

Integrated characterisation of carotid atherosclerosis - from magnetic resonance imaging to plaque macrophage profiling

Joshua Tian-Yun Chai

BA MA MB BChir MRCP(UK)



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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Joshua T. Chai

**RDM Division of Cardiovascular Medicine, and
Linacre College, University of Oxford**

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Abstract

Cardiovascular morbidity and mortality remain the most serious global public health challenge. With the evolution in global socio-economic activities and the associated lifestyle changes, there is an impending epidemic of obesity, insulin resistance, metabolic syndrome, and diabetes mellitus, all of which fuel premature coronary artery disease and stroke. In the post-statin era, despite considerable efforts in anti-atherosclerotic drug development, a significant number of these potential compounds with initial success in early phase clinical studies failed to translate to clinical outcome benefits. This shows a disparity of various biomarkers and molecular disease mechanisms, and highlighted the inadequacy of current methods of risk stratification and atherosclerosis characterisation.

Atherosclerosis imaging such as by MRI does not only serve as ‘gate keepers’ in the contemporary drug development process; but in combination with modern “omics”, would allow precise disease stratification with resolution never achieved before. This *integrated* characterisation of atherosclerosis will generate novel insights about plaque biology, bridge the gap between molecular insights and clinical outcomes, and help achieve the goal of stratified, personalized, medicine.

This thesis hypothesized that plaque *compositional heterogeneity* such as lipid content is associated with specific gene expression profiles or signatures in human carotid plaque macrophages in a spatially distinct pattern; and that this macrophage *functional heterogeneity* may in turn play a role in altering response to anti-atherosclerotic treatment. To test these hypotheses, human subjects with carotid atherosclerosis were recruited before carotid surgery and scanned using novel MRI T₂ mapping technique. Explanted carotid plaques were then examined by immunohistochemistry-guided laser capture microdissection and microarray analysis.

T₂ mapping was shown to be able to accurately and reproducibly discriminate all major plaque components, and in particular, quantify plaque lipid content with excellent histological validation. Symptomatic plaques were shown to contain larger lipid-core by T₂ mapping compared to asymptomatic plaques, despite similar degree of luminal stenosis. Macrophages from the same cohorts of plaques were procured by immuno-LCM and they were shown to display distinct gene expression profiles based on their micro-anatomical locations and clinical presentations; importantly, macrophages from plaques with high lipid content detected by T₂ mapping were found to activate specific interferon / STAT1 pathways. Finally, using nicotinic acid as an example, the functional significance of plaque macrophage heterogeneity was shown *in vitro* to affect response to pharmacological intervention.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Atherosclerosis and the residual risk

1.1.1 Epidemiology

Cardiovascular disease (CVD) has become the most serious public health burden globally. Despite an improved understanding and earlier detection of the major cardiovascular risk factors, mortality and morbidity associated with atherosclerotic complications continued to rise at an alarming rate. In the Western world CVD causes 4.3 million deaths each year, representing nearly half (48%) of all-cause mortality in Europe.¹ A similar trend is also observed in the rapidly-developing countries, such as in China, where the proportion of death caused by CVD in its vast 1.3 billion population compared with all other causes of mortality was estimated to have increased from 12.8% in 1951 to 35.8% in 1990.² With the changing patterns of global socio-economic activities and associated changes in lifestyle, we are facing an impending epidemic of obesity, insulin resistance, metabolic syndrome, and type II diabetes mellitus (T2DM).³ This constellation of metabolic abnormalities confers even further risk for the development of microvascular and macrovascular atherothrombotic complications, which can lead to premature coronary artery disease and stroke.

Over the past twenty years, 3-hydroxy-methylglutaryl coenzyme A (HMG Co-A) reductase inhibitors, statins, have become the first-line treatment of dyslipidaemia. Landmark clinical trials have shaped our understanding in cardiovascular (CV) risk reduction in both statin-naïve^{4,5} and statin-treated⁶⁻⁸ patients in both primary and secondary prevention. Cumulative evidence from clinical trials involving more than 170,000 patients, have indisputably shown the beneficial effects of statins in the reduction of cardiovascular event rates.⁹ The large meta-analysis conducted by the Cholesterol Treatment Trialists' collaboration established a 12% reduction in CV

events for every 1 mmol/L decrease in LDL-c during the first year of treatment; with an additional 25% reduction for each subsequent year (overall CV event reduction of 22% per 1 mmol/L decrease in LDL-c), indicating the ongoing benefits in terms of CV event reduction accrued over time with persistent statin treatment.⁹ Importantly, it raised serious debate about the previously held notion of a threshold of LDL-c below which further CV risk reduction would be futile.

However, despite increasing evidence from clinical trials and pre-clinical studies that treatment with statins achieves substantial LDL-c reduction and consequently contributed to up to 30% reduction of cardiovascular mortality,¹⁰ there remains a significant residual CV risk with continued high rates of CV events in patients optimally or near-optimally treated with statins.⁶⁻⁸ Recent reports corroborated the association between dyslipidemia and vascular events despite statin therapy,^{11,12} and it was estimated that an *annual* residual CV risk of 4-9% remains a current reality in patients with established coronary artery disease.¹³

1.1.2 The post-statin era and its challenges

To combat this residual risk and to further reduce CV event rates in statin-treated and / or patients intolerant to statin therapy, there is an ongoing drive to develop new or adjunctive therapeutic strategies. Although many chemical compounds have multiple actions and simultaneously target many different aspects of atherogenesis, conceptually there are three broad strategies that are being pursued: (1) further reduction of LDL-c with non-statin agents, such as ezetimibe¹⁴ or proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors;¹⁵ (2) treatment targeting non-LDL-c parameters such as increasing HDL-c (e.g. with cholesteryl-ester transfer protein (CETP) inhibitors)¹⁶ or reducing triglyceride (e.g. with PPAR α agonists such as the fibrates¹⁷ and dual PPAR α / γ agonists the glitazars);¹⁸ and (3) novel biologic

therapies to intervene in key biological processes involved in atherosclerosis such as inflammation with, for example, CCR2 antagonists,¹⁹ p38 MAPK inhibitors,²⁰ and IL-1 β antagonists.²¹

Strong evidence from epidemiological studies exists to support the so-called “HDL hypothesis”. In a landmark analysis from the Framingham study cohort by Gordon *et al.*, an inverse relationship was observed between HDL-c level and increased CV risk,²² and the protective effect of HDL-c was further confirmed by 4 prospective studies.²³ A recent meta-analysis of 20 randomized-controlled trials with more than 540,000 person-years follow-up provided convincing evidence that low levels of HDL-c is an independent CV risk factor in that low HDL-c remains significantly and independently associated with increased risk despite statin treatment.²⁴ Nonetheless, Mendelian randomization studies seemed to favour low HDL-c being a robust association to CV events rather than a causative factor *per se*.^{25,26}

In support of HDL hypothesis, *in-vitro* and animal experiments have provided some mechanistic insights by which HDL might exert its atheroprotective effects. In addition to its direct anti-inflammatory effects,²⁷⁻³¹ HDLs, through its most important structural protein Apo A-I, are believed to be the first step in the reverse cholesterol transport (RCT) pathway, which receives cholesterol from peripheral cells via ATP-binding cassette transporter proteins (ABCA1 / ABCG1) and ferry cholesterol to the liver, where a proportion is secreted in bile.³² Despite the epidemiological and pre-clinical data, and although exogenous infusion of HDL-like particles in earlier human studies did appear to be encouraging,^{33,34} therapeutic intervention targeting HDL-cholesterol have not shown clinical benefit. For instance, trials of CETP inhibitors, which target the exchange of triglycerides from plasma VLDL and LDL with cholesteryl ester from HDL, suffered repeated setbacks with development terminated in Phase III due to off-target effects (torcetrapib),³⁵ and lack of efficacy

(dalcetrapib).³⁶ Likewise, a promising new class of cardiometabolic agents, the glitazars, which act as transcriptional regulators by their dual PPAR- α/γ agonism to simultaneously target diabetes, dyslipidaemia (especially triglycerideamia), cardiovascular risk,³⁷ and showed very encouraging preclinical and phase II studies,¹⁸ had also suffered ill-fate with development terminated in late-phase trials due to specific safety concerns³⁸ or lack of efficacy.³⁹ But perhaps one of the more surprising setbacks in recent time has been the resurgence and apparent “failure” of nicotinic acid.

1.1.3 The resurgence and fall of nicotinic acid

For over fifty years, nicotinic acid (NA) has been known to exert favourable effects on plasma lipoproteins.⁴⁰ It reduces atherogenic lipoproteins LDL-c, VLDL-c, and Lp(a), and is the most efficacious drug (except for CETP inhibitors) to raise plasma HDL-c (by up to 30%).⁴¹ The Coronary Drug Project⁴² had shown benefits of NA in reducing cardiovascular events and mortality in patients with coronary heart disease, a finding supported by 2 recent meta-analyses.^{43,44} However, it was not until in 2003 that the G-protein coupled receptor (GPCR) GPR109A (also known as hydroxy-carboxylic acid (HCA) receptor 2 (HCA₂), HM74a, or NIACR1) was found to be a receptor target of NA.⁴⁵⁻⁴⁷ Two years later, GPR109A was de-orphanised when ketone body 3-hydroxy-butyrate was found by Taggart, *et al.* to be its endogenous ligand.⁴⁸ Since then, many other synthetic agonists of GPR109A had been developed (Acipimox, Acifran, MK-0354, etc.).⁴⁹ In addition to its lipoprotein-mediated effects, activation of GPR109A with NA also antagonises known inflammatory pathways in adipocytes⁵⁰ and in some leukocytes.⁵¹ Therefore, with its “pleiotropic” potential, niacin has re-entered the forefront and has fuelled accelerated clinical studies, as well as basic research into the biological role of GPR109A and its downstream signalling pathways.

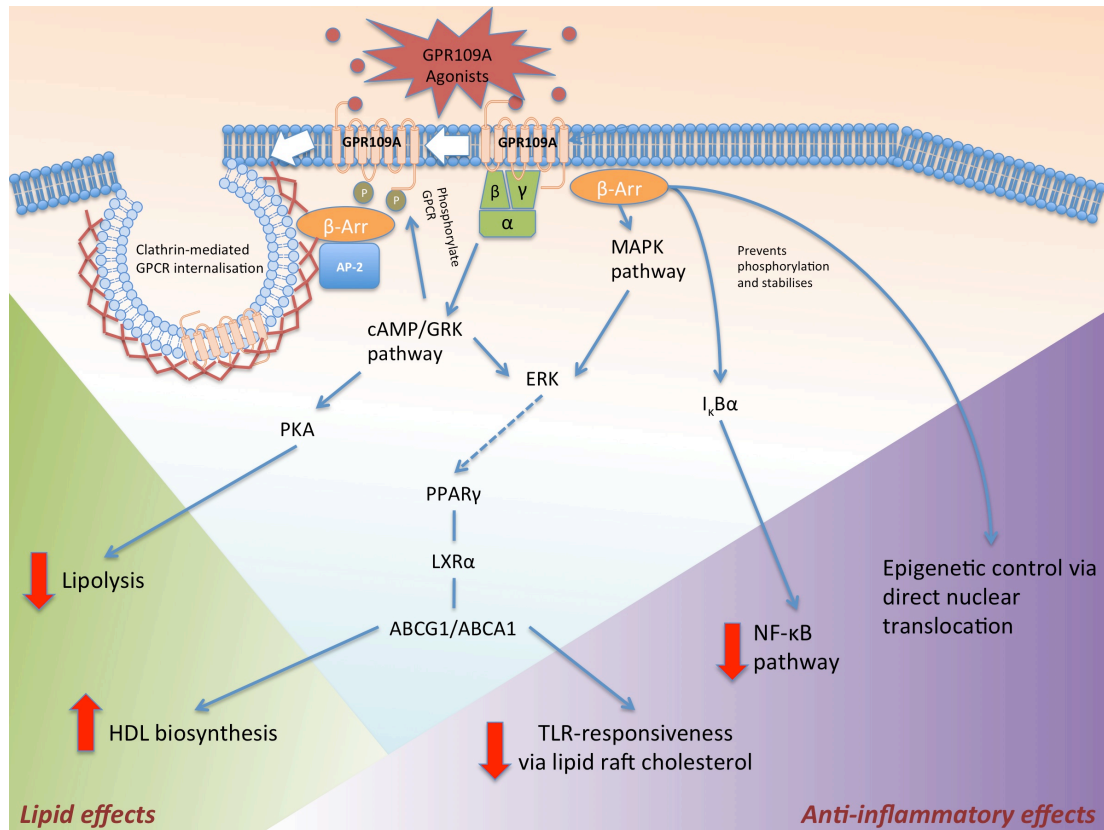


Figure 1.1 Putative signalling mechanisms downstream of GPR109A activation

GPR109A signalling is mediated by at least two known cellular pathways – one via the coupling of $G_{i/o}$ protein and the classic G-protein cAMP/GRK pathway; the other via the recently discovered β-arrestin pathway. The latter has recently been shown to mediate epigenetic control via direct nuclear translocation. This non-linearity of G-protein couple signaling highlighted the important concept of *biased agonists*. Reproduced from:- Chai JT, Digby JE and Choudhury RP. GPR109A and vascular inflammation. *Curr Atherosclerosis Rep.* 2013;15(5):325

NA has a variety of “pleiotropic” actions that are of potential relevance to processes of atherosclerosis progression and regression. In adipocytes⁵² and certain non-foam cell ‘basal-state’ mononuclear cell lines,^{53,54} NA activates the reverse cholesterol transport apparatus, namely PPAR γ , LXR α , and the ABC transporter proteins, which are known to be responsible for *in vitro* cholesterol unloading⁵⁵ and, in turn, are directly implicated in the regression of experimental atherosclerosis.^{56,57} These observations raise the possibility that effects of NA on atherosclerotic plaque regression and cardiovascular risk reduction may, in part, be due to direct effects on reverse cholesterol transport through foam cell cholesterol unloading.

Nevertheless, despite previous trials showing that NA reduces atherosclerosis, estimated from coronary angiography,^{58,59} carotid ultrasound⁶⁰⁻⁶³ and MRI,⁶⁴ recent clinical outcome trials were again disappointing with one abandoned due to lack of efficacy,⁶⁵ while another larger Phase III trial also failed to demonstrate any clinical benefit in terms of CV risk reduction in addition to optimal statin treatment; instead, it even suggested an increase of serious adverse effects.⁶⁶

1.2 Emerging therapy and the need of stratified medicine

1.2.1 Common theme for recent drug development failures

As seen from various examples above, there had been considerable efforts in anti-atherosclerotic drug development over the last decade. However, for a majority of these compounds, despite convincing mechanistic biology in pre-clinical studies, and promising early phase / surrogate biomarker clinical studies, they mostly fell at the final hurdle in translating into clinical outcome benefits. One potential reason is that in the post-statin era, any new pharmacological agent not only has to show clinical benefits on its own as monotherapy, but also has to demonstrate *incremental benefits* in addition to statin as an adjunctive therapy; since it is no longer ethically acceptable to conduct clinical trials randomizing statin therapy in high risk patients, and that recent American Heart Association / European Society of Cardiology cholesterol treatment guidelines has resulted in progressive lowering of LDL-c treatment threshold, new chemical compounds are stacked against an ever increasing challenge in meeting the bar of efficacy in clinical outcome trials with statin-treated patients.

Another potential reason for development failure, which is particularly important for agents targeting non-LDL-c mediated aspects of athero-protection such as elevation or modification of plasma HDL and for new biologic therapies, is that the trial population might just be too heterogeneous for detection of small incremental benefits in addition to statin; in other words, the current available “platform” in drug evaluation might be too crude to allow adequate patient / risk stratification, and that refinement of the target treatment group might allow benefit signals to be detected above noise, whilst minimising potential harmful side effects from these powerful new generation anti-atherosclerotic agents.

This apparent *disconnect* between early-phase imaging biomarker studies and clinical outcome trials in the current drug evaluation platform revealed a current incomplete understanding of how imaging features (surrogate biomarkers) really translate to changes at a molecular level in relation to pathology and potential therapeutic targets. For example, Libby, *et al.* has recently challenged the notion of using ^{18}F FDG-PET to image plaque inflammation by showing that it was hypoxia rather than inflammation that primarily drives the ^{18}F FDG-PET signal.⁶⁷ To confound the problem even further, current non-invasive atherosclerosis imaging techniques such as visualization of stenosis by computed tomography (CT) based contrast lumenography, measurement of carotid intimal-medial thickness (CIMT) by ultrasound and estimation of total plaque volume / burden by vascular MRI are inadequate to interrogate plaque *compositional heterogeneity*. This is important because new aggressive anti-atherosclerotic agents that target the lipid pathway might work by extracting lipid from lipid-rich necrotic core (LRNC) in atherosclerotic plaques, with replacement by fibrous tissue, and little effect on the total plaque volume. With new generations of biologic agents that directly target plaque biology near the clinical horizon,^{20,21} existing imaging techniques may not provide sufficient information for rational patient selection or response to treatment.

1.2.2 Plaque biology and macrophage as target for characterisation

To tackle the gap in our current understanding between imaging features and molecular changes at the target tissue level would first require better understanding of plaque biology and novel tools to study plaque constituent cells in the context of human atherosclerosis. Deposition of lipid from ApoB-containing lipoproteins in the subendothelial space, which eventually coalesce into lipid-rich necrotic core (LRNC),

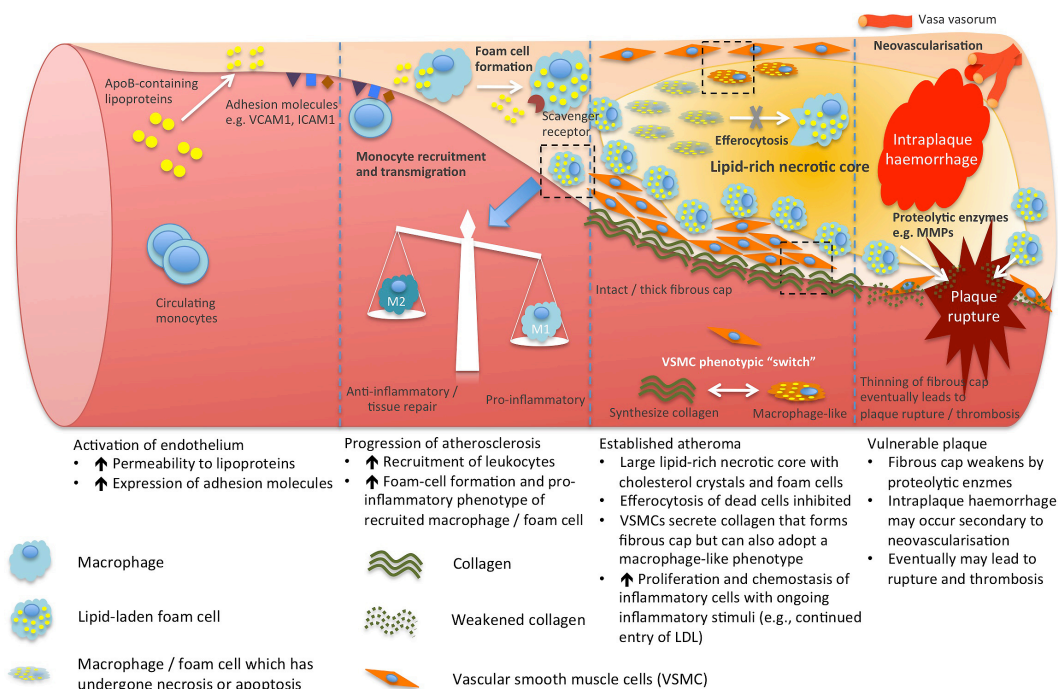
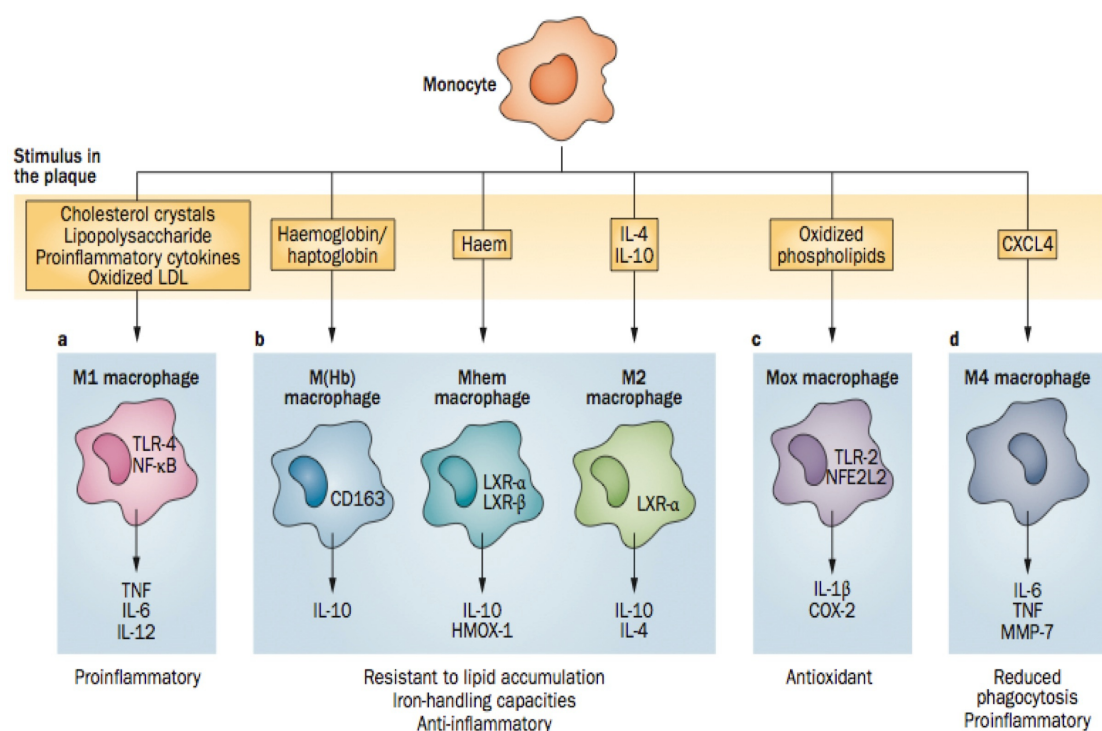


Figure 1.2 *Diagrammatic illustration of the involvement of macrophages in different stages of atherosclerosis progression*

From left to right: circulating monocytes are recruited at activated endothelium via a range of adhesion molecules and are transmigrated into the subendothelial space; here they differentiate into macrophages, which take up lipids via scavenger receptors as macrophage-derived foam cells. In established atheroma, a coalesced lipid-rich necrotic core, which is predominantly composed of lipid-laden foam cells, expands and perpetuates as efferocytosis of dead cells from macrophages is inhibited, and proliferation and chemostasis of inflammatory cells further enhanced plaque inflammation. Eventually, vascular smooth muscle cells (VSMCs) change and adopt a more macrophage-like phenotype, with degradation of the overlying fibrous cap, culminating in plaque rupture and its thrombotic complications. Intraplaque haemorrhage from unstable neovascularity within plaque, with subsequent extravasation of blood, also helps accelerate plaque progression. This effect is at least partly by physical expansion, and partly by the supply of large phospholipid load from cell membranes components derived from extravasated blood cells. Reproduced from:- Choudhury RP, Chai JT, Fisher EA. Chapter 16.13.1 Biology and pathology of atherosclerosis. Oxford Textbook of Medicine (in advanced stage of editorial review)

is a hallmark of atherogenesis; and monocytes / macrophages are involved in the all stages of this process from initiation, progression, sustainment, and even experimental regression of atherosclerosis.⁶⁸ As such, plaque macrophage has been seen as an attractive candidate not only in plaque characterisation but also potential therapeutic target.

Both blood monocytes and plaque macrophages are comprised of heterogeneous cell populations; an important characteristics of the monocyte / macrophage lineage cells are their *functional heterogeneity* and *plasticity* – i.e. their remarkable ability to adapt their functional phenotypes in response to signalling cues and their micro-environment.^{69,70} Interestingly, not only can plaque composition influence functional phenotype of macrophages, macrophage subtypes - owing to their intrinsic activities (such as phagocytosis, proteolysis, or secretion of pro- and anti-inflammatory factors) - can conversely influence plaque structure and its natural history.⁷¹ The contemporary views of macrophage plasticity still borrow heavily from the T-lymphocytes *T_H1:T_H2 paradigm* i.e. T_H1 cytokines such as TNFα, IL-1β, IFNγ inducing polarization into “M1” proinflammatory macrophages and T_H2 cytokines such as IL-4, IL-13 inducing polarization into alternatively activated “M2” (or ‘reparative’) macrophages. It is, however, increasingly recognized that characterising plaque macrophages into discreet “classes” by their expressions of a few cell surface markers are problematic, and perhaps over-simplistic, and may not correlates to their *in vivo* functional roles.⁷¹



Phenotype	Traditional classification	Polarized by	Markers	
			In mice	In humans
M1	Proinflammatory, classically activated	T _H 1 cytokines (IFN- γ , TNF, IL-1 β) or lipopolysaccharide	IL-1 β , TNF, IL-6, IL-12, IL-23, CXCL9, CXCL10, CXCL11, arginase II	IL-1 β , TNF, IL-6, IL-12, IL-23, CXCL9, CXCL10, CXCL11
M2a	Anti-inflammatory, alternatively activated	T _H 2 cytokines (IL-4, IL-13)	Arginase I, resistin-like α , Ym1, Ym2, MMGL, stabilin-1, CD163	MMR, IL-1RA, factor XIIIa, CD200R, CCL18, stabilin-1, CD163
M2b	Alternatively activated	Combinations of immune complexes with IL-1 β or lipopolysaccharide	IL-10 ^{high} , IL-12 ^{low}	IL-10 ^{high} , IL-12 ^{low}
M2c	Alternatively activated	IL-10, TGF- β , or glucocorticoids	Arginase I	MMR
M4	N/A	CXCL4	MMP-7, S100-A8, MMR	MMP-7, S100-A8, MMR
Mox	N/A	Oxidized phospholipids	HO-1, Sulfiredoxin-1, TR, NFE2L2	HO-1, sulfiredoxin-1, TR, NFE2L2
M(Hb)	N/A	Haemoglobin-haptoglobin complexes	CD163, MMR	CD163, MMR
Mhem	N/A	Haem	CD163, ATF-1	CD163, ATF-1
HA-mac	N/A	Haemoglobin-haptoglobin complexes	CD163 ^{high} , HLA-DR ^{low}	CD163 ^{high} , HLA-DR ^{low}

Abbreviations: ATF-1, cyclic AMP-dependent transcription factor-1; CCL18, C-C motif chemokine ligand 18; CXCL, C-X-C motif chemokine ligand; HLA, human leukocyte antigen; HO-1, haem oxygenase 1; MMGL, C-type lectin domain family 10 member A (also known as MGL-1); MMP-7, matrix metalloproteinase 7; MMR, macrophage mannose receptor; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; N/A, not applicable; TGF- β , transforming growth factor- β ; T_H1, type 1 T helper cells; T_H2, type 2 T helper cells; TNF, tumour necrosis factor; TR, thioredoxin reductase 1, cytoplasmic.

Figure 1.3 Diagram and table showing the proposed macrophage plasticity

Chinetti-Gbaguidi G, Colin S, Staels B. Macrophage subsets in atherosclerosis. *Nat Rev Cardiol.* 2015;12(1):10-7.

Importantly, although the general properties of macrophages are conserved between species, their cell surface markers are not. For example, the M2 phenotype is defined by the expression of arginase I and Ym1 (chitinase 3-like protein 3) in mice, but not in humans.⁷² Moreover, there are very few cell surface markers that are specific, selective, and exclusive for an individual given phenotype, which further highlights the significant overlap between respective phenotypes.⁷¹ (see Figure 1.3) Therefore, an unbiased genome-wide analytic approach of macrophage heterogeneity using functional genomics is more likely to yield useful biological insights than qualitative description of macrophage phenotypes in plaque characterisation.

In addition to characterising plaque macrophages by cell surface markers, the functional phenotypes tend also to reflect their distribution in the plaque in relation to certain micro-anatomical features. For example, M1-like classically activated macrophages were reported to be found in the plaque shoulder, where their inflammatory phenotype and secretion of proteolytic enzymes may contribute to plaque destabilisation in symptomatic plaques; while a mixture of M1-like and M2-like cells were seen inside area rich in macrophages foam cells.⁷³

Interestingly, this distinct yet overlapping pattern of distribution of plaque macrophage subsets challenges our current understanding of monocyte recruitment and the relationship between M1 and M2-like macrophages. In particular, it raises at least three possible scenarios: (1) different macrophage subsets being recruited from distinct pre-programmed blood monocyte subsets;⁷⁴ (2) infiltrated blood monocytes differentiate and locally proliferate into distinct functional phenotypes,⁷⁵ or (3) macrophage subsets sequentially evolve or 'switch' from M1 to M2 macrophages in a spatial-temporally defined manner.⁷⁶ Recent evidence in mice suggested that local proliferation of macrophages is the predominant mechanism of replenishment of

lesional macrophages in experimental advanced murine atherosclerosis and this was orchestrated by the plaque micro-environment.⁷⁵ However, the hypothesis of a macrophage phenotypic 'switch' is attractive not only in plaque characterisation but also as a potential therapeutic target. Indeed, experimental murine models of atherosclerosis regression have shown that rapid reversal of hyperlipidaemia by either transplantation of aorta from hyperlipidaemic donor to normolipidaemic recipient mice, or by a genetic switch in *Reversa* mice, resulted in broad transcriptomic changes, or a "switch", towards less inflammatory M2 phenotypes.^{57,77} Nevertheless, whether these findings in mice can be translated to humans remain unclear.

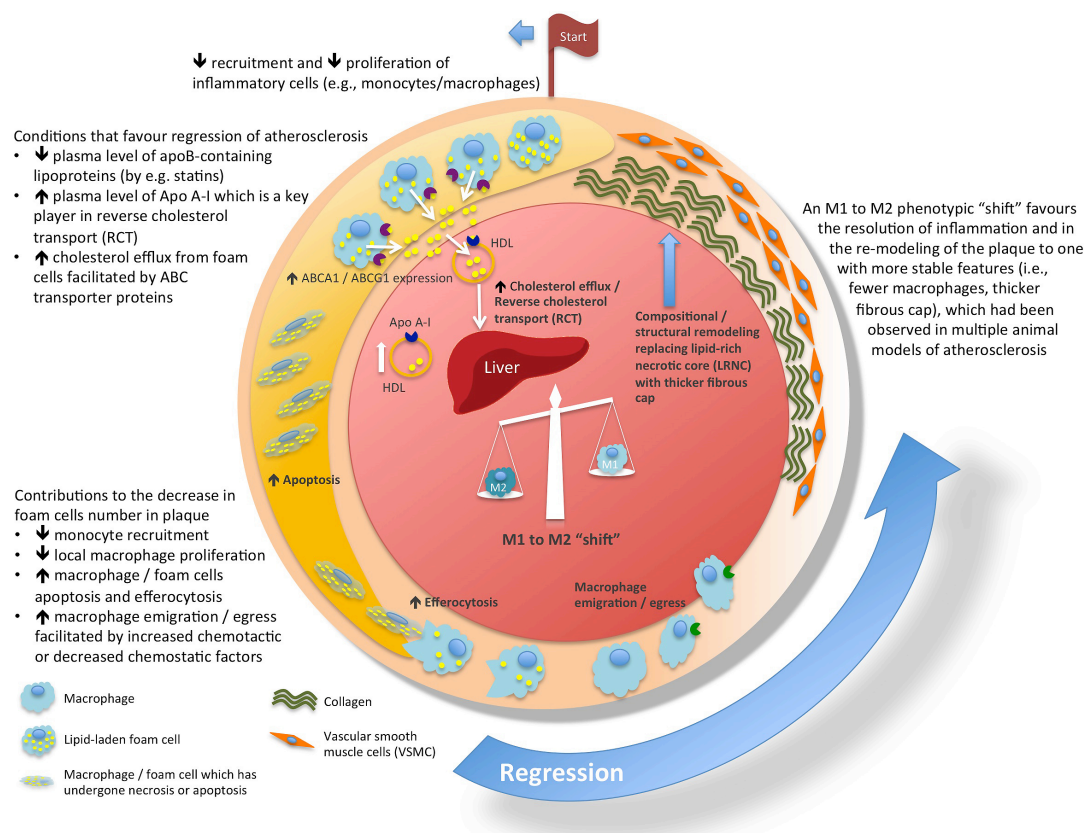


Figure 1.4 *Diagrammatic illustration of the potential processes in experimental atherosclerosis regression*

Anti-clockwise: reversal of hyperlipidaemia reduces recruitment and proliferation of plaque inflammatory cells; simultaneously, favourable plasma lipoprotein composition encourages reverse cholesterol transport and secretion of cholesterol by the liver. With inhibition of apoptosis and efferocytosis reversed, clearance of inflammatory cells in the vessel walls contributes to a shift of macrophage phenotypes from a more inflammatory (M1) to a more reparative (M2) subtype. Macrophage emigration or egress from murine vessel wall has also been observed via CCR-7 and has been hypothesized to be important in atherosclerosis regression. The M1 to M2 phenotypic "shift" favours resolution of plaque inflammation and stabilization of vessel wall. Reproduced from:- Choudhury RP, Chai JT, Fisher EA. Chapter 16.13.1 Biology and pathology of atherosclerosis. Oxford Textbook of Medicine (in advanced stage of editorial review)

1.2.3 Drug discovery in the era of stratified medicine

At the turn of the millennium, with the completion of the Human Genome Project, the notion of personalized medicine has gathered momentum with progressive enthusiasm coming from all disciplines in the biomedical world. However, it can be argued that at the level of clinical practice, personalized medicine is in fact nearly as old as the recorded history of medicine. This is because although clinical knowledge might have changed, clinical practice – in its most basic incarnation giving the right treatment to the right patient at the right time – has not really changed since the time of Hippocrates.^{78,79} However, with the ‘new’ language of the “omics” (functional genomics, proteomics, and metabolomics), different phenotypes of a disease process can be defined or ‘stratified’ with precision and detail never imagined possible just over a decade ago, and this paves the way to modern stratified medicine. Stratified medicine is nearly a clinical reality in oncology with tyrosine kinase inhibitor imatinib (Glivec®) being one of the first “designer” drugs specifically designed to inhibit the bcr-abl fusion protein that is characteristic for Philadelphia chromosome-positive leukaemia.⁸⁰ The use of trastuzumab (Herceptin®) to treat HER2-positive breast cancer has also transformed the prognosis of millions of cancer patients.⁸¹ In neurology, MRI and PET brain imaging and other biomarkers enable specific diagnoses of dementia syndromes and could potentially be used to stratify healthy individuals at risk of developing dementia for early preventative interventions.⁸²

Discovery of new anti-atherosclerotic drug has proved to be a risky and costly affair. It was estimated that for a single compound to gain regulatory approval and reach the market, a monetary investment in excess of £650 million (\$1 billion USD) and time investment of 10 years or more are needed from compounds inception.⁸³ With the limitations of time, cost, and risk of programme failure, drug discovery

programmes would benefit from robust “intermediate endpoints” to allocate resources to the most promising compounds before committing to large-scale randomized-controlled trials that involve thousands of patients for sometimes up to 5+ years.⁸³ Although non-invasive atherosclerosis imaging fulfills such a role as the “gate-keeper” of the finite clinical research resources, imaging approaches alone cannot provide the mechanistic insights obtainable by the complementary modern “-omics”. Targeted transcriptomic ($\pm$ proteomic) analysis from well characterised patient cohorts guided by imaging, will not only broaden our understanding in plaque biology, but will also generate potential biomarker candidates for disease stratification, for monitoring response to treatment, and even allow identification of new therapeutic target. Importantly, an integrated platform of patient phenotype or atherosclerotic plaque stratification allows *integration* of molecular mechanism and intermediate mechanistic insights gleaned from target tissue imaging at the point of patient selection into clinical outcome trials. This approach allows precise refinement, or enrichment, of the target population, greatly enhancing statistical power to detect benefit signal by reducing signal noise from heterogeneity, which in turns means potentially exposing less patients to harmful or undesirable side effects of these new powerful biologic therapy.

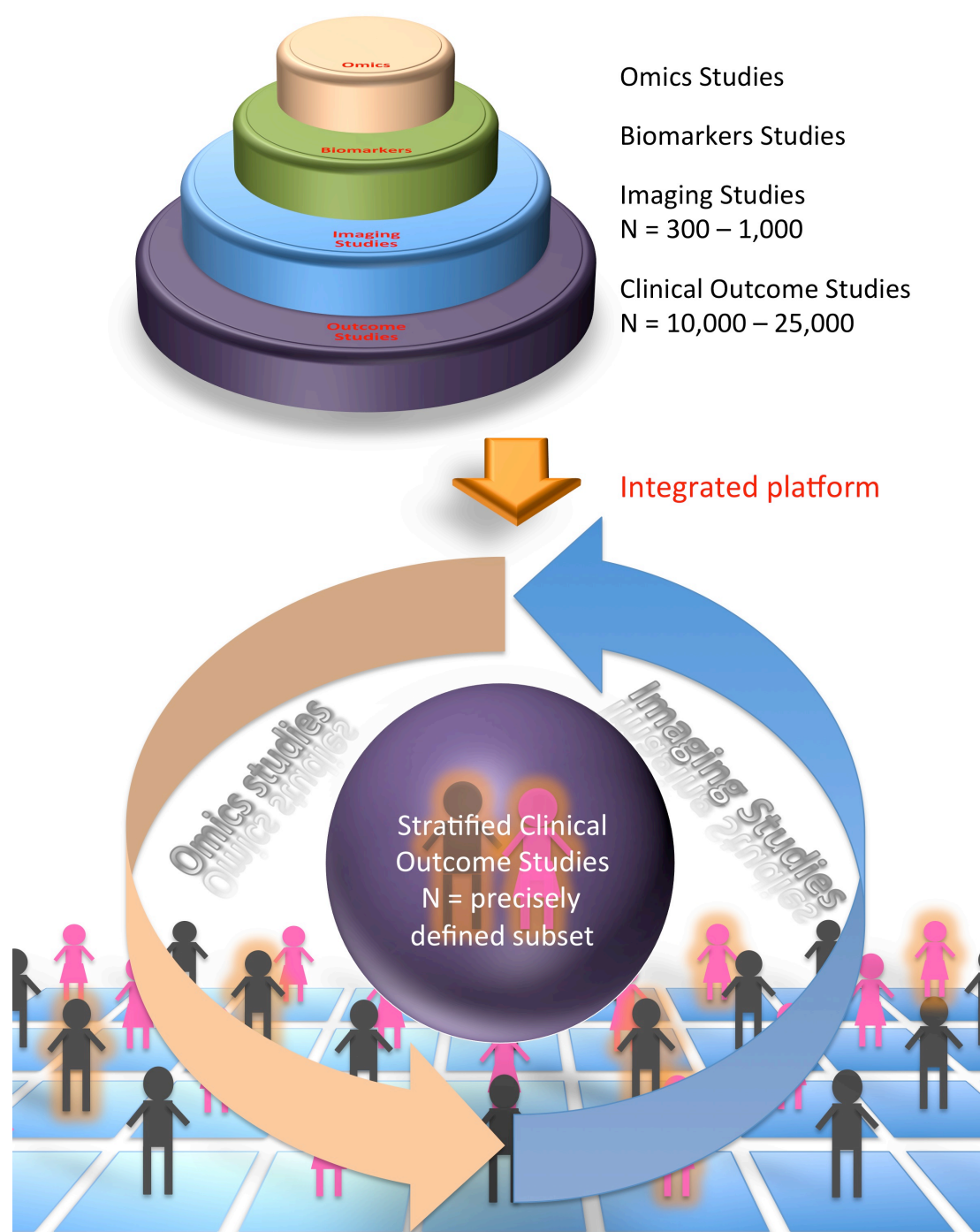


Figure 1.5 *Current approach with the drug development pyramid*

Top: hierarchical process *versus* an integrated platform (below) where data from imaging and omics are used to precisely define patient selection in clinical outcome studies.

1.3 The complementary roles of imaging and the “omics”

1.3.1 Current role of imaging in plaque characterisation

Non-invasive vascular wall imaging and characterisation of atherosclerosis has long been the aspiration of cardiovascular clinicians. Over the last decade, cardiovascular magnetic resonance imaging (MRI) has emerged to be a powerful tool for atherosclerotic plaque characterisation. ‘Lumenography’, either by means of invasive X-ray arteriography or non-invasive computed tomography (CT) or MR angiography, still plays a central role in the characterisation of acute ischaemic vascular syndromes as these techniques can define the severity and site of vascular stenoses, thereby guiding therapeutic interventions. However, the lesions of atherosclerosis develop within the arterial wall, and a substantial proportion of myocardial infarctions and strokes result from complication of ‘vulnerable’ lesions that do not cause severe luminal stenosis. Vessel *wall* imaging therefore remains the most promising tool to detect vulnerable plaques and potentially unlock the therapeutic window for timely intervention, particularly in the asymptomatic – or subclinical – cohort.

1.3.2 Vulnerable plaques from an imaging perspective

Early pathology studies established some key determinants of plaque vulnerability in coronary and carotid atherosclerosis.⁸⁴⁻⁸⁷ MRI was first applied to vessel characterisation by Toussaint and colleagues.⁸⁸ Yuan *et al*/ subsequently undertook a series of multi-contrast MRI studies, using T1, T2, proton-density (PD), and time-of-flight (TOF) sequences, to demonstrate how *in-vivo* MR imaging can distinguish many of the key plaque components in vulnerable carotid plaques.^{89,90} This led to an American Heart Association (AHA) plaque classification based on MRI.⁹⁰ The use of

gadolinium contrast further improves quantification of lipid-rich core, detection of thin fibrous cap,⁹¹ and potentially reflects degree of plaque neo-vascularisation via dynamic contrast flux.^{92,93}

This multi-contrast technique in vascular MR imaging has been shown to accurately delineate different plaque components, namely fibrous cap, lipid-rich necrotic core, intra-plaque haemorrhage, and calcification with high sensitivity and specificity.⁹⁴ (Figure 1.6) Recent prospective imaging studies have shown that in asymptomatic populations, vulnerable plaque features such as thin or ruptured fibrous cap, large lipid-rich necrotic core (LRNC), and the presence of intra-plaque haemorrhage (IPH), not only predict future vascular events in the same (downstream) vascular territory (e.g. carotid atherosclerosis and future TIA or strokes),^{95,96} but some of these MRI-identifiable plaque features may prospectively predict future overall cardiovascular outcomes.⁹⁷ Hellings *et al.* prospectively followed up 818 patients in the Athero-Express biobank for 3 years after carotid endarterectomy, and those with IPH at baseline had a significantly higher risk in reaching composite cardiovascular endpoints of cardiovascular death, myocardial infarction, stroke, and vascular interventions (3-years Kaplan-Meier estimate of 30.6% vs 17.2%, HR = 1.7; 95% CI, 1.2 to 2.5).⁹⁷

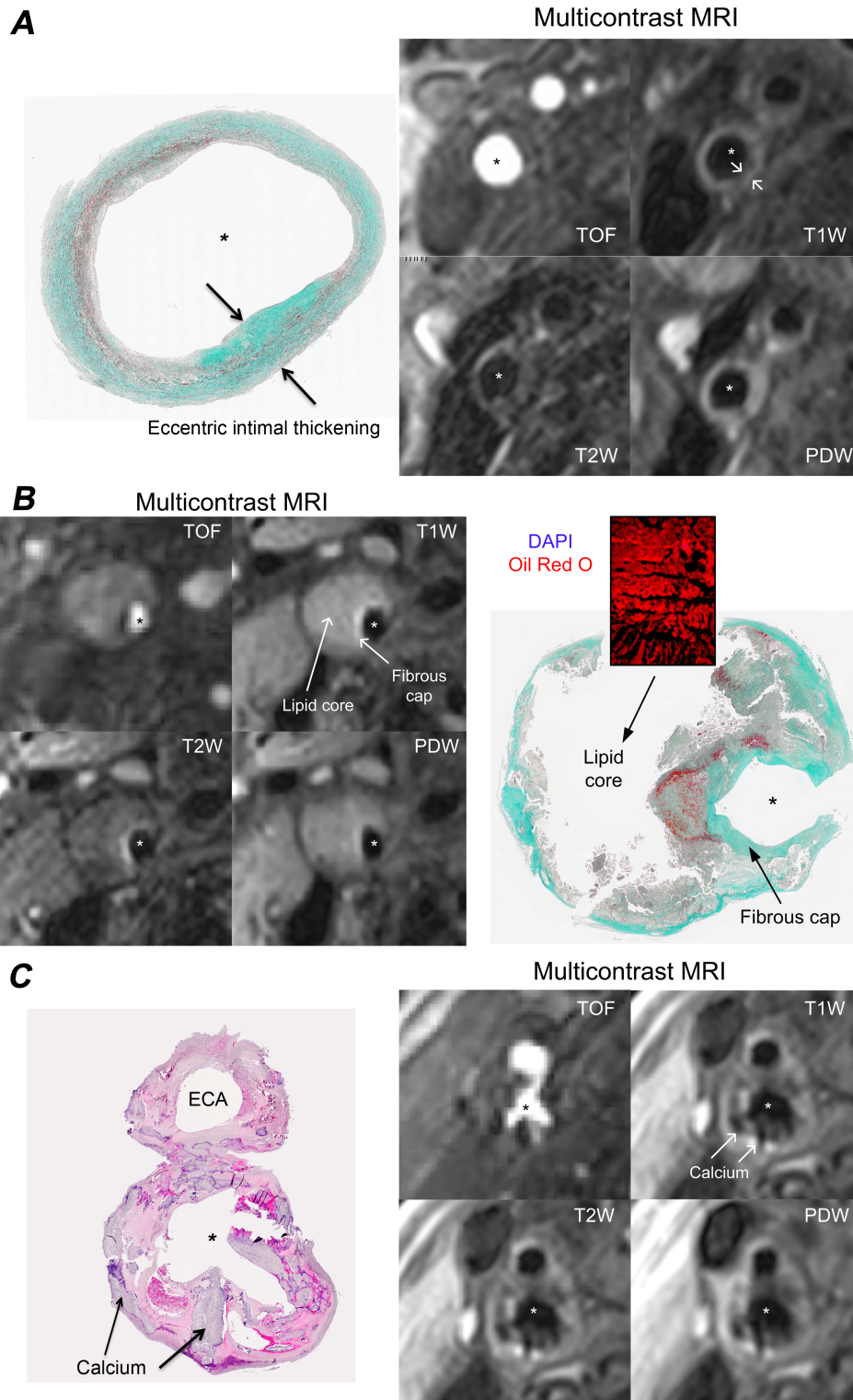


Figure 1.6 Multicontrast MRI and corresponding histology

From top to bottom: (A) an AHA type I-II plaque showing eccentric intimal / vessel wall thickening; (B) an AHA type IV-V plaque showing the presence of large lipid-rich necrotic core; and (C) an AHA type VII plaque showing extensive calcification. Adopted from Chai JT, Biasioli L, Li L, Jezard P, Handa A, Choudhury RP. Clinical vascular magnetic resonance imaging at high field (Chapter 1) *Vascular and Endovascular Consensus Update*, BIBA publishing, 2014

3T MRI scanners are now widely available. Compared to 1.5T MRI, most common in routine clinical use, 3T offers a theoretical doubling of signal-to-noise ratio (SNR), which can be used to shorten scan time, obtain higher spatial resolution, or to extend coverage.⁹⁸ Higher SNR confers particular advantage in atherosclerosis or vascular wall imaging due to the small size of the imaging target and the need for high in-plane resolution to interrogate plaque composition; and to allow adequate suppression of flowing blood signal in the lumen. Several groups have confirmed improvement in SNR close to that of the theoretical limit for 3T over 1.5T,⁹⁹⁻¹⁰¹ and that the well-validated criteria for identification of vulnerable or unstable plaque at 1.5T are generally applicable at 3T as well.^{102,103} With new imaging opportunities, however, high field MRI also brings with it technical challenges. One such challenge is increased field inhomogeneity for high field MR systems compared to that at lower field strengths.⁹⁸ This is especially problematic in complex anatomical regions like the neck, which may require adaptation of imaging sequences to compensate for these inhomogeneities. Another important consideration is specific absorption rate (SAR). Radiofrequency (RF) energy deposition is frequency dependent, and so will result in more energy deposition at higher field strengths for a given MRI protocol,⁹⁸ potentially limiting the overall scan time efficiency.

1.3.3 MRI in drug development

Given the high resolution of MRI and its ability to distinguish different soft tissue components within small imaging targets, MRI is uniquely placed to evaluate changes not just in plaque volume and burden, but changes in plaque composition over time. Corti *et al.* were amongst the first to utilise serial MRI to demonstrate reduction of carotid and aortic atherosclerosis after statin treatment over 12 months.¹⁰⁴ In a later investigation by the same group, no additional benefit on plaque regression was seen with aggressive high dose (80mg) simvastatin compared to

conventional dose (20mg) simvastatin, despite the high dose group achieving a significantly lower LDL-c.¹⁰⁵ Lee *et al.* demonstrated with 1.5T MRI that the athero-protective effect of statin treatment is rapid by showing an early regression in carotid and aortic atherosclerosis, as well as improved physiological parameters such as improved aortic distensibility, within 3 months of statin initiation.¹⁰⁶ The same group later also investigated the effects of high-dose nicotinic acid on atherosclerosis regression in the Oxford Niaspan Study with MRI and showed that in statin-treated patients with low HDL-cholesterol, high-dose (2g daily) nicotinic acid significantly reduced carotid atherosclerosis in 12 months.⁶⁴

To test the lipid-depletion hypothesis in atherosclerosis regression, Zhao *et al.* conducted a prospective MRI study on carotid plaque composition over a time-course of three years.¹⁰⁷ In this study, patients with measurable lipid-rich necrotic core (LRNC) in their baseline multi-contrast MRI were started on a combination of high-intensity lipid-lowering therapies and annual serial MR imaging was performed for three years. The time-course showed that %LRNC decreased by about 3% per year in the first two years and decreased from a total of 14.2% to 7.4% after three years of treatment, supporting the hypothesis that plaque lipid depletion is associated with the athero-regressive effects of lipid-lowering therapy. Utilising the higher resolution offered by 3T MRI, Migrino *et al.* recently showed that 6 months of statin treatment resulted in significant reduction of carotid plaque volume, not detectable by conventional 2D ultrasound carotid intima-media thickness (CIMT).¹⁰⁸ Significantly, it also showed a parallel reduction of LRNC, which is in keeping with the lipid-depletion hypothesis in atherosclerosis regression previously described,¹⁰⁹ and the reduction of LRNC reported with rosuvastatin.¹¹⁰

Vessel wall MRI, either on its own or as part of a multimodal imaging approach, has been used in a number of non-statin drug trials. Ayaori *et al.* used MRI to show that

bezafibrate induced plaque regression in thoracic and abdominal aorta and the vessel wall area correlated with the overall triglyceride decrease and HDL-c increase.¹¹¹ In the multi-modal dal-PLAQUE imaging study,¹¹² MRI was combined with ¹⁸FDG-PET/CT, which measure glucose uptake in plaques as a surrogate marker for plaque inflammation.^{113,114} Over 24 months of treatment with the cholesteryl ester transfer protein inhibitor dalcetrapib, MRI-derived total vessel area, as well as plaque inflammation measured by PET/CT was reduced in the treatment group compared to placebo. Although the development of dalcetrapib was halted after the dal-OUTCOMES phase III trial,³⁶ in a sub-study of dal-PLAQUE, dynamic contrast-enhanced (DCE-) MRI, which measured neovascularisation, was correlated with ¹⁸FDG-PET uptake signal.¹¹⁵ A significant, but weak inverse relationship between inflammation measured as ¹⁸FDG uptake by PET and plaque perfusion by DCE-MRI was found, which would be consistent with recent observations that plaque hypoxia, which causes increase in macrophage glycolysis (and enhanced ¹⁸FDG uptake), may precede plaque neovascularization.^{67,116}

Cellular inflammation, especially related to macrophages, is a key target in atherosclerosis imaging.¹¹⁷⁻¹¹⁹ MRI techniques utilising ultrasmall superparamagnetic iron oxide (USPIO, 15 – 25 nm) can reveal plaque macrophage infiltration thus allowing measurement of plaque inflammation (macrophages phagocytose and accumulate USPIO that had entered atherosclerotic plaques through perturbed / dysfunctional endothelium).¹²⁰ In the ATHEROMA study,¹²¹ Tang *et al.* showed that aggressive lipid-lowering therapy with 80mg atorvastatin over 3 months is associated with significant reduction of USPIO-defined plaque inflammation. USPIO-enhanced or other molecular MRI techniques may be a useful imaging biomarker for the screening and assessment of response to emerging therapeutic interventions that directly target plaque biology such as inflammation, which may not have discernible effect on plaque volume or burden over conventional trial duration.

MRI has also been used in drug trials involving non lipid-modifying therapy, such as that of thiazolidinedione rosiglitazone,¹²² and most recently, of bisphosphonate.¹²³ Bisphosphonate is the drug of choice in the treatment of osteoporosis. It inhibits osteoclast activity and prevents bone resorption. It has been observed that etidronate reduces vascular calcification and atherosclerosis¹²⁴⁻¹²⁶. Kawahara *et al.* recently reported significant reduction of thoracic and abdominal aortic atherosclerosis on MRI using a combination of atorvastatin and etidronate, which was superior to either drug used alone.¹²³

1.3.4 Quantitative T₂ mapping and lipid quantification

Although multicontrast MRI has been used to demonstrate lipid depletion after statin treatment in carotid plaques,^{107,108,110} its limited, semi-quantitative nature and the need of extensive post-acquisition processing to co-localize and align images of different contrast weightings acquired in multiple sequences greatly limited its clinical application in quantitative plaque characterisation such as lipid quantification. Recently, Biasioli *et al.* described an alternative method by using quantitative T₂ mapping to determine the physical composition of the tissue contained within each given voxel, hence allowing differentiation of different tissue components in carotid plaques.¹²⁷ Unlike conventional multicontrast MRI, this technique does not rely on the presence of a coalesced pool of lipid to be quantifiable because it interrogates the actual T₂ time constant of each plaque component in a *voxel-by-voxel* basis (see Chapter 2 - Method); as such it could theoretically provide superior sensitivity and reproducibility over conventional multicontrast MRI. T₂ map segmentation was shown to demonstrate good agreement with conventional multicontrast MRI in plaque classification according to AHA criteria.¹²⁷ However, whether this promising technique can be validated at a histological level and whether T₂ mapping has any

useful clinical application was unknown and this thesis would attempt to validate this technique using histology and seek to evaluate its clinical usefulness.

1.3.5. Dissecting plaque macrophage heterogeneity with laser capture microdissection

Given the importance of inflammation and macrophage infiltration in atherosclerosis, understanding macrophage functional heterogeneity is a key priority in plaque characterisation. However, one key obstacle to plaque macrophage profiling is the challenge to separate and isolate different subsets of macrophage within human atherosclerotic plaque for transcriptomic profiling. One approach is to procure cells based on an ever-expanding range of macrophage markers; but as detailed in Section 1.2.2, very few of these markers are specific and exclusive for any individual subset so procuring cells with this approach is arguably arbitrary. Another approach, knowing macrophage's plasticity driven by local environmental cues, is to procure cells based on their location in relation to the local micro-environment or anatomy. This approach bypasses the crude distinction based on non-exclusive cell markers and potentially better reflects the bilateral influence exerted between macrophage phenotypes and its local micro-environment. To tackle this challenge, however, requires adaptation and refinement of a special technique – laser-capture microdissection.

Laser capture microdissection (LCM) was first developed almost 2 decades ago at the National Institute of Health in the US as an innovative tool for molecular profiling of specific cell populations from tissue sections.^{128,129} It allows procurement, or enrichment, of specific cell types based on cell morphology, immuno-phenotype, or micro-anatomical location.¹³⁰ LCM is considered at present the only technology

capable of isolating subpopulations of cells from heterogeneous tissue sections without perturbing the “molecular state” of the cells. This is because the median half-life of mammalian mRNA has been shown to be only 9 hours,¹³¹ and alternative techniques of cell isolation such as magnetic-assisted cell sorting (MACS) or fluorescence-activated cell sorting (FACS), although useful in sorting cells in suspension (e.g. circulating blood cells), would require pre-treatment with enzymatic digestion for disaggregation of solid tissue for up to 6 hours, which could profoundly alter gene and protein expression of cells from their *in-situ* states.¹³²

Historically, LCM was mainly deployed in cancer research with a wide ranging of malignancies studied ranging from colorectal cancer¹³³, lung cancer,¹³⁴ breast cancer,¹³⁵ endometrial cancer,¹³⁶ hepatocellular cancer,¹³⁷ renal cell cancer,¹³⁸ gastric cancer,¹³⁹ parathyroid cancer,¹⁴⁰ skin cancer,¹⁴¹ and even more recently vaccine development in cervical cancer.¹⁴² A recent study by Callegaria *et al.* used LCM, directly coupled with quantitative RT-PCR, to demonstrate that a single human papillomavirus (HPV) genotype is associated with each biologically separate cervical in-situ neoplasia (CIN) 3 lesion obtained at biopsy, supporting the theory that only one virus type causes each independent CIN lesion; and in aid of vaccine development confirmed that HPV 16 is the most common culprit genotype representing over half of all cases.¹⁴²

Another active area of research taking advantage of the power of LCM is neurology and neuropsychiatry. Specific anatomical regions of the brain has been laser microdissected and studied in Parkinson's disease,¹⁴³ Alzheimer's disease,¹⁴⁴ cerebellar ataxia,¹⁴⁵ multisystem atrophy,¹⁴⁶ multiple sclerosis,^{147,148} motor neuron disease,¹⁴⁹ depression,¹⁵⁰ schizophrenia,¹⁵¹ or even cerebral changes associated with ageing.¹⁵² However, LCM-enabled research in atherosclerotic disease is distinctly lacking. This is in part due to the fact that while solid tumour against

background normal stroma, and various brain structures, can be readily differentiated visually by simple nuclear stain, or even without staining, in laser capture microscopy, differentiation of different cell types involved in the process of atherosclerosis (e.g. macrophages, lymphocytes, smooth muscle cells, endothelial cells, etc.) requires the challenging adaptation of standard LCM techniques with immunohistochemistry to facilitate “immuno-LCM”.

The Fisher laboratory in New York University was one of the pioneers in the use of immuno-LCM to study lesional macrophages in mice.¹⁵³ In mice, immuno-LCM coupled with downstream gene or protein expression analysis has since been used to study various stages of atherogenesis including atherosclerosis regression,^{57,77} and the influence of diabetes on lesional macrophage lipid handling.¹⁵⁴ In humans, however, the often challenging sample quality from biopsy / surgical / post-mortem specimen resulted in extremely limited success of immuno-LCM.

1.3.6 Transcriptome profiling - gene expression microarray

The power of LCM lies on its wide range of compatible downstream applications. Since Francis Crick published his reductionism-based linear transcription-translation-phenotype model,¹⁵⁵ for nearly 60 years, his ‘central dogma’ of molecular biology has been the standard ‘unshakable’ foundation in biology and life sciences. In recent years, however, with the discovery of non-coding regulatory RNA (e.g. microRNA),¹⁵⁶ post-translation modification of protein,¹⁵⁷ hereditary epigenetic control of gene expression,^{158,159} and especially following the ENCODE project,^{160,161} this linear view of cellular machinery from genes to proteins to phenotypes is somehow challenged.¹⁶² In spite of this, analysis of the transcriptome remains one attractive strategy to catch a glimpse on at least one facet of cellular function and its regulation.

Gene expression profiling with microarray allows high throughput simultaneous analysis of tens of thousands of genes through the use of modern silicon chips¹⁶³ or beads array technology;¹⁶⁴ and over the past decade this technology has allowed investigators to study the molecular heterogeneity in different disease states in aid of disease stratification and prognostication, for example, in renal cell cancer,¹⁶⁵ prostate cancer,¹⁶⁶ inflammatory bowel disease,¹⁶⁷ hypertension,¹⁶⁸ HIV infection,¹⁶⁹ and cardiomyopathy.¹⁷⁰ It has also been used to monitor response to long-term treatment following renal transplants,^{171,172} liver transplants,¹⁷³ and of cardiac transplants.¹⁷⁴ There are currently at least three commercially available gene expression profile-based prognostication kits available that is designed to support clinicians and their patients in clinical decision making in early breast cancer. These include Oncotype DX[®], MammaPrint[®], and Prosigna[™] assays, and some of these genomic assays have already been incorporated in major international and national management guidelines.¹⁷⁵

One key technological development that enabled wider application of microarray transcriptome profiling is the robust linear amplification of small quantity of starting RNA from often very small and precious clinical samples.¹⁷⁶ Two broad linear RNA amplification strategies are currently commercially available on the market, one is based on *in-vitro* transcription (IVT) first described by van Gelder;¹⁷⁷ recently, another RNA amplification system based on the linear isothermal amplification of double-stranded cDNA that encompasses a unique RNA/ DNA heteroduplex at one end using the RNA-dependent DNA polymerase activity has been developed by Nugen Technologies Inc.¹⁷⁸ Independent comparative evaluation study has confirmed that both strategies achieved comparable linear RNA amplification while the Nugen system is more tolerant of partially degraded RNA.¹⁷⁹ The Microarray Quality Control project has since shown robust inter-platform and inter-laboratory results between

microarray platforms in terms of differentially expressed genes validated by quantitative RT-PCR.¹⁸⁰

Transcriptome profiling has seen an important emerging role in discovering novel target for drug development. For example, DNA hypomethylation is one of the major epigenetic alterations in human cancers; and novel hypomethylation targets in pancreatic cancer had been identified using global gene expression profiling.¹⁸¹ Likewise, the dynamics of interferon- β treatment response in patients with multiple sclerosis with varying degree of treatment resistance due to development of anti-IFN β neutralizing antibodies had recently been evaluated using transcriptome profiling in a pharmacodynamics study.¹⁸² The potential toxic side-effects of a drug and its mechanism of toxicity can also be studied using transcriptomics to guide the transition from preclinical studies to clinical trials.¹⁸³⁻¹⁸⁵

An emerging approach in vaccine development has also seen increased utilization of gene expression profiling. Heterogeneity of vaccine response has long perplexed immunologists. For example, 10% to 15% of adults fail to respond to 3 doses of hepatitis B virus (HBV) vaccine.¹⁸⁶ Transcriptomic studies not only facilitate correlation of the genome and transcriptome to immunological measures of vaccine responsiveness,¹⁸⁷ but importantly might allow *prediction* of the immunogenicity or efficacy of vaccines through “system vaccinology” approach.¹⁸⁸ This approach has been applied in the development of vaccines against influenza,¹⁸⁹ malaria,¹⁹⁰ tuberculosis,¹⁹¹ and even potential future development of an HIV vaccine.¹⁹²

1.4 Summary

In summary, with the rise of global burden of atherosclerotic disease and the formidable challenge to combat 'residual risks' in the post-statin era, we urgently need to remodel our current ethos in drug development. Atherosclerosis imaging such as by MRI does not only serve as 'gate keepers' in the contemporary hierarchical drug development process; but in combination with the modern "omics", would allow precise disease stratification by defining clinical cohorts for targeted treatment with resolution never achieved before. This *integrated* characterisation of atherosclerosis will generate novel insights about the biology of progression – and possibly regression – of human atherosclerosis, and would bridge the gap between molecular insights and clinical outcome; and help achieve the goal of stratified medicine. In this thesis, a novel *platform* of human atherosclerotic plaque characterisation will be examined in detail - highlighting the complementary roles of a novel vessel wall MR imaging technique (T_2 map) and plaque macrophage transcriptome analysis using laser capture microdissection (LCM-omics). The functional consequence of this heterogeneity will also be examined in the context of drug development.

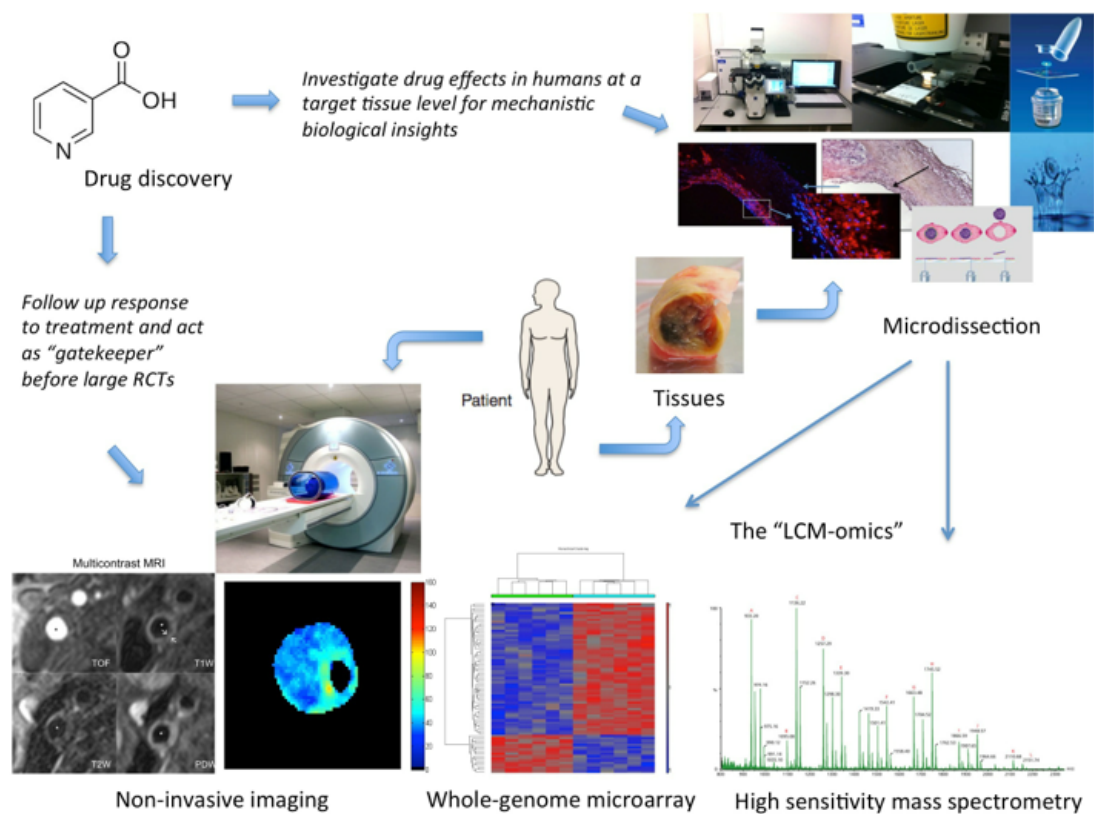


Figure 1.7 Flow diagram to illustrate the complementary role of imaging and LCM-omics in drug discovery

Anti-atherosclerotic drug response can be evaluated at the target tissue level by non-invasive MR imaging e.g. by sing T₂ mapping and by examination of the plaque macrophage transcriptome / proteome / metabolome from explanted plaque tissue using LCM-omics

1.5 Hypothesis

Plaque compositional heterogeneity such as lipid content can be identified with T2 mapping, and is associated with specific gene expression profiles or “signatures” in human atherosclerotic lesional macrophages explanted from the same plaques. In addition, there may be spatially-distinct variation in gene expression according to the micro-anatomical location of the macrophage within the plaque, in relation to other biologically important components.

1.6 Experimental aims

Chapter 3 – To develop and histologically validate MRI T₂ mapping as a tool to study plaque compositional heterogeneity and to evaluate its clinical application in plaque lipid quantification.

Chapter 4 – To develop and apply immuno-LCM (immunohistochemistry-guided laser-capture microdissection) as a tool to study functional heterogeneity of plaque macrophages using gene expression microarray.

Chapter 5 – To evaluate the functional consequence of macrophage heterogeneity in the context of response to drug therapy by using nicotinic acid as an example.

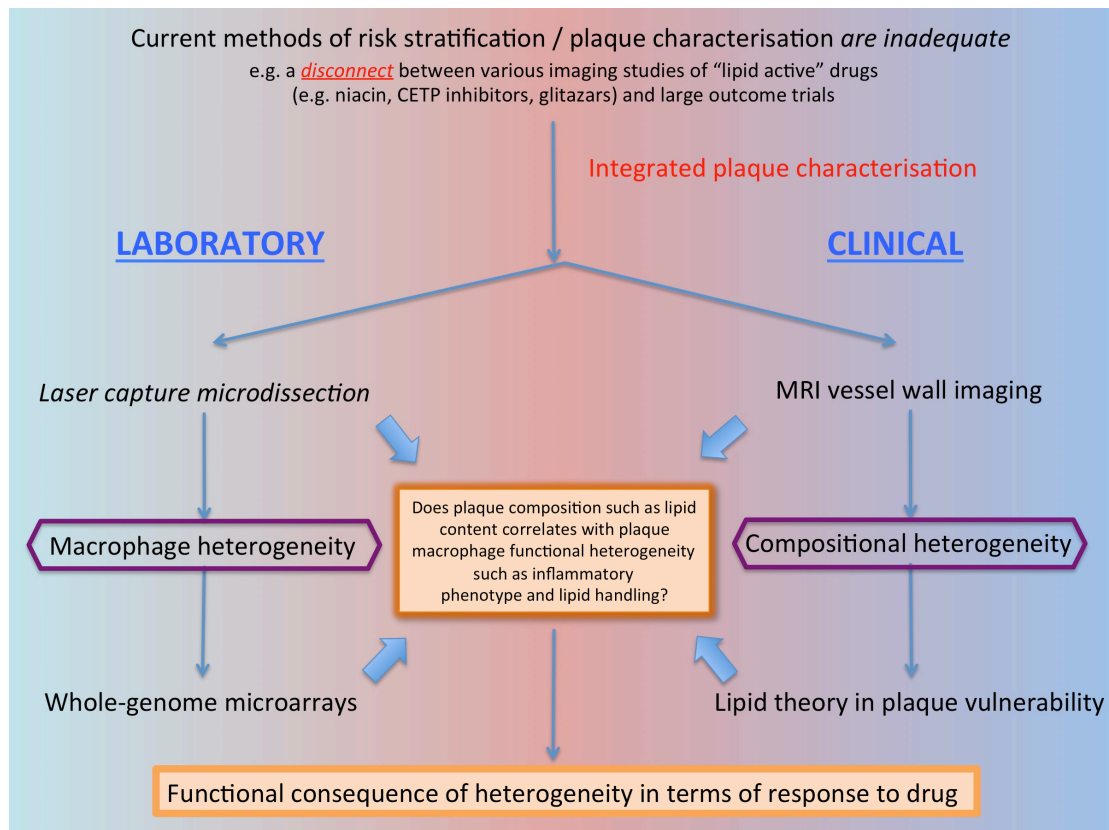


Figure 1.8 *Experimental aims*

Diagram illustrating how the different experimental aims complement one another to test the central hypothesis. The different chapters in this thesis will investigate plaque compositional heterogeneity and macrophage cell heterogeneity using clinical imaging and laboratory techniques, respectively – in the hope to understand the functional consequence of such heterogeneities in terms of response to pharmacological therapy.

CHAPTER 2

GENERAL METHODS

2.1 Cell treatment and assay techniques

2.1.1 Cell culture and treatment

THP-1 cells were purchased from American Type Culture Collection (ATCC, Teddington, UK) and maintained in RPMI 1640 medium (Sigma-Aldrich, Poole, UK) until treatment, at a density between $4\text{--}8 \times 10^5$ cells/mL, supplemented with fetal bovine serum (10%, Invitrogen, Paisley, UK) and 2-mercaptoethanol (0.05 mM, Sigma-Aldrich, Poole, UK), in a humidified atmosphere of 95% air / 5% CO₂ at 37°C. Cells were treated with nicotinic acid (NA) (Sigma-Aldrich, Poole, UK) at 1×10^{-3} to 1×10^{-6} M or PPAR γ agonist GW1929 (2×10^{-6} M, Tocris Bioscience, Bristol, UK) versus vehicle-only controls.

2.1.2 Cholesterol loading and efflux assay

THP-1 monocytic cells were seeded in 96-well or 6-well plates at 1×10^6 cells/mL density and allowed to differentiate for 48 hours into basal macrophages, in the presence of 50 ng/mL phorbol myristate acetate (PMA). For cholesterol loading and foam cell induction, basal macrophages were washed once with warm PBS and serum-starved for 12 hours, followed by incubation with acetylated (Ac-) LDL (50 mg/mL, Biomedical Technologies, Inc, USA) for 48 hours (Figure 2.1 shows cell treatment regime in a diagram). Foam cell formation was confirmed by Oil-red-O (Sigma-Aldrich, Poole, UK) staining in sample wells (Figure 2.2). Cholesterol efflux was assessed using HDL3 (50 mg/mL, Meridian Life Science, USA) and apolipoprotein (Apo) AI (10 mg/mL, Sigma-Aldrich, Poole, UK) as cholesterol acceptors and a non-radioactive enzymatic method (Amplex Red Cholesterol Assay kit, Invitrogen, Paisley, UK) modified from Robinet, *et al.*¹⁹³ In brief, after the indicated treatment period, cell culture supernatants were collected and stored for later analysis; while foam cells were washed in cold PBS twice and cellular cholesterol

extracted by incubation with hexane:isopropanol (3:2) solvent mixture with gentle agitation for 30 minutes. This solvent mixture was then transferred to 1.5 mL Eppendorf microtubes for suction evaporation with the dried cholesterol residue reconstituted in an enzyme-compatible solvent system isopropanol:Nonidat P40 (9:1, Roche, Burgess Hill, UK), supplemented with 20 U/mL of bovine catalase (Sigma-Aldrich, Poole, UK) to quench intrinsic peroxide activity. Extracted cellular and supernatant cholesterol content was then determined using Amplex Red Cholesterol Assay kit (Invitrogen, Paisley, UK) as per manufacturer's instruction.

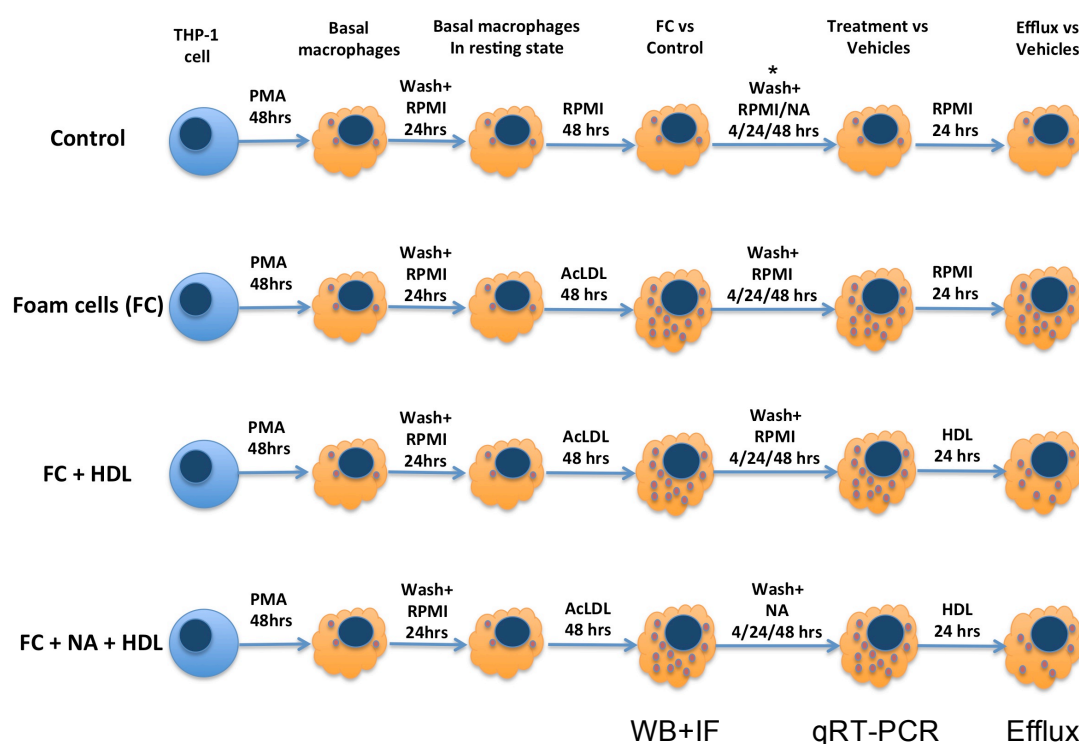


Figure 2.1 Diagrammatic representation of cell treatment in in-vitro cholesterol loading and efflux experiments

All treatment groups had identical exposure of PMA and identical culture duration. Cell treatments were identical in qRT-PCR, cholesterol efflux, Western blot (WB), and immunofluorescence (IF) staining experiments; in qRT-PCR experiments, treatment with nicotinic acid (NA) and PPAR γ agonist GW1929 were applied at the end (at asterisk *) in line with foam cell (FC) group to ensure robust comparison. Reproduced from: [Chai JT, et al. PLoS One. 2013;8\(5\):e62934.](#)

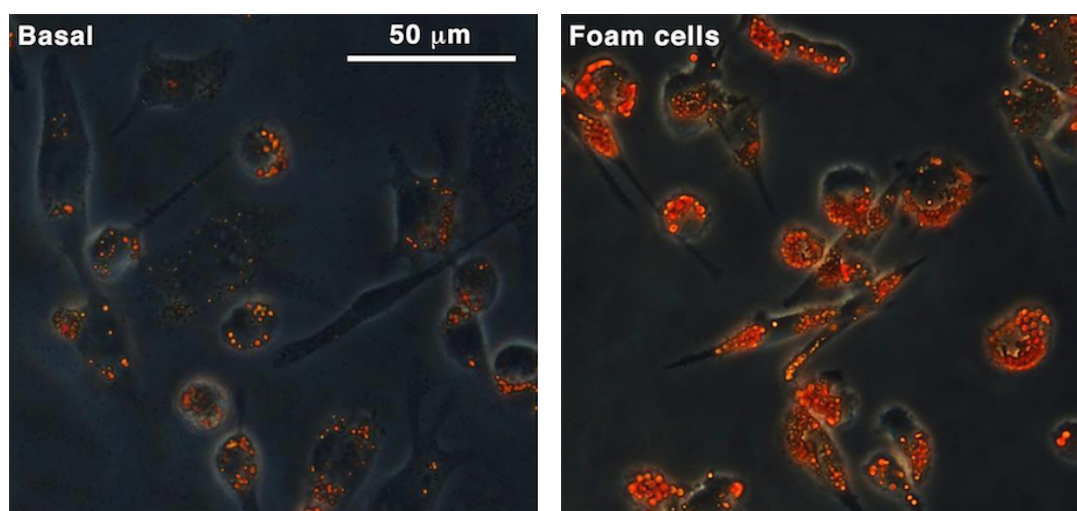


Figure 2.2 *Oil-red-O staining to confirm lipid loading and foam cell formation*

Phase-contrast microscopy of THP-1 cell stained with Oil-red-O (lipid droplets appear bright orange). Successful lipid loading with Ac-LDL and foam cell formation was confirmed in sample wells. Reproduced from: [Chai JT, et al. PLoS One. 2013;8\(5\):e62934.](#)

2.1.3 PPAR transcription factor binding assay

After treatment, THP-1 basal macrophages and foam cells were washed and total nuclear protein lysate was prepared using Nuclear Extraction Kit (Millipore, Watford, UK). Nuclear protein contents were quantified by BCA assay (Thermo Scientific, Hemel Hempstead, UK) (see section 2.3) and 3 μg of total nuclear protein per sample was used to evaluate PPAR transcription factor binding activity using PPARα, δ, γ Complete Transcription Factor Assay Kit (Cayman Chemical, Michigan, USA). In brief, a specific double-stranded DNA (dsDNA) sequence containing the peroxisome proliferator response element (PPRE) was immobilized onto the wells of a 96-well ELISA plate, PPARs contained in the nuclear extract bind to the PPRE and PPARα, PPARδ, and PPARγ were selectively detected using specific primary antibodies. A secondary antibody conjugated to HRP was added to provide a sensitive colorimetric readout at 450 nm as per manufacturer's instruction.

2.1.4 Cyclic-AMP assay

To determine the optimal treatment time period, basal macrophages were first incubated with NA at 1×10^{-4} M with duration ranging from 0 to 24 hours. Cellular cAMP concentration was determined using the Parameter™ Cyclic AMP ELISA kit (R & D Systems, Abingdon, UK) as per manufacturer's instruction. To evaluate Gi/o-protein involvement, cells were pre-incubated with Gi/o inhibitor pertussis toxin (100 ng/mL, Calbiochem, Merck, Nottingham, UK) versus vehicle for 18 hours, prior to treatment with NA, for the optimal time period determined previously.

2.2 RNA techniques

2.2.1 RNA extraction from cells in culture

After cell treatment, cells were washed with cold PBS, and lysed with 350 μ L Buffer RLT (with added β -mercaptoethanol) from Qiagen® (Crawley, UK) RNEasy mini kit on ice using cell scrappers. Total RNA was prepared using Qiagen® RNEasy mini spin columns as per manufacturer's instruction.

2.2.2 RNA extraction from *ex-vivo* human tissue

After cells were laser-captured on Zeiss PALM AdhesiveCap (for Laser capture microscopy (LCM) – see section 2.5), 350 μ L Buffer RLT containing β -mercaptoethanol from Qiagen® (Crawley, UK) RNEasy micro kit was added to the laser-captured elements in the AdhesiveCap. The AdhesiveCap tubes were then closed and incubated in an “upside-down” position for 30 minutes at room temperature. The lysates were then centrifuged at 12,000 rpm for 5 minutes. Samples were then stored at -80°C for batch RNA extraction and purification. Total RNA was prepared using Qiagen® RNEasy micro spin columns as per

manufacturer's instruction with on-column DNase I digestion to remove genomic DNA contamination.

2.2.3 DNase treatment to remove genomic DNA

LCM samples were treated by DNase I on-column incubation to remove genomic DNA (gDNA) contamination. 10 μ L of DNase I stock solution was mixed with 70 μ L Buffer RDD (Qiagen®) and mixed by pipetting. DNase I is especially sensitive to physical denaturation so vortexing was avoided. The working DNase I solution was then added to RNEasy micro spin-columns during the purification process for 15 minutes at room temperature.

2.2.4 RNA quantification and quality control by NanoDrop spectrophotometer

1 μ L of the eluted RNA samples were assessed by NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, USA) for RNA concentration and purity (Figure 2.3). In addition to concentration, each sample measurement also contained the 260/280 ratio as well as 260/230 ratio. Samples with 260/280 > 2.0 and 260/230 > 1.8 were considered good quality RNA samples suitable for downstream applications. The detection limit of nucleic acid on the NanoDrop 1000 Spectrophotometer is 2 ng/ μ L, and the quality control ratios described above become unreliable below 10 ng/ μ L according to the manufacture's manual. Therefore for RNA samples extracted from LCM samples (often has quantity in the picogram range), RNA concentrations and qualities were assessed using the Agilent RNA 6000 Pico LabChip® on the Agilent Bioanalyzer 2100 (Figure 2.4).

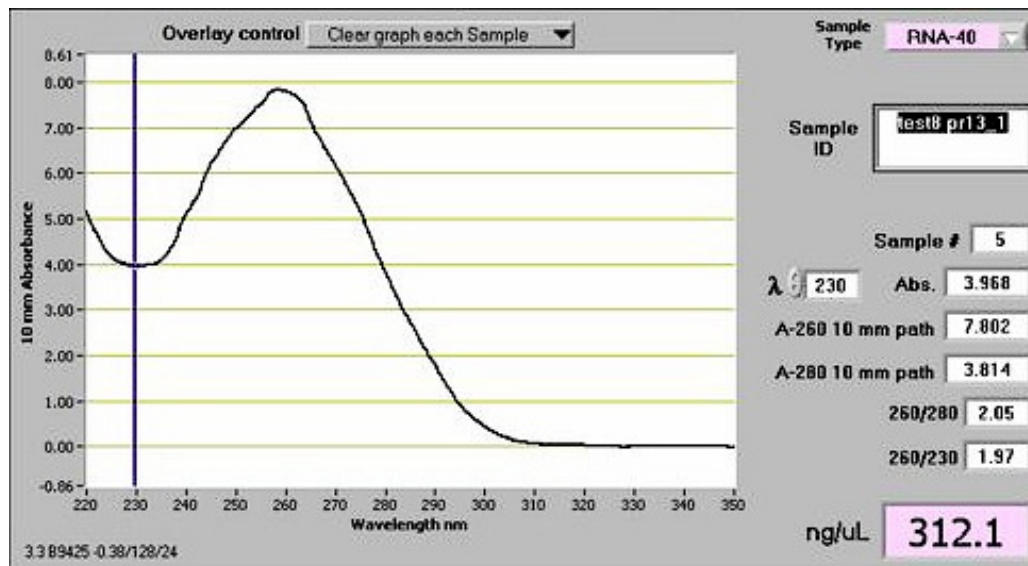


Figure 2.3 *NanoDrop spectrophotometer*

Typical tracing of RNA NanoDrop analysis from RNA purified from cells in culture. The 260/280 ratio of 2.05 (> 2.0) and 260/230 ratio of 1.97 (> 1.8) indicated good quality RNA for downstream application.

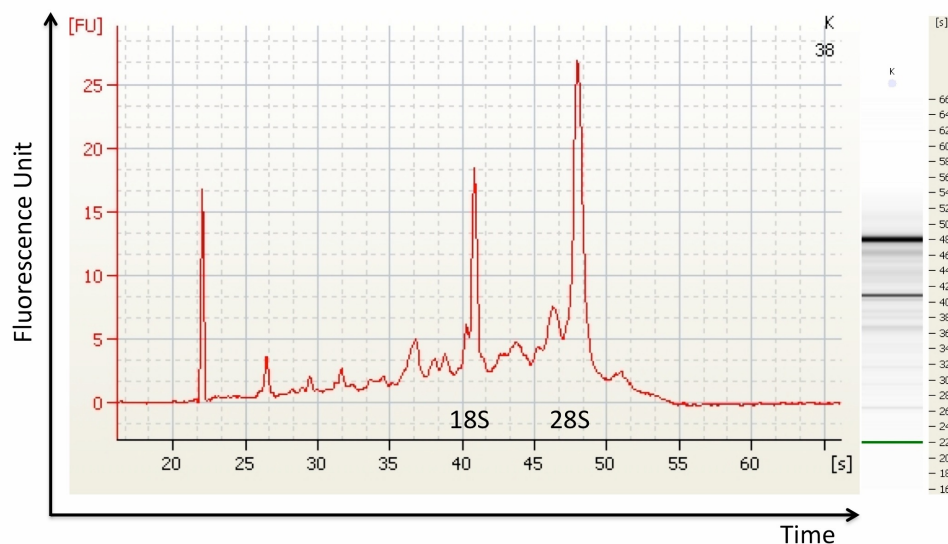


Figure 2.4 *Pico 6000 LabChip® analysed on Agilent Bioanalyzer 2100*

An example of bioanalyzer tracing from RNA purified from laser capture microdissection samples. This was from RNA extracted from a post-LCM remnant section yet still showed an excellent calculated RIN of 7.3. The 18S and 28S peaks were distinct and prominent from the baseline.

2.2.5 RNA quantification and quality control by Agilent Bioanalyzer 2100

Agilent LabChip® and Bioanalyzer® technologies are validated tools for RNA quantification and quality control which utilize micro-fluidic and on-chip gel electrophoresis. The RNA 6000 Pico LabChip® has a total RNA qualitative range of 50-5000 pg/μL. The Pico LabChip® was first prepared according to manufacture's instruction, 1 μL of samples were first heat-denatured at 70°C for 2 minutes and then cooled on ice before loaded onto the LabChips® and run on the Agilent Bioanalyzer 2100 within 5 minutes. Agilent Bioanalyzer does not only report RNA quality based on the conventional 18S and 28S ribosomal peaks, it calculates the RNA integrity number (RIN) using the whole electrophoretic trace by proprietary algorithm. RIN is reported in a scale of 1 – 10, with pure intact RNA being 10 and completely degraded RNA 1. Samples with RIN of 6 - 7 or above (good to excellent) were considered to be of high quality for RNA amplification, biotinylation, (see section 2.2.9) and gene expression microarray analysis (see section 2.2.10).

2.2.6 Reverse transcription (cDNA generation)

1 μg of purified RNA was reverse transcribed using the Qiagen® QuantiTect® Reverse Transcription Kit with oligo dTs and random hexamers as primers with the following protocol: 1 μg of template RNA and 2 μL of genomic DNA (gDNA) wipeout buffer was made up to 14 μL with nuclease-free H₂O. Samples were then incubated on a heat block at 42°C for 2 minutes for DNase to remove genomic DNA contamination. To each reaction, a master mix consisting of (per reaction) 1 μL of primer (oligo dTs), 1 μL of reverse transcriptase, and 4 μL of reaction buffer (containing random hexamers) were added according to manufacturer's instruction. After centrifugation at 2500 rpm for 30 seconds, the reaction plates / tubes were returned to the heating block at 42°C for 40 minutes followed by 90°C for 2 minutes

to complete cDNA synthesis. cDNA was stored at -80°C until ready for further downstream applications.

2.2.7 PCR primer design

Primers for genes of interests identified were either purchased as Taqman™ Gene Expression Assay primers directly from Applied Biosystems (Warrington, UK), or custom-designed for the SYBG Green dye chemistry. Exon and intron sequences were identified using the online Ensembl Genome Browser (<http://www.ensembl.org/>) and primer sequences were designed using Primer Express® version 3.0 from Applied Biosystems (Warrington, UK). Online sequence alignment search tool Primer-BLAST from the US National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) was used to avoid non-specific primer targets, which may result in false positive qRT-PCR results. Specific primer sequences were then sent to Applied Biosystems (Warrington, UK) to be custom-made. Where possible, the following guidelines were adhered to when designing the primers:-

- Primer length: 18-30 nucleotides
- Primer melting temperature: 52°C – 63°C, maximum difference = 3°C
- Avoid runs of >3 Gs or Cs at the 3' end
- Avoid a T at the 3' end and a G at the 5' end
- Avoid >3 Gs in a row and >5 of any one nucleotide in a row
- Avoid >4 di-nucleotide repeats
- Avoid complementarity within a primer sequence and between primer pairs
- Target GC content: 40-60%
- To avoid amplification of target from contaminating gDNA, where possible, primers were designed to span exon-exon junctions (intron splice sites) in the target mRNA.

To further ensure primer target specificity and PCR reaction efficiencies, all primers custom-designed for the SYBR Green system underwent a melt-curve analysis (Figure 2.5) ; as well as agarose gel electrophoresis to check amplification product.

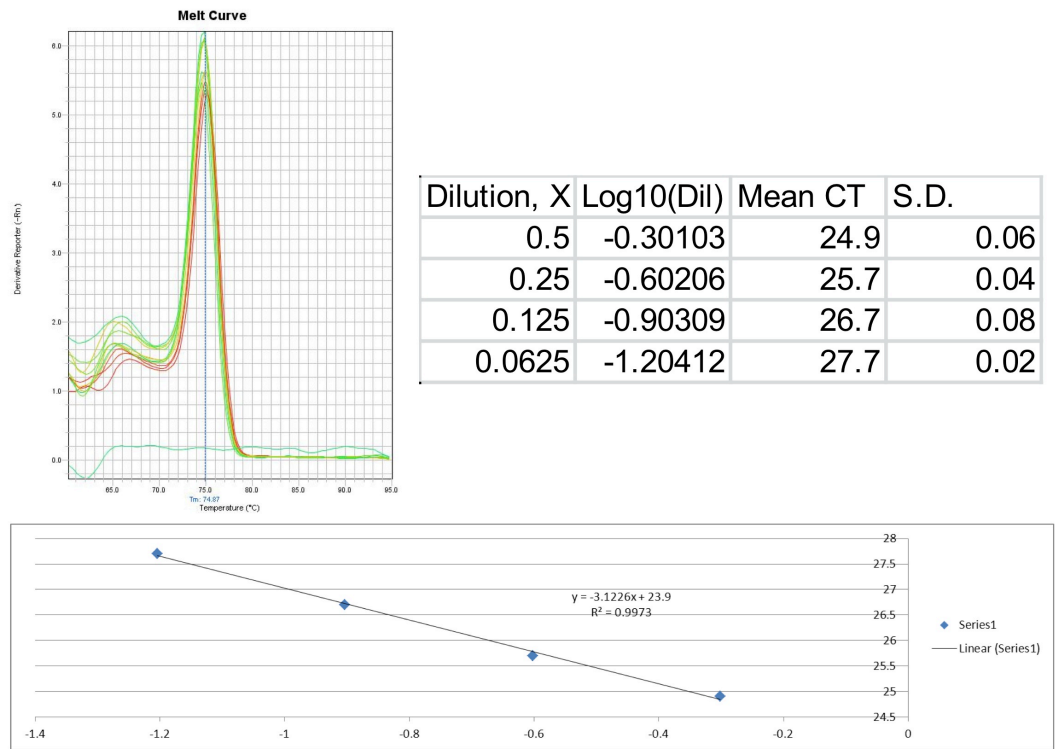


Figure 2.5 *Melt curves for custom primers*

An example of melt-curve analysis – these were the melt curves from qRT-PCR reactions using primers against PPAR γ and the SYBR Green system. The melt curves were represented mathematically as the negative first derivatives. Serial dilutions of starting template cDNA confirmed a linear relationship between cycle threshold (CT) and log (dilutional factor).

A list of primers used is detailed below:-

Table 2.1

Target	Forward (5' to 3')	Reverse (3' to 5')
Cyclophilin	GGCCGATGACGAGCC	TGTCTTTGGAACCTTTGTCTGCAA
PPARγ	GATTTCACTATGGAGTTCATGCTTGT	CATCTGTCATAGATAAGCTTCAATCTGA
NR1H3 (LXRα)	AAGCCCTGCATGCCTACGT	GCATCCGTGGGAACATCAG
ABCA1	GACAAATAAAATCAAGGATGGGTACTG	AGACGTACCGCATGTCCTCAA
ABCG1	TGTCGGCACATCTGAAGCTT	TGTCAGTATCTCCTTGACCATTTCC
GPR109A	GCGTTGGGACTGGAAGTTTG	GCGTTTCATAGCCAACATGA

Table 2.2

Target	Reference sequence (Assay ID)
GAPDH	Hs02758991_g1
TNFα	Hs00174128_m1
MCP-1	Hs00234140_m1
CD36	Hs00169627_m1
CD68	Hs02836816_g1
ACTA2 (α-Actin)	Hs00426835_g1
HMOX1	Hs01110250_m1
CD163	Hs00174705_m1
RGS5	Hs01555176_m1
ADAMTS8	Hs00199836_m1
FABP4	Hs01086177_m1
LEP	Hs00174877_m1
TIMP3	Hs00165949_m1
MMP1	Hs00899658_m1

Table 2.1 and 2.2 Primers sequences and Taqman™ Assay ID

Table 2.1 shows the sequences of custom-designed primers used with the SYBR Green dye system; Table 2.2 shows the Assay ID of target gene used in the Taqman™ Gene Expression Assay.

2.2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

Real-time PCR was carried out with 1 μL of cDNA in a 10 μL reaction mix using custom primers with SYBR Green PCR master mix, or with TaqmanTM Gene Expression assays (Applied Biosystems, Warrington, UK). SYBR Green I is a fluorescent dye that absorbs blue light ($\lambda_{\text{max}} = 497\text{nm}$), it becomes strongly fluorescent and emits green light ($\lambda_{\text{max}} = 520\text{nm}$) when bound to double-stranded DNA. It allows real-time quantification of PCR amplification product (amplicon) by the intensity of the fluorescence signal given out at each PCR cycle without the need of sequence specific probe.

TaqmanTM Gene Expression assays utilize a different dye reporter chemistry; each TaqmanTM primer set also contains an amplicon sequence specific fluorogenic probe which in turn contains a reporter dye and a quencher within itself held in close proximity. The quencher normally prevents the emission of fluorescence from the reporter dye. During each progressive PCR cycle, DNA polymerase cleaves the fluorogenic probe that hybridizes to the specific amplicon between the reporter dye and the quencher and releases the reporter dye. The increase in fluorescence from the reporter occurs only if the target sequence is complementary to the probe and is amplified during PCR, and is directly proportional to the amount of reporter cleaved.

For qRT-PCR using SYBR Green system, the following reagents were added to each well of the 96-well PCR plate:-

- 1 μL cDNA
- 0.25 μL (100mM) forward primer
- 0.25 μL (100mM) reverse primer
- 3.5 μL nuclease-free water
- 5 μL SYBR Green master mix

This made up a total volume of 10 μ L for each reaction. Technical duplicates were performed for each sample to ensure accurate pipetting. Each qRT-PCR run also includes “no template” negative control (using 1 μ L nuclease-free water instead of 1 μ L cDNA template) to exclude reagent contamination. qRT-PCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Warrington, UK) with cycling parameters set as follow:

Initial stage

- 95°C for 10 minutes (activation of AmpliTaq Gold® DNA polymerase)

Followed by 40 cycles of PCR

- 95°C for 15 seconds (denaturing)
- 60°C for 1 minute (annealing / extension)

Melt curve stage (see section 2.2.7)

- 95°C for 15 secs then stepwise increase of temperature from 50°C to 99°C

For qRT-PCR using Taqman™ Gene Expression Assay and FAM™ dye, the following reagents were added to each well of the 96-well PCR plate:-

- 1 μ L cDNA
- 0.5 μ L (100mM) Taqman™ Gene Expression Assay primers (+ reporter)
- 3.5 μ L nuclease-free water
- 5 μ L Taqman™ X2 Universal Master Mix

This made up to a total volume of 10 μ L for each reaction. Technical duplicates were performed for each sample to ensure accurate pipetting. Each qRT-PCR run also includes “no template” negative control (using 1 μ L nuclease-free water instead of 1

μL cDNA template) to exclude reagent contamination. qRT-PCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Warrington, UK) with cycling parameters described above, without the melt curve stage.

In real-time PCR experiments, the reactions are characterized by the point in time during cycling when amplification of a PCR product achieves a fixed level of fluorescence, rather than the final quantity of PCR product accumulated after a fixed number of cycles. An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed. In the initial cycles of PCR, there is no significant change in fluorescence signal. This level of fluorescence signal in the predefined initial PCR cycles is called the baseline. First, the software generates a baseline-subtracted amplification plot by calculating a mathematical trend of the normalized fluorescent reporter signal (R_n values corresponding to the baseline cycles). Then, an algorithm searches for the point on the linear phase of the amplification plot at which the baseline-corrected normalized fluorescent reporter signal crosses a set threshold. The cycle at which this happens is defined as the cycle threshold C_T . Quantitation was performed by comparative C_T ($\Delta\Delta C_T$) method,¹⁹⁴ normalized to the housekeeping genes (HKG) cyclophilin or GAPDH. $\Delta\Delta C_T$ is defined by the following formula:-

$$\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{control})$$

where $\Delta C_T(\text{sample}) = C_T(\text{sample}) - C_T(\text{HKG})$;

and $\Delta C_T(\text{control}) = C_T(\text{control}) - C_T(\text{HKG})$;

Since amplification product doubles with each cycle of PCR, a drop of C_T value by 1 represent a 2-fold increase in the starting cDNA template. Therefore, using the $\Delta\Delta C_T$ method, relative gene expression can be defined as $2^{-\Delta\Delta C_T}$.

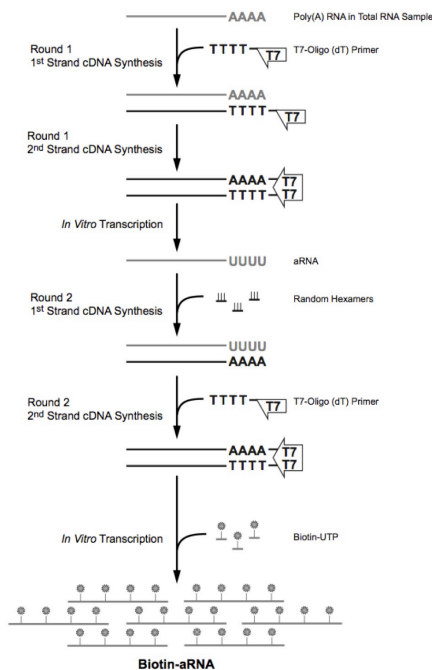
2.2.9 RNA amplification and biotinylation

Linear RNA amplification was carried out as described by Kurn¹⁷⁸ using the Ovation[®] Pico WTA V2 kit (PN3302) from Nugen Technologies Incorporation. Amplification and biotinylation was carried out as part of a commercial genomic core service provided by the Cambridge Genomic Services, Department of Pathology, University of Cambridge, UK. The Ovation Pico WTA System V2 is a whole transcriptome RNA amplification system based on Ribo-SPIA[®] technology, initiating at both the 3' end and randomly throughout the entire transcriptome. (Figure 2.6) Ribo-SPIA[®] technology is a three-step process that generates micrograms of cDNA from nanograms of total RNA. Amplification occurs across the whole transcript, resulting in successful amplification of mRNA species, even including highly degraded samples.

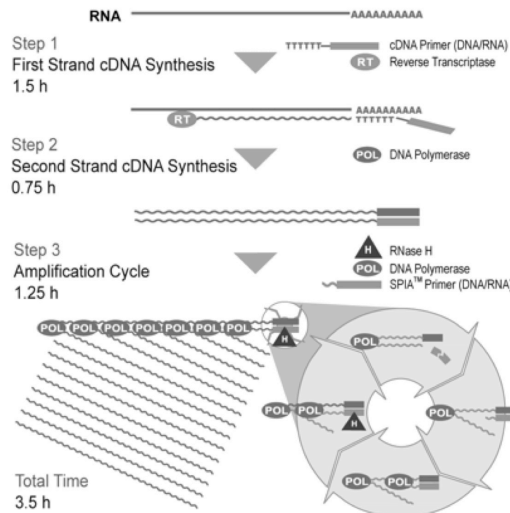
(1) *First Strand Synthesis*: Single stranded cDNA is prepared from total RNA using a unique mix of chimeric DNA/RNA primers and reverse transcriptase. This mix contains both random as well as poly T primers to achieve comparable representation across the entire transcriptome. The primer has a 3' DNA portion that hybridizes to the mRNA and a unique 5' RNA portion that does not hybridize to the mRNA. The resulting cDNA/mRNA complex includes a unique RNA sequence at the 5' end of the cDNA.

(2) *Second Strand Synthesis*: After partial fragmentation of mRNA in the cDNA/mRNA complex, DNA polymerase generates a second strand, including DNA complementary to the 5' RNA unique sequence incorporated into the first strand, resulting in a double-stranded cDNA with an RNA/DNA heteroduplex of unique sequence at one end.

(3) *SPIA Amplification*: The isothermal amplification step uses an additional DNA/RNA chimeric primer, DNA polymerase and RNase H in an isothermal reaction. RNase H removes the unique RNA sequence in the double-stranded cDNA revealing a site for binding the DNA/RNA chimeric primer. DNA polymerase synthesizes cDNA starting at the 3' end of the primer and displacing the existing forward strand. RNA at the 5' end of the newly synthesized strand is again cleaved by RNase H, exposing the priming site for initiation of the next round of DNA synthesis. The entire process is repeated continuously with multiple DNA polymerase molecules participating in DNA synthesis along the same template molecule, leading to the rapid accumulation of micrograms of amplified cDNA products. Biotin labeling of amplified cDNA was carried out using the Encore[®] BiotinIL module (PN4210) from Nugen Technologies Incorporation according to manufacturer's instruction.



Van Gelder RN, et al. *PNAS* 1990
(Eberwine's technique)



Oligo-dT AND random priming for 1st strand synthesis – reduced 3'-bias
No IVT so no intermediate RNA-DNA-RNA step

Kurn, N, et al. *Clinical Chemistry* 2005
(Nugen technique)

Figure 2.6 Comparison between conventional modified Eberwine's technique (left) and the Nugen technique (right) of linear RNA amplification

Conventional linear RNA amplification technique such as modified Eberwine's in-vitro transcription (IVT) technique requires the intermediate RNA → DNA → RNA steps and its initial reverse transcription and first strand synthesis utilizes oligo-dT primer only, hence more susceptible to 3'-bias in partially degraded samples. In contrast, the Nugen technique utilizes both oligo-dT and random priming for first strand synthesis, which reduce 3'-bias; and uses a proprietary SPIA DNA/RNA chimeric primer to directly amplify strands of cDNA.

2.2.10 Microarray

24 human RNA samples extracted from carotid plaque macrophages using laser capture microdissection (n=12 from symptomatic group and n=12 from asymptomatic group) were submitted to Cambridge Genomic Services, Department of Pathology, University of Cambridge, UK, for amplification (see section 2.2.9) and hybridization to two Illumina Human HT12 v4.0 BeadChips (Illumina, San Diego, USA). Samples were randomly allocated to the two BeadChips to minimize “chip effect”. BeadChips were then scanned using Illumina BeadChip Reader on the same day on the same reader. GenomeStudio V2010.1 (Illumina, San Diego, USA) was used for data extraction.

2.3 Protein techniques

2.3.1 Protein extraction from cells in culture

Protein lysis buffer was first made up by dissolving a Complete™ protease inhibitor cocktail tablet (Roche, Burgess Hill, UK) in 10 ml of CelLytic™ mammalian cell lysis reagent (Sigma-Aldrich, Poole, UK). Cells in culture dish were then harvested and lysed with 200 µL of the protein lysis buffer using cell scraper on ice. Protein lysate were stored in -80°C until use.

2.3.2 Protein quantification

Protein concentration was determined using standard bicinchoninic acid (BCA) assay (Thermo Scientific, Hemel Hempstead, UK), according to manufacturer’s instruction.

The BCA protein assay is a two-step technique that utilizes the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction), coupled with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. In the first step, Cu^{2+} is reduced to Cu^{1+} in the presence of sodium hydroxide, copper (II) sulphate, and sodium potassium tartrate. The amount of Cu^{1+} generated is directly proportional to the protein content of the sample. In the second step, bicinchoninic acid (BCA) reacts with the Cu^{1+} to form a BCA/copper complex, which exhibits strong linear absorbance at 562nm that can be detected and quantified by a microplate reader. The protein concentration of the sample can then be calculated from a standard curve using protein (bovine serum albumin) standards ranging from 25 to 2000 $\mu\text{g/ml}$. The working solution was first made up by mixing 50ml Reagent A with 1 ml Reagent B. 25 μL of each sample or standard was pipetted into a microplate with technical duplicates. 200 μL of working solution was then added to each well. This was mixed using an orbital shaker for 30 seconds, followed by incubation at 37°C for 30 minutes. After allowing the microplate to cool to room temperature, the absorbance was read using a microplate reader at 562nm.

2.3.3 Gel electrophoresis

12 μg of total lysate protein was diluted with sample loading buffer made up with 900 μL RIPA buffer (Sigma-Aldrich, Poole, UK), 100 μL NuPAGE® 10x Sample Reducing Agent (Invitrogen, Paisley, UK), and 10 μL of protease inhibitor (Roche, Burgess Hill, UK) to a final sample loading volume of 30 μL . These were heat-denatured at 70°C for 5 minutes and loaded in pre-casted Precis Bis/Tris 4-12% NuPage® gel (Invitrogen, Paisley, UK) for electrophoresis. 6 μL of a full-range pre-stained protein ladder (10 – 250 kDa) was also loaded in the first lane of each gel to determine the molecular weight of the electrophoretic separation product. Gels were run at 200V for 50 minutes using a Bio-Rad Power Pac 3000 (Bio-Rad, Hemel Hempstead,

Hertfordshire, UK), in running buffer made up of NuPAGE® MOPS SDS Running Buffer (Invitrogen, Paisley, UK) with NuPAGE® Antioxidant (Invitrogen, Paisley, UK) added to prevent oxidation of sample and formation of thio/S-S bonds.

2.3.4 Western blot

Protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot® dry transfer system (Invitrogen, Paisley, UK) according to manufacturer's instruction. Membrane was then washed in PBS-Tween for 5 minutes, before blocking with 5% non-fat cow milk in PBS-Tween for 2 hours rocked slowly at room temperature. After 3x PBS-Tween wash, the membrane was probed with rabbit anti-human GPR109A (1:1000, Imgenex, USA) primary antibody in PBS-Tween +5% BSA at 4°C overnight. After three washes, the membrane was incubated with anti-rabbit IgG HRP secondary antibody (1:2000, GE Healthcare, Amersham, UK) at room temperature for 1 hour. Antibody chemiluminescent complexes were formed using ECL advanced detection kit (GE Healthcare, Amersham, UK) and visualized with a UV trans-illuminator (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). To re-probe with a different primary antibody, the membrane was incubated with 1:10 stripping buffer (Invitrogen, Paisley, UK) for 15 minutes in room temperature to "strip off" the bound antibody chemiluminescent complexes. It was then washed with PBS-Tween and re-probed with rabbit anti-human primary antibody against β -tubulin (1:1000, Abcam, Cambridge, UK) using procedures already described above as loading control. Protein band signal intensity was analysed using ImagePro Plus version 6.1 (MediaCybernetics, Silver Spring, MD, USA).

2.4 Histological techniques

The objectives of histological techniques are (i) to provide high-quality histological staining with accurate location information to allow location matching with MRI images (see section 2.6.2); (ii) to preserve high-quality intact RNA / protein for subsequent isolation by laser capture microdissection (LCM, see section 2.5); and (iii) to preserve antigens and to facilitate immunohistochemistry, to be coupled with (ii) for “immuno-LCM”. These conflicting demands require careful treatment of delicate and precious clinical tissue samples (see section 2.5.3).

2.4.1 *Ex-vivo* human atherosclerotic plaque

Human atherosclerotic plaques were freshly collected at the time of carotid endarterectomy (see section 2.6 for clinical study), plaque samples were cooled on ice-cold sterile PBS immediately and washed further with sterile PBS to remove blood. Plaques were divided at the point of maximal stenosis. Half of each plaque was embedded in Optimal Cutting Temperature (OCT) medium (VWR, Lutterworth, Leicestershire, UK) and snap-frozen in crushed dry-ice ethanol slurry. The other half of each plaque was submerged in 4% PBS-buffered paraformaldehyde (VWR, Lutterworth, Leicestershire, UK) and processed with the following protocol:-

- 4% PBS-buffered paraformaldehyde at 4°C for 48 hours
- 10% formic acid at room temperature with daily inspection until decalcification complete (usually 1-3 days)
- Wash in water overnight to remove residual formic acid
- Store in 70% ethanol at 4°C until ready for batch tissue processing

Batch tissue processing was performed using an automated tissue processor and specimens were carefully cut into 5 mm segments in tissue cassettes with particular attention to tissue orientation. Careful drawings were made to facilitate subsequent location matching in relation to the carotid bifurcation as the anatomical landmark. Tissues in cassettes were rotated through the automated tissue processor overnight for graded ethanol dehydration, clearing with HistoClear (National Diagnostics, Yorkshire, UK), and paraffin impregnation. Samples were embedded in paraffin block the following day.

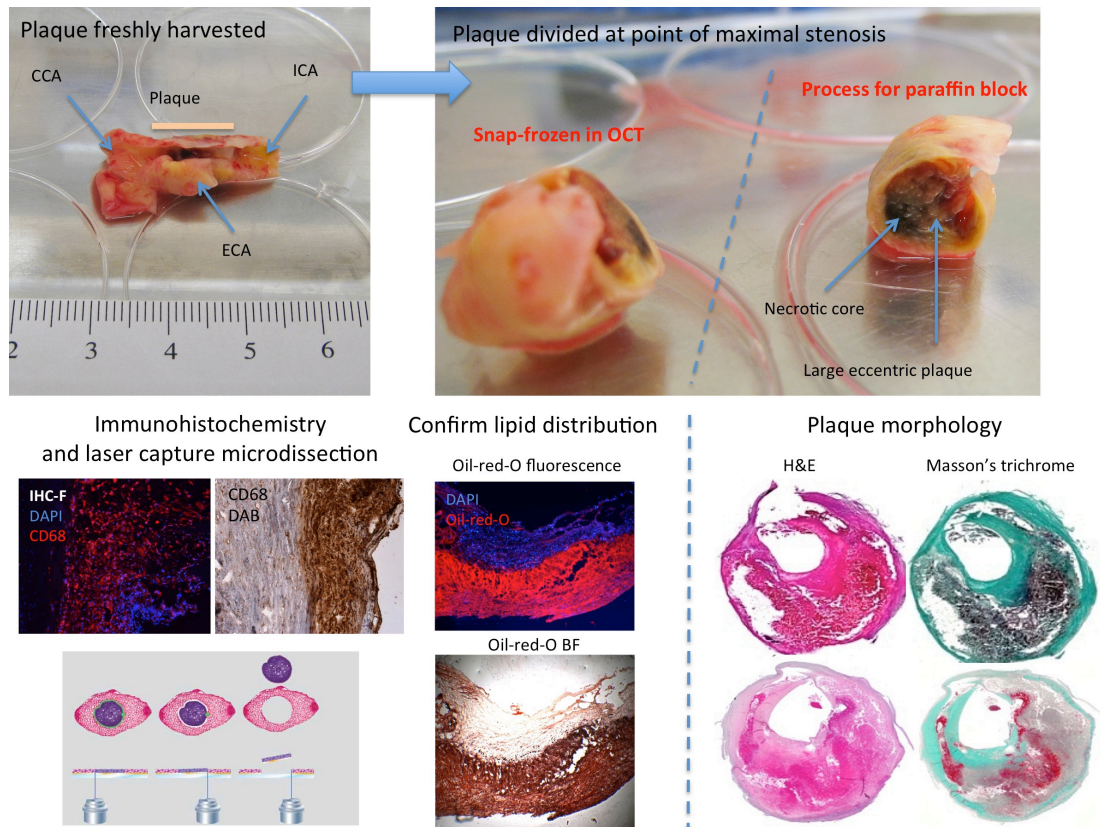


Figure 2.7 *Human carotid atherosclerotic plaque*

Fresh human carotid plaques harvested at endarterectomy and divided into two halves. One half was processed for formalin fixed paraffin embedded (FFPE) block and sectioned for H&E and trichrome stains for plaque morphology; another half was snap-frozen and cryosection obtained for Oil-red-O stains for lipid distribution, immunohistochemistry, and laser capture microdissection. CCA = common carotid artery; ICA = internal carotid artery; ECA = external carotid artery; BF = bright-field; IHC-F = fluorescent immunohistochemistry; DAB = 3,3'-diaminobenzidine; LCM schematic image from Carl Zeiss Microscopy, Germany

2.4.2 Plaque morphology and histological assessment

Formalin fixed, paraffin embedded (FFPE) sections were cut at 5 µm thickness, and frozen sections were cut at 10 µm thickness. Cryosections were post-fixed in 4% paraformaldehyde for 30 minutes in room temperature, washed in PBS before use. Sections were stained with haematoxylin and eosin (H&E), and Masson's trichrome for plaque morphology using the following protocols:-

Haematoxylin and eosin

1. Clear in HistoClear (National Diagnostics, Yorkshire, UK) x2
2. Rehydrate in graded ethanol 100%, 96%, 70%, then tap water
3. Gill 2 (progressive) Haematoxylin (Sigma Aldrich, Poole, UK) for 2.5 minutes
4. Wash in tap water until clear
5. Rinse in 70% ethanol
6. Eosin Y Alcoholic (Sigma-Aldrich, Poole, UK) 1 minute
7. Rinse in tap water, then dehydrate in graded ethanol 70%, 96%, 100%
8. Clear in HistoClear (National Diagnostics, Yorkshire, UK) x2
9. Mount in Neo-mount (Merck-Millipore, Darmstadt, Germany)

Masson's trichrome (Merck-Millipore, Darmstadt, Germany)

1. Step 1 and 2 as per H&E protocol above
2. Rinse in 1% acetic acid briefly
3. Azophloxine solution for 9 minutes
4. Rinse in 1% acetic acid for 30 seconds
5. Tungstophosphoric acid orange G solution for 2 minutes
6. Rinse in 1% acetic acid for 30 seconds

7. Light green SF solution for 2 minutes
8. Rinse in 1% acetic acid for 30 seconds
9. Step 7 to 9 as per H&E protocol above

Masson's trichrome was used for its ability to visualise collagen in the fibrous tissue (green), as well as fibrin and erythrocytes from intra-plaque haemorrhage (bright red), Fibrin also appeared bright pink in H&E. Lipid-rich area without previous evidence of haemorrhage appeared unstained / pale in both H&E and Masson's trichrome stain. Calcium deposits were unstained in Masson's trichrome, but stained blue by haematoxylin in H&E. Stained slides were scanned using an Olympus dotSlide virtual microscopy system or Hamamatsu NanoZoomer digital pathology slide scanner. Plaque types were determined according to the modified AHA plaque classification system.⁹⁰ Lipid Area (%) was calculated by manual segmentation of the lipid-rich necrotic core with the method described by Choudhury *et al.*¹⁹⁵ using ImageJ (public license, National Institute of Health, USA), confirmed by Oil-red-O staining (see section 2.4.5). Since intraplaque haemorrhage was observed exclusively inside or as part of the lipid rich core, the total Lipid Area (%) calculation combined lipid rich core area *with* and *without* intraplaque haemorrhage.

2.4.3 Immunocyto- / immunohistochemistry (ICC/IHC)

Cultured cells or freshly cut, air-dried, cryosections were fixed in ice-cold acetone for 10 minutes, washed with PBS-Tween, and blocked with serum-free protein blocking solution (Dako, Cambridge, UK) for 2 hours in room temperature. They were then incubated with a primary antibody at 4°C overnight. After washing, cells/cryosections were incubated with a secondary antibody at room temperature for 1 hour (see Table 2.3 and 2.4 for primary and secondary antibodies used). After washing, cell nuclei were counterstained with Slowfade anti-fade reagent with DAPI (Invitrogen, Paisley,

UK). Images were then taken using a Leica DM2500 microscope with QImaging MicroPublisher 5.0 RTV image sensor. Images were analysed using ImagePro Plus version 6.1 (MediaCybernetics, Silver Spring, MD, USA).

To ensure antibody binding specificity, appropriate IgG isotype control stainings were performed for monoclonal primary antibodies. For polyclonal primary antibodies, the peptides used to raise the antibodies were purchased from the antibody manufacturer for competitive peptide blocking experiment. This was performed by pre-incubating the polyclonal antibody with its raising peptide in a 1:20 ratio for 1 hour at room temperature before application of this mixture in the primary antibody incubation step described above.

Table 2.3

Antibody	Clone	Specificity	Species	Supplier	Concentration
CD68	KP-1	Mouse	Human	Dako	1:200
α-Actin	1A4	Mouse	Human	Dako	1:100
GPR109A	Polyclonal	Rabbit	Human	Imgenex	1:200
Adipophilin	Polyclonal	Goat	Human	Santa Cruz	1:100
Ki67	Polyclonal	Rabbit	Human	Abcam	1:100

Table 2.4

Antibody	Clone	Specificity	Species	Supplier	Concentration
AF 647	Polyclonal	Donkey	Mouse	Invitrogen	1:1000
AF 594	Polyclonal	Donkey	Rabbit	Invitrogen	1:1000
AF 488	Polyclonal	Donkey	Goat	Invitrogen	1:1000

Table 2.3 and 2.4 *Primary and Secondary antibodies*

Table 2.3 showed the primary antibodies used in this thesis including clone information, species they were raised in, supplier and concentration used; Table 2.4 showed the fluorescent secondary antibodies used throughout the thesis. AF = Alexa Fluor

For bright-field IHC, Ready-to-use Vectastain® Universal Quick ABC kit (Vector Laboratories, Peterborough, UK), and peroxidase substrate ImmPACT™ DAB (3,3'-diaminobenzidine, Vector Laboratories, Peterborough, UK) were used as per manufacturer's instruction. Immunohistochemistry for laser capture microdissection ("immuno-LCM") requires special consideration and modification of the techniques, which will be covered separately in section 2.5.3.

2.4.4 Confocal microscopy

Confocal fluorescence microscopy was performed using a Leica SP-5 laser-scanning confocal microscope fitted with a 63x 1.3NA oil-immersion objective (Zeiss, Cambridge, UK). Images were processed using Leica LAS AF Lite software and analysed using ImagePro Plus version 6.1 (MediaCybernetics, Silver Spring, MD, USA).

2.4.5 Lipid distribution

In lipid co-staining experiments, the immediately adjacent cryosection or cultured cells on coverslips were fixed in 4% paraformaldehyde for 1 hour, and stained with 36% solution of Oil-red-O (ORO) in triethyl phosphate (Sigma-Aldrich, Poole, UK) for 30 minutes. ORO staining was detected either on bright-field or using Texas Red excitation filter (540–580 nm) in epifluorescence.

2.5 Laser capture microdissection (LCM)

2.5.1 General consideration

LCM is compatible with different tissue preparations (e.g. frozen or paraffin tissue blocks, even from un-mounted archival histological slides) and a diverse range of downstream applications (functional genomic, proteomic, or lipidomic analysis). There are two major LCM systems on the market: (1) those using a near infra-red laser (e.g. Arcturus LCM systems from Life Technologies, USA) to melt or “wet” a thermolabile polymer layer that adhere and capture the cells of interest, and (2) those using a UV-cutting laser (e.g. PALM Microbeam LCM system from Carl Zeiss, Germany) which thermo-ablates and cuts out cells of interest, and then “catapult” the cells onto a capture device in a “no-touch” manner. Historically, LCM was developed as a tool for cancer research. Trogan, *et al.* was the first to apply LCM in atherosclerosis research in mice using an Arcturus LCM system.^{153,196} Since then, only a small number of studies had utilized LCM to study human atherosclerosis.¹⁹⁷⁻²⁰¹ The main challenge of LCM is the conflicting requirements on the actual LCM procedures, namely *cell specificity* and *RNA integrity*. While the key advantage of LCM lies of its ability of procure specific cell types, the more specific the procurement is, the longer the procedure time it leads, which in turn adversely affect the quality of RNA obtained, which has direct implication for researchers aiming to use gene expression microarrays for functional genomic analysis. In the following sections, optimization of the LCM equipment and modified immuno-LCM protocol will be discussed.

2.5.2 Equipment optimization

A PALM Microbeam laser micro-dissection system (Carl Zeiss, Germany) was employed for the LCM experiments. The laser cutting and catapult function of the system was first calibrated to the Carl Zeiss PALM MembraneSlide™ (Nuclease-free) PEN microscopic slide (Cat no. 415190-9081-000) used throughout the experiment for the thickness of the glass and membrane. The thickness of LCM tissue sections is another important consideration since a thicker section will contain more cellular material (e.g. RNA and protein) and less LCM cuts are then needed to obtain the same unit amount of biological material; this in theory shortens the procedure time and improves the overall RNA quality. Nonetheless, the thicker the tissue section, the more difficult it is to cut and pressure catapult (LPC) the cut elements onto the capture device, and often multiple attempts of LPC with higher energy (that could potentially be harmful to RNA) are needed for each thicker, heavier, cut elements, which inadvertently increases procedure time. A tissue section thickness of 15 µm was empirically determined to be the optimal trade-off between cutting speed and cellular material obtained. Optimal laser settings were determined empirically: cut = 60, laser pressure catapult (LPC) = 28, focus = 78, speed = 06. LPC was achieved using Carl Zeiss AdhesiveCap™ Opaque (Cat no. 415190-9201-000), which is a nuclease-free microtube with an opaque dry adhesive material coated in the inner surface of the cap, mounted as capture device.

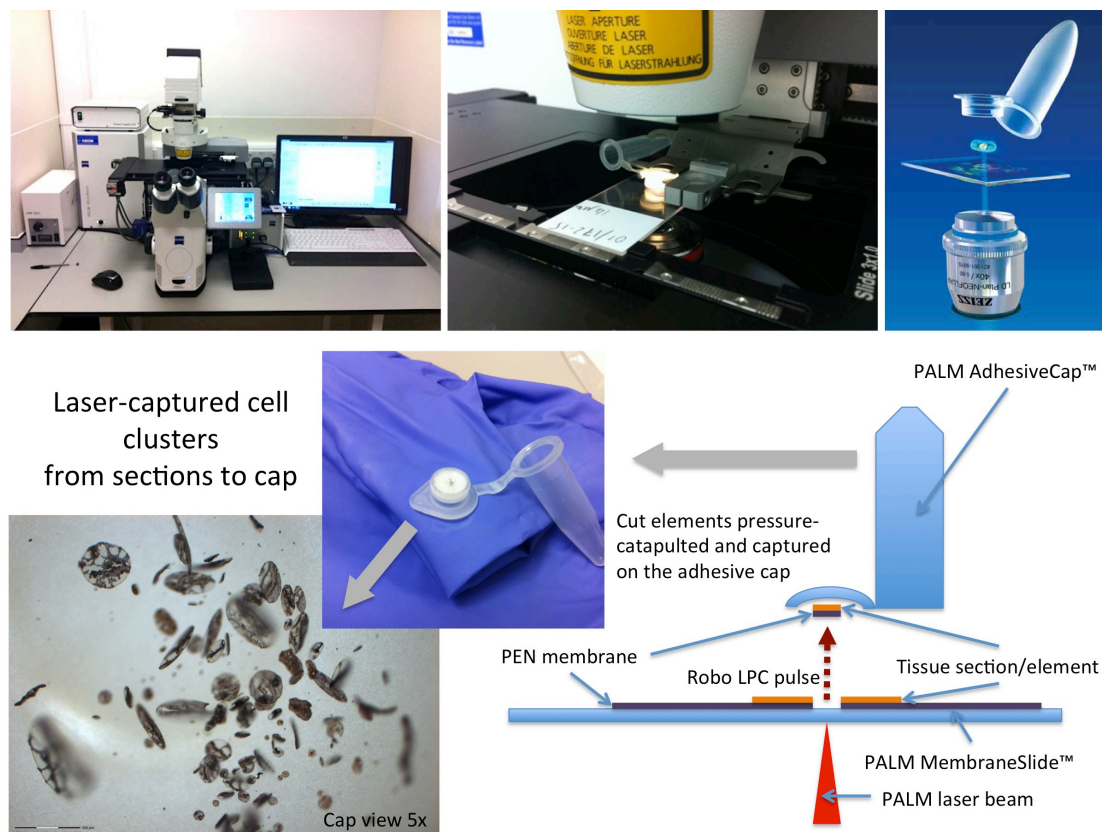


Figure 2.8 *Laser capture microdissection*

Schematic diagram showing the set-up and principles of laser capture microdissection. Top left: Carl Zeiss PALM Microbeam system; top middle: close-up showing how the AdhesiveCap™ is mounted and held over the MembraneSlide™; top right: illustration of the PALM laser beam (© Carl Zeiss GmbH, Germany); bottom right: illustration of the cutting and catapulting mechanisms; bottom middle and left: examination of the cut elements adhered to the cap using Cap View function on the PALM RoboSoftware system.

2.5.3 Optimization for cell capture specificity (Immuno-LCM)

In order to procure plaque macrophages and smooth muscle cells for downstream analysis, an immuno-LCM protocol for capturing CD68-positive and alpha-actin-positive cells was developed. Preliminary experiments demonstrated that direct immunostaining on LCM sections, despite adhering to meticulous decontamination precaution and using RNase inhibitors in all aqueous solutions, severely degraded RNA quality (average RIN < 3.0). This was most likely due to re-activation of intrinsic RNases within macrophages in an aqueous environment during staining. A “guideslide” approach was adapted and modified from Feig, *et al.*⁷⁷ In order to discriminate cells based on immuno-phenotype and micro-anatomical location, one frozen section (15 µm) was stained for Masson trichrome (see section for 2.4.2) for plaque morphology, and 2 immediately adjacent frozen sections (15 µm) were stained with primary antibodies against CD68 and smooth muscle alpha actin in a rapid immuno-staining protocol as below:-

Rapid immunohistochemistry for LCM “guideslide”:-

1. Air-dry cryosections in room temperature for 5 minutes
2. Fix in ice-cold acetone for 5 minutes
3. Re-hydrate slides in PBS-T (PBS-Tween 20, 0.5%)
4. Block with 0.3% hydrogen peroxide for 10 minutes
5. Wash in PBS-T
6. Block with DAKO serum free protein blocking solution 10 minutes
7. Drain off excess solution
8. Incubate with primary antibodies diluted with 2.5% normal horse serum for 10 minutes (Vectorstain ABC Universal Quick Kit – RTU (PK-7800))

- a. Dako mouse anti-human CD68 – 1:150 at room temperature
- b. Dako mouse anti-human alpha-actin – 1:100 at room temperature
9. Wash in PBS-T
10. Incubate with pre-diluted secondary antibody for 5 minutes
11. Wash in PBS-T
12. Incubate with pre-diluted ABC reagent for 5 minutes
13. Wash in PBS-T
14. Apply peroxidase substrate ImmPACT DAB (SK-4105) for up to 2 minutes
15. Counterstain with haematoxylin for 1.5 minutes (see section 2.4.2)
16. Wash, dehydrate in graded ethanol, and mount

Total staining procedure time: **60 minutes**

The trichrome stained and immuno-stained sections were then used as templates, or “guideslides”, to navigate and perform LCM on the next (up to) 15 serial LCM sections (15 µm each). This rapid IHC protocol is essential for the successful application of the “guideslide” LCM approach. This is because the specimen has to remain mounted to the specimen head in the cryostat during the entire duration of staining and subsequent LCM in order to maintain precise position and orientation of each of the subsequent serial sections. This would otherwise be impossible with conventional overnight IHC preparation as the specimen orientation would have changed during specimen mounting / dismounting from cryostat.

2.5.4 LCM slide preparation

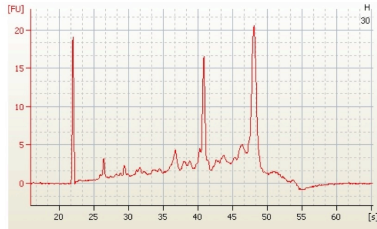
LCM nuclease-free PEN MembraneSlide™ (Carl Zeiss, Germany) was pre-cooled inside the cryostat chamber down to -20°C. Up to three 15 µm cryosections were thaw-mounted on each MembraneSlide™ by warming up small areas on the pre-chilled slide using a gloved finger on the back of slides. Each thaw-mounted section would spontaneously re-freeze within 30 seconds as the slide air-dried within the cryostat chamber. This is to ensure the minimum amount of thaw-time (and minimal degree of RNase reactivation and RNA degradation) before the sections were fixed and dehydrated. LCM slides were kept inside the dry air in the cryostat chamber at -20°C until ready and each slide was *individually* processed following the Zeiss LCM protocol as followed:-

Cresyl violet stain for individual LCM slide:-

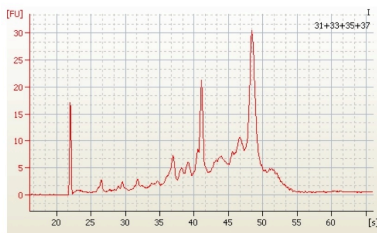
1. Prepare cresyl violet staining solution freshly by dissolving 0.5g of cresyl violet (Sigma-Aldrich, Poole, UK) in 50% ethanol and filter before use. All solutions used should be made with DEPC-treated Milli-Q water.
2. Each LCM PEN MembraneSlide™ is *individually* fixed in ice-cold 70% ethanol for 2 minutes.
3. Stain with ice-cold cresyl violet staining solution (prepared above) for 2 minutes.
4. Brief wash in ice-cold 70% ethanol with a few dips
5. Complete wash and dehydrate in ice-cold 100% ethanol with a few dips
6. Air-dry in a fume hood for 2 minutes or until completely dry

7. Once completely dehydrated, RNA is stable in room temperature for at least a few hours (validation data available from Carl Zeiss Microscopy, Germany)

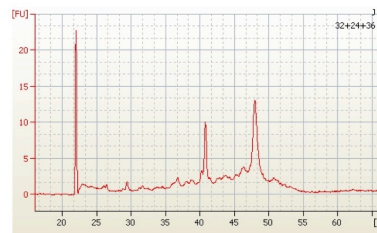
Cresyl violet was chosen as the nuclear stain of choice because it is entirely alcohol-based; so staining with cresyl violet allows minimal exposure to an aqueous environment, which would otherwise reactivate intrinsic RNases within macrophages. In theory, RNA degradation starts the moment an LCM slide is removed from the cryostat chamber and goes through the staining process; therefore, as a quality control measurement the time taken from the beginning to cresyl violet stain to post-LCM cell lysis (stain-to-lysis time) was recorded. The median stain-to-lysis time for each LCM sample was 1 hour (range 30 minutes to 2 hours). Using this protocol there was no significant LCM-associated RNA degradation.



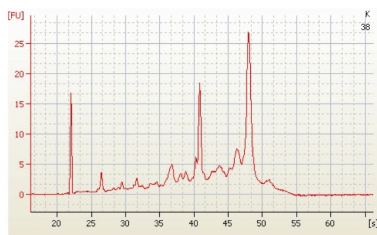
Pre-LCM tissue
(whole section freshly cut from frozen block)
RIN 7.0



LCM – SMCs
RIN 7.0



LCM – Macs
RIN 7.1



Post-LCM tissue
(whole remnant section on PEN membrane lysed)
RIN 7.3

Figure 2.9 RNA quality control before and after LCM

Examples of Agilent Bioanalyzer tracing from the sample plaque tissue – before LCM (upper) obtained from a whole section freshly cut from the frozen block; compared with LCM samples (middle) for plaque macrophages (Macs) and smooth muscle cells (SMCs), and a whole remnant post-LCM section lysed from PEN membrane (lower). There is no significant drop in RIN (around 7.1) between these samples.

2.5.5 Laser-capture microdissection using PALM RoboSoftware

Using the trichrome-stained and immuno-stained guideslides, cell clusters of interest were identified by manual selection on PALM RoboSoftware (version 4.5, Carl Zeiss GmbH, Germany). For each cell type, a total combined area of interest (AOI) between $3 - 5 \times 10^6 \mu\text{m}^2$ was harvested from the subsequent (up to) 15 serial LCM sections. RNA extraction from LCM samples was covered in section 2.2.2. The resulting RNA yield was approximately 5 – 10 ng of total RNA per cell type per plaque.

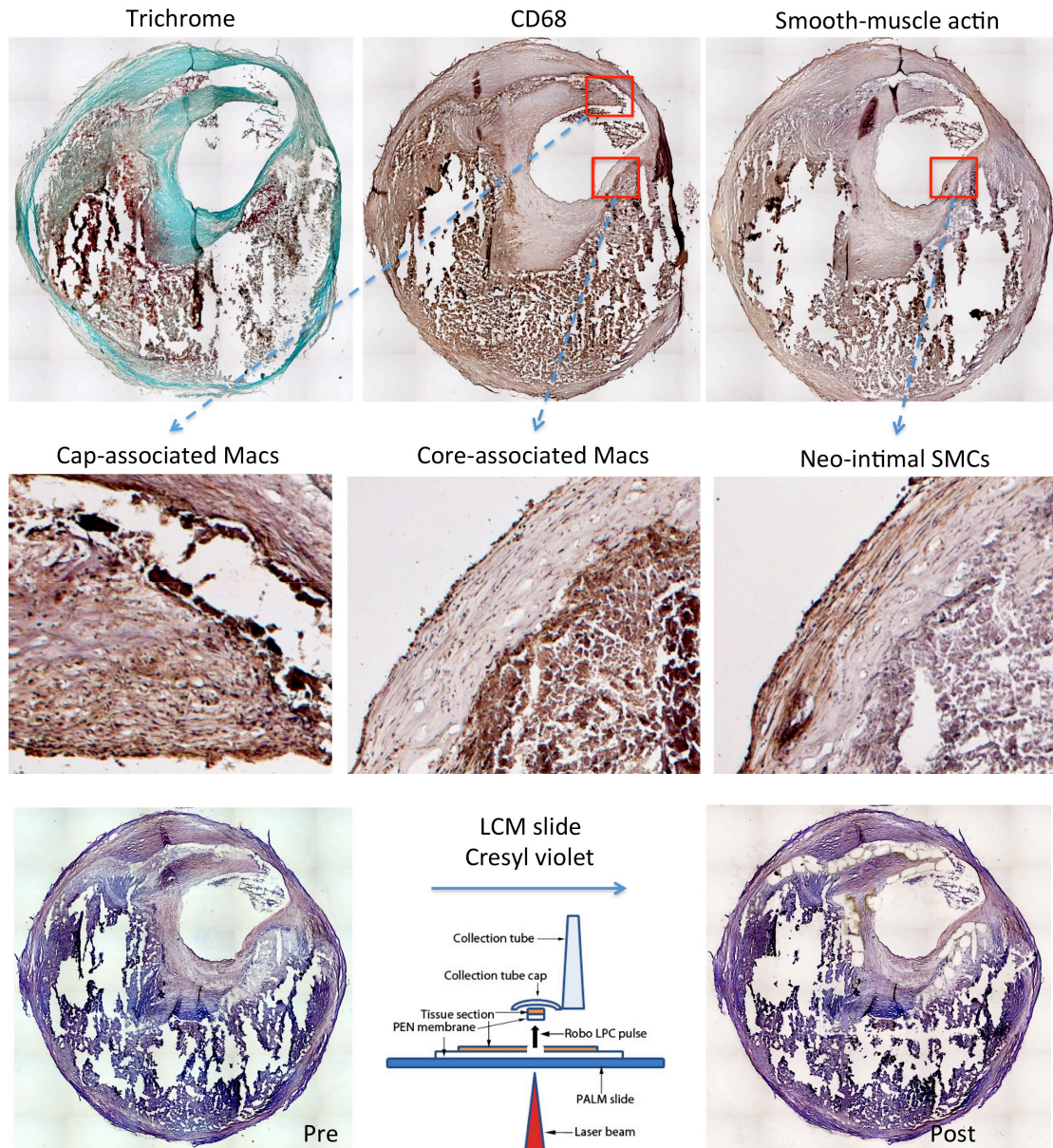


Figure 2.10 *Immuno-LCM using guide-slides*

Guide-slides (top panel) provide anatomical and immuno-phenotypical information to locate plaque macrophages and neo-intimal smooth muscles cells. Closer examination (middle panel) confirmed immunohistochemistry staining conformed with expected cell morphology of the selected cell types. Bottom panel showed cresyl violet stained LCM section on PEN slide (without coverslips – hence lack of colour contrast) before (left) and after (right) LCM. “Cut-out” elements on the post section (after LCM) corresponded to cell clusters captured by LCM.

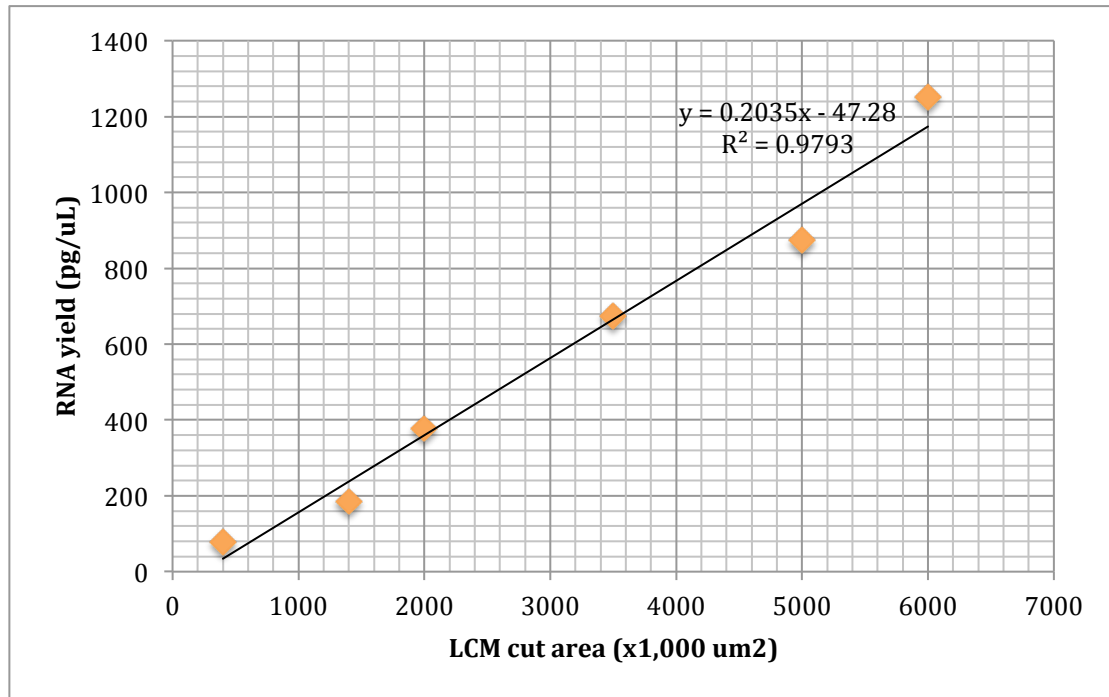


Figure 2.11 *Total RNA yield vs. LCM capture area*

A graph showing the yield of RNA against the total LCM capture area of interest given the section thickness of 15 μm . Since section thickness is thinner than the typical size of a human macrophage, counting the number of stained nuclei is not a reliable method of quantifying cellular material captured by LCM. Instead, the LCM capture area (given the same section thickness) is a more robust and reliable measure of LCM output.

2.6 Clinical study

2.6.1 Human Carotid Study

Ethics approval was obtained from UK National Research Ethics Services (REC reference: 09/H0606/95) and local research committee prior to the beginning of the study and the study ethics conformed to the Declaration of Helsinki. All patients recruited were under the routine clinical care at Oxford University Hospital under Vascular Surgery team. In those patients where we obtained surgical carotid endarterectomy samples, they were collected after routine pre-planned carotid surgery with full informed consent and stored in accordance to the Human Tissue Act (2004). Recruitment criteria are adult patients (> 18 years), with symptomatic or asymptomatic carotid disease (see below), with 50-99% carotid stenosis according to NASCET criteria, or 70-99% according to ESCT criteria. Symptomatic carotid plaques are defined as the culprit plaques deemed to have given rise to either a minor cerebrovascular accident (CVA) or a transient ischaemic attack (TIA) as assessed by the clinical team, supported where appropriate by clinical brain CT or MRI imaging. Asymptomatic carotid plaques are defined as those who had no documented clinical symptoms and degree of stenosis matched to the symptomatic cohorts.

2.6.2 Carotid Wall MRI

Patients were scanned on a Siemens Verio 3T scanner at the Oxford Acute Vascular Imaging Centre (AVIC) before scheduled carotid endarterectomy as part of their routine clinical care. Black-blood cross sectional 2D images of the carotid arteries were acquired with the Machnet 4-channel phased-array carotid coil. Scanning sequence for T2 mapping and plaque segmentation was performed according to the method described by Biasioli *et al.*¹²⁷ In brief, a novel black-blood technique based

on non-selective Delay Alternating with Nutation for Tailored Excitation (DANTE) pulse trains²⁰² was combined with a chemical-shift-selective fat saturated multiecho spin-echo (MESE) sequence to acquire 5 slices with 14 echoes in ~4 min. Acquisition parameters: TR = 2000 ms, TE = 9-127 ms, partial Fourier = 5/8, FOV = 128 × 128, matrix size = 384 × 384, slice thickness = 2 mm, slice gap = 2 mm. DANTE parameters: FA = 8°, number of pulses = 120, interval between pulses = 0.5 ms, Gz = 18 mT/m and gradient duration = 0.4 ms. This sequence was then repeated consecutively with a 2mm off-set; resulting in a total coverage of 10 slices of 2 mm thickness, i.e. 2 cm of the target carotid artery in 8 minutes.

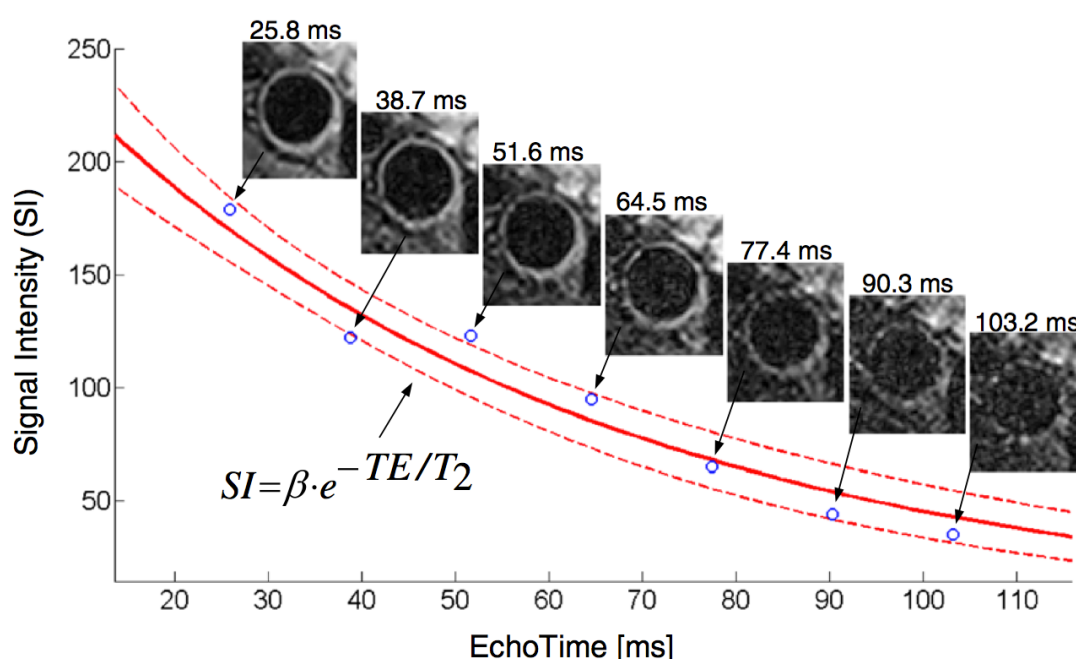


Figure 2.12 T_2 mono-exponential relaxation decay curve of normal carotid artery

Signal intensity (SI) of individual voxel in the Multi-SE image series is fitted to a mono-exponential curve to derive T_2 constant of the individual plaque components. Reproduced from: Biasioli L, Lindsay AC, Chai JT, Choudhury RP, Robson MD. In-vivo quantitative T_2 mapping of carotid arteries in atherosclerotic patients: segmentation and T_2 measurement of plaque components. *J Cardiovasc Magn Reson.* 2013 Aug 16;15:69

2.6.3 MRI T_2 map and segmentation

T_2 maps of the carotid arteries were generated using mono-exponential nonlinear fitting,¹²⁷ and lumen and external vessel boundary were segmented using a semi-automated procedure. In essence, signal intensities and the corresponding echo times were used to derive the T_2 time constant describing the T_2 decay curve in a *voxel-by-voxel* basis. The T_2 time constant is an absolute physical property of the tissue occupying that specific voxel and reflects the proton density characteristic of the target issue independent of scanning platform at a given field strength. Lipid core without haemorrhage has shorter T_2 than normal vessel wall and fibrous tissue, whereas intraplaque haemorrhage infiltrating the lipid core yields longer T_2 than all other plaque components. Since intraplaque haemorrhage was found exclusively inside or as part of the lipid rich necrotic core, the total Lipid Area (%) was calculated by combining lipid rich core area *with* and *without* intraplaque haemorrhage (same criteria applied to histology – see section 2.4.2). The optimal lipid segmentation thresholds were determined empirically by choosing T_2 threshold values that produced the highest correlation and achieved the lowest root-mean-square-error (RMSE) calculated ($T_2 < 42$ ms for lipid area without haemorrhage and $T_2 > 90$ ms for lipid area with haemorrhage) using standard leave-one-out cross-validation model²⁰³ against histology.

2.6.4 MRI and histology location matching and lipid quantification

Location matching was done by 2 independent investigators (JTC and LB). One investigator (LB) blinded to histology determined the location on each plaque for potential matching based on T_2 map and its quality. Plaque types were determined based on the modified AHA classification,⁹⁰ and Lipid Area (%) were calculated using segmentation algorithm (see section 2.6.3). Distances from carotid bifurcation and one unrelated T_1 -weighted guidance image per location at specific locations were

then passed on to a second independent investigator (JTC) to obtain histology, who then determined histological plaque type and calculate histological Lipid Area (%) independently (see section 2.4.2).

2.7 General statistics

Data were first analyzed for normality using Kolmogorov-Smirnov test and D'Agnostino and Pearson omnibus test. Unless otherwise stated, data were analysed with parametric tests with significance set at $P < 0.05$. Data were presented using mean or median depending on their modes of distribution. In Gaussian distribution, means were further quantified by standard error of the mean ($\pm$ SEM) unless stated otherwise. Statistical software packages used for analysis include GraphPad Prism 5 and IBM SPSS Statistics v19.

2.8 Bioinformatics

Illumina BeadChip microarray data was extracted using GenomeStudio V2010.1 (Illumina, San Diego, USA). GenePattern software suite from Broad Institute, Cambridge, MA, USA, was used for most of the analysis described below. Specific methodologies are discussed in Chapter 4.

2.8.1 Raw data handling

Raw output files (.idat files) from Illumina's array scanning software (BeadScan) were processed and converted to non-proprietary format using the "Illumina Expression File Creator" module in GenePattern (Broad Institute, Cambridge, MA, USA). Raw data were normalized to housekeeping controls and were collapsed from probe IDs to gene names for downstream analysis.

2.8.2 Hierarchical clustering

Unsupervised hierarchical clustering was performed using the "HierarchicalClustering" module in GenePattern (Broad Institute, Cambridge, MA, USA). Clustering results were viewed and exported using the "HierarchicalClusteringViewer" module in Java runtime environment (Oracle Corporation, USA).

2.8.3 Principal component analysis

Principal component analysis (PCA) was performed using the "PCA" module in GenePattern (Broad Institute, Cambridge, MA, USA). PCA results were viewed and explored using the "PCAVIEWER" module in Java runtime environment (Oracle Corporation, USA).

2.8.4 Differentially expressed genes and heat maps

Each sample was assigned a group and was analysed using the “ComparativeMarkerSelection” module in GenePattern (Broad Institute, Cambridge, MA, USA). Lists of genes differentially expressed between groups were generated and explored using the “ComparativeMarkerSelectionViewer” module in Java runtime environment (Oracle Corporation, USA). For each differentially expressed gene, the statistical significance of the difference was calculated using two-tailed *t-test* with the resulting P values. To account for multiple hypothesis testing, and to avoid falsely identifying non-marker genes as marker genes, False Discovery Rate (FDR) adjusted P values (Q values) were also computed. Fold change (FC) was calculated as the normalized expression ratio of a gene between two groups. Heat maps of the most differentially expressed genes illustrating expression difference between groups were generated using the “HeatMapView” module in GenePattern (Broad Institute, Cambridge, MA, USA) with the list of most differentially expressed genes generated above. Unless specified otherwise, significant differential expression was defined as $P < 0.05$, $Q < 0.25$, $FC > 1.5$.

2.8.5 Gene set enrichment analysis

To explore and extract biological insights from the gene expression differences, gene set enrichment analysis (GSEA) was performed using standalone GSEA Java application V2.1.0 from Broad Institute, Cambridge, MA, USA. Instead of focusing on a handful of isolated differentially expressed genes, this powerful analytical method focuses on gene sets, which are carefully curated group of genes that share common biological functions, chromosomal locations, or regulations.²⁰⁴ The purpose of GSEA is to evaluate the statistically significant, concordant, similarity or difference of a ranked list of differentially expressed genes between two biological states (e.g. symptomatic *versus* asymptomatic carotid atherosclerosis), and compared it with an

a priori defined gene set of known biological state collated from prior biological knowledge, e.g. from published literature, public databases of biochemical pathways (e.g. the KEGG, BioCarta, and GO databases) or co-expression in previous experiments. The real power of the GSEA method is not only its freely available software package, but its resource of an online database containing 10,295 gene sets known as the Molecular Signature Database (MSigDB, accessible at www.broadinstitute.org/gsea/msigdb/), which are sub-divided into 7 major curated collections (C1 to C7). Therefore, GSEA is an analytical technique, which attempts to interrogate whether a sample gene expression profile, or gene “signature”, matches gene sets with known biological function based on statistical methods. The detailed statistical methodologies of GSEA was described by Subramanian, *et al.*²⁰⁴ but can be summarized in three steps:-

i. Calculation of an enrichment score (ES)

First the list of differentially expressed genes from samples between two biological states were ranked to generate list *L*. Member of an *a priori* defined set of genes *S* are checked against list *L* to see if members from *S* are randomly distributed throughout list *L* (i.e. pathway not enriched) or if members from *S* are primarily found at the top or bottom of list *L* (i.e. pathway enriched and reflect phenotypic distinction associated with the gene set). An enrichment score (ES) is calculated by walking down the list *L*, increasing a running-sum statistic when a gene in *S* is encountered, and decreasing when a gene absent from *S* is encountered. The ES is the maximum deviation from zero encountered in the random walk, which corresponds to a weighted Kolmogorov-Smirnov-like statistic.

ii. Estimation of the significance of the ES

An empirical phenotype-based permutation test procedure is then used to estimate the statistical significance (nominal P value) of the ES, which aims to preserve the complex gene-gene correlation structure of the gene expression data and provides a more biologically reasonable assessment of significance than would be obtained by gene-based permutation alone.

iii. Adjustment for multiple hypothesis testing

The ES for each gene set is normalized to account for the size of the gene set to yield the normalized enrichment score (NES). The estimated significance level is then adjusted for multiple hypothesis testing using the False Discovery Rate (FDR) approach with respect to each NES. The FDR is the estimated probability that a set with a given NES represent a false positive finding. As a discovery and hypothesis generating exploratory tool the cut-off of FDR for GSEA is set to 25% (Q value < 0.25). As a more stringent cut-off might lead to potentially significant results being overlooked. (See www.broadinstitute.org/cancer/software/gsea/wiki/index.php/FAQ)

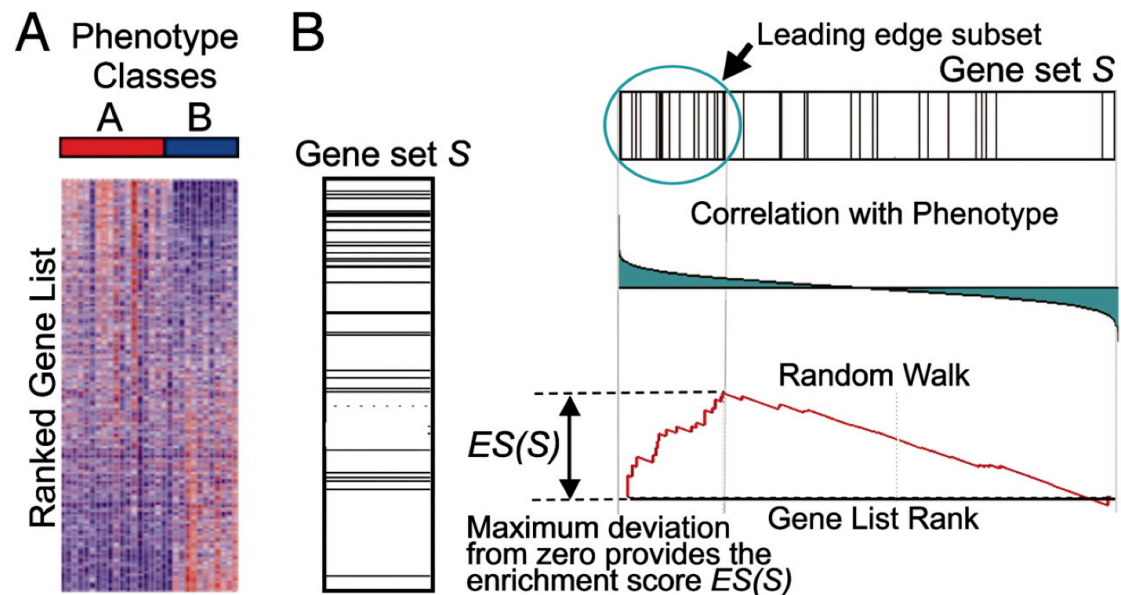


Figure 2.13 Diagram illustrating the overview of GSEA

(A) List of differentiated genes between phenotypic classes are ranked to produce list L ; (B) Enrichment score (ES) is calculated by performing running-sum statistic walking down list L against a predefined gene set S . ES is the maximum deviation from baseline with the leading edge subset defined as the contributing subset of genes before the ES reaches its peak. Reproduced from: Subramanian, *et al. Proc Natl Acad Sci U S A* 2005;102(43):15545-50

CHAPTER 3

IN-VIVO CAROTID PLAQUE CHARACTERISATION

USING MAGNETIC RESONANCE IMAGING:

QUANTITATIVE T_2 MAPPING

3.1 Introduction

Lipid deposition in the subendothelial space from retention of apoB-containing plasma lipoproteins is a key event involved in the initiation and progression of atherosclerosis.¹¹⁷ Large pathological series in coronary²⁰⁵ and carotid⁸⁷ atherosclerosis had demonstrated that *vulnerable* plaques in general contain larger lipid-rich necrotic core (LRNC), amongst other high-risk features such as thinner fibrous cap, infiltration of inflammatory cells, and the presence of intraplaque haemorrhage. It is therefore logical that one of the key goals in risk-stratifying vulnerable plaques – and “*vulnerable patients*”^{206,207} – would be to non-invasively measure and quantify lipid-rich core in atherosclerotic lesions using advanced vessel wall imaging.

Multicontrast MRI is an established technique in plaque characterisation, validated *qualitatively* on histology using the modified AHA plaque type classification system.⁹⁰ However, *quantitative* lipid core detection on multicontrast MRI requires injection of gadolinium-based contrast agent,^{91,107,208} which is not always feasible in patients with renal insufficiency, as is frequently co-existing in patients with vascular disease.²⁰⁹ Moreover, plaque characterization on multicontrast MRI relies on tissue contrast relative to the adjacent sternocleidomastoid muscle, making it strongly dependent on the specific system and acquisition parameters. The analysis of multicontrast MRI is therefore difficult to standardize and needs subjective and extensive post-acquisition interpretation. Finally, multicontrast MRI is affected by image intensity inhomogeneity and suffers from blurring artifacts that are inherent to its acquisition strategy and can strongly affect plaque characterization.²¹⁰ All these limitations have prevented the widespread adoption of *in-vivo* multicontrast MRI as a plaque characterization tool for clinical and research purposes.

Biasioli, *et al.* recently reported a quantitative T_2 mapping method that has shown broad agreement with plaque segmentation using conventional multicontrast MRI.¹²⁷ This technique obtains compositional data on a *voxel-by-voxel* basis, which raises an important possibility of lipid quantification across the entire plaque, and without the need for a coalesced core to be discernible as in the case for multicontrast MRI. Moreover, plaque T_2 mapping addresses the need for an absolute physical measurement that can be standardized among different MRI systems and widely adopted in multi-centre studies.

The observations that (i) quantitative MRI T_2 mapping appeared to be as good as conventional multicontrast technique in characterising plaque type; (ii) large LRNC being shown to be an important feature of vulnerable plaques; and (iii) current anti-atherosclerotic drugs in clinical use might alter plaque composition indiscernible on current MRI technique, raise the important potential use of quantitative T_2 mapping in quantifying plaque composition *on a per voxel basis*, especially for plaque lipid content. Therefore, this chapter would first seek to validate carotid T_2 mapping in lipid quantification using histology and then to evaluate its potential application in relation to clinical presentation.

3.2 Hypothesis and aims

To test the hypothesis that MRI T_2 mapping is capable of discriminating different plaque components and, by using histology as the gold standard for validation, can characterise carotid atherosclerosis both (i) *qualitatively* by classifying plaque type based on modified AHA plaque type classification; and (ii) *quantitatively* by calculating plaque lipid content, which tracked with the clinical presentation of the patients.

The experimental aims of this chapter were:-

- 1) To develop and explore the use of MRI T_2 mapping to discriminate different plaque components such as lipid-rich necrotic core (LRNC), fibrous tissue, calcification, and intraplaque haemorrhage.
- 2) To compare the accuracy and consistency of T_2 mapping in plaque type classification based on the modified AHA plaque classification system⁹⁰ using location matched histological sections.
- 3) To test the potential of quantitative assessment of plaque lipid content using T_2 mapping and its agreement with histology.
- 4) To explore whether T_2 mapping has the sensitivity to discern difference of plaque lipid content between different clinical presentations.
- 5) To evaluate the sensitivity and specificity of T_2 mapping as an imaging tool in the clinic.
- 6) To establish the scan-rescan reproducibility of T_2 mapping technique.

3.3 Methods

General methods used in this chapter had been discussed in Chapter 2. Only methods and techniques specific to this chapter are further discussed below.

3.3.1 Study population

Ethical approval was obtained from National Research Ethics Services (NRES) and local R&D committee prior to commencement of the study. 40 patients awaiting carotid endarterectomy at Oxford University Hospitals NHS Trust were recruited from November 2011 to June 2014. Patients were scanned at the Oxford Acute Vascular

Imaging Centre (AVIC) ≤ 24 hours before surgery. Carotid plaques were obtained at operation. Patients were adult (> 18 years), had recently symptomatic (median time from index event 2 weeks) or asymptomatic carotid disease, with 50 - 99% carotid stenosis according to NASCET, or 70 - 99% according to ESCT criteria. Plaques were defined as 'culprit' plaques where they were deemed to have given rise to either a minor cerebrovascular accident (CVA) or a transient ischaemic attack (TIA) as assessed clinically and supported, where available, by brain MRI / computed tomography imaging. Asymptomatic carotid plaques were those that had no documented clinical symptoms, but with an indication for carotid endarterectomy based on % luminal stenosis.

3.3.2 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) before scheduled carotid endarterectomy. Bright-blood Time-of-Flight (TOF) angiography of the carotid arteries was acquired using 3D Fast Low Angle Shot (FLASH) with flip angle = 25° , TR = 20 ms, TE = 3.3 ms, FOV = $200 \times 150 \text{ mm}^2$, matrix size = 384×288 and slice thickness = 1 mm. 74 TOF consecutive slices were imaged in order to localize the carotid bifurcation and the atherosclerotic plaque. Multi-slice carotid T_2 maps were generated from 14 images with echo times TE = 9 - 127 ms acquired using a novel DANTE-MESE sequence that combines black-blood preparation based on non-selective Delay Alternating with Nutation for Tailored Excitation (DANTE) pulse trains²⁰² with chemical-shift-selective fat saturated Carr-Purcell-Meiboom-Gill multiecho spin-echo (MESE) sequence. MESE parameters were TR = 2000 ms, TE = 9 - 127 ms, partial Fourier = 5/8, FOV = $128 \times 128 \text{ mm}^2$, matrix size = 384×384 , slice thickness = 2 mm and slice gap = 2 mm. DANTE parameters were FA = 8° , number of pulses = 120, interval between pulses = 0.5 ms,

$G_z = 18$ mT/m and gradient duration = 0.4 ms. DANTE-MESE acquired 5 slices in 4 min and was then repeated consecutively with a 2 mm off-set resulting in a total coverage of 10 slices of 2 mm thickness, i.e. 2 cm of the target carotid artery acquired in 8 min. Head movement was limited by a head holder and patients were instructed not to move and to try not to swallow during each 4-min acquisition.

3.3.3 Data Analysis

DANTE-MESE image quality was assessed by an experienced reviewer (LB) who inspected the visibility of the carotid lumen and outer wall boundaries and rated them from 1 (poor) to 5 (excellent) according to standard procedures.⁸⁹ Slice locations with quality <3 were rejected due to low signal-to-noise ratio and/or motion artefacts.

T_2 maps of the carotid arteries were generated *voxel-by-voxel* using mono-exponential nonlinear fitting,¹²⁷ and lumen and external vessel boundaries were segmented using a validated semi-automated procedure.²¹¹ As reported previously, LRNC without intraplaque haemorrhage (IPH) had shorter T_2 than normal vessel wall and fibrous tissue, whereas with recent IPH into the core, T_2 was longer than all other plaque components.¹²⁷ Therefore, to take into account the minority of plaques with recent haemorrhage into the core, segmentation used dual T_2 thresholds, a lower one (T_{2L}) indicating the maximum T_2 value associated with lipid and a higher one (T_{2H}) above which IPH was recorded inside the plaque lipid core. Voxels with T_2 values below T_{2L} or above T_{2H} were thus classified as LRNC. Significant IPH ($T_2 > T_{2H}$) was defined as >5% of the total cross-sectional area.

In order to use the data most efficiently, segmentation performance was estimated by 'leave-one-out' cross-validation. Lipid Area (%) was computed for all possible combinations of T_{2L} (range 30 - 50 ms) and T_{2H} (range 70 - 120 ms) to find the pair of

threshold values that achieved the optimal lipid segmentation on T_2 maps, i.e. the highest Pearson correlation coefficient R_{CV} against lipid quantification on histology. All algorithms were implemented in Matlab (MathWorks). Plaque type was also classified based on T_2 mapping data using the modified AHA scheme.⁹⁰

3.3.4 Histology

Atherosclerotic plaques were collected during carotid endarterectomy and divided into two halves at the point of maximal stenosis: one half was snap-frozen and the other half processed for paraffin embedding. Paraffin sections (5 μm) were obtained at 1mm intervals for histological examination using haematoxylin and eosin (Sigma Aldrich) and Masson's trichrome (Merck-Millipore), which identified collagen in the fibrous tissue (green), as well as fibrin and erythrocytes from IPH (bright red). Lipid-rich area without previous evidence of IPH appeared transparent / unstained or pale in both H&E and Masson's trichrome stain. Stained slides were scanned using the NanoZoomer (Hamamatsu) virtual pathology system. Frozen sections (20 μm) on 20 randomly selected locations were obtained for Oil-red-O (Sigma-Aldrich) staining to confirmed lipid distribution. Since IPH occurred exclusively inside, and as part of, the LRNC, the boundary of IPH against LRNC was often impossible to define on histology (see Figure 3.6 later). The total histological Lipid Area (%) was hence calculated by combining lipid area *with* and *without* IPH. Total vessel cross-sectional area and Lipid Area (%) were obtained by manual segmentation using ImageJ (NIH), as previously described.¹⁹⁵ Plaque type was classified histologically using the AHA scheme.⁹⁰

3.3.5 Location matching

One investigator (LB) blinded to histology measured the distance from carotid bifurcation of the available T_2 map locations and provided the corresponding T_1 -weighted images to a second investigator (JTC) who obtained histology at the specific distance from bifurcation using T_1 -weighted images to further optimize matching based on local vessel morphology (blinded and without reference to T_2 maps).

3.3.6 Scan-rescan reproducibility

Scan-rescan reproducibility of lipid quantification by T_2 mapping was studied in 10 additional carotid plaques. Patients were taken out of the scanner after the first scan and left sitting for 2 minutes before the second scan. Both scans acquired 10 contiguous slices to cover the entire plaque and assess the reproducibility of Lipid Area (%) per plaque.

3.4 Results

3.4.1 Patient characteristics

T₂ maps with histology and overall quality rating ≥ 3 were achieved for 26 out of 40 plaques, 15 symptomatic and 11 asymptomatic. The overall median age was 69 years (range 43 to 90 years) with overall mean stenosis measured by duplex ultrasound of $82.5 \pm 1.9\%$ (range 70 – 95%). Patient characteristics are summarized in Table 3.1. The two groups were similar with the exceptions that symptomatic patients were older than asymptomatic patients (73 vs. 60 years, $P = 0.046$), and that symptomatic plaques were more likely to be right-sided compared to asymptomatic plaques (R:L = 6.5:1 for symptomatic vs. 1:1.2 for asymptomatic, $P = 0.038$). There was no significant difference between genders, major cardiovascular risk factors or medications on admission between groups.

3.4.2 AHA classification

Figure 3.1 showed how T₂ mapping could clearly differentiate various major plaque components. To evaluate further how accurately T₂ mapping can determine plaque types, the modified AHA plaque classification system⁹⁰ was used to compare directly against histological classification. Figure 3.2 showed the plaque types as determined by T₂ map against histology, which showed good agreement (80.8%) between T₂ map and histology with Cohen $\kappa = 0.73$. Mismatches were caused by difficulty in ascertaining the age of IPH. As extravasated blood ages and organises, not only does its T₂ signal changes,⁸⁹ but also its histological appearance as organisation replaces fibrin with a more collagen-like extracellular matrix structure.²¹²

	Symptomatic	Asymptomatic	Significance, P =
Total	15	11	
M:F	2.75 : 1	2.67 : 1	ns
Median age (range)	73 (49 – 90) yrs	60 (43 – 89) yrs	0.046 *
CV risks			
Hypertension	14 (93.3%)	9 (81.8%)	ns
Hypercholesterolaemia	12 (80.0%)	7 (63.6%)	ns
Smoking	7 (46.7%)	4 (36.4%)	ns
Diabetes mellitus	4 (26.7%)	4 (36.4%)	ns
Previous CAD / CVA	4 (26.7%)	6 (54.5%)	ns
Medication at time of CEA			
Aspirin / antiplatelets	12 (80%)	8 (72.7%)	ns
Statins	14 (93.3%)	10 (90.9%)	ns
Beta-blockers	4 (26.7%)	3 (27.3%)	ns
Calcium antagonists	5 (33.3%)	3 (27.3%)	ns
ACE inhibitors / ARBs	6 (40.0%)	5 (45.5%)	ns
Anticoagulation	3 (20.0%)	0 (0%)	ns
Duplex ultrasound scan			
Right : Left	6.5 : 1	1 : 1.2	0.038 *
Mean % stenosis ± SEM	81.3 ± 2.5%	84.1 ± 3.0%	ns

Table 3.1 **Patient characteristics for the study**

The symptomatic and asymptomatic groups were well matched. Symptomatic patients were older than asymptomatic patients (median age 73 *versus* 60 years, P = 0.046). The most striking difference is the right sided preponderance in symptomatic carotid stenosis (Right : Left = 6.5 : 1, symptomatic), while side predilection was not seen in asymptomatic carotid stenosis (Right : Left = 1 : 1.2, asymptomatic).

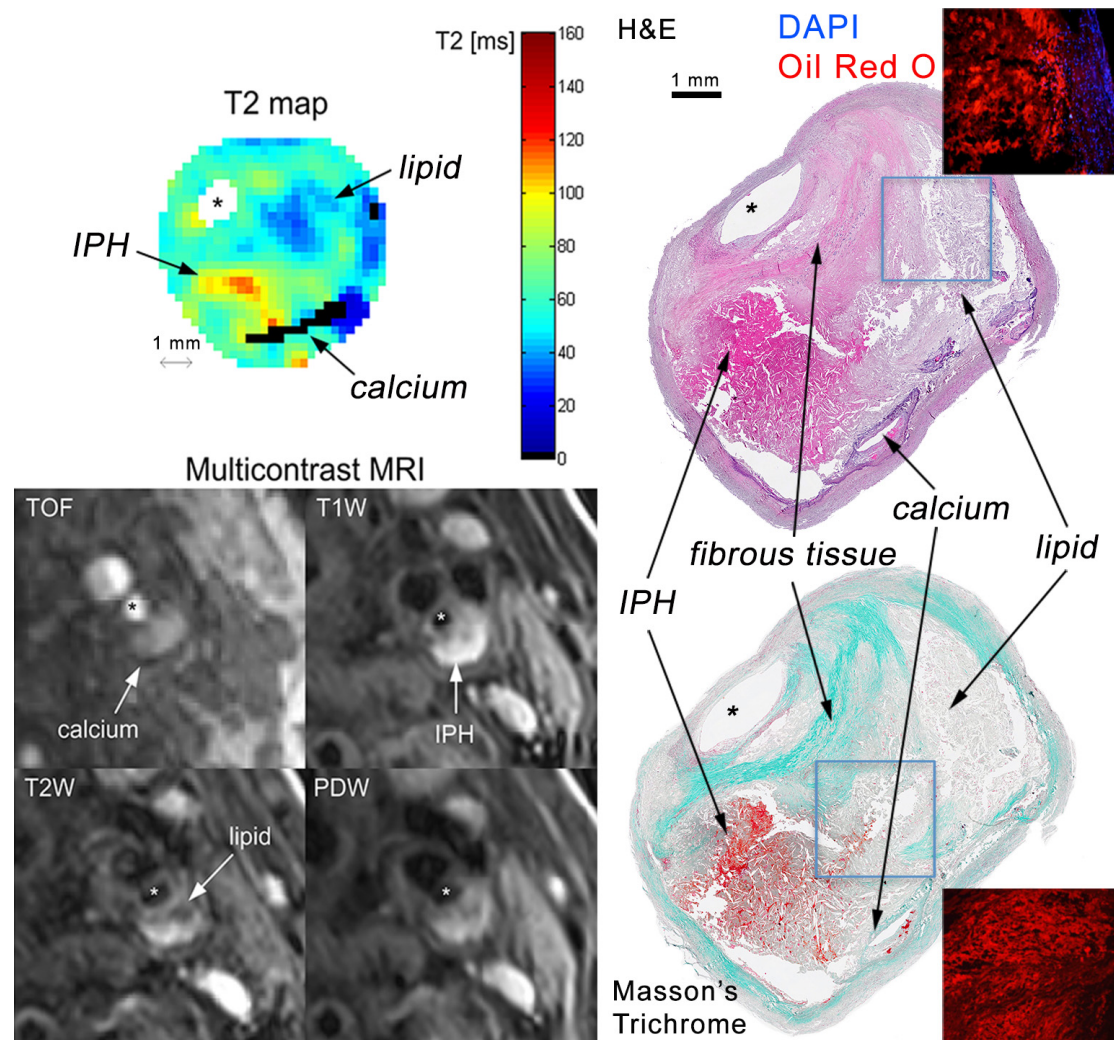


Figure 3.1 Comparison of T_2 map, multicontrast MRI, and histology

T_2 map accurately identifies different plaque components of an AHA type VI plaque showing the presence of lipid, IPH and calcium. Lipid was further confirmed on Oil-Red-O staining on adjacent frozen section. DAPI = 4',6-diamidino-2-phenylindole fluorescent nuclear stains. * denotes lumen.

Histology	CMR (T2 maps + TOF)				
	IV-V	VI	VII	VIII	Total
IV-V	7	2	-	-	9
VI	1	7	-	-	8
VII	1	-	4	1	6
VIII	-	-	-	3	3
Total	9	9	4	4	26

Figure 3.2 *Agreement in AHA plaque type classification between T_2 map (+TOF) and histology*

There was excellent agreement between the two methods (80.8%) with a Cohen κ = 0.73. Mismatches were caused by difficulty in ascertaining the age of IPH.

3.4.3 Lipid quantification

Next the potential of using T_2 map for quantitative evaluation of plaque lipid content was explored (Figure 3.3). Varying according to the length of each carotid atherosclerotic lesion, an average of 2.3 slices of T_2 map data were obtained per plaque, yielding 60 matched slices. Using leave-one-out cross-validation, the combination $T_{2L} = 42$ ms and $T_{2H} = 90$ ms produced maximum $R_{CV} = 0.85$ ($P < 0.001$) against Lipid Area (%) measured on histology (Figure 3.4A), thus achieving optimal T_2 map segmentation for lipid quantification. Additionally, root-mean-square error $RMSE_{CV}$ was minimum (10.5%); and Bland-Altman analysis confirmed a high degree of agreement between T_2 maps and histology with only 0.03% under-estimation on T_2 maps (Figure 3.4B).

For technical validation, several comparisons of T_2 maps and tissue stains were made for each *plaque*. For biological applications, the ‘unit of analysis’ would be per plaque rather than per slice. Grouping individual slice locations from each plaque on the same scatter plot, data points from the same plaque were shown to be relatively closely clustered together (Figure 3.5A), indicating that matched slices from the same plaque tend to contain similar proportions of lipid. The relationship between T_2 map and histology was therefore examined using the average calculated Lipid Area (%) *per plaque* (instead of per slice location). Lipid Area (%) by T_2 map and by histology maintained a strong correlation ($R = 0.83$, $P < 0.001$) (Figure 3.5B).

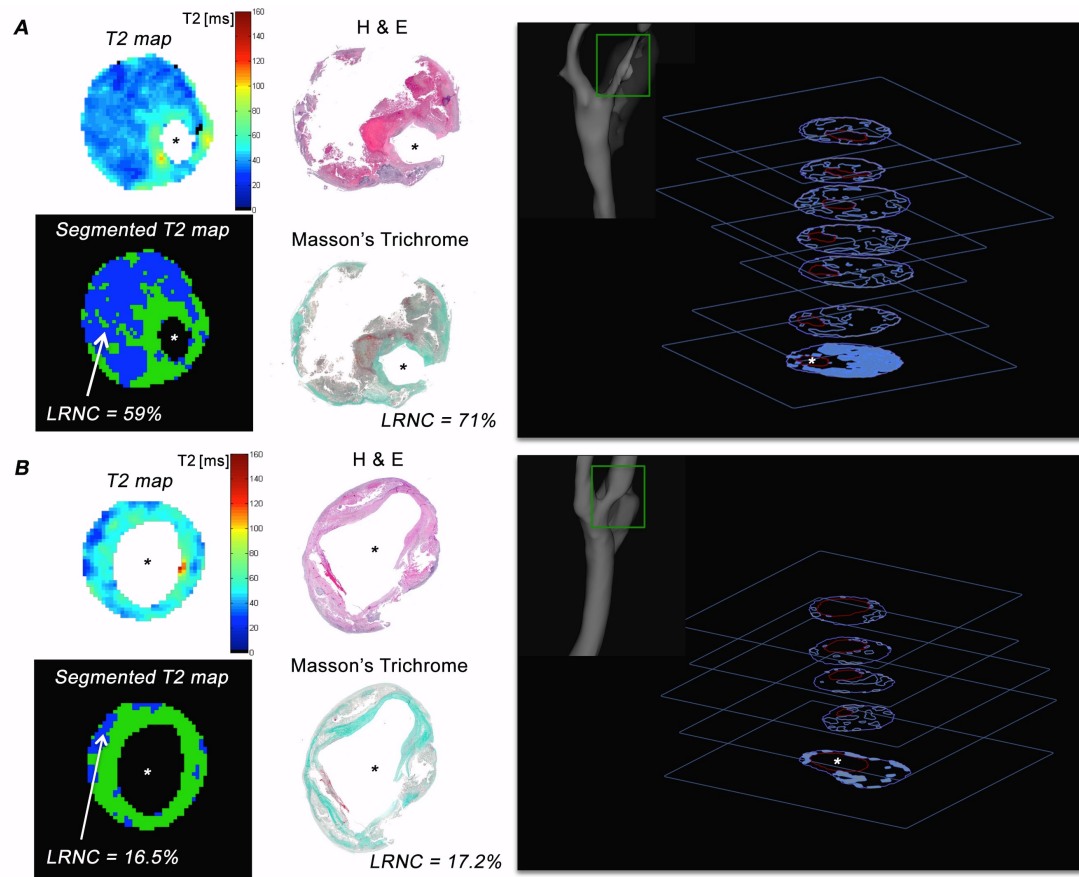
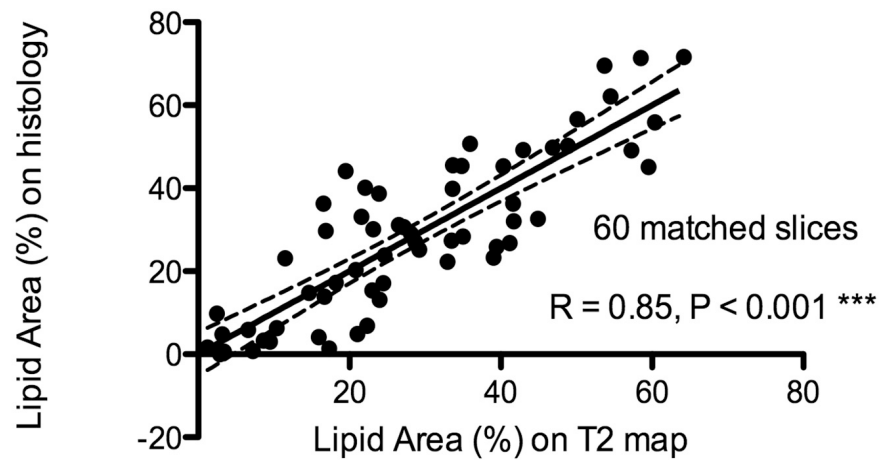


Figure 3.3 **Quantification of plaque lipid content**

A: A plaque with large LRNC; and **B:** A plaque with multiple smaller lipid-rich pools; with their matching histology. Right panels for **A** and **B** show 3D representations of lipid distribution. 3D slices with blue contours showing lipid segmentation generated from DANTE-MESE data from illustrative plaques. Lumen was marked with asterisk * and outlined red in 3D slices.

A



B

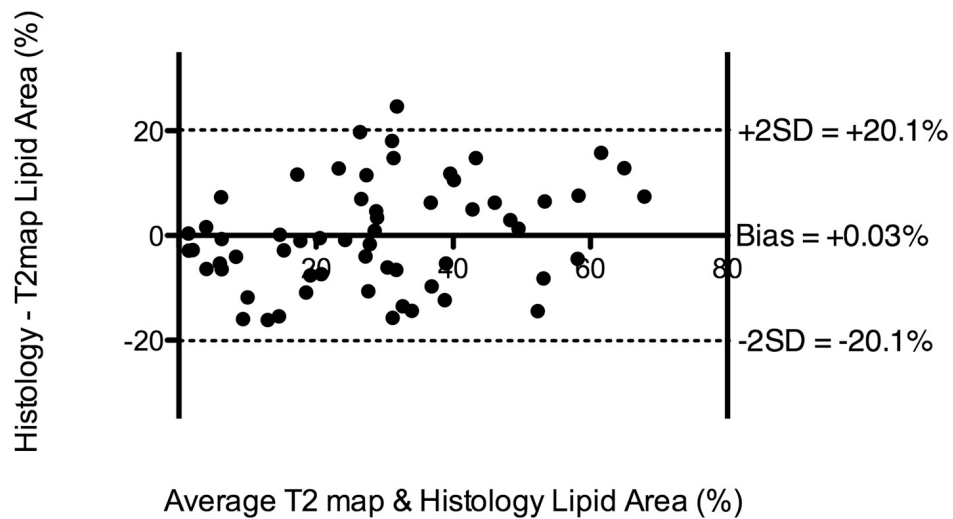


Figure 3.4 Correlation of Lipid Area (%) between T_2 mapping and histology

A: Scatter plot of Lipid Area (%) by *individual slices* showed excellent correlation with $R_{CV} = 0.85$, $P < 0.001$; **B:** Bland-Altman analysis showed good agreement between histology and T_2 map with minimal bias.

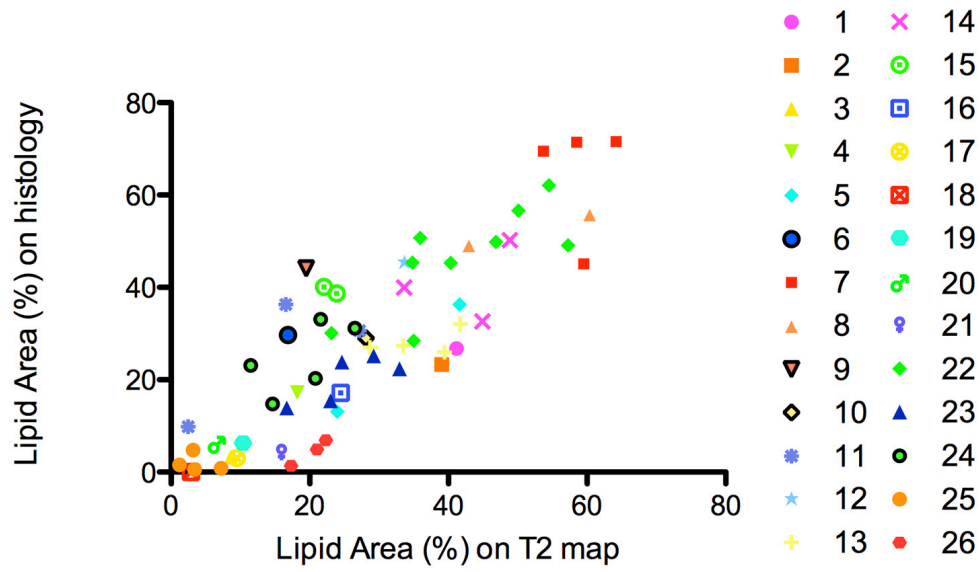
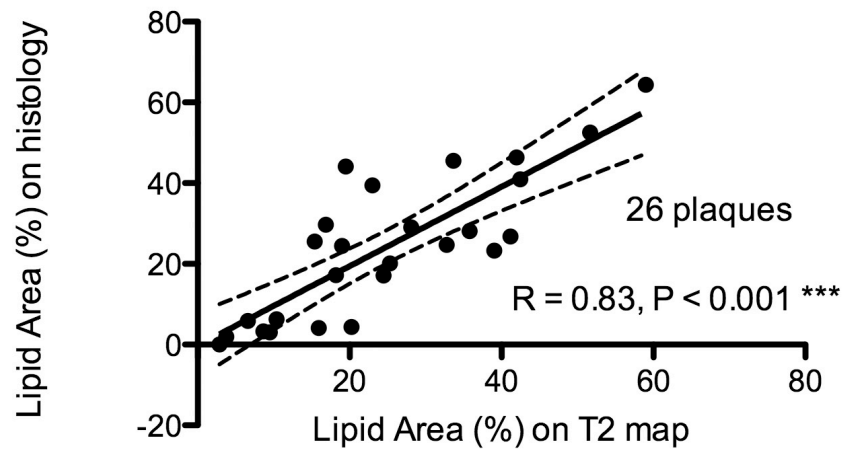
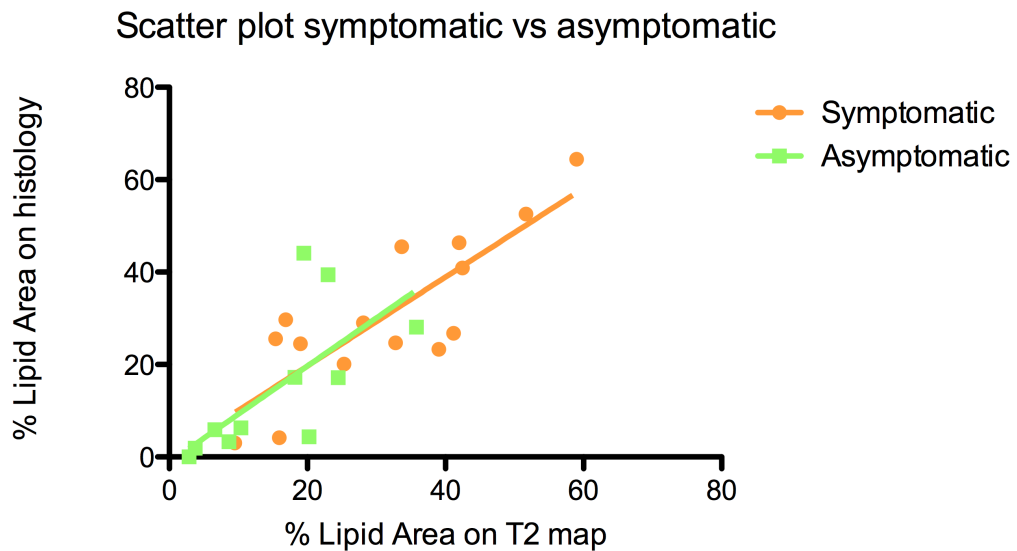
A**B**

Figure 3.5 *Plaque-by-plaque correlation of Lipid Area (%)*

A: Scatter plot of Lipid Area (%) to show slices from the same *plaque* (individually coloured – see legend) tend to contain similar proportions of lipid; **B:** Strong correlation maintained with *plaque by plaque* comparison with $R = 0.83$, $P < 0.001$.

3.4.4 Symptomatic versus Asymptomatic

Given the observed precision of the T₂ mapping technique, the next objective was to test whether lipid content varied between symptomatic and asymptomatic plaques. First, using linear regression, the validity of this comparison was tested and the measurement correlation was shown to be maintained and comparable in both symptomatic and asymptomatic plaques (linear regression lines overlapping) (Figure 3.6). Symptomatic plaques were found to have significantly higher Lipid Area (%), compared to asymptomatic plaques ($31.5 \pm 3.7\%$ vs. $15.8 \pm 3.1\%$, $P = 0.005$, Figure 3.7A). This was despite a similar degree of carotid stenosis ($81.3 \pm 2.5\%$ symptomatic vs. $84.1 \pm 3.0\%$ asymptomatic, $P = \text{n.s.}$), and only a modest difference in terms of plaque volume ($128.0 \pm 6.0 \text{ mm}^3$ symptomatic vs. $105.6 \pm 9.4 \text{ mm}^3$ asymptomatic, $P = 0.04$). In addition, using an established definition of 'large' LRNC as $\geq 25\%$ of cross-sectional area,^{87,213} symptomatic plaques were shown to have significantly larger LRNC using T₂ mapping alone ($P = 0.003$, Figure 3.7B). Finally, the Receiver Operating Characteristic (ROC) area under curve (AUC) was 0.79 ($P = 0.01$), indicating that lipid quantification by T₂ mapping has a good ability to discriminate between symptomatic and asymptomatic plaques in this clinical cohort. ROC analysis confirmed the optimal cut-off for LRNC to be indeed $\sim 25\%$ (sensitivity = 67%, specificity = 91%) (Figure 3.7C).

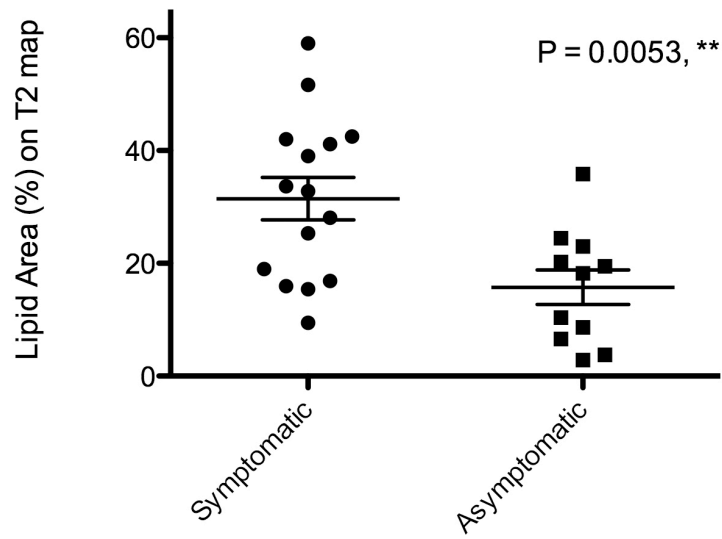


The slope and y-intercept of the two regression lines are **not** significantly different ($P = 0.84$ for slope, $P = 1.00$ for intercepts)

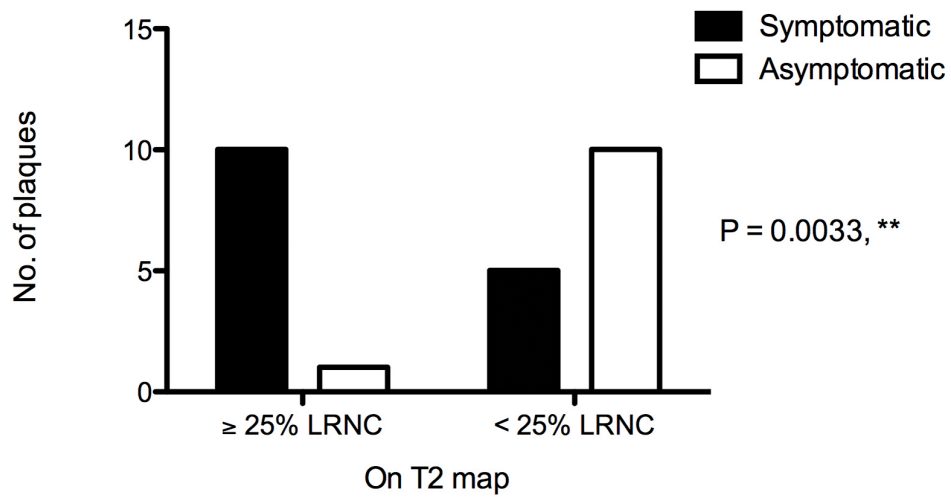
Figure 3.6 *Validity of comparison between symptomatic and asymptomatic groups*

Linear regression lines in the symptomatic group and asymptomatic group overlapped with no statistical difference – i.e. the T_2 map and histological Lipid Area (%) correlation is maintained between symptomatic and asymptomatic plaques.

A



B



(Figure legend overleaf)

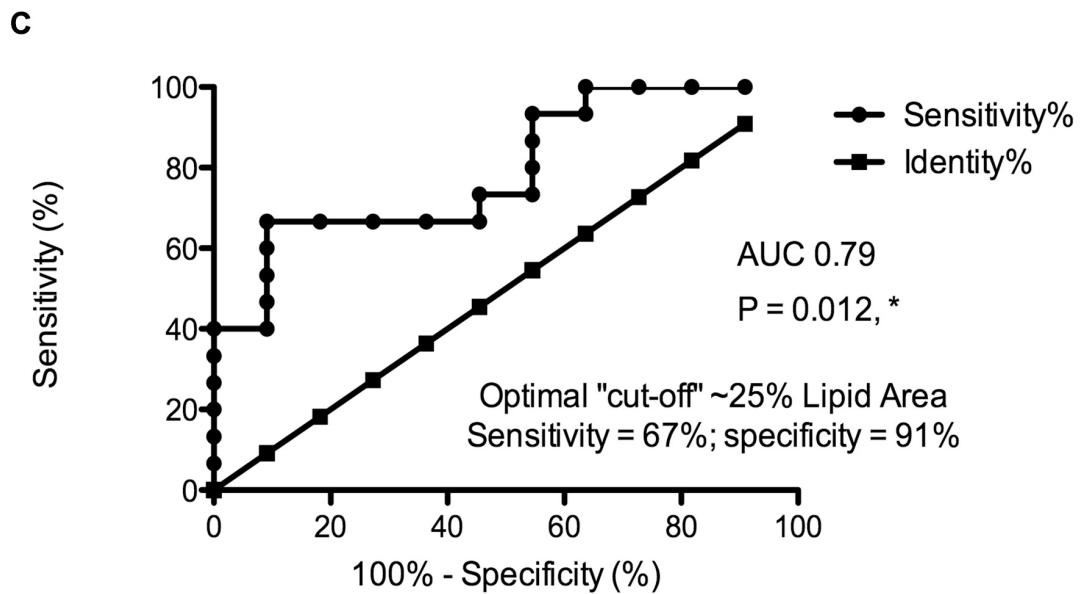


Figure 3.7 *Symptomatic plaques contain more lipid than asymptomatic plaques*

A: Symptomatic plaques contained significant more lipid than asymptomatic plaques ($31.5 \pm 3.7\%$ vs. $15.8 \pm 3.1\%$, $P = 0.005$) despite similar degree of luminal stenosis; **B:** Chi-squared test using an established cut-off of $>25\%$ to define a 'large' LRNC showed similar results; **C:** ROC curve analysis showed that T_2 mapping has a fair to good ability to discriminate between symptomatic and asymptomatic plaques.

3.4.5 Intraplaque haemorrhage

In order to clarify whether the inclusion of IPH in the calculation with a second T_2 threshold had skewed the data, the prevalence of IPH and its contribution to the total Lipid Area (%) was examined. 16 of the total 60 slices contained significant (>5%) IPH. Of these, only 7 had IPH infiltrating >50% of the lipid core. Thus IPH made a relatively minor contribution to the total lipid measurement. Figure 3.8 shows the co-presence of IPH and lipid-filled macrophage foam cells. Next, T_2 maps were then re-analysed using a single threshold of $T_2 < 42\text{ms}$ for lipid segmentation, thus effectively excluding IPH (long T_2) from the LRNC, and correlation with histology similar to the dual T_2 threshold approach ($R = 0.79$ vs. 0.85) was obtained. In fact, if those 7 slices with large (>50%) IPH were excluded from the cohort, the correlation of the remaining 53 slices using a single threshold ($T_2 < 42\text{ ms}$) went back to $R = 0.85$.

3.4.6 Scan-rescan reproducibility

1 of the 10 carotid plaques scanned to evaluate reproducibility was rejected due to low image quality during rescan. For the remaining 9 plaques, scan-rescan reproducibility was excellent with Intraclass Correlation Coefficient (ICC) = 0.89 (95% CI $0.59\text{-}0.98$) and coefficient of variation = 8.9% (plaque-by-plaque comparison).

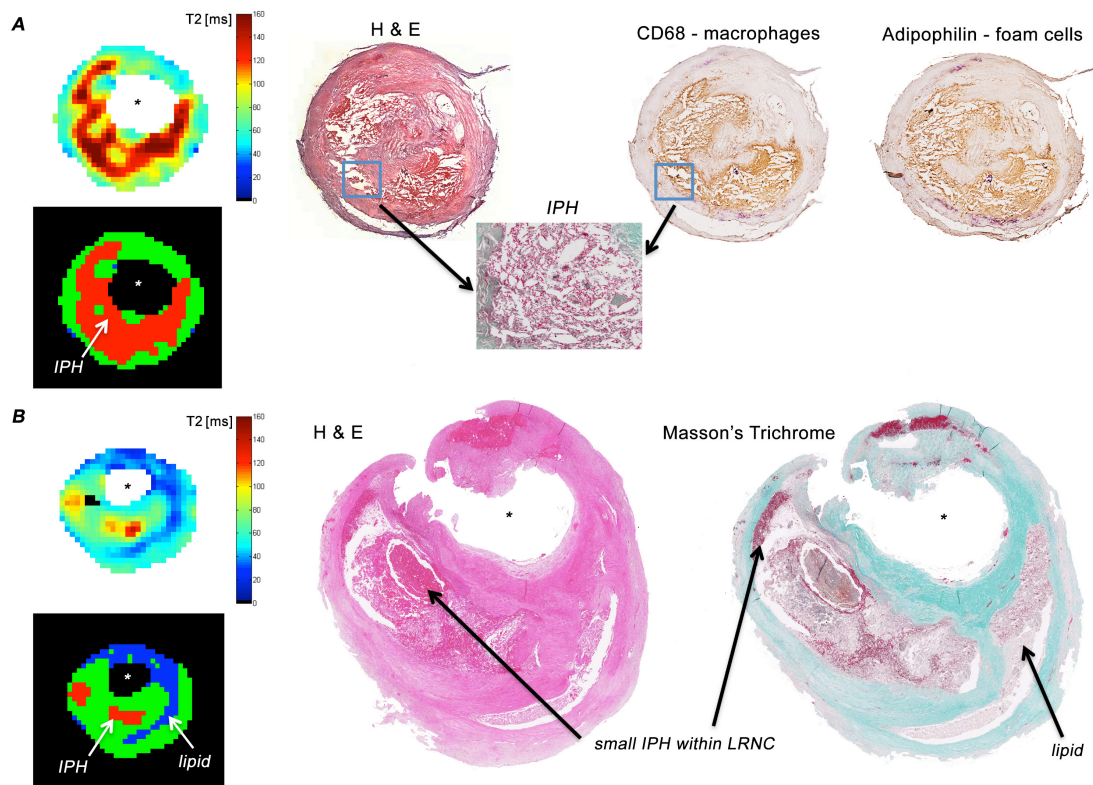


Figure 3.8 *Intraplaque haemorrhage is structurally integral to the LRNC*

A: Large IPH in background LRNC. Erythrocytes and fibrin staining on Masson's Trichrome confirmed IPH. Co-presence of macrophage and lipid droplets confirmed using standard immunohistochemistry against CD68 and adipophilin (Chai JT, *et al. PLoS ONE* 2013;8(5):e62934); **B** Small IPH, structurally continuous with background LRNC, detected by T₂ mapping and histology.

3.5 Discussion

This work makes two principal findings. Firstly, *in vivo* T₂ mapping, on a voxel-by-voxel basis, and at near-microscopic resolution, enables accurate and highly reproducible quantification of plaque lipid, compared with histology. Secondly, lipid quantification by T₂ mapping can distinguish recently symptomatic plaques with good sensitivity and specificity.

Deposition and retention of cholesterol-rich lipoproteins is a cardinal feature of atherosclerosis, occurring from the earliest foam cell lesions through to the complex plaques of advanced disease. Where the rate of deposition exceeds the rate of clearance, cholesterol and cholesterol derivatives can accumulate within the plaque. The presence of a large LRNC (especially associated with a thin overlying fibrous cap) has been associated with high risk of rupture and atherothrombotic complications.^{87,213} Furthermore, the presence of lipid in the plaque is a driver of local inflammation²¹⁴ and altered gene expression.²¹⁵ Because of these central roles, quantification of plaque lipid has been a key aim of various atherosclerosis imaging techniques such as intravascular ultrasound (IVUS),²¹⁶ near infra-red spectroscopy (NIRS),²¹⁷ cardiac computed tomography (CT),²¹⁸ as well as vascular MRI.⁹¹

Since the first description of Toussaint *et al*⁸⁸ MR techniques have been applied for the detection of LRNC, which produce low signal intensity on T2-weighted images.²¹⁹ However, a significant limitation of this approach is low sensitivity, such that only a minority of plaques assessed in this way show a LRNC. In a survey of 100 patients with varying extents of coronary atheroma, only 20% had a discernable LRNC in the carotid artery on MRI.²²⁰ The optimal multicontrast approach requires intravenous contrast injection^{91,107} and extensive post-processing. In contrast, the proposed approach provides quantitative data on a *per voxel* basis and semi-automated lipid

segmentation by simple T_2 thresholding that is validated here. Since the validation required tissue obtained by endarterectomy (undertaken for severe stenotic disease), our series contains relatively large lesions (of AHA classes IV-VIII on histology). In this context, plaque lipid was detectable in virtually all cases.

‘Atherosclerosis regression’ is a term used to denote favourable remodelling of plaque, for instance in response to aggressive reduction in plasma level of atherogenic lipoproteins.¹⁰⁸ Normalisation of plasma lipid profile in genetically-modified hyperlipidemic mice with atherosclerosis leads to marked loss of plaque lipid and deposition of fibrous tissue,²²¹ with similar changes inferred in man.²²² This has important implications in the clinic because some anti-atherosclerotic or anti-lipid agents may well change plaque lipid content, without necessarily altering total plaque volume. This suggests that accurate quantification of plaque lipid might prove useful for patient stratification and selection for intensive lipid lowering therapies and / or monitoring of response to treatment, including in clinical trials. The technique validated here shows excellent scan-rescan reproducibility and would be well suited to serial application in the context of a lipid-intervention trial. This may be important since several new classes of lipid lowering therapies have been developed (including Lp(a) and apoB anti-sense; MTP inhibition; CETP inhibitors; PCSK9 inhibitors) but considerations of cost-effectiveness, mode of administration and, in some cases, potential toxicity mean that they are unlikely to be used indiscriminately in patients with atherosclerotic vascular disease. Methods for rational stratification are therefore needed.

Applying T_2 map in the symptomatic and asymptomatic clinical cohorts, this study confirmed that *despite having similar degree of carotid stenosis, and only a modest (21.2%) difference in terms of plaque volume*, symptomatic plaques had 99.4% larger LRNC than asymptomatic plaques. A ROC curve analysis confirmed this technique having a good ability to distinguish between symptomatic and asymptomatic plaques in the current clinical cohorts and that the optimal cut-off was indeed a LRNC of around 25% of the cross-sectional area, in agreement with various large-scale histological studies in both carotid⁸⁷ and coronary²²³ literature. The ability to accurately quantify plaque lipid might also prove useful in informing decisions between carotid surgery and stenting; since a large LRNC is considered to be an important factor determining the amount of stenting-related downstream debris^{224,225} and has been shown to increase risk of stroke following carotid stenting.^{225,226} 3D rendering of lipid distribution in relation to local vessel anatomy using quantitative T_2 mapping data may also assist interventional procedural planning to minimize such risks (Figure 3.9).

The main limitation of the current T_2 mapping method is the sensitivity to motion artefacts of the DANTE-MESE sequence, a fact reflected in the relatively high overall rejection rate (35%) of completed scans. In order for this technique to fulfil its full clinical potential a number of motion correction strategies are in development. Furthermore, it remains a challenge to measure IPH alone (Figure 3.10). This is because as extravasated blood ages and organises, not only does its T_2 value change,⁸⁹ but also its histological appearance as organisation replaces fibrin with a more collagen-like extracellular matrix structure.²¹²

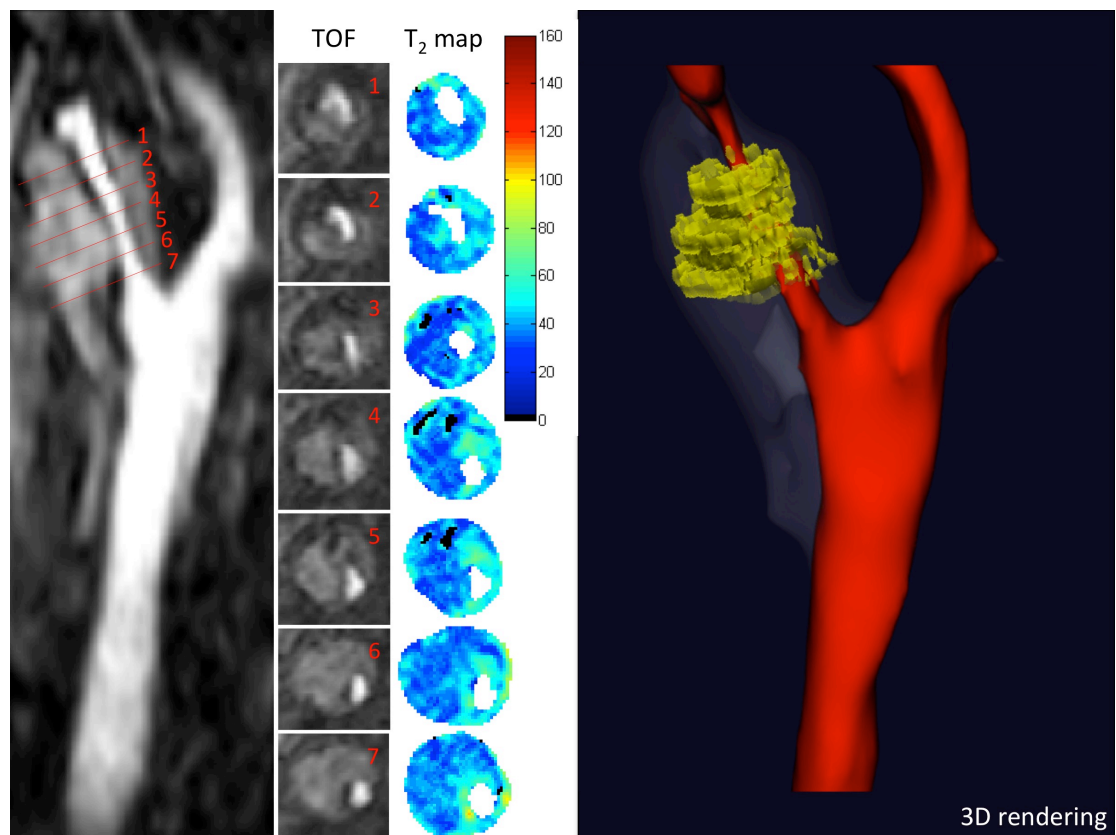


Figure 3.9 **3D carotid vessel wall rendering**

Illustration of how vessel anatomy from Time-of-Flight (TOF) data (left) can be superimposed with T_2 map compositional data (middle) to produce 3D model (right) of the vessel anatomy and lipid distribution. On the 3D reconstruction inner red indicates lumen, blue indicates lipid pool, and outer green indicates the outer vessel boundary.

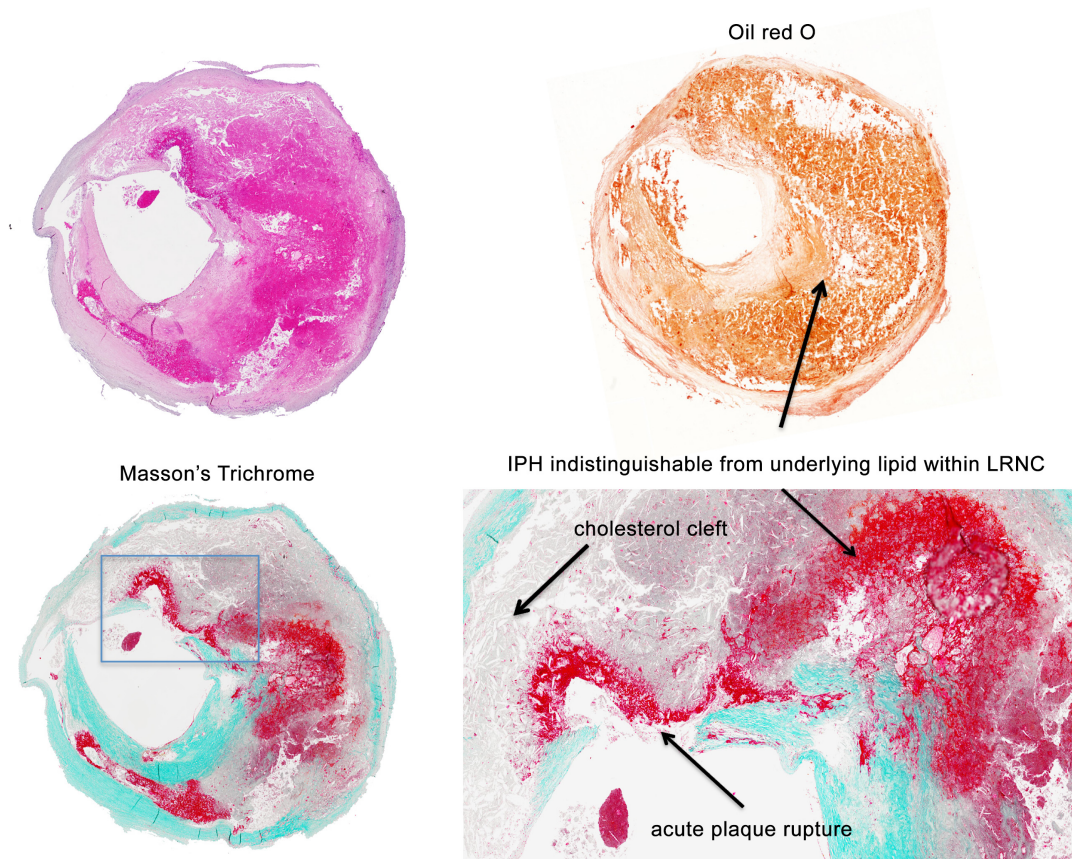


Figure 3.10 *An example of fresh intraplaque haemorrhage from rupture*

Blood entered the plaque and lipid core under systolic blood pressure. In acute IPH, it is often too early for T_2 value to rise and the border between IPH and the underlying lipid is histologically *indistinguishable* from rest of LRNC on Oil red O as haemorrhage infiltrated deep into and within the lipid core.

3.6 Conclusions

In conclusion, the work presented in this chapter has shown, for the first time, the histological validation of *in-vivo* multi-slice T₂ mapping in plaque characterisation and its ability to accurately quantify plaque lipid content. Not only can T₂ mapping distinguish between symptomatic and asymptomatic plaques based on their lipid content, this study also confirmed that despite similar degree of carotid stenosis, and only modest difference in plaque volume, symptomatic plaques indeed contain significantly more lipid compared to asymptomatic plaques. T₂ mapping technique allows for better plaque characterisation and, more importantly, the potential in evaluating response to new anti-atherosclerotic treatment and could potentially help stratify risk / patient selection in future cardiovascular clinical trials.

CHAPTER 4

PLAQUE MACROPHAGE GENE EXPRESSION

PROFILING USING LASER-CAPTURE

MICRODISSECTION

4.1 Introduction

Having validated T₂ mapping as a novel tool to characterise plaque compositional heterogeneity and established that symptom-related plaques contain higher lipid content compared to non symptom-related plaques, this chapter explores the heterogeneity of plaque functional characteristics and the extent to which these change between lipid-rich and lipid-poor plaques. Macrophages are capable of functional heterogeneity and plasticity, which allow them to readily adapt their phenotypes in response to signalling cues and their microenvironment.⁷¹ As discussed in Chapter 1.2.2, this implies a bilateral influence between plaque macrophages and their local microenvironment. Not only can plaque macrophages adapt according to their micro-anatomical locations, but subsets of differentiated plaque macrophages might secrete signalling factors and orchestrate a range of local responses concerning inflammation, its resolution, lipid handling, angiogenesis, as well as secretion of proteolytic enzymes that can degrade overlying fibrous cap to influence plaque structural integrity and evolution.⁷¹

Macrophage subsets are conventionally described by a handful of cell surface markers,⁷⁰ along a polarization spectrum where one end denotes M1 proinflammatory phenotype and the other end M2 anti-inflammatory / reparative phenotype; in recent years, however, it has emerged that although these distinct polarized phenotypes could readily be induced and studied *in-vitro* (see Figure 1.3), a wider spectrum of intermediate phenotypes exist *in-vivo* in both mice^{69,227} and humans.^{201,228} In addition, at least observed in mice, macrophage local proliferation seems to play an important role in macrophage accumulation and sustainment of inflammation in advanced atherosclerotic plaques, as opposed to predominantly recruitment from blood monocytes as seen in early atherosclerotic lesions.⁷⁵ These observations further highlighted the complexity of plaque macrophage functional

heterogeneity in human atherosclerosis, especially in response to the complex microenvironment often seen within those advanced atherosclerotic lesions.

Deposition and retention of cholesterol-rich lipoproteins is a cardinal feature of atherosclerosis, occurring from the earliest foam cell lesions through to the complex plaques of advanced disease. Where the rate of deposition exceeds the rate of clearance, cholesterol and cholesterol derivatives can accumulate within the plaque as cholesterol crystals within a lipid-rich necrotic core (LRNC).²²⁹ In mice, these cholesterol crystals had been shown to induce inflammation by stimulating caspase-1-activating NLRP3 inflammasome, which results in cleavage and release of IL-1.²³⁰ In addition to M1 and M2 phenotypes, murine macrophages exposed to oxidized phospholipids can also be driven to express the alternative Mox phenotype, which is triggered via transcription factor NFE2L2.²³¹ Mox macrophages typically express NFE2L2-mediated redox-regulatory genes such as haem oxygenase 1 (Hmox1),²³¹ which is also a recognized marker associated with intraplaque haemorrhage,²³² alongside M(Hb) macrophage marker CD163.²³³ In contrast, macrophages located in the plaque cap or shoulder, which is considered a more unstable region within the plaque where most ruptures occur,^{86,234,235} has been shown to express overlapping inflammatory and alternative macrophage markers,⁷³ making it even more challenging to dissect the functional roles of this expanding repertoire of heterogeneous plaque macrophage populations.

In order to understand the function of specific macrophage subsets in relation to (1) their local microenvironment (which might be discernable by novel non-invasive imaging) and (2) without bias by the conventional pre-defined M1:M2 paradigm, a new technique in immunohistochemistry-guided laser-capture microdissection (immuno-LCM) was adapted, refined, and coupled with linear RNA amplification to achieve location-specific plaque macrophage whole transcriptome profiling.

The history of LCM development was discussed in Chapter 1.3.5. LCM is a powerful technique that is compatible with a wide range of downstream RNA and protein applications. However, one key challenge in immuno-LCM with regards to transcriptome analysis revolves around the competing demands between achieving cell procurement specificity and the implications for conserving RNA quality. This chapter details bespoke techniques that were developed, optimized and validated for immuno-LCM-transcriptomics for human plaque macrophages. Combined with contemporary bioinformatics approaches this enabled study of the many differentially activated biological pathways in various subsets of macrophages obtained from the same cohort of patients who underwent carotid MRI imaging in Chapter 3.

4.2 Hypotheses and aims

To test the hypotheses that (1) macrophages are phenotypically different in symptom-related plaques compared to non symptom-related plaques, and that their functional gene expression profiles vary depending on their respective locations within the plaques; and (2) plaque compositional heterogeneity, in particular plaque lipid content as measured by non-invasive MRI T_2 mapping technique, might be directly correlated with activation of specific inflammatory or lipid-handling biological pathways in subsets of plaque macrophages, in both symptom-related and non symptom-related plaque, detectable at a transcriptome level.

The experimental aims of this chapter were:-

- 1) To adapt and refine the techniques of immunohistochemistry-guided laser-capture microdissection (immuno-LCM), with emphasis on how to achieve the best possible quality RNA for downstream analysis.
- 2) To ensure immuno-LCM cell procurement specificity.
- 3) To study the transcriptome of plaque macrophages according to plaque symptom status.
- 4) To examine the transcriptome of plaque macrophages according to different plaque regions.
- 5) To evaluate whether plaque lipid content, as derived from MRI T_2 mapping as per Chapter 3, might be a driver of plaque inflammation as predicted by various murine models detailed above.^{71,230}

4.3 Methods

Isolation and procurement of plaque macrophages with high specificity was critical to this series of experiments. The main methods used in this chapter have been discussed in Chapter 2. However, optimization and refinement of immunohistochemistry-guided laser-capture microdissection, and how this was coupled with downstream gene expression analysis and bioinformatics are discussed in more detail below.

4.3.1 Human carotid samples quality control

Since the same cohort of patients (plaques) was used for (i) histological validation of T_2 map (in Chapter 3) and (ii) immuno-LCM experiments described in this chapter, there were competing demands for tissue processing. For RNA analysis, swift tissue handling/processing and snap freezing was preferable to preserve high quality RNA; however to facilitate MRI-histology location matching, meticulous observation and documentation of macroscopic plaque features and distance from *ex-vivo* anatomical landmarks such as the carotid bifurcation – often including detailed drawings with tape measure – was needed. As such, each carotid plaque was collected freshly in operating theatre as soon as explanted and was washed in ice-cold sterile PBS to immediately remove blood. Plaque was then briefly inspected and divided at the level of maximal stenosis, with the proximal half of the plaque (including the bifurcation as the matching landmark) processed for paraffin embedding for histology (see Chapter 2.4.1) and the distal half snap immediately frozen in Optimal Cutting Temperature (OCT) medium (VWR, Lutterworth, Leicestershire, UK) using crushed dry-ice ethanol slurry for immuno-LCM. Time for inspection and documentation of macroscopic plaque features for MRI location matching (Chapter 2.6.4) was kept to a minimum to avoid RNA degradation. To minimize technique variation, immuno-LCM was carried

out in one large batch within 3 months towards the end of the study; as samples were collected in a period spanning three years, in order to ensure consistency of RNA quality, 2 early plaque samples were used as internal reference to check RNA quality using Agilent Bioanalyzer (see Chapter 2.2.5) at 6 monthly intervals to ensure minimal RNA degradation. RIN variations on these quality checks were ≤ 0.5 RIN value throughout the duration of the study.

4.3.2 Optimization of immuno-LCM specificity and LCM cell types

Equipment optimization and general methods of immuno-LCM were discussed in Chapter 2.5. In order to ascertain high degree of cell procurement specificity with immuno-LCM, preliminary LCM trial was first performed using guide-slides stained against CD68 (1:200, clone KP-1, Dako, Cambridge, UK) and smooth muscle alpha-actin (1:100, clone 1A4, Dako, Cambridge, UK). RNA was then extracted from (1) CD68-positive cells; (2) smooth muscle alpha actin expressing cells; and (3) whole mount tissue section from the same LCM sections. Differential enrichment of CD68 vs smooth muscle alpha actin gene on quantitative RT-PCR was then used as a proxy to assess cell procurement specificity. After immuno-LCM specificity was confirmed, the main batch of immuno-LCM experiments was carried out to procure the following cell types: (1) macrophages within or in the immediate border of the lipid-rich necrotic core (core-associated macrophages); (2) macrophages in the superficial cap or shoulder region of the plaques (cap-associated macrophages); (3) neo-intimal smooth muscle cells as internal controls (Figure 4.1).

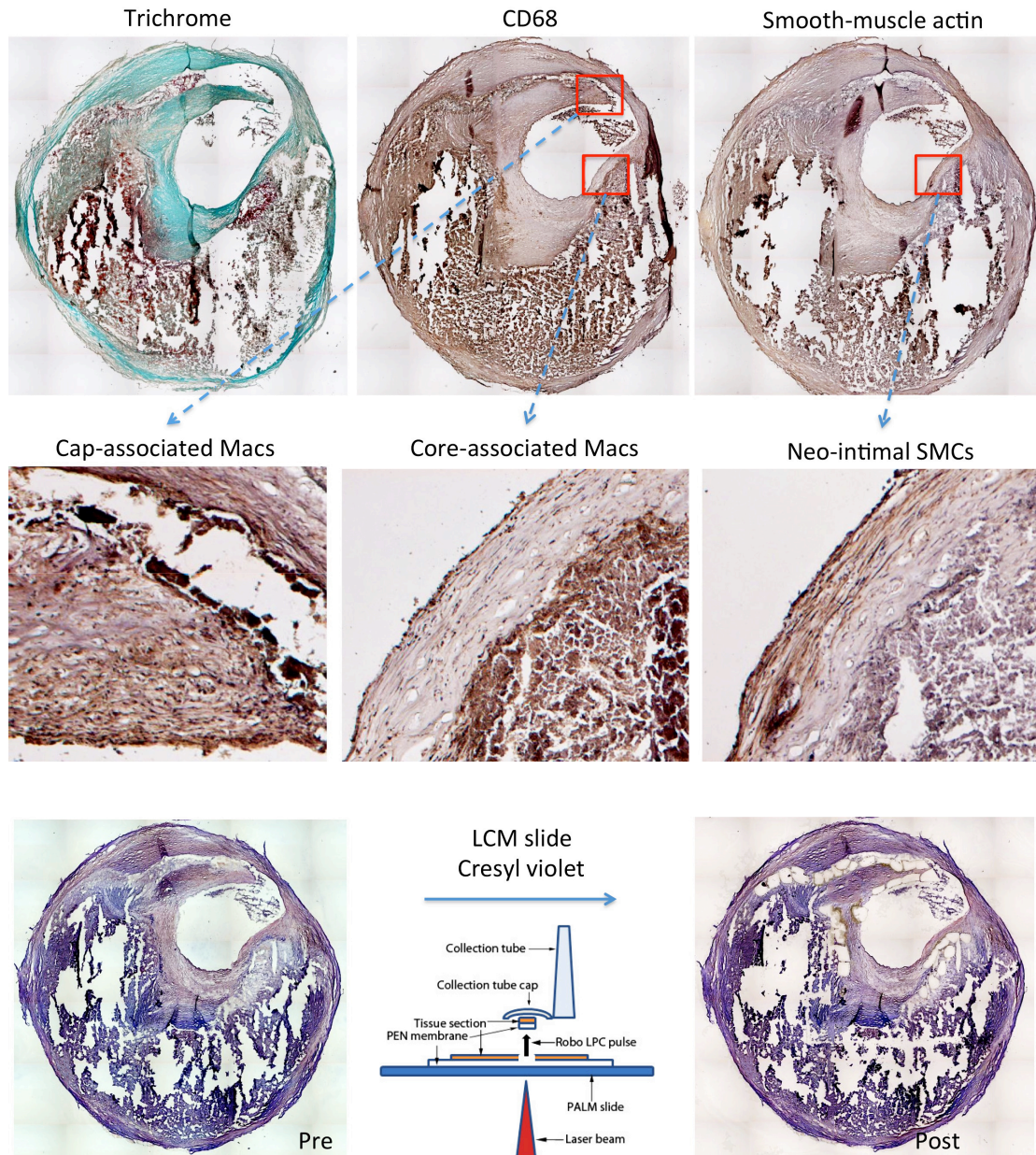


Figure 4.1 *Immuno-LCM using guide-slides*

Guide-slides (top panel) provide anatomical and immuno-phenotypical information to locate plaque macrophages and neo-intimal smooth muscles cells. Closer examination (middle panel) confirmed immunohistochemistry staining conformed with expected cell morphology of the selected cell types. Bottom panel showed cresyl violet stained LCM section on PEN slide before (left) and after (right) LCM. “Cut-out” elements on the post section (after LCM) corresponded to cell clusters captured by LCM. Images of LCM PEN slides were taken dry without coverslips and liquid mountant, hence the low colour contrast seen here.

4.3.3 RNA quality control for immuno-LCM samples

Laser-captured cell clusters were catapulted onto AdhesiveCap™ Opaque (Cat no. 415190-9201-000, Carl Zeiss Microscopy, Germany), using a 'no-touch' approach. The duration of time each LCM slide was stained, dehydrated, and exposed to room temperature was carefully controlled to maintain high RNA quality. The mean "stain-to-lysis" time (the total duration of a LCM slide from leaving of the cryostat chamber to cell lysis) was 68 ± 21 minutes. Cells were directly lysed with 350 μ L of RLT buffer (RNEasy Micro Kit, Qiagen, Crawley, UK) by inverting the AdhesiveCap microtube for 30 minutes in room temperature. Samples were then spun at 12,000 rpm for 5 minutes and stored at -80 °C for batch RNA extraction and purification as per Chapter 2.2. As part of the quality control for the LCM staining protocol, an unstained pre-LCM section was to obtain for a pre-LCM RIN, and a stained post-LCM remnant section was also obtained for a post-LCM RIN. The difference in pre-LCM RINs, post-LCM RINs, and the LCM sample RINs gave an indication of the extent of RNA degradation and where in the immuno-LCM process it had occurred. Immuno LCM was attempted in a total of 39 plaques (20 symptomatic and 19 asymptomatic), of which 11 plaques were excluded at the guide-slide preparation stage due to excessive calcification and / or lack of macrophage clusters. Immuno LCM was completed for 28 plaques (15 symptomatic and 13 asymptomatic). Four (14%) plaques had RNA quality below the minimum threshold of $RIN < 5.0$ and were excluded from downstream analysis. Table 4.1 shows the RNA quality of the remaining 24 LCM samples from human plaque macrophages and corresponding pre-LCM whole section QC RINs.

Plaque status	Sample ID	RNA code	Cell type	LCM Area ($\times 10^6 \mu\text{m}^2$)	RNA conc (pg / μL)	RIN value
Symptomatic	C063F1	32	Core macrophages	8	204	7.1
Symptomatic	C063F1	30	Whole section QC	-	309	7.0
Symptomatic	C060F1	68	Core macrophages	3.1	195	5.4
Symptomatic	C060F1	70	Whole section QC	-	1226	6.8
Symptomatic	C057F2	71	Core macrophages	5.7	634	7.5
Symptomatic	C057F2	77	Whole section QC	-	3563	7.8
Symptomatic	C034F1	79	Cap macrophages	4.3	754	6.0
Symptomatic	C034F1	80	Core macrophages	5.1	1812	7.0
Symptomatic	C034F1	86	Whole section QC	-	3882	8.0
Symptomatic	C035F1	87	Cap macrophages	4.1	565	6.9
Symptomatic	C035F1	93	Whole section QC	-	1888	8.1
Symptomatic	C027F2	103	Core macrophages	3.6	1236	5.5
Symptomatic	C027F2	110	Whole section QC	-	2328	8.9
Symptomatic	C023F1	112	Cap macrophages	3.2	929	6.3
Symptomatic	C023F1	120	Whole section QC	-	1583	8.9
Symptomatic	C021F1	122	Cap macrophages	4	495	8.1
Symptomatic	C021F1	127	Whole section QC	-	3173	8.0
Symptomatic	C019F1	137	Core macrophages	5.6	648	6.0
Symptomatic	C019F1	143	Whole section QC	-	1248	7.0
Symptomatic	C011F1	151	Cap macrophages	5.5	1205	7.5
Symptomatic	C011F1	155	Core macrophages	5.9	2148	6.8
Symptomatic	C011F1	159	Whole section QC	-	7536	6.2
Asymptomatic	C049F1	160	Core macrophages	5.9	964	6.6

Asymptomatic	C049F1	164	Whole section QC	-	4107	5.7
Asymptomatic	C024F2	165	Core macrophages	4.9	919	5.2
Asymptomatic	C024F2	170	Whole section QC	-	527	6.5
Asymptomatic	C070F1	171	Cap macrophages	6.4	233	6.7
Asymptomatic	C070F1	177	Whole section QC	-	2979	7.3
Asymptomatic	C018F1	178	Cap macrophages	6	1359	8.6
Asymptomatic	C018F1	179	Core macrophages	7.6	1493	7.4
Asymptomatic	C018F1	186	Whole section QC	-	10339	6.2
Asymptomatic	C017F2	189	Cap macrophages	6.8	5365	6.2
Asymptomatic	C017F2	194	Core macrophages	5.5	1644	6.0
Asymptomatic	C017F2	196	Whole section QC	-	6119	8.4
Asymptomatic	C055F2	212	Cap macrophages	4.8	464	7.0
Asymptomatic	C055F2	219	Whole section QC	-	2416	6.1
Asymptomatic	C040F2	220	Cap macrophages	6.4	723	6.7
Asymptomatic	C040F2	226	Whole section QC	-	444	6.1
Asymptomatic	C052F1	228	Core macrophages	6.5	1213	5.7
Asymptomatic	C052F1	235	Whole section QC	-	3230	8.5
Asymptomatic	C068F2	236	Cap macrophages	7.9	631	7.8
Asymptomatic	C068F2	246	Whole section QC	-	580	7.9
Asymptomatic	C046F1	247	Cap macrophages	8.2	2703	6.5
Asymptomatic	C046F1	252	Whole section QC	-	429	8.0

Table 4.1 **Quality control of human carotid plaque macrophages RNA**

The average RIN was similar between macrophages procured from symptomatic and asymptomatic plaques (6.26 ± 1.07 vs. 6.49 ± 0.93 , ns). The average pre-LCM sample quality control RIN was 7.27 ± 0.96 . The average RIN drop attributable to immuno-LCM protocol was 1.05 ± 1.03 . (mean $\pm$ SD)

4.3.4 Linear RNA amplification and microarray

24 RNA samples were submitted to Cambridge Genomic Services, Department of Pathology, University of Cambridge, UK, for amplification (see Chapter 2.2.9) and hybridization. The 2 x 2 experimental grid contained two reciprocal comparisons: macrophages from symptomatic plaques vs. macrophages from asymptomatic plaques; and core-associated macrophages vs. cap-associated macrophages, as defined in Chapter 4.3.2. Figure 4.2 showed the experimental grid and cell type distribution.

	Core-associated macrophages	Cap-associated macrophages	Total
<i>Symptomatic</i>	7	5	12
<i>Asymptomatic</i>	5	7	12
Total	12	12	24

Figure 4.2 LCM 2 x 2 sample grid for microarray experiment

Successfully amplified RNA was hybridized to two Illumina Human HT12 v4.0 BeadChips (Illumina, San Diego, USA). Samples were then randomly allocated to the two BeadChips to minimize “chip effect”. BeadChips were then scanned using Illumina BeadChip Reader on the same day with the same reader. GenomeStudio V2010.1 (Illumina, San Diego, USA) was used for data extraction.

4.3.5 Approach of LCM-transcriptomic analysis

Figure 4.3 outlines schematically the key steps undertaken in the LCM-transcriptomic experiment using a flowchart from tissue immunohistochemistry-guided laser capture microdissection to analysis of gene expression data. Each of the key steps in the flowchart, except those already described in Chapter 4.3.3 and 4.3.4, will be discussed in more detail in the subsequent sections.

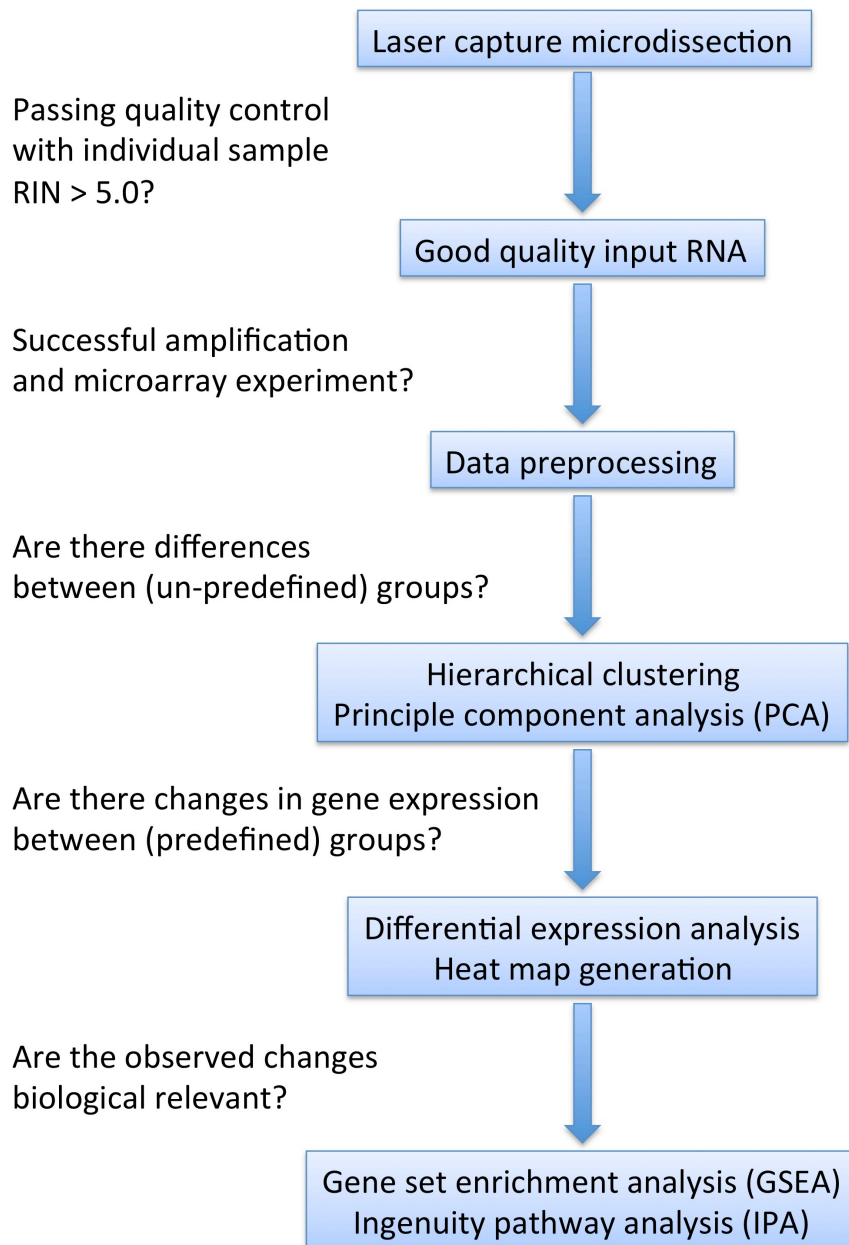


Figure 4.3 **Key steps in LCM-transcriptomic experiment**

LCM samples with sufficient RNA quality were subjected to linear amplification and microarray hybridization. Data were first pre-processed to allow for further analysis. Unsupervised hierarchical clustering and principal component analysis (PCA) was used to see if there was a difference of gene expression pattern in the experimental groups. Tool marker selection was then used to generate a list of differentially expressed genes between groups and a corresponding heat map for data visualization. To further explore the biological significance of the difference in gene expression, gene set enrichment analysis and ingenuity pathway analysis were used to identify biological pathways or processes that were significantly positively or negatively correlated in different macrophage subsets (core vs. cap) in either symptomatic or asymptomatic plaques.

4.3.6 Generation of expression data files

After the BeadChip was scanned, raw fluorescence intensity data (.idat) files for each sample were extracted using GenomeStudio V2010.1 (Illumina, San Diego, USA). Each idat file contained the raw fluorescence value for each probe for each biological sample on the BeadChip. Probe unique identifiers were then “mapped” to gene symbols using the correct array annotation manifest file in GenePattern (Broad Institute, Cambridge, MA, USA) using the “Illumina Expression File Creator” pipeline using default settings. Where multiple probes for each gene were present, they are individually represented in the expression data file. The resulting output files generated were (1) a tab-delimited text file (.gct) file containing the entire dataset of gene expression values for each sample; and (2) multiple class definition files (.cls) for each class comparison. The latter contained the grouping or phenotypic class that each sample belonged to in each comparison. While the “cls” files were not needed for unsupervised clustering and principal component analysis, they were necessary for differential gene expression and pathway analysis (see below).

4.3.7 Unsupervised hierarchical clustering

The methods of hierarchical clustering were discussed in Chapter 2.7.2. In unsupervised hierarchical clustering, no class definition was supplied to the clustering algorithm. Clustering was achieved mathematically by first calculating the distance matrix between gene expression datasets. Once this matrix of distances was computed, repeated agglomerative hierarchical processing cycles began, where the two closest (most similar) datasets were joined by a node or branch of a tree diagram. The output tree therefore clustered similar datasets close to each other, and the connecting branch would lengthen as their similarity decreases.

4.3.8 Principal component analysis

Principal component analysis (PCA) is a widely used statistical method to elucidate the co-variance structure of datasets. It does so by mathematically computing the eigenvectors and eigenvalues of the data covariance matrix, and allows reduction of the dataset dimensionality and representation of the co-variance of multi-dimensional datasets in a 3-dimensional space. The computational process is equivalent to finding the axis system (principal directions) in which the co-variance matrix is diagonal, i.e. showing the greatest variance. The eigenvector with the greatest eigenvalue is the direction of the greatest variation; the one with the second greatest eigenvalue is the (orthogonal) direction with the next highest variation and so on. This orthogonal transformation converts a set of observations (e.g. gene expression data) that are possibly correlated into a set of values of linearly uncorrelated variables called the principal components. The total number of principal components will be equal or less than the number of variable in the datasets (in this case genes). PCA analysis was performed using the PCA pipeline in GenePattern (Broad Institute, Cambridge, MA, USA) for the top three principal components – which accounts for 99% of the variance in the datasets.

4.3.9 Differential gene expression analysis and heat maps

Each sample was assigned a group and was analysed using the “ComparativeMarkerSelection” pipeline in GenePattern (Broad Institute, Cambridge, MA, USA). Lists of genes differentially expressed between groups were generated and explored using the “ComparativeMarkerSelectionViewer” module in Java runtime environment (Oracle Corporation, USA). For each differentially expressed gene, the statistical significance of the difference was calculated using two-tailed *t-test* with the resulting P values. To account for multiple hypothesis testing, and to avoid falsely identifying non-marker genes as marker genes, False Discovery Rate (FDR)

adjusted P values (FDR Q values) were also computed. Fold change (FC) was calculated as the normalized expression ratio of a gene between two groups. Heat maps of the most differentially expressed genes illustrating expression difference between groups were generated using the “HeatMapView” module in GenePattern (Broad Institute, Cambridge, MA, USA) with the list of most differentially expressed genes generated above. Unless specified otherwise, significant differential expression was defined as $P < 0.05$, $FDR < 0.25$, $FC > 1.5$.

4.3.10 Pathway analysis

4.3.10.1 Gene set enrichment analysis (GSEA)

The principles and methods of gene set enrichment analysis were discussed in Chapter 2.7.5.

4.3.10.2 Ingenuity Pathway Analysis

Ingenuity® Pathway Analysis (IPA) is a web-based software application by Qiagen Silicon Valley, Redwood City, CA, USA, for analysis, integration, and interpretation from data generated from “omics” experiments. It helps identify key regulators and activity to explain expression patterns, and predict downstream effects on biological and disease processes. It does so by leveraging the power of the Ingenuity® Knowledge Base, which integrates information from published scientific journals, curated pathway databases (e.g. Gene Ontology), and public molecular databases (e.g. Entrez), to generate a unique Ingenuity® Ontology that links patient phenotypes, disease mechanisms, cellular mechanisms, molecular mechanisms, and sequence mechanisms in a vertically structured meta-database.

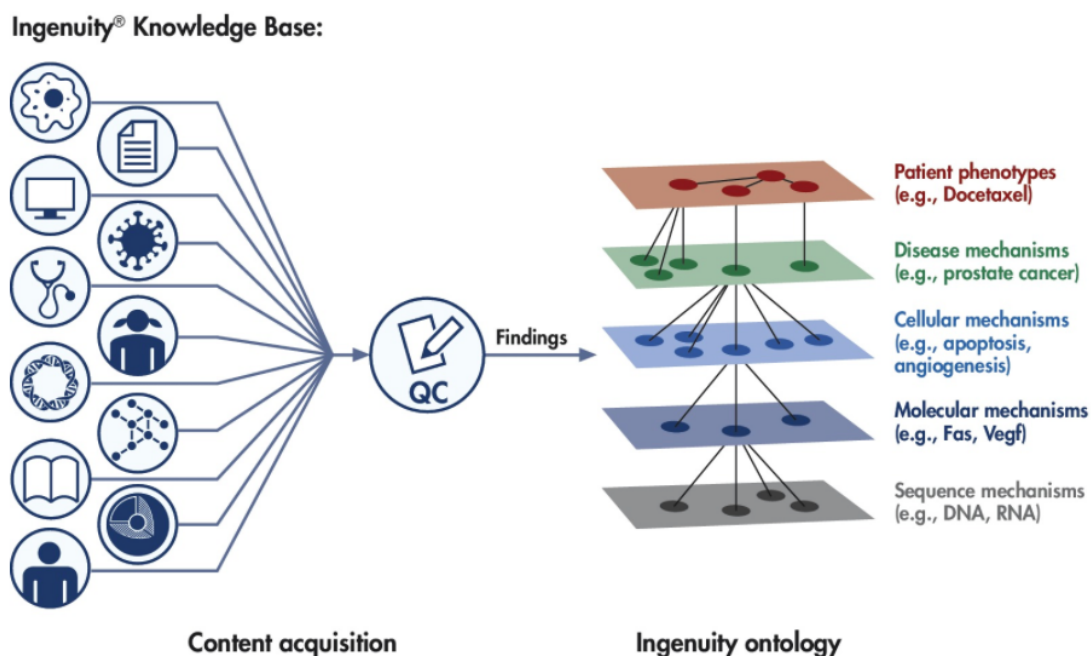


Figure 4.4 The Ingenuity® Knowledge Base

The Ingenuity® Knowledge Base regularly updates and obtains data from different sources. There are four categories of how content acquisition is organized: (1) Expert Findings: experimentally demonstrated findings that are manually curated and reviewed from the full-text of articles from top scientific journals; (2) ExpertAssist Findings: manually reviewed, automatically extracted findings from the abstracts of a broad range of recently published papers; (3) Expert Knowledge: knowledge of signaling and metabolic pathways, drug/target/disease relationships manually curated by in-house experts; (4) Supported third-party information: manually reviewed content from selected public and private databases. This enormous amount of information was used to construct the Ingenuity® Ontology that makes information computationally accessible. Ingenuity® Knowledge Base currently contains >4,000 curated journal articles and > 8.5 million findings. Figure reproduced from Qiagen's website <http://www.ingenuity.com/science/knowledge-base>

4.4 Results

4.4.1 RNA quality control and RNA yield from LCM

The average RIN was similar between macrophages procured by immuno-LCM from symptomatic and asymptomatic plaques (6.26 ± 1.07 vs. 6.49 ± 0.93 , $P = \text{n.s.}$). The average pre-LCM sample quality control RIN was 7.27 ± 0.96 . The average RIN drop attributable to the immuno-LCM protocol was 1.05 ± 1.03 (mean $\pm$ SD), which is consistent comparable with that reported in the literature. The average RIN of neointimal smooth muscle cells procured by immuno-LCM was 4.89 ± 0.92 , significantly lower than that of plaque macrophages. This difference likely reflected that anatomically neo-intimal smooth muscle cells were in close contact with the vessel lumen and were therefore more exposed to degradation caused by tissue handling during surgery and plaque processing; whereas plaque macrophages, whether cap associated or core associated subsets, were more protected by the overlying / surrounding tissue stroma. In addition, neointimal smooth muscle cells were often found in area with dense collagen fibers. This tougher / denser tissue required higher laser energy to cut and catapult during LCM, which might have further contributed to RNA degradation. There was no significant difference between RNA qualities between core-associated and cap associated macrophages (6.35 ± 0.78 vs. 7.03 ± 0.78 , $P = \text{n.s.}$)

RNA yield was influenced by the total laser-capture area and the thickness of the tissue sections. In general terms, thinner sections (e.g. 8 - 10 μm) are easier to cut but contain less cellular material resulting in less RNA yield; conversely, thicker sections (e.g. 15 - 20 μm) contain more cellular material per unit area with higher RNA yield but sometimes require multiple pass of the laser beam or increased laser energy to efficiently cut and catapult the tissue fragment against gravity to the

AdhesiveCaps. With extensive and successive rounds of optimization, a tissue section thickness between 12 - 15 μm was found to be the ideal balance between RNA yield and laser cutting efficiency. Figure 4.5 showed the relationship between LCM area and RNA yield. To allow sufficient spare capacity for downstream applications, about $5 \times 10^6 \mu\text{m}^2$ per sample were laser-captured from 15 μm thick tissue sections to yield an average of 10ng of total RNA eluted to a final concentration of 1 ng / μL following the RNEasy Micro kit protocol (Qiagen, Crawley, UK).

Pre-LCM sample RIN (mean $\pm$ S.D.)	7.27 $\pm$ 0.96
Average post-LCM RIN (<i>symptomatic</i> , mean $\pm$ S.D.)	6.26 $\pm$ 1.07
Average post-LCM RIN (<i>asymptomatic</i> , mean $\pm$ S.D.)	6.49 $\pm$ 0.93
Average RIN drop due to my LCM protocol (mean $\pm$ S.D.)	1.05 $\pm$ 1.03

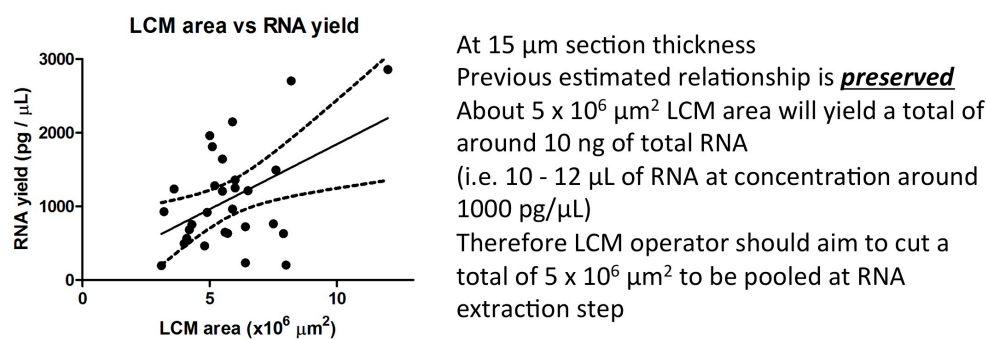


Figure 4.5 **LCM statistics for RNA yield and quality**

4.4.2 Cell procurement is highly specific with immuno-LCM

To ascertain cell procurement specificity, macrophages and smooth muscle cells identified using the immuno-LCM protocol for this study were analysed using quantitative RT-PCR to check for specificity. Differential expression of CD68 and α -actin genes was used to assess cell procurement specificity. First, it was shown that CD68 transcript was selectively enriched in laser-captured cells from plaque macrophage (CD68 immuno-positive) area compared to whole tissue sections (Figure 4.6A). Subsequently, LCM was used to separately procure cells from plaque macrophage (CD68 immuno-positive) area and smooth muscle cell (smooth muscle α -actin immuno-positive) area from the same plaque samples. Figure 4.6B showed the differential enrichment of CD68 and α -actin transcripts in cells from their respective LCM procurement area. This demonstrated that the immuno-LCM protocol used in this study was capable of procuring different cell types with high degree of specificities.

4.4.3 Microarray validation using quantitative RT-PCR

Since the quantity of RNA extracted from immuno-LCM was very small, RNA was subjected to amplification prior to microarray experiment. To ensure RNA amplification maintained high fidelity and linearity, single gene quantitative RT-PCR was carried out on the original, unamplified, RNA for 8 genes (N = 6 per group) for both symptomatic *versus* asymptomatic, and for cap *versus* core comparisons (Figure 4.7 and 4.8). Genes were chosen based on the microarray data with those representing a significant fold-change (>1.5) between groups. Validation PCR confirmed good correlation between microarray results and single gene quantitative RT-PCR. With the confirmation that the amplification has been technically successful and that the microarray data represented the unamplified transcriptome in its *ex-vivo* biological state, further downstream analysis was performed as per Figure 4.3.

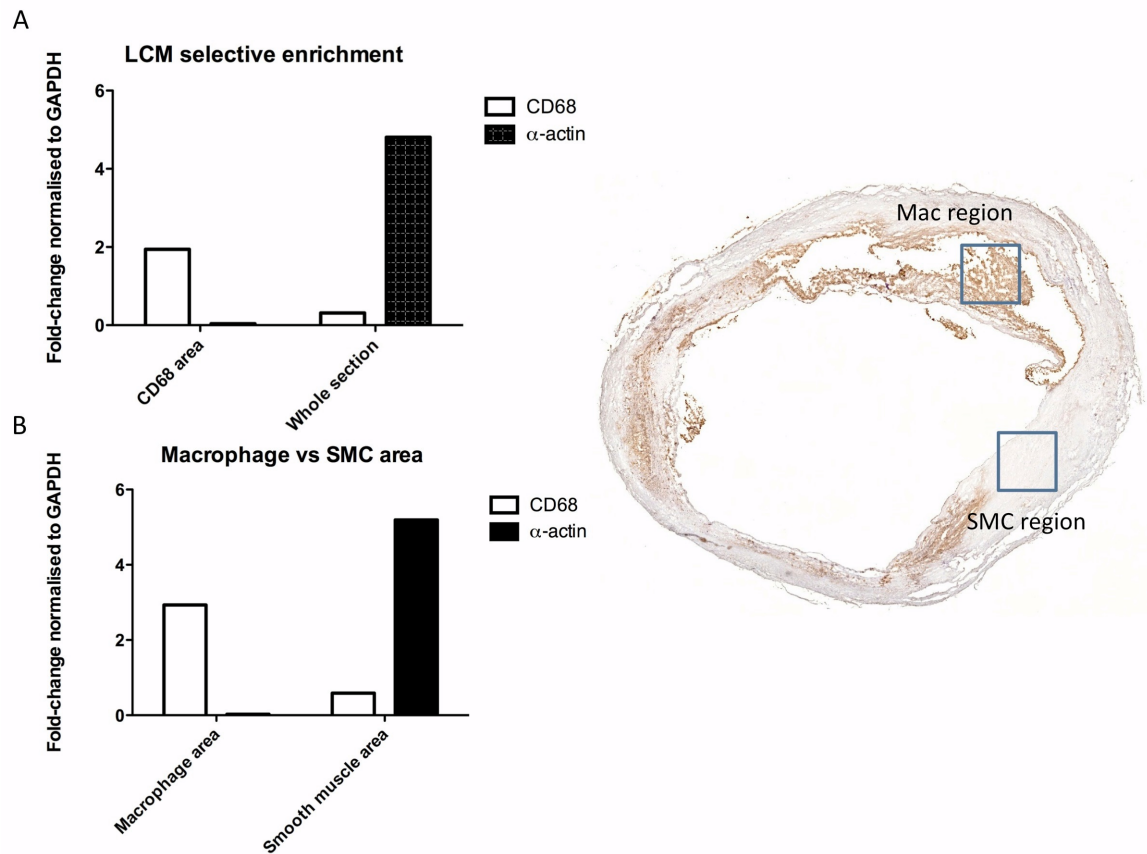


Figure 4.6 Selective enrichment by LCM

A. Selective enrichment of CD68 transcripts in plaque macrophage (CD68 immuno-positive) region compared to whole tissue section. **B.** Differential expression of CD68 and α -actin transcripts from cells procured from macrophage (Mac) region vs. smooth muscle cells (SMC) (smooth muscle α -actin immuno-positive) region.

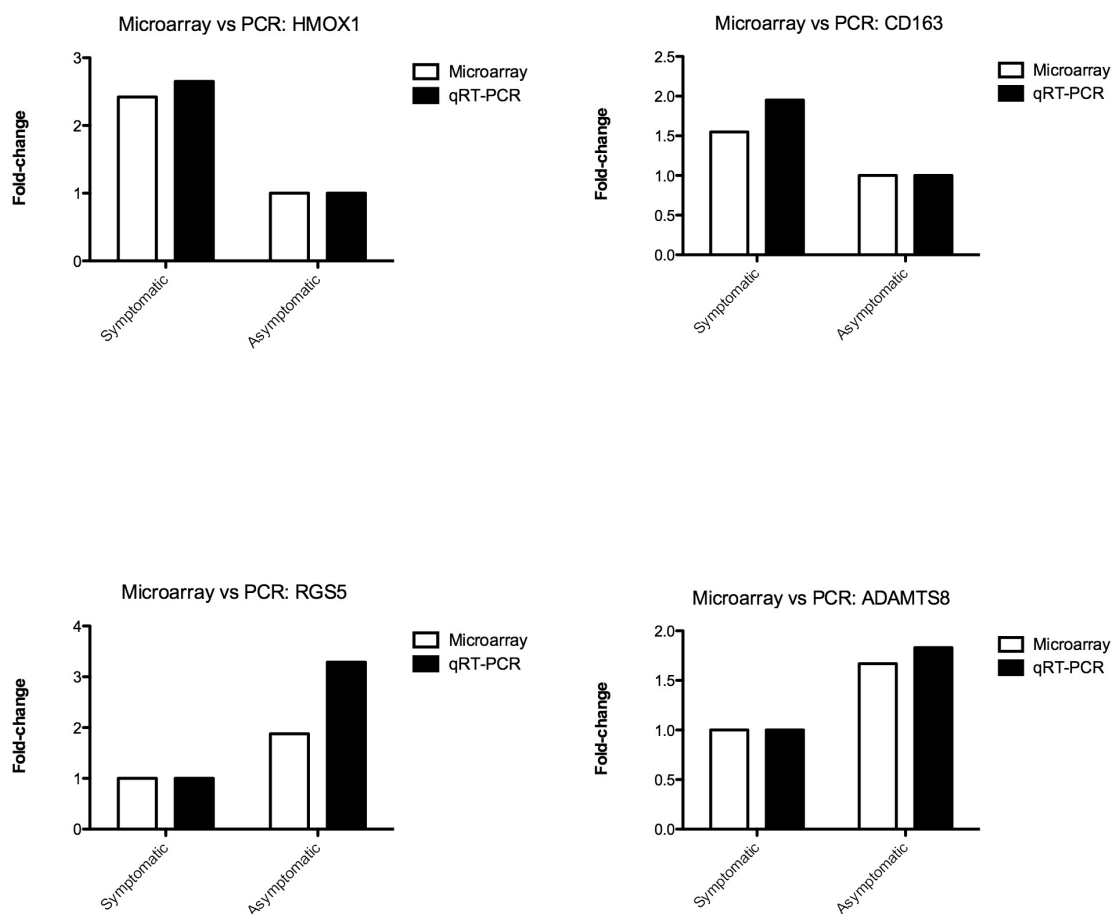


Figure 4.7 Single gene validation PCR of microarray experiment

Symptomatic versus asymptomatic comparison

Single gene PCR for 4 genes was performed on the unamplified RNA. The results showed excellent correlation with the microarray findings of the same biological samples. (N = 6 per group for qRT-PCR and 12 per group for microarray). HMOX-1: haemoxygenase-1. CD163: scavenger receptor cysteine-rich type 1 protein M130. RGS5: regulator of G-protein signaling 5. ADAMTS8: ADAM metalloproteinase with thrombospondin type 1 motif 8.

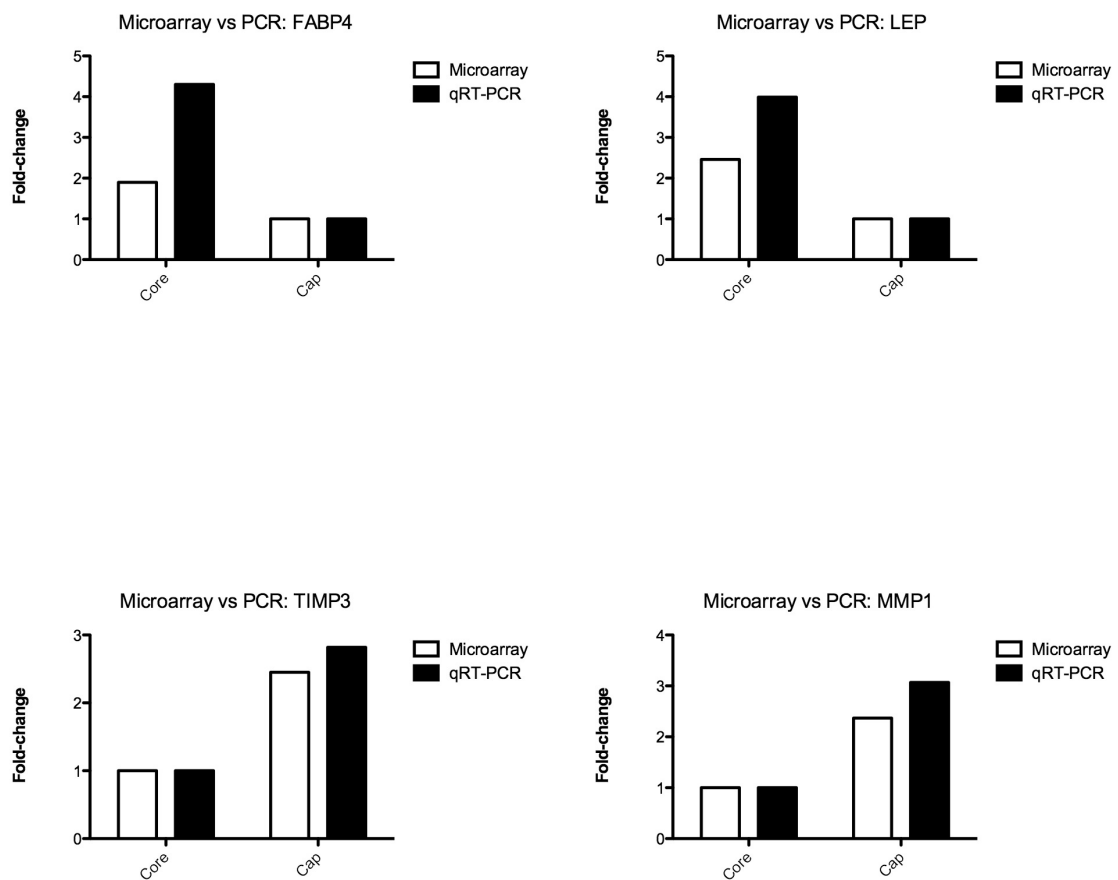


Figure 4.8 Single gene validation PCR of microarray experiment

Core versus cap comparison

Single gene PCR for 4 genes was performed on the unamplified RNA. The results showed excellent correlation with the microarray findings of the same biological samples. (N = 6 per group for qRT-PCR and 12 per group for microarray). FABP4: fatty acid binding protein 4. LEP: leptin. TIMP3: tissue inhibitor of metalloproteinases 3. MMP1: matrix metalloproteinase 1.

4.4.4 Unsupervised hierarchical clustering

As a first step in the exploratory analysis of the microarray data, unsupervised agglomerative hierarchical clustering of samples were performed with the aim to find natural / biological similarities between samples and to detect outliers. The term “unsupervised” refers to the way clustering was performed using statistical methods based on similarities between datasets with no prior knowledge of the identity of the biological samples / groupings.

Agglomerative hierarchical clustering is a “bottom-up” approach. Each gene expression datasets began in its own cluster, and pairs of clusters were merged as one moved up the hierarchy. Pairing or merging of clusters (columns) was determined by the distance matrix (a measure of similarity between gene expression profiles) by calculating the Pearson’s correlation between gene expression profiles. Clusters with the highest Pearson’s correlation (most similar) were grouped to form a new cluster. Clusters were then paired in recursive rounds of merging based on the average distance between all members of one cluster and all members of another cluster (average linkage) until a single cluster emerged. The results of hierarchical clustering are represented as a dendrogram, or inverted tree diagram, where the individual “leafs” at the bottom represents individual datasets (samples). They are connected in “nodes” (union of clusters), and eventually all connected to the “root” at the top to represents the entirety of all clustered datasets. The heights of the “nodes” represents the similarity or distance between two or more merged sub-clusters.

Figure 4.9 shows the unsupervised agglomerative hierarchical clustering of our datasets using Pearson correlation for distance matrix calculation and average linkage in cluster pairing.

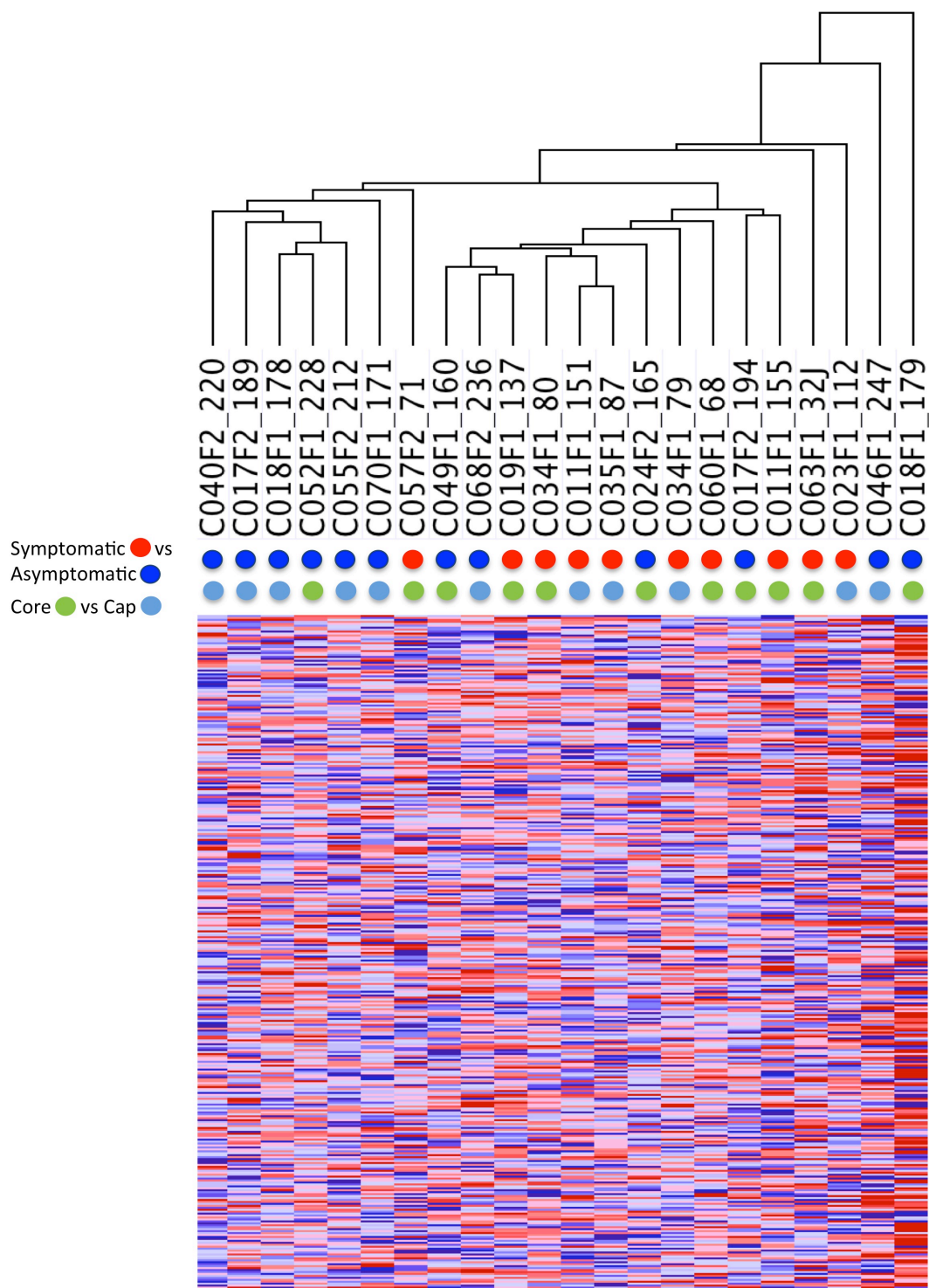


Figure 4.9 *Unsupervised agglomerative hierarchical clustering*

The dendrogram of the transcriptomes from human plaque macrophages. Symptom status and macrophage location were included beneath the “leafs” using coloured circles to highlight the clustering results.

As an initial explorative analysis, hierarchical clustering demonstrated that there was a fair degree of clustering of gene expression profiles, or transcriptomes, between macrophages harvested from symptomatic plaques compared to that from asymptomatic plaques. On the other hand, clustering appeared to reflect poorly the location from which the macrophages were harvested from; there was a lack of clustering of transcriptomes between core-associated and cap-associated macrophages. These results reflected the greater variance between macrophage transcriptomes according to symptom status (i.e. with greater biological signal); and the more subtle (i.e. with less biological signal) or more heterogeneous (i.e. with more noise) transcriptome changes based on macrophage locations.

Given the patient characteristics of this study and the heterogeneity of plaque macrophages, the findings above were not entirely surprising. To further explore the co-variance structure of the datasets, principal components analysis (PCA) was performed.

4.4.5 Principal components analysis (PCA)

Principal components analysis demonstrated that 99% of the variance in the datasets was accounted for by the first three principal components. Visual assessment of the PCA plots (Figure 4.10) confirmed that macrophage transcriptomes from symptomatic and asymptomatic plaques showed separation except for 4 outliers (marked by white arrowheads). The separation plane was marked by red dotted line. As predicted and in agreement with the hierarchical clustering, transcriptomes from core-associated and cap-associated macrophages showed poor separation in PCA analysis (Figure 4.11).

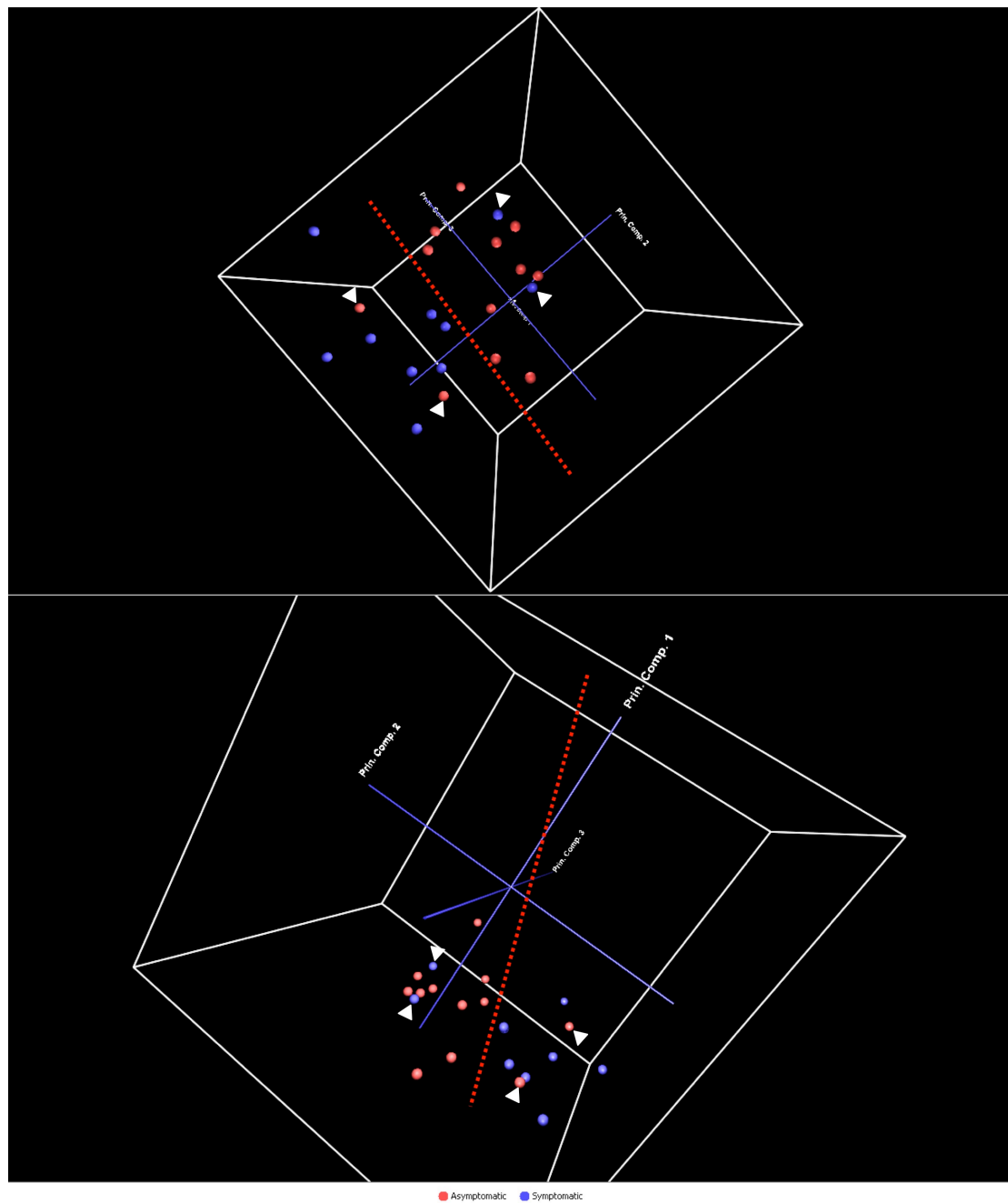


Figure 4.10 *PCA analysis of macrophage transcriptomes from symptomatic plaques compared to asymptomatic plaques*

Each red dot represented a dataset from a symptomatic plaque and each blue dot represented a dataset from an asymptomatic plaque. White arrowheads indicated outliers. Red dotted line represented the line / plane of separation. There was visual separation on the PCA plot except the 4 outliers.

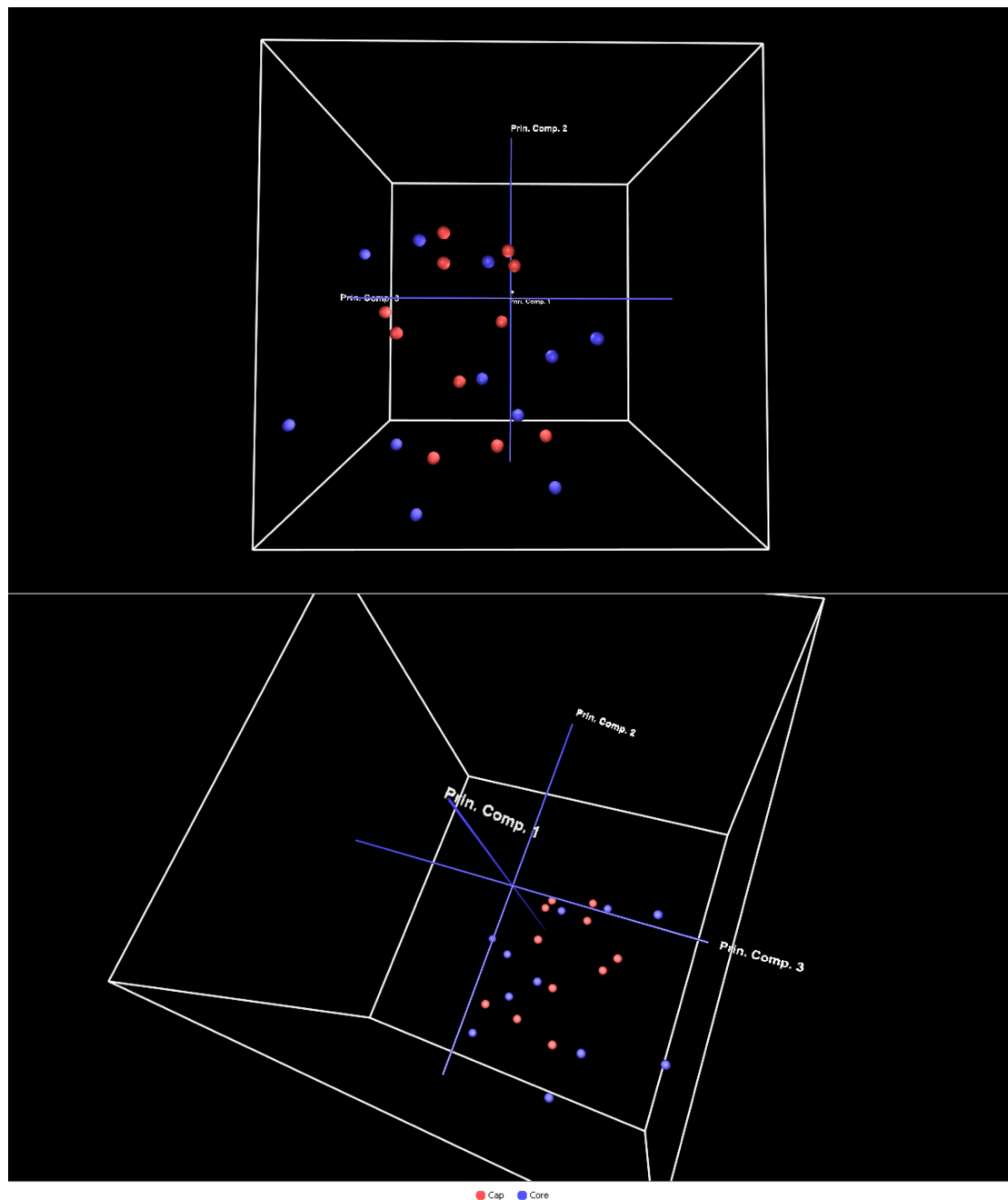


Figure 4.11 *PCA analysis of transcriptomes from core-associated macrophages compared to cap-associated macrophages*

Each red dot represented a dataset from cap-associated macrophages and each blue dot represented a dataset from core-associated macrophages. There was poor visual separation of the datasets.

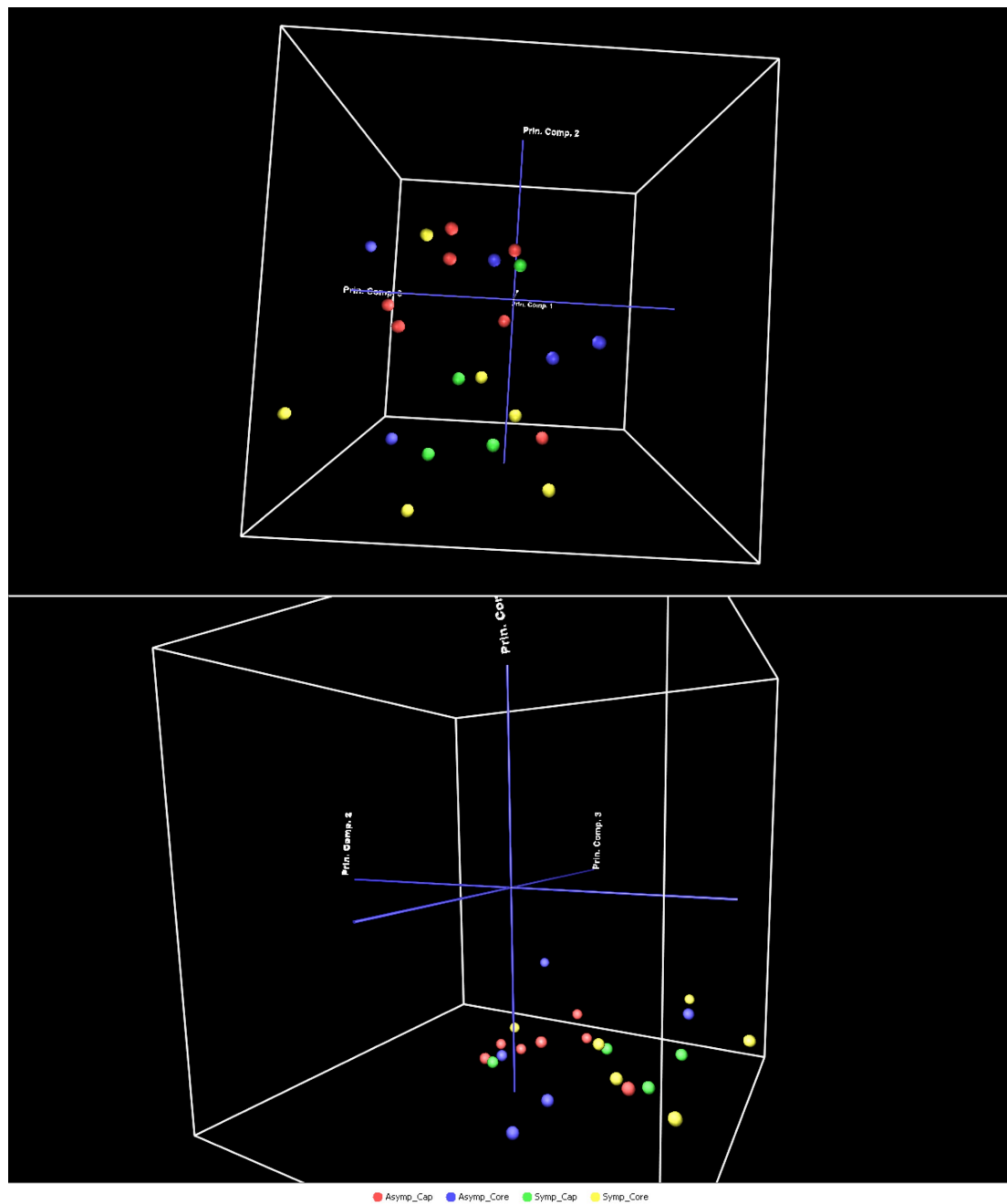


Figure 4.12 *PCA analysis of all 4 subgroups*

Each red dot represented a dataset from asymptomatic cap-associated macrophages; each blue dot represented a dataset from asymptomatic core-associated macrophages; each green dot represented a dataset from symptomatic cap-associated macrophages; and each yellow dot represented a dataset from symptomatic core-associated macrophages. There was poor visual separation of the datasets.

The results of the principal components analysis were in agreement with the hierarchical clustering analysis described earlier in Chapter 4.4.4, suggesting a greater biological signal or difference existing between macrophage transcriptomes based on symptom status (symptomatic *versus* asymptomatic) rather than on macrophage locations (core- *versus* cap-associated) within the plaques. Figure 4.12 confirms that further division of the datasets into 4 subgroups: asymptomatic, core; asymptomatic, cap; symptomatic, core; and symptomatic, cap, for downstream analysis was unlikely to yield statistically significant biological signal as the datasets became overlapping with very little separation on visual PCA assessment. Further analyses were then primarily focused to identify differentially expressed genes and pathways between the two phenotypic comparisons: symptomatic *versus* asymptomatic; and core- *versus* cap-associated.

4.4.6 Identification of differentially expressed genes

Differentially expressed genes were identified using the “ComparativeMarkerSelection” pipeline in GenePattern (Broad Institute, MIT, Cambridge, MA, USA). Lists of genes differentially expressed between groups were generated and explored using the “ComparativeMarkerSelectionViewer” module in Java runtime environment (Oracle Corporation, USA). Preliminary exploration of the dataset using established parameters ($P < 0.05$, $FDR < 0.25$, and $FC > 1.5$) to define statistical significance revealed no significantly differentially expressed gene between groups. In view of the limited sample size and heterogeneity of the human plaque macrophages procured by LCM, the dataset was further explored using statistical parameters $P < 0.05$ and $FC > 1.5$ only.

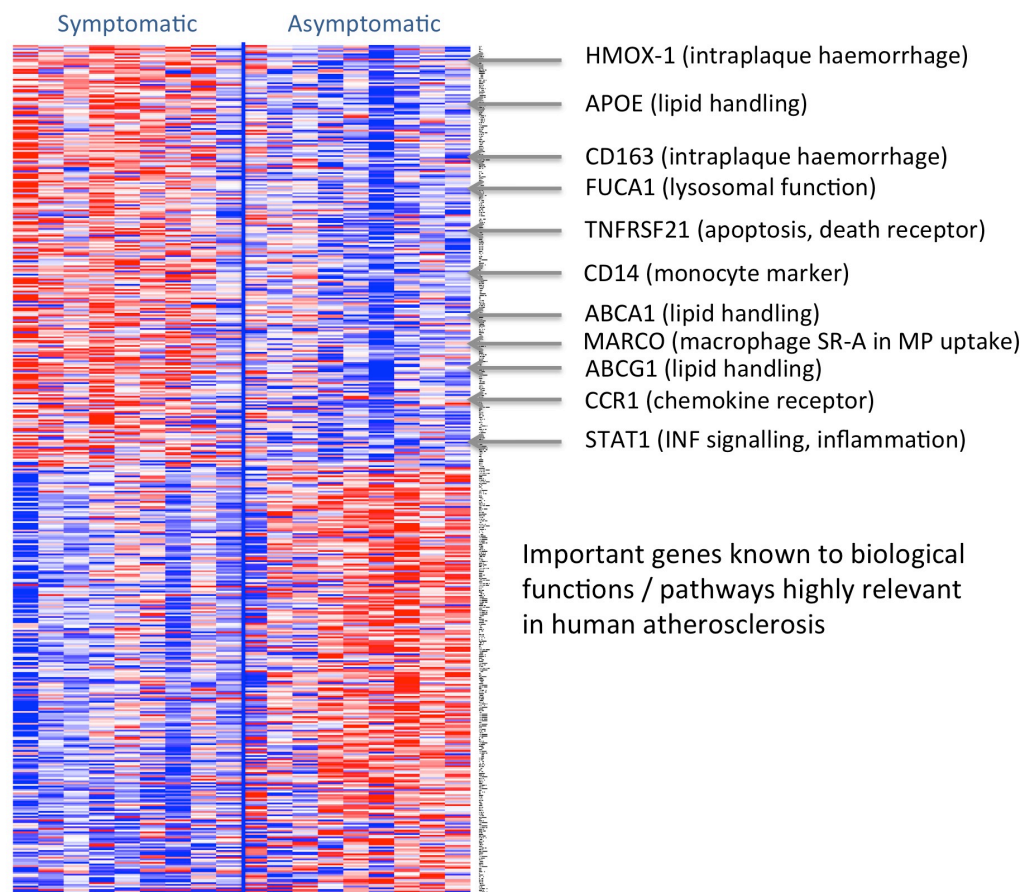


Figure 4.13 Heat map of the top 500 most differentially expressed genes in macrophages from symptomatic versus asymptomatic human carotid plaques

Each column represents a separate biological sample and each row represents a gene. The heat map illustrates the most differentially expressed (by fold-change alone) top 250 genes in symptomatic group and top 250 genes in asymptomatic group. Red indicates upregulation whereas blue indicates downregulation of a gene of interest.

Analysis of the human plaque macrophage microarray data revealed 42 genes that were significantly differentially expressed ($P < 0.05$, $FC > 1.5$) between symptomatic and asymptomatic group, of which 34 genes were upregulated in symptomatic group and 8 genes were upregulated in asymptomatic group.

Likewise, 44 genes were found to be significantly differentially expressed ($P < 0.05$, $FC > 1.5$) between core and cap macrophages, of which 11 genes were upregulated in the core macrophages and 33 genes were upregulated in the cap macrophages.

Gene ID	Rank	P-value	FDR	Fold Change
CR1	1	6.00E-04	0.900752522	1.81820925
CSF2RA	2	4.00E-04	0.900752522	1.512398389
PQLC3	3	0.00159968	0.900752522	1.574755775
CXCL10	4	0.00239952	0.900752522	1.909782122
MGAT4A	5	0.0029994	0.900752522	1.510095281
LOC653907	6	0.00239952	0.900752522	1.730322055
ASGR1	7	0.00619876	0.900752522	1.739362037
HS.197143	8	0.00539892	0.900752522	1.545918493
HS.30495	9	0.00339932	0.900752522	1.727772326
MRPS36	10	0.00539892	0.900752522	1.502515847
FAH	11	0.00519896	0.900752522	1.5400894
TNFRSF21	12	0.01059788	0.900752522	1.549606755
TMEM26	13	0.01079784	0.900752522	1.6218646
S100A9	14	0.00859828	0.900752522	1.587839723
P2RY1	15	0.01119776	0.900752522	1.685068964
IL18BP	16	0.01219756	0.900752522	1.678962078
KCNK13	17	0.012997401	0.900752522	1.642014905
CMKLR1	18	0.015596881	0.900752522	1.55020655

SIGLEC14	19	0.018396321	0.900752522	1.601572339
CXCL9	20	0.013597281	0.900752522	1.657413536
CA12	21	0.018996201	0.900752522	1.614450008
FOS	22	0.022195561	0.900752522	1.629033513
PRKCB1	23	0.025394921	0.900752522	1.5238851
S100A8	24	0.026794641	0.900752522	1.915434896
CD163	25	0.019996001	0.900752522	1.546048701
CCL8	26	0.033593281	0.900752522	1.828997247
CPVL	27	0.048990202	0.900752522	1.513454744
IL2RA	28	0.031593681	0.900752522	1.767966574
ADM	29	0.034593081	0.900752522	1.911592218
LOC730415	30	0.042791442	0.900752522	1.600649679
CTSL1	31	0.038392322	0.900752522	1.590778704
GLUL	32	0.045990802	0.900752522	1.721693621
PFKFB4	33	0.015996801	0.900752522	1.532377586
PLIN2	34	0.048990202	0.900752522	1.617896454

Table 4.2 34 genes significantly upregulated in plaque macrophages from symptomatic group (symptomatic *versus* asymptomatic; $P < 0.05$, $FC > 1.5$).

Gene ID	Rank	P-value	FDR	Fold Change
LOC651125	1	0.00359928	0.900752522	1.560245284
LOC653629	2	0.0019996	0.900752522	1.64190372
MIAT	3	0.012797441	0.900752522	1.560269193
ZNF215	4	0.01239752	0.900752522	1.519695181
LOC440345	5	0.021395721	0.900752522	1.621604727
CTSK	6	0.034393121	0.900752522	1.785485013
C3ORF15	7	0.00919816	0.900752522	1.628015537
PRMT1	8	0.029194161	0.900752522	1.840706057

Table 4.3 8 genes significantly upregulated in plaque macrophages from asymptomatic group (symptomatic *versus* asymptomatic; $P < 0.05$, $FC > 1.5$)

Gene ID	Rank	P-value	FDR	Fold Change
PRKCB1	1	0.00459908	0.999813551	1.670631436
SULT1A1	2	0.00339932	0.999813551	1.931961736
PLTP	3	0.01079784	0.999813551	1.518531662
TSPAN13	4	0.016796641	0.999813551	1.674445005
OAS1	5	0.035392921	0.999813551	1.601367537
ADM	6	0.036392721	0.999813551	1.941899218
BMP6	7	0.019596081	0.999813551	1.697444475
LEP	8	0.040391922	0.999813551	2.463812463
IL2RA	9	0.043591282	0.999813551	1.697894633
TXNDC5	10	0.020995801	0.999813551	1.612682596
PDK4	11	0.045190962	0.999813551	2.18225935

Table 4.4 11 genes significantly upregulated in core macrophages (core *versus* cap macrophages; P < 0.05, FC > 1.5).

Gene ID	Rank	P-value	FDR	Fold Change
ZBTB46	1	4.00E-04	0.999813551	1.763491082
CD8A	2	0.00339932	0.999813551	1.618356037
SKAP1	3	0.00419916	0.999813551	1.630506939
CD69	4	0.00479904	0.999813551	1.540462308
LOC653867	5	0.00519896	0.999813551	1.569682849
ADARB1	6	0.00459908	0.999813551	1.576606427
RPPH1	7	0.004999	0.999813551	1.542176075
MXRA5	8	0.00459908	0.999813551	2.034827375
RNU1F1	9	0.00519896	0.999813551	1.513684686
RN5S9	10	0.014997001	0.999813551	1.519576976
RNU1A3	11	0.01159768	0.999813551	1.595263061
COL6A3	12	0.019996001	0.999813551	1.716722865
GPER	13	0.0039992	0.999813551	1.627866992
C10ORF105	14	0.00379924	0.999813551	1.588882717

FNDC1	15	0.020395921	0.999813551	1.969494701
COMP	16	0.00719856	0.999813551	2.623943384
PMEPA1	17	0.014197161	0.999813551	1.501108534
TPR	18	0.00559888	0.999813551	1.816930839
SNORD46	19	0.021995601	0.999813551	1.632965377
SFRP4	20	0.036192761	0.999813551	2.428838074
RNU1-5	21	0.035792841	0.999813551	1.785582029
RNU1-3	22	0.036592681	0.999813551	1.772166856
LOC728649	23	0.038592282	0.999813551	1.509702582
RNU1G2	24	0.040591882	0.999813551	1.795155263
TIMP3	25	0.037192561	0.999813551	2.447272887
COL11A1	26	0.036992601	0.999813551	2.055902355
C6ORF186	27	0.042591482	0.999813551	1.540379338
PKIB	28	0.046190762	0.999813551	1.766845144
F2RL1	29	0.016596681	0.999813551	1.521339527
PTGDS	30	0.042591482	0.999813551	1.687117401
DPT	31	0.035192961	0.999813551	3.899770837
ST5	32	0.00459908	0.999813551	1.664796515
ABL2	33	0.048390322	0.999813551	1.560819598

Table 4.5 33 genes significantly upregulated in cap macrophages (core *versus* cap macrophages; P < 0.05, FC > 1.5).

To further explore the differentially expressed genes in the subgroups, the dataset was re-analysed by comparing each subgroup against the rest of the samples for differential gene expression. 48 genes were found to be upregulated in “Symptomatic/Core” group, 61 genes in “Symptomatic/Cap” group; whereas 6 genes were found to be upregulated in “Asymptomatic/Core” group, and 28 genes in “Asymptomatic/Cap” group.

Gene ID	Rank	P-value	FDR	Fold Change
HS.133181	1	4.00E-04	0.862080785	1.827561433
PRKCB1	2	6.00E-04	0.862080785	1.852252842
LOC653907	3	1.00E-03	0.862080785	1.758203606
CR1	4	0.0059988	0.862080785	1.646327356
IL18BP	5	1.00E-03	0.862080785	1.995676291
CXCL10	6	0.01159768	0.862080785	1.683208327
CMKLR1	7	0.00839832	0.862080785	1.636218849
PLTP	8	0.00539892	0.862080785	1.575890729
PLA2G7	9	0.01119776	0.862080785	1.587311051
LEP	10	0.00579884	0.862080785	3.214383419
MPEG1	11	0.015196961	0.862080785	1.531812102
ESM1	12	0.01059788	0.862080785	1.933824091
P2RY1	13	0.01219756	0.862080785	1.771152959
HS.197143	14	0.020195961	0.862080785	1.50141359
CD163	15	0.019596081	0.862080785	1.521684483
BRSK1	16	0.036992601	0.862080785	1.500444505
PPAP2B	17	0.025794841	0.862080785	1.50091336
SULT1A1	18	0.01079784	0.862080785	1.978861268
FUCA1	19	0.034993001	0.862080785	1.523736321
FAH	20	0.016996601	0.862080785	1.510978092
TSPAN13	21	0.024195161	0.862080785	1.736623516
IL2RA	22	0.0089982	0.862080785	2.081247407

ADM	23	0.019996001	0.862080785	2.171217144
EDG1	24	0.026194761	0.862080785	1.610799797
PKD2L1	25	0.038792242	0.862080785	1.5191985
STARD8	26	0.016196761	0.862080785	1.540046062
GLUL	27	0.024995001	0.862080785	2.01000168
SIGLEC14	28	0.038792242	0.862080785	1.598657118
CD163L1	29	0.021995601	0.862080785	1.624656159
EDN1	30	0.020195961	0.862080785	1.986572698
RGL1	31	0.015996801	0.862080785	1.641533291
ENPP2	32	0.023395321	0.862080785	1.528484925
IGFBP3	34	0.018196361	0.862080785	2.752387799
HS.30495	35	0.047790442	0.862080785	1.575001001
HMOX1	36	0.00979804	0.862080785	3.522205426
S1PR1	37	0.048390322	0.862080785	1.514352308
IGF1	38	0.044591082	0.862080785	1.598291399
S100A8	39	0.044591082	0.862080785	1.990112599
LOC162632	40	0.044191162	0.862080785	1.504392024
GCNT1	41	0.044991002	0.862080785	1.561190117
AKR1C4	42	0.042591482	0.862080785	1.748709256
OKL38	43	0.031993601	0.862080785	1.597334635
LOC100132336	44	0.035592881	0.862080785	1.641862191
FLJ20489	45	0.039392122	0.862080785	2.241211141
SLC48A1	46	0.041791642	0.862080785	1.693173645
PDK4	47	0.035392921	0.862080785	2.498900409
SPIC	48	0.025594881	0.862080785	1.729051203

Table 4.6 48 genes significantly upregulated in core macrophages from symptomatic carotid plaques ("Symptomatic/core" *versus* the rest; P < 0.05, FC > 1.5).

Gene ID	Rank	P-value	FDR	Fold Change
ABCG1	1	0.00959808	0.862080785	1.592030942
UBE2Z	2	0.0039992	0.862080785	1.534678359
PTGER4	3	8.00E-04	0.862080785	1.638480538
CADM1	4	0.0109978	0.862080785	1.583126317
TIMM10	5	2.00E-04	0.862080785	1.558273231
EML4	6	0.00779844	0.862080785	1.541253339
TLR8	7	0.00359928	0.862080785	1.567297919
OLR1	8	0.00159968	0.862080785	2.240073916
SERPINA1	9	0.00339932	0.862080785	1.799213419
SLC4A7	10	6.00E-04	0.862080785	1.767701131
EPOR	11	6.00E-04	0.862080785	1.681591671
LILRA5	12	0.01179764	0.862080785	1.615455262
CD69	13	0.00759848	0.862080785	1.680395163
CLDN1	14	0.01179764	0.862080785	1.739480662
ZNF525	15	0.014597081	0.862080785	1.541651797
PDPN	16	0.025194961	0.862080785	1.531987765
LOC653879	17	0.01059788	0.862080785	1.535460146
MT1F	18	0.012597481	0.862080785	1.951740707
KCNK13	19	0.017796441	0.862080785	1.591110195
CCL4L1	20	0.013797241	0.862080785	1.751913856
DUSP10	21	0.042591482	0.862080785	1.640539687
IL28RA	22	0.028794241	0.862080785	1.548009348
ATAD1	23	0.016996601	0.862080785	1.500792416
TIMP1	24	0.046390722	0.862080785	1.520986112
OTUD1	25	0.014597081	0.862080785	1.697105682
HSPH1	26	0.023795241	0.862080785	1.516580739
ITGB7	27	0.033593281	0.862080785	1.682461155
AQP9	28	0.030193961	0.862080785	1.505251683
THEX1	29	0.00379924	0.862080785	1.614881178
LRRCC1	30	0.020795841	0.862080785	1.557406592
MRPS36	31	0.014397121	0.862080785	1.569388886
MT1X	32	0.018996201	0.862080785	1.717245577

LOC645638	33	0.00759848	0.862080785	1.965053766
FNDC1	34	0.021395721	0.862080785	2.096646646
CCR2	35	0.017596481	0.862080785	1.623128152
RSPO3	36	0.01119776	0.862080785	1.644204188
SNRNP200	37	0.045790842	0.862080785	1.5425861
RBMS3	38	0.01219756	0.862080785	1.760966712
MFAP5	39	0.034793041	0.862080785	1.795141728
RPL14	40	0.048390322	0.862080785	1.561596091
MARCO	41	0.042591482	0.862080785	1.874976073
TCTEX1D1	42	0.013197361	0.862080785	1.51743292
NHP2	43	0.01019796	0.862080785	1.618722311
HIST1H2AJ	44	0.047990402	0.862080785	1.504849798
GINS2	45	0.00679864	0.862080785	1.562773826
NAT6	46	0.025394921	0.862080785	1.511174289
COMP	47	0.028794241	0.862080785	2.878301728
TPR	48	0.020395921	0.862080785	1.94900439
MT1G	49	0.020795841	0.862080785	1.979049685
MT1H	50	0.027994401	0.862080785	2.064468059
RPS6KA2	51	0.004999	0.862080785	1.700343028
TIMP3	52	0.049590082	0.862080785	2.95607285
TDP1	53	0.048390322	0.862477364	1.504550449
PFKFB4	54	0.035592881	0.862080785	1.798757534
SNORA12	55	0.0079984	0.862477364	1.54215352
HIST1H2AK	56	0.016996601	0.862477364	1.503438254
PLEKHA2	57	0.038392322	0.862477364	1.648527416
MAP3K4	58	0.040991802	0.862477364	1.61259738
GPR98	59	0.01079784	0.862600934	1.908314344
CBLN3	60	0.049990002	0.86424177	1.984753752
LOC651101	61	0.042991402	0.86695783	1.795235892

Table 4.7 61 genes significantly upregulated in cap macrophages from symptomatic carotid plaques (“Symptomatic/Cap” *versus* the rest; P < 0.05, FC > 1.5).

Gene ID	Rank	P-value	FDR	Fold Change
TBCEL	1	0.024195161	0.862080785	1.580499693
HLA-DOB	2	0.00279944	0.862080785	1.673523618
HLA-DRB5	3	0.039192162	0.862080785	2.096904767
FCRLA	4	0.00659868	0.862080785	1.821146646
HS.566008	5	0.047190562	0.862080785	1.558521073
XBP1	6	0.020195961	0.862080785	2.775844792

Table 4.8 6 genes significantly upregulated in core macrophages from asymptomatic carotid plaques (“Asymptomatic/Core” *versus* the rest; P < 0.05, FC > 1.5).

Gene ID	Rank	P-value	FDR	Fold Change
KLF8	1	4.00E-04	0.862080785	1.519284664
LOC644979	2	0.00459908	0.862080785	1.55901828
GTF2A1L	3	0.00619876	0.862080785	1.989281776
ZBTB46	4	0.00559888	0.862080785	1.668149005
ATP9A	5	0.020795841	0.862080785	1.717435698
LOC442572	6	0.00759848	0.862080785	1.515817373
ARAP3	7	0.01019796	0.862080785	1.664423995
MYO1D	8	0.026794641	0.862080785	1.627240054
SPTBN1	9	0.032993401	0.862080785	1.521157684
LOC653629	10	0.00879824	0.862080785	1.656774088
CTTN	11	0.015196961	0.862080785	1.641577077
TACC2	12	0.01079784	0.862080785	1.691112317
RASL11B	13	0.027594481	0.862080785	1.521218665

SEMA6A	14	0.012597481	0.862080785	1.568511037
MSX1	15	0.01219756	0.862080785	2.089915535
FBLN7	16	0.037792442	0.862080785	1.54633603
SEMA3A	17	0.028994201	0.862080785	1.519926742
ADAMTS8	18	0.039792042	0.862080785	2.064302616
ADAMTSL2	19	0.033193361	0.862080785	1.920778979
C3ORF15	20	0.029794041	0.862080785	1.800313762
MAB21L2	21	0.049190162	0.862080785	1.502530231
SCARNA13	22	0.043991202	0.862080785	1.5465602
OLFML2A	23	0.046790642	0.862080785	1.540920497
LTBP4	24	0.048390322	0.862080785	1.695552095
NKTR	25	0.048590282	0.862080785	1.513646374
C14ORF102	26	0.029594081	0.862080785	1.565438656
RPP38	27	0.021995601	0.862080785	1.533465428
CSAD	28	0.035592881	0.862080785	1.652632812

Table 4.9 28 genes significantly upregulated in cap macrophages from asymptomatic carotid plaques (“Asymptomatic/Cap” *versus* the rest; $P < 0.05$, $FC > 1.5$).

4.4.6.1 Interpretation of differential gene expression analysis

Analysis of gene expression in human plaque macrophages procured in the juxta-core and cap regions in symptomatic and asymptomatic plaques revealed distinct gene expression profiles. Whereas core macrophages in symptomatic plaques (“Symptomatic/core”) differentially expressed genes involved in inflammation (e.g. CR1, IL18BP, CXCL10, etc.), intraplaque haemorrhage / haem metabolism (e.g. CD163, HMOX1, SLC48A1, etc.), and lipid metabolism (e.g. PLA2G7, PLTP, LEP, etc.); on the opposite end of the spectrum, cap macrophages in asymptomatic plaques (“Asymptomatic/cap”) differentially expressed genes involved in fibrous cap integrity (e.g. ADAMTS8, ADAMTSL2), and cell motility (e.g. MYO1D, SPTBN1).

However, since the design of this study set out to procure plaque macrophages based on clinical presentation and micro-anatomical location without bias to pre-defined macrophage “subsets” (based on cell surface markers), there was substantial intrinsic heterogeneity within each procured macrophage populations; as such, conventional statistical approach to define individual differentially expressed genes becomes insensitive, as shown by the calculated high FDR (all Q-values > 0.85), which would normally result in statistical rejection of the differentially expressed candidate genes. In addition, each biological pathway is controlled by a number of key genes, which are tightly regulated in a coordinated fashion with up- or down-regulation simultaneously; therefore, the coordinated regulation of a number of key genes, each up- or down-regulated even by a small degree (without triggering individual statistical significance threshold), might be more important and representative than large fold-change of one or two genes in terms of biological pathway activation.

Therefore, having explored candidate genes that were differentially expressed in core and cap macrophages procured from symptomatic and asymptomatic human carotid plaques, pathway analysis was performed to further evaluate the biological significance of these findings.

4.4.7 Pathway analysis using GSEA

Gene set enrichment analysis (GSEA) was used to identify if any biological pathways of interest were significantly enriched within the dataset. The principle and method of GSEA was discussed in Chapter 2.7.5 in details. In brief, GSEA is a computational method to determine if a ranked *gene set*, rather than individual genes, shows statistically significant concordance when compared to curated, annotated, gene sets that are associated with known biological function, or pathophysiological states. These are catalogued in public databases, such as the Kegg, Biocarta, Reactome, and the Broad Institute, MIT.

4.4.7.1 Enriched pathways in macrophages from symptomatic plaques

GSEA was first performed comparing expression profiles from macrophages obtained from 'symptomatic' *versus* 'asymptomatic' plaques. At conventional thresholds of $P < 0.05$ and $FDR < 0.25$, 379 pathways were significantly enriched in macrophages from symptomatic plaques, of which 13 pathways were highly significantly enriched with $P < 0.01$ and $FDR < 0.25$. In contrast, only 1 pathway was significantly enriched in macrophages from asymptomatic plaques with thresholds of $P < 0.01$ and $FDR < 0.25$.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
BANDRES_RESPONSE_TO_CARMUSTIN_MG MT_24HR_DN	33	1.5144116	0.033797216	0.24937546
REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_DEGRADATION	206	1.5140097	0.026717557	0.24914505
SARRIO_EPITHELIAL_MESENCHYMAL_TRANSITION_DN	147	1.5139024	0.012	0.24810326
PETROVA_PROX1_TARGETS_DN	64	1.513802	0.016736401	0.24718794
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_MITOTIC_PROTEINS	69	1.5136893	0.03125	0.2463167
REACTOME_S_PHASE	101	1.5127031	0.035019454	0.24781145
DORN_ADENOVIRUS_INFECTION_12HR_UP	28	1.5124867	0.02008032	0.2471742
TSUNODA_CISPLATIN_RESISTANCE_DN	50	1.5124478	0.032128513	0.24602576
TAKAO_RESPONSE_TO_UVB_RADIATION_UP	85	1.5120571	0.011428571	0.2457558
SCHURINGA_STAT5A_TARGETS_UP	21	1.5118508	0.036217302	0.24498342
WENG_POR_TARGETS_LIVER_UP	38	1.5114162	0.003752345	0.24482584
TIEN_INTESTINE_PROBIOTICS_6HR_UP	54	1.5113629	0.014925373	0.24383962
POOLA_INVASIVE_BREAST_CANCER_UP	272	1.5105557	0.012024048	0.24515723
GENTILE_UV_HIGH_DOSE_UP	24	1.5104305	0.035714287	0.24435522
SESTO_RESPONSE_TO_UV_C1	69	1.5102129	0.03508772	0.2439206
DER_IFN_GAMMA_RESPONSE_UP	70	1.5097867	0.038610037	0.24402307
REACTOME_PROCESSING_OF_CAPPED_INTRONLESS_PRE_MRNA	22	1.5091698	0.017208412	0.24326351
REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	120	1.5088958	0.04085603	0.24288505
WAKABAYASHI_ADIPOGENESIS_PPARG_RX_RA_BOUND_36HR	138	1.5084497	0.011605416	0.24298202
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL_CIS	120	1.508349	0.02851711	0.24210994
KAYO_AGING_MUSCLE_DN	121	1.5081569	0.030075189	0.24145012
SCHURINGA_STAT5A_TARGETS_DN	15	1.5078896	0.013133208	0.24111278
KYNG_RESPONSE_TO_H2O2_VIA_ERCC6_DN	46	1.5067153	0.01923077	0.24314699
GNATENKO_PLATELET_SIGNATURE	46	1.5066727	0.005758158	0.24098971
KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	38	1.5064203	0.015686275	0.24055682
REACTOME_INHIBITION_OF_THE_PROTEOLYTIC_ACTIVITY_OF_APC_C_REQUIRED_FOR_THE_ONSET_OF_ANAPHASE_BY_MITOTIC_SPLINDLE_CHECKPOINT_COMPONENTS	22	1.5058587	0.034883723	0.24117362
MUNSHI_MULTIPLE_MYELOMA_UP	79	1.5053751	0.0227704	0.24156426
SESTO_RESPONSE_TO_UV_C0	106	1.5053335	0.022944551	0.24063353
MARKS_ACETYLATED_NON_HISTONE_PROTEINS	15	1.5049043	0.015009381	0.2407039
RASHI_RESPONSE_TO_IONIZING_RADIATION_6	79	1.5040202	0.007843138	0.24226007
REACTOME_RNA_POL_I_TRANSCRIPTION_INITIATION	25	1.5036091	0.036398467	0.24238
KYNG_ENVIRONMENTAL_STRESS_RESPONSE_UP	56	1.5027336	0.017612524	0.24168336
MELLMAN_TUT1_TARGETS_DN	47	1.5025938	0.007858546	0.24104062
LU_IL4_SIGNALING	90	1.5022625	0.013944224	0.2409626
YANG_BREAST_CANCER_ESR1_LASER_DN	48	1.5022347	0.017110266	0.23996416
AMUNDSON_GENOTOXIC_SIGNATURE	101	1.5013996	0.007692308	0.24125974
ALONSO_METASTASIS_UP	188	1.5013155	0.015503876	0.2403954
BYSTROEM_CORRELATED_WITH_IL5_DN	63	1.5001993	0.011811024	0.24148981
WINTER_HYPOXIA_METAGENE	232	1.4997333	0.015655577	0.24174967

BRUNO_HEMATOPOIESIS	64	1.4996777	0.003976143	0.24084347
LENAOUR_DENDRITIC_CELL_MATURATION_DN	127	1.4992911	0.01775148	0.24092096
BIOCARTA_DC_PATHWAY	22	1.4987441	0.049382716	0.241345
BURTON_ADIPOGENESIS_PEAK_AT_2HR	51	1.4985673	0.042424243	0.24090992
SENESE_HDAC2_TARGETS_UP	108	1.4985275	0.019193858	0.23996566
GAVIN_FOXP3_TARGETS_CLUSTER_T7	95	1.4968079	0.018656716	0.2427119
MOOTHA_TCA	16	1.4961965	0.007707129	0.24340804
REACTOME_TRAF6_MEDIATED_INDUCION_OF_NFKB_AND_MAP_KINASES_UPON_TLR7_8_OR_9_ACTIVATION	72	1.4960093	0.007797271	0.24196623
VILIMAS_NOTCH1_TARGETS_DN	20	1.495908	0.030303031	0.24127449
FARMER_BREAST_CANCER_CLUSTER_7	19	1.4957123	0.021653544	0.24078323
KEGG_ALLOGRAFT_REJECTION	38	1.4955549	0.04989605	0.24020645
REACTOME_SYNTHESIS_OF_PIPS_AT_THE_GOLGI_MEMBRANE	17	1.4951563	0.022132797	0.24044634
ONDER_CDH1_TARGETS_1_DN	158	1.495079	0.015873017	0.23970631
CHEN_LVAD_SUPPORT_OF_FAILING_HEART_DN	42	1.4950229	0.018255578	0.23792985
RHODES_CANCER_META_SIGNATURE	62	1.4945414	0.015414258	0.2363265
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	26	1.4943504	0.035433073	0.2358642
REACTOME_PHOSPHORYLATION_OF_THE_A_PC_C	20	1.4939685	0.046875	0.2358952
GESERICK_TERT_TARGETS_DN	20	1.4939443	0.036072146	0.23503676
DASU_IL6_SIGNALING_UP	58	1.4939307	0.011605416	0.23417188
GAZDA_DIAMOND_BLACKFAN_ANEMIA_PROGENITOR_UP	39	1.4937316	0.007751938	0.23292744
YIH_RESPONSE_TO_ARSENITE_C3	35	1.4936087	0.016096579	0.23245001
CHEN_LUNG_CANCER_SURVIVAL	27	1.4933848	0.022900764	0.23218825
REACTOME_DESTABILIZATION_OF_MRNA_BY_BRF1	17	1.4930067	0.033932135	0.23127273
TOMIDA_METASTASIS_UP	26	1.4929792	0.026768642	0.23047328
ZHONG_SECRETOME_OF_LUNG_CANCER_AND_FIBROBLAST	130	1.4929476	0.023076924	0.22966711
RUAN_RESPONSE_TO_TROGLITAZONE_DN	19	1.4921572	0.02584493	0.23068951
WENG_POR_TARGETS_GLOBAL_UP	19	1.4920156	0.02631579	0.23015365
FONTAINE_FOLLICULAR_THYROID_ADENOMA_DN	67	1.4919815	0.01980198	0.2294156
BIOCARTA_BCELLSURVIVAL_PATHWAY	15	1.4914824	0.021825397	0.22983897
CHANDRAN_METASTASIS_TOP50_UP	34	1.4913754	0.011560693	0.22933173
REACTOME_PREFOLDIN_MEDIATED_TRANSFER_OF_SUBSTRATE_TO_CCT_TRIC	26	1.491109	0.030131826	0.22747213
MA_MYELOID_DIFFERENTIATION_UP	39	1.4907746	0.025145067	0.22744882
NIKOLSKY_OVERCONNECTED_IN_BREAST_CANCER	22	1.49074	0.008	0.22668043
REACTOME_REGULATION_OF_HYPOXIA_INDUCIBLE_FACTOR_HIF_BY_OXYGEN	24	1.4905448	0.025242718	0.22641693
HILLION_HMGAT1B_TARGETS	91	1.4899417	0.043643262	0.22712646
OKAMOTO_LIVER_CANCER_MULTICENTRIC_OCCURRENCE_UP	24	1.4896686	0.02259887	0.22696845
BIOCARTA_CHEMICAL_PATHWAY	22	1.489556	0.030425964	0.2263852
SHIN_B_CELL_LYMPHOMA_CLUSTER_5	17	1.489431	0.03629032	0.22589697
LI_AMPLIFIED_IN_LUNG_CANCER	177	1.4893279	0.03041825	0.22452997
TOOKER_GEMCITABINE_RESISTANCE_UP	76	1.4888519	0.025	0.22507975
LI_DCP2_BOUND_MRNA	86	1.4884146	0.037523452	0.22539687

BIOCARTA_STRESS_PATHWAY	25	1.4879861	0.01980198	0.22554268
HAHTOLA_MYCOSIS_FUNGOIDES_CD4_UP	64	1.4875959	0.03929273	0.22579724
JAIN_NFKB_SIGNALING	73	1.4875232	0.017110266	0.22513114
SPIRA_SMOKERS_LUNG_CANCER_UP	38	1.4872758	0.03646833	0.2250347
TAKAO_RESPONSE_TO_UVB_RADIATION_DN	97	1.4870822	0.02457467	0.22471853
LE_NEURONAL_DIFFERENTIATION_DN	19	1.4870613	0.043222003	0.22399001
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	136	1.4870183	0.049115915	0.22334948
KAMIKUBO_MYELOID_CEBPA_NETWORK	28	1.4870132	0.048879836	0.22259767
CHIANG_LIVER_CANCER_SUBCLASS_UNANOTATED_DN	182	1.4867957	0.04085603	0.22241662
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_9	73	1.4862001	0.011650485	0.22160402
DAIRKEE_TERT_TARGETS_UP	348	1.4861504	0.02457467	0.22094706
YAMAZAKI_TCEB3_TARGETS_DN	205	1.4861398	0.013513514	0.22023246
REACTOME_RNA_POL_II_TRANSCRIPTION	95	1.4860086	0.036190476	0.2198617
ZAMORA_NOS2_TARGETS_UP	68	1.485183	0.023483366	0.22046506
REACTOME_MRNA_PROCESSING	149	1.485126	0.030303031	0.21992205
POS_HISTAMINE_RESPONSE_NETWORK	32	1.4849325	0.023715414	0.21891688
HU_ANGIOGENESIS_DN	37	1.4845054	0.024667932	0.21925087
REACTOME_BIOSYNTHESIS_OF_THE_N_GLYCAN_PRECURSOR_DOLICHOL_LIPID_LINKED_OLIGOSACCHARIDE_LLO_AND_TRANSFER_TO_A_NASCENT_PROTEIN	29	1.4844391	0.014	0.21866849
PUIFFE_INVASION_INHIBITED_BY_ASCITES_DN	135	1.4841058	0.019011406	0.21795739
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_DN	194	1.4839447	0.01178782	0.2176802
MORI_MATURE_B_LYMPHOCYTE_UP	87	1.4839203	0.015748031	0.2170306
BIOCARTA_MAPK_PATHWAY	86	1.4835997	0.03006012	0.21702048
REACTOME_LIPID_DIGESTION_MOBILIZATION_AND_TRANSPORT	44	1.4832237	0.013565891	0.21719252
RUTELLA_RESPONSE_TO_CSF2RB_AND_IL4_DN	300	1.4827158	0.019193858	0.2178182
XU_GH1_EXOGENOUS_TARGETS_DN	117	1.4825554	0.015873017	0.21753094
DAZARD_RESPONSE_TO_UV_NHEK_UP	235	1.4822882	0.020833334	0.21748675
LEI_MYB_TARGETS	312	1.4822828	0.011976048	0.21682101
IVANOVA_HEMATOPOIESIS_LATE_PROGENITOR	496	1.4820384	0.036398467	0.216793
SUNG_METASTASIS_STROMA_DN	51	1.4816551	0.031620555	0.21695335
WILLIAMS_ESR2_TARGETS_UP	27	1.481504	0.019723866	0.21663009
REACTOME_HDL_MEDIATED_LIPID_TRANSPORT	15	1.4815004	0.04780115	0.21596341
KEEN_RESPONSE_TO_ROSIGLITAZONE_UP	36	1.4814512	0.038387716	0.21540117
MONNIER_POSTRADIATION_TUMOR_ESCAPE_DN	334	1.4814088	0.015686275	0.2147971
REACTOME_CITRIC_ACID_CYCLE_TCA_CYCLE	23	1.4805017	0.019607844	0.21620697
SCHUHMACHER_MYC_TARGETS_UP	78	1.4802965	0.025096525	0.21608828
GERHOLD_ADIPOGENESIS_DN	64	1.4795368	0.019607844	0.21671641
OUELLET_OVARIAN_CANCER_INVASIVE_VS_LMP_UP	114	1.4793952	0.040229887	0.21638207
KIM_ALL_DISORDERS_DURATION_CORR_DN	140	1.4787568	0.029795159	0.2165559
BIOCARTA_CASPASE_PATHWAY	23	1.4784873	0.030181086	0.21659812
ROVERSI_GLIOMA_COPY_NUMBER_DN	53	1.4784226	0.035580523	0.21612366

ZHENG_RESPONSE_TO_ARSENITE_DN	18	1.4781826	0.0251938	0.21611792
BASSO_CD40_SIGNALING_UP	101	1.4780802	0.046875	0.2156953
DAZARD_UV_RESPONSE_CLUSTER_G1	65	1.4779023	0.04664179	0.21543191
REACTOME_PPARA_ACTIVATES_GENE_EXPRESSION	103	1.4775715	0.023076924	0.21573046
WANG_SMARCE1_TARGETS_DN	350	1.4775501	0.019193858	0.21512176
KEGG_HISTIDINE_METABOLISM	29	1.4775462	0.04	0.21449094
DAZARD_UV_RESPONSE_CLUSTER_G4	19	1.4772506	0.044921875	0.21404888
WILENSKY_RESPONSE_TO_DARAPLADIB	29	1.4768528	0.027504912	0.21359116
VIETOR_IFRD1_TARGETS	22	1.4765894	0.048262548	0.21354622
LUI_THYROID_CANCER_PAX8_PPARG_DN	43	1.4765024	0.042671613	0.21311542
MOREAUX_B_LYMPHOCYTE_MATURATION_BY_TACI_DN	66	1.4764687	0.038986355	0.21256477
QI_HYPOXIA	132	1.4762601	0.013833992	0.2125172
REACTOME_LYSOSOME_VESICLE_BIOGENESIS	23	1.4762306	0.025145067	0.21196736
REACTOME_PROTEIN_FOLDING	51	1.4760199	0.03409091	0.21183062
HUANG_GATA2_TARGETS_UP	140	1.4759978	0.026666667	0.21127923
REACTOME_INHIBITION_OF_VOLTAGE_GATED_CA2_CHANNELS_VIA_GBETA_GAMMA_SUBUNITS	25	1.4758431	0.02016129	0.21095425
REACTOME_MRNA_SPLICING_MINOR_PATHWAY	40	1.4757552	0.03088803	0.21054809
KEGG_BLADDER_CANCER	42	1.4755396	0.017612524	0.21040916
MOREAUX_MULTIPLE_MYELOMA_BY_TACI_DN	154	1.4754444	0.043643262	0.20945515
BIOCARTA_41BB_PATHWAY	17	1.4753077	0.047528517	0.20917158
DUTTA_APOPTOSIS_VIA_NFKB	32	1.475265	0.04085603	0.20868266
NEWMAN_ERCC6_TARGETS_UP	25	1.4751762	0.026052104	0.20824033
LEONARD_HYPOXIA	43	1.4751759	0.04183267	0.20766027
ZHAN_MULTIPLE_MYELOMA_SUBGROUPS	30	1.475007	0.023483366	0.20745635
CUI_TCF21_TARGETS_DN	29	1.4748987	0.03307393	0.20714368
LIU_VAV3_PROSTATE_CARCINOGENESIS_UP	87	1.4734627	0.02366864	0.20865281
BOGNI_TREATMENT_RELATED_MYELOID_LEUKEMIA_UP	29	1.4734343	0.023121387	0.20817469
REACTOME_TRIF_MEDIATED_TLR3_SIGNALING	69	1.4731424	0.01764706	0.20769478
VANTVEER_BREAST_CANCER_POOR_PROGNOSIS	49	1.4730688	0.012396694	0.20725554
WANG_CLIM2_TARGETS_DN	171	1.4727656	0.029296875	0.20730276
PARK_APL_PATHOGENESIS_DN	50	1.4726825	0.026465029	0.20690325
BIOCARTA_TNFR1_PATHWAY	29	1.4725682	0.03256705	0.20598906
MARTORIATI_MDM4_TARGETS_FETAL_LIVER_UP	206	1.4721305	0.011538462	0.2057992
LIM_MAMMARY_LUMINAL_PROGENITOR_UP	57	1.4719672	0.012096774	0.20557551
MOREIRA_RESPONSE_TO_TSA_DN	18	1.4712352	0.0331384	0.20616435
KAAB_FAILED_HEART_VENTRICLE_DN	41	1.4710413	0.042226486	0.20553333
BURTON_ADIPOGENESIS_9	88	1.4708221	0.023121387	0.20541517
SAKAI_CHRONIC_HEPATITIS_VS_LIVER_CANCER_UP	80	1.470796	0.04255319	0.20494291
PID_GMCSF_PATHWAY	37	1.4701467	0.021611001	0.20526472
CHEN_LIVER_METABOLISM_QTL_CIS	85	1.4695363	0.037181996	0.2054382
MEINHOLD_OVARIAN_CANCER_LOW_GRADE_DN	20	1.4690769	0.03875969	0.20599625

PODAR_RESPONSE_TO_ADAPHOSTIN_DN	16	1.4687729	0.02811245	0.20517322
FAELT_B_CLL_WITH_VH3_21_DN	48	1.4685446	0.030947777	0.2040882
MULLIGHAN_MLL_SIGNATURE_1_UP	359	1.4684162	0.027504912	0.20378599
HOLLEMAN_ASPARAGINASE_RESISTANCE_ALL_DN	23	1.4682978	0.035363458	0.20355381
REACTOME_ADP_SIGNALLING_THROUGH_P2RY1	25	1.4682935	0.031434186	0.2030452
GROSS_HYPOXIA_VIA_ELK3_AND_HIF1A_UP	136	1.4681828	0.007984032	0.20276564
BURTON_ADIPOGENESIS_1	33	1.467741	0.030947777	0.20314172
ACEVEDO_NORMAL_TISSUE_ADJACENT_TO_LIVER_TUMOR_DN	336	1.4676385	0.03809524	0.20285079
WELCSH_BRCA1_TARGETS_DN	135	1.4673189	0.039252337	0.20298167
KYNG_RESPONSE_TO_H2O2	71	1.4672376	0.007490637	0.20268361
SEIDEN_MET_SIGNALING	19	1.4671715	0.030947777	0.20233423
HILLION_HMGA1_TARGETS	89	1.467052	0.047348484	0.20213027
GOLUB_ALL_VS_AML_UP	24	1.4667897	0.01980198	0.20219159
REACTOME_ASPARAGINE_N_LINKED_GLYCOSYLATION	79	1.466692	0.037475344	0.20184667
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_1	67	1.4655514	0.02631579	0.20274721
VALK_AML_CLUSTER_16	23	1.4649193	0.028513238	0.20343277
NADLER_OBESITY_UP	61	1.4648751	0.011583012	0.20301926
HOFFMANN_SMALL_PRE_BII_TO_IMMATURE_B_LYMPHOCYTE_UP	69	1.4639871	0.034285713	0.2045973
VANTVEER_BREAST_CANCER_ESR1_DN	228	1.4637508	0.029013539	0.2040997
GOLDRATH_HOMEOSTATIC_PROLIFERATION	163	1.4634342	0.034548946	0.20387019
WOOD_EBV_EBNA1_TARGETS_DN	47	1.463171	0.033864543	0.20340283
LANG_MYB_FAMILY_TARGETS	29	1.4629971	0.02851711	0.2032227
SYED ESTRADIOL_RESPONSE	19	1.4625384	0.020715632	0.20385297
MARSON_FOXP3_TARGETS_UP	63	1.4619178	0.011450382	0.20459151
BROCKE_APOPTOSIS_REVERSED_BY_IL6	139	1.4616885	0.017208412	0.2045624
MCDOWELL_ACUTE_LUNG_INJURY_UP	45	1.4614757	0.044573642	0.20455568
GERY_CEBP_TARGETS	125	1.4612154	0.013833992	0.2041349
LIU_IL13_PRIMING_MODEL	15	1.4611065	0.038986355	0.20388213
RUAN_RESPONSE_TO_TNF_DN	83	1.4609498	0.03256705	0.20374888
DAIRKEE_CANCER_PRONE_RESPONSE_BPA_E2	116	1.4608184	0.029850746	0.20214301
KORKOLA_EMBRYONAL_CARCINOMA_UP	37	1.460512	0.02952756	0.20231996
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	100	1.4604472	0.039215688	0.20193464
UEDA_CENTRAL_CLOCK	85	1.4604089	0.013282732	0.20154794
JIANG_VHL_TARGETS	124	1.4602784	0.036679536	0.20086876
JIANG_HYPOXIA_VIA_VHL	31	1.4601492	0.039399624	0.20063289
REACTOME_NUCLEOTIDE_LIKE_PURINERGIC_RECEPTORS	15	1.4593568	0.027944112	0.20082903
REACTOME_INSULIN_SYNTHESIS_AND_PROCESSING	21	1.4593482	0.0234375	0.20037423
TSAI_RESPONSE_TO_IONIZING_RADIATION	148	1.4590073	0.01764706	0.20065059
RASHI_RESPONSE_TO_IONIZING_RADIATION_1	43	1.4583751	0.0415879	0.20050232
BIOCARTA_ERK_PATHWAY	28	1.4582645	0.03359684	0.20022093
ZHAN_MULTIPLE_MYELOMA_DN	41	1.4581132	0.030947777	0.20010114
PID_CASPASE_PATHWAY	51	1.4580185	0.01934236	0.19985874

TARTE_PLASMA_CELL_VS_B_LYMPHOCYTE_DN	37	1.4578419	0.02952756	0.19890516
BIOCARTA_FAS_PATHWAY	30	1.4577131	0.023391813	0.19872707
BOYLAN_MULTIPLE_MYELOMA_PCA3_DN	66	1.4575433	0.026717557	0.1986528
LEE_METASTASIS_AND_RNA_PROCESSING_UP	17	1.4571538	0.036750484	0.19903913
HOLLEMAN_PREDNISOLONE_RESISTANCE_ALL_DN	19	1.4569472	0.018903593	0.19894706
ZHANG_RESPONSE_TO_CANTHARIDIN_UP	18	1.4567795	0.034979425	0.19886063
HINATA_NFKB_TARGETS_KERATINOCYTE_UP	91	1.4564337	0.031558186	0.1986593
SWEET_KRAS_ONCOGENIC_SIGNATURE	88	1.4563377	0.023121387	0.19839925
IVANOVA_HEMATOPOIESIS_INTERMEDIATE_PROGENITOR	138	1.4563175	0.030245747	0.19801195
DUNNE_TARGETS_OF_AML1_MTG8_FUSION_UP	48	1.4562664	0.03742204	0.1976743
YAGI_AML_SURVIVAL	122	1.4558709	0.009652509	0.19807208
BIOCARTA_NKCELLS_PATHWAY	20	1.4556777	0.049115915	0.19768667
PID_BCR_5PATHWAY	65	1.455161	0.031496063	0.19785883
GERHOLD_ADIPOGENESIS_UP	49	1.4549737	0.04315197	0.19773614
SCHAEFFER_PROSTATE_DEVELOPMENT_6HR_UP	167	1.4549301	0.027237354	0.19740449
BIOCARTA_CD40_PATHWAY	15	1.4541649	0.028957529	0.19806807
BIOCARTA_RACCYCD_PATHWAY	26	1.4527202	0.023529412	0.20055285
MORI_LARGE_PRE_BII_LYMPHOCYTE_DN	57	1.4512357	0.02745098	0.2023926
IVANOVA_HEMATOPOIESIS_MATURE_CELL	268	1.4504292	0.03137255	0.20333493
CAFFAREL_RESPONSE_TO_THC_UP	31	1.4503958	0.033834588	0.20298463
CAFFAREL_RESPONSE_TO_THC_24HR_5_UP	31	1.4501041	0.033333335	0.2031656
SWEET_LUNG_CANCER_KRAS_UP	468	1.4491867	0.03088803	0.20433636
BIOCARTA_UCALPAIN_PATHWAY	18	1.4489548	0.039215688	0.20438658
WELCSH_BRCA1_TARGETS_UP	191	1.4488534	0.036538463	0.2041474
HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN	76	1.4488207	0.04158416	0.20379569
AKL_HTLV1_INFECTION_UP	25	1.4482987	0.017681729	0.20442164
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_BLACK_UP	34	1.4479564	0.03968254	0.20428368
WU_HBX_TARGETS_3_UP	18	1.4477054	0.038240917	0.20430772
VARELA_ZMPSTE24_TARGETS_UP	39	1.4475158	0.027184466	0.20340799
GENTILE_UV_RESPONSE_CLUSTER_D2	40	1.4474478	0.048076924	0.20315273
KYNG_DNA_DAMAGE_BY_GAMMA_RADIATION	78	1.4472003	0.038854804	0.20240137
CAFFAREL_RESPONSE_TO_THC_24HR_5_DN	56	1.4469845	0.049115915	0.2024113
DEMAGALHAES_AGING_UP	55	1.4468279	0.029239766	0.20189594
WALLACE_PROSTATE_CANCER_RACE_UP	277	1.4463493	0.04637097	0.20208217
FOURNIER_ACINAR_DEVELOPMENT_LATE_2	263	1.4461242	0.04483431	0.20215346
KIM_WT1_TARGETS_12HR_DN	195	1.4458019	0.017142856	0.2023901
ZHAN_LATE_DIFFERENTIATION_GENES_UP	32	1.4453802	0.031496063	0.20200361
FARMER_BREAST_CANCER_APOCRINE_VS_BASAL	319	1.4451637	0.015533981	0.20213637
PID_RETINOIC_ACID_PATHWAY	29	1.4451462	0.035019454	0.20175757
SASSON_RESPONSE_TO_GONADOTROPHINS_UP	87	1.4449071	0.025145067	0.20176932
SHAFFER_IRF4_TARGETS_IN_MYELOMA_VS_MATURE_B_LYMPHOCYTE	99	1.4444689	0.035363458	0.20221493
NELSON_RESPONSE_TO_ANDROGEN_UP	85	1.4442828	0.032882012	0.20177758

CASORELLI_ACUTE_PROMYELOCYTIC_LEUKEMIA_UP	164	1.4442427	0.017821781	0.20146737
ELVIDGE_HIF1A_AND_HIF2A_TARGETS_UP	38	1.4439226	0.0407767	0.20125072
SHEPARD_BMYB_MORPHOLINO_UP	197	1.443773	0.033333335	0.2011494
ROSS_AML_WITH_CBFB_MYH11_FUSION	50	1.4437128	0.033203125	0.20086503
RUTELLA_RESPONSE_TO_HGF_VS_CSF2RB_AND_IL4_UP	393	1.4430394	0.03952569	0.2018507
CHENG_RESPONSE_TO_NICKEL_ACETATE	41	1.4424179	0.040384617	0.20256771
FERRANDO_LYL1_NEIGHBORS	15	1.4421353	0.041257367	0.20235863
FARMER_BREAST_CANCER_APOCRINE_VS_LUMINAL	309	1.442035	0.02	0.20134556
BROWN_MYELOID_CELL_DEVELOPMENT_UP	157	1.4419136	0.0390625	0.20117326
REACTOME_SPHINGOLIPID_DE_NOVO_BIOSYNTHESIS	25	1.4416708	0.025	0.20127709
BRACHAT_RESPONSE_TO_METHOTREXATE_DN	25	1.4416382	0.033203125	0.20096435
BIOCARTA_CHREBP2_PATHWAY	42	1.4416214	0.026871402	0.20061138
LINDSTEDT_DENDRITIC_CELL_MATURATION_D	68	1.4414831	0.024761904	0.20049341
PENG_GLUTAMINE_DEPRIVATION_UP	38	1.4411407	0.043222003	0.20073918
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	24	1.4399216	0.04950495	0.2008437
VANTVEER_BREAST_CANCER_METASTASIS_DN	111	1.4396454	0.038383838	0.19955207
HIRSCH_CELLULAR_TRANSFORMATION_SIGNATURE_UP	236	1.4396262	0.015564202	0.19922654
RODWELL_AGING_KIDNEY_UP	443	1.4396198	0.023483366	0.19886665
ST_TUMOR_NECROSIS_FACTOR_PATHWAY	28	1.4395857	0.040697675	0.19855791
REACTOME_INTEGRATION_OF_ENERGY_METABOLISM	118	1.4393116	0.02734375	0.19863829
HOLLMANN_APOPTOSIS_VIA_CD40_UP	190	1.439101	0.045454547	0.19868264
ZUCCHI_METASTASIS_DN	44	1.4389151	0.048543688	0.19865057
KYNG_DNA_DAMAGE_DN	192	1.4388145	0.041420117	0.19847967
FUJII_YBX1_TARGETS_UP	42	1.4385655	0.03877551	0.19859533
HUMMERICH_SKIN_CANCER_PROGRESSION_UP	88	1.4385365	0.033398822	0.1978999
WANG_CISPLATIN_RESPONSE_AND_XPC_DN	218	1.4384984	0.011952192	0.19759431
GOTZMANN_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_DN	203	1.4381257	0.04950495	0.19801383
BRACHAT_RESPONSE_TO_CAMPTOTHECIN_DN	41	1.4367781	0.032882012	0.19957037
THUM_SYSTOLIC_HEART_FAILURE_UP	393	1.4365736	0.034	0.19956228
GENTILE_UV_RESPONSE_CLUSTER_D1	18	1.4362411	0.048582997	0.19975875
BERENJENO_ROCK_SIGNALING_NOT_VIA_RHOA_UP	29	1.4359007	0.03529412	0.19934379
ZAMORA_NOS2_TARGETS_DN	94	1.4346354	0.036821704	0.20057382
GRUETZMANN_PANCREATIC_CANCER_UP	351	1.4343752	0.04133858	0.20079619
HOSHIDA_LIVER_CANCER_SUBCLASS_S1	232	1.4340945	0.021568628	0.20070386
KYNG_ENVIRONMENTAL_STRESS_RESPONSE_NOT_BY_UV_IN_OLD	24	1.4340758	0.048732944	0.20037952
MARIADASON_RESPONSE_TO_BUTYRATE_SULINDAC_4	20	1.4337964	0.031189084	0.19987436
HOFMANN_CELL_LYMPHOMA_UP	50	1.4334409	0.03187251	0.20000558
CHANDRAN_METASTASIS_TOP50_DN	44	1.4323373	0.022988506	0.20117053
SAMOLS_TARGETS_OF_KHSV_MIRNAS_DN	57	1.4319996	0.027079303	0.20142905
HOEBEKE_LYMPHOID_STEM_CELL_DN	83	1.4319347	0.04509804	0.20119178
SHIN_B_CELL_LYMPHOMA_CLUSTER_9	18	1.431624	0.016563147	0.2003746

LI_INDUCED_T_TO_NATURAL_KILLER_UP	290	1.4315621	0.035019454	0.20015976
LIU_SOX4_TARGETS_UP	131	1.4309552	0.033333335	0.20066133
THEILGAARD_NEUTROPHIL_AT_SKIN_WOUND_DN	219	1.4303721	0.049212597	0.20074165
AKL_HTLV1_INFECTION_DN	64	1.4300462	0.029821074	0.20071188
OSWALD_HEMATOPOIETIC_STEM_CELL_IN_COLLAGEN_GEL_UP	225	1.4299767	0.015936255	0.20050228
TONG_INTERACT_WITH_PTTG1	52	1.4291956	0.03307393	0.20020553
SEKI_INFLAMMATORY_RESPONSE_LPS_UP	74	1.4289377	0.04183267	0.20034075
FERRANDO_T_ALL_WITH_MLL_ENL_FUSION_UP	86	1.4285892	0.028355388	0.20067781
GOLDRATH_ANTIGEN_RESPONSE	335	1.4284855	0.049115915	0.20055318
PEDERSEN_METASTASIS_BY_ERBB2_ISOFORM_4	105	1.4276938	0.02173913	0.20108652
PID_ERBB1_DOWNSTREAM_PATHWAY	103	1.4273788	0.03550296	0.20064856
RAY_TARGETS_OF_P210_BCR_ABL_FUSION_DN	16	1.4270401	0.04950495	0.2006602
LINDSTEDT_DENDRITIC_CELL_MATURATION_C	68	1.4267913	0.035363458	0.20086169
KAUFFMANN_DNA_REPLICATION_GENES	137	1.4267441	0.04606526	0.20062631
BERTUCCI_MEDULLARY_VS_DUCTAL_BREAST_CANCER_UP	191	1.4263692	0.0390625	0.2010609
HOLLMANN_APOPTOSIS_VIA_CD40_DN	250	1.4263337	0.03522505	0.20078944
RUTELLA_RESPONSE_TO-CSF2RB_AND_IL4_UP	326	1.4259839	0.031746034	0.20084253
SEITZ NEOPLASTIC_TRANSFORMATION_BY_8P_DELETION_DN	30	1.425102	0.04133858	0.20160203
WANG_RECURRENT_LIVER_CANCER_DN	16	1.4250776	0.04863813	0.20131202
COATES_MACROPHAGE_M1_VS_M2_DN	70	1.4247619	0.041749503	0.20151909
LIU_SOX4_TARGETS_DN	291	1.4246192	0.034951456	0.20143603
WALLACE_JAK2_TARGETS_UP	24	1.4244909	0.049523808	0.20130771
REACTOME_REGULATION_OF_KIT_SIGNALING	17	1.4239879	0.04191617	0.2003151
LABBE_TARGETS_OF_TGFB1_AND_WNT3A_UP	109	1.4230118	0.044573642	0.19989794
APRELIKOVA_BRCA1_TARGETS	49	1.4226924	0.03696498	0.19949424
MCBRYAN_PUBERTAL_BREAST_4_5WK_DN	185	1.4224294	0.02016129	0.19929878
REACTOME_MAPK_TARGETS_NUCLEAR_EVENTS_MEDIATED_BY_MAP_KINASES	30	1.4223742	0.041904762	0.19908077
SHETH_LIVER_CANCER_VS_TXNIP_LOSS_PAM1	221	1.4219369	0.023210831	0.1984217
SHEPARD_CRUSH_AND_BURN_MUTANT_UP	186	1.4217001	0.032818533	0.19790219
BURTON_ADIPOGENESIS_12	30	1.4214846	0.041420117	0.19796763
JIANG_HYPOXIA_CANCER	78	1.4214509	0.04606526	0.19773677
REACTOME_TRANSCRIPTIONAL_REGULATION_OF_WHITE_ADIPOCYTE_DIFFERENTIATION	70	1.4205791	0.04109589	0.19819407
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_AND_BRAIN_QTL_TRANS	175	1.4204888	0.03646833	0.19744013
JOHNSTONE_PARVB_TARGETS_2_UP	135	1.4203668	0.020833334	0.19735296
AGUIRRE_PANCREATIC_CANCER_COPYNUMBER_DN	217	1.4193898	0.04597701	0.19844371
WOOD_EBV_EBNA1_TARGETS_UP	110	1.4193314	0.044487428	0.19824389
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_GREEN_UP	23	1.4188923	0.041825093	0.19780645
LUI_THYROID_CANCER_CLUSTER_1	49	1.41838	0.024	0.19751209
RIGGINS_TAMOXIFEN_RESISTANCE_DN	214	1.4180951	0.027613413	0.1977499
PID_TRAIL_PATHWAY	28	1.4175515	0.03952569	0.19781253

KEGG_CHEMOKINE_SIGNALING_PATHWAY	188	1.417496	0.015873017	0.19759427
HOFFMANN_SMALL_PRE_BII_TO_IMMATURE_B_LYMPHOCYTE_DN	50	1.4174112	0.023483366	0.19744635
SESTO_RESPONSE_TO_UV_C5	46	1.417382	0.048828125	0.19719689
REACTOME_G1_PHASE	37	1.4165213	0.04255319	0.19677778
WEIGEL_OXIDATIVE_STRESS_BY_TBH_AND_H2O2	35	1.4148192	0.03929273	0.19754297
BIOCARTA_MET_PATHWAY	37	1.4146954	0.03353057	0.1974791
GROSS_HYPOXIA_VIA_ELK3_DN	150	1.4146127	0.04330709	0.19732976
REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION	91	1.4145036	0.033333335	0.1971607
MAHAJAN_RESPONSE_TO_IL1A_UP	81	1.4122285	0.042462844	0.1990607
LEIN_OLIGODENDROCYTE_MARKERS	69	1.4122274	0.038986355	0.1987765
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	59	1.4114577	0.03937008	0.19960748
PID_ILK_PATHWAY	45	1.4080392	0.027504912	0.1998721
GAL_LEUKEMIC_STEM_CELL_DN	231	1.4069612	0.033009708	0.19990104
ZHONG_SECRETOME_OF_LUNG_CANCER_AND_ENDOTHELIUM	65	1.4068204	0.045454547	0.19955413
UEDA_PERIPHERAL_CLOCK	159	1.405421	0.031496063	0.19868033
NIELSEN_LEIOMYOSARCOMA_UP	17	1.4051892	0.026209677	0.19779873
ZHANG_BREAST_CANCER_PROGENITORS_DN	140	1.4042321	0.0407767	0.19634518
PID_TNFPATHWAY	44	1.4034581	0.033009708	0.196922
BILD_HRAS_ONCOGENIC_SIGNATURE	248	1.4033405	0.032520324	0.19688185
REACTOME_MAP_KINASE_ACTIVATION_IN_TLR_CASCADE	46	1.402121	0.04054054	0.1962722
MAGRANGEAS_MULTIPLE_MYELOMA_IGLL_VS_IGLK_UP	40	1.4018964	0.021359224	0.19587065
WHITFIELD_CELL_CYCLE_G2	165	1.3993015	0.03353057	0.19697462
JAATINEN_HEMATOPOIETIC_STEM_CELL_DN	213	1.39919	0.041420117	0.19688626
NOUZOVA_METHYLATED_IN_APL	58	1.398552	0.028409092	0.19746159
KEEN_RESPONSE_TO_ROSIGLITAZONE_DN	104	1.3963083	0.04158416	0.1981765
OUYANG_PROSTATE_CANCER_PROGRESSSION_UP	19	1.3951851	0.04527559	0.19938174
REACTOME_NUCLEAR_EVENTS_KINASE_AND_TRANSCRIPTION_FACTOR_ACTIVATION	24	1.3950818	0.047709923	0.19928803
WIERENGA_STAT5A_TARGETS_UP	204	1.3950083	0.03515625	0.19892493
IGLESIAS_E2F_TARGETS_UP	150	1.3938861	0.04901961	0.19956821
LUI_THYROID_CANCER_PAX8_PPARG_UP	43	1.3927048	0.03245436	0.20002839
YORDY_RECIPROCAL_REGULATION_BY_ET51_AND_SP100_UP	21	1.3924973	0.037523452	0.20007755
CADWELL_ATG16L1_TARGETS_DN	60	1.392442	0.023121387	0.19991656
RICKMAN_TUMOR_DIFFERENTIATED_MODALITY_VS_POORLY_UP	112	1.3922454	0.039447732	0.19974175
LEE_LIVER_CANCER_DENA_UP	59	1.3920181	0.04892368	0.1996334
DELASERNA_MYOD_TARGETS_DN	55	1.3898811	0.03929273	0.20043284
IWANAGA_CARCINOGENESIS_BY_KRAS_UP	154	1.3848802	0.05	0.20179583
LASTOWSKA_COAMPLIFIED_WITH_MYCN	40	1.3847269	0.039033458	0.20135136
LIM_MAMMARY_STEM_CELL_DN	404	1.3836287	0.04296875	0.20130512
JIANG_HYPOXIA_NORMAL	296	1.3829882	0.04263566	0.20116593
BANDRES_RESPONSE_TO_CARMUSTIN_MG	159	1.3821402	0.03256705	0.20184582
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	44	1.3819085	0.04423077	0.20206337

HAN_SATB1_TARGETS_DN	421	1.3806916	0.041501977	0.2020063
YANG_BCL3_TARGETS_UP	347	1.375523	0.030991735	0.20453852
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_IN TERACTION	265	1.370787	0.046709128	0.20557475
ZHU_CMV_8_HR_DN	53	1.3706459	0.042424243	0.20538308
FARMER_BREAST_CANCER_BASAL_VS_LUL MINAL	315	1.3697442	0.036190476	0.205419
YAGI_AML_RELAPSE_PROGNOSIS	35	1.3693016	0.04536862	0.20547073
SANSOM_APC_TARGETS_REQUIRE_MYC	196	1.3692548	0.04296875	0.20533429
WANG_ESOPHAGUS_CANCER_VS_NORMAL_ UP	116	1.3672936	0.027777778	0.20669007
NAKAMURA_ADIPOGENESIS_LATE_UP	99	1.3586439	0.041501977	0.20981163
GREGORY_SYNTHETIC_LETHAL_WITH_IMATI NIB	139	1.3500751	0.031189084	0.21257612
HELLER_SILENCED_BY_METHYLATION_UP	277	1.3488044	0.04255319	0.21298103
ACEVEDO_FGFR1_TARGETS_IN_PROSTATE_ CANCER_MODEL_UP	280	1.3425481	0.033663366	0.21520826

Table 4.10 *GSEA analysis of enriched pathways in symptomatic plaques*

379 biological pathways significantly enriched in macrophages from symptomatic plaques. Statistical threshold set as $P < 0.05$, $FDR\ Q < 0.25$. NES = normalized enrichment score.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
WENG_POR_TARGETS_LIVER_UP	38	1.5114162	0.003752345	0.24482584
GNATENKO_PLATELET_SIGNATURE	46	1.5066727	0.005758158	0.24098971
RASHI_RESPONSE_TO_IONIZING_RADIATION _6	79	1.5040202	0.007843138	0.24226007
MELLMAN_TUT1_TARGETS_DN	47	1.5025938	0.007858546	0.24104062
AMUNDSON_GENOTOXIC_SIGNATURE	101	1.5013996	0.007692308	0.24125974
BRUNO_HEMATOPOIESIS	64	1.4996777	0.003976143	0.24084347
MOOTHA_TCA	16	1.4961965	0.007707129	0.24340804
REACTOME_TRAF6_MEDIATED_INDUCION_ OF_NFKB_AND_MAP_KINASES_UPON_TLR7_ 8_OR_9_ACTIVATION	72	1.4960093	0.007797271	0.24196623
GAZDA_DIAMOND_BLACKFAN_ANEMIA_PRO GENITOR_UP	39	1.4937316	0.007751938	0.23292744
NIKOLSKY_OVERCONNECTED_IN_BREAST_ CANCER	22	1.49074	0.008	0.22668043
GROSS_HYPOXIA_VIA_ELK3_AND_HIF1A_UP	136	1.4681828	0.007984032	0.20276564
KYNG_RESPONSE_TO_H2O2	71	1.4672376	0.007490637	0.20268361
YAGI_AML_SURVIVAL	122	1.4558709	0.009652509	0.19807208

Table 4.11 The top 13 biological pathways most highly enriched in macrophages from symptomatic plaques. Statistical threshold set as $P < 0.01$, $FDR\ Q < 0.25$. NES = normalized enrichment score.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
REACTOME_TERMINATION_OF_O_GLYCAN_BIOSYNTHESIS	24	1.884635	< 0.001	0.014480328

Table 4.12 Only 1 biological pathway was significantly enriched in macrophages from asymptomatic plaques with $P < 0.01$ and $FDR\ Q < 0.25$. NES = normalized enrichment score.

Of those pathways significantly enriched in macrophages from symptomatic plaques, they can be broadly categorized into upregulation of pathways involving:

- (1) inflammation,
- (2) lipid metabolism,
- (3) hypoxic response,
- (4) cell proliferation,
- (5) apoptosis,
- (6) antigen presentation,
- (7) cellular energetics, as shown in Table 4.13 below:-

Biological function	Enriched curated gene sets e.g. Kegg, Biocarta, Reactome, etc.	Enriched Gene Ontology (GO) gene sets
(1) Inflammation	Reactome TRAF6 mediated induction of NFkB and MAP kinases upon TLR7, 8, 9 activation	GO IKappaB kinase NFKappaB cascade
	Reactome TRIF mediated TLR3 signaling	GO Adaptive immune response
	Reactome MAPK targets unclear events mediated by MAP kinases	GO inflammatory response
	Reactome MAP kinase activation in TLR cascade	GO immune system process
	PID TNF pathway	GO defence response
	Kegg Toll-like receptor signaling pathway	GO chemokine activity
	Biocarta MAPK pathway	GO cytokine activity
	Biocarta TNFR1 pathway	GO chemokine receptor binding
	Reactome lipid digestion mobilization and transport	GO protein amino acid lipidation
	Reactome HDL mediated lipid transport	GO lipid biosynthetic process
(2) Lipid metabolism	Reactome PPARA activates gene expression	GO lipid raft
	Reactome transcriptional regulation of white adipocyte differentiation	GO lipid metabolic process

		GO membrane lipid metabolic process
(3) Hypoxic response	Reactome regulation of hypoxia inducible factor HIF by oxygen	
	Winter hypoxia metagene	
	Gross hypoxia via ELK3 and HIF1A up	
	Elvidge HIF1A and HIF2A targets up	
	Jiang hypoxia normal	
(4) Cell proliferation	Reactome S phase	GO positive regulation of cell proliferation
	Reactome G1 phase	GO homeostasis of number of cells
	Reactome activation of the pre replicative complex	
	Reactome E2F mediated regulation of DNA replication	
(5) Apoptosis	Biocarta caspase pathway	GO regulation of protein stability
	Biocarta FAS pathway	
	PID caspase pathway	
	Dutta apoptosis via NFkB	
	Hollmann apoptosis via CD40	
(6) Antigen presentation	Reactome antigen processing ubiquitination proteasome degradation	GO antigen binding
	Reactome MHC class II antigen presentation	
	Biocarta DC pathway	
	Lindstedt dendritic cell maturation	
(7) Cellular energetics	Reactome TCA cycle and respiratory electronic transport	GO generation of precursor metabolites and energy
	Reactome citric acid cycle TCA cycle	
	Reactome integration of energy metabolism	

Table 4.13 ***Upregulated biological functions in symptomatic plaques***

Top 7 biological functions upregulated in human carotid plaque macrophages from symptomatic as opposed to asymptomatic plaques; as revealed by significantly enriched corresponding gene sets in the curated databases such as Kegg, Biocarta, Reactome, etc. in the middle column) and Gene Ontology (GO) database in the right column. Threshold for all significant pathways set as $P < 0.05$, FDR < 0.25 .

4.4.7.2 Enriched pathways in core *versus* cap macrophages

To evaluate gene set enrichment in plaque macrophage subsets based on micro-anatomical location, GSEA was performed on core *versus* cap macrophages. At conventional thresholds of $P < 0.05$ and $FDR < 0.25$, none of the pathways were significantly enriched either in core or cap macrophages. On further inspection of the datasets, 15 gene sets were enriched at $P < 0.05$ and 2 of these significantly enriched at $P < 0.01$ in core macrophages but these did not fulfill $FDR < 0.25$ threshold; likewise, 8 gene sets were enriched at $P < 0.05$ and 2 of these significantly enriched at $P < 0.01$ in cap macrophages but these also did not fulfill $FDR < 0.25$ threshold.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
Gene sets enriched in <i>core</i> macrophages				
WANG_CLASSIC_ADIPOGENIC_TARGETS_OF_PPARG	25	1.7091866	0.005870841	1
REACTOME_SYNTHESIS_SECRETION_AND_INACTIVATION_OF_GLP1	18	1.666416	0.014198783	1
REACTOME_INCRETIN_SYNTHESIS_SECRETION_AND_INACTIVATION	21	1.63266	0.016393442	1
ICHIBA_GRAFT_VERSUS_HOST_DISEASE_D7_DN	39	1.6137489	0.021186441	1
REACTOME_LIPID_DIGESTION_MOBILIZATION_AND_TRANSPORT	44	1.5822108	0.014112903	1
STEGEER_ADIPOGENESIS_UP	21	1.5754874	0.009940358	1
KEGG_NITROGEN_METABOLISM	23	1.5664947	0.02258727	1
BIOCARTA_ERYTH_PATHWAY	15	1.5403708	0.03373016	1
LIU_LIVER_CANCER	30	1.5088805	0.030241935	1
WOO_LIVER_CANCER_RECURRENCE_DN	78	1.4968517	0.047131147	1
REACTOME_METABOLISM_OF_POLYAMINES	15	1.4968022	0.043933053	1
HASLINGER_B_CLL_WITH_MUTATED_VH_GENES	18	1.486346	0.021097047	1
FERRARI_RESPONSE_TO_FENRETINIDE_UP	19	1.4677336	0.040983606	1
SPIELMAN_LYMPHOBLAST_EUROPEAN_VS_ASIAN_2FC_DN	19	1.4160488	0.046747968	1
ZHAN_MULTIPLE_MYELOMA_CD1_VS_CD2_DN	50	1.4043485	0.04312115	1
Gene sets enriched in <i>cap</i> macrophages				
SABATES_COLORECTAL_ADENOMA_SIZE_UP	21	1.6792744	0.001956947	1
BIOCARTA_CYTOKINE_PATHWAY	21	1.643262	0.018218623	1
BIOCARTA_IL12_PATHWAY	21	1.6137017	0.003960396	1
JOHANSSON_GLIOMAGENESIS_BY_PDGF_DN	20	1.4726485	0.037190083	1
MEISSNER_BRAIN_HCP_WITH_H3K4ME2_AND_H3K27ME3	57	1.4660553	0.03469388	1
WOTTON_RUNX_TARGETS_DN	29	1.4516805	0.02173913	1
HOWLIN_CITED1_TARGETS_1_UP	33	1.3919983	0.041825093	1
GAVIN_FOXP3_TARGETS_CLUSTER_P7	82	1.3916888	0.034883723	1

Table 4.14 *GSEA analysis of enriched pathways in core vs. plaques*

Enriched pathways in core and cap macrophages at $P < 0.05$ statistical threshold. Unfortunately, none of these pathways fulfill $FDR < 0.25$ criteria. NES = normalized enrichment score.

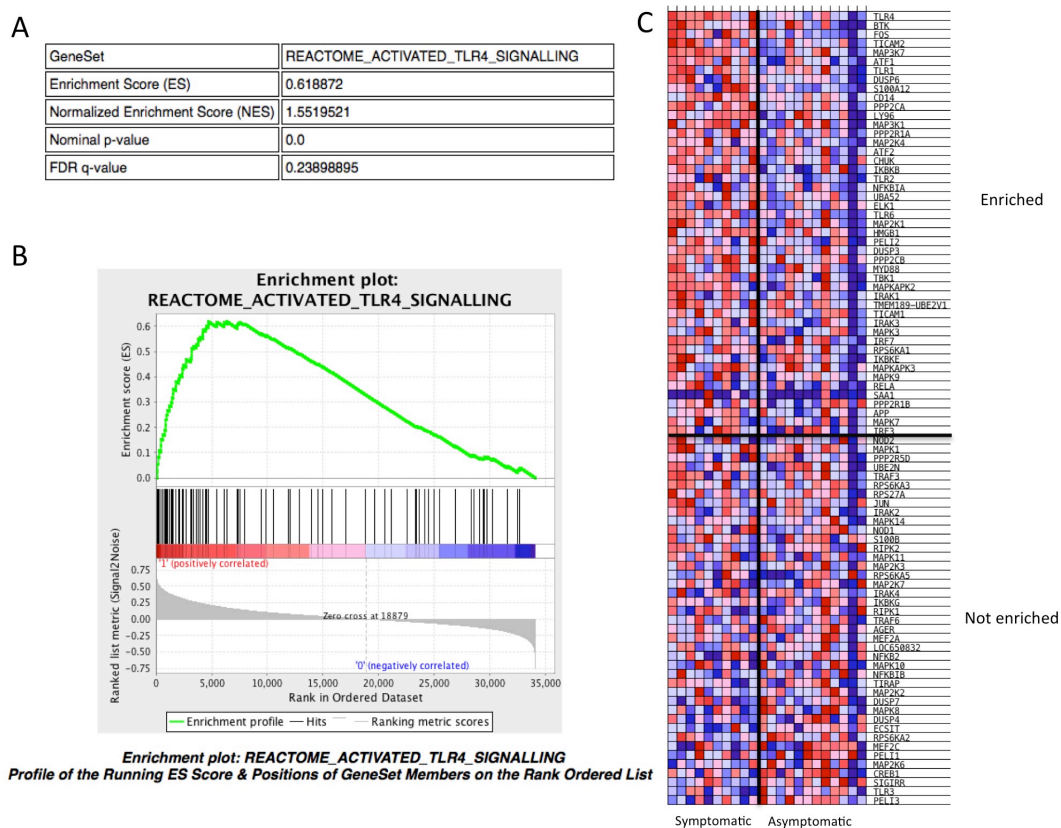


Figure 4.14 An illustration of a *highly significantly enriched* pathway (“Reactome Activated TLR4 Signaling”) in macrophages from symptomatic plaques. P value < 0.001, and FDR < 0.25 (A). B: Each individual gene in the gene sets (vertical black line) was plotted against the “Reactome Activated TLR4 Signaling” gene set with more genes clustered to the left of the plot, indicating a positive correlation with the gene set. C: The resulting heat map generated showing the enriched genes in the gene set and those that are not enriched in the “Symptomatic vs Asymptomatic” comparison. Each column represents a separate biological sample and each row represents a gene in the gene set. Red indicates upregulation and blue indicates downregulation of the specific gene.

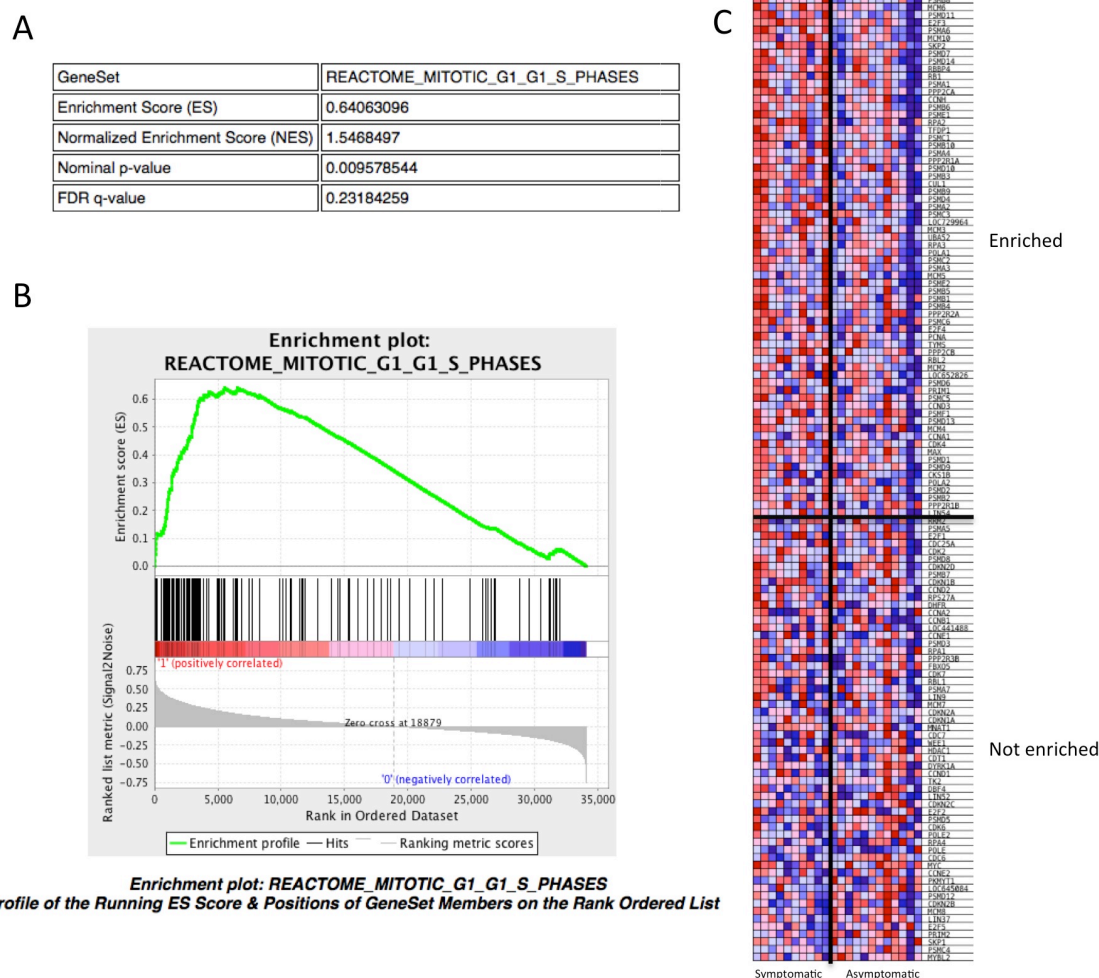


Figure 4.15 An illustration of a *significantly enriched* pathway (“Reactome Mitotic G1 G1 S Phases”) in macrophages from symptomatic plaques. P value < 0.01, and FDR < 0.25 (A). B: Each individual gene in the gene sets (vertical black line) was plotted against the “Reactome Mitotic G1 G1 S Phases” gene set with more genes clustered to the left of the plot, indicating a positive correlation with the gene set. C: The resulting heat map generated showing the enriched genes in the gene set and those that are not enriched in the “Symptomatic vs Asymptomatic” comparison. Each column represents a separate biological sample and each row represents a gene in the gene set. Red indicates upregulation and blue indicates downregulation of the specific gene.

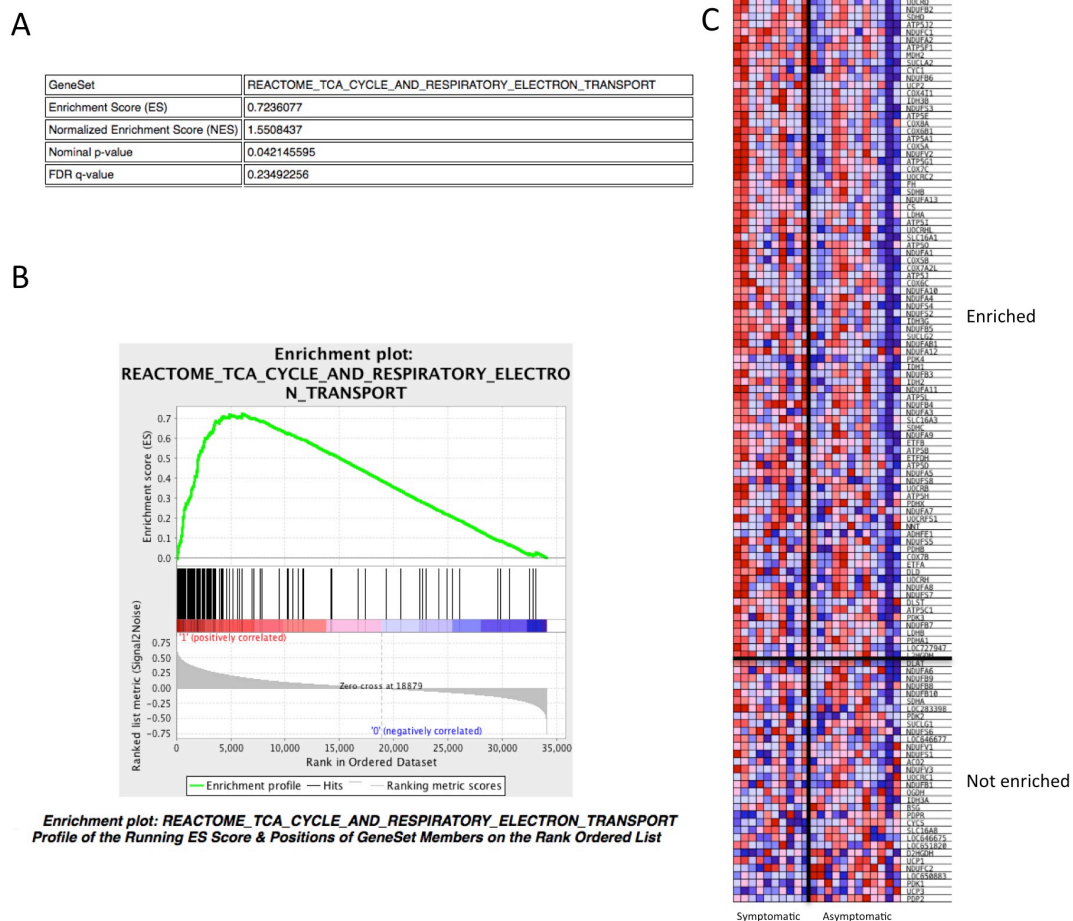


Figure 4.16 An illustration of an enriched pathway (“Reactome TCA cycle and Respiratory Electron Transport”) in macrophages from symptomatic plaques. P value < 0.05, and FDR < 0.25 (A). B: Each individual gene in the gene sets (vertical black line) was plotted against the “Reactome TCA cycle and Respiratory Electron Transport” gene set with more genes clustered to the left of the plot, indicating a positive correlation with the gene set. C: The resulting heat map generated showing the enriched genes in the gene set and those that are not enriched in the “Symptomatic vs Asymptomatic” comparison. Each column represents a separate biological sample and each row represents a gene in the gene set. Red indicates upregulation and blue indicates downregulation of the specific gene.

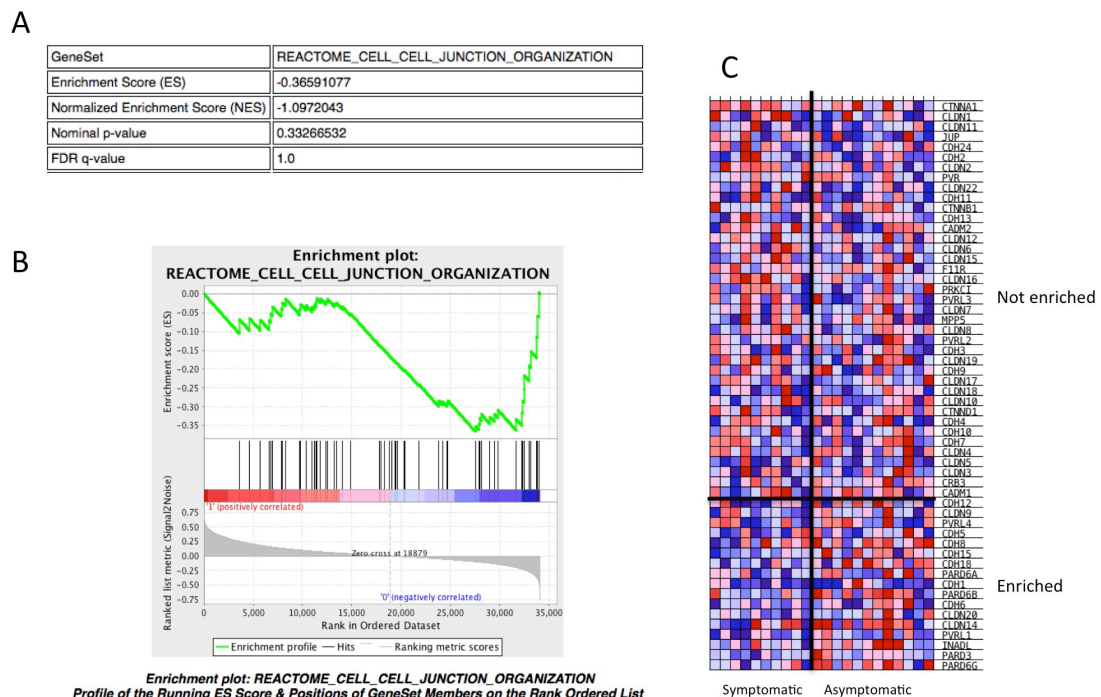


Figure 4.17 An illustration of a biological pathway (“Reactome Cell-Cell Junction Organization”) not enriched in macrophages from symptomatic plaques. P value 0.33, and FDR 1.0 (A). B: Each individual gene in the gene set (vertical black line) was plotted against the “Reactome Cell-Cell Junction Organization” gene set with genes randomly scattered across the plot, indicating no significant correlation with the gene set. C: The resulting heat map generated showing no distinct cluster of gene regulation. Each column represents a separate biological sample and each row represents a gene in the gene set. Red indicates upregulation and blue indicates downregulation of the specific gene.

4.4.7.3 Enriched pathways in subgroup analysis

GSEA was also performed in subgroup analysis: “Symptomatic/Core”, “Symptomatic/Cap”, “Asymptomatic/Core”, and “Asymptomatic/Cap”, were used in 4 permutations for comparison. The computed comparisons were as follow:-

1. “Symptomatic/Cap” *versus* “Asymptomatic/Cap” – to test the gene expression profiles of cap macrophages in acute vs non-acute plaques.
 - 183 statistically significant gene sets enriched in cap macrophages in acute plaques at $P < 0.05$, $FDR < 0.25$, in which 11 gene sets were enriched at $P < 0.01$ and $FDR < 0.25$. In contrast, 1 gene set was statistically enriched in cap macrophages in non-acute plaques at $P < 0.01$ and $FDR < 0.25$. [see Table 4.15 - 4.17]
2. “Symptomatic/Core” *versus* “Asymptomatic/Core” – to test the gene expression profiles of core macrophages in acute vs non-acute plaques.
 - No statistically significant gene set enrichment at $P < 0.05$, $FDR < 0.25$. 31 gene sets reached $P < 0.01$ in acute plaques but did not meet $FDR < 0.25$ threshold.
3. “Symptomatic/Core” *versus* “Symptomatic/Cap” – to test the gene expression profiles of core vs cap macrophages in acute plaques.
 - No statistically significant gene set enrichment at $P < 0.05$, $FDR < 0.25$. At $P < 0.01$, 2 gene sets were enriched in core macrophages and 16 gene sets were enriched in cap macrophages in acute plaques, but none meet the $FDR < 0.25$ threshold.

4. “Asymptomatic/Core” *versus* “Asymptomatic/Cap” – to test the gene expression profiles of core vs cap macrophages in non-acute plaques.

- No statistically significant gene set enrichment at $P < 0.05$, $FDR < 0.25$. At $P < 0.01$, 23 gene sets were enriched in core macrophages and 1 gene set was enriched in cap macrophages in non-acute plaques, but none meet the $FDR < 0.25$ threshold.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
SAKAI_CHRONIC_HEPATITIS_VS_LIVER_CANCER_UP	80	1.5004746	0.033027522	0.24962582
NIELSEN_GIST_AND_SYNOVIAL_SARCOMA_DN	20	1.5004169	0.036960986	0.24865687
KEGG_HOMOLOGOUS_RECOMBINATION	26	1.4998183	0.026717557	0.2482393
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_7	74	1.4993283	0.043052837	0.2482348
REACTOME_PURINE_METABOLISM	32	1.4993149	0.047445256	0.24722424
KEGG_ONE_CARBON_POOL_BY_FOLATE	17	1.4992199	0.019313306	0.24657993
WIELAND_UP_BY_HBV_INFECTION	98	1.4984518	0.04743083	0.24565116
GARY_CD5_TARGETS_DN	416	1.4983891	0.04779412	0.24479444
COATES_MACROPHAGE_M1_VS_M2_UP	77	1.498134	0.013779528	0.24447308
PID_CD8TCRPATHWAY	53	1.4973329	0.016064256	0.24453288
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	128	1.4971054	0.040935673	0.24404702
REACTOME_RNA_POL_III_TRANSCRIPTION_INITIATION_FROM_TYPE_2_PROMOTER	23	1.495738	0.037328094	0.24702609
GROSS_HYPOXIA_VIA_ELK3_ONLY_UP	33	1.4947462	0.04263566	0.24743843
OKAMOTO_LIVER_CANCER_MULTICENTRIC_OCCURRENCE_UP	24	1.494058	0.043726236	0.24634029
LANG_MYB_FAMILY_TARGETS	29	1.4927437	0.04696673	0.24916057
REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION	91	1.4922913	0.007920792	0.24849002
SCHLINGEMANN_SKIN_CARCINOGENESIS_TPA_UP	39	1.4918779	0.00589391	0.24880382
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_AND_BRAIN_QTL_TRANS	175	1.4891915	0.03954802	0.2495433
THILLAINADESAN_ZNF217_TARGETS_UP	40	1.4887851	0.015873017	0.24972919
REACTOME_SIGNAL_AMPLIFICATION	31	1.4885978	0.019646365	0.24936232
HERNANDEZ_ABERRANT_MITOSIS_BY_DOCETAXEL_4NM_UP	22	1.4878564	0.01632653	0.24865352
HASLINGER_B_CLL_WITH_11Q23_DELETION	23	1.4877483	0.014256619	0.24806665
ZHAN_V2_LATE_DIFFERENTIATION_GENES	43	1.4866855	0.042801555	0.24542798
BIOCARTA_FAS_PATHWAY	30	1.4866645	0.039215688	0.24462332
BILANGES_RAPAMYCIN_SENSITIVE_GENES	36	1.4866372	0.021825397	0.24383584
REACTOME_OXYGEN_DEPENDENT_PROLINE_HYDROXYLATION_OF_HYPOXIA_INDUCIBLE_FACTOR_ALPHA	17	1.4863956	0.033195022	0.24360909
REACTOME_NUCLEOTIDE_BINDING_DOMAIN_LEUCINE_RICH_REPEAT_CONTAINING_G_RECEPTOR_NLR_SIGNALING_PATHWAY	43	1.4860259	0.030592734	0.24196208

KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	137	1.485816	0.03	0.24176802
LIU_SOX4_TARGETS_DN	291	1.4855804	0.01814516	0.24173027
BIOCARTA_EIF4_PATHWAY	23	1.4853991	0.017175572	0.2413392
PID_NFKAPPABATYPICALPATHWAY	17	1.4851664	0.04238921	0.24094974
THEILGAARD_NEUTROPHIL_AT_SKIN_WOUND_UP	76	1.4837854	0.03929273	0.2417004
NOUZOVA_METHYLATED_IN_APL	58	1.4826146	0.012121212	0.24157575
PID_GMCSF_PATHWAY	37	1.4808366	0.035643563	0.24182953
KOKKINAKIS_METHIONINE_DEPRIVATION_96HR_DN	72	1.4791657	0.03359684	0.24501835
CAFFAREL_RESPONSE_TO_THC_24HR_5_UP	31	1.4788841	0.03468208	0.24475808
BIOCARTA_GSK3_PATHWAY	27	1.4784435	0.041322313	0.24430129
BIOCARTA_EIF_PATHWAY	16	1.4776341	0.01734104	0.24456906
BIOCARTA_TNFR2_PATHWAY	18	1.4771616	0.038314175	0.24499354
BIOCARTA_GCR_PATHWAY	19	1.4768343	0.018	0.24501283
PIONTEK_PKD1_TARGETS_UP	37	1.4762343	0.04743083	0.24504824
YIH_RESPONSE_TO_ARSENITE_C3	35	1.4752998	0.015180266	0.24680948
LEE_LIVER_CANCER_MYC_UP	52	1.4738364	0.009380863	0.24607648
SCHURINGA_STAT5A_TARGETS_DN	15	1.47374	0.007920792	0.24555516
REACTOME_DOWNSTREAM_TCR_SIGNALING	35	1.4735658	0.040076334	0.24507432
SANSOM_APC_TARGETS_REQUIRE_MYC	196	1.4727306	0.027184466	0.24519356
HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN	76	1.4724995	0.03305785	0.24498515
TAVOR_CEBPA_TARGETS_UP	47	1.4723217	0.026052104	0.243946
DOANE_BREAST_CANCER_CLASSES_DN	34	1.4719888	0.04233871	0.24410176
REACTOME_ACTIVATED_TLR4_SIGNALING	88	1.4717095	0.04255319	0.24252713
PID_FASPATHWAY	38	1.4702531	0.023060797	0.23957598
GEISS_RESPONSE_TO_DSRNA_UP	38	1.4692719	0.032629557	0.23997344
NIELSEN_LEIOMYOSARCOMA_UP	17	1.4690268	0.019522777	0.2398659
BIOCARTA_MPR_PATHWAY	33	1.4682046	0.040733196	0.23855712
ROSS_AML_WITH_CBFB_MYH11_FUSION	50	1.4680756	0.031558186	0.23842262
REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	88	1.4674851	0.020134227	0.23577273
GAZDA_DIAMOND_BLACKFAN_ANEMIA_PROGENITOR_UP	39	1.4672475	0.014736842	0.23496306
LINDSTEDT_DENDRITIC_CELL_MATURATION_A	66	1.4662416	0.034764826	0.23517162
HINATA_NFKB_TARGETS_KERATINOCYTE_UP	91	1.4648747	0.01705757	0.23399225
REACTOME_DESTABILIZATION_OF_MRNA_BY_KSRP	17	1.4646901	0.04511278	0.23373228
REACTOME_ADP_SIGNALING_THROUGH_P2RY1	25	1.4645596	0.004115226	0.23349936
REACTOME_TRAF6_MEDIATED_NFKB_ACTIVATION	20	1.4644078	0.039832287	0.2331578
BOGNI_TREATMENT_RELATED_MYELOID_LEUKEMIA_UP	29	1.4640045	0.039583333	0.23157565
VERHAAK_AML_WITH_NPM1_MUTATED_UP	180	1.4631249	0.008639309	0.23244488
ZHAN_MULTIPLE_MYELOMA_DN	41	1.4628038	0.024904214	0.23254305
MOREIRA_RESPONSE_TO_TSA_UP	28	1.4618803	0.044401545	0.2332369
SA_MMP_CYTOKINE_CONNECTION	15	1.461746	0.033898305	0.23223796
REACTOME_MITOTIC_G2_G2_M_PHASES	73	1.4613994	0.04771372	0.23177128

CHESLER_BRAIN_QTL_CIS	70	1.4612508	0.03875969	0.23091011
KEGG_TYROSINE_METABOLISM	42	1.4610546	0.025477707	0.23011073
PID_ERBB1_INTERNALIZATION_PATHWAY	40	1.4605713	0.045652173	0.22986591
RASHI_RESPONSE_TO_IONIZING_RADIATION_6	79	1.4602448	0.012422361	0.2287052
DITTMER_PTHLH_TARGETS_DN	73	1.4593395	0.04024145	0.22865249
CHOW_RASSF1_TARGETS_UP	27	1.4593287	0.013207547	0.22815798
BERTUCCI_MEDULLARY_VS_DUCTAL_BREAST_CANCER_UP	191	1.4591808	0.0390625	0.2267907
BIOCARTA_CCR5_PATHWAY	17	1.4584318	0.046184737	0.22618337
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_DN	30	1.45832	0.019880716	0.22530927
PID_PI3KPATHWAY	49	1.4572011	0.029069768	0.22582522
THEILGAARD_NEUTROPHIL_AT_SKIN_WOUND_DN	219	1.4547468	0.04743833	0.22806007
PID_IL3_PATHWAY	27	1.4542497	0.026262626	0.22750741
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	97	1.4503974	0.046413504	0.22359505
XU_RESPONSE_TO_TRETINOIN_UP	16	1.450148	0.024856597	0.22355121
KEGG_P53_SIGNALING_PATHWAY	67	1.4501227	0.026530612	0.22312953
GAZDA_DIAMOND_BLACKFAN_ANEMIA_ERYTHROID_UP	24	1.4500693	0.011904762	0.2227448
RHEIN_ALL_GLUCOCORTICOID_THERAPY_UP	73	1.4494152	0.031835206	0.22400029
SEIDEN_ONCOGENESIS_BY_MET	83	1.4493601	0.048732944	0.22365162
VANHARANTA_UTERINE_FIBROID_WITH_7Q_DELETION_DN	35	1.4493003	0.02268431	0.22333454
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	31	1.4490556	0.04536862	0.22328067
IVANOVA_HEMATOPOIESIS_MATURE_CELL	268	1.4487342	0.043052837	0.2228764
LIM_MAMMARY_LUMINAL_PROGENITOR_UP	57	1.4486942	0.006507592	0.22244148
REACTOME_MYD88_MAL_CASCADE_INITIATED_ON_PLASMA_MEMBRANE	78	1.4484295	0.04296875	0.22189167
PID_P75NTRPATHWAY	69	1.4480209	0.02783726	0.22210063
OZANNE_API1_TARGETS_UP	17	1.447068	0.04109589	0.22259495
KUROZUMI_RESPONSE_TO_ONCOCYTIC_VIRUS	42	1.4466532	0.023255814	0.22247727
CAIRO_LIVER_DEVELOPMENT_DN	222	1.4462671	0.012903226	0.22226205
NEMETH_INFLAMMATORY_RESPONSE_LPS_UP	87	1.4449958	0.0385439	0.22120807
PID_EPOPATHWAY	34	1.4446619	0.03544776	0.22132042
RAMALHO_STEMNESS_DN	74	1.4441143	0.035555556	0.22115992
HUMMERICH_BENIGN_SKIN_TUMOR_UP	17	1.4439896	0.039748956	0.2209435
KEGG_GALACTOSE_METABOLISM	26	1.4432163	0.04592902	0.22203201
HOLLEMAN_PREDNISOLONE_RESISTANCE_B_ALL_UP	21	1.4424454	0.04016064	0.22258244
PID_NECTIN_PATHWAY	30	1.4421413	0.0295858	0.22220452
BIOCARTA_MET_PATHWAY	37	1.442001	0.049115915	0.22209352
ASTIER_INTEGRIN_SIGNALING	55	1.4416281	0.035714287	0.22091037
WENG_POR_TARGETS_LIVER_UP	38	1.4414192	0.029106028	0.22053862
WANG_RESPONSE_TO_ANDROGEN_UP	29	1.4410565	0.04699248	0.22028203
ZHANG_RESPONSE_TO_CANTHARIDIN_UP	18	1.4400878	0.010909091	0.22071525
REACTOME_THE_ROLE_OF_NEF_IN_HIV1_REPLICATION_AND_DISEASE_PATHOGENESIS	27	1.4398305	0.017429193	0.22028458

MYLLYKANGAS_AMPLIFICATION_HOT_S POT_17	19	1.439636	0.030368764	0.21978028
GARY_CD5_TARGETS_UP	454	1.4385782	0.039447732	0.2205906
PID_RB_1PATHWAY	65	1.4377849	0.034816246	0.22111124
REACTOME_PROCESSING_OF_CAPPED_ INTRONLESS_PRE_MRNA	22	1.4373555	0.03937008	0.22092952
REACTOME_INHIBITION_OF_VOLTAGE_G ATED_CA2_CHANNELS_VIA_GBETA_GA MMA_SUBUNITS	25	1.4357975	0.032319393	0.22227493
REACTOME_TRAF6_MEDIATED_INDUCTI ON_OF_NFKB_AND_MAP_KINASES_UPO N_TLR7_8_OR_9_ACTIVATION	72	1.4356995	0.04563492	0.22206119
WANG_TARGETS_OF_MLL_CBP_FUSION _UP	43	1.4334208	0.038934425	0.22277315
KEGG_HEMATOPOIETIC_CELL_LINEAGE	87	1.4329708	0.00621118	0.22273977
ODONNELL_TARGETS_OF_MYC_AND_TF RC_UP	79	1.432641	0.033962265	0.22294584
RAO_BOUND_BY_SALL4_ISOFORM_A	170	1.4312278	0.03846154	0.22345254
REACTOME_LOSS_OF_NLP_FROM_MITO TIC_CENTROSOMES	51	1.430138	0.044989776	0.2226032
SEKI_INFLAMMATORY_RESPONSE_LPS_ UP	74	1.4294922	0.025	0.22257671
NIKOLSKY_BREAST_CANCER_16Q24_AM PLICON	46	1.4292458	0.022	0.22258072
CHIARADONNA_NEOPLASTIC_TRANSFO RMATION_KRAS_CDC25_UP	56	1.4259602	0.04040404	0.2220088
LIM_MAMMARY_STEM_CELL_DN	404	1.4249346	0.044806518	0.22195834
PID_CXCR3PATHWAY	42	1.4239037	0.03125	0.22169723
DASU_IL6_SIGNALING_UP	58	1.4238466	0.013215859	0.22137108
HIRSCH_CELLULAR_TRANSFORMATION_ SIGNATURE_UP	236	1.4236281	0.02811245	0.22096872
KEGG_N_GLYCAN_BIOSYNTHESIS	46	1.4234729	0.01992032	0.22090912
MAGRANGEAS_MULTIPLE_MYELOMA_IG G_VS_IGA_DN	24	1.4228687	0.027559055	0.22083287
KYNG_DNA_DAMAGE_BY_UV	60	1.4228482	0.037401576	0.22047697
DIAZ_CHRONIC_MEYLOGENOUS_LEUKE MIA_DN	116	1.422349	0.04945055	0.22024696
HAHTOLA_SEZARY_SYNDROM_UP	94	1.4210801	0.049407113	0.2191794
PID_INTEGRIN_A4B1_PATHWAY	33	1.4206486	0.041431263	0.21810874
REACTOME_RECRUITMENT_OF_MITOTIC _CENTROSOME_PROTEINS_AND_COMPL EXES	58	1.4205273	0.046413504	0.2175818
GNATENKO_PLATELET_SIGNATURE	46	1.4186628	0.04819277	0.2191334
MARIADASON_RESPONSE_TO_BUTYRAT E_SULINDAC_4	20	1.4185771	0.033797216	0.21859246
NAKAJIMA_EOSINOPHIL	30	1.4174118	0.024242423	0.21877249
PID_NFAT_3PATHWAY	54	1.4171227	0.04288499	0.2181551
KEGG_APOPTOSIS	88	1.4165134	0.04761905	0.21726908
MURAKAMI_UV_RESPONSE_1HR_UP	17	1.4165024	0.027888447	0.21657588
FRASOR_RESPONSE_TO_SERM_OR_FUL VESTRANT_UP	21	1.4104728	0.03195489	0.22001459
GAUSSMANN_MLL_AF4_FUSION_TARGE TS_F_DN	33	1.4100572	0.025490196	0.22009514
KEGG_CHEMOKINE_SIGNALING_PATHW AY	188	1.4098258	0.008658009	0.22022524
CASORELLI_ACUTE_PROMYELOCYTIC_L EUKEMIA_UP	164	1.4097352	0.04330709	0.21997505
SESTO_RESPONSE_TO_UV_C1	69	1.4081304	0.049484536	0.22057857
GALLUZZI_PERMEABILIZE_MITOCHONDR IA	43	1.4076462	0.033771105	0.21999054
AZARE_STAT3_TARGETS	22	1.4074045	0.03937008	0.21907768

REACTOME_SYNTHESIS_OF_GLYCOSYL PHOSPHATIDYLINOSITOL_GPI	17	1.4064907	0.01532567	0.2193801
TONKS_TARGETS_OF_RUNX1_RUNX1T1_ FUSION_HSC_DN	182	1.4056698	0.012396694	0.21895196
DORSAM_HOXA9_TARGETS_UP	33	1.4055322	0.041825093	0.21850961
WANG_RESPONSE_TO_GSK3_INHIBITOR _SB216763_UP	356	1.4028182	0.04509804	0.21986079
LU_IL4_SIGNALING	90	1.4009844	0.026168223	0.22113451
RICKMAN_METASTASIS_DN	248	1.4000518	0	0.21978721
BOSCO_ALLERGEN_INDUCED_TH2_ASS OCIATED_MODULE	143	1.399208	0.034416825	0.21949981
WILCOX_PRESPONSE_TO_ROGESTERON E_UP	141	1.3975996	0.049603175	0.21932152
JAATINEN_HEMATOPOIETIC_STEM_CELL DN	213	1.3961684	0.030592734	0.21951778
PID_EPHRINBREVPATHWAY	30	1.3954701	0.049713194	0.21980964
YOSHIOKA_LIVER_CANCER_EARLY_REC URRENCE_DN	59	1.3936652	0.028846154	0.2193087
KYNG_ENVIRONMENTAL_STRESS_RESP ONSE_NOT_BY_GAMMA_IN_WS	33	1.3921841	0.027253669	0.21958093
MARKEY_RB1_CHRONIC_LOF_DN	117	1.3908545	0.032119915	0.22003877
PENG_GLUTAMINE_DEPRIVATION_UP	38	1.3896791	0.040084388	0.22036932
GRANDVAUX_IRF3_TARGETS_DN	19	1.3876691	0.028138528	0.22067685
MCCABE_HOXC6_TARGETS_CANCER_D N	19	1.3870937	0.047528517	0.2199954
BIOCARTA_DEATH_PATHWAY	33	1.3863924	0.026970955	0.22023848
KASLER_HDAC7_TARGETS_1_UP	184	1.3843033	0.03250478	0.21984145
BROCKE_APOPTOSIS_REVERSED_BY_IL 6	139	1.3828446	0.043052837	0.22002867
PID_INTEGRIN2_PATHWAY	29	1.3821644	0.031055901	0.21924181
KEGG_GLYCOSYLPHOSPHATIDYLINOSIT OL_GPI_ANCHOR_BIOSYNTHESIS	25	1.3804765	0.030612245	0.21899275
AMIT_EGF_RESPONSE_120_MCF10A	42	1.3795819	0.038961038	0.21913294
COULOUARN_TEMPORAL_TGFB1_SIGNA TURE_UP	103	1.3785965	0.037894737	0.21920714
HUTTMANN_B_CLL_POOR_SURVIVAL_U P	259	1.3769187	0.023109244	0.22049394
RUTELLA_RESPONSE_TO_HGF_DN	223	1.3744978	0.046468403	0.22187953
ST_FAS_SIGNALING_PATHWAY	63	1.3713486	0.040169135	0.22166935
KEGG_LYSINE_DEGRADATION	44	1.3708233	0.03846154	0.2221355
KYNG_DNA_DAMAGE_UP	220	1.3707255	0.039138943	0.2220319
YEGNASUBRAMANIAN_PROSTATE_CAN CER	120	1.3703079	0.03137255	0.22193906
ROZANOV_MMP14_TARGETS_DN	34	1.3673761	0.041484717	0.22320653
SUBTIL_PROGESTIN_TARGETS	35	1.3613145	0.040948275	0.22491102
WIERENGA_STAT5A_TARGETS_UP	204	1.3555409	0.036809817	0.22698487
WEIGEL_OXIDATIVE_STRESS_BY_TBH_A ND_H2O2	35	1.3518505	0.03557312	0.22919609
REACTOME_TRANSPORT_OF_INORGANI C_CATIONS_ANIONS_AND_AMINO_ACID S_OLIGOPEPTIDES	94	1.347688	0.04661017	0.22864047
KEGG_CELL_ADHESION_MOLECULES_C AMS	134	1.3349037	0.04183267	0.23416021
LABBE_TGFB1_TARGETS_DN	107	1.3309225	0	0.23688203
KEGG_CYTOKINE_CYTOKINE_RECEPTO R_INTERACTION	265	1.3061987	0.021186441	0.24928865

Table 4.15 183 significantly enriched pathways in cap macrophages in symptomatic, acute carotid plaques. Threshold $P < 0.05$, FDR < 0.25 threshold.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
REACTOME_MHC_CLASS_II_ANTIGEN_P RESENTATION	91	1.4922913	0.007920792	0.24849002
SCHLINGEMANN_SKIN_CARCI NOGENESIS_TPA_UP	39	1.4918779	0.00589391	0.24880382
LEE_LIVER_CANCER_MYC_UP	52	1.4738364	0.009380863	0.24607648
SCHURINGA_STAT5A_TARGETS_DN	15	1.47374	0.007920792	0.24555516
REACTOME_ADP_SIGNALLING_THROUG H_P2RY1	25	1.4645596	0.004115226	0.23349936
VERHAAK_AML_WITH_NPM1_MUTATED_ UP	180	1.4631249	0.008639309	0.23244488
LIM_MAMMARY_LUMINAL_PROGENITOR _UP	57	1.4486942	0.006507592	0.22244148
KEGG_HEMATOPOIETIC_CELL_LINEAGE	87	1.4329708	0.00621118	0.22273977
KEGG_CHEMOKINE_SIGNALING_PATHW AY	188	1.4098258	0.008658009	0.22022524
RICKMAN_METASTASIS_DN	248	1.4000518	0	0.21978721
LABBE_TGFB1_TARGETS_DN	107	1.3309225	0	0.23688203

Table 4.16 Top 11 significantly enriched pathways in cap macrophages in symptomatic, acute carotid plaques. Threshold $P < 0.01$, FDR < 0.25 threshold. NES = normalized enrichment score.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
REACTOME_TERMINATION_OF_O_GLYC AN_BIOSYNTHESIS	24	1.8485806	< 0.001	0.025674254

Table 4.17 Only 1 significantly enriched pathways in cap macrophages in asymptomatic, non-acute carotid plaques. Threshold $P < 0.01$, FDR < 0.25 threshold. NES = normalized enrichment score.

Subgroup analysis confirmed that while the gene expression profiles of core macrophages between symptomatic and asymptomatic plaques were similar, gene expression profiles of cap macrophages between symptomatic and asymptomatic plaques were highly significantly different. Of those pathways significantly enriched in cap macrophages from symptomatic plaques, they can be broadly categorized into upregulation of pathways involving:-

(1) Inflammation

- Reactome activated TLR4 signaling
- Reactome TRAF6 mediated NFkB activation
- Reactome MYD88 cascade initiated on plasma membrane
- PID NFkB atypical pathway
- PID GM-CSF pathway
- Biocarta CCR5 pathway
- Coates macrophage M1 vs M2 UP
- Azare STAT3 targets

(2) Adhesion molecule / cell signaling

- Reactome cell surface interactions at the vascular wall
- PID integrin A4B1 pathway
- PID integrin2 pathway
- Kegg cytokine-cytokine receptor interaction
- Kegg cell adhesion molecules CAMS
- Kegg chemokine signaling pathway

(3) Apoptosis

- PID FAS pathway
- Kegg apoptosis
- Biocarta FAS pathway
- Biocarta death pathway

(4) Antigen presentation

- Reactome MHC class II antigen presentation

(5) **Cell proliferation**

- Reactome mitotic G2 G2 M phases
- Reactome recruitment of mitotic centrosome proteins and complexes

(6) **Matrix metalloproteinase function**

- SA MMP cytokine connection
- Rozanov MMP14 targets DN

The above activated biological functions in cap macrophages from symptomatic plaques are consistent with current understanding of the pathophysiological processes of plaque instability - with cap macrophages involved in cell-cell signaling and likely responsible in the secretion of proteolytic enzymes, which over time degrade the fibrous cap and lead to cap rupture, with subsequent thrombotic clinical complication.

4.4.8 Enriched pathways in subgroup with diabetes mellitus

Patients with diabetes mellitus do not only show accelerated atherosclerosis but the pathophysiology of plaque progression and distribution is distinct from non-diabetic subjects, often presenting as diffused disease distributing across multiple vascular territory.^{236,237} Since a subgroup of the study cohort had a documented history of diabetes (8 out of 24), the dataset was re-analysed to evaluate biological pathways up- or downregulated in plaque macrophages from diabetic *versus* non-diabetic subjects. GSEA revealed 57 pathways *downregulated* in diabetic patients compared to non-diabetic patients with $P < 0.01$, albeit none reached $FDR < 0.25$ threshold.

Of the 57 downregulated pathways in diabetic patients, they can be broadly categorized according to the following biological functions:-

(1) Hypoxia

- Biocarta HIF pathway
- PID VEGFR 1 and 2 pathway

(2) Cellular energetics

- Reactome pyruvate metabolism and citric acid TCA cycle
- Mootha gluconeogenesis

(3) Nuclear signaling

- Reactome nuclear signaling by ERBB4
- PID RXR VDR pathway
- Biocarta PPARa pathway

(4) Inflammation

- PID Toll endogenous pathway
- PID p38 alpha beta pathway
- Ruan response to TNF Troglitazone UP

Pathway name	Size in gene set	NES	P-val	FDR Q-val
PID_RXR_VDR_PATHWAY	26	1.7329096	0.002066116	1
LIEN_BREAST_CARCINOMA_METAPLASTIC_VS_DUCTAL_UP	81	1.7025828	0	1
HUMMERICH_BENIGN_SKIN_TUMOR_UP	17	1.6994619	0.0056926	0.9483567
RUAN_RESPONSE_TO_TNF_TROGLITAZONE_UP	17	1.6986217	0.001941748	0.7654746
REACTOME_PYRUVATE_METABOLISM_AND_CITRIC_ACID_TCA_CYCLE	44	1.6834996	0	0.8075097
SMID_BREAST_CANCER_RELAPSE_IN_LUNG_UP	21	1.6831999	0.001968504	0.6955378
JAEGGER_METASTASIS_UP	43	1.672558	0.002020202	0.69563967
IKEDA_MIR30_TARGETS_DN	26	1.6669729	0.001937985	0.6645593
CHEN_LUNG_CANCER_SURVIVAL	27	1.65344	0.006097561	0.72351336
REACTOME_POST_CHAPERONIN_TUBULIN_FOLDING_PATHWAY	17	1.6425195	0	0.77287155
GRANDVAUX_IRF3_TARGETS_DN	19	1.6387632	0.004	0.7397808
LIU_BREAST_CANCER	25	1.6361241	0.002096436	0.6591183
LIAN_NEUTROPHIL_GRANULE_CONSTITUENTS	24	1.6354685	0.009727626	0.6195652
GROSS_HYPOXIA_VIA_ELK3_ONLY_UP	33	1.6309502	0.003960396	0.61708283
PID_P38ALPHABETAPATHWAY	30	1.6288933	0.002012072	0.59691054
MOOTHA_GLYCOGEN_METABOLISM	20	1.6275072	0.002020202	0.57203573
LOPEZ_MBD_TARGETS_IMPRINTED_AND_X_LINKED	17	1.6226666	0.002008032	0.58147985
PID_TOLL_ENDOGENOUS_PATHWAY	25	1.616265	0	0.60073805
BIOCARTA_KERATINOCYTE_PATHWAY	46	1.6138604	0	0.5925655
MOOTHA_GLUONEOGENESIS	31	1.6133049	0.00998004	0.5696139
CHEN_ETV5_TARGETS_SERTOLI	20	1.6115901	0.005725191	0.5582896
PID_DNAPK_PATHWAY	16	1.6100221	0	0.5462128
BIOCARTA_PPARA_PATHWAY	57	1.5980722	0.004048583	0.5916646
WANG_RECURRENT_LIVER_CANCER_DN	16	1.5971388	0.008032128	0.57783556
MYLLYKANGAS_AMPLIFICATION_HOTSPOT_17	19	1.5963445	0.006097561	0.5635086
SMIRNOV_CIRCULATING_ENDOTHELIOCYTES_IN_CANCER_UP	157	1.5946028	0.002008032	0.55711555
BIOCARTA_RHO_PATHWAY	32	1.5897621	0.004024145	0.5585544
BIOCARTA_HIF_PATHWAY	15	1.5892824	0.001996008	0.54490507
PIONTEK_PKD1_TARGETS_UP	37	1.5872548	0.008032128	0.5268027
GALE_APL_WITH_FLT3_MUTATED_DN	16	1.5869957	0.006185567	0.51346207
REACTOME_FORMATION_OF_TUBULIN_FOLDING_INTERMEDIATES_BY_CCT_TRIC	20	1.5855993	0.001992032	0.49527177
PID_ALPHASYNNUCLEIN_PATHWAY	33	1.5803266	0.002083333	0.5034232
REACTOME_PREFOLDIN_MEDIATED_TRANSFER_OF_SUBSTRATE_TO_CCT_TRIC	26	1.5802114	0.008032128	0.4918576
BIOCARTA_CERAMIDE_PATHWAY	22	1.577656	0.00407332	0.49635184
WILSON_PROTEASES_AT_TUMOR_BONE_INTERFACE_UP	21	1.5741959	0.005976096	0.5059969
KEGG_HEDGEHOG_SIGNALING_PATHWAY	56	1.5692533	0.004115226	0.5289848
PID_AMB2_NEUTROPHILS_PATHWAY	41	1.5634614	0.00204918	0.53572077
BIOCARTA_MPR_PATHWAY	33	1.562236	0.006302521	0.52166396
REACTOME_SULFUR_AMINO_ACID_METABOLISM	24	1.5589654	0.00929368	0.5194353
LEE_LIVER_CANCER_HEPATOBLAST	16	1.554261	0.005813954	0.52011806

QI_HYPOXIA	132	1.545177	0.005940594	0.52114
REACTOME_SEMA3A_PAK_DEPENDENT_AXON_REPULSION	15	1.5442092	0.005988024	0.51886934
BREDEMEYER_RAG_SIGNALING_NOT_VIA_ATM_DN	55	1.5427961	0.00984252	0.51925075
KAAB_FAILED_HEART_VENTRICLE_DN	41	1.5422877	0.008097166	0.5136875
PID_PS1PATHWAY	45	1.5416646	0.004237288	0.50144553
MURAKAMI_UV_RESPONSE_6HR_UP	37	1.5415257	0.006134969	0.49461642
FONTAINE_THYROID_TUMOR_UNCERTAIN_MALIGNANCY_DN	25	1.5239991	0.007272727	0.53839993
HEDENFALK_BREAST_CANCER_BRACXD_DN	19	1.5232556	0.00204499	0.5357982
KEGG_AMYOTROPHIC_LATERAL_SCLEROSIS_ALS	53	1.5137528	0.006185567	0.5268015
REACTOME_NUCLEAR_SIGNALING_BY_ERBB4	37	1.5056542	0.007874016	0.5217888
LEE_AGING_NEOCORTEX_UP	88	1.5028763	0.008438818	0.51702005
VERHAAK_GLIOBLASTOMA_MESENCHYMAL	213	1.5011265	0.002105263	0.5076006
GRAESSMANN_RESPONSE_TO_MC_AND_SERUM_DEPRIVATION_DN	82	1.493432	0.008438818	0.47813442
GENTILE_UV_RESPONSE_CLUSTER_D7	38	1.480224	0.008264462	0.46617064
DELASERNA_MYOD_TARGETS_DN	55	1.4760146	0.008080808	0.46143195
PID_VEGFR1_2_PATHWAY	69	1.4536192	0.008316008	0.44046122
BOYLAN_MULTIPLE_MYELOMA_PCA3_UP	73	1.408254	0.00589391	0.43338785

Table 4.18 Pathways downregulated in diabetes mellitus

57 pathways downregulated in plaque macrophages from patients with diabetes mellitus. Threshold $P < 0.01$ but none of these pathways reached $FDR < 0.25$ cut-off. NES = normalized enrichment score.

4.4.9 Enriched pathways against plaque lipid content by T₂ mapping

As validated and discussed in Chapter 3, T₂ mapping accurately and reproducibly quantifies plaque lipid content in human carotid plaques. One of the major hurdles preventing widespread clinical adaptation of such non-invasive imaging techniques is the lack of direct correlation of imaging biomarkers to molecular pathways or targets amenable to pharmacological intervention. In order to explore the molecular correlation of plaque lipid content as detected by T₂ mapping, the whole dataset was re-analysed using Lipid Area (%) derived by T₂ mapping alone, regardless of clinical status.

First, the dataset was divided into two groups: plaques with <25% lipid area (small LRNC) and plaques with ≥25% lipid area (large LRNC) – 25% being the optimal threshold in the literature and also proven to be consistent in our study cohort (see Chapter 3.4.4 and Figure 3.7B). GSEA revealed 72 pathways significantly enriched at $P < 0.05$ and $FDR < 0.25$ in plaques with large LRNC with the most significantly enriched pathway being “PID Interferon gamma pathway” with $P < 0.01$ and $FDR < 0.25$.

To further refine the enriched pathways, GSEA was repeated on core macrophages only – since they are in direct contact with plaque lipid and most likely to be affected by changes in lipid content – 28 pathways was significantly enriched at $P < 0.01$, of which 14 of them were involved in TNF / interferon / STAT signaling pathways.

Finally, instead of using a pre-defined “cut-off” of 25% lipid area to divide the dataset, the whole dataset from core macrophages was re-analysed using raw Lipid Area (%), as directly measured by T₂ mapping, as a *continuous variable* for GSEA. This approach quantitatively evaluates the degree of pathway enrichment / activation as

the lipid content increases in the plaques, without bias to any pre-defined parameter. 5 pathways were found to be significantly ($P < 0.05$, $FDR < 0.25$) enriched as plaques lipid content increases, of which three of them were involved in interferon signaling, and the most significantly enriched pathway being interferon alpha response pathway ($P < 0.01$, $FDR < 0.25$).

This provided, for the first time, direct evidence showing plaque lipid content positively correlated with activation of inflammatory pathways in macrophages in the same clinical cohort of plaques/patients; and showed how an non-invasive imaging biomarker can be directly correlated to biological pathways / targets amenable to pharmacological intervention.

Pathway name	Size in gene set	NES	P-val	FDR Q-val
EINAV_INTERFERON_SIGNATURE_IN_CANCER	27	1.5974699	0.022633744	0.24895108
REACTOME_ANTIGEN_PROCESSING_CROSS_PRESENTATION	74	1.5732976	0.016129032	0.24769165
REACTOME_ER_PHAGOSOME_PATHWAY	59	1.5717558	0.016194332	0.24965818
TAVOR_CEBPA_TARGETS_UP	47	1.5717279	0.004065041	0.24687399
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	55	1.5709977	0.024	0.2463786
HAHTOLA_CTCL_CUTANEOUS	26	1.5705765	0.018442623	0.24506004
CAFFAREL_RESPONSE_TO_THC_24HR_5_UP	31	1.5540613	0.011904762	0.24928048
BENPORATH_PROLIFERATION	137	1.553627	0.044145875	0.24844381
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	41	1.5528826	0.024640657	0.24814849
PRAMOONJAGO_SOX4_TARGETS_UP	49	1.5527544	0.022357723	0.2463354
PID_IFNGPATHWAY	39	1.5519499	0.004081633	0.24644986
JACKSON_DNMT1_TARGETS_UP	77	1.5517613	0.022	0.24478626
ALCALA_APOPTOSIS	86	1.5514604	0.018404908	0.24355878
REACTOME_CDK_MEDIATED_PHOSPHORYLATION_AND_REMOVAL_OF_CDC6	47	1.5512561	0.030120483	0.24198502
REACTOME_CELL_CYCLE_CHECKPOINTS	112	1.5490972	0.03187251	0.24446274
FULCHER_INFLAMMATORY_RESPONSE_LECTIN_VS_LPS_DN	430	1.548959	0.008163265	0.24282087
CHANG_IMMORTALIZED_BY_HPVS1_DN	64	1.5477376	0.013435701	0.24470448
LENAOUR_DENDRITIC_CELL_MATURATION_DN	127	1.5461832	0.013916501	0.24752015
PELLICCIOTTA_HDAC_IN_ANTIGEN_PRESENTATION_DN	49	1.5458522	0.03629032	0.24645306
REACTOME_P53_INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT	50	1.5455699	0.020491803	0.24527808
CHUNG_BLISTER_CYTOTOXICITY_UP	126	1.545433	0.022088353	0.24368535
ZHU_CMV_24_HR_UP	87	1.5448447	0.012121212	0.24345483
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	264	1.5445395	0.014084507	0.24254717
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	100	1.5444627	0.020408163	0.24088882
XU_AKT1_TARGETS_6HR	26	1.5440959	0.031120332	0.24004014
REACTOME_REGULATION_OF_IFNA_SIGNALING	24	1.542886	0.044989776	0.23990346
GOLUB_ALL_VS_AML_DN	24	1.5418373	0.023762377	0.24112871
RUTELLA_RESPONSE_TO_HGF_VS_CSF2_RB_AND_IL4_UP	393	1.5417224	0.02745098	0.23973913
VILIMAS_NOTCH1_TARGETS_DN	20	1.5396484	0.024242423	0.24396884
TARTE_PLASMA_CELL_VS_PLASMABLAST_DN	302	1.539056	0.03984064	0.24409102
VERHAAK_AML_WITH_NPM1_MUTATED_UP	180	1.5389129	0.014285714	0.24257474
KEGG_PROTEASOME	46	1.5386915	0.038617887	0.24146692
NOJIMA_SFRP2_TARGETS_DN	25	1.5384821	0.011976048	0.2401832
REACTOME_CITRIC_ACID_CYCLE_TCA_CYCLE	23	1.5381352	0.020876827	0.23947226
LIAN_LIPA_TARGETS_6M	72	1.537518	0.012244898	0.23943807
BIOCARTA_MPR_PATHWAY	33	1.5356623	0.010162601	0.24276935
REACTOME_GLUCOSE_TRANSPORT	38	1.535104	0.038306452	0.24261639
REACTOME_AUTODEGRADATION_OF_THE_E3_UBIQUITIN_LIGASE_COP1	49	1.5345644	0.022494888	0.24240388
DAZARD_RESPONSE_TO_UV_SCC_UP	114	1.534419	0.0332681	0.24121417

ZHU_CMV_ALL_UP	114	1.5342325	0.018108651	0.24002223
PAPASPYRIDONOS_UNSTABLE_ATEROSCLEROTIC_PLAQUE_UP	50	1.5341467	0.037623763	0.23866367
KEGG_NITROGEN_METABOLISM	23	1.5327405	0.03869654	0.24065809
PID_TOLL_ENDOGENOUS_PATHWAY	25	1.5318885	0.01443299	0.24166556
BIOCARTA_CDC42RAC_PATHWAY	16	1.5317639	0.021912351	0.24032849
BOYVAULT_LIVER_CANCER_SUBCLASS_G3_UP	184	1.5315595	0.045908183	0.23925987
MOOTHA_HUMAN_MITODB_6_2002	418	1.5311809	0.044088177	0.23857982
RUAN_RESPONSE_TO_TROGLITAZONE_UP	24	1.5310782	0.032520324	0.23723798
BIOCARTA_41BB_PATHWAY	17	1.530749	0.031055901	0.23647171
SHIPP_DLCL_VS_FOLLICULAR_LYMPHOMA_UP	45	1.5297024	0.03006012	0.2372898
KEGG_CITRATE_CYCLE_TCA_CYCLE	32	1.5290573	0.024439918	0.23798706
LY_AGING_OLD_DN	55	1.5287704	0.035643563	0.23721232
SHARMA_PILOCYTIC_ASTROCYTOMA_LOCATION_UP	24	1.5282121	0.01622718	0.23721
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_13	160	1.5256032	0.048387095	0.24064209
REACTOME_DNA_REPLICATION	183	1.5252736	0.04901961	0.23997411
REACTOME_CROSS_PRESENTATION_OF_SOLUBLE_EXOGENOUS_ANTIGENS_ENDOSOMES	48	1.524808	0.04819277	0.23686178
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B	22	1.523689	0.04347826	0.23851733
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_YELLOW_UP	26	1.5234045	0.021912351	0.23777136
REACTOME_DESTABILIZATION_OF_MRNA_BY_BRF1	17	1.5217061	0.018329939	0.24077147
SESTO_RESPONSE_TO_UV_C3	20	1.5208337	0.037037037	0.24182242
PARK_HSC_VS_MULTIPOTENT_PROGENITORS_DN	17	1.5202718	0.033464566	0.24185173
BIOCARTA_TID_PATHWAY	19	1.5200062	0.030991735	0.24112174
REACTOME_DESTABILIZATION_OF_MRNA_BY_AUF1_HNRNP_D0	52	1.5198694	0.03846154	0.23999318
BRACHAT_RESPONSE_TO_METHOTREXATE_DN	25	1.5179381	0.00610998	0.24394432
AMIT_SERUM_RESPONSE_20_MCF10A	21	1.5176522	0.029126214	0.24329337
KOINUMA_COLON_CANCER_MSI_DN	16	1.5167948	0.034	0.24429756
HU_ANGIOGENESIS_DN	37	1.5165755	0.045816734	0.24344231
BIOCARTA_ACTIN_PATHWAY	20	1.5157374	0.04040404	0.24418472
JISON_SICKLE_CELL_DISEASE_UP	178	1.5156785	0.028	0.24295089
CHOW_RASSF1_TARGETS_DN	29	1.5148889	0.022540983	0.24364522
BIOCARTA_TOLL_PATHWAY	35	1.5145446	0.01632653	0.24314828
REACTOME_REGULATION_OF_APOPTOSIS	57	1.5142158	0.049382716	0.24273741
REACTOME_REGULATION_OF_GLUCOKINASE_BY_GLUCOKINASE_REGULATORY_PROTEIN	27	1.5120791	0.042857144	0.24728873

Table 4.19 72 significantly enriched pathways in macrophages from carotid plaques containing large (>25%) LRNC as detected by MRI T₂ mapping. Threshold P < 0.05, FDR < 0.25. NES = normalized enrichment score.

4.4.10 Leading-edge analysis

As explained in the methods in Chapter 2.7.5, GSEA was performed by comparing the list of differentially expressed genes between two biological states (list L) against a pre-defined gene set (set S). An enrichment score (ES) is calculated by walking down the list L – the running sum statistics increases if a member present in set S is encountered, whereas the running sum decreases when a member is not in set S is encountered. Genes encountered before ES reaches its plateau (see Figure 2.13) are defined as in the “leading edge”. The leading-edge subset can be interpreted as the core of a gene set that accounts for most of the enrichment signal.

Leading edge analysis is therefore a useful tool, which helps identify specific genes that contribute to the enrichment signal in multiple biological pathways. In order to elucidate the candidate genes that link plaque lipid content to plaque instability and progression to clinical event (i.e. to acute, symptomatic, plaques), leading edge analysis was performed on the following GSEA datasets:-

1. The 379 significantly enriched gene sets in macrophages from symptomatic plaques. $P < 0.05$, FDR < 0.25 .
2. The 72 significantly enriched gene sets in macrophages from plaques with “large” lipid rich core ($>25\%$ by T_2 mapping). $P < 0.05$, FDR < 0.25 .
3. The 5 significantly enriched gene sets in macrophages from plaques with larger lipid content using T_2 map Lipid Area (%) as a continuous variable. $P < 0.05$, FDR < 0.25 .

Only one gene was found to be present in the top 10 contributing genes in the leading edge analysis of all three GSEA listed above – and it was Signal Transducer and Activator of Transcription (STAT1). In fact, STAT1 was the top ranking gene,

present in the leading edge, and contributed to the enrichment signal in the highest number (35 out of 379) of the significantly enriched gene sets in macrophages from symptomatic plaques. It was also present in the leading edge in 4 out of 5 significantly enriched gene sets in macrophages from plaque with larger lipid content using T₂ map Lipid Area (%) as a continuous variable for analysis.

4.4.11 Exploration of upstream effectors and downstream functions using Ingenuity® Pathway Analysis

To further explore the upstream effectors and downstream functional consequence of the LCM-transcriptomic microarray findings, and to assess cross-platform agreement and consistency of the bioinformatic analysis, differentially expressed gene lists were uploaded to Ingenuity Pathway Analysis (IPA) software (Qiagen, Silicon Valley, Redwood City, CA, USA). The principles and methods of IPA were discussed in section 4.3.10.

The upstream regulator analysis is based on prior knowledge of expected effects between transcriptional regulators and their target genes stored in the Ingenuity® Knowledge Base, which takes into account the potential transcription factor regulatory sites amongst the up/down regulated genes in the candidate differential expression gene list, and their direction of change, and predict the cascade of upstream transcriptional regulators that can explain the observed changes in gene expression in a given biological state.

The analysis generates two measurements, an “overlap P-value”, which is calculated using Fisher’s Exact Test to measure statistically significant overlap between the dataset genes and the genes regulated by a transcriptional regulator; and an “activation z-score”, which infer the activation states of the predicted transcriptional regulators. A z-score of >2 or <-2 is considered significant.

IPA downstream effects analysis, on the other hand, identifies cellular functions that are anticipated to increase or decrease given the observed gene expression changes. It is based on expected causal effects between genes and functions and are derived from the literature in the Ingenuity® Knowledge Base, which take into consideration the directionality of the observed gene expression changes.

To mirror the GSEA analyses performed in section 4.4.7-10, IPA was performed on 3 separate and independent comparisons:

1. Macrophages from symptomatic *versus* asymptomatic plaques
2. Macrophages from plaques with large lipid core (>25% %Lipid Area on MRI T₂ map) *versus* those with small lipid core
3. Macrophages from core *versus* cap plaque regions

Upstream Regulator	Molecular Type	Predicted Activation State	Activation z-score	Overlap P-val
IL1B	cytokine	Activated	3.138	5.35E-06
TNF	cytokine	Activated	3.046	3.52E-11
IL1	group	Activated	2.922	7.88E-07
PDGF BB	complex	Activated	2.903	5.05E-06
IL1A	cytokine	Activated	2.733	1.68E-07
RELA	transcription regulator	Activated	2.587	6.32E-04
CEBPA	transcription regulator	Activated	2.451	9.79E-09
NFKB1	transcription regulator	Activated	2.416	4.00E-06
PI3K (family)	group	Activated	2.401	4.64E-07

NFkB (complex)	complex	Activated	2.264	3.50E-06
E. coli B5 lipopolysaccharide	chemical	Activated	2.219	3.32E-03
NFKBIA	transcription regulator	Activated	2.215	5.47E-05
AKT1	kinase	Activated	2.201	2.55E-03
ERK	group	Activated	2.193	2.11E-03
Ins1	other	Activated	2.173	1.61E-03
IRF7	transcription regulator	Activated	2.170	4.93E-04
AGT	growth factor	Activated	2.169	8.82E-06
PPARA	nuclear receptor	Activated	2.169	3.78E-05
ERK1/2	group	Activated	2.156	1.08E-04
HIF1A	transcription regulator	Activated	2.128	1.68E-06
Forskolin	chemical toxicant	Activated	2.099	1.15E-03
Peptidoglycan	chemical	Activated	2.060	3.16E-06
EHF	transcription regulator	Activated	2.000	2.81E-04
ARNT2	transcription regulator	Activated	2.000	9.31E-03
RAF1	kinase	Activated	2.000	9.94E-03
TGM2	enzyme	Activated	2.000	1.26E-02
FAS	transmembrane receptor	-	1.981	6.63E-04
MAPK14	kinase	-	1.981	3.94E-03
NOS2	enzyme	-	1.974	6.67E-03
STAT5B	transcriptional regulator	-	1.964	4.21E-03
STAT1	transcriptional regulator	-	1.957	4.59E-03
IL18	cytokine	-	1.952	2.15E-03
MYD88	other	-	1.935	5.13E-06
SOCS3	phosphatase	-	-1.945	2.30E-04
ERBB3	kinase	Inhibited	-2.000	3.25E-04
IL13	cytokine	Inhibited	-2.065	1.69E-02
INSIG1	other	Inhibited	-2.236	5.51E-05
Nitrofurantoin	chemical drug	Inhibited	-2.236	5.35E-04

Table 4.20 List of IPA-derived upstream regulators in macrophages from symptomatic plaques. 31 significant predicted upstream regulators, together with 8 upstream regulators relevant in atherosclerosis that narrowly missed the significant threshold of z-score > 2 or <2.

In symptomatic plaques, IPA predicted upstream regulators are in agreement with GSEA results, involved mainly in inflammation (e.g. IL1B, IL1A, TNF, NFkB), lipid metabolism (e.g. PPARA), hypoxic response (e.g. HIF1A). One of the most important genes predicted by GSEA leading edge analysis in section 4.4.10, STAT1, also appeared high in the list of upstream activators but narrowly missed significance z-score threshold of +/-2 (z-score = 1.957). Another candidate gene FAS, involved in control of apoptosis, again appeared high in the list but also narrowly missed significance z-score threshold (z-score = 1.981). IPA-predicted downstream cellular functions listed below were also in broad agreement with GSEA-generated results in section 4.4.7-10. The predicted increased downstream cellular functions can be categorized as:-

1. Cellular movement, immune cell trafficking, and inflammatory response
2. Cell cycle, proliferation and mitogenesis
3. Cell death and survival
4. Cell-to-cell signaling and interaction
5. Metal and mineral metabolism (calcium in particular)

Cellular Function	Predicted Activation State	Activation z-score	P-val
Homing of cells	Increased	2.977	8.10E-08
Chemotaxis of cells	Increased	2.830	2.20E-07
Chemotaxis of phagocytes	Increased	2.646	4.80E-09
Chemotaxis of myeloid cells	Increased	2.639	4.06E-08
Synthesis of nitric oxide	Increased	2.516	6.13E-04
Proliferation of vascular endothelial cells	Increased	2.442	6.69E-05
Chemotaxis of granulocytes	Increased	2.423	5.72E-07
Engulfment of cells	Increased	2.400	7.11E-05
Lymphocyte migration	Increased	2.389	9.83E-05
Adhesion of blood cells	Increased	2.249	4.21E-03
Chemotaxis of neutrophils	Increased	2.204	1.68E-06
Mitogenesis	Increased	2.190	1.55E-04
Cell death of tumor cells	Increased	2.186	3.69E-03

Phagocytosis of cells	Increased	2.124	1.14E-05
Development of epithelial tissue	Increased	2.053	2.90E-05
Proliferation of endothelial cells	Increased	2.045	6.78E-07
Quantity of metal	Increased	2.029	1.98E-09
Quantity of Ca²⁺	-	1.979	5.48E-09
Migration of phagocytes	-	1.970	8.39E-05
Release of nitric oxide	-	1.969	5.84E-04
Recruitment of T lymphocytes	-	1.969	2.99E-04
Concentration of Ca²⁺	-	1.967	2.88E-03
Chemotaxis of monocytes	-	1.964	1.06E-03
Migration of mononuclear leukocytes	-	1.962	3.18E-05
Chemotaxis of mononuclear leukocytes	-	1.947	2.23E-03

Table 4.21 List of IPA-derived downstream effects and predicted cellular functions based on gene expression changes found in macrophages from symptomatic plaques. 17 significantly increased cellular functions was listed, together with 8 other relevant increased cellular functions that narrowly missed z-score threshold.

Consistent with GESA result in section 4.4.9, IPA upstream analysis from plaques with large (>25% %Lipid Area on T₂ map) *versus* small lipid core showed significant upstream activation of interferon pathways as shown in Table 4.22 and 4.23 below.

Upstream Regulator	Molecular Type	Predicted Activation State	Activation z-score	Overlap P-val
IFNG	cytokine	Activated	3.917	9.56E-12
IFNL1	cytokine	Activated	3.552	7.73E-18
IFNA2	cytokine	Activated	3.439	2.66E-14
TGM2	enzyme	Activated	3.162	1.54E-07
PRL	cytokine	Activated	3.092	8.33E-12
Interferon alpha	group	Activated	2.374	2.38E-07
IFNA1/IFNA13	cytokine	Activated	2.364	6.39E-13
PAF1	other	Activated	2.219	5.07E-06
TNF	cytokine	Activated	2.204	1.57E-08
EIF2AK2	kinase	Activated	2.2	1.35E-05
P38 MAPK	group	Activated	2.194	8.73E-04
IRF3	transcription regulator	Activated	2.189	6.70E-07
IgG	complex	Inhibited	-2	3.76E-03
SOCS3	phosphatase	Inhibited	-2.236	8.51E-09
MAPK1	kinase	Inhibited	-2.345	2.61E-07
SOCS1	other	Inhibited	-2.39	1.23E-08
IL1RN	cytokine	Inhibited	-2.985	1.16E-10

Table 4.22 List of 17 significant IPA-derived upstream regulators in macrophages from plaque with large lipid core (>25% Lipid Area on T₂ map).

Cellular Function	Predicted State	Activation	Activation z-score	P-val
Proliferation of cells		Increased	2.977	2.05E-07
Immune response of cells		Increased	2.830	2.50E-03
Relapsing-remitting multiple sclerosis		Increased	2.646	2.81E-05
Chemotaxis of phagocytes		Increased	2.639	6.78E-06
Inflammatory response		Increased	2.516	3.38E-04
Chemotaxis of myeloid cells		Increased	2.442	9.61E-06
Chemotaxis of granulocytes		Increased	2.423	4.85E-06

Table 4.23 List of 7 significant IPA-derived downstream cellular functions in macrophages from plaque with large lipid core (>25% Lipid Area on T₂ map).

Finally, IPA was performed on the core *versus* cap macrophages comparison. Consistent with the GSEA results in Chapter 4.4.7.2, significant differential gene expression signals between core and cap macrophages were difficult to detect, likely due to heterogeneity within macrophage subsets. There was only one significant upstream regulator activated in cap macrophages, MAPK1 (z-score = 2.219, P = 0.0012) and none in core macrophages. In terms of IPA-predicted downstream cellular function, necrosis and apoptosis were increased in cap macrophages (with z-score 2.371 and 2.339; P = 2.02E-05 and 5.81E-06, respectively); while angiogenesis was increased in core macrophages (z-score = 2.07, P = 1.3E-12). Interestingly, lipid pathways enriched in core macrophages in GSEA did not show up in IPA.

4.5 Discussion

In this chapter immunohistochemistry-guided laser capture microdissection (immuno-LCM) was optimized to procure cap- and core-associated macrophages from symptomatic and asymptomatic human carotid plaques for gene expression profiling, which facilitated identification of functional, biological, pathways in plaque macrophages, that are differentially activated and are associated with different micro-anatomical locations within plaques, as well as clinical symptom status of the plaques. Further analysis was then performed to explore specific pathways activated in macrophages from plaques containing larger amount of lipid, readily detectable by MRI T₂ mapping described in Chapter 3, and provided a novel bridge between non-invasive imaging biomarker and molecular targets.

The key findings of this Chapter were:

- I. Immuno-LCM was able to procure plaque macrophages from different plaque locations with high degree of specificity; and with resulting high quality RNA compatible with downstream microarray gene expression profiling.
- II. Technical validation PCR using unamplified RNA revealed that the microarray results were a true representation of the gene expression changes in human plaque macrophages.
- III. Unsupervised analyses such as PCA and hierarchical clustering showed that there were greater biological signals or differences between plaque macrophages based on symptom status, as opposed to plaque location.
- IV. Differential gene expression analysis identified a number of genes that showed significant upregulation in different plaque locations and symptom-statuses.
- V. Pathway analysis using GSEA and the upstream/downstream predictive algorithms of IPA revealed a number of biological processes (both expected

and novel) that were significantly upregulated in macrophages from symptomatic plaques. They can be grouped in 7 broad categories: (1) inflammation; (2) lipid metabolism; (3) hypoxic response; (4) cell proliferation; (5) apoptosis; (6) antigen presentation; and (7) cellular energetics.

- VI. Using T₂ map Lipid Area (%) as an independent variable, GSEA identified significant enrichment of interferon / STAT1 pathways in macrophages from plaques with large lipid content.
- VII. Subsequent leading edge analysis of the enriched gene sets in macrophages from (a) symptomatic plaques; (b) plaques with large lipid core as defined by >25% LRNC “discreet” cut-off on T₂ map; and (c) plaques with an increasing lipid content based on the raw Lipid Area (%) as continuous variable, revealed that STAT1 was the highest ranking gene that contributed the most to the enrichment signal.

The power of immuno-LCM in procuring highly specific sub-populations of cells from heterogeneous tissue samples has long been appreciated in cancer research since late 1990s.²³⁸ In subsequent years, it was adopted to atherosclerosis research in mice,¹⁵³ and more recently, in humans.¹⁹⁸ Now with the ability to couple with microarray whole-genome expression profiling, “immuno-LCM-omics” has been anticipated to show great potential in untangling the complex functional heterogeneity of immune cells in atherosclerotic lesions. However, a number of recent human immuno-LCM studies failed to fully realize this potential because of either (1) only a small panel of genes were studied using quantitative RT-PCR instead of microarray,^{201,239} potentially due to higher quality of input RNA required for microarray experiments; or (2) immune cells such as macrophages were indiscriminately procured from plaques without reference to their micro-anatomical locations within plaques.^{200,240} One study did attempt to address the micro-anatomical location issue by simultaneous analysis of the same human vascular tissues using gene and tissue

microarray,²⁴¹ but this study used whole plaque homogenates for gene microarray, without the cellular specificity provided by immuno-LCM.

This chapter described the challenging journey taken to optimize immuno-LCM with a “guide-slide” approach to achieve high quality RNA in a location-specific manner, which then allowed coupling with downstream whole-genome expression profiling to generate unique, location-specific, macrophage gene expression profiles based on clinical symptom status (i.e. symptomatic plaques vs asymptomatic plaques). As expected, this location information turned out to be crucially important in the interpretation of microarray findings. In 2013, Lee, *et al.* reported increased fatty-acid binding protein 4 (FABP4), leptin, and activation of the adipokine/PPAR signalling pathways in ruptured human carotid plaques compared to stable plaques.²⁴⁰ Differential gene expression and pathway analysis of the current dataset described in this chapter revealed that instead of differentiating between ruptured vs. stable plaques, leptin was in fact differentially expressed in lipid core-associated macrophages, and that adipogenesis and PPAR signalling pathways were amongst the most significantly activated pathways in lipid core-associated macrophages, with the highest ranking gene within the most significantly enriched pathway (Classic adipogenic targets of PPAR γ) being FABP4. As such, the increased expression of FABP4 and leptin in ruptured plaques that Lee, *et al.* reported may have simply reflected the fact that high-risk, vulnerable, plaques contained more lipid-core associated macrophages, which is consistent with the MRI T₂ mapping finding (Chapter 3) that symptomatic plaques, in general, contain higher lipid content.

The 7 categories of biological functions identified by GSEA in macrophages between symptomatic and asymptomatic plaque included both expected and novel biological insights. Plaque inflammation has long been shown to be a driver for atherosclerosis progression and thrombotic complication.¹¹⁷ With that notion¹⁸FDG-PET has been

established over a decade ago to image plaque inflammation in carotid atherosclerosis,^{113,118} and its signal has been shown to correlate to the quantity of plaque macrophage infiltration.¹¹⁴ However, there have been some debates in recent years about the origin of the ¹⁸FDG-PET signal and macrophage hypoxia as been suggested as an alternative explanation to inflammatory activity *per se*.⁶⁷ In the current study, both inflammation and hypoxic responses were shown to be upregulated in macrophages derived from symptomatic plaques; this may potentially explain and reconcile these previous findings. In addition, processes related to cellular energetics such as the Krebs cycle were also enhanced in macrophages from symptomatic plaques. This did not only indicate the increased metabolic demand of active inflamed macrophages, but highlighted the intersection or “cross-talk” between metabolic and inflammatory pathways that might be amenable to new therapeutics to simultaneously target metabolic and inflammatory disorders.^{37,242}

Perhaps more importantly, not only was apoptosis increased in symptomatic plaques-derived macrophages, cell proliferation was also enhanced. This is a novel finding in humans, which is in agreement with a recent report in mice suggesting that while monocyte recruitment is the main mechanism of macrophage accumulation in early atherosclerosis, local proliferation of lesional macrophages dominates advanced atherosclerotic lesions.⁷⁵ In fact, the importance of cell proliferation in monocyte/macrophage lineage of immune cells in both atherosclerosis and myocardial infarction has been increasingly recognized. A recent report used similar GSEA techniques to demonstrate that both inflammation and cell proliferation processes were activated in peripheral blood monocytes *prior to* their infiltration in the infarcted myocardium; and this response is evolutionarily conserved between mice and humans.²⁴³ Indeed, immunohistochemistry staining using a cell proliferative marker Ki-67 confirmed co-localisation of Ki-67 signals with macrophage marker CD68 within human carotid atherosclerosis (Figure 4.19).

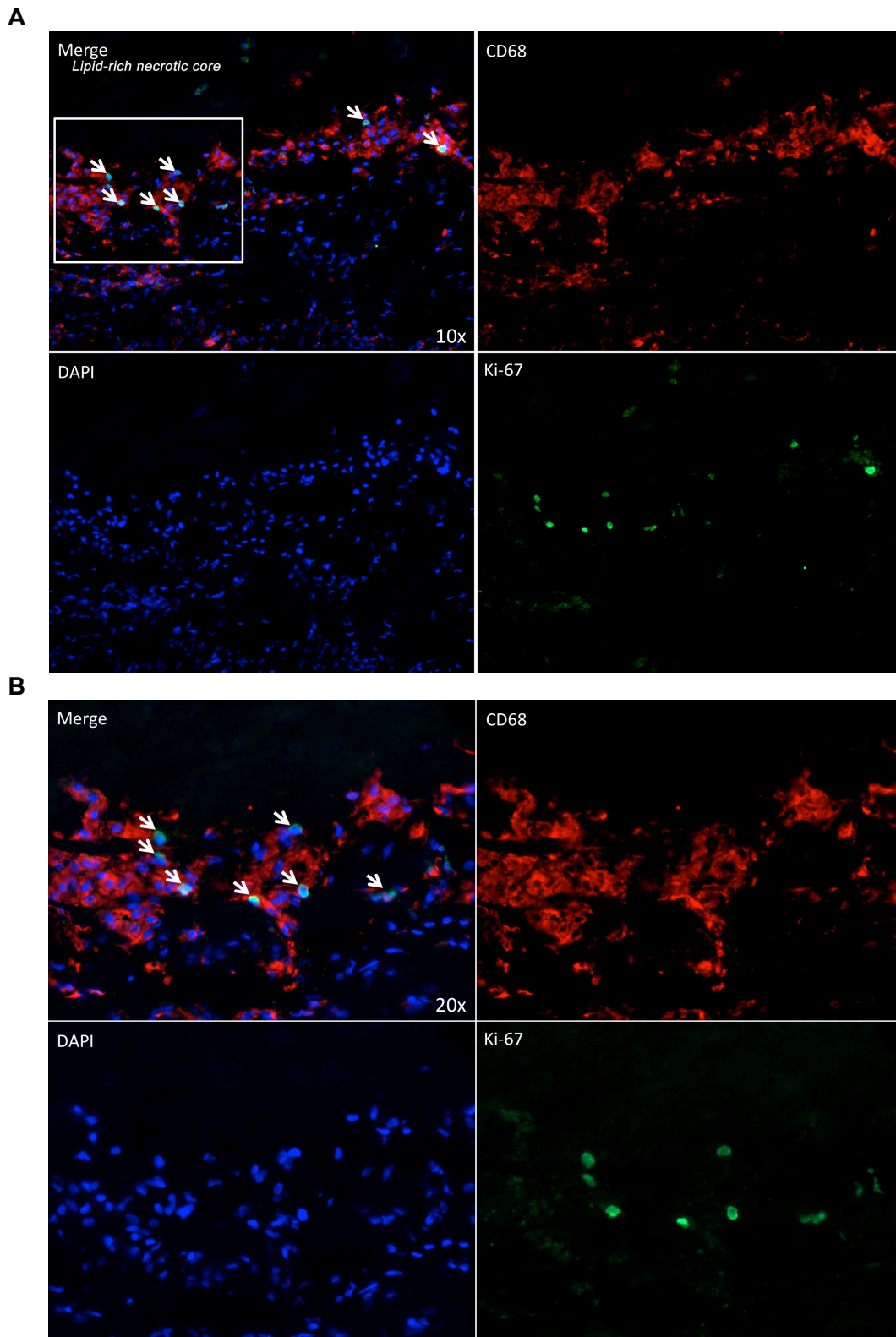


Figure 4.19 Co-expression of proliferative marker *Ki-67* in human carotid plaque macrophages

Ki-67 (green) is co-expressed in the nuclei of some CD68-positive plaque macrophages (red) at the border zone of LRNC interfacing the overlying fibrous cap. Nuclear counterstaining with DAPI (blue). Boxed area in A shown as B at 20x.

Another novel finding of this Chapter was that, for the first time, a non-invasive MRI imaging biomarker was shown to directly correlate with molecular functional marker at a gene level. Macrophages from plaques with high lipid content, as revealed by T₂ mapping, differentially activate their inflammatory pathways and, in particular, interferon/STAT1 pathways. A recent study cross-examined the upregulated candidate genes from Gene Expression Omnibus repository (GEO) deposited microarray datasets generated from both human coronary and carotid atherosclerosis, and discovered that not only were there increased expression of multiple chemokines, adhesion molecules and matrix-remodeling molecules commonly detected in both plaque types but they correlated with the presence of putative STAT1 binding sites in their promoters, strongly predicting the involvement of STAT1 in plaque development.²⁴⁴ The same group then went on to identify a set of STAT1-dependent genes that were synergistically affected by interactions between IFN γ and TLR4 in vascular cells manipulated *in vitro*, and demonstrated the expression of STAT1 in human carotid atherosclerosis.²⁴⁵ In fact, at least shown to be true in tumour development, canonical IRF/STAT signaling pathways are activated by both type I ($-\alpha/\beta$) and type II ($-\gamma$) interferons and TLR signaling to skew macrophage function toward the M1 phenotype (via STAT1) or by IL-4 and IL-13 to skew toward the M2 phenotype (via STAT6).^{246,247}

Both type I and type II IFN pathways were shown in the current study to be activated in macrophages with increasing lipid content, with IFN α pathways being most significantly enriched in macrophages with the highest lipid content. Type II interferon (IFN γ) is more well known to be pro-atherogenic;²⁴⁸ and while type I interferons (IFN α/β) are historically better known in fighting viral infection,²⁴⁹ a recent study has shown that IFN α priming of THP1 cells induced increased oxLDL uptake, and promoted macrophage foam cells formation.²⁵⁰ Furthermore, peripheral blood mononuclear cells (PBMCs) from in patients with systemic lupus erythematositis

(SLE) were shown to have increased scavenger receptor-A (SR-A) expression, which was positively correlated with IFN α signaling activity.²⁵⁰ A similar phenomenon seems to play a role in peripheral blood mononuclear cells from patients with HIV, as their PBMCs possess a type I IFN profile with an increase in lipid uptake, accompanied by increased SR-A expression.²⁵¹ A growing body of evidence thus supports a role for the type I IFN (α/β) in promoting foam cell formation and atherogenesis,²⁵² and novel biologic therapy is currently in development targeting IFN α/β receptor.²⁵³

Last but not least, genome-wide association studies (GWAS) had identified multiple single nucleotide polymorphisms on the p arm of chromosome 9 at position 21 (9p21) as a susceptibility locus;^{254,255} with recent meta-analysis confirmed 9p21 being one of the most established and replicated susceptibility locus cluster for coronary artery disease, myocardial infarction, and type 2 diabetes.²⁵⁶ For example, in the GRACE prospective genetic study in patients with acute coronary syndrome (ACS), not only was the high-risk C allele of rs1333049 on chromosome 9p21 being an independent risk factor for the index ACS event, this high-risk allele was significantly and independently associated with recurrent MI or cardiovascular death within 6 months after the index ACS episode.²⁵⁷ However, chromosome 9p21 has long been thought to be a “gene desert” and the biological underpinnings of these genetic associations with coronary artery disease were unclear. Recently, Harismendy, *et al.* identified elements within 9p21 that can act as long-range regulator for STAT1.²⁵⁸ In human vascular endothelial cells, interferon- γ activation strongly affects the structure of the chromatin and the transcriptional regulation in the 9p21 locus, including STAT1-binding, long-range enhancer interactions and altered expression of neighbouring genes.²⁵⁸ Using “immuno-LCM-omic” techniques, the current study provided corroboration in humans of the likely role of STAT1 by bridging the gap between population sequencing and *in vitro* assays; and provided a possible *tissue-*

level explanation for the 9p21 GWAS findings. It confirmed the crucial involvement of STAT1 in human plaque inflammatory cells *ex-vivo*, especially within high-risk atherosclerotic plaques with large lipid-rich necrotic core. This is consistent with recent findings that high-risk alleles at 9p21 interfere with STAT1 bindings and downstream CDKN2B expression and that the loss of CDKN2B promotes atherosclerosis by increasing the size and complexity of the lipid-laden necrotic core through impaired efferocytosis.^{258,259}

There are a number of limitations in the immuno-LCM and gene expression analysis methods used in this Chapter. Firstly, because of the conflicting demand in speed in immuno-LCM between cell procurement specificity and RNA quality (more specific cell procurement -> longer preparation and procedural time in immuno-LCM -> more RNA degradation -> worse quality of resulting RNA), the current protocol of immuno-LCM developed for this Chapter utilized a “guide-slide” approach and as such, only larger clusters of plaque macrophages can be procured accurately. In plaques where macrophages were sparse or scattered, this method could not be used reliably. In addition, since decalcification readily destroys RNA, plaques that contain large calcium deposits were excluded from LCM experiment, as cryosectioning were almost always impossible.

Although powerful, pathway analysis such as GSEA or IPA relied on databases of biological pathways curated from published literature. The greatest amount of work carried out in this area at present relates to the fields of cancer research, and as such there is an over-representation of cancer-related pathways in the analysis. It is therefore possible that there could potentially be many other relevant biological processes as yet unidentified or under-represented in existing databases. Finally, transcriptomic analysis only examined one facet of the functional genome – there

exist a complex multi-layer regulatory network involving miRNA, dynamics of transcript or protein degradation, as well as post-translation modification that could alter the flux of metabolic pathways, and hence the functional effects.

4.6 Conclusion

In conclusion, immuno-LCM gene expression profiling of plaque macrophages successfully identified important biological processes that are significantly upregulated in recently symptomatic plaques. The ability to examine different subsets of plaque macrophages based on their micro-anatomical locations further enhanced the understanding of macrophage heterogeneity in advanced human atherosclerotic lesions. Combining T_2 mapping data from Chapter 3, immuno-LCM gene expression profiling identified unique activation of interferon/STAT1 pathways in carotid atherosclerosis, which correlated with the amount of plaque lipid; this demonstrated potential therapeutic targets and provided a molecular “handle” to a non-invasive imaging biomarker, highlighting the complementary roles of imaging and “-omics” in the integrated platform in stratified medicine and drug discovery.

CHAPTER 5

FUNCTIONAL SIGNIFICANCE OF PLAQUE

MACROPHAGE HETEROGENEITY -

THE EFFECTS OF NIACIN ON MACROPHAGE-

DERIVED FOAM CELLS

5.1 Introduction

Having shown the functional heterogeneity of plaque macrophages in Chapter 4 and its direct correlation to compositional data derived from T₂ map in Chapter 3, an important question remained to be addressed – i.e. what is the functional significance of high plaque lipid content and inflammatory activation of plaque macrophages, especially in the context of drug development? This chapter will attempt answer this question using nicotinic acid, and its G-protein coupled receptor GPR109A, as an example to illustrate the functional consequence of plaque macrophage heterogeneity.

As discussed in general introduction in Chapter 1, nicotinic acid (NA) has been known for over five decades to exert favourable effects on plasma lipoproteins.⁴⁰ It reduces atherogenic lipoproteins LDL-c, VLDL-c, and Lp(a), and potently raises plasma HDL-c (by up to 30%).⁴¹ In addition to these recognized effects on plasma lipoproteins, it has also emerged that NA has a variety of “pleiotropic effects” that are of potential relevance to processes of atherosclerosis progression and regression.²⁴²

GPR109A (also known as hydroxy-carboxylic acid 2 receptor or HM74a) which is the receptor for nicotinic acid, has been shown to mediate an expanding repertoire of these potentially therapeutic actions.^{260,261} This receptor belongs to a family of Gi-protein-coupled cell surface receptors that are expressed in adipocytes and immune cells in both human and rodent species.²⁶⁰ Recent studies have reported potent anti-inflammatory effects of NA in both human adipocytes⁵⁰ and monocytes,⁵¹ which are mediated via GPR109A-dependent mechanisms. The relevance of these observations has been enhanced by a recent study in mice, which has shown that NA reduces progression of atherosclerosis, via GPR109A on myeloid cells, without affecting plasma lipoproteins.²⁶²

In adipocytes⁵² and certain non-foam cell ‘basal-state’ mononuclear cell lines,^{53,54} NA also activates the reverse cholesterol transport apparatus, namely PPAR γ , LXR α , and the ABC transporter proteins, which are known to be responsible for *in vitro* cholesterol unloading⁵⁵ which, in turn, are directly implicated in the regression of experimental atherosclerosis.^{56,57} These observations raise the possibility that effects of NA on atherosclerotic plaque regression and cardiovascular risk reduction may, in part, be due to direct effects on foam cell cholesterol unloading.

However, despite numerous imaging trials having shown that NA reduces atherosclerosis, estimated from coronary angiography;^{58,59} carotid ultrasound⁶⁰⁻⁶³ and MRI,⁶⁴ two recent large outcome trials, AIM-HIGH⁶⁵ and HPS2-THRIVE,²⁶³ both failed to show additional clinical efficacy in statin-treated patients. The discrepancy between promising results in pre-clinical animal studies, early-phase imaging studies, and the failure to translate into general clinical outcome benefits highlighted not only the need of an *integrated* drug development platform for more precise patient stratification in drug evaluation (see section 1.2.3 in Chapter 1), but prompted an exploration on how this discrepancy may potentially be reconciled.

The observations that, NA [1] plays a role in atherosclerosis regression in humans and [2] has the capacity to act directly on monocyte / macrophage function by up-regulating proteins that are involved in cellular cholesterol efflux, raise the important possibility that NA may exert its atheroprotective effects, via its receptor GPR109A, in foam cells to induce cholesterol efflux, leading to plaque regression. Since the size of the plaque lipid pool directly contributes to the extent of lipid-laden foam cells formation *in-vivo*, it raises the question whether plaque macrophages’ response to NA varies depending on the “lipid” context / phenotypes – i.e. do lipid-laden foam cells respond to NA differently compared to non-foam cells “basal-state”

macrophages? Accordingly, this chapter sought to test the effects of NA on human macrophage-derived foam cells and explore its cellular mechanisms of action in the context of lipid handling and atherogenesis.

5.2 Hypothesis and aims

To test the hypothesis that nicotinic acid (NA) might exert differential response *in-vitro* in basal state macrophages *versus* macrophage-derived foam cells.

The experimental aims of this chapter were:-

- 1) To confirm the anti-inflammatory effects of NA in basal-state macrophages.
- 2) To explore the effects of NA in terms of cholesterol efflux in macrophage-derived foam cells.
- 3) To examine the effect of NA on the reverse cholesterol transport apparatus in both basal-state macrophages and foam cells
- 4) To study the putative downstream signalling pathways of GPR109A upon NA stimulation
- 5) To evaluate the potential consequence of lipid-loading on the expression of NA receptor GPR109A *in vitro* and *ex-vivo*

5.3 Methods

General methods used in this chapter had been discussed in Chapter 2.

5.4 Results

5.4.1 NA suppresses LPS-induced transcription of pro-inflammatory mediators

The response to NA in basal state (i.e. non-foam cell) macrophages was first examined. TLR-4 agonist lipopolysaccharide (LPS) was used as an inflammatory stimulus. THP-1 macrophages were incubated with LPS (50 ng / mL) for 18 hours, followed by 1×10^{-4} M NA for 24 hours. Previous dose-response experiments (1×10^{-3} to 10^{-6} M) established optimal dosing of NA and excluded detrimental effects on cell viability.⁵¹ Pre-incubation with LPS increased mRNA transcription of pro-inflammatory cytokines: MCP-1 by 80-fold ($P < 0.001$, $n = 3$ per group); and TNF α by 10-fold ($P < 0.001$, $n = 3$ per group) (Figure 5.1 A). As was previously reported, NA treatment significantly attenuated this LPS-induced transcriptional up-regulation by $76.6 \pm 12.2\%$ for MCP-1 ($P < 0.01$, $n = 3$ per group), and by $56.1 \pm 11.5\%$ for TNF α ($P < 0.01$, $n = 3$ per group), confirming the anti-inflammatory effect of NA on basal non-foam cell macrophages.

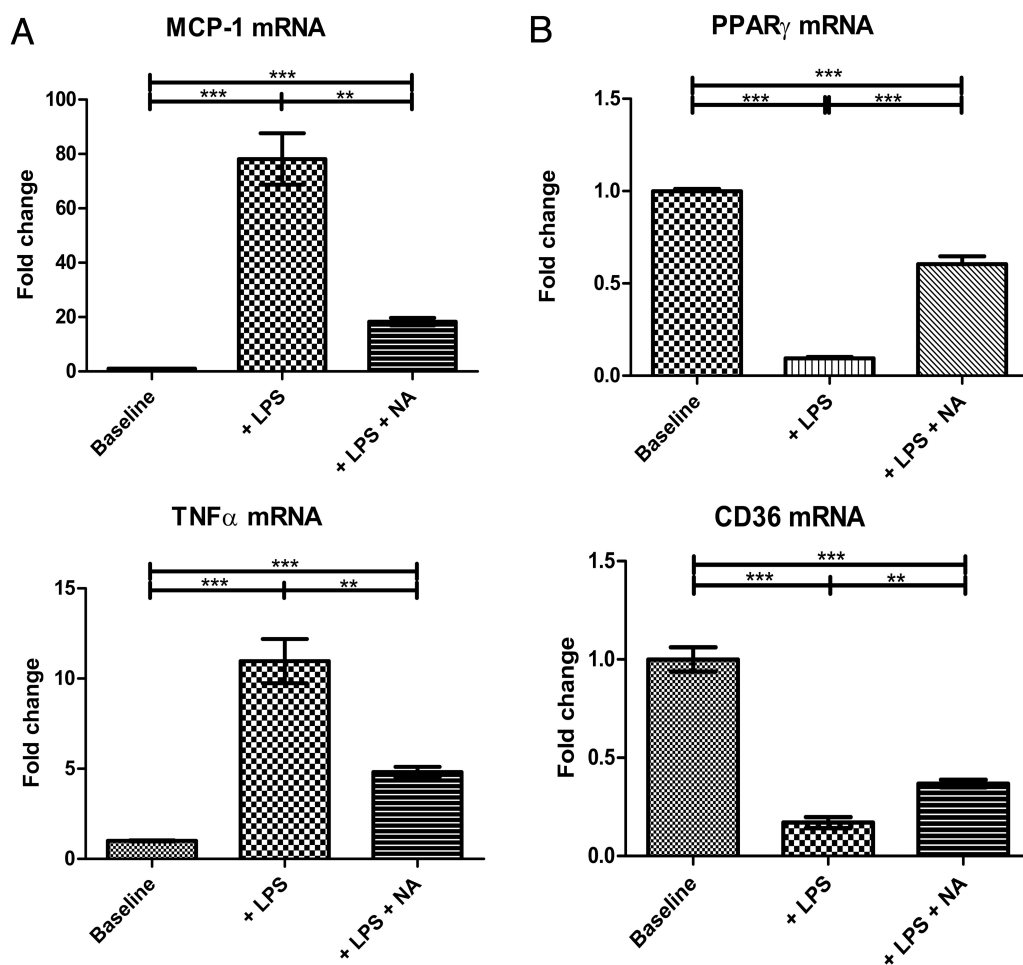


Figure 5.1 Effects of nicotinic acid (NA) on mRNA transcription of MCP-1, TNF α , PPAR γ , and CD36

Basal THP-1 macrophages were pre-incubated with LPS prior to administration of NA versus vehicle controls. **A** LPS increased mRNA transcription of MCP-1 and TNF α , while NA treatment attenuated this LPS-induced up-regulation (n = 3 per group). **B** LPS suppressed mRNA transcription of PPAR γ and its downstream effector CD36, yet treatment by NA effectively reversed it (n = 3 per group). * P < 0.05, ** P < 0.01, *** P < 0.001, ns = non significant.

5.4.2 NA restores LPS-induced transcriptional suppression of PPAR γ in basal macrophages

PPAR γ contributes to the regulation of reverse cholesterol transport apparatus and cellular lipid metabolism. Pre-incubation with LPS was first shown to suppress PPAR γ and its downstream effector CD36 mRNA transcription by 90% ($P < 0.001$, $n = 3$ per group) and 83% ($P < 0.001$, $n = 3$ per group), respectively (Figure 5.1 B). This LPS-induced transcriptional suppression was effectively reversed by treatment with NA which increased PPAR γ by $536.5 \pm 46.4\%$ ($P < 0.001$, $n = 3$ per group) and CD36 by $116.8 \pm 19.8\%$ ($P < 0.01$, $n = 3$ per group).

5.4.3 GW1929 but not NA promotes cholesterol efflux from macrophage-derived foam cells

Activation of PPAR γ facilitates reverse cholesterol transport (RCT) by up-regulation of ABC transporter proteins via recruitment and dimerization of another nuclear transcription factor, LXRo.²⁶⁴ Since NA activated PPAR γ in basal macrophages, one hypothesis would be that NA might promote RCT in macrophage-derived foam cells. To test this hypothesis, basal macrophages were induced into foam cells, confirmed by Oil-red-O staining in sample wells (Figure 5.2 A). Foam cells were treated with NA, GW1929 (a selective PPAR γ agonist) or vehicles only for 4, 24, or 48 hours, followed by cholesterol efflux for 24 hours facilitated by cholesterol acceptor HDL3 or ApoAI in the culture medium. As anticipated, direct activation of PPAR γ by GW1929 resulted in significantly higher cholesterol efflux as demonstrated by a reduction in extracted cellular cholesterol by $37.7 \pm 3.1\%$ in GW1929 + HDL group compared to incubation with HDL alone ($P < 0.01$, $n = 2$ per group); and by $15.6 \pm 4.3\%$ in GW1929 + ApoAI group compared to incubation with ApoAI alone ($P < 0.05$, $n = 2$ per group) (Figure 5.2 B).

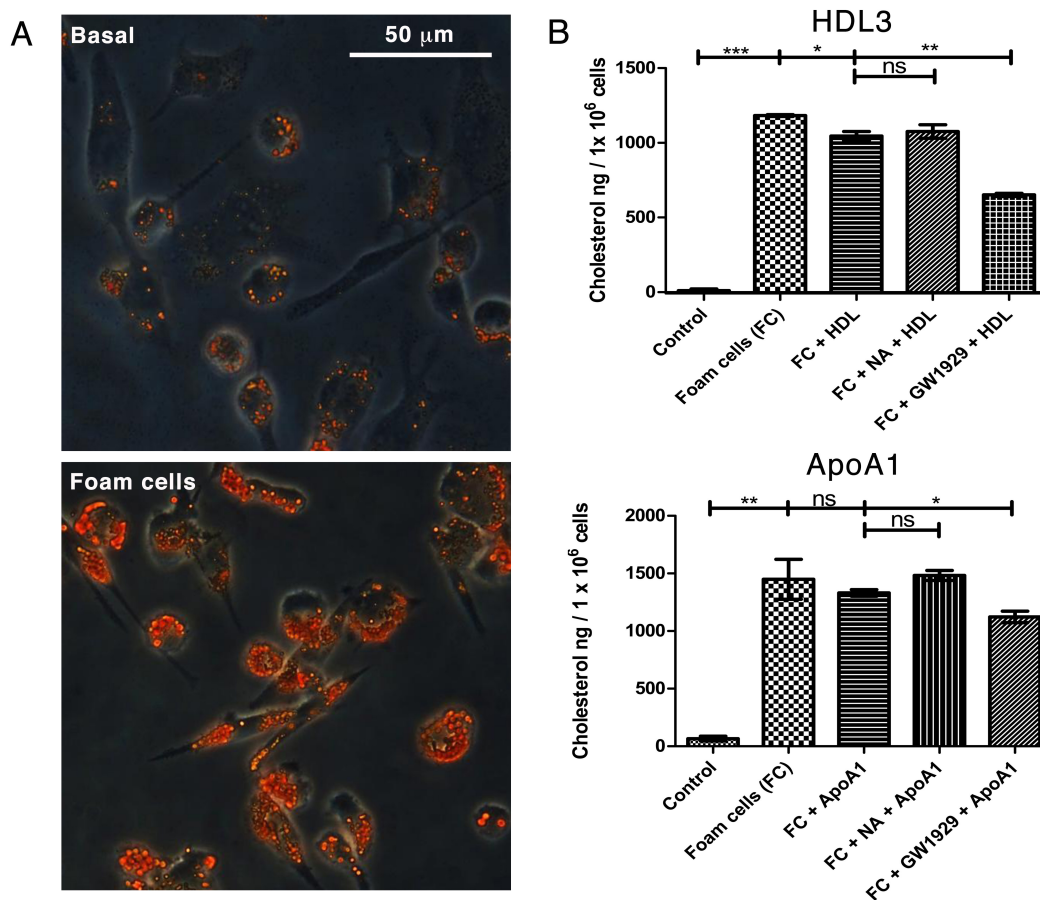


Figure 5.2 Foam cell induction and the effects of NA and GW1929 on foam cell cholesterol efflux

A Phase-contrast microscopy of Oil-red-O staining of THP-1 cells in basal (upper) and foam cells (lower) states, confirming foam cell induction. **B** Foam cells were treated with NA, GW1929, or vehicles only for 24 hours, followed by cholesterol efflux facilitated by HDL3 or ApoA1 in the culture medium. Cholesterol efflux was studied by measuring cellular cholesterol content after hexane:isopropanol extraction. GW1929 treatment reduced extracted cellular cholesterol in GW1929 + HDL group versus with HDL alone; and in GW1929 + ApoA1 group versus with ApoA1 alone ($n = 2$ per group). NA treatment had no effect on extracted cellular cholesterol content at a range of NA concentration 1×10^{-3} to 10^{-6} M compared to either HDL3 or ApoA1 alone groups. (Representative data at 1×10^{-4} M NA concentration shown). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = non significant.

Corresponding cholesterol contents in efflux supernatants were increased by $15.8 \pm 2.7\%$ in GW1929 + HDL group ($P < 0.05$, $n = 2$ per group); though no significant change was detected in the GW1929 + ApoAI group. Nicotinic acid treatment had no effect on either extracted cellular cholesterol or supernatant cholesterol content at a range of NA concentration 1×10^{-3} to 10^{-6} M compared to either HDL or ApoAI alone groups. (Representative data at 1×10^{-4} M NA concentration of 24 hours treatment shown in Figure 5.2 B; no difference was detected in 3 repeat experiments, each with $n = 4$ per group. Data from other time points shown in Figure 5.3).

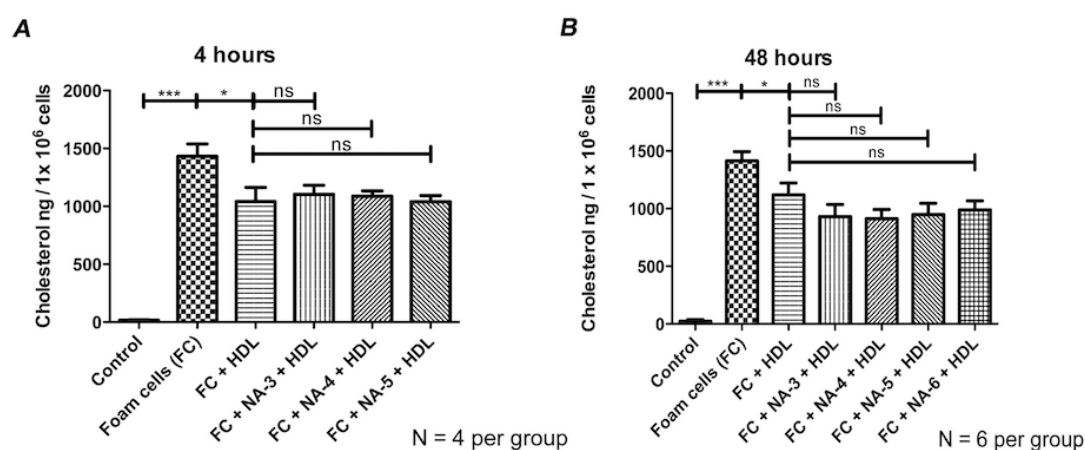


Figure 5.3 *Effect of NA on foam cells at different concentration and time points*

Cholesterol efflux was studied by measuring extracted cholesterol content after hexane:isopropanol extraction. Nicotinic acid (NA) has no effect on cholesterol efflux compared to using HDL alone at 4 hours (**A**) and at 48 hours (**B**). NA concentration -3 = 1×10^{-3} M, -4 = 1×10^{-4} M, -6 = 1×10^{-6} M. Time indicated duration of NA treatment.

5.4.4 Effect of NA and GW1929 on reverse cholesterol transport apparatus:

LXR α , ABCA1 and ABCG1

In order to elucidate why NA had no effect on RCT in foam cells, the effect of NA and GW1929 on genes in the PPAR γ -LXR α -ABC transporters pathway was investigated. First, the lipid “responsiveness” of PPAR γ , NR1H3 (for LXR α), and ABCA1 was confirmed using qRT-PCR. mRNA transcription of all three genes were significantly up-regulated by cholesterol loading. Immediately following 48 hours of 50 μ g / mL ac-LDL incubation, PPAR γ mRNA transcript was significantly increased by 4.3-fold ($P < 0.001$); NR1H3 by 3.6-fold ($P < 0.001$); and ABCA1 by 4.7-fold ($P < 0.01$) (Figure 5.4 A). However, after resting these induced foam cells in standard media (RPMI supplemented with 10% fetal bovine serum and 0.05 mM 2-mercaptoethanol) for 24 hours after induction, the mRNA transcript levels of all 3 genes returned to baseline or below-baseline levels. This time point was adopted for subsequent experiments given the intention to test the potential for gene up-regulation upon treatment. Basal macrophages and foam cells were treated with 1×10^{-4} M NA or 2×10^{-6} M GW1929 versus vehicles for 4 or 24 hours. In basal macrophages, NA significantly up-regulated ABCG1 mRNA transcription at 24 hours by $92.1 \pm 11.8\%$ ($P < 0.001$, $n = 3$ per group). In foam cells, however, NA showed no effect on transcriptional regulation (Figure 5.4B). This contrasts with direct PPAR γ activation using GW1929, where significant transcriptional activation was seen with both NR1H3 and ABCG1 in both basal macrophages and foam cells at 4 hours and 24 hours.

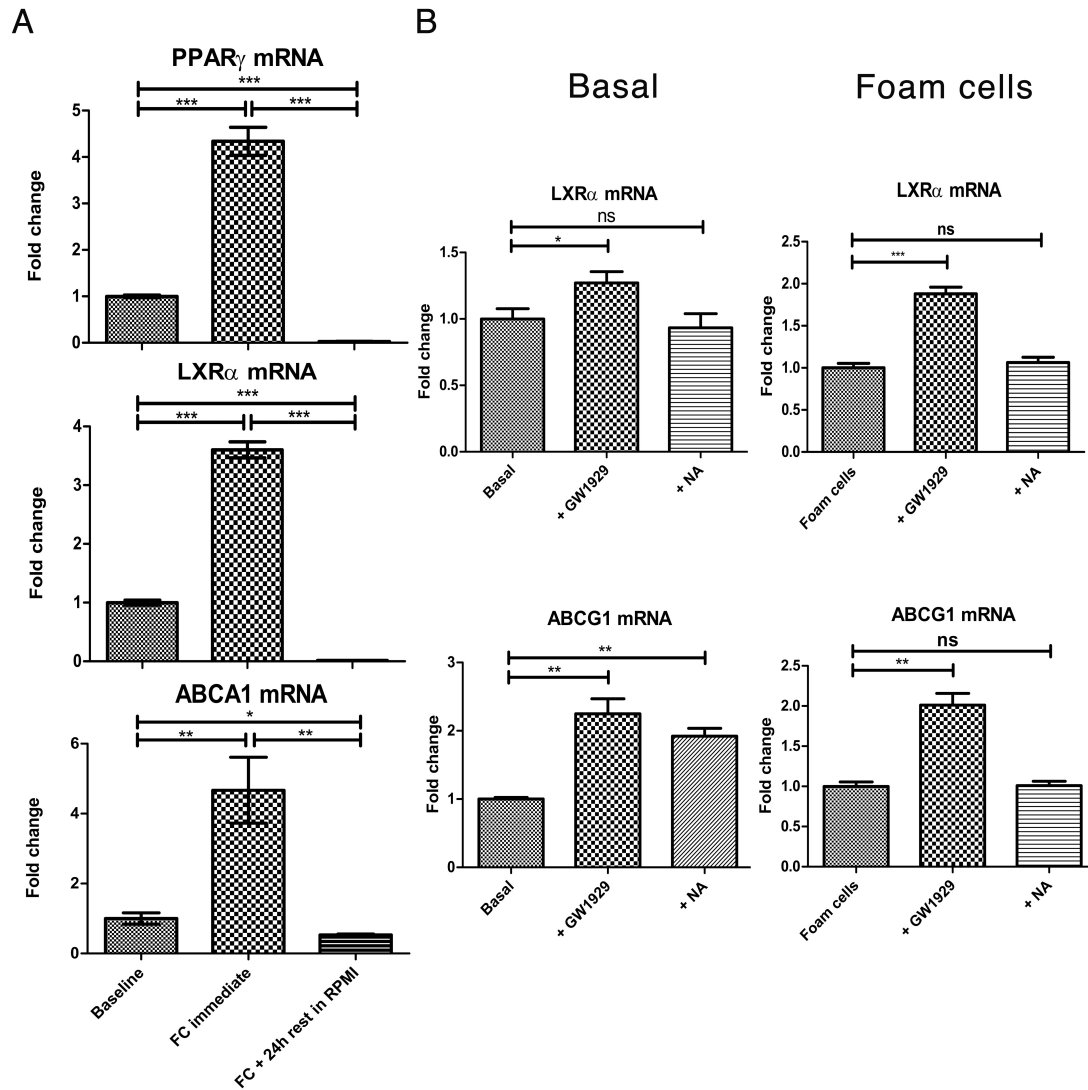


Figure 5.4 Effects of NA and GW1929 on LXR α , ABCA1 and ABCG1

A mRNA transcription of all three genes were significantly up-regulated immediately following cholesterol loading but returned to baseline or below-baseline level after resting in standard RPMI media for 24 hours. **B** In basal macrophages, NA significantly up-regulated ABCG1 mRNA transcription ($n = 3$ per group); this effect was not seen in foam cells. PPAR γ activation using GW1929 significantly up-regulated mRNA transcription with both NR1H3 and ABCG1 in both basal macrophages and foam cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = non significant.

5.4.5 PPAR γ pathway is activated by NA in basal macrophages but not in foam cells

In view of the discrepancy of ABC transporters activation between GW1929 and NA treatment, the putative cellular mechanisms by which ABCA1 and ABCG1 may be regulated by NA was further explored. Having already established that NA could reverse LPS-induced suppression of PPAR γ and CD36 mRNA transcription in basal THP-1 macrophages, direct PPAR α , δ , γ transcription factor binding activity (TFBA) by 1×10^{-4} M NA activation in basal macrophages and foam cells was tested using ELISA, in the absence of LPS. PPAR α TFBA was significantly increased in foam cells compared to basal macrophages by $36.3 \pm 6.2\%$ ($P < 0.001$, $n = 6$ per group), whereas PPAR δ and PPAR γ TFBA were both significantly diminished in foam cells compared to basal macrophages by $25.8 \pm 3.6\%$ ($P < 0.001$, $n = 6$ per group), and $26.9 \pm 5.1\%$ ($P < 0.001$, $n = 6$ per group), respectively. Interestingly, NA significantly increased PPAR γ TFBA in basal macrophages by $20.8 \pm 4.5\%$ ($P < 0.01$, $n = 3$ per group) (Figure 5.5 A). This PPAR γ activation in basal non-LPS stimulated macrophages by NA was further confirmed by quantitative RT-PCR showing up-regulation of PPAR γ downstream effector CD36 by $73.7 \pm 12.6\%$ ($P < 0.01$, $n = 3$ per group). In contrast, no effect on PPAR γ TFBA or CD36 mRNA transcription was seen in foam cells.

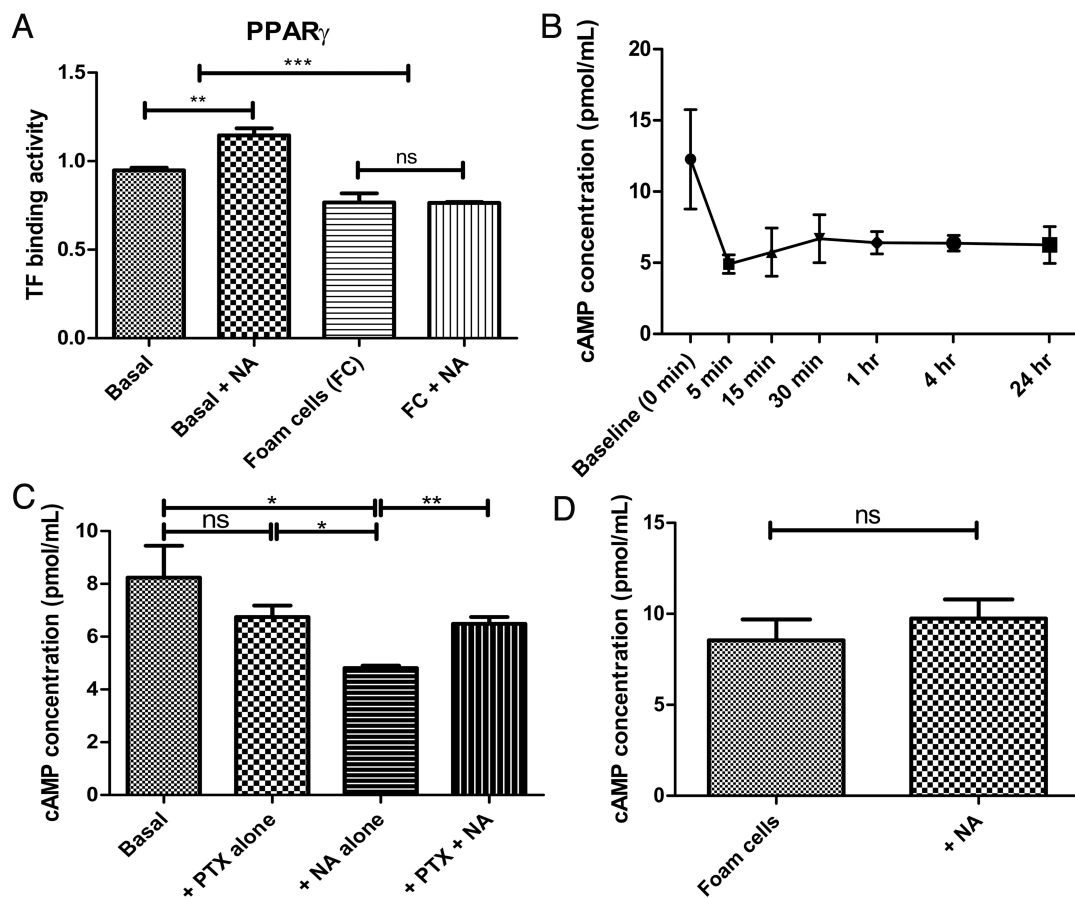


Figure 5.5 Effects of NA on PPAR γ and cAMP pathways in basal versus foam cells

A PPARs nuclear binding ELISA showed that cholesterol loading reduced PPAR γ transcription factor binding ($n = 6$ per group); and that NA significantly increased PPAR γ binding in basal macrophages ($n = 3$ per group) but not in foam cells. **B** NA (1×10^{-4} M) rapidly reduced cAMP level and this effect persisted for 24 hours. **C and D** Pre-incubation with pertussis toxin (PTX) or vehicles for 18 hours followed by NA. In basal macrophages, cAMP level diminished in NA alone group compared to control ($n = 3$ per group) and this effect was abolished by PTX pre-incubation. In contrast, NA did not affect cAMP level in foam cells ($n = 3$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = non significant.

5.4.6 Cyclic AMP is suppressed by NA in basal macrophages, but not in foam cells

Another potential mechanism for NA-induced activation of ABC transporters is via G-protein mediated cyclic AMP regulation.²⁶⁵ NA activates GPR109A, a G_i -protein coupled receptor, and reduces cAMP in adipocytes.⁴⁶ cAMP level in basal THP-1 macrophages was found to drop within 5 minutes after the application of 1×10^{-4} M NA and this reduction persisted for at least 24 hours (Figure 5.5 B). Basal macrophages and foam cells were then pre-incubated with 100 ng / mL pertussis toxin (PTX, to inhibit $G_{i/o}$ coupling) or vehicle-only for 18 hours followed by 1×10^{-4} M NA for 1 hour. In basal macrophages, cAMP level was significantly diminished in NA alone group compared to control by $41.5 \pm 14.8\%$ ($P < 0.05$, $n = 3$ per group) and this effect was abolished by PTX pre-incubation, confirming the role of $G_{i/o}$ -protein signaling involved in NA activation (Figure 5.5 C). In contrast, NA did not affect cAMP level in foam cells (Figure 5.5 D).

5.4.7 GPR109A expression is lost in foam cells *in-vitro*

Since neither cAMP nor PPAR γ pathways were influenced by NA in foam cells, the expression pattern of GPR109A was examined in cell culture. Using quantitative real-time RT-PCR, GPR109A mRNA was shown not to express in undifferentiated THP-1 cells but expression was seen after differentiation into basal macrophages (Figure 5.6 A). Treatment with NA for 24 hours, as expected with G-protein coupled signaling, caused desensitization and significant down-regulation of GPR109A mRNA transcription by $70 \pm 16.9\%$ ($P < 0.01$, $n = 3$ per group). Surprisingly, foam cell induction alone caused an even more dramatic down-regulation of GPR109A mRNA transcription by $87 \pm 17\%$ compared to basal macrophages ($P < 0.01$, $n = 3$ per group). To confirm this finding at a protein level, Western blot and fluorescence immunocytochemistry were performed on basal macrophages and foam cells. Figure 5.6 B shows the loss of protein expression of GPR109A in total cell lysates from foam cells compared to basal macrophages in a Western blot; whereas Figure 5.6 C and Figure 5.6 D demonstrate the expression of GPR109A on the cell surface in cultured THP-1 basal macrophages (5.6 C), and the down-regulation of GPR109A expression after foam cell induction (5.6 D).

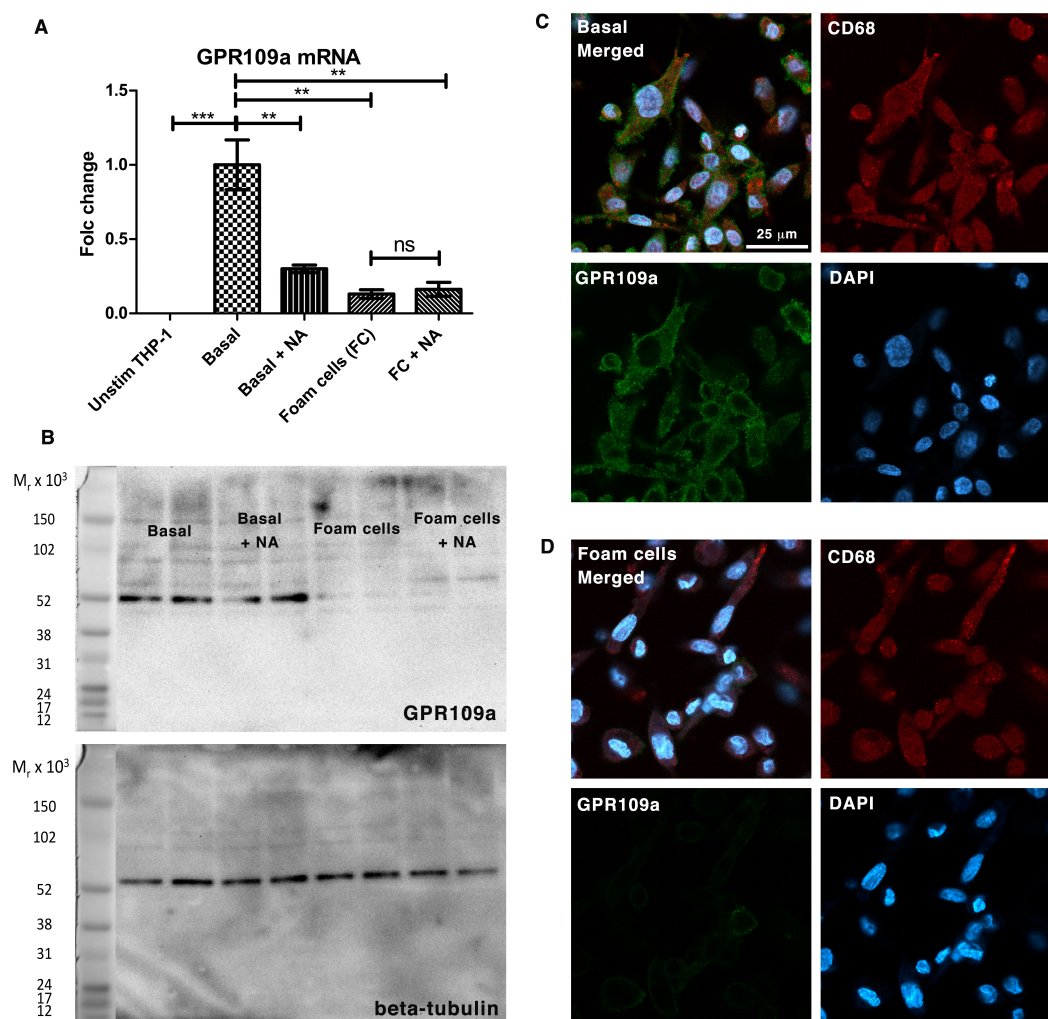


Figure 5.6 *Down-regulation of GPR109A in human macrophage-derived foam cells*

A GPR109A mRNA was not expressed in undifferentiated THP-1 cells but expression was seen after differentiation into basal macrophages. Treatment with NA for 24 hours caused significant down-regulation of GPR109A mRNA transcription ($n = 3$ per group). Foam cells induction down-regulated GPR109A mRNA transcription compared to basal macrophages ($n = 3$ per group). **B** Western blot showed the loss of protein expression of GPR109A in total cell lysates from foam cells compared to basal macrophages. **C and D** Confocal fluorescence immunocytochemistry demonstrated the expression of GPR109A on the cell surface in cultured THP-1 basal macrophages (**C**), and the down-regulation of GPR109A expression after foam cell induction (**D**).

5.4.8 GPR109A is expressed in human carotid plaques but its expression is down-regulated in carotid plaque foam cells

Finally, to ascertain whether these *in-vitro* findings are of potential clinical relevance, *ex-vivo* human carotid atherosclerotic plaques from endarterectomy were analysed. Five recently symptomatic carotid plaques were obtained for histological and fluorescence immunohistochemistry. At the region of maximal luminal stenosis, 10 μ m cryosections were double-stained with antibodies against CD68 and GPR109A; with the immediately adjacent section stained with Oil-red-O to visualise plaque lipid distribution. As shown with Figure 5.7 A, CD68-positive cells were consistently observed clustering at the interface between the lipid-rich area and the overlying fibrous cap layer; and a sub-population of these CD68-positive cells also expressed GPR109A. More importantly, GPR109A co-expression was observed in CD68-positive cells outside of the lipid-rich area, whereas CD68-positive cells within the lipid-rich area, which are highly likely to represent macrophage-derived foam cells, do not co-express GPR109A. Figure 5.7 B and 5.7 C show confocal images of the co-expression of GPR109A and CD68 in basal macrophages outside of lipid core (5.7 B), and the markedly diminished GPR109a co-expression in CD68-positive macrophages inside the lipid-rich area (5.7 C).

To further confirm this finding, a second set of co-localisation experiment was performed using antibodies against CD68, GPR109A, and a lipid-droplets associated protein adipophilin to identify foam cells. Figure 5.8 B shows that GPR109A-positive cells have low content for lipid droplets i.e. are non-foam cell macrophages; whereas Figure 5.8 C demonstrated CD68-positive cells with high lipid-droplets content i.e. “foam cells” macrophages do not co-express GPR109A.

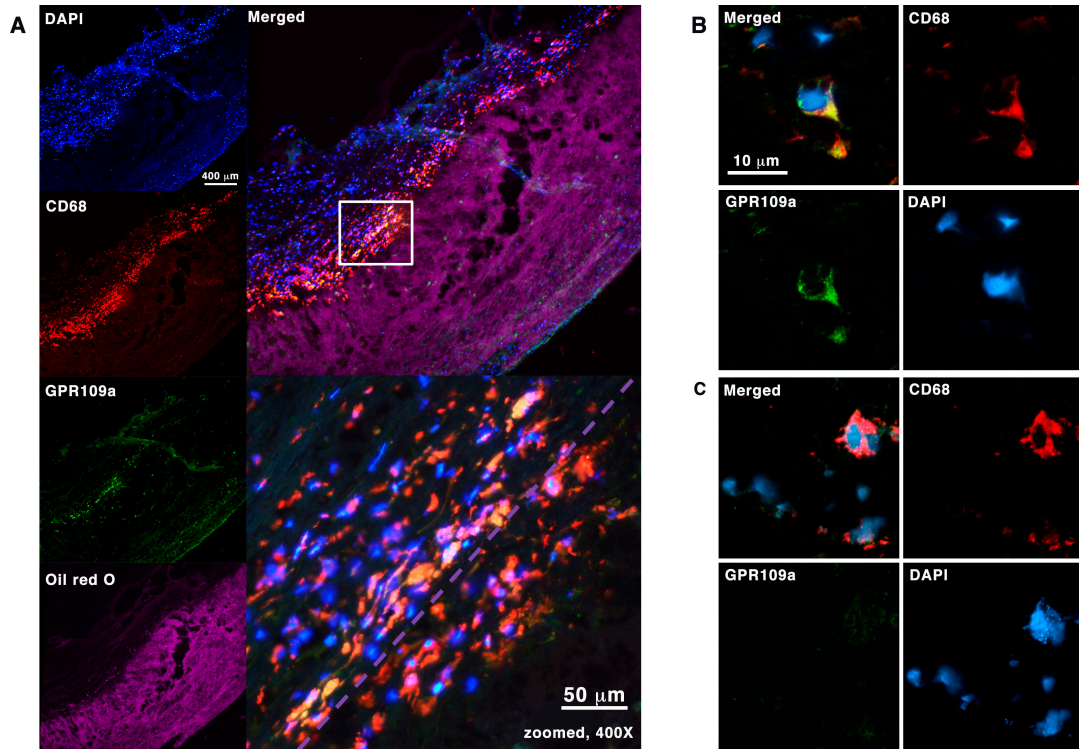


Figure 5.7 *Expression of GPR109A in ex-vivo human carotid plaques*

A Fluorescence immunohistochemistry staining with antibodies against CD68 and GPR109A. Immediately adjacent 10 μm cryosection was stained with Oil Red O for lipid distribution and visualized using Texas Red excitation filter (540 – 580 nm) in epifluorescence. CD68-positive cells were seen clustering at the interface between lipid-rich region and the overlying fibrous cap. GPR109A co-expression was seen in a sub-population of these CD68-positive cells outside of the lipid-rich region (yellow). Purple dotted line represents the boundary of the lipid-rich region as seen in the image above. **B and C** Loss of GPR109A expression in lipid-rich region. Confocal fluorescence images of CD68-positive cells outside of lipid-rich region are shown in **B**; and those within the lipid-rich region, likely to represent foam cells, are shown in **C**.

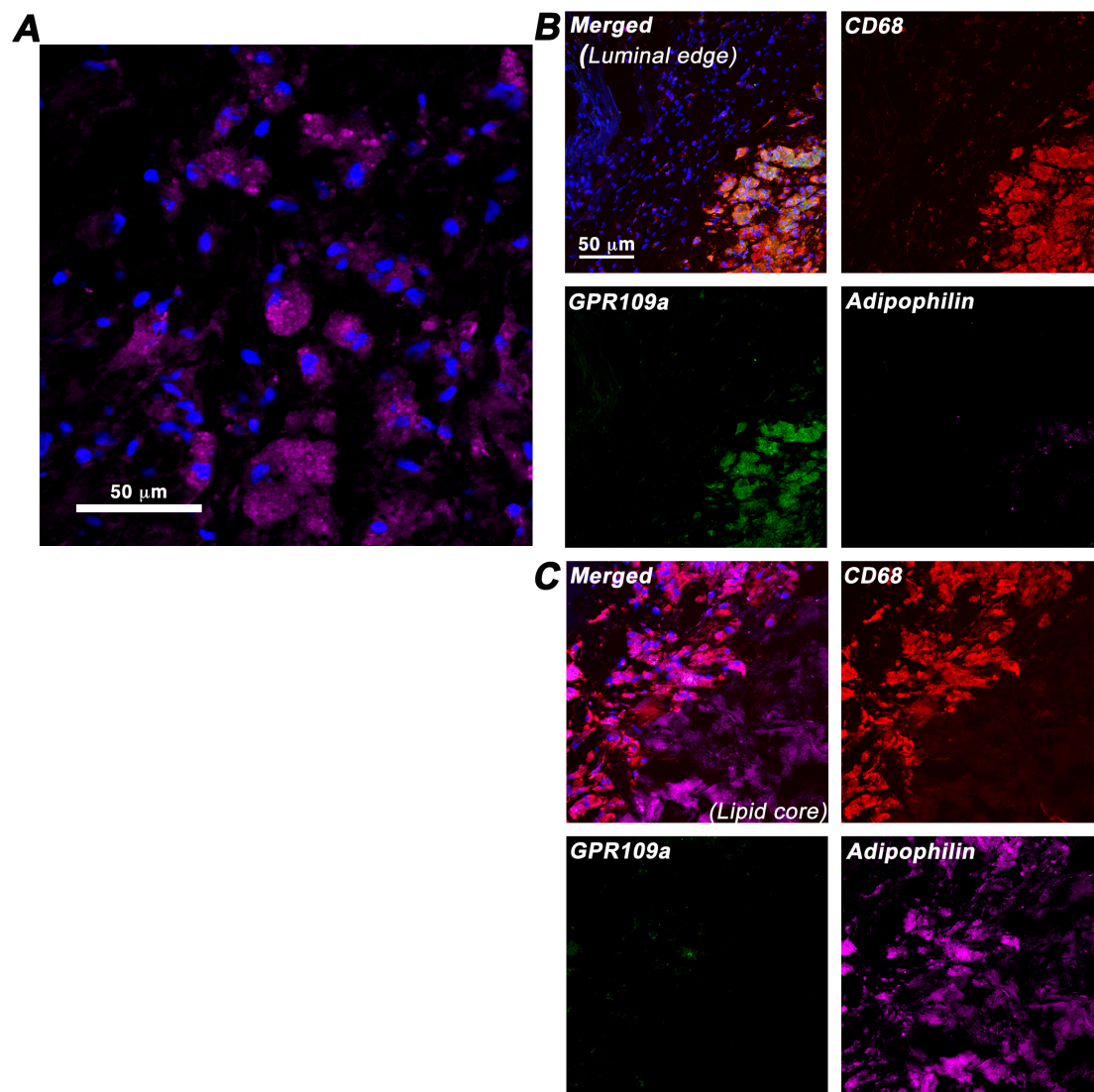


Figure 5.8 *Down-regulation of GPR109A in ex-vivo lipid-laden plaque foam cells*

Adipophilin (perilipin 2) staining is specific to intra-cellular lipid-droplet associated protein and represents a sensitive marker of foam cells. **A** High-powered confocal image showed the outline of intra-cellular lipid droplets. **B** GPR109A is strongly co-expressed in CD68-positive plaque macrophages without extensive lipid-loading i.e. non-foam cells; whereas plaque foam cells, which showed strong co-expression of CD68 and adipophilin due to extensive lipid-loading as shown in **C**, do not co-express GPR109A.

5.5 Discussion

Nicotinic acid drives atherosclerosis regression in humans and acts on monocytes via GPR109A to up-regulate mediators of cholesterol efflux. Recent observations in mice suggested that atherosclerosis progression can be attenuated by nicotinic acid independently of any effect on lipoproteins.²⁶² These observations suggested that nicotinic acid may exert anti-atherosclerotic effects by directly promoting cholesterol efflux from foam cells. In this chapter, this hypothesis was tested and it was demonstrated that, while PPAR γ agonism enhanced cholesterol efflux, NA failed to do so due to down-regulation of the receptor GPR109A in foam cells as compared with basal macrophages. Using samples obtained at carotid endarterectomy, a similar phenomenon was shown to occur *in-vivo*. These findings imply that direct NA-promotion of cholesterol efflux from foam cells does not contribute to atherosclerosis regression that has been documented with this drug. The patho-physiological relevance of GPR109A expression in monocytes / macrophages and its subsequent down-regulation in foam cells in the context of plaque regression remain to be established.

The effect of NA on peroxisome proliferator-activated receptors (PPARs), which are members of a nuclear receptor protein family that control transcription cascades in both glucose and lipid metabolism, was examined. Both PPAR α and PPAR γ are implicated in the regulation of cellular lipid metabolism,²⁶⁶ with PPAR γ -LXR α -ABC transporters pathway being the most studied.²⁶⁴ Consistent with previous *in-vitro* studies in non-foam cell macrophage/monocytic cell lines,^{53,54} NA was shown to exert a stimulatory effect on PPAR γ expression and nuclear activation with enhanced transcription of one of its downstream effector, CD36; and of the cellular cholesterol efflux ABC transporters. As observed recently in mice by Lukasova *et al.*,²⁶² enhanced transcription with ABCA1 with NA was not detected, but ABCG1

transcription was significantly up-regulated by NA in basal macrophages. However, NA loses this stimulatory effect on the RCT apparatus as a result of GPR109A receptor down-regulation after foam cell induction, as well as being unable to affect functionally cellular cholesterol efflux in cultured macrophage-derived foam cells.

NA also suppressed cAMP in non-stimulated, basal monocytic cells, through a mechanism that was pertussis toxin-sensitive, which deactivates G_i and G_o G-proteins. Although this is contrary to Rubic *et al*,⁵³ who showed a rise in cAMP associated with enhanced cholesterol efflux from basal macrophages by NA, results shown in this chapter are in agreement with several other groups who have observed NA-mediated inhibition of the cAMP response^{267,268} and similar effects are also seen in HM74a-transfected CHO-K1,⁴⁶ 293EBNA,⁴⁵ HEK293 and 3T3L1 adipocytes,²⁶⁹ which are at least partially ascribed to inhibition of adenylate cyclase. This classic cAMP response observed in basal monocytic cells was shown to be absent in transformed macrophage-derived foam cells.

This chapter showed, for the first time, the expression of GPR109A in a subpopulation of CD68-positive plaque macrophages in human carotid plaques. Moreover, these GPR109A and CD68 co-expressing cells appear to cluster just outside of lipid-rich region in plaques at the interface between the lipid pool and the overlying fibrous cap. These findings are supported by a recent animal study in LDLR^{-/-} mice, which reported co-expression of a red fluorescent protein (RFP) transgene placed under GPR109A promoter control and a macrophage marker MOMA-2 in murine aortic plaques.²⁶² These findings provide a plausible target in human plaques for the direct GPR109A-mediated anti-atherosclerotic effects of NA described recently.^{51 262} Adipophilin (perilipin 2) has recently been established as a sensitive marker of monocyte lipid loading^{270,271} and that its protein expression is increased in symptomatic carotid atherosclerosis.²⁷² Using immunofluorescence

against adipophilin, GPR109A expression in symptomatic human carotid plaques was confirmed to be down-regulated in CD68-positive cells that are laden with intracellular lipid-droplets, characteristic of foam cells.

Crucially, this down-regulation of GPR109A upon lipid-loading appeared to be a evolutionarily conserved cross-species phenomenon. Skogsberg, *et al.* used a transgenic mice model to examine the transcriptional changes in murine aortic plaque tissue in atherosclerosis progression and regression.²⁷³ In this study, the authors analysed the transcriptome of whole aortic sections serially in carefully characterised stages of progression as well as regression of atherosclerosis; and discovered a panel or network of cholesterol-responsive genes that respond to rapid lowering of plasma cholesterol with a genetic switch. Importantly, re-analysis of the raw microarray data (GEO accession number: GSE38574) revealed parallel results to this chapter, in which GPR109A was indeed expressed differentially at a time point before rapid expansion of the plaque lesions (weeks 30 *versus* 40) caused by the accelerated or deregulated lipid loading of lesional foam cells already present at 30 weeks (P = 0.025, FDR = 0.078, fold-change 1.73).

There are limitations in this study, for which attempts were made to address. In particular, GPR109A and GPR109B receptors are highly homologous and are 95% identical on the amino acid level.²⁷⁴ The detection of specific GPR109A expression in Western Blot and on immunohistochemistry was thus challenging. However, the same anti-GPR109A antibody was shown in a recent study to preferentially targets GPR109A such that its binding on a Western Blot was substantially attenuated when GPR109A was specifically knocked down using siRNA.⁵¹ In addition, although GPR109A and GPR109B share high degree of homology, only GPR109A binds nicotinic acid with high affinity. Another limitation is that in the cholesterol efflux study, free cholesterol and esterified cholesterol (EC) was not evaluated separately, since

early study has estimated that up to 30% of the cellular EC pool in THP-1 foam cells was derived directly from un-degraded AcLDL-derived EC, hence bypassing the normal hydrolysis / re-esterification cycle.²⁷⁵ Therefore, attempts to separately evaluate free cholesterol and EC would be confounded and would make a valid interpretation difficult.

In addition, one interesting observation from the RCT gene expression experiment also remained unexplained. After the initial up-regulation following Ac-LDL incubation, both PPAR γ and its heterodimerisation partner LXR α mRNA transcription appeared to be down-regulated (beyond baseline) in the resting phase (Figure 5.4 A). Although no clear explanation was found from the literature, subsequent complete PPAR α , γ , δ transcription factor binding ELISA assay also demonstrated a down-regulation of PPAR γ transcription factor activity following the same foam cell induction protocol (Figure 5.5 A), which was reassuring. Peculiarly, PPAR α transcription factor activity was enhanced by the same foam cell treatment. Therefore, although the PPAR regulatory mechanisms remain illusive, the foam cell induction protocol used in this study, with its resting phase, produced viable foam cells that displayed diverse PPAR responses. Moreover, stimulation of induced foam cells with GW1929 elicited the anticipated effect of LXR α and ABCG1 mRNA transcription up-regulation (Figure 5.4B, right column), further supporting that the induced foam cells behaved appropriately. The transcriptional regulation of nuclear factor PPAR α , γ , and δ is complex^{3,276} and beyond of scope of this current study. It would be appropriate to postulate that Ac-LDL, being itself a pro-inflammatory stimulus, may stimulate the release of pro-inflammatory cytokine such as TNF- α and IL-1 β , which is known to down-regulate PPAR γ mRNA transcription.^{277,278} During the resting phase, when the overwhelming lipid loading stimulus was withdrawn, the pro-inflammatory after-effect might have contributed to the “over-shot” phenomenon as seen with PPAR γ mRNA transcription and that of its partner LXR α .

Besides its potential pharmacological value, there remain important biological questions as to the physiological role of GPR109A, for which the cognate ligand is believed to be β -hydroxybutyrate.⁴⁸ β -hydroxybutyrate is a ketone body that is produced from acetyl-CoA in hepatocytes and is an alternative energy source to the brain and, to a lesser extent, the heart when glucose availability is low during fasting or starvation. Activation of GPR109A in adipocytes inhibits fatty acid release, possibly acting as a negative feedback against excessive lipolysis in starvation.²⁷⁹ Indeed plasma concentration of ketones can change over several orders of magnitude under normal physiology.²⁶⁰ However, the physiological function of GPR109A in immune cells is still largely unknown. Activation of GPR109A by the bacterial fermentation product, butyrate, has been shown to exert tumour-suppressing effects in colon cancer,²⁸⁰ while in cerebral hypoxia, β -hydroxybutyrate is shown to be neuroprotective.²⁸¹⁻²⁸³ Although this may reflect adaptations to metabolic substrate utilisation / energetics, immune cell modulation via GPR109A is another plausible explanation.

The co-expression of GPR109A in CD68-positive immune cells in human plaques at the interface between lipid pool and the overlying fibrous cap may suggest a regulatory cross-talk between 'metabolic' and 'inflammatory' pathways, which is increasingly appreciated.²⁸⁴ The anti-inflammatory effects of NA in both adipocytes⁵⁰ and stimulated monocytes,⁵¹ those being the two principal cell types bearing GPR109A, was previously shown. Each of these cell types is implicated in inflammatory pathologies relating to obesity and / or high caloric intake. It is therefore plausible that a metabolic mediator that is regulated and released by the liver over a large dynamic range in starvation might be involved in the suppression of inflammation in these cell types. Although no direct measurement of β -hydroxybutyrate has been made in atherosclerotic plaque, the selective expression

of GPR109A in macrophages suggests that it, or an alternative ligand, may have a role in immunomodulation in that context.

It is perhaps important to allude to the potential mechanisms of GPR109A down-regulation in foam cells. As with most G-protein coupled signaling, GPR109A is negatively regulated upon receptor stimulation. Ligand binding of GPR109A causes activation of G-protein coupled receptor kinases (GRKs), which in turn phosphorylate the occupied receptor.²⁸⁵ The phosphorylated GPR109A is then susceptible to binding of regulatory adaptor protein β -arrestins. In addition to direct attenuation of GPCR-signalling (desensitization), binding of β -arrestins also recruits AP2 and clathrin to facilitate endocytosis via clathrin-coated pits and recycling of desensitized GPCR.²⁸⁶ Moreover, it has recently been shown that β -arrestins contribute to the PGD₂-mediated cutaneous flushing associated with NA,²⁸⁷ and antagonize NF- κ B signaling,²⁸⁸ both independent of classic G-protein signaling. As a result, although little is currently known with regards to β -arrestin signaling in foam cells, its involvement in G-protein coupled receptor down-regulation, and its direct involvement in downstream cell signaling, make β -arrestins an attractive target for future investigation.²⁴²

5.6 Conclusion

In conclusion, this chapter showed that despite its potent anti-inflammatory effect on human macrophages, nicotinic acid has no direct effect on reverse cholesterol transport in macrophage-derived foam cells; and this may be explained by the down-regulation of its receptor GPR109A upon foam cell transformation. This implies that direct NA-promotion of cholesterol efflux from foam cells does not contribute to the atherosclerosis regression, which has been demonstrated with this drug. Given the cellular distribution of GPR109A / CD68 co-expressing macrophages in human carotid plaque, and its apparent cross-species conservation, the patho-physiological significance of GPR109A expression in human plaque macrophages and its down regulation in foam cells remains to be elucidated. More importantly, it demonstrated differential drug response in basal-state macrophages as opposed to lipid-laden macrophage-derived foam cells, whose formation reflects local lipid content and its effect on gene / protein expression, detectable by vessel-wall imaging and LCM transcriptomic techniques, respectively, described in earlier chapters.

CHAPTER 6

GENERAL DISCUSSION

6.1 Original hypothesis and aims

In the post-statin era, there is an urgent unmet clinical need to rethink the current paradigm in drug discovery and in the delivery of stratified medicine. The complementary roles of atherosclerosis imaging combined with modern “omics” could provide disease stratification by defining clinical cohorts for targeted treatment with high degree of resolution.

This thesis hypothesized that this *integrated* characterisation of atherosclerosis would generate novel insights and understandings in plaque biology, which would help bridge the gap between molecular mechanisms and clinically measurable outcomes. In particular, this thesis sought to examine whether plaque *compositional heterogeneity* such as lipid content, discernable by MRI T₂ mapping, is associated with specific gene expression profiles in human atherosclerotic plaque macrophages in a spatially distinct pattern, reflecting macrophage *functional heterogeneity* within plaques, using immuno-LCM techniques. This thesis further hypothesized that this functional heterogeneity of plaque macrophages might play an important role in determining response to pharmacological interventions.

6.2 Summary of key findings

The key findings of this thesis are summarized below:-

- 1) MRI T₂ mapping could accurately and reproducibly provide *in vivo* human carotid plaque tissue characterisation, and readily discriminated different plaque components such as lipid-rich necrotic core, fibrous tissue, calcification. This was validated using histology *qualitatively* based on AHA

- classification system; and *quantitatively* in plaque lipid assessment. (Chapter 3)
- 2) Symptomatic plaques were found to contain significantly more lipid compared to asymptomatic plaques, based on T_2 mapping, despite having similar degree of luminal stenosis and only a modest difference in plaque volume. Receiver operating characteristic (ROC) curve analysis demonstrated T_2 mapping to have a good ability to discriminate between symptomatic and asymptomatic plaques. (Chapter 3)
 - 3) Immuno-LCM was able to procure plaque macrophages and achieve high quality RNA in a location-specific manner, which then allowed coupling with downstream whole-genome expression profiling to generate unique, location-specific, macrophage gene expression profiles based on clinical symptom status. (Chapter 4)
 - 4) Pathway analysis using GSEA, and subsequently confirmed using the upstream / downstream predictive algorithms of IPA, revealed a number of biological functions that were significantly upregulated in macrophages from symptomatic plaques. They can be grouped in 7 broad categories: (1) inflammation; (2) lipid metabolism; (3) hypoxic response; (4) cell proliferation; (5) apoptosis; (6) antigen presentation; and (7) cellular energetics. (Chapter 4)
 - 5) Combining T_2 map %Lipid Area data from Chapter 3, GSEA identified significant enrichment of inflammatory pathways, in particular, interferon / STAT1 pathways, in macrophages that correlated with plaques lipid content. (Chapter 4)

- 6) Nicotinic acid is known to have anti-atherogenic properties by virtue of its anti-inflammatory effects and it promotes reverse cholesterol transport in adipocytes and basal-state macrophages. However, it had no direct effect on reverse cholesterol transport in macrophage-derived foam cells *in vitro*; and this might be explained by the down-regulation of its receptor GPR109A upon macrophage foam cell transformation. (Chapter 5)
- 7) *Ex vivo* GPR109A expression was found in human carotid plaque macrophages but its expression was down-regulated in lipid-rich region, where macrophages existed predominantly as lipid-laden foam cells. This highlighted the complex functional consequences of plaque macrophage heterogeneity in terms of response to drug treatment. (Chapter 5)

6.3 Future directions

This thesis focused on the concept of an *integrated* platform for characterisation of human atherosclerosis that could potentially lead to future improvement in disease stratification and treatment delivery; the key findings in this thesis has opened up the possibility of a number of interesting areas for future development both in the imaging part and LCM-omics part of the study.

For the imaging platform, MRI T_2 mapping described in this thesis is a new technique that requires further optimization before ready for the clinic. For example, the current DANTE-MESE sequence is strongly susceptible to motion artifacts, a fact reflected in the relatively high overall rejection rate (35%) of completed scans. In order for this technique to fulfil its full clinical potential a 'navigator' based motion correction strategy is in development. In addition, a novel 10-channel phased array surface coil

is currently being designed that will increase signal-to-noise ratio and superior-inferior coverage compared to the 4-channel coil used in the current study. In addition, automated 3-dimensional vessel wall anatomical rendering derived from isometric MR dataset is currently in development, which will allow evaluation of not only the quantity of lipid but the distribution of lipid to be appreciated in relation to local vessel anatomy. This ability might prove useful in informing decision between carotid surgery and stenting; since a large LRNC is considered to be an important factor determining the amount of stenting-related downstream debris^{224,225} and has been shown to increase risk of stroke following carotid stenting.^{225,226} This may eventually assist interventional procedural planning to minimize such risk.

Furthermore, although a previous attempt was made to correlate carotid atherosclerosis with plaque burden in other vascular territories, such as aorta and coronary arteries,²²⁰ the relationship of plaque lipid content and distribution in relation to symptom status described in this thesis have not yet been demonstrated in the coronary vessels. Intravascular ultrasound (IVUS), optical coherence tomography (OCT), and near-infrared spectroscopy (NIRS) are all promising tools to interrogate coronary plaque composition, especially fibrous cap thickness and lipid-core size;^{289,290} in fact NIRS was recently used in the YELLOW trial to show that short-term intensive statin therapy did result in reduction in coronary plaque lipid content.²⁹¹ Nonetheless the invasive nature of these imaging modalities limited their use in clinical trials. An important extension of the T_2 mapping technique described in this thesis is to see whether carotid lipid content can be correlated to coronary lipid using technique such as NIRS. Once validated, ongoing trials such as YELLOW II²⁹² can be supplemented with a non-invasive imaging modality as a surrogate to relate coronary plaque lipid to HDL function, and alterations in macrophage gene expression. T_2 mapping can also be adapted to study other peripheral vascular diseases.

In terms of the functional genomic aspect of vascular cells involved in atherosclerosis, this thesis primarily focused on macrophages but neo-intimal vascular smooth muscle cells (VSMCs) were also procured during the same batch immuno-LCM procedures in the same cohorts of patients. Meanwhile, T₂ mapping data can be further analysed to extract other quantitative information such as fibrous tissue content or even the extent of IPH; therefore, once the transcriptomic analysis of VSMCs can be obtained, further correlation with other quantitative T₂ mapping data might be evaluated. For example, comparison could be made to look at the transcriptomes of VSMCs and to correlate that to the plaque fibrous tissue content.

An important question in functional genomic is how data at a transcriptomic level relate to protein and metabolic functions. After all, mammalian mRNA only has a median half-life of 9 hours compared to 46 hours for cellular protein.¹³¹ As such, during the entire immuno-LCM experiment, a parallel set of samples was procured, both from macrophages and VSMCs, from the same cohort of patients. These samples will be subjected to standard liquid chromatography and tandem mass spectrometry for proteomic analysis.¹⁹⁷ One can then attempt to map peptides to Gene Ontology functions and integrate transcriptomic and proteomic data using pathway analysis tools such as IPA.

Recently, we have shown that in acute myocardial infarction, activated pathways of inflammation and proliferation were conserved between humans and mice in peripheral monocytes, prior to recruitment.²⁴³ Similar cross-species analysis of immuno-LCM driven plaque macrophage transcriptomes can be applied to our current human data, against murine models of atherosclerosis, with published array data from the GEO repository.^{273,293}

Last but not least, in terms of the potential applications of this *integrated* platform of plaque characterisation discussed in this thesis, the current study identified activation of the interferon / STAT1 pathways in human carotid plaques that correlated to high lipid content. The functional significance of STAT1 in human atherogenesis is complex, but as discussed earlier, supporting evidence suggests it plays a regulatory role in the susceptible locus identified by multiple GWAS studies. Not only can LCM-omic techniques provide unique validation of GWAS data at a *target-tissue level*, MRI T₂ mapping could be utilized to stratify patient cohorts, and to identify patients likely to benefit from novel biologic therapy, such as interferon- α/β antagonist,²⁵³ currently in pre-clinical development. Finally, local delivery of anti-inflammatory agents such as STAT1 inhibitor fludarabine,^{294,295} via drug coated balloon²⁹⁶ or biodegradable vascular scaffold drug delivery platform²⁹⁷ might allow direct delivery of potent drugs to target immune cells at the “core” of the fatty vulnerable plaques (balloon inflation in effect caused controlled dissection and may allow diffusion of drug directly into plaques), without incurring the toxic systemic side effects.

6.4 Final conclusion

Combating the residual risk of thromboembolic complications in atherosclerosis remained a significant challenge in the post-statin era; nevertheless, the very idea that atheroma are dynamic entities with progression – and regression – that could be non-invasively tracked in time – is no longer a dream. At this juncture in medical history, novel technologies are emerging that will soon not only make the delivery of stratified or personalized medicine a reality, but at a resolution never imagined before. This thesis delved into the concept of an *integrated* platform of atherosclerosis characterisation in humans and its potential advantages; its findings provided evidence of the superior ability of MRI T₂ mapping to accurately interrogate plaque composition, and with modern functional genomic techniques, directly linking plaque lipid quantity to activation of specific inflammatory pathways in human plaque macrophages. This serves to reinforce the complementary roles of imaging and the genome-wide multi-layer bioinformatics in the current “omics” era.

CHAPTER 7

APPENDICES

7.1 Timing and venue of research

The work described in this thesis was conducted between April 2011 and March 2015, in the RDM Division of Cardiovascular Medicine, Levels 5 & 6, West Wing, John Radcliffe Hospital, Oxford; the Acute Vascular Imaging Centre (AVIC), John Radcliffe Hospital, Oxford; the Wellcome Trust Centre for Human Genetics, Oxford; Techniques of immunohistochemistry-guided laser capture microdissection were refined at Zeiss Microscopy Labs, Carl Zeiss Microscopy GmbH, Munich, Germany.

7.2 Research funding

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7.3 Attributions

The author is extremely grateful to the contributions to this body of research made by colleagues and collaborators.

Dr Linqing Li and Dr Luca Biasioli designed the MRI sequences used in T_2 mapping. Ms Melanie Jones, Mrs Juliet Semple, and Dr Erica Dall'Armellina performed the carotid MRI at AVIC. Dr Francesca Galassi helped develop the 3D MR carotid models. Professor Ashok Handa coordinated collaboration with a team of vascular

surgeons, namely, Mr Chris Darby, Professor Alison Halliday, Professor Linda Hands, Mr Timothy Magee, Mr Jeremy Perkins, and Mr Ed Sideso. Dr Mohammad Alkhalil assisted in patient recruitment and sample collection. Dr Andrew Jefferson performed and taught me confocal microscopy. Dr Janet Digby taught me cellular and general laboratory techniques and helped in *in vitro* experiments.

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This thesis is dedicated to my wife Michelle, and my son Alfred.

7.5 Awards relating to this body of research

- Finalist of American Heart Association Melvin Judkins Young Investigator Award in Cardiovascular Radiology and Intervention (2015)
- Winner of Best Presentation Award by a Young Investigator, European Atherosclerosis Society (2015)

7.6 Published abstracts arising from this period of research

Chai JT, Biasioli L, Li L, Handa A, Perkins J, Hands L, Sideso E, Jezard P, Robson MD, Choudhury, RP. Lipid-rich Core Quantification in Carotid Atherosclerosis Using MRI T2 Mapping - Relation to Clinical Presentation and Plaque Macrophage Activation. *Circulation* 2015; 132: A11833

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7.7 Publications arising from this period of research

Book Chapters

Choudhury RP, Chai JT, Fisher EA. Biology and pathology of atherosclerosis (Chapter 13.1) *Oxford Textbook of Medicine / Oxford Textbook of Cardiovascular Medicine*. Oxford University Press, 2016 (in editorial review)

Chai JT, Biasioli L, Li L, Jezard P, Handa A, Choudhury RP. Clinical vascular magnetic resonance imaging at high field (Chapter 1). *Vascular and Endovascular Consensus Update* book, BIBA publishing, 2014

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Alkhalil M, Chai JT, Choudhury RP. Plaque imaging to refine indication for emerging lipid lowering drugs. *Eur Heart J Cardiovascular Pharmacotherapy*. (in preparation, 2016)

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Chai JT, Digby JE, Choudhury RP. GPR109A and vascular inflammation. *Curr Atheroscler Rep*. 2013 May;15(5):325

Original research

Chai JT, Ruparelia, N, Biasiolli L, Gardner L, Handa A, Choudhury RP. Laser-capture microdissection revealed distinct macrophage gene expression profiles in human carotid plaques that correlated with plaque lipid content measured by MRI (*in preparation, 2016*)

Chai JT, Biasiolli L, Li L, Handa A, Perkins J, Hands L, Sideso E, Jezzard P, Robson MD, Choudhury RP. Quantification of lipid-rich core in carotid atherosclerosis using T2 mapping with MRI – relation to clinical presentation (*in review, submitted to JACC Cardiovascular Imaging, 2016*)

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CHAPTER 8

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