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DEPARTMENT OF MEDICINE
EXPERIMENTAL CARDIOVASCULAR RESEARCH UNIT
Karolinska Institutet, Stockholm, Sweden

IDENTIFICATION OF INFLAMMATORY GENES INVOLVED IN THE PATHOGENESIS OF HUMAN AND EXPERIMENTAL ATHEROSCLEROSIS

Hanna E Agardh

Karolinska Institutet

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ABSTRACT

Atherosclerosis is a chronic systemic inflammatory disease of large and medium sized arteries, developing slowly and silently over decades. The disease is usually not apparent until occurrence of a sudden clinical symptom, such as myocardial infarction (MI) or stroke. Several classical risk factors have been established to play a role in the progression of disease over a long period of time. However, markers recognizing vulnerable patients being at risk of having an event in the near future are lacking. Thus, more knowledge about the ongoing complex pathogenesis is needed for identification of potential biomarkers and therapeutic targets of atherosclerosis. Patients with carotid atherosclerosis experiencing cerebral symptoms within one month before undergoing carotid endarterectomy (CEA) are classified having vulnerable plaques.

Based on the classification above, I show in this thesis that mRNA levels of enzymes in the leukotriene 5-lipoxygenase pathway, 5-lipoxygenase (5-LO) and leukotriene A4 hydrolase (LTA4H), are associated with plaque vulnerability.

Gene expression can be investigated on a single target level using real-time PCR or by analyzing thousands of genes simultaneously, using global transcription microarrays. Based on correlations to microarrays we argue for using total RNA mass in normalization of real-time PCR data, when analyzing heterogeneous human specimen.

To identify new candidates of plaque vulnerability an unbiased approach was used - transcript profiles of symptomatic plaques were compared to asymptomatic plaques, demonstrating an increase of fatty acid binding protein 4 (FABP4), which was associated with vulnerability, independent of age or gender. FABP4 localize mainly to the numerous macrophages present in the atherosclerotic plaque. This study suggests FABP4 to play a role in plaque vulnerability and to be a potential valuable biomarker within the carotid atherosclerotic plaque.

To determine if any atherosclerosis-related changes can be detected in circulating cells the transcriptome of leukocytes in the circulation from an experimental atherosclerotic model *Apoe*$^{-/-}$was analyzed. Surprisingly, we also here identify FABP4 as a marker in neutrophils and monocytes reflecting atherosclerotic lesion progression. Moreover, I observe human monocytes and neutrophils from the circulation to be positive for FABP4. Our findings make FABP4 in circulating cells interesting for functional investigations, and an appealing and easy accessible biomarker target for potential future translation into clinical purposes.

In conclusion, I have studied inflammatory genes being involved in the pathogenic process during atherosclerosis using human and experimental models. In brief, we demonstrate that human vulnerable plaques display increased mRNA levels of 5-LO and LTA4H, and FABP4. In addition, the latter is shown in an experimental model, to be a potential valuable biomarker in circulating leukocytes reflecting the extent of atherosclerotic lesion. Our discoveries in the human plaque may be of future clinical relevance to identify vulnerable plaques, whereas FABP4 in leukocytes potentially could be useful for recognizing asymptomatic patients before onset of symptoms.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred in the text by their corresponding roman numerals (I-IV).

I. Qiu, H., Gabrielsen, A., **Agardh, H.E**., Wan, M., Wetterholm, A., Wong, C.H., Hedin, U., Swedenborg, J., Hansson, G.K., Samuelsson, B., Paulsson-Berne G., and Haeggström JZ. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. *Proc Natl Acad Sci U S A.* 2006 May 23;103(21):8161-6

II. Folkersen, L., Kurtovic, S., Razuvaev, A., **Agardh, H.E**., Gabrielsen, A., and Paulsson-Berne, G. Endogenous control genes in complex vascular tissue samples. *BMC Genomics*. 2009 Nov 10;10:516

III. **Agardh, H.E**., Folkersen, L., Ekstrand, J., Marcus, D., Swedenborg, J., Hedin, U., Gabrielsen, A., and Paulsson-Berne, G. Expression of fatty acid–binding protein 4/aP2 is correlated with plaque instability in carotid atherosclerosis. *Journal of Internal Medicine*. 2011 Feb;269(2):200-10

IV. **Agardh H.E.**, Gertow K., Salvado Duro D., Hermansson A., van Puijvelde GH., Haeggström J., Hansson GK, Paulsson-Berne G. and Gabrielsen A. Fatty acid binding protein 4 in circulating leukocytes mirrors lesion progression in experimental atherosclerosis. *Manuscript*

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LIST OF ABBREVIATIONS

5-LO	5-Lipoxygenase
5-HPETE	5-Hydroxyperoxy eicosatetraenoic acid
AA	Arachidonic acid
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARE	Adipocyte regulatory elements
AUC	Area under curve
BiKE	Biobank of Karolinska Endarterectomies
CAD	Coronary artery disease
CD	Cluster of diffrentiation
cDNA	Complementary deoxyribonucleic acid
CEA	Carotid endarterectomy
CRP	C-reactive protein
CT	Cycle threshold
CVD	Cardiovascular disease
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECST	European Carotid Surgery Trial
ELISA	Enzyme-linked immunosorbent assay
FABP	Fatty acid binding protein
FLAP	5-Lipoxygenase activating protein
FRS	Framingham risk score
FSE	Fat specific elements
GM-CSF	Granulocyte macrophage colony stimulating factor
HKG	Housekeeping gene
IL	Interleukin
IPH	Intraplaque hemorrhage
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
Lp-PLA2	Lipoprotein associated phospholipase 2
LT	Leukotriene
LTA4H	Leukotriene A4 hydrolase
MCP-1	Monocyte chemotactic protein
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PMN	Polymorphonuclear
PPRE	Peroxisome proliferator response elements
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SMC	Smooth muscle cell
SR-A1	Scavenger receptor A
TIA	Transient ischemic attack
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organisation
VLDL	Very low density lipoprotein

PREFACE

Cardiovascular disease is the leading cause of death and disability in the world. Although a large proportion is preventable, the incidence continues to rise mainly because preventive measures are inadequate. The most common types of CVD, cerebrovascular disease and coronary artery disease, have severe consequences for the affected individual and results in huge costs for the society. The main underlying pathological process driving these diseases is atherosclerosis. Atherosclerosis derives from the Greek words, *athere* meaning gruel, and *skleros* meaning hard.

Atherosclerosis was for decades believed to be just a passive accumulation of lipids within the arterial vessel. Today the disease is recognized to be far more complex, involving the interplay between locally infiltrated immune cells and lipids in the plaque, along with systemic inflammation. Although several classical risk factors have been identified, there is still a need for better screenings/markers/measures to find patients in high risk of cardiovascular events and ultimately prevent future events.

This thesis focuses on identification of markers associated with plaque vulnerability and markers associated with atherosclerotic plaque progression, from initiation of fatty streaks to formed advanced plaques, which may cause clinical symptoms. Three out of the four included papers have been based on human carotid endarterectomies from our Biobank at Karolinska Endarterectomies (BiKE). In addition, experimental murine models have been used to study pre-chosen genes as well as to analyze disease progression over time. All my investigations have primarily been based on mRNA, using real-time PCR to study pre-selected genes, and microarray technology to analyze thousands of potential targets. In addition, I have localized the different proteins to the specific cell types. Some of the findings uncover potential involvement of markers in plaque vulnerability, while other illustrates potential biomarkers which may be of value for clinical screening of atherosclerotic lesion progression.

In this thesis I will give an overview of the atherosclerotic disease process including a discussion about the vulnerable plaque and ways to identify it. Furthermore, I will discuss the main relevant cell types and targets for the studies included in this thesis and the importance of my findings.

1 INTRODUCTION

1.1 ATHEROSCLEROSIS- AN OVERVIEW

Atherosclerosis is a progressive systemic inflammatory disease of the arterial system, affecting large and medium sized arteries, and the main underlying cause of cardiovascular diseases (CVD), such as cerebrovascular disease or coronary artery disease (Hansson 2005). Despite advances in risk factor management, CVD is the leading cause of mortality worldwide accounting for around 17.3 million victims 2008, which represents 30% of all global deaths according to World Health Organization (WHO). Of these deaths approximately 6.2 million were due to stroke and 7.3 million due to coronary heart disease (WHO). If the current trend continues, this number is predicted to increase to approximately 25 million deaths by 2020 (Dahlöf 2010; Lloyd-Jones 2010).

Epidemiological studies have established several risk factors for CVD of which the most important are hypertension, hypercholesterolemia, smoking and diabetes (Dahlöf 2010) (Mehta, Saldeen et al. 1998). Despite these known risk factors, there is a great need to identify additional markers to improve identification of patients at high risk before onset of symptoms (Eagle, Ginsburg et al. 2010). Although several inflammatory mediators, such as soluble CD40 ligand (sCD40L), adiponectin, C-reactive protein (CRP) and matrix metalloproteinase 9 (MMP-9), associate with increased cardiovascular risks, they only rarely add to the risk score models available (Packard and Libby 2008). One exception is CRP although it is being under debate of its potential utility (Libby and Ridker 2004; Scirica and Morrow 2006). In addition, several other markers are being thoroughly investigated in large patient cohort studies.

A healthy vessel wall consists of three different layers: intima, media and adventitia. The innermost layer, the intima, is composed of endothelial cells facing the lumen of the vessel, as well as some loose connective tissue and occasional smooth muscle cells (SMC) and macrophages. The middle layer, the media, is of importance for contractility of the vessel and consists almost solely of smooth muscle cells. The outer layer, the adventitia, is composed of SMCs, fibroblasts, connective tissue, immune cells and vasa vasora, which supply the vessel with oxygen and nutrients. Between all the three different layers elastic laminae are present. It is within the intima of large and medium sized arteries that atherosclerotic lesions, also called plaques, are formed (Lusis 2000), a process referred to as atherogenesis.

Historically atherosclerosis was thought to develop due to passive accumulation of lipids in the vessel wall (Brown and Goldstein 1983). Another theory dominating the scene was the response to injury hypothesis, explaining lesion development as a cause of migrating and proliferating smooth muscle cells upon endothelial damage (Ross 1976). However, the picture changed in the mid-eighties, when active immune cells were demonstrated to be present within the plaque (Jonasson, Holm et al. 1986) and today atherosclerosis is considered to be a chronic inflammatory systemic disease (Hansson and Hermansson 2011).

1.1.1 Atherogenesis

Formation of fatty streaks starts to form when low density lipoprotein (LDL) particles present in the circulation diffuse into the intimal layer of the vessel or are transported through the endothelial cell layer of arteries (von Eckardstein and Rohrer 2009). The LDL particle carries the large protein ApoB100 which is negatively charged and interacts with positively charged proteoglycans present in the extracellular matrix. This step has been shown to be important in initiating early atherosclerosis (Skalen, Gustafsson et al. 2002; Tabas, Williams et al. 2007).

Once in the subendothelial space, the LDL particles are trapped and prone to undergo oxidative modifications by enzymatic attacks by lipoxygenases and myeloperoxidases, or by reactive oxygen species (Berliner and Heineke 1996) (Figure 1). As a result of the oxidation, the modified components (e.g. truncated lipids and reactive aldehydes) will activate endothelial cells and macrophages to express adhesion molecules (Cybulsky and Gimbrone 1991; Nakashima, Raines et al. 1998) and chemokines (Zernecke and Weber 2010). In addition to the modified LDL, sheer stress at arterial branching points is believed to cause further activation and lead to expression of selectins on the endothelial wall, in turn initiating leukocyte migration into the vessel wall (Eriksson, Xie et al. 2001).

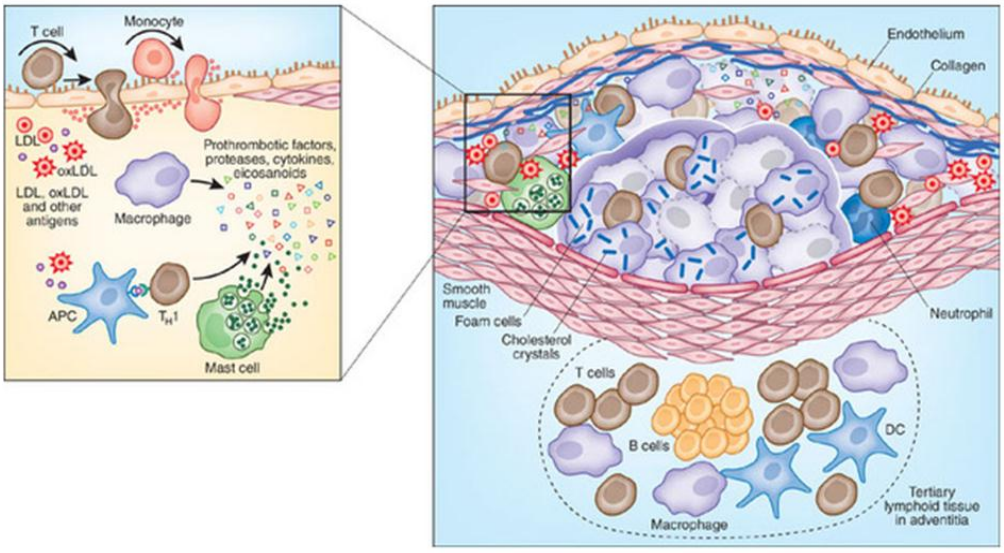

Figure 1. The plaque has a core of lipids, including cholesterol, living and apoptotic cells and a fibrous cap with smooth muscle cells and collagen. Plasma lipoproteins accumulate in the subendothelial region. Several types of cells of the immune response are present throughout the atheroma including macrophages, T cells, mast cells and DCs. The atheroma builds up in the intima, the innermost layer of the artery. Outside the intima, the media contains smooth muscle cells, and further abluminally, the adventitia continues into the surrounding connective tissue. Here, immune cells accumulate outside the advanced atheroma and may develop into tertiary lymphoid structures with germinal centers. *Reprinted by permission from Macmillian Publishers Ltd: Nature Immunology, Hansson et al. 2011 Mar;12(3):204-12. Copyright 2012.*

Infiltrating monocytes are further stimulated and differentiate into macrophages essential for lesion development (Smith, Trogan et al. 1995). The oxidation of LDL present in the intima will increase its binding to scavenger receptors, especially CD36 and SR-A1, on the macrophages. The increased endocytosis of cholesterol eventually turns these macrophages into foam cells, which eventually will die and form the necrotic core (Goldstein, Ho et al. 1979; Kunjathoor, Febbraio et al. 2002).

Besides monocytes, several other different cell types including T cells, B cells, mast cells, neutrophils and dendritic cells, are present within the lesion (Hansson and Hermansson 2011). The plaque eventually becomes more and more complex, containing a lot of necrotic and apoptotic cells constituting the necrotic core, which is covered by a cap separating the lesion from the blood stream.

1.1.2 The plaque

Studies have shown that acute clinical manifestations of atherosclerotic disease, such as myocardial infarction (MI) or stroke, are mainly a consequence of acute disruption, rupture or erosion of the plaque. The rupture leads to exposure of thrombogenic plaque components to the bloodstream with subsequent thrombus formation. The newly formed thrombus may occlude the whole lumen, giving rise to MI, or cause distal embolization as seen in symptomatic atherosclerotic carotid disease (Spagnoli, Mauriello et al. 2004). In addition, it has been shown that the coronary plaque phenotype in patients with coronary artery disease (CAD) significantly correlate with the carotid plaque composition (Zhao, Zhao et al. 2011). Thus, presumably investigations of lesions at one site in the vasculature, as the carotid lesion in our studies, reflect lesion phenotype at other sites.

Histology of plaques obtained during endarterectomy or post-mortem has associated certain lesion characteristics with adverse clinical events. These plaques are referred to as unstable, vulnerable or high-risk plaques and defined as plaques with a high risk to cause local thrombosis with unstable clinical symptoms such as MI, unstable angina or cerebrovascular events (Schaar, Muller et al. 2004). The most common plaque phenotype, the classically defined vulnerable plaque will be described in more detail in coming section. However, it is important to stress that these rupture-prone plaques are not the only vulnerable plaques. All types of atherosclerotic plaques with high likelihood of thrombotic complications and progression should be considered as vulnerable plaques. Basically, there are three types of vulnerable plaques:

1) Classical plaques prone to rupture
2) Plaques with superficial erosion (Farb, Burke et al. 1996)
3) Plaques with calcified nodules protruding the lumen (Virmani, Kolodgie et al. 2000).

The mechanism to which carotid atherosclerosis results in cerebrovascular symptoms are less understood than those linking coronary disease and MI. Plaque erosion, which is common in the coronaries, was only recently described in carotid lesions and shown to be present in 10% of patients with strokes or TIA (Spagnoli, Mauriello et al. 2004). Finally, plaques with calcified nodules are least well understood and represent the least common cause of luminal thrombus, accounting for 2-5% of coronary thrombi (Virmani, Kolodgie et al. 2000).

The lesion itself may be of stenotic or non-stenotic nature. However, non-stenotic plaques are far more frequent throughout the arterial tree and also the majority of plaques causing MI (Ambrose, Tannenbaum et al. 1988).

1.1.3 The classical vulnerable plaque

The main three features of the classically defined vulnerable plaque are:

- Large lipid core
- Thin fibrous cap
- Infiltration of inflammatory cells.

The lipid core is composed mainly by cholesterol and lipids remaining from dead foam cells (Davies, Richardson et al. 1993). Due to the rich presence of tissue factor produced by macrophages, this part of the lesion is extremely thrombogenic (Fernandez-Ortiz, Badimon et al. 1994). Autopsy studies have shown that lipid cores of ruptured plaques are larger in size than from non-ruptured (Virmani, Kolodgie et al. 2000). From a biomechanical point of view a large soft lipid core is disadvantageous, unable to carry the mechanical forces, which instead are concentrated in the thin cap overlying the lesion, making it more vulnerable (Richardson, Davies et al. 1989).

The cap covering the lesion consists of extra cellular matrix components such as, proteoglycans, collagen and elastin. There are few or no smooth muscle cells within the cap of a vulnerable plaque, the cap of carotid plaques have shown to have fewer macrophages than coronary plaques (Virmani, Kolodgie et al. 2000). The mean carotid cap thickness in plaque rupture (72±15 μm) is around three times as thick as in the coronary cap, which measures 23±19 μm near rupture sites and <65 μm for the other 95% of the cap (Burke, Farb et al. 1997; Virmani, Kolodgie et al. 2000). The cap thickness is a net effect of the balance between matrix synthesis by smooth muscle cells and matrix degradation by metalloproteoases by inflammatory cells such as macrophages (Galis, Sukhova et al. 1994).

Inflammatory cells present in the plaque are another hallmark of the classically defined plaque. Especially the numbers of present macrophages vastly outnumbers other cell types, such as T cells, neutrophils and mast cells. Macrophages infiltrating the fibrous cap are known to weaken the cap by secreting and activating matrix-degrading proteases. Compared to stable plaques, vulnerable plaques have been shown to contain abundant amounts of proteases (such as MMP-1, MMP-3 and MMP-9), these proteases are particularly active in the vulnerable regions of the plaque, such as the cap (Galis, Sukhova et al. 1994). Morover, neutrophils have also been associated with carotid plaque vulnerability (Ionita, van den Borne et al. 2010).

In addition, neovascularization with intra-plaque bleedings is commonly observed. The importance of intraplaque hemorrhage (IPH) for plaque progression was elucidated in 2003 (Kolodgie, Gold et al. 2003). IPH was demonstrated to contribute to cholesterol deposition and macrophage infiltration, which in turn result in enlargement of the necrotic core. It was speculated that neovascularization of leaky microvessels in the

plaque could be responsible for the IPH. Later it was shown that an increased density of intimal leaky microvessels in the coronary lesion associate with vulnerable plaques (Sluimer, Kolodgie et al. 2009). This also holds true in carotid plaques, where vascularity correlates with IPH and presence of symptoms (Mofidi, Crotty et al. 2001). The leakiness is due to compromised integrity of the endothelium of the microvessels.

1.1.4 The vulnerable patient

Knowing that the vulnerable plaques are not the only culprit factors for developing cardiovascular events Naghavi *et al*. published a consensus statement in 2003, suggesting a new term for identification of patients being at risk of having an event in the near future, "the vulnerable patient". The term "vulnerable patient" takes additional factors besides the vulnerable culprit lesion into account when estimating outcome, such as the vulnerable blood prone to thrombosis and the vulnerable myocardium, (Naghavi, Libby et al. 2003). The consensus document encouraged translational research for the identification and treatment of vulnerable patients, resulting in numerous studies trying to identify biomarkers.

1.2 TOOLS TO IDENTIFY VULNERABILITY

1.2.1 Biomarkers

A biomarker is defined as a *"characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"*(2001). The ultimate goal when studying a biomarker, whether the marker is used to screen for disease, diagnose a disease, inform about prognosis or guide treatment response, is that it should affect clinical management.

In CVD, cholesterol markers for cardiovascular events have been thoroughly investigated due to the crucial role of lipids in the development of atherosclerosis, unfortunately, the prognostic value of these markers is low since more than half of CVD events occur in patients with normal cholesterol levels (Ridker, Rifai et al. 2002). Many other potential biomarkers are shown to be quite unstable, one example being lipoprotein-associated phospholipase A2 (Lp-PLA2). Since Lp-PLA2 serum levels falls significantly after a stroke or a MI it is considered to be less robust for long term risk stratification shortly after primary events (Elkind, Leon et al. 2009). Contrary to LpPLA2, the serum levels of the myofibrillar protein troponin is rising after having an MI and is considered to be one of the most valuable standard diagnostic markers in clinical use. Assays for high sensitivity cardiac troponin have improved the early diagnostic accuracy for acute myocardial infarction as well as assessment of risk outcomes for acute coronary syndromes (Scirica 2010; Christenson and Phillips 2011).

Another biomarker that has gained a lot of attention is C-reactive protein (CRP), a marker of ongoing inflammation. CRP has been shown to be a predictive marker for cardiovascular events among healthy people and add to the Framingham risk score (Ridker, Rifai et al. 2002). Based on the famous JUPITER (Justification for the Use of Statins in Primary Prevention) trial statins were demonstrated to be beneficial even in the absence of hyperlipidemia, resulting in lowered CRP and a significant decrease in

cardiovascular events (Ridker, Danielson et al. 2009). CRP is thought controversial since it is not specific for atherosclerosis and is upregulated during most infections and other inflammatory diseases, such as rheumatoid arthritis (Anderson, Caplan et al. 2012).

Non- invasive and invasive imaging of plaques has also been investigated to identify patients at risk. Although ongoing improvements have been made for imaging, there are still apparent limitations. One example of this is that many potentially dangerous vulnerable plaques tend to associate with compensatory remodeling, making current available imaging techniques impossible to base a clinical decision on (Pasterkamp, Schoneveld et al. 1998). The PROSPECT (Providing Regional Observations to Study Predictors of Events in the Coronary Tree) a prospective plaque imaging study followed the natural history of plaques throughout the coronary tree for the occurrence of future cardiovascular events and concluded that acute coronary syndromes often occurred at sites of mild coronary artery stenosis and did not necessarily have to involve luminal narrowing (Stone, Maehara et al. 2011). However, lesion-related risk factors for such events are poorly understood. Taken together, the continuous search for identification of markers of plaque instability is important. Table I summarizes the main desirable features of a good biomarker in atherosclerotic disease:

Table I. *Optimal features of a good biomarker*

All Biomarkers	*Biomarkers for screening of Vulnerable CVD patients*
• Explicit for a specific disease	• Known reference limits
• Add to clinical assessment	• Add to known risk score such as FRS
• Sampling acceptable to the patient	• Change in total risk as seen with AUC risk calculations
• Correlation between change in marker and pathology	• Change management of the patient
• Stable product	
• Single measurement representative	
• Applicable to both genders, different ages and ethnicities	
• Replicated in separate cohort	

CVD: Cardiovascular Disease; FRS: Framingham Risk Score; AUC: Area Under the Curve.

1.2.2 Biobanking

Biobanks are organized resources of biological tissue specimens, which are linked with clinical characteristics and can either be population or disease based (Yuille, van Ommen et al. 2008). The Swedish Act on Biobanks (SF 2002:297) defines the concept “Biobank” as "biological material from one or several human beings collected and stored indefinitely or for a specified time and whose origin can be traced to the

human or humans from whom it originates". All patients participating are required to receive informed consent since its introduction in the Nuremberg Code after the World War II. Informed consent requires that the research subject should know “the nature, duration, and purpose of the experiment; the method and means by which it is to be conducted; all inconveniences and hazards reasonable to be expected; and the effects upon his health or person which may possibly come from his participation in the experiment” (http://ohsr.od.nih.gov.ezp-prod1.hul.harvard. edu/guidelines/ nuremberg. html). These requirements have later been supplemented in many variants of the Declaration of Helsinki.

During the last decade the numbers of biobanks have increased significantly. In Sweden alone, it is estimated that total sample numbers stored are around 50-100 million human samples, increasing with around 3-4 million samples per year. Handling of biological sample is covered by the Swedish Act on Biobanks, however, information that is gained during analysis is strictly regulated by other laws, such as Patientjournallagen (the Swedish Act of Medical Records), Personuppgiftslagen (the Swedish Act of Personal Information), Sekretesslagen (the Swedish Official Secrets Act) and Etiklagen (the Swedish Act on Ethics) (biobanks.se).

Biobanks are an important resource for:

- Identifying intricate mechanisms and patterns of complex disorders. Depending on the timing of collection of specimens, the biobanks open up an opportunity to study disease prior to, during and after, onset of disease at a molecular level.
- Investigations of rare disorders
- Validations of findings made in experimental models

One of the most famous and first examples of a population based cardiovascular biobank is the Framingham Heart Study initiated in 1951(Dawber, Meadors et al. 1951) Periodically monitoring and registering cases of CVD among the 28 000 participating subjects gave rise to many novel insights behind the pathophysiology behind CVD and resulted in the Framingham risk score model for risk stratification of CVD (Dawber, Meadors et al. 1951). The model is in use and so far the best available risk model in clinical practice.

Considering that atherosclerosis is a systemic disease (Libby, Ridker et al. 2002; Hansson 2005), one could speculate that local plaque characteristics represent plaque development in other sites of the vasculature tree (Vink, Schoneveld et al. 2001). Thus, lesions obtained during surgery might both help us to dissect the pathogenesis behind the disease and to investigate markers predictive for future cardiovascular events depending on the design of the study.

1.2.3 Biobank of Karolinska Endarterectomies, BiKE

In this thesis, three out of four papers are based on data from our Biobank of Karolinska Endarterectomies (BiKE). BiKE was established 2001 in collaboration

between the Experimental Cardiovascular Research Group and the Department of Vascular Surgery. BiKE holds blood samples and carotid lesions removed during carotid endarterectomy (CEA) on consecutive patients operated at the Karolinska Hospital. CEA is recommended for patients with severe and symptomatic lesions based on several clinical trials addressing benefits and risks in different groups of patients with this procedure (1991) (ECST 1998). In these trials the degree of stenosis was used as a factor to predict risk, therefore stenosis has been used in the clinic to select patients for surgery. As already mentioned, however, a growing body of evidence has suggested the morphology to play a greater role than size in predicting vulnerability and subsequent events (Fuster, Moreno et al. 2005). Patients with severe unilateral carotid stenosis, but without symptoms were also operated based on results from randomized trial (Halliday, Harrison et al. 2010).

In addition to tissue samples BiKE contains gene expression profiles and a clinical database, which includes a broad range of clinical information about the patients, such as, medications, echogenicity, laboratory tests, antrophometric data and comorbidities. The clinical data can be linked to gene expression data for investigations. Moreover, follow-up data and genotype data for the patients have been collected (Folkersen, Persson et al. 2012). Currently BiKE contains around 400 lesions of which around 150 have been subjected to gene expression microarrays. This large collaboration, that BiKE constitutes, has over the years generated several publications investigating different aspects of atherosclerosis. For further reading on these topics, see the Table II.

Table II. *Overview of all publications that use data from BiKE*

Authors	**Main target/finding**	**Role of BiKE**
Qiu et al. 2006	5-LO and LTA4H associate with plaque vulnerability **(Paper I)**	Essential role for all human expression data and IHC
Tran et al. 2007	HSPG2 is reduced in human carotid lesions	Central role as source of all immunostaining and gene expression material
Mälarstig et al. 2008	IRF5 is expressed in the lesions and is effected by proximal SNPs	Source of eQTL measurements
Folkersen et al. 2009	Real-time PCR comparison with microarrays **(Paper II)**	Central role as source for all gene expression data
Folkersen et al. 2009	CAD risk phenotype and chromosome 9p21	Central role together with other biobanks
Olofsson et el 2009	Tnfsf4 is increased in murine atherosclerosis	A role in human gene expression data together with other biobanks
Ueland et el. 2009	DKK-1 levels in	Plaque mRNA

	atherosclerosis, mechanistic and clinical studies	measurements supporting main data
Breland et al. 2010	MCP-4 levels, increased in symptomatic carotid atherosclerosis	*In vitro* plaque mRNA measurements supporting main data
Diez et al. 2010	Theoretical overview of network analysis methods	Methodology exemplified by using BiKE expression data
Folkersen et al. 2010	Association of genetic risk variants	Central role for all measurements
Gabrielsen et al. 2010	TBXAS1 is increased in murine atherosclerosis and correlates with macrophage markers	Major role on macrophage marker correlation
Gertow et al. 2010	ALOX15B is investigated in human carotid atherosclerosis	Central role as a source of all gene expression data
C4D 2011	GWAS, identifying new risk SNPs for MI	Finding expression quantitative trait loci effects (eQTL) of novel GWAS risk-SNPs
Agardh et al. 2011	FABP4 associate with plaque vulnerability **(Paper III)**	Central role as source of all expression data and IHC data
Razuvaev et al. 2011	Investigation of 317 known atherosclerotic genes	Central role as source of all expression measurements
Wang et al 2011	IGFs and IGF-binding proteins in human carotid atherosclerosis	Central role as source of all gene expression data
Folkersen et al. 2012	Prediction of ischemic events	Central role as source of all gene expression measurements
Paulsson-Berne et al. 2012	Commentary to Agardh *et al*. 2011	

5-*LO: 5-Lipoxygenas; LTA4H: Leukotriene A4 Hydrolase; IHC: Immunohistochemistry; HSPG2: Heparan Sulphate Proteoglycan; IRF5: Interferon Regulatory Factor 5; CAD: Coronary Artery Disease; DKK-1: Dickkopf-related protein 1; MCP-4: Monocyte Chemoattractant Protein 4; TBXAS1: Thromboxane A Synthase 1, ALOX15B: Arachidonate 15-Lipoxygenase, GWAS: Genome wide Association Study; FABP4: Fatty Acid Binding Protein 4, IGF: Insulin Like Growth Factor; eQTL: Quantitative Trait Loci Effects; BiKE: Biobank of Karolinska Endarterectomies; SNP: Single Nucleotide Polymorphism.*

1.2.4 OMICS

The central dogma in molecular biology, going from DNA via RNA to protein, gives ample opportunities for investigations at several unique levels in the search for new biomarkers (Figure 2). Through the Human Genome Project, the full human genome was sequenced and by 2004 the estimated numbers of protein coding genes was around 20 000-25 000 (2004). This revolution was accompanied by development of high-throughput methods providing immense capability for analyzing large number of genes, whether being on DNA (genomics), RNA (transcriptomics) or protein level (proteomics). By using all these approaches, functional genomics aims to give a more complete picture how functions arise from the genome. The concept behind *omics* approaches is that a given biological system is best analyzed by looking at global profiles rather than individual gene level. Coming section will focus on transcriptomics as the papers in my thesis are mainly based on investigations of RNA.

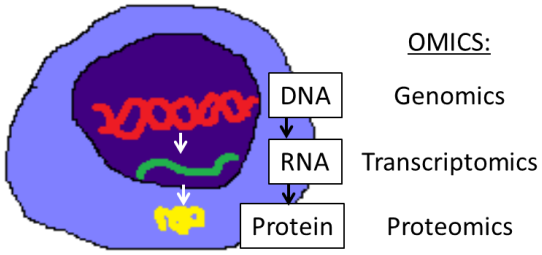

Figure 2. Biological systems can be analyzed by the help of OMICS: DNA by genomics, RNA by transcriptomics and proteins by proteomics.

1.2.4.1 Transcriptomics

The definition of a transcriptome is the relative expression level of all transcripts in a cell or tissue at a given time point. Over the past 35 years reverse transcription and sequencing have provided insight into the transcribed regions of the genome, going from the well-known ribosomal, transfer and messenger RNAs to more recently discovered non-coding classes such as small interfering RNA(siRNA) and microRNA(miRNA) (Li and Liu 2011). Despite that only around 25% of the human genome consists of protein-coding genes, there is evidence indicating that the greater part of the genome is transcribed to some sort of the mentioned RNA transcript (Birney, Stamatoyannopoulos et al. 2007). Regardless of whether the RNA is translated into protein or has direct RNA-based functions, it is the first step in realizing the information content of the genome making it a highly interesting target to investigate.

Conversely to the genome, the transcriptome is extremely dynamic and varies depending on factors such as, cell type, developmental stage of the cell and surrounding milieu. Hence, expression profiling of the transcriptome gives a biological snap shot of the transcript levels in the cell at a particular time or condition. For this reason many

studies have analyzed the transcriptome as means to dissect complex molecular processes behind a disease. Typically, specimens from healthy and diseased patients are compared, or tissue biopsies are used to compare different stages and forms of a disease. In my thesis plaque tissue and circulating cells have been investigated for identification of new biomarkers of atherosclerotic disease. In our studies the monocytes and neutrophils turned out to be important cell types, therefor I will introduce them in the next section.

1.3 RELEVANT TARGETS

1.3.1 Immune Cells

Immune cells are classified either as tissue residing or blood borne. The blood borne cells are further distinguished based on their histological appearance, either polymorphonuclear (PMN) or mononuclear (Figure 3). The PMNs constitute; *neutrophils* that phagocytize microbes during inflammation; *eosinophils* that participate during allergic and parasitic infections; and *basophils* involved in allergic reactions. Lymphocytes and monocytes instead make up the mononuclear cell compartment. *Monocytes* are recruited from the circulation during late stages of acute inflammation and during chronic inflammation, and differentiate into phagocytic macrophages in the extravascular tissue. *Lymphocytes*, T and B cells, are specialized cells of the adaptive immune system, T cells being important for antigen-specific responses and B cells for antibody production (Abbas & Lichtman, 2009). In my thesis there are above all two cell types of interest for our findings, neutrophils and the monocytes/macrophages. These will be briefly introduced in the sections below.

Figure 3. Typical cell nucleus of a PMN neutrophil(left) and a mononuclear monocyte(right).

1.3.2 Neutrophils

Neutrophils are the most abundant leukocytes in the circulation and also one of the most short lived cell types, dying within a few hours of inflammatory initiation. They are usually associated with acute inflammation and are thus among the first to respond to tissue damage and invading microorganisms. They phagocytose and with the help of reactive oxygen species (ROS) they destroy microbes. In addition, the cytoplasm of the neutrophils is filled with different types of granules containing presynthesized enzymes, such as cytotoxic myeloperoxidase (MPO), elastases and matrix metalloproteases (MMPs). These will both be secreted and/or come into contact with the microbes upon formation of the phagosome. In addition, the neutrophils are predominant producers of inflammatory mediators called leukotrienes, known to

mediate inflammatory responses such as vasodilation, increased vascular permeability and further neutrophil recruitment (Haeggstrom and Funk 2011).

1.3.3 Monocytes and Macrophages

Monocytes originate from the bone marrow and constitute between 3 to 8% of all leukocytes in the circulation. In contrast to neutrophils they are long lived, typically present in the circulation for a couple of days before entering into tissues where they can differentiate into macrophages and undergo cell division. Macrophages respond nearly as fast as neutrophils to microbes. In addition to being a phagocytic cell, macrophages present antigens to lymphocytes /cells of the adaptive system and are producers of cytokines. Several subsets of both monocytes and macrophages have been identified during the last decade and can be divided into both pro- and anti-inflammatory subsets based on their cytokine secretion and surface markers (Abbas & Lichtman). The classically activated macrophages (M1) are known to drive inflammation by secreting pro-inflammatory cytokines such as IL1β, IL-6 and IL-12, whereas, it has been suggested that CD163 positive macrophages (M2) play a role in the resolution of inflammation. M2 are found in high number in inflamed tissues and are differentiated by mediators, such as IL-4 and IL-13 (M2a) or IL-10 (M2b) or LPS (M2c) (Sulahian, Hogger et al. 2000; Schaer, Boretti et al. 2002; Martinez, Sica et al. 2008). For a simplified version of the subsets, see figure 4 below.

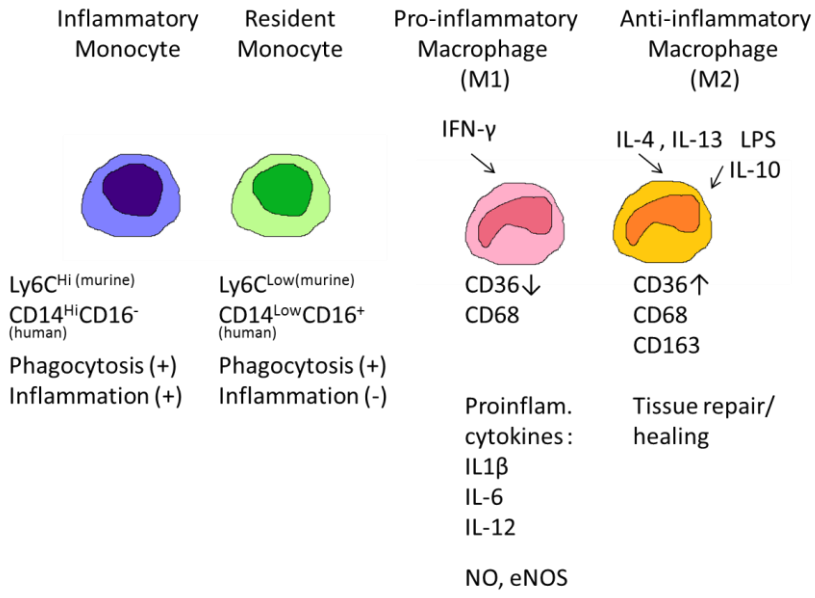

Figure 4. Overview showing subsets of monocytes and macrophages.
The murine inflammatory monocyte expresses Ly6C^{high}, whereas the human counterpart expresses CD14^{+}CD16^{-}. The murine anti-inflammatory, resident monocyte, express a lower level of Ly6C (human counterpart CD14^{low}CD16^{+}). The surrounding milieu effects the transition to either a pro-inflammatory M1 macrophage or a wound healing, repairing M2 macrophage phenotype. Typically IFN-γ drives the differentiation into M1. M1 secretes pro-inflammatory cytokines such as, IL1β, IL-6 and IL-12 as well as nitrogen reactive species (NRS). IL-4 and IL-13 instead pushes the monocyte into anti-inflammatory M2 phenotype expressing CD163. In addition to these, several other macrophage subsets have been found. The scavenger receptor CD36 are more highly expressed on M1 than on M2 macrophages, whereas CD68 is present on both subtypes.

In addition to these two classical subsets of macrophages, several other populations have been described. The so called Mox macrophage was recently introduced, shown to be induced by oxidized phospholipids (Kadl, Meher et al. 2010). Thereafter the M4 macrophage was found displaying a different transcriptome to the classically described M1 and M2 macrophages, and showed lower expression of scavenger receptors plus increased levels of cholesterol efflux (Gleissner, Shaked et al. 2010). These subsets are probably not the last ones to be introduced in the macrophage field.

1.3.4 Fatty acid binding proteins

Fatty acid binding proteins (FABPs) are a family of, so far, nine identified proteins (Smathers and Petersen 2011) of which the first member was found in 1972 (Ockner, Manning et al. 1972). The members were historically regarded to be tissue specific therefore their names derive from their site of expression. However, this nomenclature is slightly misleading due to the fact that the different family members were later shown to be present at several locations. Subsequently an extra numerical nomenclature was added (Storch and Thumser 2010) (see Table III). Furthermore, the expression of the FABPs in a given cell type seems to reflect the cells lipid-metabolizing capacity. In for example adipocytes and cardiomyocytes, where fatty acids are prominent for lipid biosynthesis, storage or breakdown, the FABP can make up between 1-5% of all soluble proteins. The amount of FABPs in the cells can be further increased during times of increased fatty acid exposure (Veerkamp and van Moerkerk 1993).

All FABPs members are 14-15kDa in size and localized in the cytoplasm of the cell, where their major function is to act as a lipid chaperon, reversibly binding and trafficking a quite broad range of lipids throughout cellular compartments (Smathers and Petersen 2011). Studies using experimental models have demonstrated FABPs to play a central role in lipid mediated processes, affecting metabolic and immune response pathways. However, their regulation, their exact biological function(s) and mechanism of action is still poorly understood.

The sequence diversity between the members of FABPs is great, sharing from only 15 up to 70% similarity. Although, structurally they are extremely conserved and consist of ten stranded anti-parallel beta sheets which form a beta barrel surrounding a water-filled interior binding pocket. In addition, two alpha helices cap the pocket and regulate the binding of ligands (Chmurzynska 2006). The family is evolutionary conserved and present in a spectrum of species including *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse and humans (Furuhashi and Hotamisligil 2008).

Table III *Members of the human fatty acid binding family*

Common Name	Gene	Expression Sites
Liver FABP	Fabp1	Liver, intestine, pancreas, kidney, lung, stomach
Intestinal FABP	Fabp2	Intestine, liver
Heart FABP	Fabp3	Heart, skeletal muscle, brain, kidney, lung, stomach, testis, aorta, adrenal gland, placenta, ovary, brown adipose tissue
Adipocyte FABP	Fabp4	Adipocyte, macrophage, dendritic cell, endothelial cell
Epidermal FABP	Fabp5	Skin, tongue, adipocyte, macrophage, dendritic cell, mammary gland, brain, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen
Ileal FABP	Fabp6	Ileum, ovary, adrenal, gland, stomach
Brain FABP	Fabp7	Brain, glial cell, retina, mammary gland
Myelin FABP	Fabp8	Peripheral nervous system, Schwann cell
Testis FABP	Fabp9	Testis, salivary gland, mammary gland

Modified from *Nature Reviews, Furuhashi et al.2008 June;7:489-503.*

1.3.4.1 Fatty Acid Binding Protein 4

Adipocyte fatty acid binding protein or FABP4 was originally found in adipocytes. The cytosolic protein makes up about 1-3% of total cytosolic protein content in adipocytes and is up-regulated around 50 times during the adipocyte differentiation process (Reese-Wagoner, Thompson et al. 1999). Later the protein has been identified in macrophages (Makowski, Boord et al. 2001), foam cells (Fu, Luo et al. 2002), dendritic cells (Rolph, Young et al. 2006) and endothelial cells (Elmasri, Karaaslan et al. 2009). Still the exact biological function of FABP4 is unknown.

The gene for FABP4 is located on chromosome 8q21human (3A1 in mice) and is transcriptionally regulated by trans-acting nuclear factors and cis-acting regulatory elements in the 5' flanking region. The TATA box is located 31 base pairs upstream of the transcription start site and two fat-specific elements (FSE) are found within the gene. Further upstream of the flanking region of FABP4 there are five adipocyte regulatory elements (ARE1, ARE2, ARE4, ARE6 and ARE7). Interestingly, around 5.5 kb upstream of the transcription site, FABP4 also comprise peroxisome proliferator response elements (PPREs), which allow for transcriptional regulation by fatty acids, insulin and PPARɣ agonists.

1.3.4.2 FABP4 in Atherosclerosis

Animal studies have shown that FABP4 is strongly linked to atherosclerotic plaque development. Crossing *Apoe*$^{-/-}$ with a knockout of FABP4, *Ap2*$^{-/-}$ resulted in a greatly reduced lesion size with or without high fat diet (Makowski, Boord et al. 2001) (Perrella, Pellacani et al. 2001). In addition, bone marrow transplantation (BMT) showed a comparable reduction in lesion size, indicating a role for FABP4 in macrophages during atherogenesis. These results highlight a separate action of FABP4 from adipocytes and provide a link between metabolic syndrome and atherosclerosis.

Macrophages lacking FABP4 also show a significant decrease of cholesterol accumulation and decreased level of secreted cytokines TNF-α, Il-1β and IL-6 upon exposure to lipoproteins (Makowski, Boord et al. 2001). Moreover, it has been shown that using an orally active small-molecule inhibitor of FABP4 effectively reduces atherosclerosis without effecting body weight, lipid or glucose metabolism in *Apoe*$^{-/-}$ (Furuhashi, Tuncman et al. 2007).

One of the first studies implying relevance of FABP4 in human atherosclerosis was observed *in vitro* when FABP4 was upregulated during differentiation of human THP-1 cells to foam cells (Fu, Luo et al. 2002).Thereafter, studies investigating the expression in human plaques are lacking until our own study was published in 2011. In addition, the role of FABP4 in the serum has recently being intensively investigated, even though it is not known whether this free form has any functional role. Serum concentration of FABP4 has been associated in the CVD field with factors such as carotid intima-media thickness in women (Yeung, Xu et al. 2007) and coronary plaque burden (Miyoshi, Onoue et al. 2010). In line with this, one of the 5 SNP variants (T-87C) identified in Caucasians leading to decreased expression, has been associated with reduced risk for coronary artery disease in a population cohort of around 8000 carriers (Tuncman, Erbay et al. 2006).

1.3.5 Leukotrienes

Leukotrienes (LTs) were first discovered in leukocytes, as indicated by their name, and are primarily formed in granulocytes, monocytes/macrophages, mast cells, and dendritic cells (Samuelsson 1979). In 1976 a great discovery was made at Karolinska Institute, showing the 5-lipoxygenase (5-LO) pathway for arachidonic acid (AA) metabolism in rabbit leukocytes (Borgeat, Hamberg et al. 1976) and subsequent studies identified leukotriene B4 (LTB4) to be the major product of the 5-LO pathway(Borgeat and Samuelsson 1979). The AA derivates are known to mediate inflammatory responses such as vasodilation, vascular permeability and neutrophil recruitment and are predominantly made by PMNs and mast cells (Funk 2001).

1.3.5.1 The 5-LO pathway

Expression of the enzyme 5-LO is essentially restricted to cells derived from the bone marrow such as, neutrophils, monocytes/macrophages, mast cells, dendritic cells and B cells (Radmark and Samuelsson 2007). Upon cell activation and LT biosynthesis, 5-LO will translocate from the cytosol to the nuclear envelope in a calcium dependent manner (Chen and Funk 2001) (Figure 5). Upregulation of the 5-LO protein has been shown during GM-CSF stimulation of human neutrophils (Pouliot, McDonald et al. 1994). Assistance of the enzyme 5-leukotriene activating protein (FLAP) seems to facilitate the interaction between 5-LO and AA (Mancini, Abramovitz et al. 1993) and to increase the efficiency in which 5-LO converts 5-hydroxyperoxy eicosatetraenoic acid (5-HPETE) into leukotriene A4 (LTA4) (Abramovitz, Wong et al. 1993). Thereafter leukotriene A4 hydrolase (LTA4H), present in most cells, catalyzes the hydrolysis of the unstable LTA4 into LTB4 (Haeggstrom and Funk 2011). LTB4 are known to bind, with high and low affinity to the BLT1 and BLT2 receptors respectively, that are present on leukocytes and when stimulated cause degranulation and chemotaxis (Tager and Luster 2003).

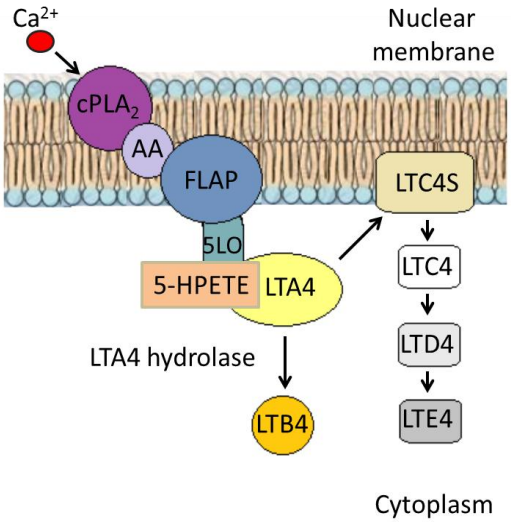

Figure 5. The leukotriene 5-LO pathway
Following activation, arachidonic acid (AA) is liberated from cPLA2 and converted to LTA4 by 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP) via 5-hydroxyperoxy eicosatetraenoic acid (5-HPETE). LTA4 is then converted to LTB4 by LTA4 hydrolase and to LTC4 by LTC4 synthase which is further converted to LTD4 and LTE4. *Adapted from: Clinical Asthma, Castro & Kraft, 2005.*

Alternatively to form LTB4, LTA4 can be conjoined with glutathione to form LTC4 in turn yielding LTD4 and LTE4. These leukotrienes are collectively called cysteinyl leukotrienes and are known for their slow and sustained muscle contracting abilities. The cysteinyl leukotrienes bind to the Cys-LT1 and Cys-LT2 receptors that are present on airway smooth muscle cells and vascular endothelial cells(Funk 2001), and are known as potent inducers of increased vascular permeability(Dahlen, Bjork et al. 1981).

1.3.5.2 5-LO and Atherosclerosis

Several interesting studies regarding 5-LO in experimental and human atherosclerosis came out starting from year 2002, highlighting a potential important role for this pathway in disease progression. In *Ldlr*$^{-/-}$ mice crossed with *5-lo*$^{-/+}$ a dramatic reduction of lesion development was observed (Mehrabian, Allayee et al. 2002) and in human atherosclerotic lesions abundant level of 5-LO was demonstrated at different stages of disease (Spanbroek, Grabner et al. 2003). Additionally, genetic studies from Iceland convincingly showed polymorphisms in the 5-LO promoter to associate with an increased atherosclerosis (Dwyer, Allayee et al. 2004), and the gene encoding FLAP to augment the risk for MI and stroke (Helgadottir, Manolescu et al. 2004). However, later publications from work in mouse models have yielded inconclusive results, for example in *Apoe* $^{-/-}$ x *5-lo*$^{-/-}$ only a minor atheroprotective effect was shown (Poeckel and Funk 2010).

2 AIMS OF THIS THESIS

The common objective for all studies performed within this thesis has been to investigate the inflammatory genes involved during different stages of human and murine atherosclerotic lesion development. More specifically the aims were to:

- Investigate atherosclerotic plaque levels of the 5-LO leukotriene pathway, both in human vulnerable plaques and in murine plaques
- Determine optimal normalization method for real-time PCR in complex carotid tissue for transcript evaluation
- Analyze the gene expression profile of human atherosclerotic plaques linked to clinical data, to identify potential biomarkers of plaque vulnerability
- Investigate possible biomarkers in circulating leukocytes reflecting progression of murine atherosclerotic lesions

3 METHODOLOGICAL CONSIDERATIONS

3.1 HUMAN SPECIMENS

The papers included in my thesis have all been conducted at least in some parts on human tissue samples including; atherosclerotic carotid lesions obtained during carotid endarterectomies (**paper I, II, III**), iliac arteries obtained from transplant donors (**paper I**) and blood cells obtained from healthy donors (**paper III and IV**).

3.1.1 Biobank of Karolinska Endarterectomies, BiKE

The BiKE biobank was established in 2001. The database consists of clinical data, plasma samples and atherosclerotic lesion removed during carotid endarterectomy (CEA) on consecutive patients operated at Karolinska Hospital. Removed carotid plaques were immediately rinsed with sterile PBS in the operation theatre and cut transversely over the most prominent region of the plaque. Three quarters of the specimen is frozen on dry ice for later isolation of RNA and one quarter, for morphological analyses, is embedded in OCT and stored in -80^{o}C.

Patient undergoing surgery usually present with symptoms such as minor stroke, transient ischemic attacks (TIA) or amaurosis fugax. In cases of more than 70 % stenosis of the carotid artery, as detected with ultrasonography, CEA is recommended. In addition, CEA is also recommended in asymptomatic men with narrowing of the carotid artery. The North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria were used for selection. In **paper III,** patients with atrial fibrillation was excluded from the data set, as their emboli causing symptoms might be of cardiac origin.

In **paper I,** a major concern was the use of suitable controls to the carotid lesions. A contra-lateral healthy artery would obviously have been an optimal normal control, but this is for obvious reasons not an option. Instead we had to settle with the best we could get, which in this case were iliac arteries from transplant donors. The adventitia and outer media of the arteries were removed before freezing. In addition, the vessels were macroscopically analyzed and excluded if signs of intimal thickening were apparent. In **paper III,** we based our investigation solely on endarterectomies and could thus avoid this discussion.

3.2 MURINE MODELS OF ATHEROSCLEROSIS

Murine models of atherosclerosis are the most commonly used experimental models for studying the processes that underlie the pathogenesis of atherosclerosis. There are several advantages with this approach. Mice are relatively cheap, have short reproducibility time with large litters and are genetically well-defined. Even before the era of transgenic technology, it was known that most mice strains are resistant to atherosclerosis even after feeding with an atherosclerotic diet. The C57BL/6 mouse, which is the standard background of all mice strains used in this thesis, is an exception and develops small fatty streaks when fed atherosclerotic diet. With the technique of genetic engineering several models of atherosclerosis prone mice strains were made available. The most commonly used today are the apolipoprotein E (*Apoe*$^{-/-}$) knock out

mouse and the LDL-receptor (*Ldlr*$^{-/-}$) knock out mouse. In addition, these two knock out models have been crossed in order to give rise to an even more susceptible disease model (Hemdahl, Gabrielsen et al. 2006).
Even though murine models give the opportunity to dissect the different molecular pathways during development of advanced plaques, there are some major limitations that should be kept in mind when performing translational studies. The experimental murine models very seldom develop plaque rupture with thrombosis, which is one of the main clinical manifestations in humans. Furthermore, the cholesterol levels in mice necessary for plaque development goes far beyond concentrations encountered in humans. In addition, the murine immune system deviates from that of humans (Libby, Ridker et al. 2011).

3.2.1 *Apoe*$^{-/-}$ mice (paper I, IV)

Apoe$^{-/-}$ mice were created in 1992 by homologous recombination in embryonic stem cells (Plump, Smith et al. 1992; Zhang, Reddick et al. 1992). Due to the lack of ApoE, needed for clearance of lipoproteins by the liver, the strain develops severe hypercholesterolemia even under normal dietary conditions. The aortic plaques develop rapidly from initial fatty streaks to more complex lesions strikingly similar to those in humans, both in phenotype and distribution. Predilection sites for atherosclerotic lesions in *Apoe*$^{-/-}$ mice are the aortic root, followed by the aortic arch, the brachiocephalic trunk, the left carotid, and subclavian and coronary arteries(Nakashima, Plump et al. 1994) (Bentzon and Falk 2010). One disadvantage with the *Apoe*$^{-/-}$ model is that the lipoprotein profile deviates from humans. Whereas LDL is the major carrier of cholesterol in the circulation, the murine lipid profile is dominated by augmented levels of VLDL. Another drawback with this knock out model is that the role ApoE plays in other processes, such as immune activation, is lost (Tenger and Zhou 2003).

3.2.2 *Apoe*$^{-/-}$ x *Ldlr* $^{-/-}$ mice (paper I)

The *Ldlr*$^{-/-}$ strain instead have a plasma lipid profile resembling that of humans. The knock out of the receptor affects the clearance of VLDL and LDL from plasma. As a result, the mice will develop atherosclerosis then fed a diet high in fat and cholesterol (Knowles and Maeda 2000). In addition, the *Ldlr*$^{-/-}$ strain has been crossed with *Apoe*$^{-/-}$ rendering a strain which develops severe hyperlipidemia and atherosclerosis on chow diet. This model has been proposed suitable to study the anti-atherosclerotic effect of compounds without having to feed the animals an atherogenic diet (Bonthu, Heistad et al. 1997).

3.3 RNA EXPRESSION ANALYSIS

Quantifying gene expression by traditional DNA methods, such as Northern blot or Southern Blot can be quite crude and will not allow to precise quantification. In all papers included in my thesis gene expression analysis has been made by using the more modern techniques, real-time PCR and microarrays. In the following sections I will introduce these two different methods.

3.3.1 Real-time PCR (paper I, II, III, IV)

Polymerase chain reaction (PCR) is a powerful technique used to amplify segment of DNA by using oligonucleotides (flanking primers) in a series of reactions catalyzed by a DNA-polymerase in the presence of the four dNTPs (Saiki, Gelfand et al. 1988). In 1993 the first attempt was made to monitor amplification of PCR products in real-time, using a video camera and inclusion of ethidiumbromide in the PCR mixture (Higuchi, Fockler et al. 1993). The emitted fluorescence was found to be proportional to the amount of product formed. A cycle number where the fluorescent signal exceeded the background signal (called "cycle threshold", CT-value) were identified and could be used to calculate starting material in a sample. Real-time PCR revolutionized the RNA expression analysis of single gene targets by collecting data during real-time.

We are using real-time PCR to investigate different levels of mRNA expression in different tissues. Briefly, the method is based on sequence specific DNA probes labeled with a fluorescent reporter dye in combination with primers. First RNA is reversely transcribed to cDNA with reverse transcriptase, before adding real-time PCR probe and primers. As the reaction starts the primers and the probes will anneal to the cDNA. From the primers, polymerisation of a new DNA strand will be initiated with help of *Taq* DNA polymerase. When the polymerase reaches the probe, which contains both a quencher and a fluorescent reporter at the opposite end, a fluorescent signal will be emitted due to physical separation of the quencher and the reporter. The fluorescence is measured and the exponential increase is used to analyze the threshold cycle.

3.3.2 Microarrays (paper I, II, III, IV)

In mRNA or microarray gene expression profiling experiment, the expression of thousands of genes is simultaneously measured to study and identify gene transcription level during for example pathogenesis of different diseases, developmental stages or efficacy of certain treatments. The microarray technology was evolved from the Southern blotting principle, where fragmented DNA is probed with a known fragment or gene. The early microarrays were first described in 1987 and consisted of cDNA spotted onto filter paper (Kulesh, Clive et al. 1987). Since then several different types of arrays have been developed, all based on a common mechanism, namely the hybridization of a sample of unknown labeled RNA sequences to a set of known sequences located on the array, so called oligonucleotides. Fluorescently labeled sequences that bind to a probe sequence will generate a signal, which then will be scanned and translated to a quantitative parameter for the RNA at the specific position (Figure 6). Depending on the type of array used, several steps such as reverse transcription, amplifications and/or fragmentations steps before the hybridization might be performed. There are also differences in the sequence length of the probes, as well as organization of the probes within the gene.

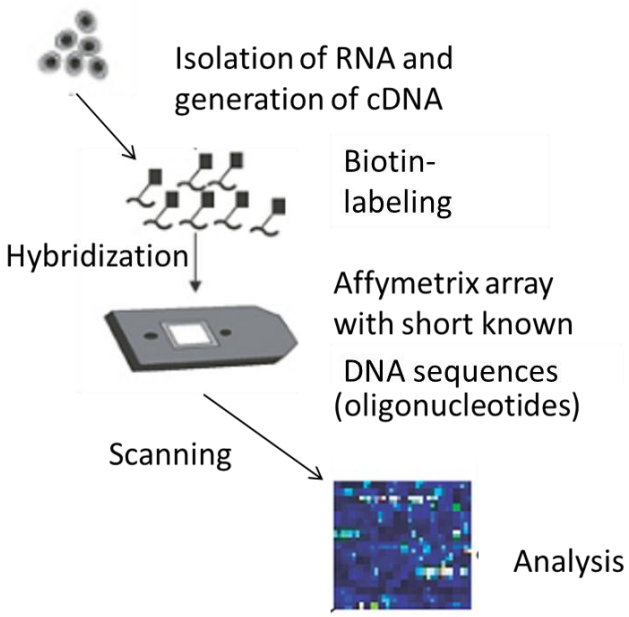

Figure 6. Overview – RNA to microarray analysis
RNA is isolated, reversely transcribed to cDNA subsequently biotin labeled and then allowed to hybridize to the microarray containing short oligonucleotides from known reference sequences. Finally, the array is scanned with a laser scanner and analyzed.

3.3.2.1 Microarrays from Affymetrix

The microarrays used in Paper II, III and IV are all from Affymetrix;

Paper II: Human Genome U133 Plus 2.0;
Paper III: Human Genome U133A
Paper IV: Mouse Genome MOE430A.

These microarrays use probe sets, consisting of groups with between 4 to13 of 25 nucleotides long probe sequences, which only matches one specific region in the gene. The different probes in a set are in turn grouped to match genomic locations in close proximity to each other. For an overview of number of transcripts/genes possible to analyze, see table IV below.

Table IV Overview of microarrays included in paper II, III and IV

	HGU133 plus 2.0	**HGU133A**	**MOE430A**
Transcripts (n)	~47 400	~18 400	
Genes (n)	>38 500	>14 500	~14 000
Probe sets (n)	>54 000	>22 000	>22 600
Feature size (um)	11	8	8
Oligonucleotide probe length	25	25	25
Probe pairs/sequence	11	11	11

HGU- Human Genome; MOE: Mouse Genome

3.4 IMMUNOHISTOCHEMISTRY (PAPER I, III, IV)

We have analyzed different proteins of interest by using immunohistochemistry. A general protocol for enzymatic staining visualized with light microscopy has been used in **paper I** and **III.** Immunohistochemistry is based on detection of a specific antigen by a primary antibody followed by incubation of a secondary, biotinylated antibody, which recognizes regions of the first antibody. Thereafter enzyme-conjugated streptavidin molecules are incubated on the section forming a complex with the bound biotin. By adding a substrate, the enzymatic activity of the bound will form a color precipitate at the sites of antigen-antibody binding. The most crucial of this method is the use of good quality antibodies with high antigen specificity. Another important aspect of this method is the use of proper controls. There are many different discussions about this topic. In **paper I** we have been using blocking peptides to ascertain the specificity of the primary antibodies. In **papers III** and **IV** we take use of isotype controls not recognizing any proteins in the sample of interest, but generated in the same species as the antibody recognizing the protein of interest. In addition one should try every step of the staining procedure, and each new batch of primary and secondary antibodies, to exclude false positive staining. In addition, it is helpful to counterstain specimens with a nuclear stain to be able to identify cells from staining artifacts. In **papers I, III** and **IV** we have used fluorescent double labeling for simultaneous detection of two antigens in the same specimen. The principle is the same as for detecting proteins with enzymatic staining, the difference is that the secondary antibody is either directly conjugated with a fluorochrome or the streptavidin molecules are conjugated with a fluorochrome. Similarly, controls are crucial to determine whether the same signal for each individual antibody is obtained both in the single and the double staining step. To counterstain the nuclei I have used the dye DAPI (4', 6-diamidino-2-phenylindole), which binds to AT-rich regions in DNA and stains the nuclei blue. Lipids and structural components in atherosclerotic lesions cause autofluoresence which may affect the detection of specific signals severely. We have managed to partly quench the autofluoresence signals by incubating the sections with Sudan Black allowing for successful use of fluorescent staining in the lesion. In **paper I** we are using fluorescence microscopy with different filter cubes to analyze our stainings and in **paper III** and **IV** I have been using confocal microscopy.

4 RESULTS AND DISCUSSION

4.1 PAPER I- 5-LO PATHWAY IN CAROTID PLAQUE INSTABILITY

Leukotrienes (LTs) belong to a family of lipid mediators and are involved in host defense and inflammatory responses. Furthermore, LTs have been found to be involved in both human and experimental atherogenesis (Mehrabian, Allayee et al. 2002; Spanbroek, Grabner et al. 2003). In humans the 5-lipoxygenase pathway has previously been shown to be expressed in the vessel wall of patients with various stages of atherosclerotic lesions in the carotid and coronary arteries (Spanbroek, Grabner et al. 2003). In **paper I** we aimed at investigating plaque levels and more specifically vulnerability in relation to three key enzymes in the LTB4-biosynthesis, namely 5-lipoxygenase (5-LO), 5-LO activating protein (FLAP), and LTA4 hydrolase. Furthermore, the levels of these enzymes were investigated in atherosclerotic murine models, the *Apoe*$^{-/-}$ and the double knockout *Ldlr*$^{-/-}$ x *Apoe*$^{-/-}$.

We show that the human carotid lesions reveal high transcript levels of 5-LO, FLAP and LTA4H as compared to control iliac arteries, confirming previous data on human atherosclerotic specimen (Spanbroek, Grabner et al. 2003). In addition, staining of the plaques demonstrate abundant expression of 5-LO, FLAP and LTA4H in the intima, co-localizing with the macrophage marker CD163. All three enzymes have a similar distribution throughout the plaque, indicating a possible degree of co-expression in the cells, presumably facilitating enzyme coupling and LTB4 production.

In agreement with our finding, macrophages represent a major source for LTB4 (Haeggstrom and Funk 2011). In addition, previous research has shown LTB4 to be a strong chemotactic agent influencing major events involved throughout the atherosclerotic lesion progression, such as recruitment of inflammatory T cells and monocytes both via chemotaxis and effects on adhesion molecules(Friedrich, Tager et al. 2003). Moreover, LTB4 increase the expression of MCP-1 which in turn enhances further monocyte recruitment (Huang, Zhao et al. 2004). This may create a vicious circle to further recruit and drive the inflammatory response in the atherosclerotic lesion, eventually leading to plaque vulnerability. Consistent with this hypothesis, the mRNA levels of 5-LO and LTA4H in the atherosclerotic plaques correlate to the occurrence of clinical symptoms. Interestingly, both 5-LO and LTA4H are significantly higher in patients with recent clinical symptoms of TIA, minor stroke and/or amaurosis fugax (see Figure 7). This is independent of plasma cholesterol levels, gender, or treatment with statins or angiotensin-converting enzyme inhibitors. However, FLAP did not associate with clinical symptoms, suggesting the existence of an additional regulatory mechanism.

Ruptured plaques causing symptoms will eventually heal (Virmani, Kolodgie et al. 2005). The time between the last recorded symptom (<1 month, 1-3 months and >3 months) and the endarterectomy presumably represents plaques at different stages of instability. Combining our data, showing the association of 5-LO and LTA4H mRNA to plaque vulnerability, and previous Icelandic studies showing 5-LO and LTA4H to be risk factors for atherosclerosis (Dwyer, Allayee et al. 2004) (Helgadottir,

Manolescu et al. 2004), strongly suggest LTB4 to be involved in local inflammatory processes that precede vulnerability and clinical symptoms.

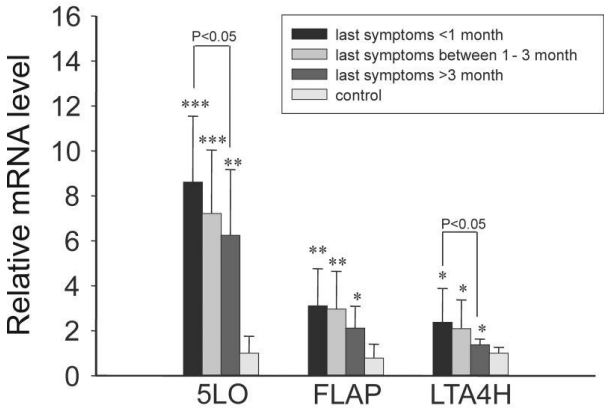

Figure 7: Correlation of 5-LO, FLAP, and LTA4H mRNA levels with clinical symptoms. Levels of 5-LO, FLAP, and LTA4H mRNA in human carotid plaques were correlated with the occurrence of symptoms of plaque instability. The mRNA levels of 5-LO and LTA4H were significantly higher in patients with symptoms within 1 month (n = 18) than in patients with the last symptoms >3 months ago (n=10). Data are presented as mean ± SD, *****, $P < 0.05$; ******, $P < 0.01$; *******, $P < 0.001$ (as compared with iliaca).

Moreover, we demonstrate that homogenate of human plaque indeed produce significant amounts of LTB4 when incubated with arachidonic acid. This synthesis is dampened by 80% after pre-incubation with the selective and tight-binding inhibitor of LTA4H, aminohydroxamic acid inhibitor (figure 8). Thus LTA4H, the enzyme that catalyzes LTB4-production, could be a potential good drug target for stabilizing the plaque and preventing associated complications.

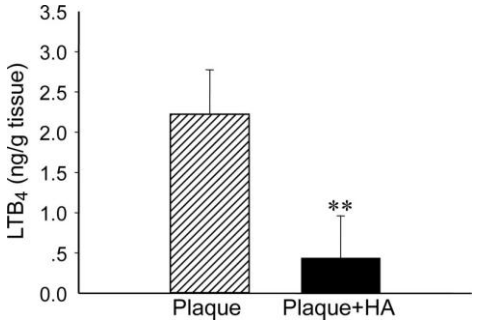

Figure 8. Synthesis of LTB4 in human carotid plaque and inhibition by an LTA4H inhibitor. Homogenates of human plaque tissue (n=8) were preincubated for 30 min in the absence (n=4) or presence (n=4) of a hydroxamic acid inhibitor of LTA4H (HA) followed by incubation with arachidonic acid. LTB4 was measured by HPLC-EIA, and data are presented as mean ±SD. ******, P <0.01

Importantly, our finding in human atherosclerotic lesions with respect to expression pattern of the proteins in the 5-LO LT-pathway shows profound differences to the murine situation. In murine aortic plaques from *Apoe*$^{-/-}$ and the *Ldlr*$^{-/-}$ x *Apoe*$^{-/-}$ we show the levels of 5-LO, FLAP and LTA4H to be essentially the same except for LTA4H, which is being up-regulated in the double knock out. Staining of 5-LO and LTA4H show the enzymes to distribute in different localizations of the murine plaque,

with 5-LO being present in the adventitia and LTA4H being present in the intima. In addition, analysis of the LT cysteinyl receptor CysLT1 show a 2.5 to 3 times fold upregulation in *Apoe*$^{-/-}$ and the *Ldlr*$^{-/-}$ x *Apoe*$^{-/-}$ as compared with control mice. Immunostaining demonstrate the CysLT1 receptor to colocalize with CD68 in the murine lesion. Also the CysLT2 receptor is upregulated 3 times in the *Apoe*$^{-/-}$ as compared to control. These receptors did not differ in level between human carotid plaques as compared to controls.

Several factors could be the reasons for this observed species difference. The human atherosclerotic plaques used in this study are from advanced stages causing clinical symptoms after decades of progression, whereas the murine lesions are less advanced. Although much knowledge has been achieved with the use of murine models, it is essential to keep in mind that the mice do not develop signs of plaque rupture and symptoms. Moreover, the multiple factors operating in initiation of disease as compared to the ones in destabilization are also likely to differ.

4.2 PAPER II- ENDOGENOUS CONTROL GENES IN CAROTID LESIONS

When determining mRNA levels in different specimens and cell line experiments, a commonly used and sensitive method is real-time PCR. The relative quantification of mRNA is usually determined by normalizing to endogenous controls, so called housekeeping genes (HKGs), to correct for amount of RNA starting material and for efficiency in the cDNA synthesis(Livak and Schmittgen 2001).

In **paper II** we wanted to investigate how different choices of endogenous controls would affect the real-time PCR results. To perform this we take advantage of the possibility to correlate our measurements to another method for measuring mRNA, namely the microarrays. In addition, we wanted to compare these two different methods used for expression analysis. The samples we are investigating are human carotid lesions from our biobank, BiKE. These tissue samples are quite heterogeneous, consisting of a various degree of inflammatory cells, smooth muscle cells and endothelial cells. Thus, the proportion between the cell types will vary between different samples.

We selected 5 endogenous control genes based on the available set of 10 HKGs from Applied Biosystems. In addition to the direct measurement of these 5 HKGs, we calculated the stability index using all combinations of two or more of the 5 HKGs. This was done to test the recommendation by Vandesompele *et al*. (Vandesompele, De Preter et al. 2002) that multiples of HKGs are beneficial to real-time PCR normalization.

Real-time PCR for 15 different genes normalized with the 5 controls or combinations were compared with probe sets from microarray expression data. For all different combinations Pearson correlations were calculated to be able to compare; 1) correlations between different genes and; 2) differences in correlations depending on the choice of using either an endogenous control, combinations of endogenous controls or no endogenous control. We demonstrate that some, but not all, genes show a good correlation between real-time PCR measurements and microarrays. Furthermore, we demonstrate that the best correlation between microarray data and real-time PCR is

obtained when only normalizing real-time PCR to the initial amount of RNA material used – the no endogenous control situation.

The reasons for non-corresponding genes can be due to systematic technical as well as biological factors. One technical factor we could identify was a coefficient of variance of duplicate real-time PCR measurements. If this was below 0.02, there was no correspondence between microarray and real-time PCR data. In spite of thorough quality control of other technical aspects of real-time PCR and microarray, we could not explain all non-corresponding genes. Another interesting aspect that could explain difference in measurements is alternative splicing and difference in probe location for the two measurements. In our setting the probe locations were different for all except 4 genes. This opens up speculation that alternative-splicing could theoretically be the reason for the remaining non-corresponding genes. However, further measurements of concurrent exonic locations would have been required to support such a claim. Instead we focused on the difference in HKGs-dependent correspondence between microarray and real-time-PCR measurements. Our results showed that there was a better correspondence between the two methods when real-time PCR is normalized to the amount of initial RNA- i.e. without the use of endogenous controls.

A useful formal definition of gene expression is that it is units of mRNA per cell. This is the level at which changes will affect the biology of a cell. To reach this definition, the methods we compare must therefore be based on one of three assumptions:

1) There exists one or more genes for which the expression is at sufficiently constant level to be proportional to the amount of cells across the sample (for real-time PCR, using endogenous controls)
2) The overall distribution of the expression levels of all genes is the same in all cells and across all samples (for microarray using the RMA-algorithm)
3) Total concentration of RNA is the same in all cells across all samples (for normalization to total RNA mass). This carries the assumption based on that the reverse transcription from RNA to cDNA is equally efficient.

It seems likely, that the first assumption is very hard to satisfy in tissue sample of mixed cell populations. We therefore conclude that introduction of HKGs may perturb the data without improving measurements in complex heterogeneous tissues, such as carotid plaques. This is in line with a previous study that speculated that normalizing to total RNA mass will be better in complex tissue (Qin, Beyer et al. 2006). In addition, we see advantages with the microarrays having the total gene expression profile to normalize against, arguing a more robust data normalization-wise as compared to real-time PCR. Nonetheless normalization using HKG is still the golden standard, therefore in coming paper III and IV we take use of this method.

4.3 PAPER III- CAROTID PLAQUE VULNERABILITY ASSOCIATE WITH FABP4

Accumulation of lipids and ongoing inflammation are hallmarks of the vulnerable plaque and play essential roles in the pathogenesis of atherosclerosis (Virmani, Kolodgie et al. 2000). The lipid core of a vulnerable plaque is demonstrated to be large, infiltrated by inflammatory cells and covered by a thin cap (Corti, Hutter et al. 2004). Within the first few days after a transient ischemic attack the risk of a stroke is increased. This risk is reduced if a CEA is performed soon after the occurrence of symptoms (Giles and Rothwell 2006). Plaques which have ruptured and caused symptoms may also heal and show regression of inflammation (Sacco, Adams et al. 2006).

The time aspects mentioned above make up the basis for our investigation in **paper III** where we base plaque vulnerability on time between last recorded symptom and CEA. By the use of microarray technology we compared the transcriptomes of the atherosclerotic lesion between asymptomatic patients and symptomatic patients with symptoms less or more than 1 month ago, revealing FABP4 mRNA to be more abundant in vulnerable plaques. This was confirmed using real-time RT-PCR (see Figure 9). The patient groups differed with regard to gender and age, however, the level of FABP4 in the lesion did importantly not associate with any of these factors. Thus, FABP4 turns out to be a potentially attractive biomarker for predicting plaque vulnerability and applicable for investigated ages and both genders.

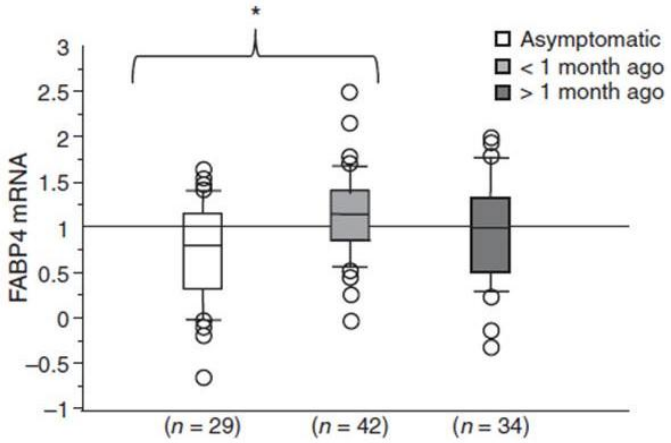

Figure 9. Expression of FABP4 mRNA in carotid endarterectomies. FABP4⁄aP2 mRNA expression as determined by real-time quantitative RT-PCR in carotid endarterectomies. Comparison between asymptomatic patients, patients with symptoms within 1 month and those experiencing symptoms more than 1 month ago. Symptoms of plaque instability are defined as transient ischemic attacks, minor stroke and/or amaurosisfugax. mRNA levels were normalized to cyclophilinA mRNA expression in each sample. *P = 0.01

At the time of our finding, studies had implicated FABP4 to integrate the inflammatory and lipid-mediated pathways. The lipid binding protein had moreover been considered to play an essential role in plaque progression as investigated in experimental models (Makowski, Boord et al. 2001; Perrella, Pellacani et al. 2001; Boord, Maeda et al. 2002; Makowski, Brittingham et al. 2005). However, studies investigating FABP4 in human atherosclerotic lesions were lacking, making our study highly relevant.

Along with association to plaque vulnerability we observe the level of FABP4 mRNA within atherosclerotic endarterectomies to correlate with markers of macrophages supporting our initial finding (Figure 10). Specifically good correlation was seen with the markers CD36, CD68 and CD163 in the plaque. Our observation is in line with a previous study made in a human THP-1 macrophage cell line, where FABP4 correlated with foam cell development (Fu, Luo et al. 2002). The same group also observed FABP4 to be related with increased inflammatory activity (Makowski, Brittingham et al. 2005). Moreover, an inverse correlation was observed between FABP4 and smooth muscle cells, giving further evidence for linking FABP4 to unstable plaque phenotype, as unstable plaques usually display a thin cap with fewer SMCs (Burke, Farb et al. 1997).

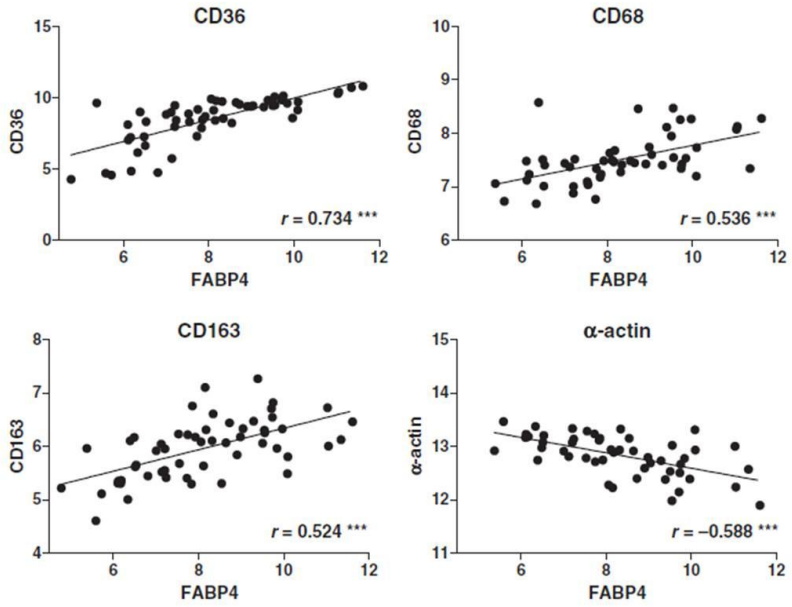

Figure 10. Correlations between FABP4 and different cells markers at mRNA level in BiKE, within the atherosclerotic lesion as extracted from microarrays. Plots show the correlation between FABP4 and macrophages (CD36, CD68 and CD163) and vascular smooth muscle cells (α-actin). Pearson correlation *** p<0.001

To identify cell types expressing FABP4 and to localize the protein in the plaque, we performed immunohistochemistry and could clearly show FABP4 to be localized in macrophages (CD36, CD68 and CD163 positive cells). FABP4 was present both in the cytoplasm and the nuclei of the cells. From our study we cannot draw any conclusions whether FABP4 is more abundant in the pro-inflammatory macrophage phenotype M1or the healing macrophage phenotype M2 or Mox. FABP4 was also shown to be present in some endothelial cells. Furthermore, we observe an association of FABP4

and T cells on mRNA level. The association between these markers reflects increased number of inflammatory cells in advanced plaques, as we did not observe any FABP4 in the T cells.

In addition to an association between FABP4 and CD36, FABP4 associates with the enzymes 5-LO and LTA4H involved in LTB4 production. LTA4H is soluble and does not translocate to the nuclear membrane, transfer of LTA4 from 5-LO to LTA4H seems to require a carrier stabilizing the molecule, which presumably could involve FABP (Zimmer, Dyckes et al. 2004). LTB4 in turn has been shown to enhance expression of CD36 (Subbarao, Jala et al. 2004) leading to increased foam cell formation. Interestingly, FABP4 also associated with adipophilin, previously shown to be increased at both RNA and protein level in symptomatic carotid plaques (Nuotio, Isoviita et al. 2007).

Adipophilin is an adipose differentiation-related protein expressed in lipid-containing cells, shown to participate in foam cell formation *in vitro* (Robenek, Buers et al. 2009) and suggested to play an inflammatory role in macrophages *in vivo* (Chen, Yang et al. 2010). The association between adipophilin and FABP4 might therefore reflect increased foam cell formation in the plaque, but a direct interaction between FABP4 and adipophilin is to date unknown. Taken together, our data strengthens a complex relation between FABP4, leukotrienes, foam cell formation and plaque vulnerability.

In conclusion, our data show FABP4 to be upregulated in symptomatic plaques as compared to asymptomatic ones. This is in line with experimental data implying FABP4 to play a major role in atherosclerotic progression and to contribute in plaque destabilization. Our study has later been confirmed by three other groups demonstrating FABP4 to correlate with plaque instability in three independent cohorts (Peeters, de Kleijn et al. 2010; Holm, Ueland et al. 2011; Saksi, Ijas et al. 2011).

4.4 PAPER IV- FABP4 IN LEUKOCYTES ASSOCIATE WITH LESION PROGRESSION

In **paper III** we identified FABP4 to be augmented within the lesion in unstable plaque phenotypes, which have given rise to symptoms. Although this finding is exciting the plaque *per se* is quite impractical to access for measuring biomarkers. Moreover it would be beneficial to identify asymptomatic patients at risk of having an event in the near future, before the actual event. We hypothesized that circulating cells might contain potential biomarkers reflecting the disease progression due to the fact that atherosclerosis is a systemic inflammatory disease. Hence, the purpose of **paper IV** was to analyze gene expression profiles in circulating cells during atherosclerotic disease progression. To investigate this we used the well-defined experimental atherosclerotic *Apoe*$^{-/-}$ model, where lesion progression from early fatty streaks to more advanced plaques can be studied.

By unbiased approach using Affymetrix microarrays, we identify FABP4 mRNA in circulating cells to associate with lesion progression (Figure 11). FABP4 has, as previously mentioned, been shown to play an important role in experimental models of

atherosclerosis. To our knowledge this is the first time that the intracellular level of FABP4 in circulating leukocytes has been reported to reflect extent of disease.

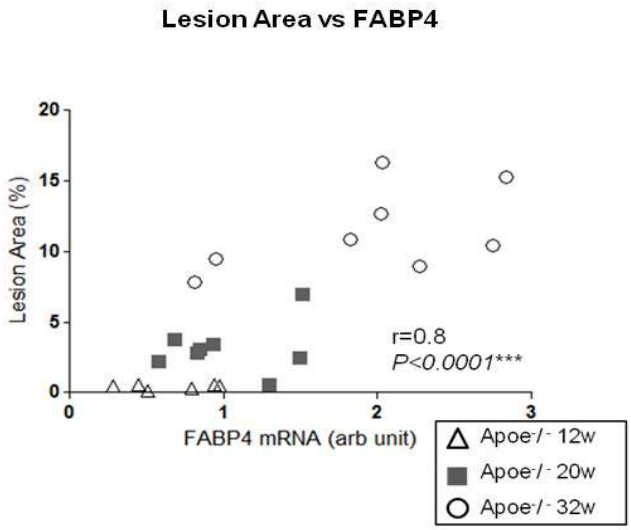

Figure 11. Level of FABP4 mRNA in circulating leukocytes correlates with extent of atherosclerotic lesion in aorta. mRNA levels of FABP4 in circulating leukocytes from *Apoe*$^{-/-}$ (12, 20 and 32 weeks of age) measured by RT-PCR TaqMan and correlated to percentage of lesion size (r= 0.800, ***p<0.0001) (n=22).

Using ELISA we confirm our finding of FABP4 within the leukocytes on protein level (Figure 12). Importantly the plasma level of FABP4 protein did not change with disease progression, indicating FABP4 to be an attractive biomarker for atherosclerosis specifically within the leukocytes.

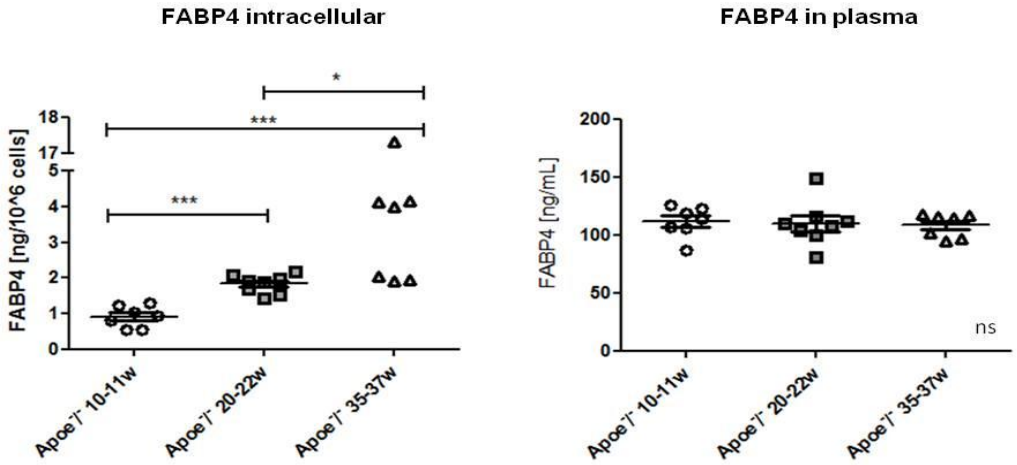

Figure 12. FABP4 protein level increases in circulating leukocytes in *Apoe*$^{-/-}$ with disease progression but is stable in plasma. Intracellular protein level in circulating leukocytes of FABP4 in *Apoe*$^{-/-}$ (10-11, 20-22 and 35-37 weeks of age female) as measured by ELISA (n=7-8 per group). FABP4 concentrations in plasma in *Apoe*$^{-/-}$ (10-11, 20-22 and 35-37 weeks of age) as measured by ELISA (n=7-8 per group). ****P*<0.0001 vs. *Apoe*$^{-/-}$ 20w, **P*<0.05 vs. *Apoe*$^{-/-}$ 32w. ns indicates not significant.

Interestingly, we demonstrate FABP4 to be mainly expressed by Ly6G-positive neutrophils in the circulation. Total neutrophil numbers in the murine model are stable throughout the ages investigated, implying that the increase seen is not linked with changes in specific leukocyte populations. Neutrophils *per se* have not until recently been thought to play a major role in atherosclerosis progression (Zernecke, Bot et al. 2008). One recent study showed the neutrophils to constitute 2% of all infiltrated leukocytes in the shoulder region of the murine plaque (Rotzius, Thams et al. 2010). We show FABP4 positive neutrophils to be localized to the subendothelial space and shoulder regions within the murine plaque.

Inflammatory Ly6C^{Hi} monocytes are robustly recruited to the atherosclerotic lesion. In our study Ly6C^{Hi} dominates over the less inflammatory Ly6C^{low} monocyte population in the circulation at less advanced stages of disease (age 12 and 20 weeks of age). During the time of early fatty streak formation (age 12 weeks of age in *Apoe*$^{-/-}$ mice) cholesterol levels are twice as high as observed at later stages. Our observations support a previous study, showing hypercholesterolemia to increase the Ly6C^{Hi} population (Swirski, Libby et al. 2007). Although, at 20 weeks of age when the cholesterol level drops by half the Ly6C^{Hi} population still dominates in the circulation, suggesting additional other factors in the inflammatory milieu to drive this subset. Due to technical issues we could not stain Ly6C together with FABP4. However, in the circulation FABP4 is localized to some extent in monocytes expressing CD68 and in the lesion FABP4 is more strongly expressed by some of these cells. This is in line with *in vitro* data showing increased levels of FABP4 during foam cell formation.

Finally, we investigate localization of FABP4 in leukocytes from human healthy donors, to see whether our finding could be of relevance for further investigations in carotid atherosclerotic patients. Indeed, we see similar expression pattern, localizing FABP4 to both monocytes and neutrophils (Figure 13).

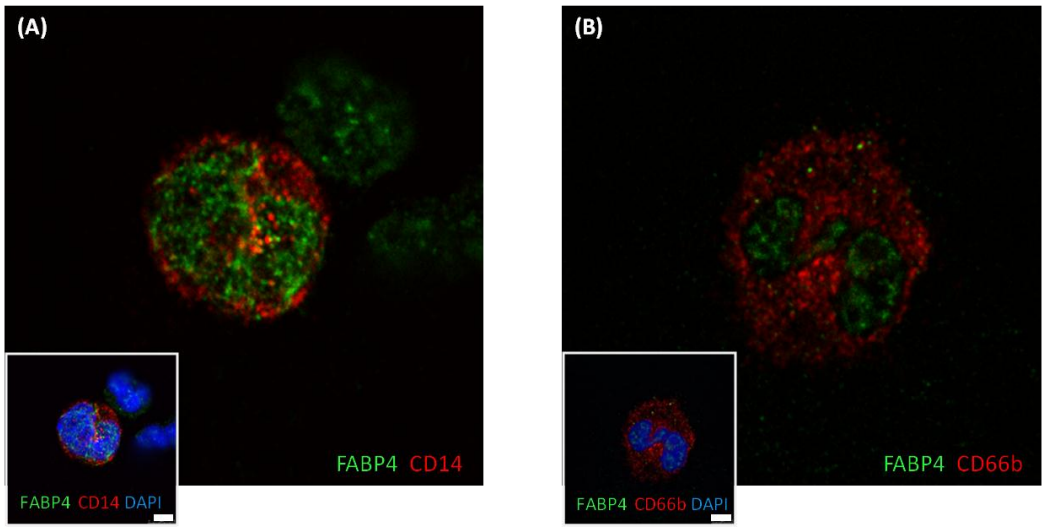

Figure 13. Co-localization of FABP4 to human neutrophils and monocytes
Human leukocytes were stained with FABP4 (green) and the neutrophil marker CD66b (A) or the monocyte marker CD14 (B). Nuclei stained blue with DAPI, as seen in the small imbedded photos in the left hand corner of A and B. White bar 5µm.

In conclusion, our results imply a role for FABP4 in the circulating leukocytes as a biomarker reflecting extent of atherosclerosis in an experimental model. This finding needs to be further investigated in human atherosclerotic patients.

5 CONCLUDING REMARKS

It is generally acknowledged that atherosclerosis is an inflammatory systemic disease of the arteries. Numerous ongoing investigations are trying to dissect and understand the complex processes behind lesion development. Although some classical risk factors have been established during the last decades, there is still a need for finding and validating new possible biomarkers involved in the pathogenesis for better identification of vulnerable plaques and vulnerable patients at risk in the near future. Combination of clinical samples and experimental models, as seen in this thesis, is essential to cover different aspects and stages of disease. My aim was to identify genes involved in the pathogenesis of human as well as experimental atherosclerosis.

In **paper I** we showed that mRNA of enzymes involved in LTB4-production, 5-LO and LTA4H, associated with plaque vulnerability in humans. Thus, suggesting a critical role of LTB4 during clinically significant stages of atherosclerosis. We conclude that LTA4H, the terminal enzyme in LTB4-synthesis, could be a potential pharmacological target for amelioration of atherosclerotic complications.

In **paper II** we compared real-time PCR and microarrays and tested different normalization methods of real-time PCR for correlations to microarrays. We conclude that normalization to total RNA mass in complex heterogeneous tissues might be better than using housekeeping genes. However, since the latter is standard we chose to use this normalization method in **paper III and IV**.

In **paper III** we demonstrated an increased mRNA level of FABP4 in human symptomatic plaques as compared to asymptomatic plaques. In addition we localized FABP4 to macrophages within the lesions. In line with **paper I,** mRNA of FABP4 associated with the enzymes 5-LO and LTA4H. We conclude that FABP4 is involved in processes leading to plaque vulnerability and could be an interesting target in future therapeutics.

In **paper IV** we investigated if mRNA in circulating cells could reflect atherosclerotic lesion progression in the atherosclerotic *Apoe*$^{-/-}$ model and found FABP4 expressed in neutrophils and monocytes to be a promising candidate. We conclude that FABP4 in circulating cells is of future interest to investigate in human atherosclerosis.

The papers presented in this thesis demonstrate that FABP4 is a player in processes involved in atherosclerosis, both within human plaque reflecting vulnerability and in circulating leukocytes in *Apoe*$^{-/-}$ reflecting extent of atherosclerotic plaque progression. Although, the exact mechanism of action still is to be unraveled. In addition to associate with plaque vulnerability, FABP4 also associates with the enzymes 5-LO and LTA4H, enzymes of the leukotriene pathway resulting in LTB4 production. The fact that all these proteins constitute markers for vulnerability makes them interesting for further clinical investigations for identification and/or amelioration of plaque vulnerability. Another attractive approach for identification of atherosclerotic disease progression could potentially be to measure the level of FABP4 in circulating leukocytes. Even though our findings need further investigations to be translated into

clinic practice, I hope that these studies included in my thesis have shared some light on these potential markers involved in the atherosclerotic disease process.

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