirating each well.

200 µL of CRP Conjugate (monoclonal antibody against CRP conjugated to horseradish peroxidase with preservatives) were added to each well, incubated for 2 hours and subsequently were washed using the same protocol. 200 µL mixture of stabilised hydrogen peroxide and stabilised chromogen (tetramethylbenzidine) were added to each well and incubated for 30 minutes away from light.

50 µL of Stop Solution (N Sulfuric acid) were added to each well and colour density was measured at 450 nm. Further readings at 570 nm were performed and subtracted from the initial readings to correct for optical imperfections in the plate. All samples and standards were performed in duplicates for statistical reasons (Figure 2.9).

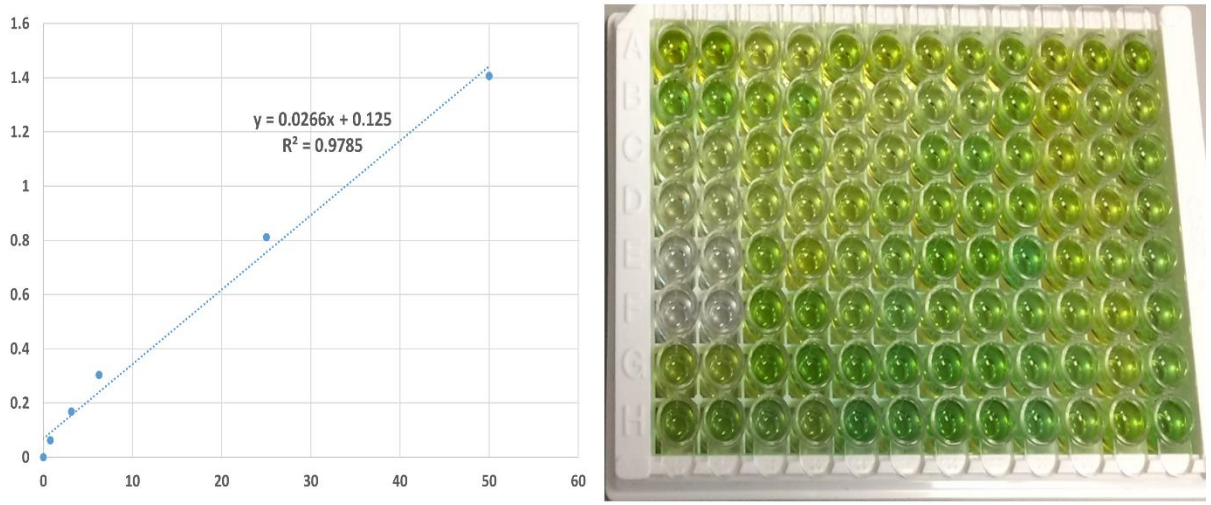


Figure 2.9 hsCRP measurements from human plasma

A: Standard curve for hsCRP concentration with best fit line added to the chart to establish the concentration of hsCRP based on the absorbance reads on the microplate reader. Using standards concentrations with their corresponding absorbance reads, an equation was derived with R^2 value of 0.978.

B: 96 microplate following the addition of sulfuric acid stop solution to visualise substrate solution producing yellow discolouration. The optical density within the wells was proportionate to the concertation of hsCRP. Standards were initially prepared and used to construct the standard curve to determine the concentrations of hsCRP in patients samples performed in duplicates.

2.6.5 PCSK9 analysis

PCSK9 was measured in plasma by ELISa using kit from R&D systems Ltd (Abingdon, UK). Human samples were prepared by adding 20 µL of plasma to 380 µL of 1:5 diluted Calibrator Diluent RD5P achieving 20-fold dilutions.

PCSK9 standards were prepared by reconstituting the human PCSK9 standards with 1:5 diluted Calibrator Diluent RD5P, producing a stock solution of 40 ng/ mL. Subsequently, serial dilutions were performed by adding 250 µL of 1:5 Calibrator Diluent RD5P to the stock solution and transferring the mixture to another Eppendorf tube. The Calibrator Diluent RD5P (1:5) served as zero standard (0 ng/ mL).

Using 96 pre-coated microplate with monoclonal antibody specific for human PCSK9, 100 µL of Assay Diluent RD1-9 were added to each well. 50 µL of either diluted plasma or standards were added and incubated for 2 hours at room temperature. Each well, subsequently, was mechanically washed 4 times with 400 µL Wash Buffer and 200 µL of polyclonal antibody specific for human PCSK9 conjugated to horseradish peroxidase with preservatives were added and incubated for another 2 hours. Following mechanical washes, 200 µL of substrate solution (mixture of stabilised hydrogen peroxide and stabilised chromogen) were added to each well and incubated for 30 minutes away from light.

A Stop Solution of 50 µL of N sulfuric acid were added to each well to stop colour development initiated by the bound antibody-enzyme reagent. Intensity of the developed colour was measured using a microplate reader set to 450 nm. Further readings at 570 nm were obtained and subtracted from the initial readings at 450 nm to correct for any optical imperfections in the plate. For statistical reasons, all samples and standards were performed in duplicates (Figure 2.10).

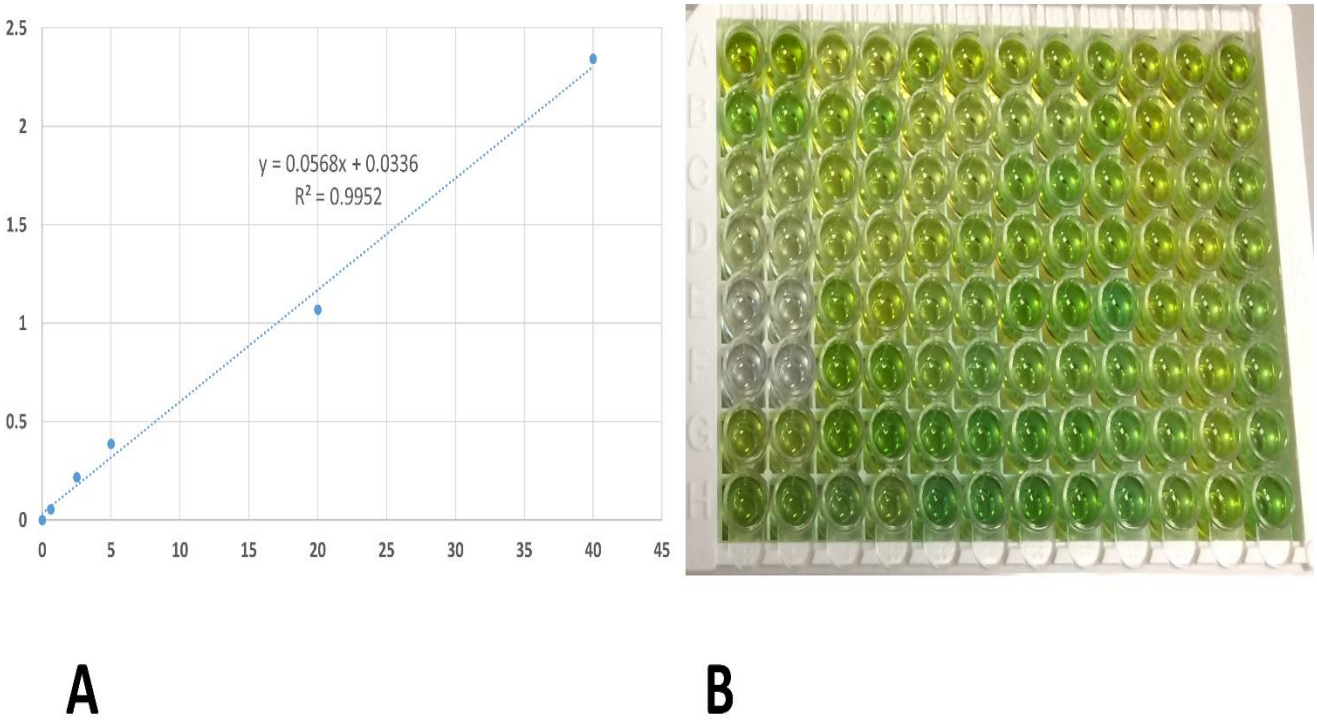


Figure 2.10 PCSK9 measurements from human plasma

A: Standard curve for PCSK9 concentration with best fit line added to the chart to establish the concentration of PCSK9 based on the absorbance reads on the microplate reader. Using standards concentrations with their corresponding absorbance reads, an equation was derived with R^2 value of 0.995.

B: 96 microplate following the addition of sulfuric acid stop solution to visualise substrate solution producing yellow colouration. The optical density within the wells was proportionate to the concertation of PCSK9. Standards were initially prepared and used to construct the standard curve to determine the concentrations of PCSK9 in patients samples performed in duplicates.

2.6.6 Cholesterol efflux capacity analysis

The cholesterol efflux capacity of HDL was determined using fluorescence-labelled cholesterol method as validated previously.[86] J774.1 cells were cultivated in RPMI 1640 medium (Sigma-Aldrich, Poole, UK) supplemented with 10% FBS (fetal bovine serum, Invitrogen, Paisley, UK) and 2-mercaptoethanol (0.05 mM, Sigma-Aldrich, Poole, UK), in a 37^{o}C incubator containing humidified atmosphere of 95% air & 5% CO_2.

Cells were subsequently split under sterile conditions and approximately 1×10^5 J774.1 cells were seeded into a 96 microplate using 100 µL of media per well. These cells were incubated for two hours in a 37^{o}C incubator containing 5% CO2 and subsequently were washed using RPMI 1640 media with no added serum forming a monolayer cells attached to the plates.

Cells labelling was performed by adding 50 µL cholesterol from dimethyl sulfoxide (DMSO) with 50 µL of Equilibration buffer to each cells per well. Following incubation overnight, the labelling reagent was removed and cells were washed by adding 200 µL of serum-free RPMI media to all the wells.

Afterward, serum samples were treated with Serum Treatment Reagent at ratio of 2:5 and incubated for 30 minutes and subsequently centrifuged at 9,000 x g for 10 minutes at 4^{o}C. The apolipoprotein B depleted supernatant was then used to treat the cells after making up its volume to 100 µL using RPMI media. Cells were incubated for 6 hours in a 37^{o}C incubator containing 5% CO2.

The quantity of cholesterol in the supernatant was measured at excitation/ emission of 482/515 nm fluorescence. The monolayer cells were solubilised by adding 100 µL of Triton-X-100 and dithiothreitol (DTT) and shaking the plate for 30 minutes at room temperature. All

wells were pipetted up and down to ensure cell debris are completely dissolve. Cholesterol quantity were measured at the same excitation/ emission wavelengths.

Cholesterol efflux capacity was calculated as the amount of effluxed cholesterol (in the media) determined as fraction of the initial cell cholesterol content (sum media and cell lysate) which was then multiplied by 100 to obtain percentage cholesterol efflux as demonstrated in the equation:

% Cholesterol Efflux =

Fluorescence intensity of the media / (Fluorescence intensity of cell lysate + media) X 100

Below is a schematic figure summarising the steps of measuring cholesterol efflux capacity (Figure 2.11)

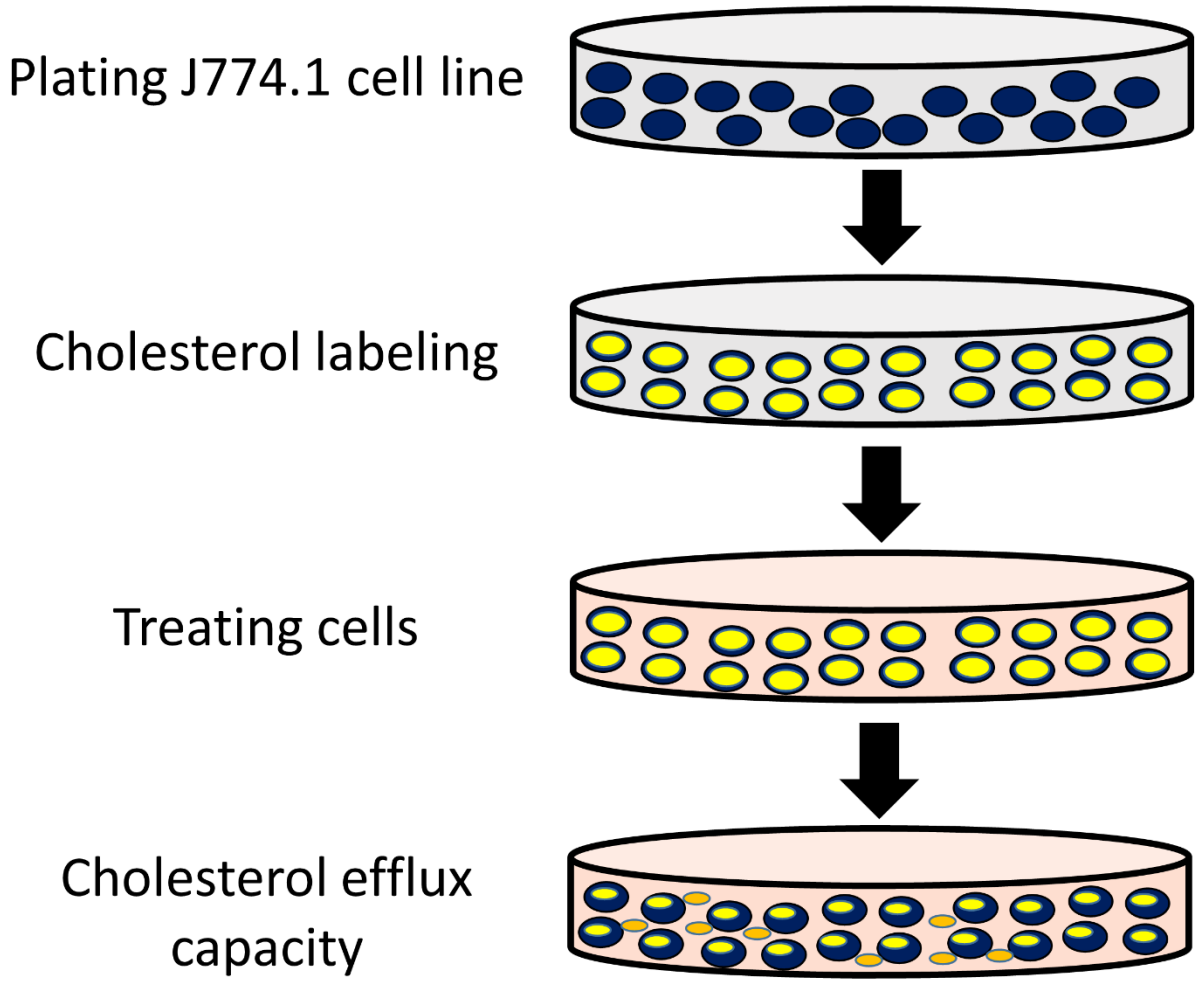


Figure 2.11 Cholesterol efflux capacity schematic diagram

J774.1 cells were initially cultivated and plated at approximately concentration of 1×10^5 per well. Cells were then labelled using fluorescence-labelled cholesterol. Subsequently, cells were treated with cholesterol acceptor using apolipoprotein B depleted serum. Efflux capacity was calculated as the ratio of effluxed cholesterol in the media to the total initial cholesterol quantity (in both cell and media).

2.6.7 Cholesteryl ester transfer protein activity analysis

Cholesteryl ester transfer protein activity was measured with a fluorometric method using a kit from Abcam (Cambridge, UK). In principal, a fluorescent self-quenched phospholipid from a donor molecule is transferred (CETP-mediated) to an acceptor molecule, resulting in an increase in fluorescence detected on microplate reader. The increase in fluoromteric intensity is proportional to the amount of transferred phospholipid.

Samples were prepared by adding 10 µL of human plasma to 90 µL of CETP Assay Buffer (EDTA) to produce 1:10 sample dilution. Standards were prepared by performing serial dilutions of 0.6 pmol Donor Molecule into 500 µL of 10% isopropanol, with isopropanol acting as blank tube (0 pmol Donor Molecule).

5 µL of CETP acceptor molecule (sodium azide and EDTA) were added to each well and the final volume was adjusted to 200 µL by adding CETP assay buffer. All wells were incubated for 30 minutes at 37ºC away from light to stabilise the signal.

The fluorescence signal was measured on microplate reader at Ex/Em 480/510 every three minutes for 140 minutes. Both samples and standards were performed in duplicates for statistical reasons. All mean absorbance values were subtracted from the blank to get the corrected absorbance and were used to obtain the amount of cholesteryl ester transferred by CETP (Figure 2.12). Subsequently, CETP activity was calculated from following:

$$\left(\frac{B}{\Delta T \times V} \right) * D$$

Where B is the amount of cholesteryl ester (donor molecule) from standard curve

ΔT is the reaction time in hours, V is the sample volume added into the reaction in each well (200 µL), and D is the sample dilution factor (1:10)

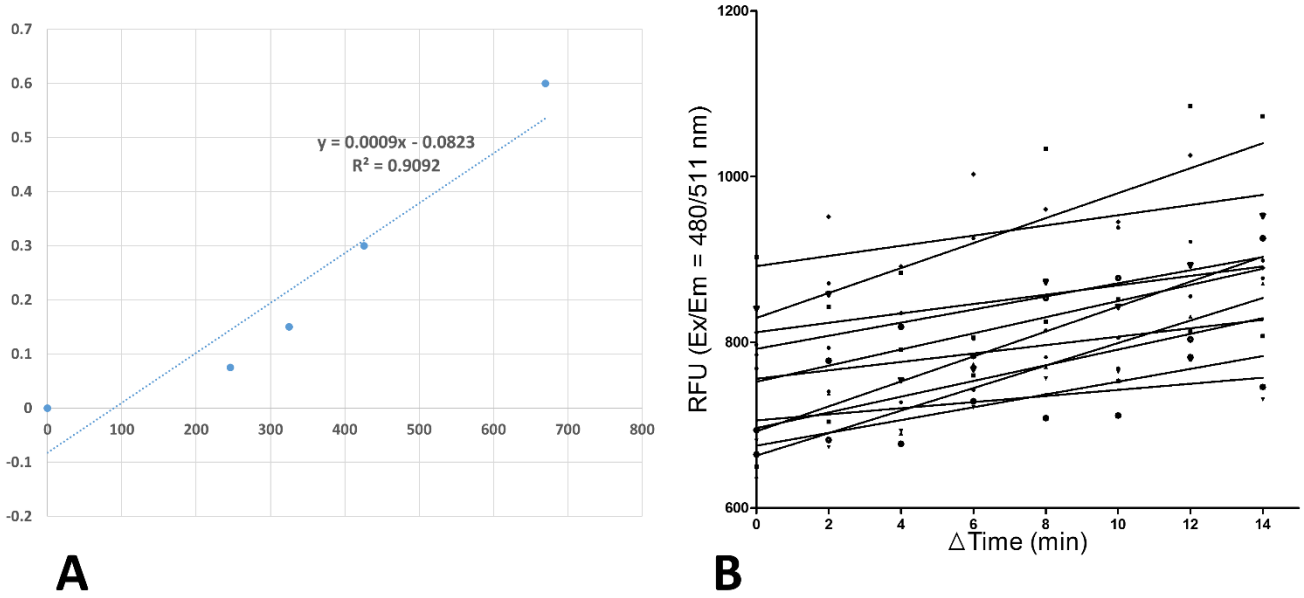


Figure 2.12 CETP activity measurement in human plasma

A: : Standard curve for CETP activity with best fit line added to the chart to establish the activity of CETP based on the absorbance reads on the microplate reader. Using standards concentrations with their corresponding absorbance reads, an equation was derived with R^2 value of 0.909.

B: The increase in fluorescent intensity was proportional to the amount of transferred phospholipid from Donor Molecule to the Acceptor Molecule via CETP over time.

Chapter 3: Quantification of carotid plaque lipid content with magnetic resonance T2 mapping in patients undergoing carotid endarterectomy

3.1 Abstract

Background

Tools to characterise asymptomatic carotid patients are urgently needed to early identify those who may benefit from invasive revascularisation prior to symptoms development. Using the degree of luminal stenosis (DS%) has not been effective, perhaps because this approach ignores the pathological process within wall of the artery. Large lipid content is a recognised feature of vulnerable plaque and a determinant of its behaviour. Therefore, quantifying carotid lipid content may be a promising method to recognise high risk asymptomatic patients.

Methods

Using a highly sensitive T2 mapping MRI technique, lipid quantity and distribution were systematically surveyed in patients scheduled for carotid endarterectomy. Lipid percentage was quantified in 50 carotid endarterectomy patients. Lipid distribution was tested, using two imaging indices (contribution of the largest lipid deposit towards total lipid (LLD %) and a newly-developed LAI 'lipid aggregation index').

Results

The bifurcation of the carotid arteries contained the maximal lipid volume irrespective of DS%. In the whole cohort (irrespective of DS%) and in subjects with DS ≥ 50%, symptomatic patients had a higher lipid percentage compared to asymptomatic ones (P=0.013 and P=0.005, respectively). Lipid distribution varied between symptomatic and asymptomatic groups where both LLD % and LAI were higher in the symptomatic patients (P=0.028 and P= 0.018, respectively) compared to the asymptomatic ones. This suggests that coalesced

lipid deposits were more likely to be associated with symptomatic events for a given plaque lipid content. There was no correlation between DS% measured on ultrasound duplex and plaque volume or lipid content ($r = -0.09$, $P = 0.53$ and $r = -0.05$, $P = 0.75$, respectively). However, there was a strong correlation in carotid plaque and lipid volume between left and right carotid arteries ($r = 0.41$, $P = 0.003$ and $r = 0.5$, $P < 0.0001$, respectively).

Conclusions

The symptomatic status of the carotid artery was associated with plaque lipid content and distribution. Importantly, the degree of luminal stenosis assessed by ultrasound did not reflect carotid plaque volume or lipid content. Plaque lipid content could be useful in guiding decisions for revascularisation options and also in stratification for intensive lipid modification given that some patients have propensity to develop lipid rich plaques in both carotid arteries.

3.2 Introduction

Carotid endarterectomy (CEA) has been established as an effective approach in reducing risk of ischaemic stroke in patients with recent symptomatic carotid artery disease based on two landmark studies, the European Carotid Surgery Trial (ESCT) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET).[186, 187] Timing of surgery and the DS% were key determinants of benefits from CEA in the symptomatic patients.[199] On the other hand, there is ongoing debate about the benefits of CEA in patients with asymptomatic carotid artery disease.[200] The Asymptomatic Carotid Artery Study (ACAS) and the Asymptomatic Carotid Surgery Trial (ACST) have suggested a potential role of CEA in asymptomatic patients with a significant degree of carotid artery luminal stenosis, i.e. > 60-70%.[201, 202] Notably, however, CEA produced a modest AAR of 5-6% in patients with asymptomatic carotid disease compared to 16% in the symptomatic group despite having a similar degree of stenosis.[201-203] Moreover, the reduction in stroke risk with evolving medical therapies has challenged the CEA-related modest AAR and questioned the strategy of unselective revascularisation for asymptomatic carotid artery disease.[9, 200] Despite the low periprocedural stroke risk with revascularisation, for example 2.3% in the CREST (Stenting versus Endarterectomy for Treatment of Carotid Artery Stenosis) trial, there is an important need to justify an invasive procedure given a similarly low annual stroke risk with optimal medical therapy of 1.4%.[200, 204, 205] Since the risk of periprocedural stroke following invasive revascularisation may be comparable to the risk of spontaneous stroke when deferring CEA, there is an increasing need to identify a subgroup of patients with asymptomatic carotid artery disease where early revascularisation may produce larger benefits that would not be offset by the risk of the procedure itself.[200]

Certainly, there is a lack of uniformity in stroke risk in patients with asymptomatic carotid artery disease. While a proportion of asymptomatic patients do not develop symptoms and

may undergo unnecessary procedures, revascularisation using stents or CEA is potentially beneficial in other subgroups within the asymptomatic carotid artery patients. In order to identify asymptomatic carotid patients with high risk of stroke, clinical and imaging features have been tested.[200] The DS% derived from ultrasound duplex has not been effective in stratifying asymptomatic patients and identifying those with high risk of stroke.[200] The lack of relationship between DS% and future stroke risk was shown in two trials of patients with asymptomatic carotid artery disease (ACAS and ACST trials).[201, 202] Importantly, using DS% as a tool to reflect the severity of the atherosclerotic plaque almost entirely disregards the pathological process that resides within the wall of the artery. The inconsistency in predicting stroke risk using DS% should not be surprising given the highly variable incursion of atherosclerotic plaque into the lumen. On the other hand, advanced atherosclerosis is more related to plaque characteristics; and features such as large lipid content is associated with high risk of plaque rupture leading to acute vascular events.[176, 206] Similarly, it is plausible that atherosclerotic plaques with more homogeneous fibrotic characteristics may carry a lower stroke risk making intensive medical therapy the optimal management for these patients.

The recently developed and validated T2 mapping MRI technique can accurately and reproducibly quantify carotid plaque lipid content on voxel by voxel basis.[167, 180] This technique offers an excellent opportunity to study carotid artery lipid content without reliance on the presence of discernible lipid core, a major limitation of the currently available multi-contrast MRI (MC-MRI) technique.[167, 180] Accordingly, this chapter would first seek to study the relationship of carotid plaques lipid content and their symptomatic status and to investigate if carotid plaque lipid content have any association with DS% or the propensity to develop in multi-site vascular territories

.

3.3 Hypotheses and aims

To test the hypotheses that: (i) carotid plaque lipid content and distribution, as determined by T2 mapping MRI technique, is associated with the symptomatic status of the carotid plaques, (ii) plaque lipid content does not reflect the degree of carotid luminal stenosis as measured by ultrasound duplex, and (iii) plaque lipid content is correlated between left and right carotid arteries.

The experimental aims of this chapter were:

1) To study the extent and distribution of carotid plaque volume and lipid content in patients scheduled for carotid endarterectomy.
2) To compare both carotid plaque lipid content and distribution between symptomatic and asymptomatic carotid patients.
3) To assess the relationship between DS% measured on ultrasound duplex and both carotid plaque and lipid volumes.
4) To establish whether some patients have propensity to develop multi-site lipid-rich plaques throughout the arterial tree.

3.4 Methods

Chapter 2 discussed the general methodology related to this chapter and only methods specific to this chapter are discussed below in further details.

3.4.1 Study population

All participants provided written informed consent and ethical approval was obtained from NRES and Oxford R&D committee prior to commencement of the study. 71 patients awaiting

carotid endarterectomy at the John Radcliffe Hospital were recruited from November 2011 to September 2015 to validate the T2 mapping technique. Patients were scanned at AVIC ≤ 24 hours before surgery. Patients were adult (>18 years) and either recently symptomatic with median time from index event of less than 2 weeks or asymptomatic carotid disease, with 50-99% carotid stenosis according to NASCET, or 70-99% according to ESCT criteria.[180, 207] Symptomatic plaques were identified as those to have given rise to either a stroke or TIA as assessed clinically and supported, where available, by brain MRI and/ or computed tomography imaging. Asymptomatic carotid plaques were deemed so if there were no documented clinical symptoms, but with an indication for carotid endarterectomy based on degree of stenosis.

3.4.2 MRI protocol

Patients were imaged on a Verio 3T scanner (Siemens Healthcare, Erlangen, Germany) with a 4-channel phased-array carotid coil (Machnet, Roden, The Netherlands). Bright-blood TOF angiography of the carotid arteries was acquired to localize carotid bifurcation and atherosclerotic plaque. Multi-slice carotid T_2 maps (5 in each acquisition) were generated from 14 images with echo times TE = 9-127 ms and repetition time TR = 2 s, acquired using the DANTE-MESE sequence. This sequence combined black-blood preparation based on non-selective DANTE pulse trains with chemical-shift-selective fat saturated MESE.[188, 208] Two acquisitions of DANTE-MESE sequence were obtained yielding 10 slices of 2 mm thickness each, covering 2 cm of the target carotid artery in 8 minutes. In order to cover the full plaque length, the obtained 10 slices were centralised on the maximum plaque volume identified on TOF and T1 weighted images.

3.4.3 Data analysis

Using mono-exponential nonlinear fitting, T_2 maps of the carotid arteries were generated *voxel-by-voxel*,[167] and a validated semi-automated procedure was used to segment both lumen and external vessel boundaries.[209] A segmentation method using T2 threshold of less than 42 ms was implemented to identify lipid as previously validated.[180] Each carotid artery (right and left) had 10 slices studied covering 2 cm of the vessel with the exception of 7 patients having only 5 slices obtained. All algorithms were implemented in Matlab (MathWorks, Natick, USA).

The analysis was performed based on the average lipid and plaque volume in all the 10 studied slices respectively and irrespective of the degree of stenosis, representing a per vessel analysis as the unit of interest. For each slice plaque lipid and plaque volume were quantified using a single voxel as one unit (0.33 x 0.33 x 2 mm). Voxels with T2 values threshold range of less than 42 ms were grouped together to quantify lipid volume (without recent intra-plaque haemorrhage), according to the published method.[180]

Lipid distribution

Using both lipid percentage and number of separate lipid deposits, lipid distribution was studied within the vessel wall. This process was undertaken using two parameters namely, LLD % and a newly devised LAI. This process was executed in an individual slice with the largest lipid volume on each side (right and left). The number of separate lipid deposits were quantified in that slice and subsequently the percentage of every individual lipid deposit out of the total lipid area of the studied slice was calculated. The hypothesis was to test if difference in lipid distribution beyond lipid content, in terms of scattered lipid deposits *vs.* coalesced 'pools', has any significance relation to the symptomatic status of the plaque.

Therefore, in the slice with the largest lipid content (out of the 10 obtained slices of the carotid artery) the largest lipid deposit was identified and its contribution (percentage LLD %) to the total slice lipid volume was then calculated. This parameter represents the first tested imaging index and the largest the LLD % the more likelihood to have coalesced lipid and *vice versa*.

In order to account for the remaining lipid deposits, after calculating LLD %, and to study their contribution and whether they add any insight towards the symptomatic status of the plaque, a second imaging index was proposed. The remainder of lipid deposits percentage were averaged (RLD %) (excluding the largest one) using the following formula:

RLD% = 100 * (1-LDD) / (number of lipid deposits-1) (For number of lipid deposits ≥ 2).

Lipid aggregation index (LAI) is the ratio of largest lipid deposit percentage (LLD %) and other lipid deposits (RLD %):

LAI = LLD% / RLD% (See Figure 3.1)

Accordingly, a plaque with a coalesced lipid would have a high LAI; whereas a plaque with multiple small deposits would have a lower LAI, even if the total lipid content was similar. There are two points related to LAI need to be highlighted. Firstly, LAI was not applicable in patients with single lipid deposit as (a) there is no merit of quantifying lipid scattering if all lipid deposits are coalesced in a single deposit and (b) the index will not be calculable. Secondly, lipid deposits of very small size (≤ 1%) were excluded to be distinguished from noise and ensure robustness of this imaging index. This process was executed by using ImagePro Plus software (Media Cybernetics, Rockville, USA). Lipid area and number of lipid particles were segmented and quantified by the author who was blinded to the origin of the data.

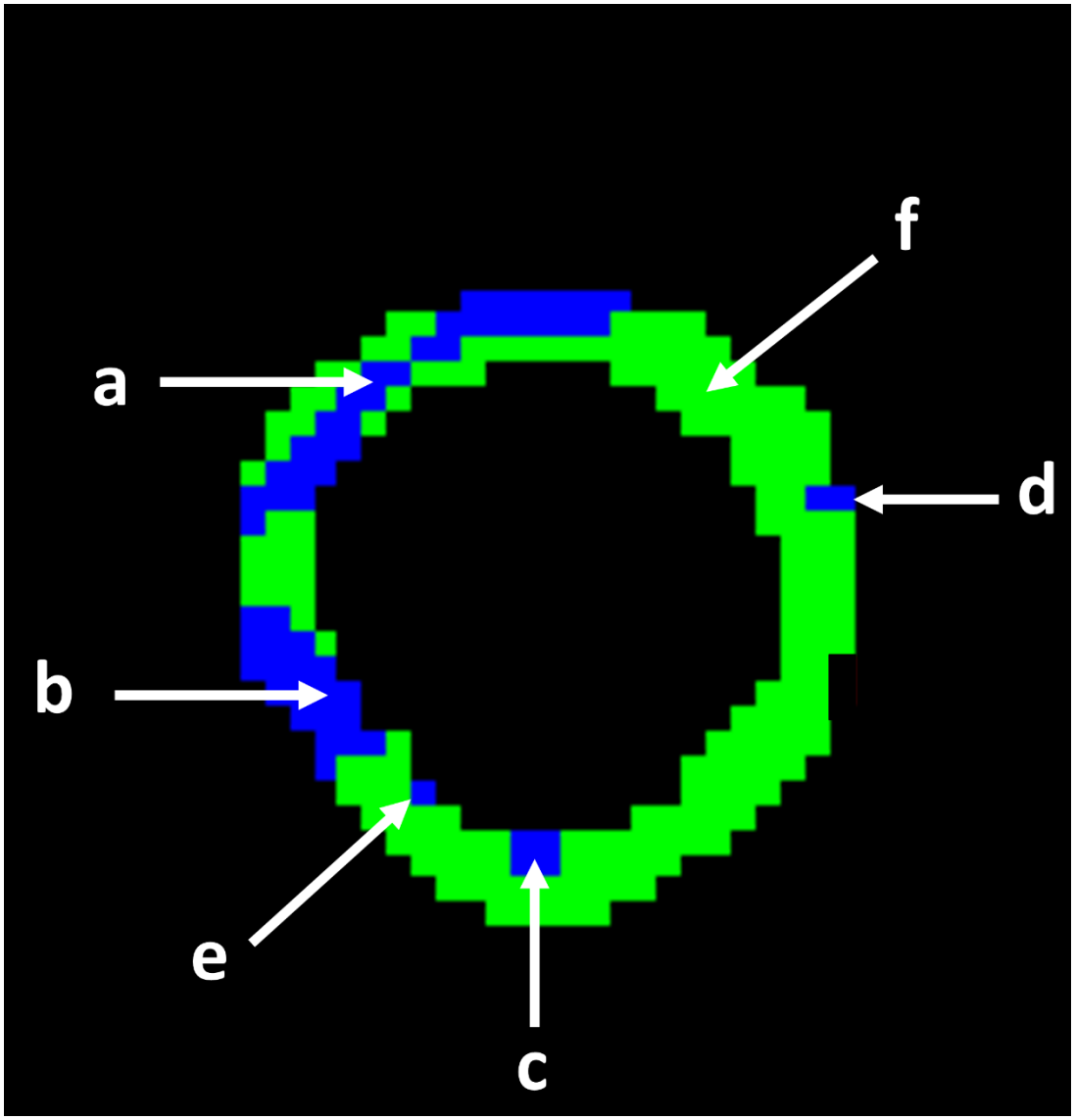


Figure 3.1 Calculation of lipid aggregation index

Five separate lipid deposits were identified and their percentage of the total vessel area was calculated and denoted as a, b, c, d and e. fibrous component was labelled as f. One deposit (e) was excluded as it counted for less than 1% of lipid content, thus, leaving total number of lipid deposit equal to 4. Deposit (a) was established as the LDD % and subsequently the remainder of lipid deposits was calculated as: RDD % = 100* (1- a) / (4 - 1). The ratio of LDD % over RDD % will yield LAI

3.4.4 Statistical analysis

Data were expressed, where appropriate, as mean ± standard deviation for normally distributed continuous variables or as median accompanied by interquartile range (IQR) for skewed continuous variables using the Shapiro-Wilk test to assess the normality distribution of the data. Categorical data were expressed as frequencies and percentages and were compared using Chi squared (χ^2) test or Fisher's Exact test where appropriate. Continuous data were compared using unpaired student t-test in normally distributed variables and Wilcoxon rank sum tests was otherwise used. Correlations were assessed using Spearman rank test. Statistical analysis was performed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) and *P*-values <0.05 were considered statistically significant.

3.5 Results

71 patients were recruited during the study period. 21 patients (29.5%) were excluded due to inadequate MRI image quality and mostly due to motion artefact. The remaining 50 patients were divided into symptomatic group of 34 patients with symptoms attributed to the downstream cerebrovascular territory and asymptomatic group of 16 patients.

The mean age ±SD was 70 ±12 years with almost three-quarters (74%) were males. The overall mean stenosis, measured by duplex ultrasound, was 80 ±10%, which was not significantly different between the 2 groups. There were no significant differences in age, gender, major cardiovascular risk factors or medications on admission between symptomatic and asymptomatic groups. Patients' clinical characteristics are summarized in Table 3.1 and categorized according to presence or absence of symptoms.

Table 3.1 Clinical characteristics of recruited patients

	Symptomatic	Asymptomatic	P-value
Total	34	16	
Male (%)	27 (79%)	10 (63%)	0.30
Mean age ± SD	71 (± 12)	67 (± 13)	0.25
Cardiovascular risk factors			
Hypertension	27 (79%)	10 (63%)	0.30
Hypercholesterolemia	20 (59%)	11 (69%)	0.50
Smoking	16 (47%)	7 (44%)	0.80
Diabetes mellitus	7 (21%)	4 (25%)	0.72
CAD disease	7 (21%)	2 (13%)	0.69
Atrial fibrillation	7 (21%)	0 (0%)	0.08
Medication at time of carotid endarterectomy			
Aspirin /antiplatelets	26 (76%)	12 (75%)	1.0
Statins	27 (79%)	13 (81%)	1.0
Beta-blockers	5 (15%)	4 (25%)	0.42
Calcium antagonists	9 (26%)	3 (23%)	0.70
ACE inhibitors / ARBs	11 (32%)	8 (50%)	0.17
Anticoagulation	4 (12%)	0 (0%)	0.29
Duplex ultrasound scan			
Right : Left	2.7 : 1	1 : 1	0.10
Mean stenosis ± SD (%)	78 ± 11%	83 ± 8%	0.08

Plaque lipid was present in (8.99 ± 1.92) slices per vessel in the 100 obtained carotid vessels. The carotid bifurcation contained the maximal lipid content, irrespective of the symptomatic status or degree of stenosis, in 75% of the vessels (56 out of 75) where the bifurcation was included in the studied vessel.

3.5.1 Difference in plaque burden and lipid content between symptomatic and asymptomatic patients

Plaque burden was estimated by calculating the average vessel wall volume across the 10 slices. Symptomatic patients had significantly larger plaque burden than asymptomatic patients irrespective of the degree of stenosis [118 (IQR 102 – 153) mm^3 *vs.* 92 (IQR 72 – 109) mm^3, P<0.001] (Figure 3.2)

In patients with moderate to severe carotid arteries stenosis (DS% ≥50%) and using the slice with largest lipid volume, symptomatic patients had larger lipid percentage than asymptomatic ones [31% (IQR 25 - 40) % *vs*. 22% (IQR 18 - 29) %, P = 0.013] (Figure 3.3, Panel A & Figure 3.4). When investigating the whole cohort of 50 patients (100 carotid vessels) and irrespective of the degree of stenosis in each vessel, lipid percentage was significantly higher in symptomatic patients when compared with asymptomatic ones [18% (IQR 12 -23) % *vs*. 13% (IQR 10 -17) %, P = 0.005] (Figure 3.3, Panel B). Similarly, vessel lipid *volume*, calculated as average lipid volume across all slices, was significantly larger in symptomatic patients compared to asymptomatic ones in the total 50 studied patients [24 (IQR 16 – 30) mm^3 *vs.* 12 (IQR 8 – 19) mm^3, P<0.001]. In the symptomatic group only (n=34), symptomatic (culprit) plaques had higher lipid percentage [18% (IQR 12 -23) % *vs*. 13% (IQR 10 -16) %, P = 0.005], higher lipid volume [24 (IQR 16 – 30) mm^3 *vs.* 13 (IQR 8 – 19) mm^3, P<0.001] and larger atherosclerotic burden [118 (IQR 102 – 153) mm^3 *vs.* 96 (IQR 77 – 120) mm^3, P<0.001] when compared to asymptomatic contralateral (non-culprit) plaques.

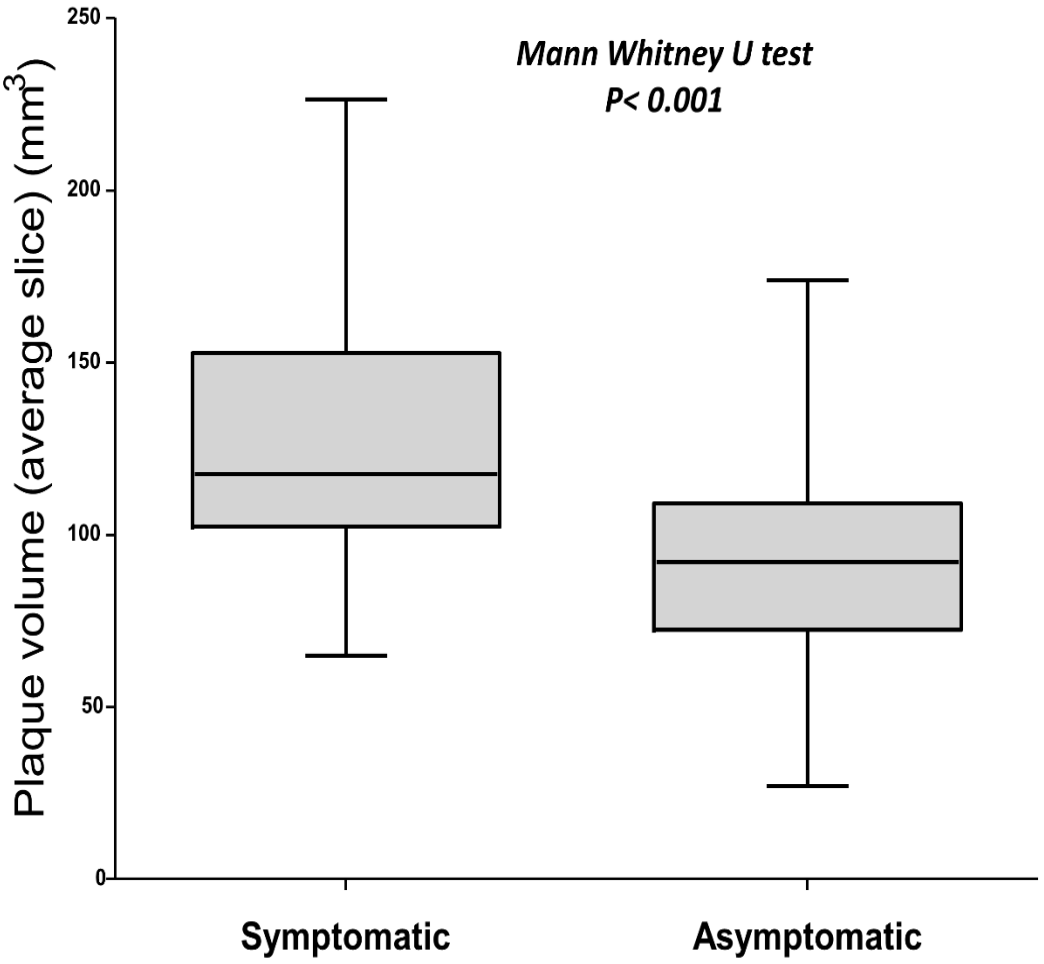


Figure 3.2 Difference in plaque burden between symptomatic and asymptomatic patients

Plaque volume, calculated as the average vessel volume in each slice, was significantly higher in patients with recent symptoms (stroke or TIA) compared with those who had no documented history of previous cerebrovascular events irrespective of DS%. Yet, both groups had similar DS% on carotid duplex ultrasound.

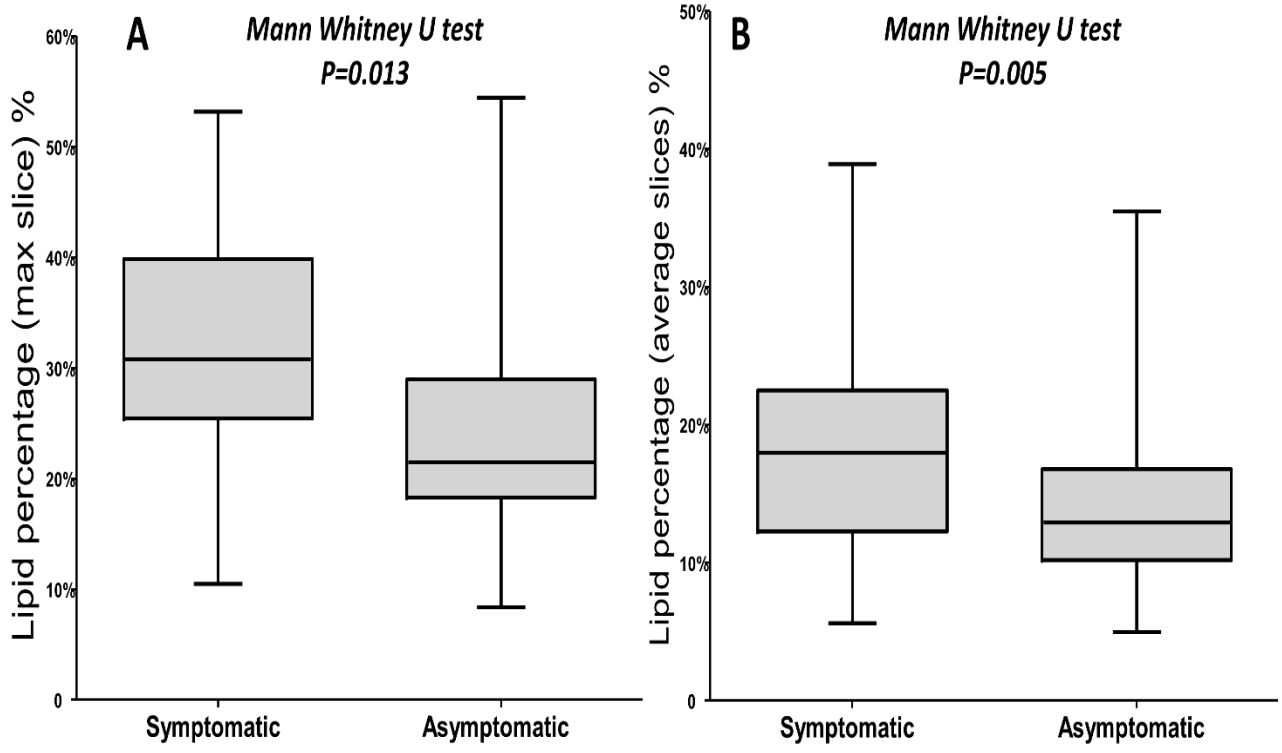


Figure 3.3 Difference in lipid percentage between symptomatic and asymptomatic patients

A: Using the slice with largest lipid volume, symptomatic patients with DS% of more than 50% had larger lipid content than their asymptomatic counterparts.

B: Similarly but investigating the whole cohort of 100 carotid artery (irrespective of DS%) symptomatic patients had more lipid percentage than asymptomatic ones as calculated by the average lipid content across all the slices.

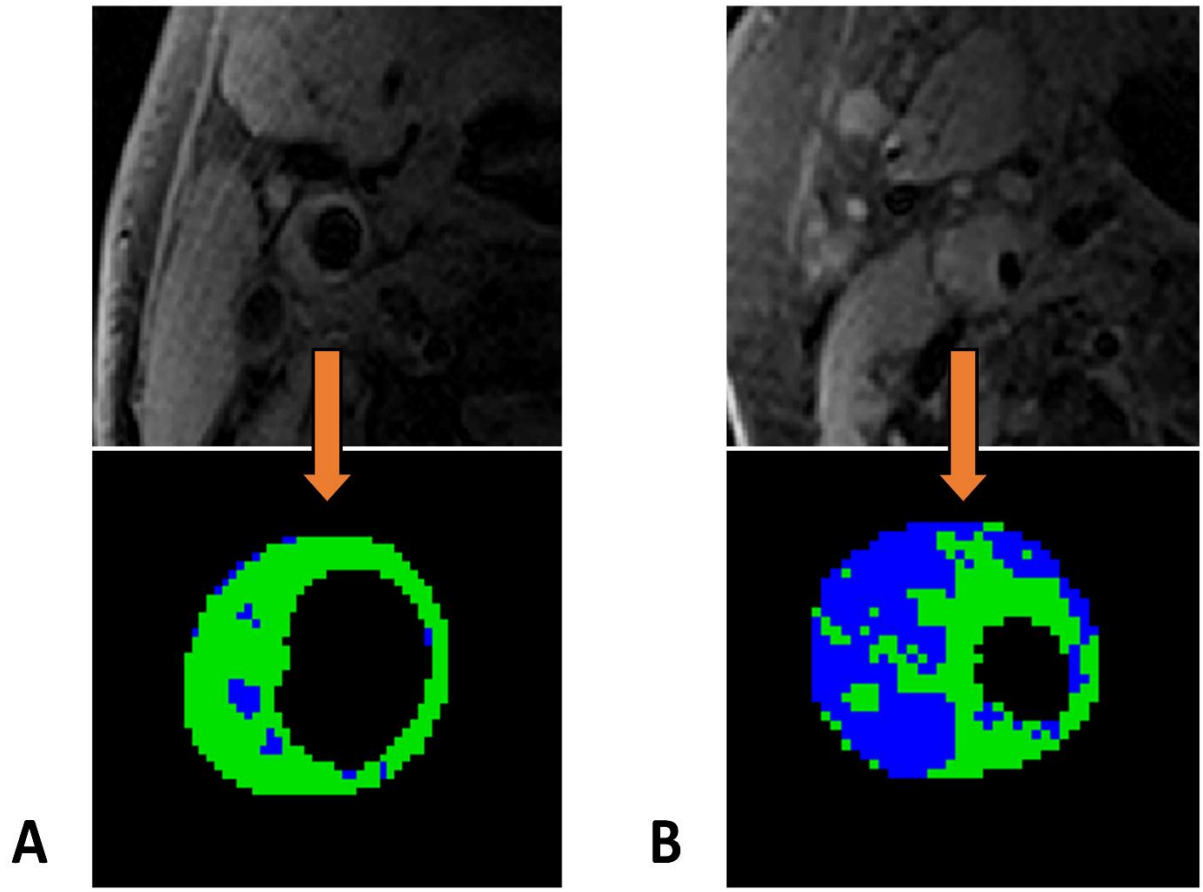


Figure 3.4 Difference in lipid content between symptomatic and asymptomatic patients

A: Asymptomatic right carotid plaque in 65 years old male with estimated 70% luminal stenosis on ultrasound duplex. Lipid content was estimated as 7.4% using T2 mapping MRI technique.

B: Right carotid artery plaque from 86 years old male who presented with left sided weakness one week prior to his carotid endarterectomy. Lipid percentage was quantified as 55.5% using T2 mapping MRI technique.

3.5.2 Difference in lipid distribution between symptomatic and asymptomatic patients

To assess lipid distribution within carotid plaque, two imaging indices were defined and namely are:

1. The ratio of largest lipid deposit over total lipid content (LLD %).
2. Lipid aggregation index (LAI)

In both cases the higher the index, the more likely was a plaque to have a large individual lipid deposit (coalesced lipid pool) while plaques with low indices were more likely to contain scattered lipid deposits of more uniform size distribution.

The median LLD % was 45% (IQR 35 - 57%) while the LAI median was 5.1 (IQR 3.7 - 8.8). Using these two indices, the significance of patterns of lipid deposition in relation to the symptom-related versus non symptom-related status of plaques was investigated:

Median LLD % was significantly higher in the symptomatic compared to the asymptomatic plaques [49% (IQR 38- 84) % *vs.* 42% (IQR 32- 53) %, P = 0.028] (Figure 3.5 & Figure 3.6, Panel A). Similarly, median LAI was significantly higher in the symptomatic compared to the asymptomatic plaques [6.2 (IQR 3.9- 30.7) *vs.* 5.0 (IQR 3.5- 7.6), P = 0.018] (Figure 3.6, Panel B), indicating that for a given quantity of plaque lipid, coalesced cores were more likely to be associated with symptomatic events.

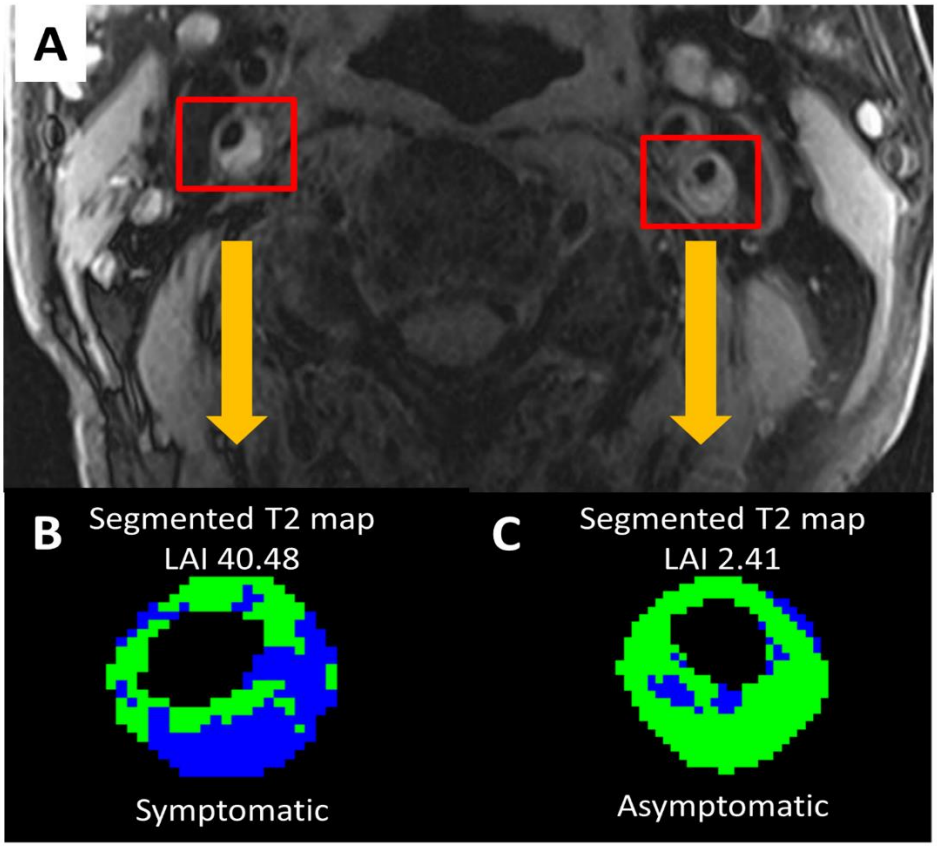


Figure 3.5 Difference in lipid distribution between symptomatic and asymptomatic patients

A: Bilateral carotid artery stenosis in 76 years old female as demonstrated on cross sectional T1-weighted image.

B: Difference in lipid distribution within the atherosclerotic plaques assessed by LAI. The right carotid plaque was the presumed culprit of a transient ischaemic event that had occurred two weeks previously, resulting in left sided facial weakness; while the left carotid plaque did not have any documented past symptoms. The higher the index in the right vessel was associated with more coalesced lipid and symptomatic plaque compared with more scattered lipid deposit in the left and asymptomatic vessel.

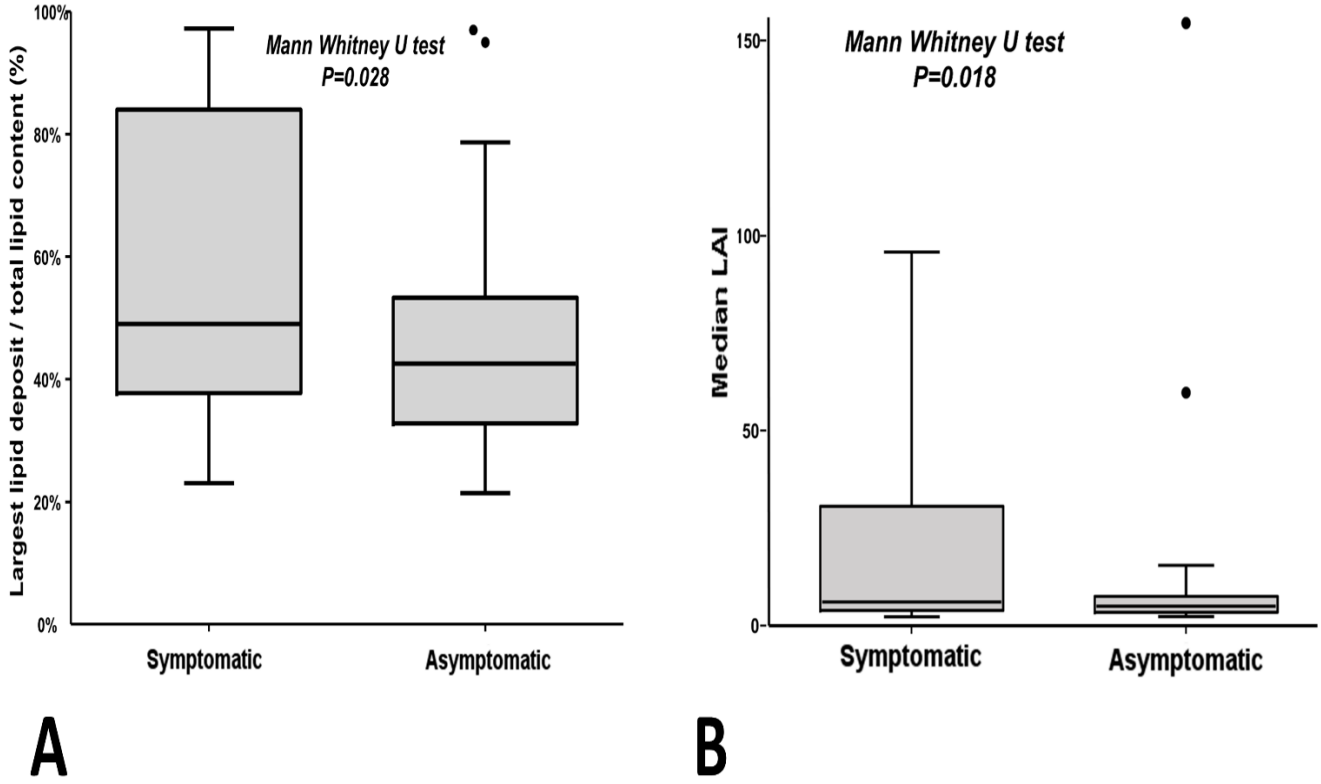


Figure 3.6 Assessment of lipid distribution using two imaging indices

A: Symptomatic patients had statistically higher median LLD % compared to asymptomatic ones.

B: Using the newly-devised lipid aggregation index, median LAI was statistically higher in symptomatic patients compared to asymptomatic ones.

Notably, there were two vessels with extremely high lipid distribution measurements, quantified using both indices, at the time of acquiring T2 mapping MRI but with corresponding plaques which had not caused symptoms at the time of imaging, but theoretically may have posed a risk for future events.

3.5.3 Relationship between T2 mapping MRI outputs and luminal degree of stenosis on ultrasound duplex

The relationship between plaque burden and composition and luminal stenosis was assessed in patients with at least a moderate degree of stenosis (DS%≥ 50%).

Carotid plaque volume had no association with degree of luminal stenosis measured on ultrasound duplex (Spearman r = -0.09, P = 0.53) (Figure 3.7, Panel A). This lack of association was present when evaluating asymptomatic patients (Spearman r= 0.04, P= 0.82) and symptomatic ones (Spearman r= -0.17, P= 0.45) separately.

Similarly, carotid lipid volume showed no correlation with and degree of stenosis (Spearman r= -0.05, P = 0.75) (Figure 3.7, Panel B). The dissociation existed when assessing asymptomatic (Spearman r= -0.01, P= 0.92) and symptomatic patients (Spearman r=0.09, P= 0.69) individually.

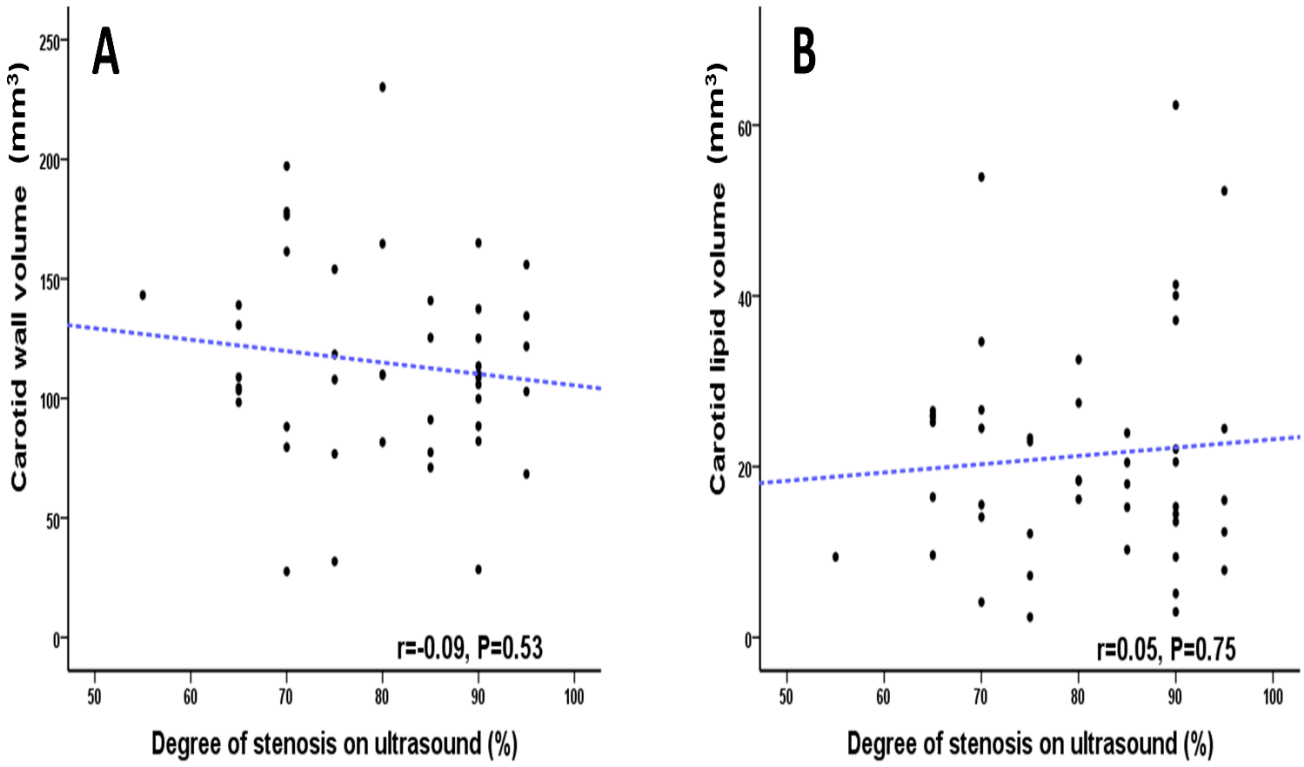


Figure 3.7 Luminal degree of stenosis and its relationship with both plaque burden and lipid volume

A: Degree of luminal stenosis as estimated on ultrasound duplex did not demonstrate any relationship with plaque burden as measured on T2 mapping MRI technique in patients with moderate degree of luminal stenosis

B: Luminal stenosis was disconnected from lipid volume as quantified on T2 mapping MRI technique.

3.5.4 Relationship between left and right carotid arteries

Plaque volume was significantly related between left and right carotid arteries irrespective of the symptomatic status and degree of luminal stenosis (Spearman r = 0.41, P = 0.003) (Figure 3.8, Panel A). This relationship was comparable when tested in asymptomatic patients (Spearman r= 0.48, P= 0.06) and symptomatic individuals (r= 0.40, P= 0.02).

There was statistically significant correlation when comparing lipid volume between left and right carotid vessels (Spearman r = 0.5, P <0.0001) (Figure 3.8, Panel B). This association was more prominent in the asymptomatic subgroup (r= 0.75, P<0.001) compared with the symptomatic one (Spearman r= 0.37, P= 0.03). Both subgroups demonstrated a significant relationship between left and right carotid arteries.

Plaque and lipid volumes in same vessel were compared in the total cohort of 100 vessels. There was a strong correlation between carotid plaque volumes and their correspondent lipid content (Spearman r = 0.69, P < 0.0001) and this relationship was more modest in the symptomatic patients (Spearman r= 0.54, P= 0.001) compared to the asymptomatic ones (Spearman r= 0.68, P<0.001). Notably large plaques had wider variations in lipid percentage than plaques with small plaque burden (Figure 3.9).

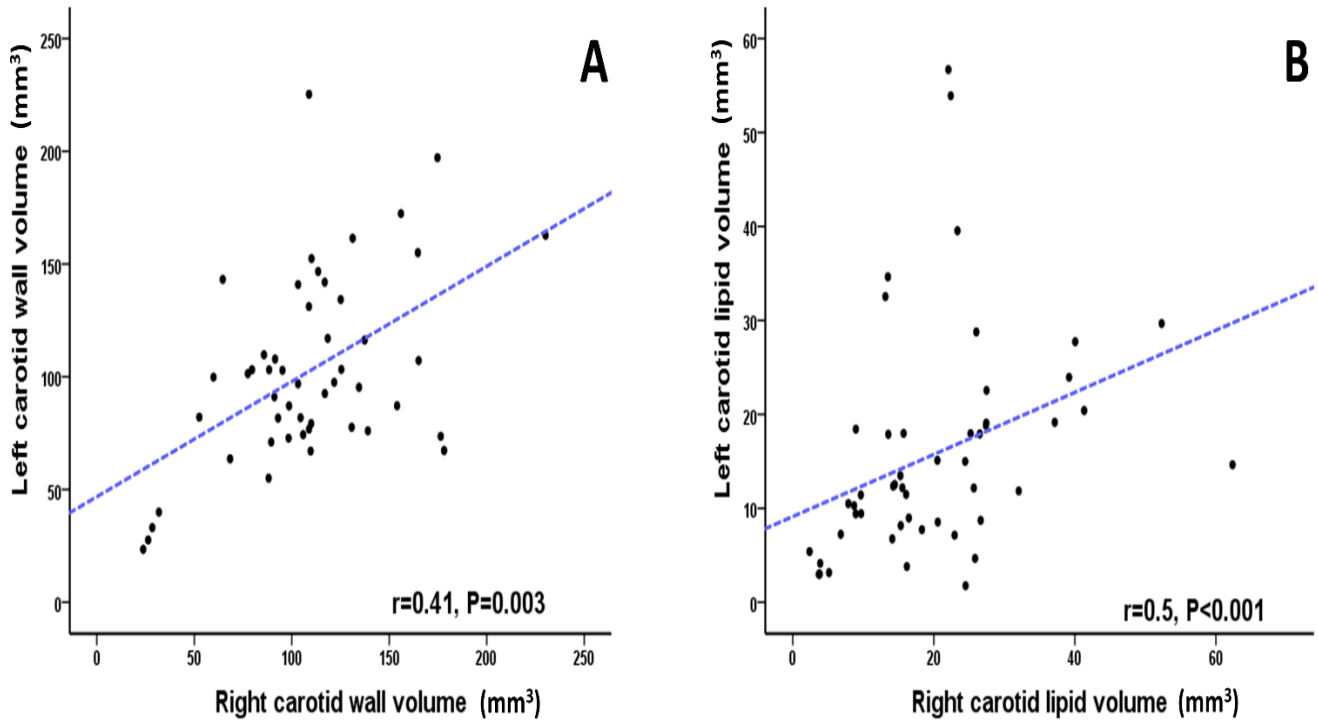


Figure 3.8 Relationship between left and right carotid arteries in patients scheduled for carotid endarterectomy

A: Scatter plot graph demonstrating the significant relationship in plaque burden between left and right carotid arteries. This relationship was present irrespective of the symptomatic status of the carotid vessel or DS%.

B: Scatter plot graph showing the significant correlation between lipid volumes of the right and left carotid arteries as quantified on T2 mapping MRI technique. This relationship was present irrespective of the symptomatic status of the carotid vessel or DS%.

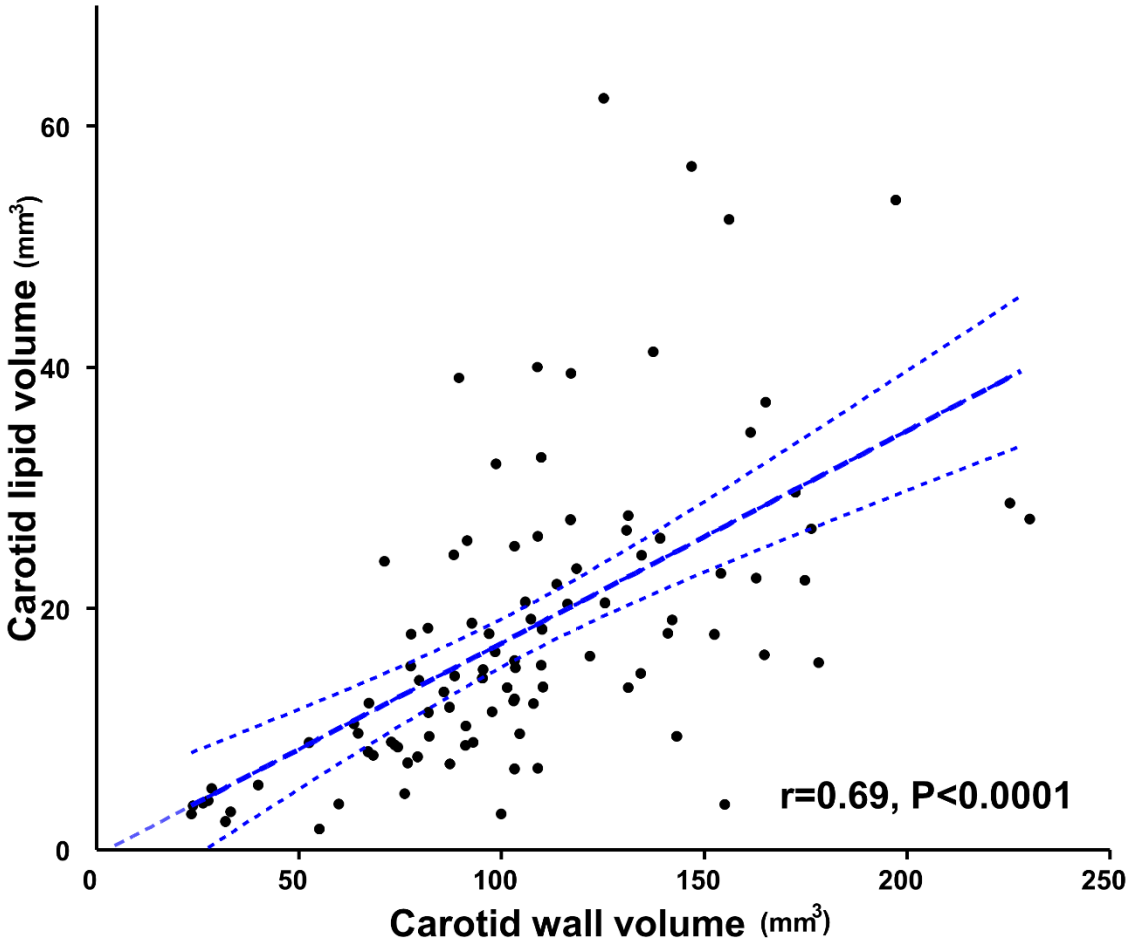


Figure 3.9 Relationship between carotid plaque volumes and their corresponding lipid volumes in patients scheduled for carotid endarterectomy

Scatter plot graph illustrating a strong correlation between plaque lipid content and plaque volume in the whole cohort of one hundred carotid arteries. This relationship was present irrespective of the symptomatic status of the carotid vessel and DS%. Importantly, with increasing plaque size, there was a wider variation in lipid content i.e. points fall further outside the 95% confidence interval.

3.6 Discussion

The current systematic quantification of carotid plaque lipid content using a highly sensitive T2 mapping MRI technique in patients scheduled for carotid endarterectomy has generated the following findings:

1. Most plaques contained lipid and this tended to be maximal at the carotid bifurcation, irrespective of the symptomatic status of the carotid artery or DS%.
2. Symptomatic patients were more likely to have larger plaque lipid *content* than asymptomatic ones.
3. Lipid *distribution* within the atherosclerotic plaques was related to the symptomatic status of the plaque, where more coalescence, as opposed to scattered lipid, was associated with prior symptoms.
4. Luminal degree of stenosis, measured by ultrasound duplex, did not reflect either plaque burden or lipid volume.
5. Plaque burden and their corresponding lipid volume were significantly related but this association was less strong in larger plaques.
6. Both plaque burden and lipid content were correlated between left and right carotid arteries.

The current approach of 'one size fits all' to intervene on all asymptomatic carotid patients is increasingly challenged, in particularly, with progresses in medical therapies.[9, 200, 205] In fact, almost 10% of patients with asymptomatic significant unilateral or bilateral carotid stenosis had a stroke over 10 years follow up.[205] The risk of undergoing carotid revascularisation needs to be justified and balances with the modest risk of spontaneous stroke in patients receiving optimal medical therapy.[204] Therefore, there is an increased need for tools to recognise patients who are at a low risk and are potentially candidates for intensive medical

therapies and those who are at higher risk of future stroke and are hypothetically candidates for invasive revascularisation.[200]

Previous studies have supported the significant role of plaque burden and composition in determining the risk of both spontaneous and periprocedural stroke.[179, 210, 211] These data, using quantitative T2 mapping technique, extend previous observations and highlighted a potential role of using plaque characteristics to establish the symptomatic status of the carotid plaque.[180] Plaque lipid content is significantly higher in symptomatic patients and in particularly those with at least moderate degree of stenosis. The management of this group with moderate disease is not well defined and plaque characteristics may play a role in determining future management. Furthermore, the distribution of lipid within the atherosclerotic plaques, in addition to lipid volume, was also associated with their symptomatic status. Coalesced lipid was linked to previous events (CVA or TIA), while scattered lipid was more likely to be associated with asymptomatic plaques. Notably, among asymptomatic patients there were two 'outliers' vessels with extremely high LAI and yet these two plaques did not demonstrate any symptoms. Prospective studies are required to show whether such plaques, with high lipid dispersion indices, are at high future risk.

Clinical decisions to determine which patients are candidates for invasive revascularisation, surgically, or with stenting, are currently based on estimating carotid DS%.[212, 213] However, this approach does not directly assess the disease process taking place in the wall of the artery but relies on the extent of encroachment of the atherosclerotic plaque into the lumen of the artery, which is unpredictable. Furthermore, this work has highlighted the disassociation between DS% and both the atherosclerotic process and the symptomatic status of the vessel. On the other hand plaque burden and lipid volume, which are features of advanced atherosclerotic disease, were reflective of the symptomatic status of the vessel. These imaging biomarkers may potentially play a role in identifying patients who are at higher risk

of developing future symptoms. Additionally, the role of plaque characteristics may also be extended to provide a basis for testing the optimal strategy for invasive revascularisation (CEA *versus* stenting) in a clinical trial context. Using intra-vascular ultrasound, carotid plaque composition, and in particular large plaque lipid volume, was associated with large amount of debris during carotid stenting and was an important element in determining periprocedural stroke.[211] The CREST trial reflected these findings where endarterectomy outperformed stenting in the symptomatic patients while both invasive strategies had comparable results in asymptomatic patients.[204] Therefore, there may be a rationale, based on these observations, to select patients for invasive vascular intervention based on non-invasive pre-operative assessment of plaque volume and lipid burden. This strategy could be test in a clinical outcomes trial.

Across all the analysed vessels, there was a positive and strong correlation between quantity of plaque lipid and plaque size. Of clinical significance, as plaques became the larger was the variability in their lipid content, further highlighting the importance of quantifying plaque lipid content at an individual plaque level. These findings matched reports using IVUS-VH and NIRs in the coronary arteries.[214] Here, using a non-invasive MRI technique the association between plaque and lipid volumes was also demonstrated. The variation in lipid content and its significant role in stent-associated periprocedural stroke highlight the potential role of T2 mapping in decision making with regards to carotid intervention.

The relationship between left and right carotid arteries of both plaque and lipid volumes was reflective of the systematic nature of atherosclerosis. This association suggests that some patients have a propensity to develop lipid-rich plaques and that atherosclerosis is potentially identifiable at multiple sites in those individuals. The comparison between left and right carotid arteries was previously evaluated in a small number of studies.[215, 216] In post mortem study of 50 patients, there was a correlation of total wall volumes, quantified on MRI

between left and right carotid arteries.[216] Similarly, in the Rotterdam Study the association between both carotid arteries in prevalence, severity and composition was assessed in stroke-free population, where a majority of patients (85%) had identifiable bilateral carotid atherosclerosis.[215] The data presented here confirmed these findings using more sensitive T2 mapping techniques and in more clinically relevant high risk atherosclerotic patients. Importantly, results from the BioImage and PESA studies have suggested that detecting atherosclerosis in different vascular sites using various vascular imaging modalities improved risk predication in comparison to conventional risk factors.[183, 184] Therefore, stratifying patients based on their burden of atherosclerosis is a promising strategy for selecting patients for future treatments, akin to using imaging for staging cancers and guiding selection of therapies.[43] Moreover, matching the nature of plaques (lipid poor or rich) using T2 mapping MRI technique, with a specific targeted therapy (lipid-lowering drugs) is a good opportunity to refine the use of expensive anti-atherosclerotic drugs.[43]

The main limitations of this report could be highlighted in two important points related to data acquisition and analysis:

Firstly, the sensitivity to motion artefacts of the DANTE-MESE sequence. This has resulted in a relatively high overall rejection rate (29.5%) of all scans. However, there has been some marginal improvements compared with the initial rejection rate of 35%.[180] In addition, there is ongoing work to develop motion correction strategies that would allow this technique to fulfil its full clinical potential.

Secondly, the LAI is not calculable in slices with a single lipid deposit and therefore it only could be applicable to slices with number of lipid deposits ≥2. However, across all analysed slices there was no slice with a single lipid deposit suggestive that this was probably a theoretical limitation.

3.7 Conclusion

The work presented in this chapter has demonstrated that carotid plaque lipid content and distribution are associated with the symptomatic status of the plaque. In addition, the degree of luminal stenosis was not reflective of symptom status, nor it was related to the plaque burden or lipid volume. Finally, the relationship between left and right carotid arteries support the notion that there are patients with propensity to develop lipid-rich plaques at multiple vascular sites. The high sensitivity of T2 mapping MRI technique may help decision making on clinical management beyond luminal stenosis. This could be applied to identify those with multi-sites lipid-rich plaques who are potential candidates for intensive lipid modifications. In addition, T2 map applicability could be extended beyond medical treatments to determine the optimal strategy for invasive revascularisation

Chapter 4: The extent of carotid plaque lipid in patients with acute coronary syndrome and the impact of high intensity statin initiation as determined by T2 mapping

4.1 Abstract

Background

LDL-cholesterol reduction with PCSK9 inhibitors, resulted in only 1-2% absolute risk reduction of combined clinical endpoints in patients with previous atherosclerotic cardiovascular disease, but who were otherwise unselected. If the potential clinical benefits of these drugs are to be realized, attention should be sharply focused on developing stratification tools to identify patients who may benefit maximally. One potential method is to use plaque lipid content to characterise atherosclerotic process in individual patients. This approach is promising since the reduction in vascular events using lipid-lowering drugs has been linked to a decrease in plaque lipid content.

Methods

Statin naïve subjects presenting with ACS underwent MRI of their carotid arteries at 3Tesla to quantify plaque lipid using T2 mapping. Patients were subsequently commenced on high dose statin and underwent second MRI after three months. Plaque composition was measured using objective semi-automated techniques.

Results

Twenty-seven patients underwent carotid MRI within 48 hours of their presentation. There was a strong correlation between left and right carotid arteries in both plaque volume ($r=0.72$, $P< 0.001$) and lipid volume ($r=0.53$, $P=0.016$). Combining data from the total 54 vessels, there was an association between plaque and lipid volumes across the whole cohort ($r=0.45$, $P=0.003$).

Following three months of high intensity statin, there was a significant reduction in vessel wall volume from [44.8 mm^3 (36.2 - 54.6)] to [44.1 mm^3 (36.1 - 50.1) (P = 0.009). Similarly, there was a significant reduction in carotid vessel lipid percentage from [10.3% (7.2 - 14.2) to 7.4% (5.4 - 10.0), P=0.002]. This reduction was mirrored by a significant increase in carotid vessel fibrous percentage from [83.3% ± 6.6 to 85.5% ± 4.8, P=0.039] between baseline and follow up.

Conclusions

The correlation of plaque burden and lipid content between left and right carotid arteries re-emphasised the systemic nature of atherosclerosis. Using a T2 mapping MRI technique, intensive statin therapy significantly depleted carotid plaque lipid content, which was replaced by fibrous tissue. These findings show that plaque imaging can be used to assess response to lipid-lowering and, potentially, as a tool to identify patients who are candidates for intensive lipid modification therapy.

4.2 Introduction

LDL-c reduction, especially with statins, has consistently been effective in reducing cardiovascular events. Each 1.0 mmol/L decrease in LDL-c has been associated with 23% reduction in these events.[14] The effects of LDL-c reduction have been further reinforced by the outcomes of the IMPROVE-IT and FOURIER trials,[39, 63] in which ezetimibe and evolocumab, respectively, produced an incremental LDL-c reduction that was associated with a further decrease in cardiovascular events. Remarkably, however, it is still unclear precisely *how* reducing LDL-c translates into improved cardiovascular outcomes at the level of the atherosclerotic plaque. One prevalent theory is that LDL-c reduction leads to a net cholesterol evacuation from atherosclerotic plaque with potential replacement of fibrous tissue. This is in turn has a 'stabilising effect' on plaques, which are then less likely to rupture and precipitate acute events.[43]

In experimental atherosclerosis, reduction in plasma LDL cholesterol has been associated with depletion of plaque lipid content and changes in plaque composition.[159, 217, 218] Libby *et al*,[159] demonstrated that low cholesterol diet, not only decreased cholesterol content within rabbit aortic plaques but also increased the deposition of collagen. Similar observations have been made in mice.[217, 218] Likewise, *ex vivo* analysis of explanted carotid endarterectomy specimens in patients treated with statins has shown depletion in lipid core with an increase in collagen content compared to placebo.[160] Further advances in non-invasive imaging modalities have allowed the study of lipid-lowering drugs on plaque composition, *in vivo.*[148, 165] In the CPC study, there was a decrease in lipid-rich necrotic core by 3.2% in the first year following LDL-c reduction.[148] Similarly, in the ORION study, there was almost 3% decrease in lipid-rich core after 24 months of combined low and high dose rosuvastatin treatment.[165] However, these studies have been fundamentally limited by the inherent insensitivity of the imaging tools.[43, 166]

MC-MRI relied on patients with sufficiently large areas of coalesced lipid to be discerned, meaning that only a minority of patients have sufficient lipid to be quantified serially.[43, 167] Because of the previous requirement for large lipid cores, earlier studies have often focused on patients with pre-identified carotid atherosclerosis.[148, 165]

T2 mapping, on the other hand, is a newly validated MRI technique that accurately identifies plaque lipid, at a voxel by voxel level basis.[180, 219] It relies on the physical properties of each voxel and is not limited by the requirement for a coalesced necrotic core within the carotid plaque for lipid to be precisely and reproducibly quantified.[180, 219] This technique has also enabled the investigation of the relationship between lipid *distribution* and the symptomatic status in the carotid arteries, beyond the volumetric quantification of vessel lipid content.[219] Therefore, the ability to quantify vessel lipid content with high sensitivity may allow the extent of carotid plaque lipid to be surveyed in high risk vascular patients who were not pre-screened for existing carotid disease. This will help to establish patients with propensity to develop lipid-rich plaques at multiple sites. These patients may be candidates for new lipid modifying drugs. In addition, T2 mapping technique may allow the effects of intensive LDL-c reduction to be quantified serially with much greater precision than has been previously possible and in patients who do not necessarily have large lipid-rich necrotic cores. Therefore, this chapter seeks (i) to investigate the extent of carotid plaque lipid in patients presenting with ACS and (ii) to test whether intensive LDL-c reduction, using an established treatment (high intensity statin), translates into rapid depletion of plaque lipid content.

4.3 Hypotheses and aims

To test the hypotheses that: (i) some patients have the propensity to develop lipid-rich atherosclerotic plaques in multiple vascular territories, and (ii) carotid plaque composition, as determined by T2 mapping MRI technique, changes with intensive LDL-c reduction. Such

changes in plaque composition may include a reduction in carotid plaque lipid percentage and an increase in plaque fibrous content.

The experimental aims of this chapter were:

1) To study the extent and distribution of *carotid* lipid content and plaque burden in patients presenting with *coronary* syndromes (ACS)
2) To investigate the effects of LDL-c reduction on carotid plaque composition after three months of high intensity statin treatment by quantifying both plaque lipid and fibrous contents at baseline and follow up.
3) To assess if there is any relationship between changes in carotid plaque burden and lipid volume following statin treatment.
4) To establish if there are any changes in carotid plaque lipid *distribution* as assessed by two imaging indices, LLD % and LAI following statin treatment (see Chapter 3 for more details)

4.4 Methods

Chapter 2 discussed the general methodology related to this chapter and only methods specific to this chapter are discussed below in further details.

4.4.1 Study population

Statin naïve patients admitted with ACS to the John Radcliffe Hospital were screened between April 2016 and November 2016. ACS was defined according to the third universal definition of myocardial infarction.[185] In detail, patients presenting with ischemic-type chest pain, associated with ECG changes and / or elevated cardiac troponin and scheduled for

invasive coronary angiography were recruited. All included subjects were adults (>18) who had not been exposed to any form of lipid-lowering drugs, including statins, at the time of admission. Patients were commenced on intensive statin therapy (atorvastatin 80 mg daily) as part of routine clinical care,[116] and were scheduled for follow up within 3- 4 months for a second MRI scan.

Ethical approval was obtained from NRES and local R&D committee prior to commencement of the study. All patients provided written informed consent prior to participation in this study.

4.4.2 MRI protocol

Both *carotid arteries* were studied at 3T (Verio, Siemens Healthcare, Erlangen, Germany) with a 4-channel phased-array carotid coil (Machnet, Roden, The Netherlands) or 10-channel phased-array carotid coil (Pulseteq, Surrey, UK). Bright-blood, TOF angiography of the carotid arteries was acquired to localize carotid bifurcation and atherosclerotic plaque. Multi-slice carotid T2 maps were generated from 14 images with echo times TE = 9-127 ms and repetition time TR = 2 s as previously described.[180, 219] Ten slices of 2 mm thickness each, covering 2 cm of both carotid arteries were acquired. Slices were centralised on the maximum plaque volume identified on TOF and T1 weighted images so full plaque length was covered within 10 slices. If no plaque was identified, then slices were centred on the carotid bifurcation. All follow up MRI scans were obtained applying identical sequence protocols and were matched with baseline MRI scans, using the carotid bifurcation as an internal landmark. A standardised patient position with shoulders pulled-down and full neck extension was coupled with a customised neck brace to minimise movement. Both left and right carotid arteries were matched separately to account for any subtle difference in neck position between baseline and follow up.

4.4.3 MRI data analysis

T2 maps of the carotid arteries were generated using mono-exponential nonlinear fitting,[167] and lumen and external vessel boundaries were segmented using a validated semi-automated procedure.[209] A segmentation method to identify plaque components was implemented using T2 threshold less than 42 ms for lipid and T2 threshold more than 42 ms but less than 90 ms for fibrous tissue as previously validated.[167, 180] Plaque components were quantified using a single voxel as one unit (0.33 x 0.33 x 2 mm) and voxels, according to T2 thresholds, were aggregated for each vessel, to yield lipid volume (without recent intra-plaque haemorrhage) and fibrous volume. Lipid and fibrous volumes of the studied vessel were expressed as the average lipid and fibrous volume obtained from all of the 10 studied slices and irrespective of the degree of stenosis. Vessel wall volume was calculated from the difference between external vessel boundary and vessel lumen. In keeping with previous studies of carotid atherosclerosis, a mean value was obtained for left and right carotid arteries and per subject was used as unit of analysis.[81, 154] Lipid distribution was assessed using two parameters namely, LLD % and a newly devised LAI (see Chapter 3 for more details). In summary, this process was executed in an individual slice with the largest lipid volume on each side (right and left). The number and percentage of separate lipid deposits were quantified in that slice. The largest lipid deposit was identified and its contribution (percentage LLD %) to the total slice lipid content was then calculated, and represented the first tested imaging index. The remainder of lipid deposits percentage were averaged (RLD %) and LAI was the ratio of LLD % and other lipid deposits RLD % as previously calculated.

4.4.4 Coronary angiogram analysis

The severity of coronary luminal stenosis was measured by two dimensional quantitative coronary angiography (Medcon QCA software, Medcon Limited, Tel Aviv). The complexity

of coronary artery atherosclerosis was scored using the modified Gensini method as previously described.[220] This cumulative numerical scoring system grades the degree of luminal stenosis (1 = 1% to 49%; 2 = 50% to 74% stenosis; 3 = 75% to 99% stenosis; 4 = 100% occlusion) which then will be multiply by a constant number according to the location of the diseased segment (proximal, mid or distal) as below:

Coronary segment		***Constant factor***
Left main stem,		5
Left anterior descending,	*Proximal*	2.5
	Mid	1.5
	Distal	1
	1st Diagonal	1
	2nd Diagonal	0.5
Left circumflex,	*Proximal*	2.5
	Distal	1
	Obtuse marginal	1
Right coronary,	*Proximal*	1
	Mid	1
	Distal	1
Posterior descending,		1
Posterolateral,		0.5

4.4.5 Statistical analysis

The Shapiro-Wilk test was used to assess whether data were normally distributed. Data were presented as mean ± standard deviation or median and interquartile range, as appropriate. Measurements at each time point were compared using paired t tests for normally distributed data or Wilcoxon signed-rank test for non-normally distributed data. Correlations were assessed using Spearman rank test. Comparison among pairwise subgroups was performed using Wilcoxon Match-Pairs Signed Rank test. Statistical analysis was performed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) and *P*-values <0.05 were considered statistically significant.

4.5 Results

54 *statin naïve* patients were screened with total of 27 subjects were excluded from the study. 7 (13%) patients had non-obstructive CAD and, therefore, their presentation was attributed to a non-coronary event, 7 patients (13%) patients were scheduled for coronary artery bypass surgery which may preclude having a follow up MRI, 2 patients (4%) had statin intolerance, 1 patient had a stroke prior to the scheduled angiogram procedure, 4 patients (7%) had contraindication to MRI, 3 patients (6%) had difficulties with follow up scan and 4 patients (7%) refused to be part of the study (Figure 4.1). A total of 27 patients were scanned at AVIC within 48 hours of their admission and included in the study. Three patients were excluded due to inadequate MRI image quality. Three patients did not complete their follow up scan and were also excluded from the analysis (Figure 4.1).

Of the remaining 21 patients, 11 patients presented with STEMI and 10 patients suffered from NSTEMI. The mean age ± SD was 60 ± 10 years with two-thirds of patients were males. Clinical characteristics of recruited patients are summarized in Table 4.1.

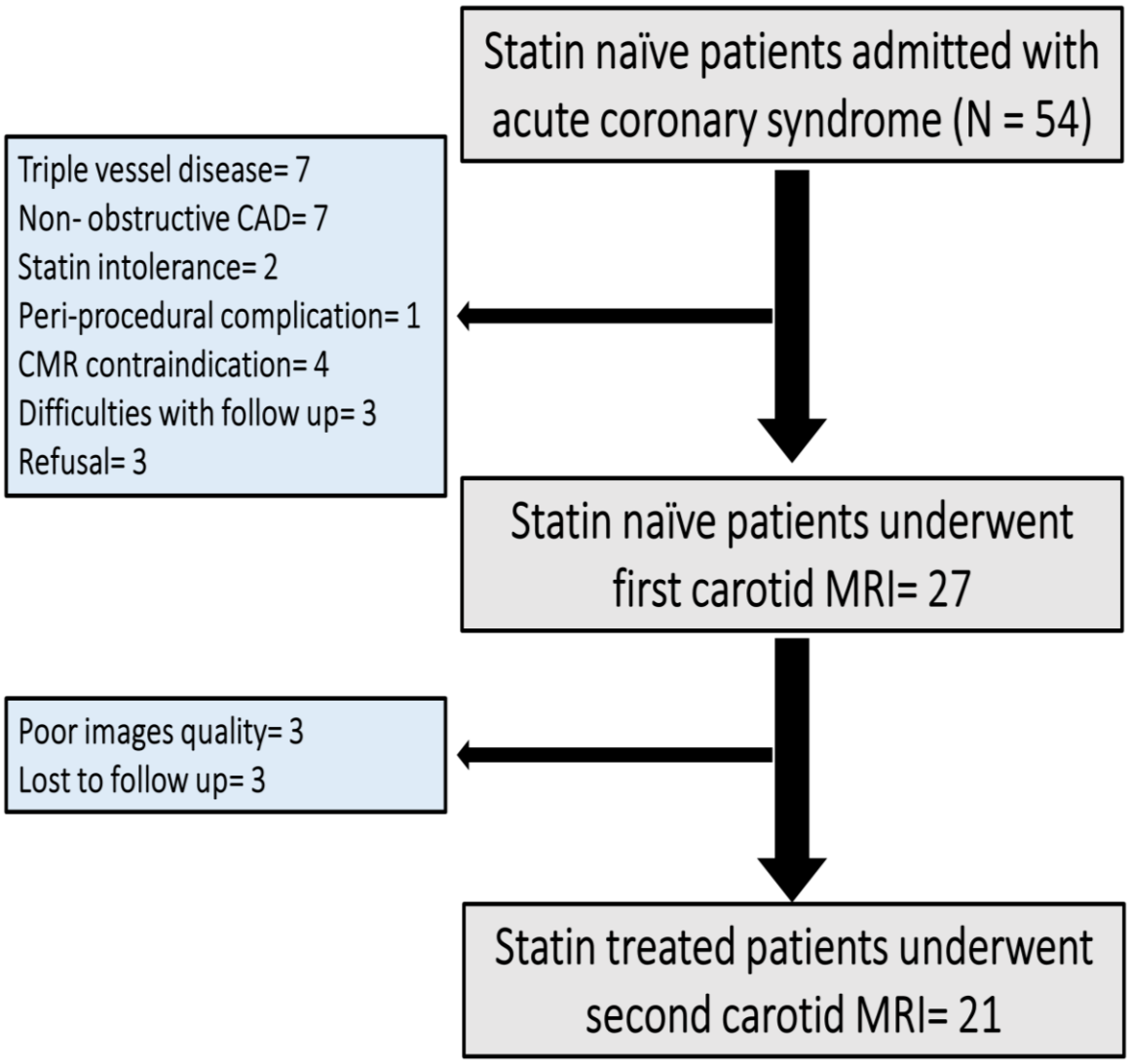


Figure 4.1 Study flow chart

54 statin naïve patients presenting with ACS were screened for study enrolment. 50% were excluded following their coronary angiogram leaving a total of 27 patients who underwent carotid MRI scan during their admission (first time point). High intensity statin treatment was commenced as part of routine clinical care and 21 out of 24 scheduled patients undertook the second carotid MRI scan (second time point). Three patients had low image qualities and were excluded from the analysis at the outset.

Table 4.1 Clinical characteristics of recruited patients

Patients characteristics, N=21		
Age (mean ±SD)		64 ± 10
Male (%)		15 (71%)
Body mass index (mean ±SD)		29 ± 5
Cardiovascular risk factors	**Hypertension**	8 (38%)
	Diabetes	1 (5%)
	Active smoking	8 (38%)
	Family history of IHD*	6 (29%)
Medication on admission	**Antiplatelet**	3 (14%)
	Beta or calcium blockers	2 (10%)
	ACE or ARB¶	7 (33%)
	Anticoagulation	0 (0%)
Presentation	**NSTEMI**	10 (48%)
	STEMI	11 (52%)
Number of disease vessels	**Single vessel**	9 (43%)
	Two vessels	10 (47%)
	Three vessels	2 (10%)
Gensini score (median, IQR)		24 (16- 36)
Troponin (ng/mL) (mean ±SD)		17 ± 21

¶ ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker

4.5.1 Extent of carotid plaque burden and lipid volumes in ACS patients

There was no significant difference in carotid lipid percentage [9.2% (7.5- 13.9) *vs.* 10.3% (4.2- 15.4), P= 0.86] or lipid volume [4.3 mm^3 (2.6- 6.1) *vs.* 5.1 mm^3 (3.0- 7.2), P= 0.55] between patients presenting with STEMI or NSTEMI, respectively. Carotid vessel wall volume was numerically higher in the NSTEMI group compared to the STEMI one but this did not reach statistical significance [50.6 mm^3 (42.7- 61.2) *vs.* 41.7 mm^3 (33.5- 48.6), P=0.07].

Similarly, there was no difference in lipid percentage (P= 0.29), lipid volume (P= 0.36) or vessel wall volume (P= 0.97) among subgroups stratified according to the number of diseased coronary arteries (defined as more than 50% degree of stenosis on quantitative angiography).

There was no significant correlation between the severity of coronary artery disease, as quantified using the modified Gensini score, and carotid lipid percentage (r= 0.39, P= 0.07), nor with carotid lipid volume (r= 0.34, P= 0.14). Likewise, there was no significant association between carotid vessel wall volume and the complexity of coronary artery disease (r= -0.12, P= 0.60).

There was a significant correlation in lipid volumes between left and right carotid arteries which was similar to that seen in patients scheduled for carotid endarterectomy (r= 0.53, P= 0.016) (Figure 4.2, Panel A). This correlation was also present when comparing vessel wall volume between left and right carotid arteries (r= 0.72, P= 0.003) (Figure 4.2, Panel B).

Combining lipid and vessel wall volumes from the whole cohort of 42 carotid arteries, there was a significant association between vessel lipid content and plaque volume (r = 0.45, P= 0.003) (Figure 4.3).

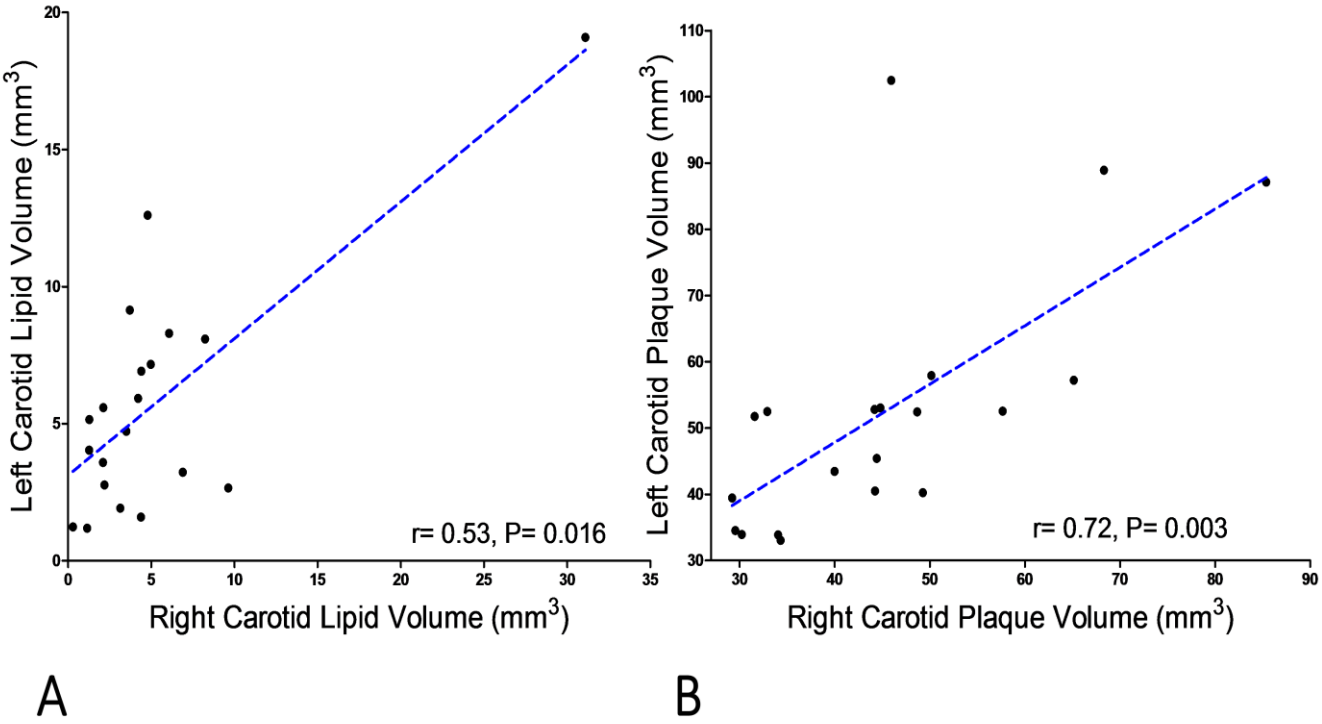


Figure 4.2 Relationship between left and right carotid arteries in patients admitted with ACS

A: Scatter plot graph demonstrating the significant correlation in lipid volumes between right and left carotid arteries as quantified on T2 mapping MRI technique. This relationship was present irrespective of mode of presentation or the number of diseased coronary arteries.

B: Scatter plot graph showing the significant relationship between left and right carotid vessel wall volume. This relationship was present irrespective of mode of presentation or the number of diseased coronary arteries.

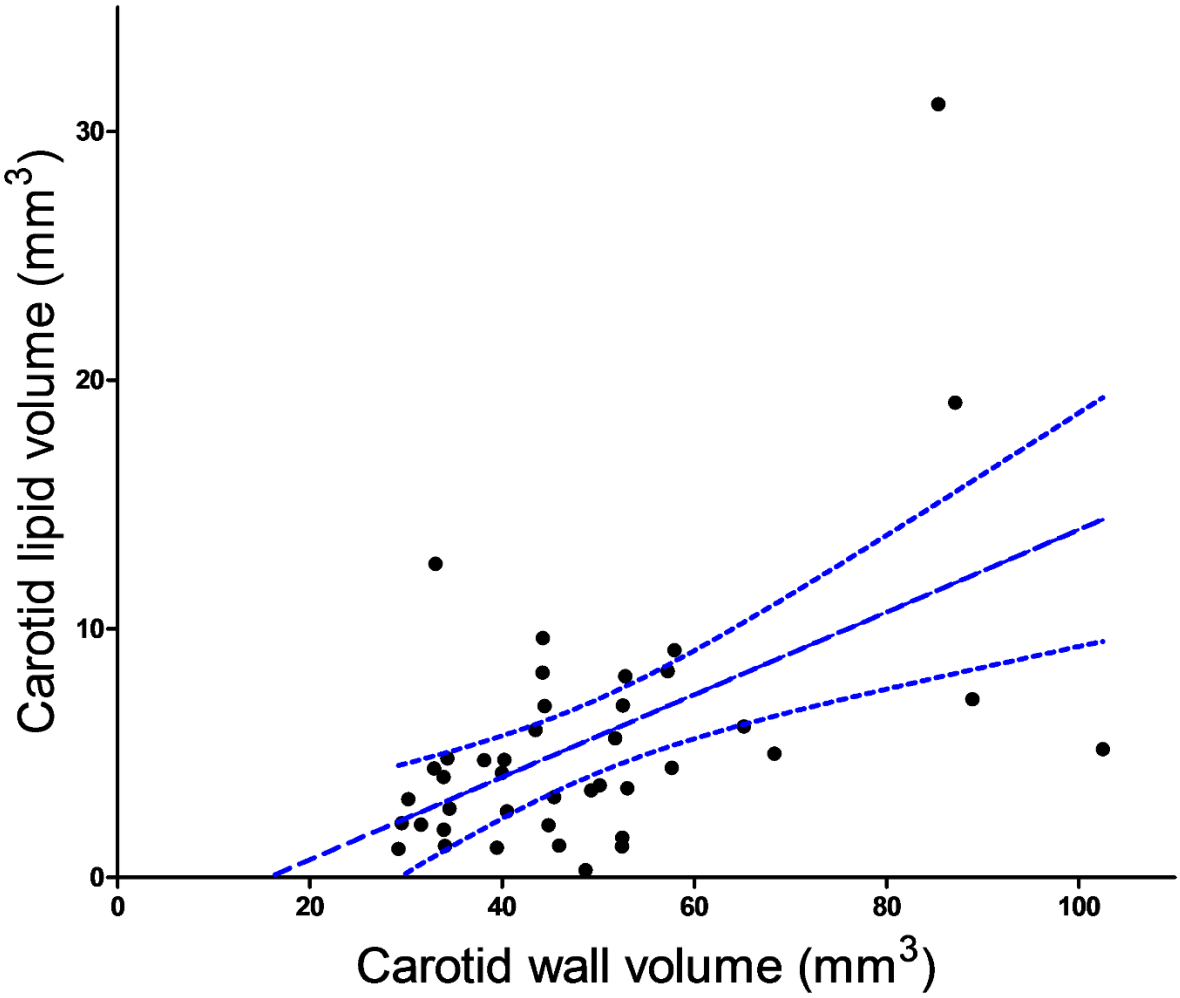


Figure 4.3 Relationship between carotid vessel wall volumes and their correspondent lipid volumes in patients admitted with ACS

Scatter plot graph illustrating the significant correlation between plaque lipid content and vessel wall volume in the whole cohort of 42 carotid arteries. This relationship was present irrespective of mode of presentation or the number of diseased coronary arteries. Notably, with larger vessel wall volume there was an increased variation in lipid content, similar to carotid disease patients scheduled for endarterectomy (see Chapter 3).

4.5.2 Change in plaque composition with high intensity statin

Following a median follow up of 107 days (IQR 100 - 137) after commencing intensive statin treatment, changes in carotid plaque composition were assessed for both lipid and fibrous contents.

Changes in plaque lipid content

There was a significant reduction in carotid vessel lipid percentage from 10.3% (7.2 - 14.2) to 7.4% (5.4 - 10.0) (P= 0.002) (Figures 4.4) & (Figure 4.5).

Similarly, there was a significant reduction in vessel lipid volume from 4.7 mm^3 (2.7 - 6.3) to 3.4 mm^3 (2.1 - 4.5) (P<0.001).

There was no difference in the reduction of lipid percentage among patients with 'early' follow up (≤ median) and those with later follow up [-3.1% (-7.2 to -0.2) *vs.* -2.3% (-4.0 to -0.1), P= 0.36].

Changes in plaque fibrous content

Compared to baseline, there was a significant increase in carotid vessel fibrous percentage following intensive statin treatment from [83.3% ± 6.6 to 85.5% ± 4.8, P = 0.039] (Figures 4.4) & (Figure 4.6).

On the other hand, there no significant change in fibrous volume over the study period [36.6 mm^3 (31.4- 47.4) *vs.* 37.4 mm^3 (31.7- 42.4), P= 0.079].

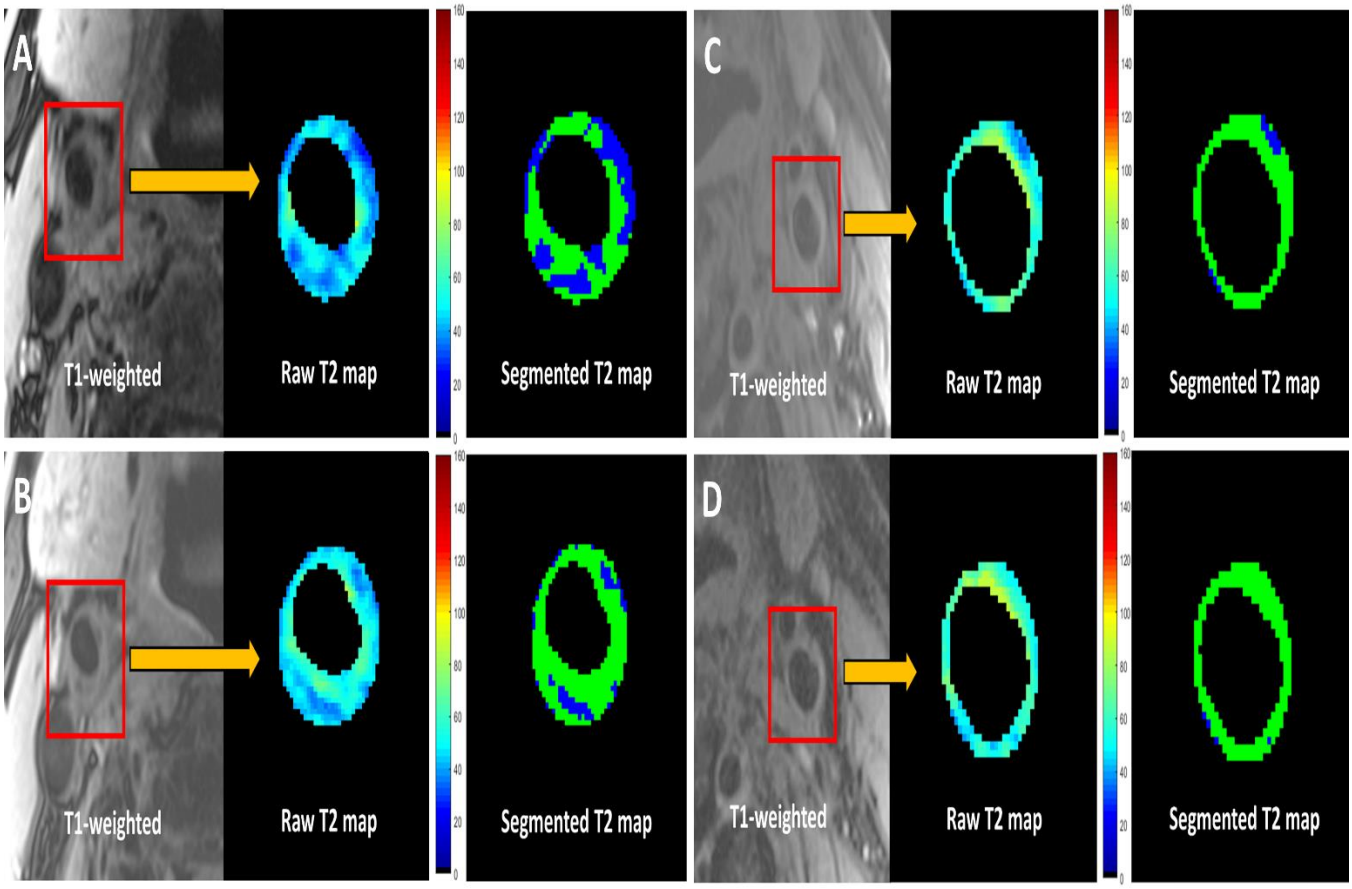


Figure 4.4 Reduction in lipid percentage as quantified on T2 mapping in two different patients

A: Cross-sectional T1-weighted image of right carotid artery plaque from 63 years old female presented with ACS with corresponding raw and segmented T2 map images.

B: Significant reduction in lipid percentage from 39% to 15% after 97 days of statin treatment.

C: Cross-sectional slice of left carotid artery from 72 years old male with ACS with matching raw and segmented T2 images.

D: After 111 days of statin treatment there was significant reduction in lipid percentage from 8.3% to 2.4%.

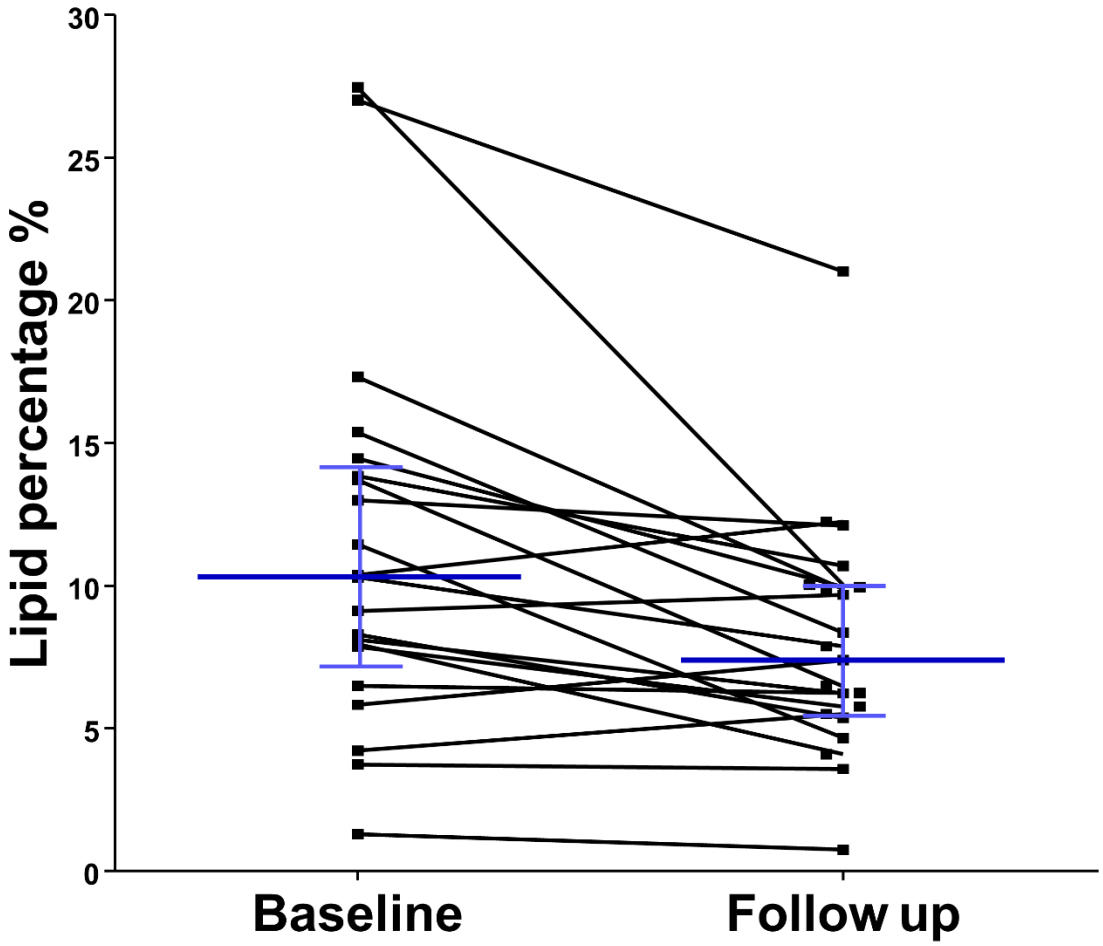


Figure 4.5 Carotid lipid percentage change over study period

Significant reduction in carotid lipid percentage in statin-naïve patients following treatment with high intensity statin. The individual variations of changes in lipid percentage suggested that most patients showed some degree of lipid percentage reduction in response to statin treatment

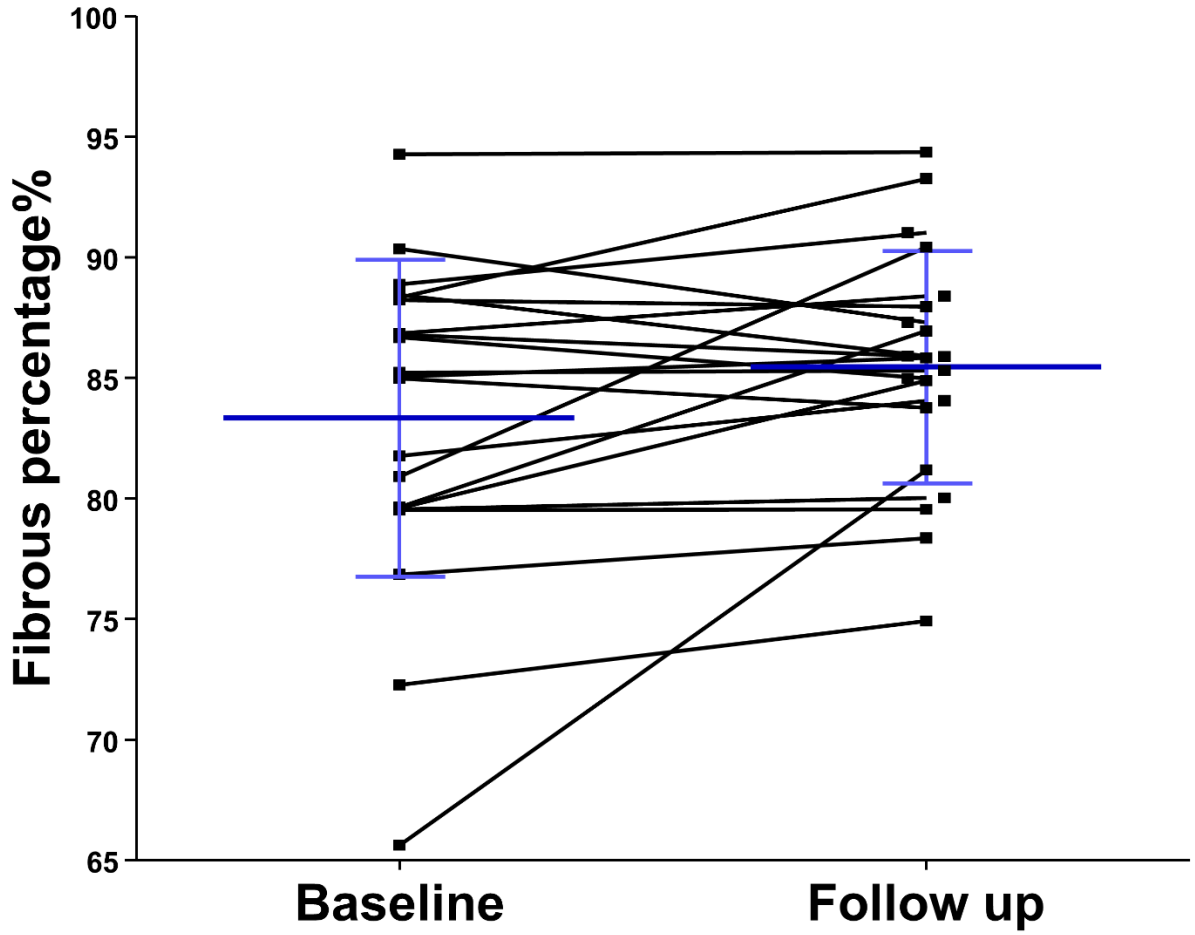


Figure 4.6 Carotid fibrous percentage change over study period

Significant increase in carotid fibrous percentage in statin-naïve patients following treatment with high intensity statin. The individual variations of changes in fibrous percentage suggested that most patients showed some degree of fibrous percentage increase in response to statin treatment.

4.5.3 Changes in carotid atherosclerosis burden following three months high intensity statin

Carotid wall atherosclerosis burden was assessed over time using

1. Mean carotid vessel wall thickness (vWT)
2. Carotid vessel wall volume
3. Carotid Lumen area.

There was a significant reduction in carotid arteries mean vWT between baseline and follow up [0.81 mm (0.76- 1.04) *vs.* 0.77 mm (0.75- 0.97), P = 0.016] (Figure 4.7, Panel A). These changes were derived from a reduction in the mean vWT in the highest quartile. There were minimal changes that took place in the lowest quartile of the mean vWT.

Similarly, there was a significant reduction in vessel wall volume between baseline and follow up [44.8 mm^3 (36.2- 54.6) *vs.* 44.1 mm^3 (36.1- 50.1), P = 0.009]. (Figure 4.7, Panel B). Similar to the mean vWT, the reduction in vessel wall volume was mainly present in the highest quartile compared to the lowest quartile of vessel wall volume.

Lumen area did not change over the study period and was comparable between baseline and follow up [42.1 mm^2 ± 12.6 *vs.* 42.1 mm^2 ± 12.5, P= 0.91].

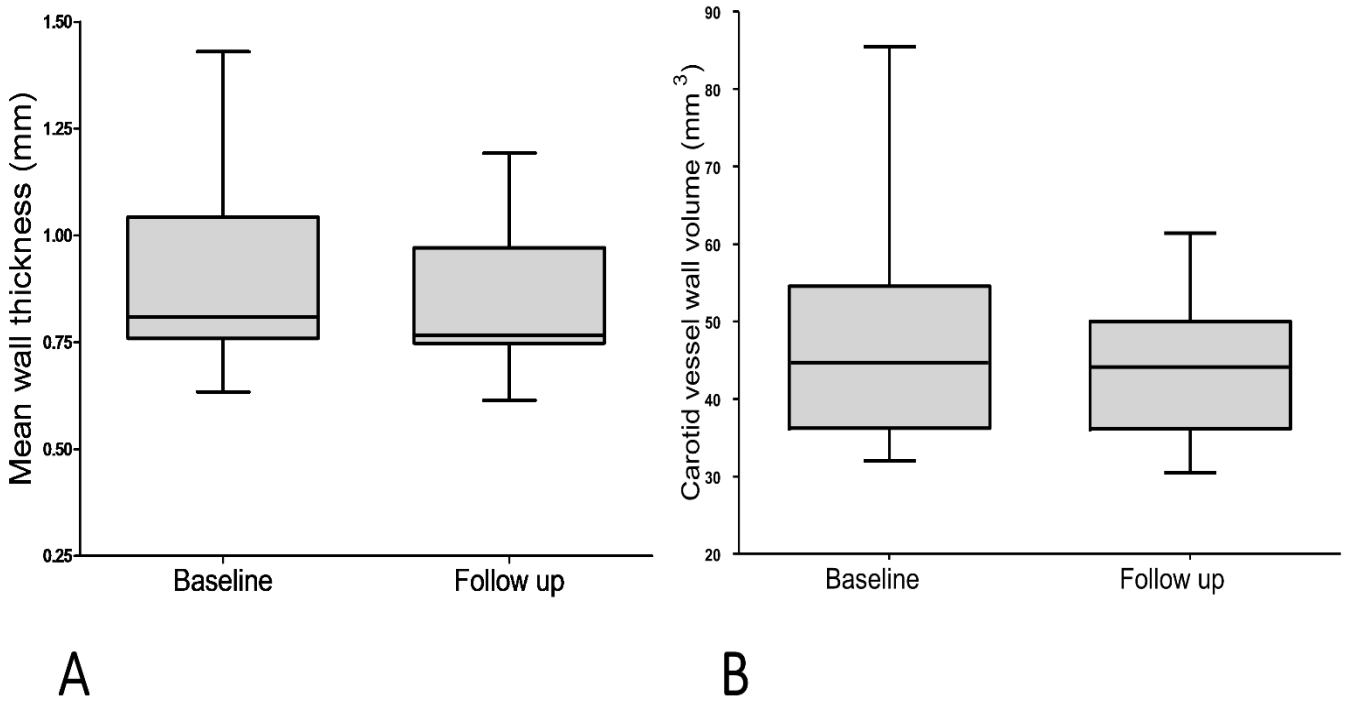


Figure 4.7 Changes in carotid atherosclerosis burden following three months of intensive statin treatment

A: Significant reduction in carotid mean vessel wall thickness when comparing baseline and follow up. The reduction was mainly affecting the highest quartile while carotid vessels with the smallest quartile vessel wall thickness stayed comparably the same over the study period.

B: Significant reduction in carotid vessel wall volume following three months statin treatment. Similarly, the reduction in vessel wall volume was mainly affecting the highest quartile while carotid vessels with the smallest quartile stayed comparably the same over the study period.

4.5.4 Relationship between changes in carotid plaque burden and lipid volume

Using a modified AHA classification of carotid vessel wall (Table 2.2, Chapter 2),[152] slices of each individual corresponding to type I and II were categorised into the '*normal*' group while other slices corresponding to type III onward of AHA classification were combined together as the '*abnormal*' group.

In the '*normal'* group, compared to baseline there were no significant changes in either carotid lipid percentage [3.1% (0.5 – 7.1) *vs.* 3.7% (0.8 – 6.6), P= 0.086] or carotid lipid volume [1.1 mm^3 (0.2 – 2.4) *vs.* 1.3 mm^3 (0.2 – 2.7), P = 0.085] (Figure 4.8, Panel A). Similarly, there was no significant changes in carotid vessel wall volume after three months of statin treatment [33.0 mm^3 (28.5 – 40.0) *vs.* 33.3 mm^3 (28.8 – 38.6), P= 0.61] (Figure 4.8, Panel B).

On the other hand, there was a significant reduction in carotid vessel lipid percentage in the *'abnormal'* group when compared to baseline [9.7% (4.1 – 18.4) *vs.* 6.2% (2.3 – 12.8), P< 0.001]. Likewise, lipid volume was significantly reduced following statin treatment in this subgroup [4.4 mm^3 (2.2 – 8.0) *vs.* 2.9 mm^3 (1.1 – 5.8), P< 0.001] (Figure 4.8, Panel A). In addition, carotid vessel wall volume followed the same trend and a significant reduction was detected between baseline and follow up [46.8 mm^3 (37.1 – 64.6) *vs.* 44.6 mm^3 (35.0 – 57.7), P< 0.001] (Figure 4.8, Panel B), suggesting that changes in both lipid content and vessel wall volume in the whole cohort were mainly derived from the reduction in areas with a significant carotid disease.

There was no significant correlation between changes (difference between baseline and follow up) in carotid vessel wall volume and lipid volume or percentage over time (r= 0.22, P= 0.34 & r= -0.18, P= 0.94, respectively).

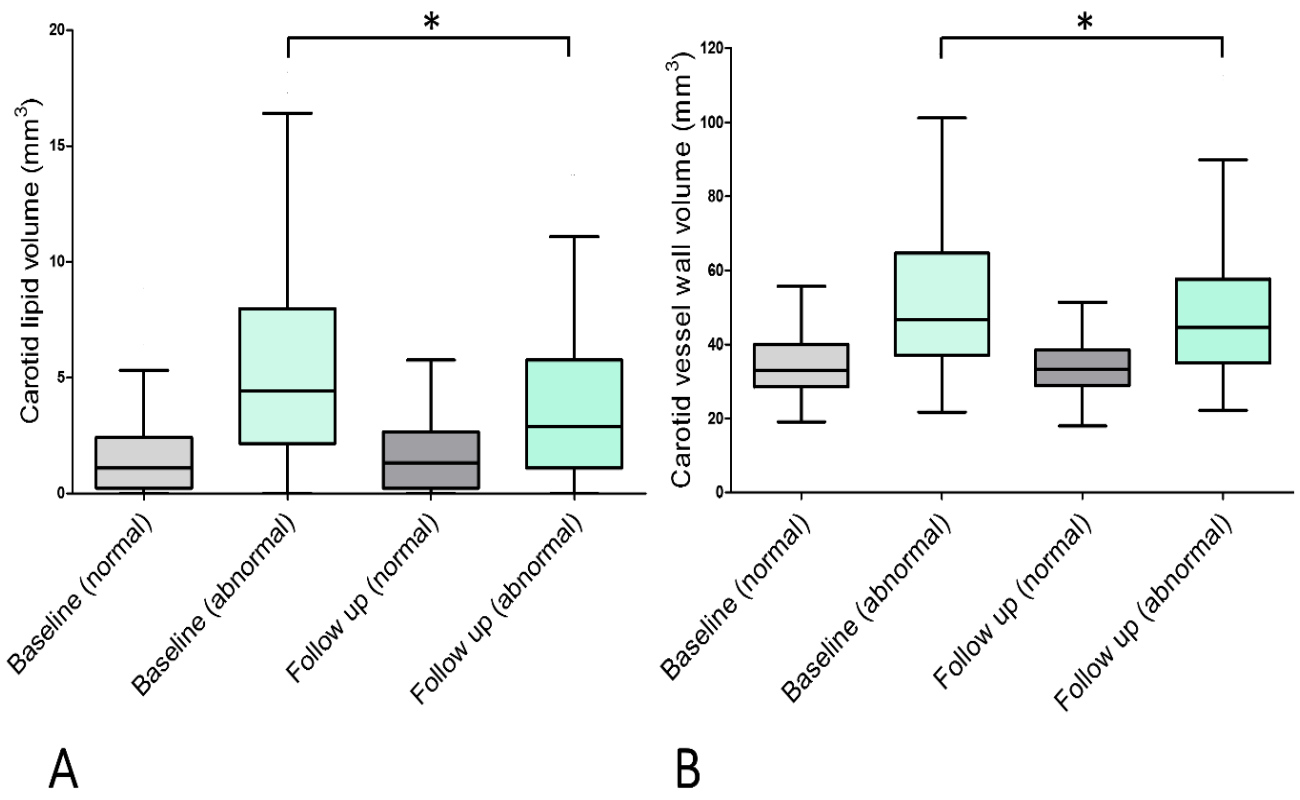


Figure 4.8 Changes in carotid lipid content and vessel wall volume stratified according to AHA classification

A: Changes in carotid lipid volume in both the '*normal'* group where no significant reduction was noted following statin treatment and the '*abnormal'* group where significant reduction in carotid lipid volume was identified compared with baseline.

B: Changes in carotid vessel wall volume in the same two groups. In the '*normal'* one, there was no detected difference in carotid vessel wall volume over study period, while the '*abnormal'* group had a significant reduction in vessel volume following three months of statin treatment.

4.5.5 Changes in lipid distribution with statin treatment

Lipid distribution was assessed using two imaging indices, LLD % and newly-devised LAI (more details in Chapter 3, data analysis).

The median baseline LLD % was 45% (30 – 53) which was significantly reduced, following three months of statin treatment, to 36% (25 – 48), P= 0.027 (Figure 4.9, Panel A) & (Figure 4.10).

Similarly, there was a significant reduction in LAI between baseline and follow up [2.9 (2.2 – 5.4) to 1.8 (1.3 – 3.0), P= 0.004] (Figure 4.9, Panel b) & (Figure 4.10).

Changes in both indices were suggestive of a 'stabilised' pattern in lipid distribution following the initiation of statin treatment. These changes were reflective of more scattered arrangement of lipid deposits.

To assess whether these lipid distribution indices were independent of the reduction in lipid content, both regression analysis and correlations were performed. Using linear regression model, adjusted for the side of carotid artery (left or right) with age and gender as background variables, changes in LLD % was not a predictor of change in lipid percentage [0.01, 95% confidence interval (0, 0.02), P= 0.17] or changes in lipid volume [0.02, 95% confidence interval (0, 0.05), P= 0.09]. On the other hand, changes in LAI was a predictor of changes in both lipid percentage [0.50, 95% confidence interval (0.16, 0.85), P= 0.006] and changes in lipid volume [1.14, 95% confidence interval (0.59, 1.69), P<0.001].

There was no significant association between change in LLD % and change in lipid percentage (r= 0.22, P= 0.18) or change in lipid volume (r= 0.16, P= 0.34). Similarly, change in LAI was not associated with change in lipid percentage (r= 0.25, P= 0.13) or change in lipid volume (r= 0.24, P= 0.15).

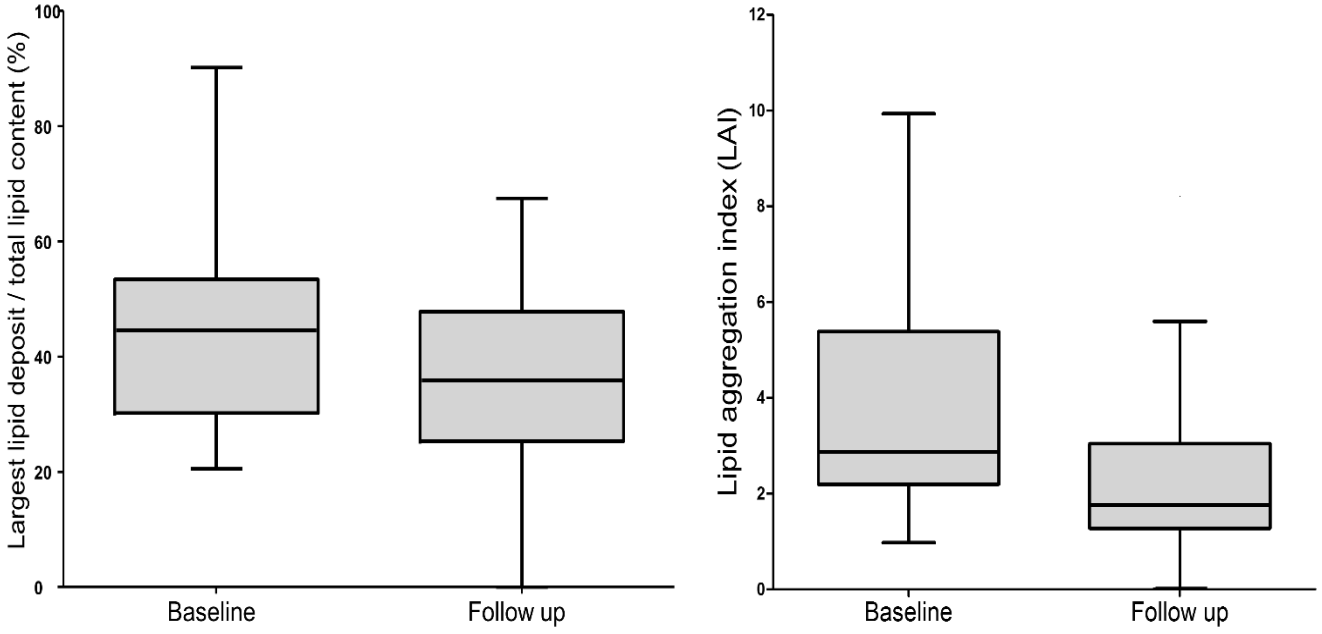


Figure 4.9 Changes in lipid distribution as assessed by LLD % and LAI

A: Following three months of statin treatment, there was a significant reduction in the contribution of largest lipid deposit out of the total lipid content.

B: Lipid aggregation index was significantly reduced over study period.

Both indices are suggestive of more scattered lipid deposits and potentially more stable plaques following statin treatment.

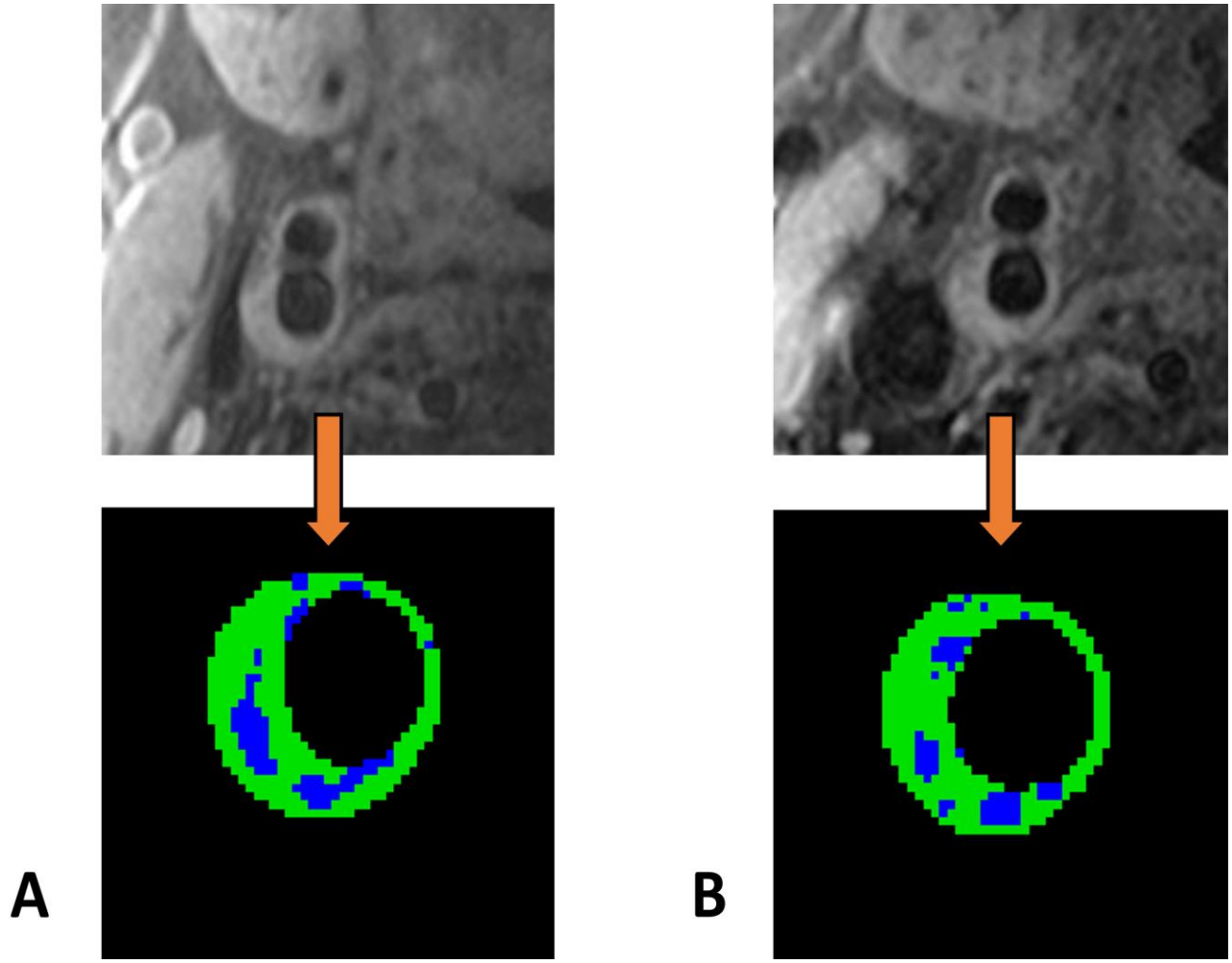


Figure 4.10 Changes of lipid distribution following statin treatment

A: Cross-sectional T1-weighted image of right carotid artery in 72 years old male who presented with unstable angina. His baseline LLD % was 52% with corresponding LAI of 6.50.

B: Following 101 days of statin treatment, patient's LLD % was decreased to 38% with corresponding LAI of 2.68. Importantly, the calculated LLD was not identical between baseline and follow up.

4.6 Discussion

The current analyses of carotid arteries using highly sensitive T2 mapping MRI technique in patients *who were not pre-screened for carotid disease* but who presented with acute coronary syndrome have produced the following findings:

1. There was a dissociation between the severity of coronary artery disease assessed using the modified Gensini score and both carotid wall volume and lipid volume.
2. There were striking similarities in the association between lipid and vessel wall volumes, and, additionally, between left and right carotid arteries irrespective of the category of disease (post ACS or endarterectomy scheduled patients).
3. Intensive LDL-c reduction was associated with significant changes in plaque composition detected after as little as three months of statin treatment. These changes included a reduction in carotid vessel lipid content coupled with an increase in fibrous percentage.
4. There was a significant reduction in lipid distribution indices suggestive of more scattered and 'stabilised' pattern following statin treatment.

The unselective approach of applying new lipid-lowering drugs have yielded a small ARR with unmanageably high NNT.[63, 105] Tools to identify a subgroup of patients that may benefit maximally are urgently needed. It is plausible that LDL-c reduction is translated into cardiovascular benefits by evacuating lipid from atherosclerotic plaques.[165, 172] Therefore, using plaque lipid content may be promising to rationalise the applications of these new lipid-lowering drugs. Individuals with multi-sites, coronary and carotid, lipid-rich plaques maybe the right candidates for these drugs. Yet, the 'disconnect' between carotid and coronary arteries in this chapter was probably anticipated. The severity of coronary artery disease quantified using angiographic-based tools has been shown to be weakly correlated with *coronary* plaque lipid measured by NIRs.[221] Such disparity in the same territory makes it

unlikely for a relationship to exist with different vascular territories. Nonetheless, a comparison between coronary and carotid lipid volumes would be a pressing question to address.

The significant correlation of carotid lipid and vessel wall volumes was strikingly similar in patients presenting with ACS and in patients scheduled for endarterectomy. Despite having different coefficients of correlation (r= 0.69 in the endarterectomy group versus r= 0.45 in the ACS one), the regression lines were similar when comparing both slopes (P= 0.90) and intercepts (P=0.18). Such similarity suggests a potentially similar pathophysiological process between lipid and plaque volumes that was common to both patient groups. The modest correlation in ACS cohort is possibly reflective of larger variations between plaque and lipid volumes but is more likely to be secondary to the tighter range of both lipid content and vessel wall volumes (Figure 4.11).

Likewise, there was a significant relationship between left and right carotid arteries imitating the same association in the endarterectomy group. This association re-emphasised the systemic nature of atherosclerosis but, more importantly, it suggests that some patients may have the propensity to develop lipid-rich plaques. Identifying this group of patients may offer an indication for emerging new lipid lowering drugs.[43] Patient stratification based on the extent and nature of atherosclerotic plaques is a promising strategy for selective aggressive treatment, akin to the imaging-based staging that is used to guide selection of cancer treatments.[43, 183, 184]

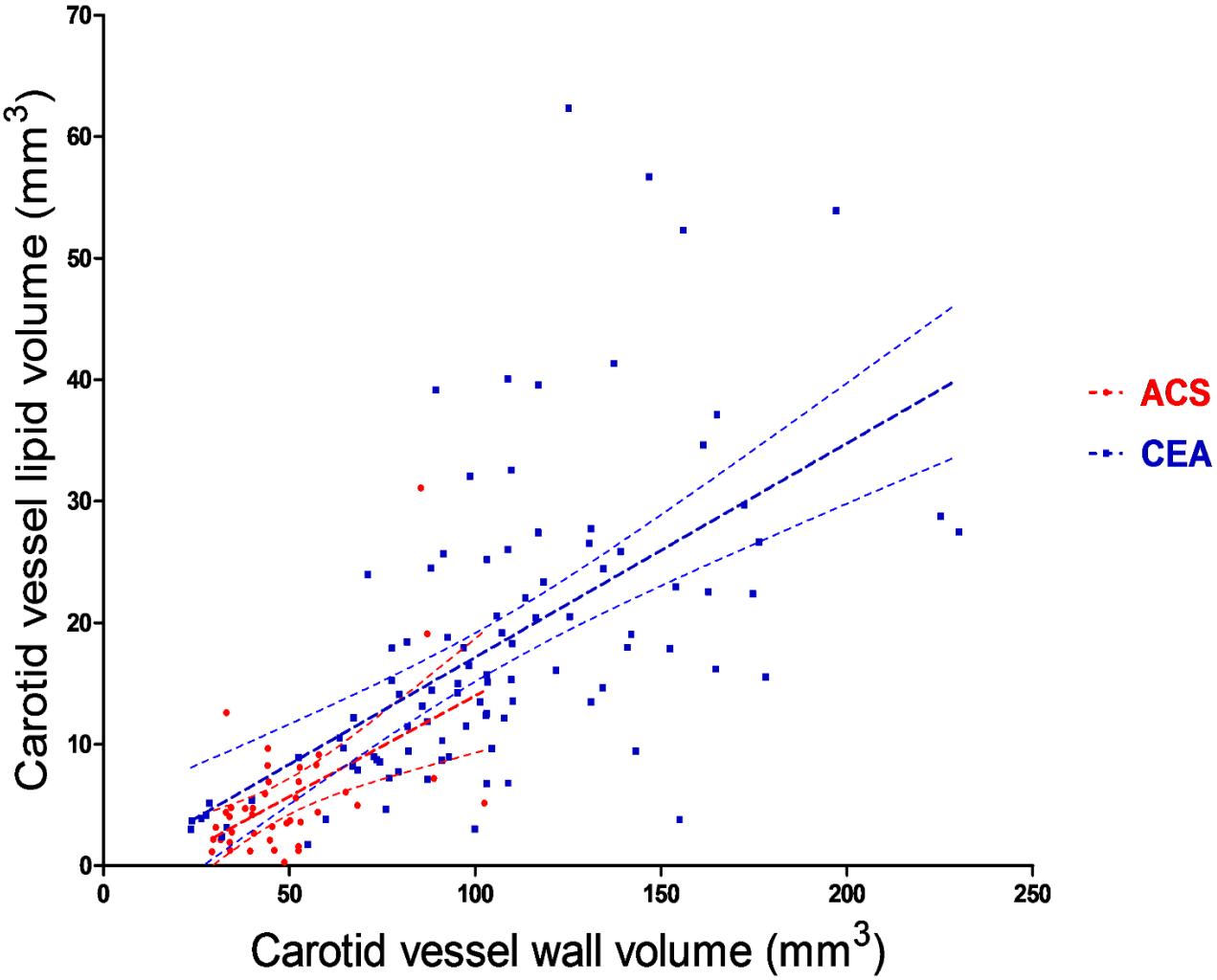


Figure 4.11 Relationship between carotid vessel wall and lipid volumes

Scatter plot figure demonstrating the striking similar relationship between carotid vessel wall and lipid volumes in two different cohorts, CEA in blue and ACS in red. Notably, both lipid and plaque volumes are larger, and with a wider range, in the CEA cohort compared to the ACS one, yet, both have comparable slopes and intercepts.

Plaque lipid depletion in response to LDL-c reduction is widely accepted as a plausible mechanism of benefits using lipid lowering drugs.[43, 148] In that model, the reduction in plaque lipid content coupled with the increase in fibrous percentage changes the status of the plaque to one less prone to rupture.[43] Changes in plaque composition have previously been demonstrated in both the ORION and CPC studies,[148, 165] but only after 12 months of LDL-c lowering treatment and in patients with pre-defined carotid disease. This is important as it reflects both the potential utility and the limited sensitivity of the previous generation of multi-contrast imaging tools used to detect 'subtle' changes within short periods of time. Moreover, it restricts the use of imaging as an approach to monitor response to lipid lowering treatment to those with identified carotid disease. Within only three months of statin treatment, reduction in *carotid atherosclerosis burden,* as quantified on MRI, was shown to occur among stable coronary patients.[154] Changes in plaque composition, on the other hand, could be demonstrated within three months of pravastatin treatment in an *ex-vivo* model of explanted carotid plaques.[160] Here, using a newly validated T2 mapping MRI technique, *in-vivo* plaque composition changes occur as early as three months. These findings are in-line with previous histological studies,[160] but they extend the findings into patients with no pre-defined carotid disease. This makes T2 mapping MRI a highly-sensitive and comprehensive tool that could be potentially applied to identify high risk patients and to guide treatment selection and to track their response to lipid lowering therapies. This technique is capable of accurate, objective, reproducible volumetric quantification of plaque lipid on a voxel-by-voxel basis.[167, 180]

Plaque lipid depletion has been examined invasively using NIRs, and non-invasively using MC-MRI techniques.[148, 172] The limitations related to the MC-MRI have restricted our capacity to study changes in plaque composition to a minority of recruited patients.[148, 165, 166] This was reflected in small proportion of screened patients, with discernible lipid core, that could be included (55% in the ORION and 27% in the CPC studies).[148, 165] This is important as changes

in plaques with no identifiable lipid core were not studied, thus, limiting the generalizability of observations of net lipid evacuation in response to LDL-c reduction.

In this study, changes in vessel lipid content were examined irrespective of plaque type or degree of stenosis. There was almost 30% relative reduction in lipid percentage in subjects presenting with ACS who were commenced on high dose statin therapy. Interestingly, this reduction was derived from areas with a significant carotid artery disease. On the other hand, there were no detected changes in lipid vessel content in structurally normal carotid artery. Moreover, changes in carotid vessel wall volume followed the same course with no detected regression in the *'normal'* group. The reduction in carotid plaque lipid content was mirrored with a significant increase in fibrous percentage resembling previously reported *in vivo* and *ex vivo* studies.[148, 160]

Lipid distribution, quantified using LLD % & LAI, was also changed following three months of statin treatment. The reduction in both indices was suggestive of more scattered pattern of vessel lipid content and potentially making atherosclerotic plaque less prone to rupture. Although changes in lipid distribution were not parallel to those of lipid content, reduction in lipid percentage and lipid volume were significant predictors of reduction in LAI. On the other hand, changes in lipid content did not predict reduction in LLD %. This is potentially related to the fact that LLD % disregards the changes of all but the largest lipid deposits while both LAI and lipid content take into account the entire lipid deposits.

Taking all these results together, direct quantification of both plaque lipid content and distribution, using T2 mapping technique, may contribute to the identification of patients at greatest risk of atherosclerosis complications and to the targeting of this patient group with aggressive lipid lowering drugs. The indiscriminate use of these drugs is not desirable, practical or affordable and even when successful, the margin of benefits is small challenging their routine use post ACS.[43, 63, 114] Therefore, lipid quantification techniques could be tested for use

as stratification tools to identify patients with high lipid percentage within atherosclerotic plaque and to guide more rational treatment by adding a second lipid modifying drug. In addition, direct quantification of plaque lipid will allow the effects of intensive LDL reduction to be monitored serially with much greater precision than has been possible previously. This will give the opportunity to assess patients' response and offer the chance to change current treatment regime accordingly. Finally, this technique could be used as a screening test for patients prior to recruitment in clinical trials based on their plaque lipid percentage. Patients with "high lipid content plaques" might benefit disproportionately from emerging agents that lower LDL very aggressively. This will rationale clinical drugs trials producing an overall cost-effective approach for expensive therapies.

The main limitation of this method is the sensitivity to motion artefacts of the DANTE-MESE sequence as previously reported in Chapter 3.[180, 219] In this study the overall rejection rate was 13% of all scans which is a significant improvement since the initial rejection rate of 35%. In addition, a causal relationship cannot be determined given the observational design of the current study. Moreover, the recruited patients post ACS were commenced on other drugs such as antiplatelet therapy which theoretically may affect the function of LDL-c.[222] Yet, this study was not a test of statin *per se* and a randomised placebo-controlled clinical trial would not have been appropriate or ethical in this patient group. Nonetheless, a group of previously-treated statin patients of whom their statin doses were not changed during their admission may serve as a control group. Such group would allow to accurately delineate statin-induced plaque lipid reduction and potentially overcome some of limitations highlighted above.

4.7 Conclusion

The work presented in this chapter has demonstrated the striking similarities of both the association between carotid vessel wall and lipid volumes, and relationship between left and

right carotid arteries irrespective of the disease category. In addition, it showed the disparity between the severity of coronary artery disease, quantified using the modified Gensini score, and both carotid vessel wall and lipid volumes. Finally, the reduction in both carotid lipid content and distribution following three months of statin treatment support the strategy of using this highly sensitive technique: (a) to identify patients with multi-sites lipid rich plaques, and (b) to track these patients' response to lipid lowering therapies. Failure to display reduction in lipid content may prompt changing or adding a second treatment for 'intractable' atherosclerosis process. The application of T2 mapping could also be used as screening test for patients prior to recruitment in clinical trials based on their plaque lipid percentage and will lay the ground for Phase 2 and/ or 4 studies of emerging lipid active agents.

Chapter 5: Relationship of plaque lipid content quantified using T2 mapping MRI technique and functional lipid indices in blood

5.1 Abstract

Background

Emerging classes of lipid-lowering therapies are highly efficacious in reducing plasma LDL-c, but when added to statin in unselected patient groups e.g. "all comers' post-ACS, the NNT was unmanageably high. Tools to guide the selection of targeted therapies are urgently needed. Candidates include new generation blood biomarkers to characterise the disease process in individual patients. These blood biomarkers are of current and real importance since drugs targeting some of these molecules and pathways are already in advanced stages of clinical development. T2 mapping MRI technique offers the opportunity to study the relationships between emerging functional measures of cholesterol metabolism and plaque lipid content as the presumed target of targeted of lipid-lowering therapies.

Methods

Statin naïve subjects presenting with ACS underwent MRI of their carotid arteries at 3Tesla scanner coupled with blood sampling for both standard and functional lipid indices. These biomarkers were repeated after three months during participants' follow up visit for carotid MRI. Over this period, study participants were commenced on high intensity statin as per routine clinical care.

Results

Twenty-one patients had analyzable paired carotid MRI and blood sampling. Following three months of intensive statin treatment, there was a significant reduction in total cholesterol,

(P=0.001), LDL-c (P<0.001), triglyceride (P=0.05), high sensitive CRP (hsCRP) (P= 0.001, LDL particle concentration (P<0.001) and cholesterol efflux capacity (P<0.001).

None of the studied functional blood biomarkers were strongly related to either *baseline* carotid plaque lipid content or its propensity to *change* with statin treatment.

With the exception of *change* in hsCRP, there was no difference in *baseline* blood lipid indices or their *changes* over time between two groups stratified according to the magnitude of reduction in lipid percentage.

Conclusions

Functional blood biomarkers did not relate to plaque lipid content nor did they predict its changes with treatment. These findings support the notion of possibly using plaque imaging as a tool for identifying patients who are potentially suitable for intensive lipid modification better and beyond single blood lipid biomarker.

5.2 Introduction

Statins have been shown repeatedly to be effective in reducing adverse cardiovascular events.[13, 15] Their benefits have been proportional to the level of plasma LDL-cholesterol reduction.[14] However, a substantial proportion of individuals on intensive statin therapy, who have achieved "normal" LDL-cholesterol level, continue to experience adverse cardiovascular events.[130] In order to address this "residual" risk, new classes of lipid-lowering drugs were added to statin to test their effects on clinical outcomes in large randomised trials.[39, 63, 104] These drugs were highly efficacious in reducing LDL-c, however, the unselected nature of adding them to "all comers" post ACS made the NNT unmanageably high.[39, 63, 105] Without a clear means of stratification, patients who stand to benefit greatly will miss an important therapeutic opportunity.

LDL-c, like other traditional risk factors, is not good at stratifying risk at individual level.[124] Moreover, using LDL-c as a single blood biomarker to assess the efficacy of added therapies to statin treatment may become less desirable since these therapies have been ineffective in a large majority of individuals.[43, 79, 100] Some of these individuals continued to be at increased risk, possibly as a result of non-LDL-c biomarkers. Therefore, tackling individuals' future risk should be determined by characterising patients beyond the level of a single risk factor i.e. LDL-c.[124] This is important since numerous factors, including lipid and non-lipid markers are intricately related to the progression of atherosclerosis.

For this purpose other blood biomarkers have been suggested as adjuvant measures of LDL-related risk to add more insights to an individual's level of risk.[223] These biomarkers ranged from non-LDL lipid indices such as HDL-c and triglycerides, to the functionally pleotropic properties of lipoprotein particles.[224-226] Moreover, markers of inflammation such as serum C-reactive protein and Lp-PLA2 to new functional lipid parameters like cholesterol efflux capacity and PCSK9 level were also proposed.[43, 227] These blood biomarkers have been

advocated as both ‘stratification’ tools and also as potential therapeutic targets given their plausible propensities to contribute to plaque lipid accumulation and/ or removal.[43, 130, 228] Nonetheless, the complex interactions amongst these different lipid and blood biomarkers made it difficult for any single measure to be adopted into routine clinical practice.[43]

Plaque lipid content has been associated with an increased risk of plaque rupture, rendering it a strong marker to recognise high risk plaques.[229] Moreover, plaque lipid depletion in response to LDL-c reduction is widely accepted as a plausible mechanism of benefits using lipid lowering drugs.[43, 172] Levels of LDL-c might be thought to predict plaque lipid content, however, it has proven difficult to ascertain or exclude any relation between plaque lipid content and conventional blood lipid markers.[43, 148] Functional blood lipid biomarkers such as PCSK9, CETP, and HDL efflux were demonstrated to have a significant role in future cardiovascular events (see Table 2.3). These biomarkers may provide further insights in to the dynamic aspects of lipid metabolism and potentially may be better representatives of plaque lipid content.[87] Major limitations in plaque imaging techniques have restricted our ability to rigorously study any relationship with blood lipid indices and confined any attempts to a minority of subjects.[148, 165, 166]

Only recently have novel vascular imaging techniques become available to accurately quantify plaque lipid content.[126, 180] Quantitative T_2 map accurately quantifies carotid plaque lipid content non-invasively and with a striking degree of accuracy.[180, 219] The ability to quantify both baseline plaque lipid content and changes over time will allow the functional significance of potential blood biomarkers to be evaluated for the first time. This is important since imaging validation of new [panels of] blood biomarkers might obviate the need for expensive imaging while opening up possibilities for precision / stratified medicine for patients.

Therefore, this chapter seeks to study the relationship, if any, between plaque lipid content, quantified on T2 mapping MRI technique, and both standard and functional lipid biomarkers; and to determine if any of these biomarkers could predict the propensity of plaque lipid content to change in response to LDL-c lowering therapy

5.3 Hypotheses and aims

To test the hypotheses that: (i) plasma lipid biomarkers will be *dissociated* from plaque lipid content as quantified using T2 mapping MRI technique and (ii) that changes in plaque lipid content, in response to intensive statin treatment, are not related to baseline lipid indices or their propensity to change using both standard lipid biomarkers (including HDL-c and triglyceride, and functional ones such as HDL efflux capacity, CETP activity and Lp(a)).

The experimental aims of this chapter were:

1) To assess the response of blood lipid indices to high dose statin therapy over three months.
2) To study the relationship, if any, between carotid plaque lipid content and both standard and functional blood lipid biomarkers.
3) To establish if baseline blood biomarkers or their tendency to change can predict changes in carotid lipid content in response to statin treatment.
4) To investigate if there are any differences in baseline blood lipid indices or their tendencies to change in relation to the magnitude of plaque lipid reduction in response to statin treatment.
5) To assess if there are any differences in plaque imaging biomarkers or their propensities to change in relation to the level of attained LDL-c with statin.

5.4 Methods

Chapter 2 discussed the general methodology related to this chapter and only methods specific to this chapter are discussed below in further details.

5.4.1 Study population

Statin naïve patients admitted to the John Radcliffe Hospital with an ACS between April 2016 and November 2016 were screened (see Figure 4.1 for more details on excluded patients). ACS was defined according to the third universal definition of myocardial infarction and more details on included subjects are in Chapter 4, study population.[185] All patients were adults (>18) and were scanned in AVIC within 48 hours of their admission. At the time of MRI scanning, venous blood samples were obtained. No patients had been exposed to any form of lipid-lowering drugs, including statins, at the time of admission but were commenced on intensive statin therapy (daily atorvastatin 80 mg) as part of routine clinical care.[116] Subsequently, patients and were scheduled for follow up within 3 months for follow up MRI scans coupled with second venous blood samples.

All included patients in this study provided written informed consent. The study protocol conformed to the ethical guidelines and was approved by the NRES and local R&D committee prior to commencement of the study.

5.4.2 MRI protocol and data analysis

MRI protocol and data analysis were described in more details in Chapter 2 & 4. In summary, T2 maps were generated using mono-exponential nonlinear fitting with semi-automated procedure to segment both lumen and external carotid vessel boundaries.[167, 209] Voxels with T2 threshold of less than 42 ms were segmented and identified as lipid component of the

carotid vessel wall.[167, 180] A mean value was obtained for left and right carotid arteries lipid percentages and was used per subject for analysis to relate lipid content to blood lipid biomarkers.[81, 154]

5.4.3 Blood lipid biomarkers assays analyses

Blood samples were obtained via venepuncture using winged butterfly system (vacuum extraction) and standard laboratory tubes. Serum and EDTA-plasma were centrifuged at 3,300 x g for 15 minutes at 4^{o} C within one hour of blood collection and stored in aliquots at −80 °C for future analyses. Standard lipid measurements (i.e. LDL-c, total cholesterol, HDL-c and triglyceride) in addition to lipoproteins particle size and concentrations were analysed using NMR spectroscopy using Axinon lipoFIT-S100 (Numares AG, Regensburg, Germany). ApoA1, hsCRP and PCSK9 were determined in plasma by ELISA using kits from R&D systems Ltd (Abingdon, UK). Cholesterol ester transfer protein activity was measured using a fluorometric method using assay kits from Abcam (Cambridge, UK). Lp(a) was measured in serum using ELISA kit from Abcam (Cambridge, UK). The cholesterol efflux capacity of HDL was determined using fluorescence-labelled cholesterol method as validated previously.[86, 87] (see Chapter 2 for more details).

5.4.4 Statistical analysis

The Shapiro-Wilk test was used to assess whether data were normally distributed. Data are presented as mean ± standard deviation or median and interquartile range, as appropriate. Measurements at each time point were compared using paired t tests for normally distributed data or Wilcoxon signed-rank test for non-normally distributed data. Categorical data between response groups were analysed by the $\chi 2$ test, while continuous variables were

assessed with unpaired t test or Mann Whitney test, where appropriate. Independent predictors of change in lipid percentage were measured using the multivariable linear regression analysis. To construct the multivariable model, only variables with a probability value <0.05 at univariable analysis were entered into the multivariable model, with age and gender as background variables. Statistical analysis was performed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) and *P*-values ≤0.05 were considered statistically significant.

5.5 Results

Over a duration of three months, 21 patients underwent two carotid MRI scans coupled with two blood samplings. The mean age ± SD was 60 ± 10 years with two-thirds of patients were males. Mean baseline LDL-c was 2.94 ± 0.85 mmol/ L with HDL-c of 1.17 ± 0.28 mmol/ L and triglyceride level of 1.76 ± 0.69. Averaged right and left carotid lipid percentage as quantified on T2 mapping was 10.3%.

5.5.1 Changes in blood lipid indices with statin

Both total cholesterol and LDL-c were significantly reduced from 176 ±38 to 133 ±50 mg/dL (P=0.001) & 109 ±30 to 71 ±34 mg/dL (P<0.001), respectively. IDL-c was also decreased from 51 ± 12 to 40 ±19 mg/dL (P= 0.012). On the other hand, there was no significant change in HDL-c from 46 ±11 to 45 ±3 mg/dL (P= 0.71). Triglycerides were reduced from 146 (112 – 191) to 107 (71 – 144) mg/dL (P=0.05).

There was no significant changes in lipoprotein particle size, including LDL, HDL and VLDL, over the study period. Unlike HDL & VLDL particles concentration, LDL was significantly reduced from 1389 ±452 to 948 ±502 nmol/L (P<0.001). This was driven by the

reduction in small LDL particles from 600 ±248 to 452 ±305 (P=0.034) with no statistical difference in the concentration of large LDL particles.

hsCRP was also significantly reduced from 7.2 ±3.6 to 2.8 ±2.5 mg/dL (P= 0.001). Lp(a) level was numerically higher compared with baseline but did not reach statistical significance [9.6 (6.1 – 14.5) *vs.* 8.8 (5.1 – 15.0), P= 0.64]. Apolipoprotein A1 was no different over the three months statin treatment [152 (139 – 201) *vs.* 144 (120 – 202), P= 0.26].

Similarly, PCSK9 concentration and CETP activity did not change with short intensive statin treatment [276 (203 – 336) *vs.* 277 (223 – 327), P= 0.55] & [17.0 ± 1.7 *vs.* 16.9 ± 1.3, P= 0.91], respectively. Interestingly, cholesterol efflux capacity was significantly reduced following statin treatment (23 ±3% *vs.* 19 ±2%, P<0.001) (see Table 5.1).

Table 5.1 Baseline blood lipid characteristics and their changes over time

Lipid biomarker			Baseline	Follow up	P value
Total cholesterol (TC) (mg/dL) mean ±SD			176 ±38	133 ±50	**0.001**
LDL-c (mg/dL) mean ±SD			109 ±30	71 ±34	**<0.001**
HDL-c (mg/dL) mean ±SD			46 ±11	45 ±3	0.71
IDL-c (mg/dL) mean ±SD			51 ± 12	40 ±19	**0.012**
Triglyceride (mg/dL) median (IQR)			146 (112 – 191)	107 (71 – 144)	**0.05**
Lipoprotein particle size (nm)	LDL-s mean ±SD		20.9 ±0.4	20.8 ±0.5	0.62
	HDL-s mean ±SD		8.8 ±0.5	8.9 ±0.5	0.09
	VLDL-s median (IQR)		48.5 (45.1 – 50.9)	48.4 (46.7 – 50.9)	0.68
Lipoprotein particle concentration	LDL-p (nmol/L) mean ±SD	Total	1389 ±452	948 ±502	**0.01**
		Large	798 ±261	587 ±506	0.08
		Small	600 ±248	452 ±305	**0.034**
	HDL-p (umol/L) mean ±SD	Total	32.0 ±5.5	31.3 ±7.2	0.65
		Large	4.3 ±3.4	4.7 ±3.4	0.34
		Small	27.7 ±5.6	26.7 ±7.4	0.55
	VLDL-p (umol/L) median (IQR)		2.1 (1.5 - 5.9)	2.5 (1.5 – 5.4)	0.37
hsCRP (mg/dL) mean ±SD			7.2 ±3.6	2.8 ±2.5	**0.001**
Lp(a) (mg/dL) median (IQR)			9.6 (6.1 – 14.5)	8.8 (5.1 – 15.0)	0.64
ApoA1 (mg/dL) median (IQR)			152 (139 – 201)	144 (120 – 202)	0.26
PCSK9 (ng/ml) median (IQR)			276 (203 – 336)	277 (223 – 327)	0.55
CETP activity (pmol/uL/hr) mean ±SD			17.0 ± 1.7	16.9 ± 1.3	0.91
Cholesterol efflux capacity mean ±SD			23 ±3%	19 ±2%	**<0.001**

5.5.2 Relationship between carotid plaque lipid content and blood lipid indices

In the first instance, *baseline* carotid plaque lipid content was compared with standard plasma lipoprotein measures, and there was no significant correlation with either total cholesterol, LDL-c, HDL-c or triglycerides. Subsequently, the pleotropic properties of lipoproteins, including the size and the concentration of the particles, were examined and similarly no association was found with *baseline* carotid plaque lipid content. Finally, emerging functional measures such as Lp(a), PCSK9, CETP activity and cholesterol efflux capacity were explored and likewise there was dissociation when compared with *baseline* plaque lipid content. The observed coefficients of correlations ranged between -0.34 to 0.35 across all standard and functional blood lipid biomarkers (Table 5.2).

None of the standard or functional blood lipid biomarkers showed any relationship with the propensity of lipid percentage to change with statin treatment. This was assessed using both *baseline* and *follow up* blood lipid measurements The observed coefficients of correlations ranged between -0.26 to 0.41 across all standard and functional blood lipid biomarkers (Tables 5.3 & 5.4).

Similarly, with the exception of hsCRP, there were dissociations between *change* in plaque lipid percentage and *change* in both standard and functional blood lipid biomarkers. The coefficients of correlations ranged between -0.42 and 0.37. *Change* in hsCRP showed modest correlation with *change* in plaque lipid percentage (r= 0.46, P=0.038) (Table 5.5).

Table 5.2 Relationship between baseline carotid plaque lipid content and baseline blood lipid indices

Baseline lipid biomarker	Coefficient of correlation (baseline lipid %)	P value
Total cholesterol	0.23	0.32
LDL-c	0.32	0.15
HDL-c	0.19	0.41
IDL-c	0.13	0.58
Triglyceride	-0.11	0.62
LDL particle size	0.01	0.98
HDL particle size	0.05	0.82
VLDL particle size	0.20	0.36
LDL particle concentration	0.21	0.36
Large LDL particle concentration	0.21	0.36
Small LDL particle concentration	0.23	0.32
HDL particle concentration	0.35	0.12
Large HDL particle concentration	0.12	0.61
Small HDL particle concentration	0.34	0.14
VLDL particle concentration	0.23	0.33
hsCRP	0.27	0.24
Lp(a)	-0.34	0.13
ApoA1	0.12	0.61
PCSK9	0.03	0.91
CETP activity	0.08	0.75
Cholesterol efflux capacity	0.35	0.12

Table 5.3 Relationship between baseline blood lipid indices and change in carotid lipid content with statin treatment

Baseline lipid biomarker	Coefficient of correlation (Change in lipid%)	P value
Total cholesterol	0.07	0.75
LDL-c	-0.08	0.74
HDL-c	0.16	0.49
IDL-c	0.13	0.59
Triglyceride	0.24	0.30
LDL particle size	-0.05	0.84
HDL particle size	0.10	0.67
VLDL particle size	-0.02	0.92
LDL particle concentration	-0.05	0.85
Large LDL particle concentration	0.07	0.75
Small LDL particle concentration	-0.22	0.34
HDL particle concentration	0.07	0.76
Large HDL particle concentration	0.22	0.34
Small HDL particle concentration	-0.12	0.61
VLDL particle concentration	0.01	0.97
hsCRP	-0.23	0.33
Lp(a)	0.26	0.25
ApoA1	0.26	0.27
PCSK9	-0.02	0.93
CETP activity	-0.16	0.5
Cholesterol efflux capacity	-0.26	0.25

Table 5.4 Relationship between follow up blood lipid indices and change in carotid plaque lipid content with statin treatment

Attained lipid biomarker	Coefficient of Correlation (Change in lipid %)	P value
Total cholesterol	0.29	0.20
LDL-c	0.28	0.23
HDL-c	0.30	0.19
IDL-c	0.29	0.21
Triglyceride	0.41	0.07
LDL particle size	0.38	0.09
HDL particle size	0.07	0.76
VLDL particle size	0.25	0.27
LDL particle concentration	0.23	0.33
Large LDL particle concentration	0.24	0.29
Small LDL particle concentration	-0.04	0.88
HDL particle concentration	0.26	0.27
Large HDL particle concentration	0.35	0.13
Small HDL particle concentration	0.25	0.29
VLDL particle concentration	0.33	0.16
hsCRP	-0.02	0.92
Lp(a)	0.22	0.34
ApoA1	0.14	0.56
PCSK9	-0.09	0.70
CETP activity	-0.04	0.88
Cholesterol efflux capacity	-0.24	0.31

Table 5.5 Relationship between change in both blood lipid indices and carotid plaque lipid content with statin treatment

Change in lipid biomarker	Coefficient of correlation (change in lipid %)	P value
Total cholesterol	0.31	0.17
LDL-c	0.36	0.11
HDL-c	0.30	0.19
IDL-c	0.19	0.41
Triglyceride	0.06	0.81
LDL particle size	0.37	0.10
HDL particle size	-0.11	0.64
VLDL particle size	0.26	0.25
LDL particle concentration	0.19	0.41
Large LDL particle concentration	0.15	0.51
Small LDL particle concentration	0.18	0.43
HDL particle concentration	0.27	0.24
Large HDL particle concentration	0.15	0.52
Small HDL particle concentration	0.14	0.56
VLDL particle concentration	0.09	0.70
hsCRP	0.46	**0.04**
Lp(a)	-0.04	0.86
ApoA1	0.02	0.93
PCSK9	-0.04	0.86
CETP activity	0.04	0.86
Cholesterol efflux capacity	0.26	0.26

5.5.3 Predictors of lipid percentage reduction

Neither the 'standard' nor 'functional' blood lipid biomarkers were significant predictors of *changes* in carotid lipid percentage when assessed at *baseline value*, *follow up value* or as *change* during study period.

Interestingly, baseline vessel wall volume was not a predictor of changes in lipid percentage [-0.05, 95% confidence interval (-0.18 to 0.09), P= 0.50] nor was change in vessel wall volume [0.12, 95% confidence interval (-0.17 to 0.41), P= 0.40] (Table 5.6, 5.7 & 5.8).

5.5.4 Blood lipid biomarkers between patient groups stratified according to the magnitude of lipid percentage reduction

Using median reduction in carotid plaque lipid percentage as cut-off (-2.4%), blood lipid biomarkers were evaluated between patients who "responded" and "did not respond" to statin treatment (reduction in lipid percentage > or ≤ median). There was no difference between the two groups in terms of age and gender distribution. Carotid plaque lipid percentage at baseline was significantly higher in the 'responders' group compared to the 'non-responders' group (13.8% *vs.* 7.2%, P = 0.002). *Baseline* and *change* over time in both standard and functional lipid biomarkers were comparable between the two groups, with the exception of *change* in hs-CRP, which was significantly larger in magnitude in the 'responders' group (Table 5.9).

Table 5.6 Predictors of change in lipid percentage using baseline blood lipid biomarkers

Baseline biomarker	*Beta* coefficient	95% confidence interval	P value
Total cholesterol	-0.03	-0.08 to 0.03	0.36
LDL-c	-0.04	-0.11 to 0.03	0.20
HDL-c	0	-0.19 to 0.19	0.97
IDL-c	-0.01	-0.18 to 0.16	0.90
Triglyceride	0.02	-0.02 to 0.06	0.34
LDL particle size	-3.16	-8.90 to 2.59	0.26
HDL particle size	0.96	-3.55 to 5.47	0.66
VLDL particle size	0.01	-0.37 to 0.40	0.94
LDL particle concentration	0	-0.01 to 0	0.63
Large LDL particle concentration	0	-0.01 to 0.01	0.96
Small LDL particle concentration	0	-0.01 to 0.01	0.43
HDL particle concentration	0	0	0.86
Large HDL particle concentration	0	0	0.43
Small HDL particle concentration	0	0	0.52
VLDL particle concentration	0.11	-0.25 to 0.47	0.53
hsCRP	0.22	-0.11 to 0.54	0.18
Lp(a)	0.02	-0.02 to 0.06	0.36
ApoA1	-0.12	-0.71 to 0.47	0.68
PCSK9	0	-0.03 to 0.02	0.82
CETP activity	0.09	-1.15 to 1.33	0.88
Cholesterol efflux capacity	-0.46	-1.08 to 0.17	0.14

Table 5.7 Predictors of change in lipid percentage using follow up blood lipid biomarkers

Attained biomarker	*Beta* coefficient	95% confidence interval	P value
Total cholesterol	0.02	-0.02 to 0.07	0.23
LDL-c	0.03	-0.03 to 0.09	0.27
HDL-c	0.08	-0.07 to 0.24	0.28
IDL-c	0.04	-0.06 to 0.15	0.41
Triglyceride	0	-0.01 to 0.02	0.59
LDL particle size	1.45	-2.72 to 5.63	0.48
HDL particle size	0.57	-3.87 to 5.00	0.79
VLDL particle size	0.04	-0.27 to 0.36	0.78
LDL particle concentration	0	0 to 0.01	0.59
Large LDL particle concentration	0	0 to 0.01	0.67
Small LDL particle concentration	0	-0.01 to 0.01	0.90
HDL particle concentration	0	0	0.39
Large HDL particle concentration	0	0	0.26
Small HDL particle concentration	0	0	0.52
VLDL particle concentration	0.02	-0.19 to 0.22	0.88
hsCRP	0.10	-0.24 to 0.45	0.54
Lp(a)	-0.01	-0.05 to 0.03	0.72
ApoA1	0.17	-0.68 to 1.02	0.67
PCSK9	-0.02	-0.04 to 0.01	0.17
CETP activity	0.12	-1.47 to 1.70	0.88
Cholesterol efflux capacity	-0.13	-1.03 to 0.77	0.77

Table 5.8 Predictors of change in lipid percentage using changes in blood lipid biomarkers with statin

Change in biomarker	*Beta* coefficient	95% confidence interval	P value
Total cholesterol	0.04	0 to 0.08	0.07
LDL-c	0.05	0 to 0.11	0.06
HDL-c	0.17	-0.04 to 0.38	0.11
IDL-c	0.05	-0.06 to 0.16	0.34
Triglyceride	0	-0.02 to 0.02	0.92
LDL particle size	2.47	-1.15 to 6.09	0.17
HDL particle size	-0.90	-7.92 to 6.14	0.79
VLDL particle size	0.02	-0.23 to 0.27	0.86
LDL particle concentration	0	0 to 0.01	0.37
Large LDL particle concentration	0	0 to 0.01	0.65
Small LDL particle concentration	0	0 to 0.01	0.43
HDL particle concentration	0	0	0.29
Large HDL particle concentration	0	0	0.96
Small HDL particle concentration	0	0	0.85
VLDL particle concentration	-0.01	-0.18 to 0.16	0.92
hsCRP	-0.19	-0.61 to 0.23	0.36
Lp(a)	-0.01	-0.05 to 0.02	0.38
ApoA1	0.24	-0.39 to 0.88	0.43
PCSK9	-0.01	-0.03 to 0.01	0.33
CETP activity	-0.02	-1.18 to 1.15	0.98
Cholesterol efflux capacity	0.35	-0.26 to 0.96	0.25

Table 5.9 Baseline and changes in standard and functional blood lipid biomarkers between responders and no-responders groups

Variables		**Responders**	**Non-responders**	**P value**
Lipid percentage change		-6.0 (-7.2, -3.1)	-0.2 (-1.1, 1.3)	**<0.001**
Age		62 ± 11	67 ± 8	0.22
Male gender		7 (64%)	8 (80%)	0.64
Imaging parameters	Baseline lipid%	13.8 (10.4, 17.3)	7.2 (4.1, 9.4)	**0.002**
	Baseline WV	44.8 (41.7, 54.0)	45.4 (33.8, 59.9)	0.83
	Change in WV	-2.8 (-5.6, 1.7)	-1.6 (-8.4, 0.2)	0.62
Standard lipid biomarkers	LDL-c	113 ± 33	105 ± 27	0.55
	Change in LDL-c	-43 (-70, -23)	-30 (-42, -16)	0.16
	TC	179 ± 43	173 ± 33	0.74
	Change in TC	-53 (-97, -26)	-29 (-50, -17)	0.23
	HDL-c	46 ± 13	47 ± 10	0.85
	Change in HDL-c	-4.0 ± 9.5	2.8 ± 8.3	0.10
	TG	146 ± 44	157 ± 69	0.66
	Change in TG	-14 (-106, 17)	-25 (-73, -6)	0.70
Lipoprotein particle size	LDL-s	20.9 ± 0.3	20.8 ± 0.4	0.63
	Change in LDL-s	-0.2 (-0.4, 0.2)	0.1 (0.0, 0.28)	0.07
	HDL-s	8.7 ± 0.4	8.8 ± 0.6	0.54
	Change in HDL-s	0.9 ± 0.4	0.2 ± 0.2	0.56
	VLDL-s	47.7 (42.9, 50.2)	48.6 (45.2, 53.9)	0.44
	Change in VLDL-s	-0.9 (-2.0, 1.5)	1.2 (-2.0, 1.9)	0.70

Table 5.9 Baseline and changes in standard and functional blood lipid biomarkers between responders and non-responders groups

Variables		**Responders**	**Non-responders**	**P value**
Lipoprotein particle number	Total LDL-p	1392 ± 401	1386 ± 524	0.98
	Change in LDL-p	-666 (-874, -374)	-487 (-797, -198)	0.67
	Large LDL-p	786 ± 207	810 ± 322	0.84
	Change in L.LDL-p	-342 (-524, -189)	-290 (-484, -78)	0.75
	Small LDL-p	622 ± 182	576 ± 314	0.68
	Change in S.LDL-p	-254 (-349, -71)	-171 (-318, -69)	0.67
	HDL-p	31729 ± 6095	32250 ± 5148	0.84
	Change in HDL-p	-1681 ± 8587	411 ± 4127	0.49
	Large HDL-p	3272 ± 3134	5034 ± 3585	0.24
	Change in L.HDL-p	221 ± 2341	472 ± 1289	0.76
	Small HDL-p	28145 ± 4991	27320 ± 6194	0.74
	Change in S.HDL-p	-1117 (-5582, 1820)	-144 (-2850, 1515)	0.89
	VLDL-p	2.1 (1.5, 5.3)	3.0 (1.5, 9.8)	0.54
	Change in VLDL-p	-0.4 (-1.7, 0.5)	-0.1 (-5.1, 1.0)	0.86
IDL-c		50 ± 12	51 ± 13	0.86
Change in IDL-c		-19 (-25, -7)	-13 (-24, -5)	0.73
Lp(a)		6.0 (4.5, 13.0)	11.1 (6.7, 16.6)	0.18
Change in Lp(a)		1.9 ± 5.2	-0.5 ± 4.5	0.28
ApoA1		141 (131, 188)	190 (142, 228)	0.18
Change in ApoA1		-23 (-32, 93)	-20 (-59, 3)	0.73

Table 5.9 Baseline and changes in standard and functional blood lipid biomarkers between responders and non-responders groups

Variables	Responders	Non-responders	P value
hsCRP	8.5 ± 2.6	5.7 ± 4.1	*0.09*
Change in hsCRP	-5.8 ± 2.8	-2.9 ± 3.2	**0.04**
PCSK9	267 (205, 438)	281 (197, 302)	0.67
Change in PCSK9	4 ± 119	22 ± 74	0.68
Cholesterol efflux capacity	23.7 ± 3.1	21.3 ± 3.0	*0.08*
Change in cholesterol efflux capacity	-4.1 ± 4.1	-2.0 ± 1.7	0.15
CETP activity	17.1 ± 1.7	16.8 ± 1.8	0.67
Change in CETP activity	0.0 ± 2.1	-0.1 ± 1.6	0.99

5.5.5 Change in plaque lipid content according to the attained plasma LDL-c

When the group was stratified according to the level of attained plasma LDL-c post-intensive statin treatment (above or below 70 mg/dL), there was no significant difference in plaque lipid response between these two groups. This was assessed using *baseline, follow up* and *change* in both lipid percentage and vessel wall volume (Table 5.10)

Table 5.10 Baseline and changes in carotid vessel wall volume and lipid content between patients with LDL-c of more and less 70 mg/dL following statin treatment

Imaging biomarker	Group 1 (LDL-c < 70mg/dL)	Group 2 (LDL-c > 70mg/dL)	P value
Base lipid percentage (%)	9.2 (6.0, 14.3)	10.4 (8.7, 20.2)	0.36
Base plaque volume (mm^3)	44.8 (34.1, 53.2)	42.7 (41.7, 70.7)	0.51
FU lipid percentage (%)	6.4 (4.9, 9.5)	10.0 (7.5, 12.2)	0.099
FU plaque volume (mm^3)	43.6 (33.3, 48.8)	45.2 (41.1, 56.3)	0.31
Change lipid percentage (%)	-2.8 (-6.6, -0.3)	-0.9 (-10.2, 1.2)	0.36
Change plaque volume (mm^3)	-1.7 (-5.9, 0.38)	-2.8 (-14.4, 2.1)	0.84

5.6 Discussion

This systematic analyses of statin effect on blood lipid indices and their relationship with carotid plaque lipid content in statin naïve ACS patients have these main findings:

1. Cholesterol efflux capacity was significantly reduced following statin treatment in this cohort.
2. There was a dissociation between *baseline* blood lipid indices and *baseline* carotid plaque lipid content.
3. *Change* in hsCRP was the only blood biomarker to relate to *change* in carotid plaque lipid content, albeit this relationship was only modest.
4. There was no significant predictor of change in carotid plaque lipid content using *baseline*, *attained* or *change* in blood lipid biomarkers following statin treatment.
5. Responders to statin treatment, defined as a reduction of plaque lipid percentage of more than median, had higher baseline lipid percentage and larger reduction in hsCRP compared to non-responders
6. There was no difference in carotid vessel wall volume or lipid content among patients with LDL-c of less or more than 70 mg/dL following statin treatment

The residual risk after moderate-intensity statin treatment was shown to be modifiable in both the PROVE-IT and TNT trials.[16, 78] This was borne out in more contemporary studies when adding ezetimibe or evolocumab to statin in reducing combined clinical events.[39, 63] However, the marginal benefits of these drugs in particularly when juxtaposed to the marked reduction in LDL-c challenged the unselective nature of applying these drugs.[79] In addition, it questioned the current approach of using LDL-c as a single biomarker to track patients' response to these drugs in the era post intensive statin treatment. The diminishing return of reduction in clinical outcomes when achieving very low LDL-c support the notion of tackling lipoprotein-related residual risk using non-LDL-c measures. LDL particle concentration was

demonstrated to refine future cardiovascular risk compared to using LDL-c as a single blood metric.[130] Therefore, in response to statin treatment changes to other non-LDL-c lipid indices may play a role in the clinical outcomes and this role is not only related to the reduction in LDL-c. Here, LDL particles concentration was significantly reduced following 3 months of intensive statin treatment, matching previous reported literature.[230]

Interestingly, there was a reduction in cholesterol efflux capacity which was theoretically suggestive of pro-atherogenic lipid profile in response to statin treatment. Yet, cholesterol efflux capacity was demonstrated to have an inverse relationship with atherosclerotic burden and incidence of cardiovascular disease.[86, 87] However, in the YELLOW II trial, almost 40% of patients showed reduction in their cholesterol efflux capacity following statin treatment.[231] Notably, hsCRP was significantly higher in patients with reduced cholesterol efflux capacity compared to those with increased cholesterol efflux in response to statin treatment.[231] Such an interaction was reported in the YELLOW II trial and was evident in the results of this report (baseline hsCRP 7.2 mg/dL).[231] This observation has two important implications, firstly it reinforces the complex interactions among blood lipid biomarkers and suggests that a single blood measurement does not estimate precisely future risk. Secondly, it highlights the inaccuracy in measuring biomarkers that are derived from risk factors at population level and which bear no relation to individual disease or response to therapy. Therefore a better stratification method that could characterise and individualise the risk beyond blood biomarkers is urgently needed.

Plaque lipid content is a promising tool since it is a feature of plaque vulnerability and is the likely target of lipid lowering drugs.[229] The relationship with blood lipid indices was previously studied but this was limited due to (a) the insensitivity of the used imaging technique and (b) the lack of studied functional lipid biomarkers which may be more reflective of plaque lipid content.[87, 148, 232] Yet using a highly sensitive T2 mapping MRI

technique,[180, 219] there was dissociation between plaque lipid content and both standard and functional lipid indices. In other words state of the art blood biomarkers cannot substitute for direct plaque lipid quantification. This is not surprising since the dynamic range of plaque lipid (potentially accumulating over months to years) was unlikely to be reflected by the relatively narrow range of lipids measures.

Although *change in hsCRP* was associated with *change in plaque lipid percentage*, this correlation was only modest (r= 0.46) and reflected both statin initiation and the resolution of inflammation post ACS. Diminishing inflammatory response post ACS was shown to have invariable contribution to the *changes in hsCRP* and made the interpretation of this association less precise.[113, 233] More importantly, none of the studied blood biomarkers, including hsCRP, was a significant predictor of changes in plaque lipid percentage. Thereby reinforcing the significant role of plaque imaging in assessing response to established or new therapies. Interestingly, changes in carotid lipid percentage in response to LDL-c reduction was not uniform across all patients. Responders tended to have higher lipid percentage at baseline and were more likely to have larger change in hs-CRP in response to intensive statin treatment. These differences matched previous reports when using invasive techniques in the coronary arteries.[172] In a *post hoc* analysis of the YELLOW trial, the magnitude of reduction in lipid content following 7 weeks of intensive statin treatment quantified using NIRs was proportional to the quantity of lipid amount at baseline.[172] Using IVUS-VH, the decrease in hsCRP was greater in patients with reduced necrotic core than those with persistent ones.[232] Results in this chapter extend these findings when applying *non-invasive* imaging technique and are indicative that statin effect on stabilizing plaques is not confined to a single blood lipid biomarker. Moreover, the intricate relationship between lipoprotein- and non-lipoprotein related risks, such as diabetes and smoking, would undermine the approach of using risk factors to estimate an individual future vascular risk.[124] T2 map is a tool to characterise the atherosclerotic disease at individual patient

level, making plaque imaging and, in particular, lipid quantification a promising strategy to recognise high risk individuals.

5.7 Conclusion

The work presented in this chapter highlighted a potential paradoxical effect of statin in reducing cholesterol efflux capacity in patients post ACS. It also showed that existing blood lipid biomarkers did not predict changes in plaque lipid content with treatment. In addition, it extended the previous observations of the intricate relationship between inflammatory markers and changes in plaque lipid content and highlighted the lack of uniform response to statin treatment across patients. Taking all these observations together render the use of a single blood measure to estimate future cardiovascular risk imprecise. On the other hand, plaque lipid quantification using T2 mapping technique can characterise the atherosclerotic disease at an individual level and is potentially a promising tool to identify high risk patients beyond currently available blood biomarkers.

Chapter 6: The relationship between perivascular adipose tissue and plaque lipid content in the carotid arteries, as determined by magnetic resonance imaging

6.1 Abstract

Background

PVAT characterisation has recently been suggested as a sensor of inflammation in adjacent arteries and has been proposed as a promising tool to early identify high risk plaques. Understanding the relationship between PVAT and features of advanced atherosclerosis may aid the use of PVAT as a risk stratification tool. MRI incorporating a dedicated water suppressed sequence is capable of identifying PVAT based on its absolute physical properties. It can also overcome difficulties when using CT in delineating PVAT due to its close proximity with vessel adventitia. Since a paracrine route is proposed as the link between PVAT and adjacent vascular wall, the relationship of the circumferential distribution of PVAT and carotid plaque lipid content, as a marker of atherosclerosis, was studied.

Methods

Plaque lipid content and PVAT were acquired at 3T MRI using T2 mapping and T1-weighted turbo-spin echo water suppressed sequences, respectively in patients scheduled for carotid endarterectomy. Following segmentation of co-registered images, PVAT and vessel wall lipid content were measured along radii of the vessel spaced at angles of 5º each and compared circumferentially.

Results

PVAT thickness of the carotid arteries was 0.33 ± 0.83 mm, while the mean thickness of carotid lipid wall content was 0.16 mm ± 0.41 mm. There was no identifiable pattern of carotid PVAT but minimal thickness was noted at 265-310º.

Symptomatic carotid vessels had larger PVAT volume compared to asymptomatic ones despite similar degree of stenosis (69 ±37 mm^3 *vs.* 38 ±27 mm^3, P= 0.048). Although

statistically significant, the correlation between the circumferential distribution of PVAT thickness and carotid wall lipid thickness in both symptomatic (r= 0.10, P= 0.003) and asymptomatic vessels (r= 0.06, P= 0.008) was merely nominal.

Conclusions

PVAT was more abundant in symptomatic carotid vessels. However, dissociation between the spatial distribution of PVAT and wall lipid content in the carotid arteries does not support the concept that PVAT has a major causal role in promoting atherosclerotic plaque via a simple paracrine route in these vessels.

6.2 Introduction

Perivascular adipose tissue (PVAT) has recently been proposed as a new imaging biomarker closely related to the development of atherosclerosis.[139, 234] The concept that PVAT has a passive role which only supports vascular structure is no longer accepted.[139, 140, 234] In fact, there is a growing body of evidence suggesting that PVAT is actively linked to vascular function with 'cross-talk' between PVAT and vascular wall.[140, 235] Moreover, the role of PVAT in secreting adipokines, such as adiponectin and subsequently stimulating nitric oxide synthase (eNOS) with its vasodilatory effects has suggested that PVAT may exert vaso-protective effects.[140, 236] However, changes in adipocyte function e.g. in obese humans and experimental mice have also shown the potential for a pro-atherogenic role of PVAT.[234, 237-240] Hypertrophic adipocytes released monocyte chemoattractant protein-1 (MCP-1) leading to monocytes migration, secreting TNF-α, and directly inhibiting insulin signalling in adipocytes.[234, 237-240] Therefore, the influence of PVAT on vascular function and the pathobiology of atherosclerosis is still to be determined.

Recently, Antonopoulos *et al*.[139] demonstrated that inflamed coronary vessels altered the attenuation of the surrounding adipose tissue on computed tomography (CT). These changes in CT attenuation were proposed to be in response to cytokines release from inflamed coronary plaques.[139] Since inflammation is strongly implicated in the process of acute plaque rupture, PVAT imaging was proposed as a tool to early identify high-risk atherosclerotic patients.[139, 241] However, for PVAT to fulfil its potential imaging role, it is crucial to have a clearer understanding of the potential of PVAT to influence the progression of atherosclerotic plaque. In other words, establishing whether PVAT is related to features of advanced atherosclerosis may add further insights into its role as a determinant of atherosclerosis risk and as a tool to stratify patients.

Advances in technology have allowed alternative *in-vivo* imaging techniques other than CT to be used to study PVAT.[242] MRI has the advantage of isolating signals from fat or water using differential suppression techniques. Accordingly, MRI is able to overcome the difficulties of separating PVAT from the vessel wall to which it is immediately adjacent. Despite the proximity, by suppressing in turn the water or fat signal it is possible to delineate these the PVAT and vessel wall clearly. Comparably, studies using CT have used an arbitrary 'anatomical box' surrounding the studied vessel to quantify PVAT.[243, 244] The delineation between PVAT and vessel adventitia is pivotal to elucidate the proposed locally regulated function and effect of PVAT on the vessel wall.[243, 245, 246] The close proximity of PVAT and vessel wall and the potential bidirectional communication through their connectivity of micro-vessels network was supportive of the paracrine route to convey adipokines to vessel wall via the vasa vasora.[247-249] Moreover, coronary segments that are insulated from adipose tissue and surrounded by cardiac muscle, termed 'myocardial bridging', consistently show absence of atherosclerosis and thus favouring the argument of PVAT role in the development of vascular disease.[249] We have recently examined the spatial correlation between PVAT and vessel wall thickness by systematic evaluation of the aorta and carotid arteries *in-vivo.*[242] There was a nominal relationship between the circumferential distribution of PVAT and vascular wall, challenging the concept that PVAT has a causal role in promoting atherosclerosis via paracrine local route. Nonetheless, the association between PVAT and vessel wall composition was not investigated and a potential relationship between plaque lipid content and PVAT needs to be surveyed.

Recent validation of T2 mapping MRI technique, and the ability to quantify plaque lipid content, have enabled exploration of a possible mechanistic role of PVAT in determining risk of atherosclerosis. Importantly, given the proposed paracrine route, any relationship between lipid content and PVAT may better be studied in adjacent segments. Additionally,

the evaluation of spatial relationship between vessel wall lipid content and adjoining perivascular adipose tissue with MRI may inform the contentious issue of whether PVAT has a protective or atherogenic role in atherosclerosis.

Therefore, this chapter seeks to study the relationship, if any, between carotid perivascular adiposity as quantified by MRI and plaque lipid content quantified on T2 mapping using a spatial distribution model, and to determine the association of carotid perivascular adiposity with the symptomatic status of atherosclerotic plaques.

6.3 Hypotheses and aims

To test the hypotheses that PVAT quantified on MRI is related to the symptomatic status of carotid atherosclerotic plaque and to assess whether PVAT is a predictor of lipid accumulation in carotid vessel wall as quantified on T2 mapping MRI technique. This relationship is tested independently from the overall volumetric quantification of PVAT and rather related to the *circumferential distribution of PVAT* and vessel wall lipid content.

The experimental aims of this chapter were:

1) To systematically study the distribution of PVAT in patients with high risk atherosclerotic disease i.e. CEA patients.
2) To assess the relationship between PVAT and the symptomatic status of carotid atherosclerotic plaque.
3) To evaluate the circumferential relationship between PVAT and vessel wall lipid content and to investigate if PVAT is a predictor of lipid retention in the carotid vessel wall as a marker of advanced atherosclerosis.

6.4 Methods

Chapter 2 discussed the general methodology related to this chapter and only methods specific to this chapter are discussed below in further details.

6.4.1 Study population

Patients scheduled for CEA between December 2016 and July 2017 at the John Radcliffe Hospital were recruited. Patients were identified from the vascular surgical waiting list or those who were already admitted to the hospital with symptoms suggestive of cerebrovascular events. All patients had 50-99% carotid stenosis according to NASCET, or 70-99% according to ESCT criteria as measured on ultrasound duplex.[180, 207] Cerebrovascular events were defined as temporary or permanent deficit in neurological functions mapping a specific vascular territory as assessed clinically and supported with either CT or MRI imaging. Asymptomatic patients were deemed so if there were no previous documented clinical symptoms, but with an indication for an endarterectomy procedure based on carotid degree of stenosis. All patients were adults (>18) and were scanned in AVIC prior to their surgical procedure.

All included patients in this study provided written informed consent. The study protocol conformed to the ethical guidelines and was approved by the NRES and local R&D committee prior to commencement of the study.

6.4.2 MRI protocol

Both *carotid arteries* were studied at 3T (Verio, Siemens Healthcare, Erlangen, Germany) with a 4-channel phased-array carotid coil (Machnet, Roden, The Netherlands). After localizing the carotid bifurcation using bright-blood TOF angiography and T1-weighted

images multi-slice carotid T2 maps were generated as previously described.[180, 219] 2 cm of both carotid arteries were imaged with 10 slices centralised on the maximum plaque volume as identified by TOF and T1-weighted images.

A water suppression sequence was applied to acquire PVAT slices and were generated using TSE black blood fat saturated cross sectional images of the carotid arteries (sequence parameters: TR 750, TE 12, FOV 150 X 150, in plane resolution 0.39 X 0.39 mm, slice thickness 2 mm). This sequence yielded 13 slices and was centred on the first DANTE-MESA sequence so PVAT slices were matched to the T2 mapping slices. Fat saturated slices spanned more than 2.5 cm of the carotid artery and the acquired images had a low signal from vessel wall separating it from the surrounding adipose tissue. The raw obtained images were subsequently processed to identify perivascular adipose tissue (Figure 2.4).

6.4.3 MRI data analysis

Lipid identification of carotid vessel wall

As previously described, T2 maps were generated using mono-exponential nonlinear fitting with semi-automated procedure to segment both lumen and external carotid vessel boundaries.[167, 209] Voxels with T2 threshold of less than 42 ms were segmented and identified as lipid component of the carotid vessel wall with a single voxel measured as 0.33 x 0.33 x 2 mm (see Chapter 2 for more details).[167, 180]

Quantification of PVAT

Using raw fat saturated images, the signal intensity of each voxel was measured and included within ROI excluding the subcutaneous adipose tissue. PVAT was segmented based on a

method using threshold intensity set at two standard deviations greater than the mean pixel intensity of the ROI. The close proximity of the coil with high signal intensity from the subcutaneous tissue would have increased the mean pixel intensity and underestimated the volume of PVAT. Voxels with signal intensity more than the set threshold were grouped together and denoted as adipose tissue.

There is a little consensus in the literature as to the anatomical definition of PVAT. The juxtaposition of the carotid arteries next to the subcutaneous adipose tissue would render the identification of PVAT more challenging. While delineating adipose tissue on MRI is dependent on the physical properties of the imaged voxels, the difference in lipid content or size of adipocytes, if any, does not allow reliable differentiation between PVAT and subcutaneous adipose tissue. On the other hand, adipose tissue is precisely distinguished from water-rich structures such as vascular wall and volumetric quantification, rather than characterisation, of adipose tissue can be achieved. Therefore, the majority of published literature relied on an anatomical and fixed distance description of PVAT. Lehman *et al.*,[250] used an arbitrary box to identify PVAT when studying PVAT of the thoracic aorta. Borders of this box were the anterior border of the vertebral body, the left costovertebral joint, and the oesophagus. In another study by the same group,[251] a threshold for PVAT surrounding the abdominal aorta was set at 5 mm away from the vessel wall. In the current study, PVAT was quantified using a single voxel as one unit (0.39 x 0.39 x 2 mm) within a circle with a diameter of two times the radius of the vessel wall. The distance from the outer boundary of the vessel wall we set up at non-fixed distance but derived from the radius of the studied artery to standardise measurements from patient to patient. This method is vessel-specific and has been used in contemporary PVAT studies.[139] The intention of this method was to include the full physiological range of normal PVAT distribution, which may have been overlooked with smaller, arbitrary thresholds. One drawback in applying this method in carotid

endarterectomy patients would be overestimating the volume of PVAT in vessels with positive remodelling. However, the used diameter in the current analysis was derived from the average of all acquired slices and potentially diminished the impact of large positively remodelled plaque in an individual slice.

PVAT in each slice of the studied vessel was quantified and overall PVAT volume was calculated as the average PVAT obtained from the 10 slices that matched T2 mapping slices (Figure 6.1).

Spatial distribution analysis

Both PVAT and vessel wall lipid content were analysed using measurements along radii of the vessel passing through cords on the vessel circumference. These cords were at evenly spaced out angles of 5º (starting from 0º to 355º; i.e. 72 cords per cross-sectional image of the vessel). The thickness measurements of both PVAT and carotid vessel wall lipid content were measured along the 72 cords around the circumference of the vessel (Figure 6.1).

The centre from where these measurements were taken was operator-defined with software-defined best-fit circle of the vessel wall. The zero angle position was defined at the lateral direction (9 o'clock) and the angle was incrementally increased in a clockwise rotation. All analyses to segment and quantify the circumferential distribution of PVAT and plaque lipid content were implemented in Matlab (MathWorks, Natick, USA).

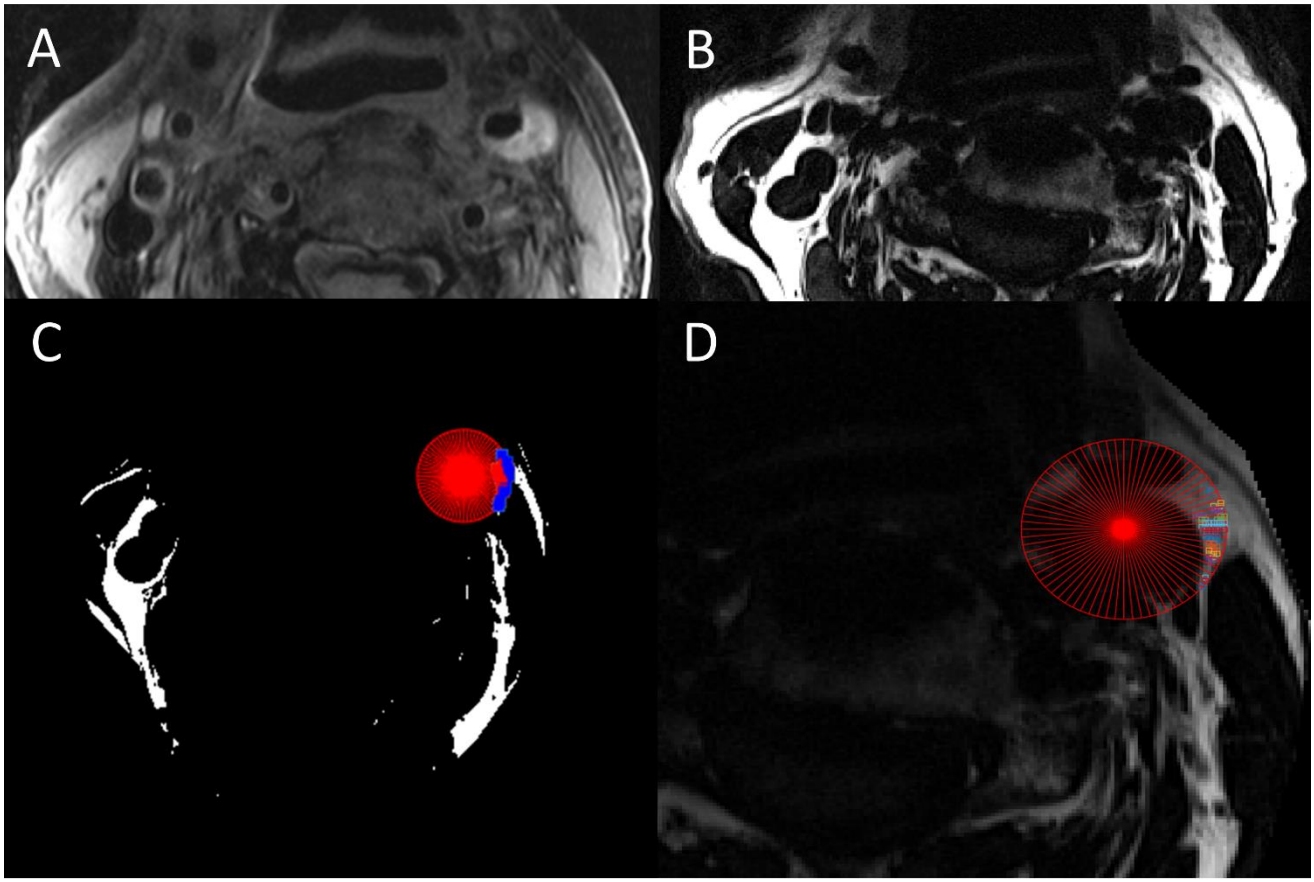


Figure 6.1 Carotid PVAT segmentation and circumferential measurement

A: Cross-sectional T1-weighted image of left common carotid artery in 88 years old male with transient right sided weakness associated with speech disturbance.

B: Water suppressed sequence to enhance adipose tissue at the same level of the left symptomatic carotid plaque.

C: Segmented image of the left carotid artery highlighting a red circle of two times the radius of the carotid artery. Voxels with high signal intensity within this circle were defined as PVAT and denoted in blue.

D: Magnified image with superimposed red circle over raw fat saturated sequence to highlight area of identified PVAT.

6.5 Results

11 patients were recruited during the study period. Two carotid vessels were excluded due to inadequate image quality. The remaining 20 carotid vessels from 11 patients were from 6 patients with symptoms attributed to the downstream cerebrovascular pathology and 5 asymptomatic ones with no previous documented symptoms. The spatial distribution analysis was executed in 200 slices yielding 14,400 adjoining vascular/ PVAT segments.

The mean age ± SD was 76 ± 9 years with 81% were males. The overall mean stenosis, measured by duplex ultrasound was 64 ± 18% with no difference in the degree of stenosis between symptomatic and asymptomatic patients. 81% of patients were already treated with statin at the time of MRI. Clinical characteristics of recruited patients are summarized in Table 6.1.

Mean lipid percentage in the whole twenty carotid arteries was 13 ±13% with average vessel wall volume of 123 ±44 mm^3. Comparably, the mean PVAT was 46 ±42 mm^3. There was no difference in PVAT volume between males and females (45 ±8 versus 62 ±23 mm^3, P= 0.42). PVAT volume had no relationship with age (r= 0.28, P=0.23).

Table 6.1 Clinical characteristics of recruited patients

Patients characteristics	Carotid endarterectomy patients
Number of patients	11
Male (%)	9 (81%)
Mean age ± SD	76 ± 9
Hypertension	9 (81%)
Hypercholesterolemia	7 (64%)
Active Smoking	2 (19%)
Diabetes mellitus	3 (27%)
CAD disease	2 (19%)
Atrial fibrillation	1 (9%)
Aspirin /antiplatelet	9 (81%)
Statins	9 (81%)
Beta-blockers	4 (36%)
Calcium antagonists	2 (19%)
ACE inhibitors / ARBs	6 (55%)
Anticoagulation	1 (19%)
Right : Left	1.75 : 1
Mean stenosis ± SD (%)	64 ±18%.

6.5.1 *Spatial distribution of PVAT and its relationship with the symptomatic status of the carotid artery*

The mean thickness of carotid wall lipid was 0.16 mm ± 0.41 mm with average carotid PVAT thickness of 0.33 ± 0.83 mm.

Changes of the *mean* carotid lipid content across different angles was largely uniform (circumferential distribution) [interquartile range (0.14 – 0.18) mm], while the *mean* PVAT thickness appeared to show wider variations [interquartile range (0.25- 0.42) mm] (Figure 6.2, Panel A & B). There was no identifiable pattern of PVAT distribution, but carotid PVAT appeared to be thinner between angles 265 − 310º (Figure 6.2, Panel A).

The individual variations of PVAT changes across different angles are presented in Figure 6.3, Panel A. There was no tangible difference in patients presenting with cerebrovascular events suggestive of symptomatic carotid plaques and those with no documented symptoms (Figure 6.3, Panel B).

Perivascular adipose tissue of the carotid arteries was significantly larger in symptomatic patients compared to those with no documented symptoms (69 ±37 mm^3 *vs.* 38 ±27 mm^3, P= 0.048) despite having similar degree of luminal stenosis (66 ±13 % vs. 62 ±24 %, P= 0.73) (Figure 6.4 & Figure 6.5). Notably there was a wide variation in the volume of PVAT in symptomatic patients.

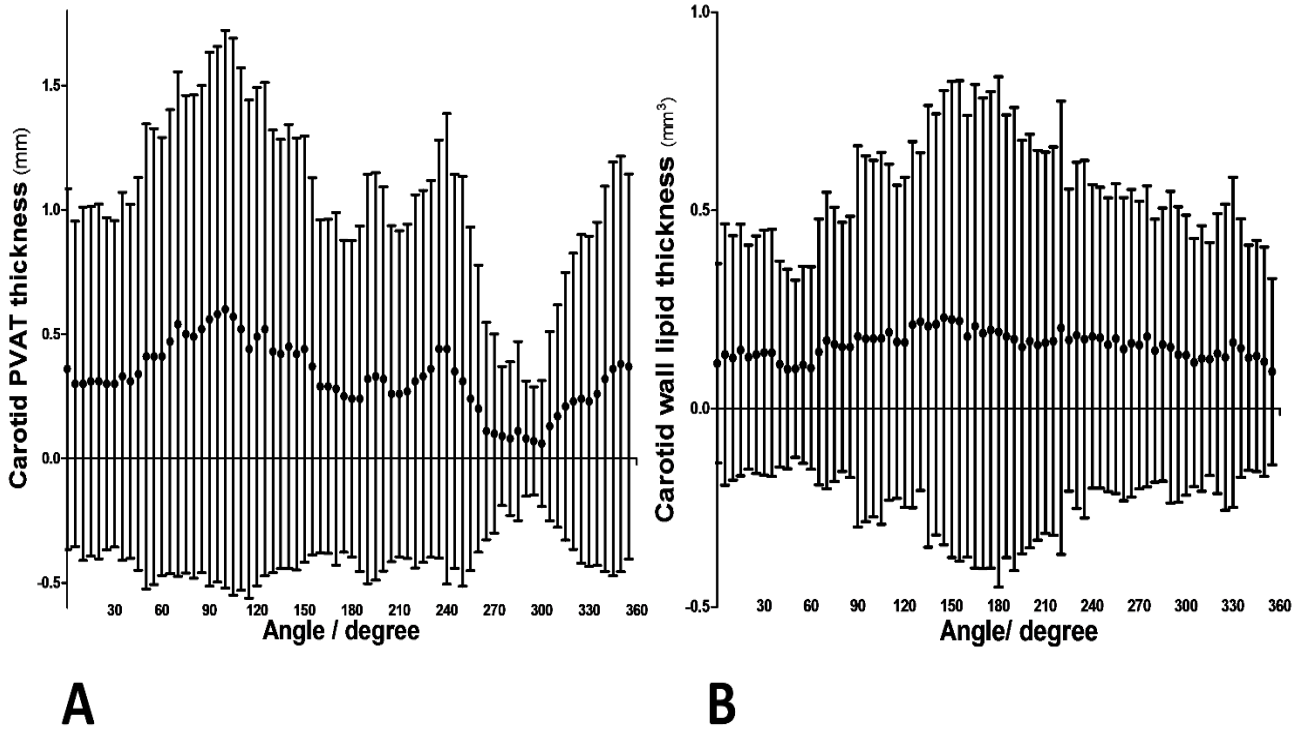


Figure 6.2 Circumferential distribution of PVAT and vessel wall lipid thickness of the carotid arteries

A: Changes of average mean carotid PVAT thickness with standard deviations of all patients across different circumferential angles. PVAT thickness is noticeably small between angles 265-310º.

B: Changes of average carotid wall lipid content with standard deviation of all patients across different circumferential angles. These changes appear to be are more uniform compared to PVAT.

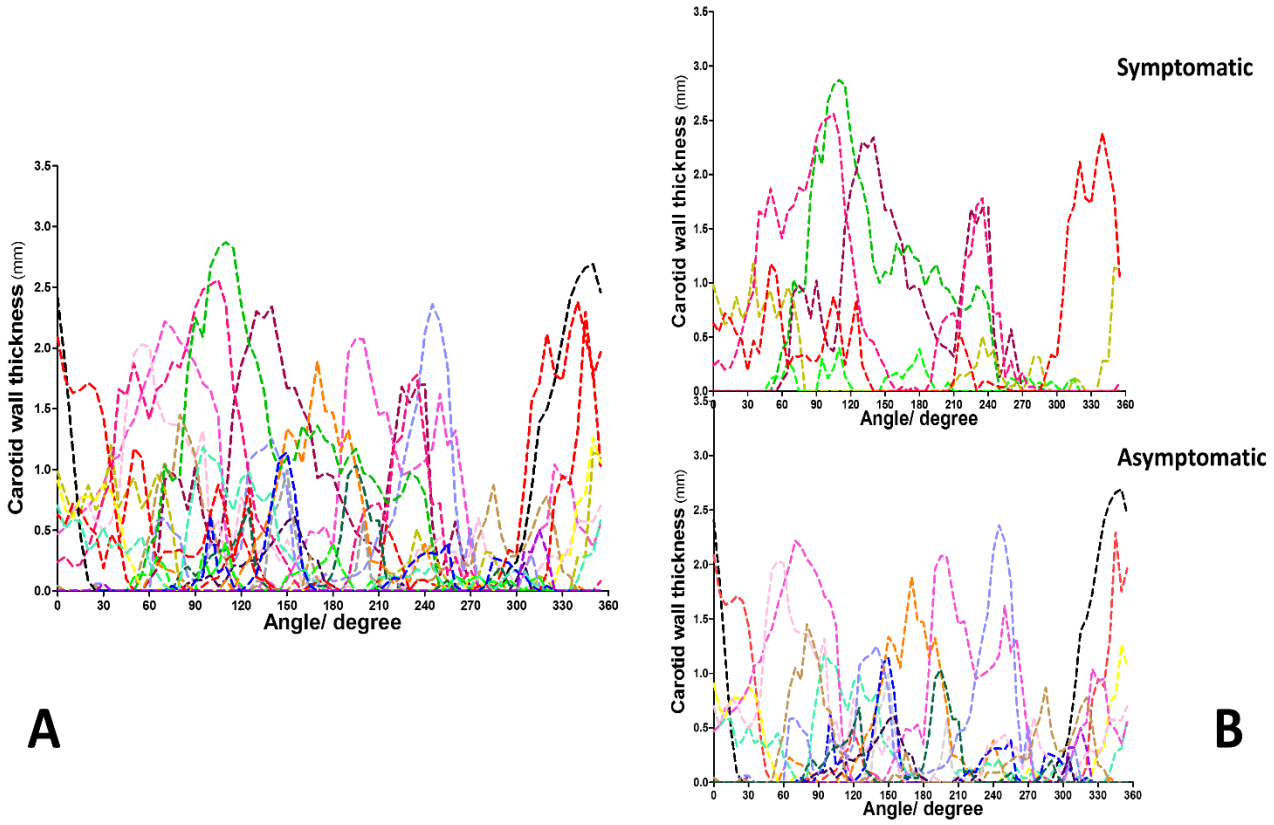


Figure 6.3 Individual distribution of PVAT thickness of the carotid arteries

A: The individual variation of PVAT thickness of all recruited patients, assigning every carotid artery into a different colour. There was no identifiable pattern of PVAT distribution across arteries.

B: Changes in PVAT thickness across different angles but stratified according to the symptomatic status of the vessel. Both symptomatic and asymptomatic carotid vessels shared the lack of uniformity in the spatial PVAT distribution.

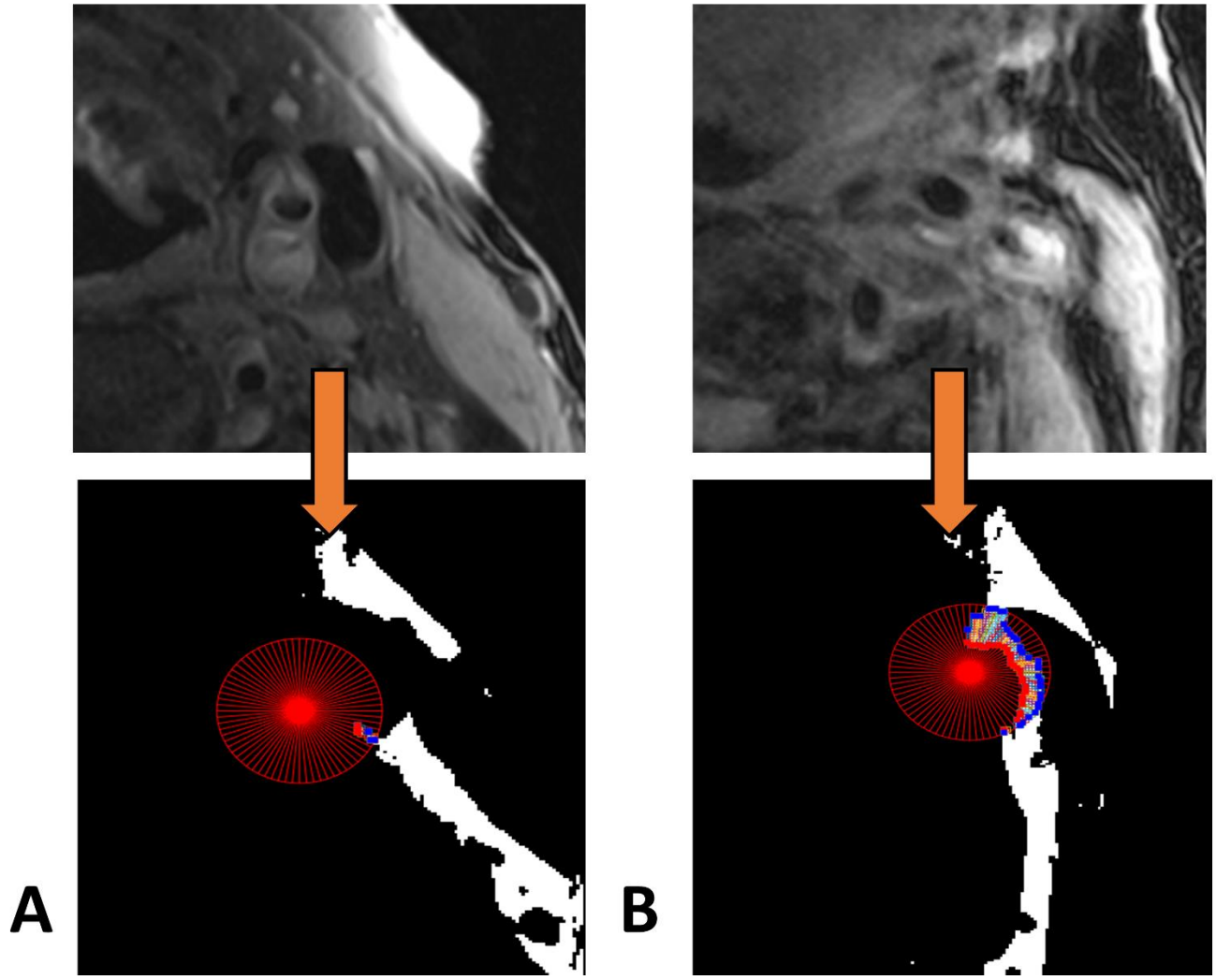


Figure 6.4 Difference in PVAT volume between symptomatic and asymptomatic patients

A: Asymptomatic left carotid plaque in 70 years old male with subtotal occlusion of the left carotid artery. PVAT volume was measured as 5.4 mm^3 using fat saturated MRI sequence.

B: Left carotid artery plaque from 63 years old male who presented with right sided weakness one week prior to his carotid endarterectomy. PVAT volume was measured at 169.7 mm^3. Notably, the degree of luminal stenosis was more than 90% in the asymptomatic patient compared to 75% in the symptomatic subject.

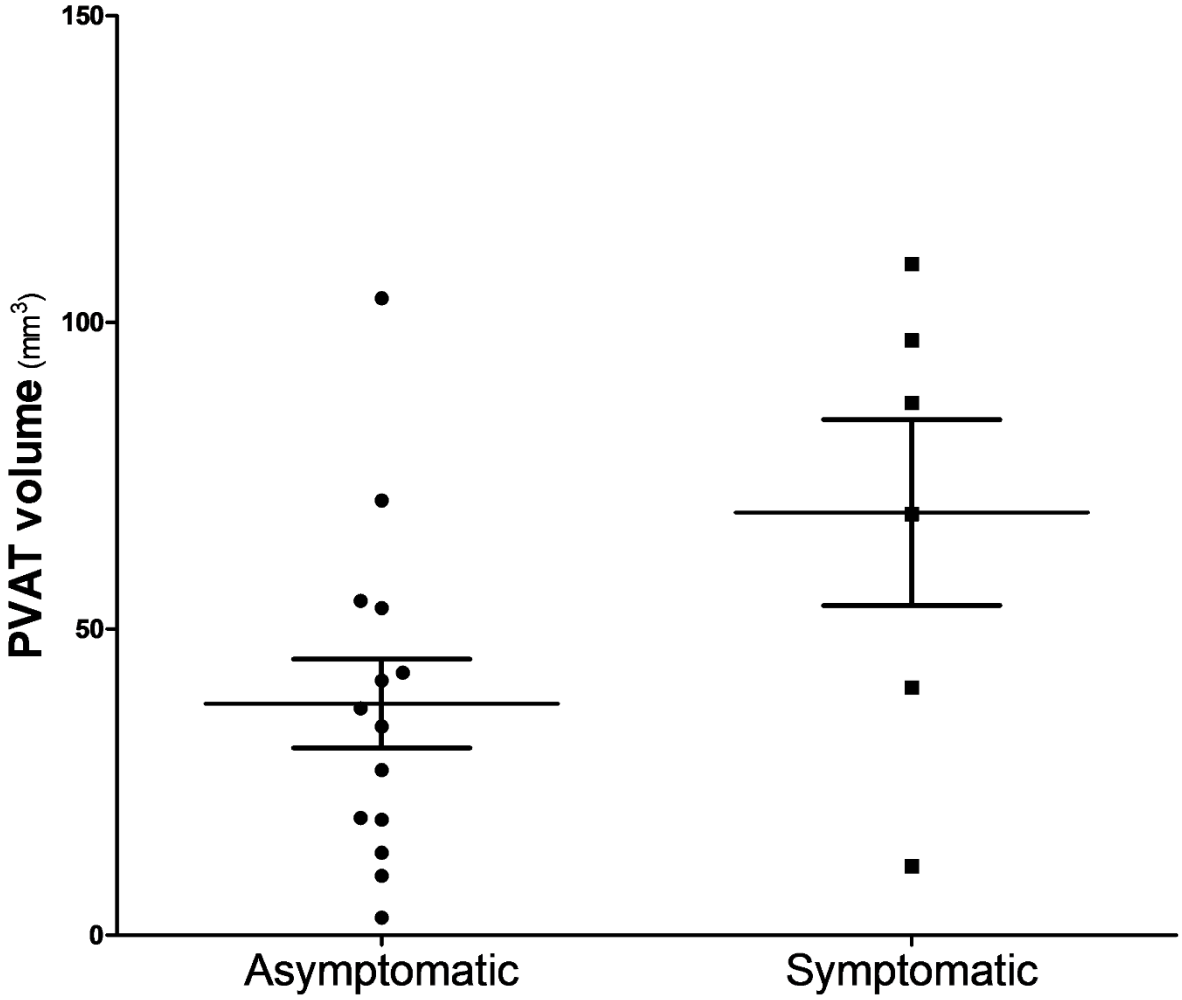


Figure 6.5 Difference in PVAT volume between symptomatic and asymptomatic carotid vessels

In the total 20 carotid vessels and irrespective of the degree of stenosis symptomatic vessels had larger PVAT volume than asymptomatic ones as measured by the average PVAT volumes across all the slices (P=0.048).

6.5.2 Spatial relationship between PVAT and vessel wall lipid content

There was a statistically significant but very weak positive correlation between PVAT thickness and carotid vessel wall lipid content (r= 0.1, P= 0.003). In addition PVAT thickness was an independent predictor of vessel wall lipid thickness, after adjusting to individual patient, side of carotid artery, location of the slice from the bifurcation and circumferential angle of the studied segment [0.056, 95% confidence interval (0.039 to 0.073), P <0.001].

When carotid vessels were stratified according to their symptomatic status of the vessel, the circumferential relationship between PVAT and vessel wall lipid thickness was almost identical (Table 6.2).

Table 6.2 Spatial relationship between PVAT and vessel wall lipid content in both symptomatic and asymptomatic patients

Symptomatic status	**Coefficient of correlation**	**P value**	**Beta coefficient**	**Confidence interval**	**P value**
Symptomatic	0.10	0.002	0.055	0.019 to 0.092	0.003
Asymptomatic	0.06	0.005	0.022	0.006 to 0.038	0.008

6.6 Discussion

Using a newly-devised methodology to quantify PVAT as identified on MRI, findings in this chapter could be summarised as follow:

1. Carotid PVAT does not have any identifiable pattern and its circumferential distribution is not uniform across individuals.
2. Symptomatic carotid plaques are associated with larger PVAT volume than asymptomatic ones.
3. The weak correlation in the angular distribution between carotid lipid wall content and PVAT in the carotid arteries, irrespective of the burden of atherosclerosis or the symptomatic status of the vessel, did not suggest a biologically important interaction.

There is an ongoing debate regarding the role of PVAT in developing atherosclerosis.[234, 245] Adipose tissue is known to secrete pro-atherogenic cytokines and, hence, it has been implicated in promoting atherosclerosis,[249] however, there is also a significant body of evidence to support a protective role of PVAT in atherosclerosis.[140, 252, 253] Factors related to the intrinsic properties of the adipose tissue and to the locations of vascular sites might have contributed to this apparent paradox.[139, 234, 235, 254] The variations in adipocyte lipid content, their stage of maturation and size may have led to variations in secretory profile and, in turn, different local effects on the vascular wall.[243, 255] More recently, PVAT has been proposed to function as a sensor rather than a driver of vascular inflammation, adding more complexity to the role of PVAT in atherosclerosis.[139] Therefore, it would be considered an over simplistic statement to broadly categorize and pre-define the role of PVAT as pro-atherogenic or vascular protective, while this role maybe more dynamic and varies according to numerous factors.[140, 234, 236-240, 256, 257]

Earlier studies have focused on the utility of CT to study PVAT and mainly fixated on its overall volumetric quantification to relate to the atherosclerosis burden.[243, 244] Using the sum of Hounsfield unit-defined adipose tissue in 5 mm segment surrounding the coronary artery, PVAT was shown to be related to the severity of coronary artery disease.[243, 244] In this chapter MRI-derived methodology has been devised to identify PVAT based on the intrinsic physical properties of adipose tissue. This has an advantage of acquiring an operator-independent image which was subsequently segmented to identify PVAT based on patient-specific methodology. Thus, overcoming the issue of close proximity of adipose tissue and vessel wall, as well as, the arbitrary anatomical distribution using a pre-defined fix borders when quantifying PVAT. [234, 243-245] While most reported studies have focused on the role of PVAT in coronary arteries and to less extent the aorta,[139, 258] carotid PVAT was surveyed here and was demonstrated not to be uniformly distributed across circumferential angles. More importantly, PVAT did not have any specific individual pattern of distribution and there were wide variations among subjects with similar category of disease i.e. carotid endarterectomy patients.

The overall volumetric quantification of PVAT was more associated with symptomatic carotid plaques compared to asymptomatic ones. Given the cross-sectional, observational design of study, it is not be possible to ascertain whether PVAT was directly involved in triggering symptoms i.e. plaque rupture. *In vitro* studies have demonstrated the ability of periaortic space-derived adipocytes to stimulate migration of inflammatory cells. In addition, there was an increase in infiltrating macrophages within PVAT in apolipoprotein E knock-out mice with subsequent increase in PVAT release of pro-inflammatory cytokines.[259, 260] Moreover, imaging studies have demonstrated an association between large PVAT volume and the burden of atherosclerosis.[243, 244, 261] In addition, in a post mortem study of 16 patients, coronary atherosclerotic burden expressed as plaque / media ratio was related to PVAT area

and degree of macrophage infiltration within PVAT.[261] In the same study, PVAT was weakly correlated with the presence of lipid core where PVAT was measured in an area of 3 mm circle around the studied coronary aretry.[261] The latter findings supported the results from this chapter but the current analysis extended the nominal association with plaque composition to be highlighted *in-vivo*. Nonetheless, these studies do not provide a causal relationship between PVAT and atherosclerosis,[250, 262] in fact the pro-atherogenic role of PVAT could be challenged and a reverse causal relationship is also plausible. Atherosclerotic plaque may incite an inflammatory process within the vessel wall that may lead into an increase in PVAT volume (inside-out theory) or change in its composition rendering it more detectable on MRI and not vice versa.[249]

Importantly, the work here related PVAT to atherosclerosis by studying adjacent segment of the carotid arteries. Examining these adjacencies was based on the theory that pro-inflammatory cytokines secreted from PVAT pass through vasa vasora or through adventitia and media to reach vessel intima. This proposed cross-talk paracrine route was challenged, contrary to previous imaging studies that related the total volumetric quantity of PVAT to atherosclerosis.[243] The local route/ effect was rigorously studied using a newly-devised method to associate PVAT to adjacent lipid content. Carotid lipid content was chosen as a surrogate of advanced atherosclerosis since the association between PVAT and vessel wall thickness did not suggest a biologically important interaction.[242] Even though there was a statistically significant correlation between PVAT and adjacent carotid wall lipid thickness, this relationship was very modest in particularly in the asymptomatic group. The correlation coefficient in both groups, symptomatic and asymptomatic, was very close to zero (0.06 and 0.10, respectively) suggesting that there was no strong determinant of the association between the two parameters. These findings do not support the hypothesis of the pro-atherogenic role of PVAT via paracrine local effect. However, few points need to be highlighted. Firstly,

while the mechanism of PVAT on atherosclerosis may still exist, anatomical changes, studied here, may not be sufficiently sensitive to detect them. Secondly, the influence of PVAT may not involve features that could be detected on MRI e.g. inflammatory cell infiltrate. Thirdly, it is plausible that the overall quantification of PVAT may not play a significant role but rather the change of adipocytes characterisation, structure and morphology is more biologically important. Therefore, further work is needed to characterise PVAT on MRI to address these limitations.

6.7 Conclusion

The results from this chapter added to the body of evidence examining the possible role of PVAT in atherosclerosis. A systematic and detailed description of PVAT of the carotid arteries in high risk atherosclerotic disease was performed. While others have used CT to study PVAT, MRI was demonstrated to act as a potential alternative imaging modality. Despite the association between symptomatic status of carotid arteries and total PVAT volume, there was a lack of a biologically important interaction between the angular distribution of PVAT and arterial wall lipid thickness. Such dissociation does not support the concept that PVAT has a causal role in promoting atherosclerosis via a local paracrine route.

Chapter 7: General discussion

7.1 Original aims and hypotheses

In the era post-statin treatment, tackling the residual risk requires reconsideration of the paradigm of non-selective application of new anti-atherosclerotic drugs and necessitates new methods to stratify patients based on their atherosclerotic disease process. This is important because without means of stratification, patients who may stand to benefit greatly will miss an important therapeutic opportunity. These therapies are already in advanced stages of clinical development and some of these drugs have shown marked effects on intermediate endpoints such as LDL-c or hs-CRP but their indiscriminate use on 'all comer' was unaffordable and undesirable.

This thesis tests the hypothesis that T2 mapping MRI technique can characterise atherosclerotic individuals and identify those who are at high risk and potentially candidates for targeted therapies. Using T2 mapping, plaque lipid volume and distribution can be used to discriminate between symptomatic and asymptomatic carotid patients. This technique can also identify patients with propensity to develop lipid-rich plaques in multi-sites vascular territories and these patients may be candidates for new anti-atherosclerotic drugs. In addition, this thesis evaluates T2 mapping technique in detecting changes in plaque composition over short period of intensive statin treatment. These changes in plaque composition were hypothesised to be dissociated from standard and functional blood lipid indices and their changes on therapy. Finally, plaque lipid content was theorised to be related to perivascular adipose tissue, a new imaging biomarker that have been implicated in the development of atherosclerosis. This was to investigate the relationship between PVAT and plaque composition given recent data demonstrating the lack of relationship between PVAT and burden of atherosclerosis, measured as arterial wall thickness in the aorta and carotid arteries.

7.2 Summary of key findings

The main findings of this thesis can be summarised as follow:

1. Plaque lipid volume and distribution quantified on T2 mapping MRI technique were related to the symptomatic status of the plaque in patients scheduled for carotid endarterectomy. Patients with recent cerebrovascular events had larger volume with more coalescent lipid distribution compared to those with no previous documented symptoms. The asymptomatic group tend to have small lipid volume with more scattered pattern of lipid distribution (Chapter 3).

2. The current biomarker for clinical decision-making i.e. the degree of luminal stenosis using ultrasound duplex did not relate to the size, composition, or more importantly to the symptomatic status of carotid plaques. The pathological process of atherosclerotic disease in the wall of the vessel is overlooked by the current clinically-utilised ultrasound duplex. Markers of advanced atherosclerotic process such as plaque lipid content may be better determinant of future behaviour of the plaque (Chapter 3).

3. T2 mapping MRI technique can identify patients with propensity to develop lipid-rich plaques at multi-sites vascular territories. These patients are potentially considered at high risk of future vascular events and may be candidates for new lipid-lowering treatments (Chapter 3&4).

4. There was a strong association between plaque burden and lipid content of the carotid arteries. With increasing plaque burden there was an increase variations in carotid plaque lipid content suggesting wide heterogeneity within these plaques. This may indicate various risk of plaque rupture and subsequent different clinical behaviours.

Such relationship was very similar irrespective of the category of disease (carotid endarterectomy *vs*. acute coronary syndrome) (Chapter 3&4).

5. LDL-c reduction using high intensity statin treatment was associated with significant changes in plaque burden and composition. These changes were detected as early as three months using T2 mapping MRI technique. Changes in plaque composition involved reduction in carotid plaque lipid percentage mirrored with an increase content of fibrous tissue. These changes have stabilising effect on plaques rendering them less likely to precipitate acute events (Chapter 4).

6. Carotid plaque lipid distribution echoed changes in plaque composition following three months of statin treatment. Using two imaging indices derived from T2 mapping MRI technique, reduction in LDL-c was associated with decrease in both indices suggestive of more scattered and potentially 'stabilised' pattern of lipid distribution following statin treatment (Chapter 4).

7. *Baseline* carotid plaque lipid content was not related to *baseline* blood lipid indices. Similarly, *changes* in carotid plaque lipid content were dissociated from *baseline*, *attained* and *changes* in blood lipid indices in response to statin treatment, with exception of change in hsCRP. However, this relationship with change in hsCRP was only modest (Chapter 5).

8. There was no significant predictor of changes in carotid plaque lipid content using *baseline*, *attained* or *change* in blood lipid biomarkers following statin treatment. These biomarker included standard lipid measurement such as LDL-c, HDL-c and

triglycerides level, in addition, they extended to emerging functional biomarkers such as PCSK9, CETP activity, Lp(a) and HDL efflux capacity. These biomarkers are of current and real importance given their plausible propensities to contribute to plaque lipid accumulation and/ or removal (Chapter 5).

9. The overall volumetric quantity of PVAT was related to the symptomatic status of the carotid plaque. Yet, there was no circumferential relationship between PVAT and adjacent carotid plaque lipid content. This would challenge the concept that PVAT has a causal role in promoting atherosclerosis via a local paracrine route (Chapter 6).

7.3 Future directions

This thesis focused on the clinical utility of a recently validated MRI imaging and the applications of this technique to characterise the atherosclerotic process beyond the traditional risk factors and the category of the disease. This technique has real clinical potentials in early identifying patients who are at high risk and offering these patients therapies that may modify the disease process preventing future clinical events. The findings from this thesis has opened up a number of interesting areas for future management of atherosclerosis.

In order for this technique to achieve its maximal potential, few technical development are required to ensure that T2 mapping is ready for the real clinical world. The main limitation was the susceptibility of DANTE-MESA sequence to motion artefacts. This was reflected by the relatively high rejection rates of 35% and 29% during the early studies.[180, 219] It is important to highlight that the rejection rate was reduced when coaching subjects about the sensitivity to motion. This was signalled by a lower rejection rate of around 13% at later

stage of patients' recruitment. Nonetheless, to address this particular issue further developments are underway to build a 'navigation' based motion correction sequence that may overcome some of the limitations of the T2 mapping technique. In addition, the relatively long acquisition time of 4 minutes means that DANTE-MESA sequence is more reliant on patients' ability to stay still during image acquisition. Therefore, methods to shorten acquisition time such as compressed sensing is an attractive solution.[263] Compressed sensing reconstructs data from under sampled measurements and therefore accelerate acquisition time.[263] Final refinements of the 10-channel phased-array carotid coil will yield a better signal to noise ratio preparing T2 mapping technique to achieve its full clinical potential.

On the clinical platform, using T2 mapping technique demonstrated a group of patients with propensity to develop lipid-rich plaques within the carotid vascular territories (left and right). However, a 'disconnect' between carotid and coronary arteries was noted. The severity of CAD assessed using angiogram-based methods have been shown to be weakly correlated with coronary plaque lipid measured by NIRs.[221] Therefore, a comparison between coronary and carotid *lipid content* would be pressing to identify a subgroup of patients with multi-sites lipid-rich plaques. These patients could subsequently be trialled for targeted therapies such PCSK9 inhibitors.

T2 mapping has the potential to early identify high risk patients and potentially offering them disease-modifying treatment to target their future risk. To address this aim, a prospective study looking at the prognostic role of quantified lipid using T2 mapping is fundamental. T2 mapping of carotid artery may prove useful in patients with at least mild to moderate carotid disease. Carotid plaque lipid content has been shown to be strongly associated with clinical vascular events.[121] In the MRI sub-study of the AIM-HIGH, there was almost 60% increase in sustaining future vascular event for 1 standard deviation increase in percent lipid core

volume.[121] This study was conducted using MC-MRI technique that is restricted to the presence of the lipid core to be discernible to be quantified. In addition the primary endpoints were derived from the main AIM-HIGH study,[36] and they were not pre-specified to address clinical outcomes specifically related to atherosclerotic disease within the carotid arteries such as disease progression and risk of stroke.

Finally, revascularisation of carotid artery disease, using stenting or endarterectomy, has been compared in large randomised trials.[204, 264] Notably, stroke risk was at least 70% higher using carotid stenting compared to endarterectomy in the symptomatic group,[204] while results of both revascularisation strategies were comparable in the asymptomatic group.[204] Carotid plaque lipid quantified using T2 mapping MRI technique was significantly higher in the symptomatic patients suggestive that there may be a rationale to select patients for revascularisation method based on non-invasive assessment of plaque lipid content. Therefore T2 mapping technique may also be useful in patients with severe carotid artery disease.

Chapter 8: Appendices

8.1 Attributions

The author of this thesis is very grateful for the contribution of colleagues and collaborators to ensure completion of this work.

Dr. Joshua Chai and Dr. Luca Biasiolli validated T2 mapping technique with histology and some of the included endarterectomy patients in Chapter 3 were recruited for this purpose. Carotid MRI scans were performed by Ms. Alison Fletcher, Mr. Peter Manley and Mrs. Juliet Semple with support from the author and Dr. Luca Biasiolli. Dr. Franesca Galassi helped developing scripts for image analyses. Professor Ashok Handa who coordinated the collaboration with the vascular team, namely Mr. Chris Darby, Professor Alison Halliday, Professor Linda Hands, Mr. Jeremy Perkins, Mr. Ed Sideso and Mr. Andrew Northeast. Dr. Naveed Akbar who helped with cell culture and ELISA measurements. In addition, to the team of interventional Cardiologists, namely Professor Rajesh Kharbanda, Professor Adrian Banning, Professor Keith Channon, Dr. Andrew Lucking and Dr. Jeremy Langrish.

8.2 Published work from this period of research

8.2.1 Review article

Alkhalil M, Chai JT, Choudhury RP. **Plaque imaging to refine indications for emerging lipid-lowering drugs.** *Eur Heart J Cardiovasc Pharmacother. 2017 Jan; 3(1): 58-67*

8.2.2 Original Research

Alkhalil M. **Low-density lipoprotein cholesterol lowering with evolocumab and outcomes in patients with peripheral artery disease: Insights from the FOURIER trial.** *Circulation. In press*

Alkhalil M, Biasiolli L, Chai JT, Galassi F, Li L, Darby C, Halliday A, Hands L, Magee T, Perkins J, Sideso E, Jezzard P, Robson MD, Handa A, Choudhury RP. **Quantification of carotid plaque lipid content with magnetic resonance T2 mapping in patients undergoing carotid endarterectomy.** *PLoS One. 2017 Jul 26; 12(7):e0181668.*

Alkhalil M, Biasiolli L, Akbar N, Galassi F, Chai JT, Robson MD, Choudhury RP. **Carotid plaque lipid reduction, determined by T2 mapping, occurs early after high-intensity statin initiation.** Submitted to *EHJ Cardiovascular Imaging.*

Alkhalil M, Edmond E, Edgar L, Digby JE, Omar O, Robson MD, Choudhury RP. **The Relationship of Perivascular Adipose Tissue and Atherosclerosis in the Aorta and Carotid Arteries, Determined by Magnetic Resonance Imaging**. *Diabetes and Vascular Disease Research. 2018 Feb 1:1479164118757923. doi: 10.1177/1479164118757923. [Epub ahead of print]*

Chai JT, Biasiolli L, Li L, Alkhalil M, Galassi F, Darby C, Halliday AW, Hands L, Magee T, Perkins J, Sideso E, Handa A, Jezzard P, Robson MD, Choudhury RP. **Quantification of Lipid-Rich Core in Carotid Atherosclerosis Using Magnetic Resonance T_2 Mapping: Relation to Clinical Presentation.** *JACC Cardiovasc Imaging. 2017 Jul; 10(7):747-756.*

Alkhalil M, Choudhury RP. **Evolocumab Added to Statins to Reduce Progression of Coronary Atherosclerosis.** *JAMA. 2017 Apr 25; 317(16):1690-1691*

Galassi F, Alkhalil M, Lee R, Martindale, P, Grau V, Choudhury RP. **3D Reconstruction of coronary arteries from 2D angiographic projections using non-uniform rational basis splines (NURBS) for accurate modelling of coronary stenoses.** Submitted to *PLoS One*.

8.2.3 Abstracts

Alkhalil M, Biasiolli L, Chai JT, Galassi F, *et al*. **Carotid plaque lipid reduction, determined by T2 mapping, occurs early after high-intensity statin initiation in patients presented with acute myocardial infarction.** Finalist Young Investigator Award, *British Society of Cardiovascular Magnetic Resonance 2018*.

Alkhalil M, Biasiolli L, Chai JT, Galassi F, *et al*. **Changes in carotid plaque lipid on T2 mapping using high intensity statin.** *British Atherosclerosis Society 2017*.

Chai JT, Biasiolli L, Li L, Alkhalil M, *et al*. **Lipid-rich core quantification in carotid atherosclerosis using MRI T2 mapping - relation to clinical presentation and plaque macrophage activation.** *American Heart Association 2015*

Chai JT, Biasiolli L, Li L, Alkhalil M, *et al*. **In vivo carotid plaque characterisation using quantitative MRI T2 mapping- histological validation and lipid quantification.** *European Atherosclerosis Society 2015.*

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