Thesis for doctoral degree (Ph.D.)
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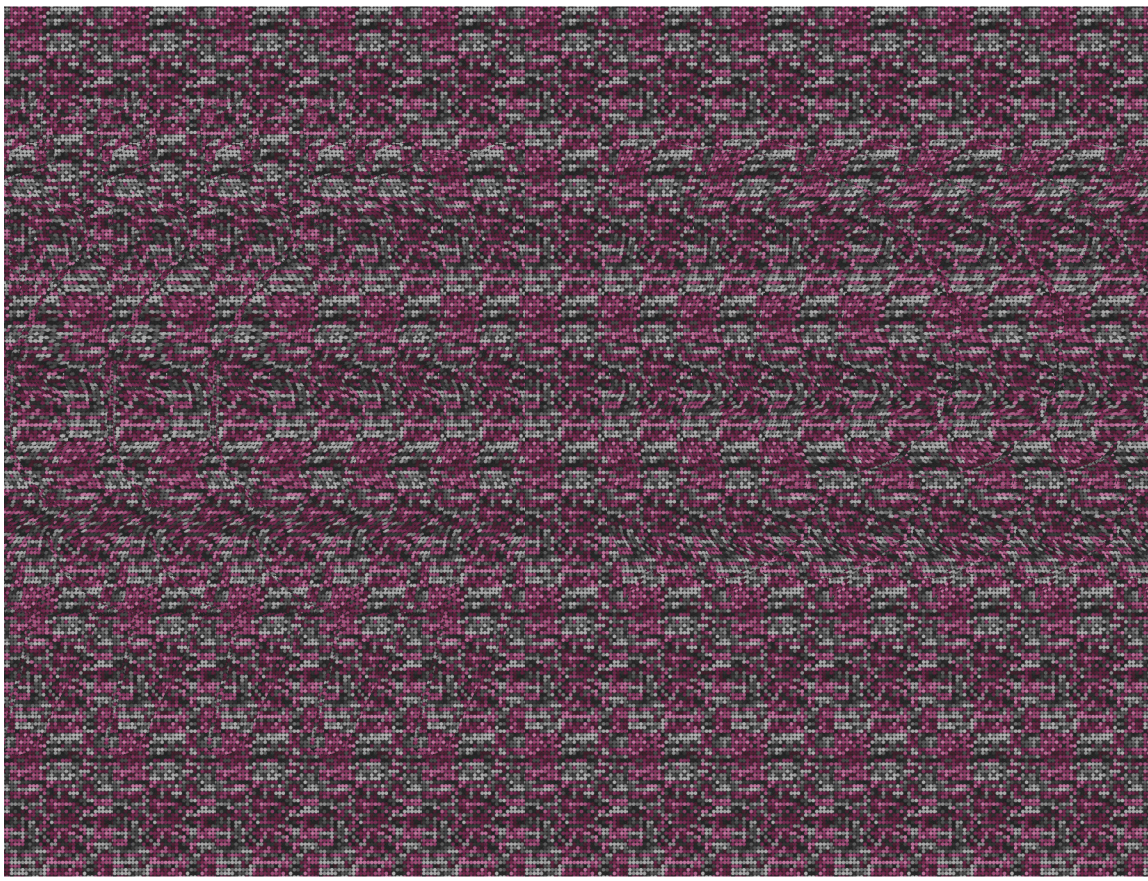
Improving stroke risk prediction and individualised treatment in carotid atherosclerosis



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Improving stroke risk prediction and individualised treatment in carotid atherosclerosis
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IMPROVING STROKE RISK PREDICTION AND INDIVIDUALISED TREATMENT IN CAROTID ATHEROSCLEROSIS

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Cover illustration: *Can you see what is hidden in the pattern? I finally did.*
Lead 1: This illustration demonstrates one of the main principles of the whole thesis, decipher 'the outcome' in a pattern of a large number of genes. (The pattern is heatmaps of gene expressions in carotid plaques, representing CALC, LRNC, IPH and MATX.)
Lead 2: This is a stereogram. To see the hidden image, try to relax your eyes and look through the image focusing behind the pattern.
Correct answer? Check the bottom of the page.*
Created by Holger Thorsin, Andrew Buckler and Eva Karlöf

**Correct answer: A brain with a stroke, (a flash to the upper left)*

IMPROVING STROKE RISK PREDICTION AND INDIVIDUALISED TREATMENT IN CAROTID ATHEROSCLEROSIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Eva Karlöf

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To Vera, Stina, Brita and Per

"When someone is searching," said Siddhartha, "then it might easily happen that the only thing his eyes still see is that what he searches for, that he is unable to find anything, to let anything enter his mind, because he always thinks of nothing but the object of his search, because he has a goal, because he is obsessed by the goal.
Searching means: having a goal.
But finding means: being free, being open, having no goal."

From Siddhartha, by Herman Hesse, 1922

POPULÄRVETENSKAPLIG SAMMANFATTNING (POPULAR SCIENCE SUMMARY IN SWEDISH)

Varje år drabbas 15 miljoner människor av slaganfall (stroke) världen över, en tredjedel dör och en tredjedel får permanenta men i form av funktionsnedsättningar i större eller mindre grad. Stroke är en av de vanligaste dödsorsakerna i världen.

Stroke definieras som en blödning eller infarkt (stopp i cirkulationen) i hjärnans kärl. Vid åderförkalkning av halspulsådern (karotisartären) kan en förträngning (stenos) uppstå och orsaka en stroke genom att delar eller partiklar av placket följer med blodflödet upp till hjärnan. Vid kranskärlssjukdom och hjärtinfarkt gäller samma mekanism. Det som avgör om detta sker är hur stabilt placket är. Åderförkalkningsplack kan vara mer eller mindre benägna att släppa partiklar, dvs mer eller mindre instabila. Man har visat att plack bestående av mycket fett, inflammatorisk vävnad med död kärna, ibland även med en blödning i placket, som täcks av en tunn bindvävskappa är mer instabila och kan orsaka stroke och hjärtinfarkt. Betydelsen av förkalkning i placket är mer omdebatterat.

Idag saknas bra metoder att fastställa vilka plack som är instabila. Internationella riktlinjer säger att förekomst av symtom och hur tät stenosen är avgör hur patienten ska behandlas; antingen med optimal medicinsk behandling endast, eller med tillägg av kirurgi av karotisstenosen. Dessa riktlinjer är över lag bra men lämnar en hel del att önska i precision då studier har visat att många patienter blir opererade i onödan och skulle statistiskt sett aldrig ha drabbats av stroke. Dessutom förekommer det tyvärr även att mindre täta karotisstenoser orsakar stroke. Därför är mer exakta metoder för att identifiera det instabila placket högt eftersökta.

Studierna i detta doktorandprojekt syftade således till att studera:

1) Om karotisplackets form och uppbyggnad (morfologi) enligt skiktröntgen (datortomografi, CTA) kunde kopplas till pågående biologi (genuttryck och biologiska processer) i motsvarande plack och därmed ge information om plackets instabilitet.

2) Om riskscores, använda i kliniken för att estimera strokerisk, gick att koppla ihop med plack-morfologi och biologiska processer.

3) Huruvida plackets genuttryck och biologiska processer gick att förutsäga utifrån CTA-bilderna på individ-nivå vilket skulle kunna innebära en metod att skräddarsy behandlingen för varje enskild persons biologi.

De huvudsakliga resurser vi hade tillgängliga var pre-operativa CT-bilder med ett mjukvaruprogram utvecklat för att få ut mer information ur CT-bilderna, kliniska uppgifter om patienterna samt BiKE (Biobank of Karolinska Endarterectomies, BiKE) en biobank av karotisplack.

Resultaten av studie I och II visade att plackmorfologin enligt pre-operativ CTA kunde kopplas ihop med aktiva biologiska processer i placket. Studie I visade att högförkalkade plack var förknippade med en biologisk profil av stabiliserande genuttryck och processer relaterade till glatta

muskelceller och extra-cellulär matrix. Inflammatoriska processer var nedreglerade. Vi kunde vidare konstatera att PRG4 var den mest uppreglerade genen i högförkalkade plack, en gen ej tidigare känd i åderförkalkning.

Studie II visade att lipid-rik nekrotisk (död) kärna hade ett signifikant samband till stroke-symtom och hade dessutom en klar inflammatorisk biologisk profil. Plack med blödning hade likaså inflammatorisk profil men visade även processer anknutna till nybildning av kärl. Plackbörda (proportionellt stor plackvolym) visade också på ett signifikant samband med förekomst av stroke eller ej samt en biologisk profil av främst inflammatoriska processer. I en kors-validerad matematisk prediktionsmodell för symtom presterade plackmorfologi signifikant bättre än stenosgrad som metod att värdera symtomgivande plack.

Studie III kunde påvisa att hög riskscoring för stroke enligt kliniska patient-parametrar associerade med en biologisk profil relaterad till inflammatoriskt svar och koagulation där genen ABCB5 noterades som en av de mest uppreglerade generna i båda riskscore-metoderna.

Studie IV resulterade i 414 gener, gediget predicerade med maskininlärning utifrån CTA-bilderna. Dessa gener och biologiska processer stämde väl överens med typisk patofysiologi för åderförkalkning. De upptränade matematiska modellerna testades på fyra 'nya' patienter där det predicerade genuttrycket stämde väl överens med det verkliga genuttrycket i karotisplacket.

Sammanfattningsvis visade våra resultat att biologiska processer associerade till plackinstabilitet kunde kopplas till karotis-plackmorfologi enligt avancerad bildanalys av CTA. De patienter som uppskattades ha högre risk för stroke hade en plack-biologi typisk för instabilitet men även ett samband till plackmorfologi enligt CTA. Slutligen, kunde vi se att bildanalys av CTA kunde predicera det faktiska genuttrycket i placket genom maskininlärning och därmed skulle denna metod kunna utgöra en icke-invasiv metod att estimera plackbiologin på individ-nivå vilket är en förutsättning för att skräddarsy en behandling för varje patient. Med dessa metoder och resultat hoppas vi kunna introducera ett sätt att identifiera det instabila placket och därmed förbättra prediktion av stroke men även en metod att precisera behandling utefter patientens egen biologi.

ABSTRACT

Background: Unstable carotid atherosclerosis causes stroke, but methods to identify patients and lesions at risk are lacking. Currently, this risk estimation is based on measurements of stenosis and neurological symptoms, which determines the therapy of either medical treatment with or without carotid endarterectomy. The efficacy of this therapy is low and higher accuracy of diagnosis and therapy is warranted. Imaging of carotid plaque morphology using software for visualisation of plaque components may improve assessment of plaque phenotype and stroke risk. These studies aimed firstly to investigate *if*, and if yes, *how*, the carotid plaque morphology with image analysis of CTA associated with on-going biology in the corresponding specimen. Secondly, if risk stratification in clinical risk scores can be linked to the aforementioned associations. Finally, if the on-going biological processes can be specifically predicted out of the CTA imaging analysis. **Methods:** Plaque features were analysed in pre-operative CTA with dedicated software. In study I and II, the plaques were stratified according to quantified high and low of each feature, profiled with microarrays, followed by bioinformatic analyses. Immunohistochemistry was performed to evaluate the findings in plaques. In study III, patient phenotype, according to clinical stroke risk scores of CAR and ABCD2 stratified the cohorts of high vs low scores which were subsequently profiled with microarrays, followed by bioinformatic analyses and correlation analyses of plaque morphology in CTA. In study IV, the microarray transcriptomes were individually coupled to morphological data from the CTA analysis, developing models with machine intelligence to predict the gene expression from a CTA image. The models were then tested in unseen patients. **Results:** In study I, stabilising markers and processes related to SMCs and ECM organisation were associated with highly calcified plaques, while inflammatory and lipid related processes were repressed. PRG4, a novel marker for atherosclerosis, was identified as the most up-regulated gene in highly calcified plaques. Study II showed that carotid lesions with large lipid rich necrotic core, intraplaque haemorrhage or plaque burden were characterized by molecular signatures coupled with inflammation and extracellular matrix degradation, typically linked with instability. Symptomatology associated with large lipid rich necrotic core and plaque burden. Cross-validated prediction model for symptoms, showed that plaque morphology by CTA alone was superior to stenosis degree. Study III revealed that a high clinical risk score in CAR and ABCD2, reflect a plaque phenotype linked to immune response and coagulation, where the novel ABCB5, was one of the most up-regulated genes. The high risk scores correlated with the plaque components matrix and calcification but no positive association with stenosis degree. Study IV resulted in 414 robustly predicted transcripts from the CTA image analysis, of which pathway analysis showed biological processes associated with typical pathophysiology of atherosclerosis and plaque instability. The model testing demonstrated a good correlation between predicted and observed transcript expression levels and pathway analysis revealed a unique dominant mechanism for each individual. **Conclusions:** Biological processes in carotid plaques associated to vulnerability, can be linked to plaque morphology analysed with CTA image analysis. Patient phenotype classified with clinical risk scores associates to plaque phenotype and morphology in CTA. The biological processes in the atherosclerotic plaque can be predicted with plaque morphology CTA analysis in this small pilot study, providing a possibility to precision medicine after validation in larger scale studies.

LIST OF SCIENTIFIC PAPERS

I. **Correlation of Computed Tomography with Carotid Plaque Transcriptomes Associates Calcification to Lesion-Stabilization**
Eva Karlöf, Till Seime, Nuno Dias, Mariette Lengquist, Anna Witasp, Håkan Almqvist, Malin Kronqvist, Jesper R. Gådin, Jacob Odeberg, Lars Maegdefessel, Peter Stenvinkel, Ljubica Perisic Matic, Ulf Hedin
Atherosclerosis. 2019 Sep;288:175-185.

II. **Carotid Plaque Phenotyping by Correlating Plaque Morphology from Computed Tomography Angiography with Transcriptional Profiling**
Eva Karlöf, Andrew J. Buckler, Moritz Lindquist Liljeqvist, Mariette Lengquist, Malin Kronqvist, Mawaddah A. Toonsi, Lars Maegdefessel, Ljubica
Perisic Matic, Ulf Hedin
Submitted, under review

III. **Clinical risk scores for stroke associate with carotid plaque vulnerability**
Katarina Wadén, Eva Karlöf, Maria Ioanna Kotopouli, Sampath Narayanan, Mariette Lengquist, Ulf Hedin, Joy Roy, Ljubica Perisic Matic
Manuscript

IV. **Virtual Transcriptomics: Phenotyping of Atherosclerosis by Decoding Plaque Biology from Non-invasive Computed Tomography Angiography Imaging**
Andrew J. Buckler, Eva Karlöf, Mariette Lengquist, T Christian Gasser, Lars Maegdefessel, Ljubica Perisic Matic, Ulf Hedin
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Manuscript accepted in Cells, 2021 May 18

Osteomodulin is a novel biomarker of cardiovascular calcification that attenuates smooth muscle cell osteogenic transition
Taxiarchis Skenteris N, Seime T, Witasp A, Karlöf E Wasilewski GB, Jaminon A, Heuschkel MA, Oduor L, Dzhanaev R, Kronqvist M, Lengquist M, Peeters F, Söderberg M, Hultgren R, Roy R, Maegdefessel L, Arnardottir H, Bengtsson E, Goncalves I, Quertermous T, Goettsch C, Stenvinkel P, Schurgers LJ, Matic L
Submitted

Patient-specific Biomechanical Analysis of the Fibrous Cap is Enabled by Histologically Validated Tissue
Characterization by CTA: A Case Study
Buckler AJ, Wanrooij M, Andersson M, Karlöf E, Matic L, Hedin U, Gasser CT
Submitted

CONTENTS

LIST OF ABBREVIATIONS

ABCD2	Age Blood pressure Clinical features Duration of TIA Diabetes
ACAS	Asymptomatic Carotid Atherosclerosis Study
ACST	Asymptomatic Carotid Surgery Trial
AUC	Area Under the Curve
AUROC	Area Under Receiver Operating Characteristics
BiKE	Biobank of Karolinska Endarterectomies
CAC score	Coronary Artery Calcium Scoring
CALC	Calcification
CAR	Carotid Artery Risk score
CAS	Carotid Artery Stenting
CEA	Carotid EndArterectomy
CKD	Chronic Kidney Disease
CTA	Computed Tomography Angiography
CVD	CardioVascular Disease
ECM	Extra Cellular Matrix
ECST	European Carotid Surgery Trial
GSEA	Gene Set Enrichment Analysis
HDL	High-Density Lipoprotein
HU	Hounsfield Units
IPH	Intra Plaque Haemorrhage
LDL	Low-Density Lipoprotein
LRNC	Lipid Rich Necrotic Core
MATX	Matrix
MRI	Magnetic Resonance Imaging
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NNT	Numbers Needed to Treat
PBMCs	Peripheral Blood Mononuclear Cells
SMCs	Smooth Muscle Cells
TIA	Transient Ischemic Attack
US	Ultrasound

1 INTRODUCTION

Fifteen million people suffers stroke each year globally, where one third dies and one third is permanently disabled[1]. Around 85% of all strokes are ischemic, out of approximately 60% is related to atherothrombotic disease in extracranial or cerebral vessels. Unstable carotid stenosis, due to atherosclerotic plaque causing thromboembolism, is a known cause of ischemic stroke and is appreciated causing approximately 10-20% of them[2]. The stability or instability of the carotid plaque is determined by the content of the plaque and the hemodynamics. However, accurate diagnostics of the plaque instability are lacking. Internationally practiced guidelines recommend stenosis degree and preceding neurological symptoms as determining factors in assessment of carotid atherosclerosis, which should be treated with either with best medical treatment (BMT) alone or BMT together with surgical carotid endarterectomy (CEA). These guidelines are based on the two large clinical carotid endarterectomy trials in Europe (European Carotid Surgery Trial; ESCT) and in North America (North American Symptomatic Carotid Endarterectomy Trial; NASCET) where operation of high grade stenosis (>50% of NASCET criteria) showed to be efficient to prevent future strokes in patients with preceding neurological symptoms, assuming the carotid plaque as the thromboembolic source[3–5]. However, numbers needed to treat (NNT) with surgery is certainly leaving room for improvements with NNT of 9-15 for symptomatic carotid stenosis[3,4] and especially for asymptomatic patients where NNT has been suggested as high as 50 to prevent one stroke[6] Concerning asymptomatic patients, two large randomised trials examining advantages of CEA compared to BMT alone, ACST-1 and ACAS, showed moderate benefit of CEA in older patients with tighter stenosis. However, these studies were performed decades ago and medical treatment of atherosclerosis has improved significantly since then. Asymptomatic carotid stenosis is, in many centres around the world, not surgically treated at all[7]. This is a difficult clinical decision since the lack of symptoms up until the moment of assessment does not leave any guarantees that the plaque will not become symptomatic, atherosclerosis being a dynamic process. In addition, the factor of significant stenosis degree has limitations, since a non-significant (<50%) carotid stenosis still can cause stroke[8–10]. This also calls for better diagnostics and more precise recommendations for therapy. On the other hand, the periprocedural stroke risk, in addition to valuable health care resources utilised also needs to be considered from both a patient safety and a socio-economical perspective. Because of these dilemmas, together with the evolving technology of imaging modalities of MRI, ultrasound and CTA, the focus has emerged to shift from the stenosis degree of the lumen to the actual atherosclerotic plaque; the vessel wall and plaque morphology. In this PhD project, we have aimed to further explore the plaque morphology in relation to the on-going biological processes but also to the patient phenotype. The biology of the plaques has never been associated with plaque morphology in CTAs on this scale before, making this project unique. Associations of the patient phenotype and risk scoring for ischemic stroke with plaque phenotype and morphology are also new to the research field. As resources for our investigations, we have had access to the biobank of carotid endarterectomies at Karolinska (BiKE) where the pathophysiology of carotid plaques has been explored using global gene microarrays and linked to clinical-, laboratory, epidemiological and imaging data from patients undergoing CEA for asymptomatic and symptomatic carotid stenosis in our unit. By extending the results from these studies into larger scale prospective studies of patients with carotid disease, the connection

between plaque morphology assessed by image analysis of CTA and plaque phenotype may become implemented in the clinical praxis and improve identification and treatment of patients with vulnerable lesions before they suffer a stroke.

2 BACKGROUND

Cardiovascular disease, epidemiology and risk factors

Cardiovascular disease (CVD) is the number one killer globally causing close to 18 million deaths annually, which amounted to 31% of deaths in 2016. Myocardial infarction and stroke are responsible for 85% of these deaths[11]. Over 75% of the deaths due to CVD is occurring in low and middle income countries [11] where the most increasing incidents are due to population growth and increasing average life span[12]. CVD is defined as coronary heart disease causing myocardial infarction or heart failure, cerebrovascular disease causing stroke (infarction or haemorrhage), peripheral arterial disease causing ischemia in legs and arms. All, but cerebral haemorrhage, are caused by atherosclerotic disease obstructing the blood vessels. But also rheumatic heart disease, congenital heart disease and vein thrombosis and pulmonary embolism are included in this definition according to WHO[13].

Well established risk factors for developing coronary heart disease are known to be smoking, hypertension, hyperlipidaemia, obesity, diabetes, age [14,15] and can be well extrapolated to risk of cerebrovascular disease and peripheral disease[16–19].

Carotid disease and stroke

The global incidence of stroke is 15 million/year and is the second most common cause of death globally[18]. In 2015, 3.3 million died from ischemic stroke and 3.4 million from haemorrhagic strokes[20]. Approximately 8-20% of all strokes are estimated to be caused by extracranial carotid artery disease[2,21]. For people 65 years and older, carotid artery disease with narrowing is prevalent in approximately 75% for men and 62% for women even though not all stenoses are symptomatic[22]. To avoid ischemic stroke caused by thromboembolism from a carotid stenosis, the patient is prescribed optimal medical treatment: smoking cessation (where applicable), change to healthier diet, exercise, controlled blood pressure, and proper blood glucose levels. In addition, antiplatelet therapy (acetylsalicylic acid or clopidogrel) and HMG-CoA reductase-inhibitors (statins) are prescribed. In some cases, surgical intervention is indicated such as either carotid endarterectomy (CEA) where the plaque in the carotid artery is excised, or carotid artery stenting (CAS).

Symptomatic carotid stenosis

Carotid stenosis can cause major or minor stroke, transitory ischemic attack (TIA), retinal emboli or amaurosis fugax (sudden reversible mono-ocular blindness or vision impairment) and is then called symptomatic stenosis.

To investigate which of the patients that should be treated more actively with surgical intervention two large multi-centre randomized controlled trials (RCT) European Carotid Surgery Trial (ECST)[3], North American Symptomatic Carotid Endarterectomy Trial Collaborators (NASCET)[4] were performed in the 1990's. Both studies aimed to investigate if CEA in symptomatic patients reduced risk for major stroke or death compared to medical treatment alone. Both studies showed that stenosis >80% in digital subtraction angiography exam benefitted from CEA compared to medical treatment alone. In NASCET the benefit of surgical intervention was seen already in less significant stenosis (50-69%) where risk reduction of stroke or death were 29% in 5 years, but those with <50% stenosis no benefit was seen. In stenosis >70% the risk reduction was so dramatic that study arm was ceased pre-maturely and those patients were referred for CEA. In ECST the stenosis degree favouring CEA was >80% (14.9% *vs.* 26.5%). However, the methodologies to define stenosis differ between the two studies, (figure 1), where ECST may have overestimated the stenosis degree[23].

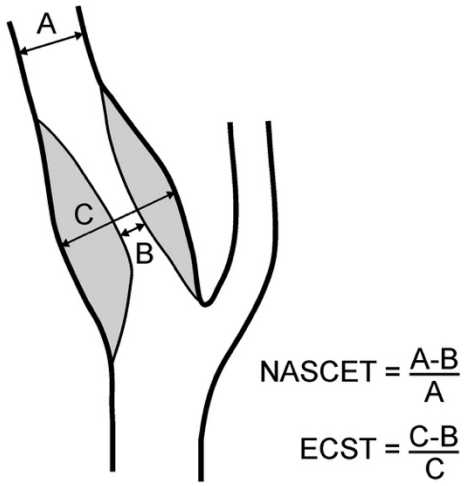


Figure 1. Illustration of stenosis showing the differences NASCET and ECST methods of calculating percentage diameter stenosis. Oates et al, Eur J Vasc Endovasc Surg (2009) 37, 251e261

These two studies from 1998 built a foundation on which international guidelines were formed both from SVS (Society for Vascular Surgery) and ESVS (European Society for Vascular Surgery), which clinicians at present use every day to evaluate symptomatic carotid stenosis. Even though these studies are of high quality the clinical practice remain diffuse; to avoid one stroke, 9 or 15 patients need to be operated (NNT=9 in stenosis 80-100%, ESCT; NNT=15 in stenosis 50-69%, NASCET). The stenosis degree according to ultrasound alone is therefore a blunt tool to select the right patient for prophylactic surgery. In addition, with a severe stenosis, called near-occlusion, (the lumen distally from the stenosis may or may not collapse) available data is limited, however this state has been considered having rather low risk of recurrent stroke and low benefit of CEA. But in a more recent study this consensus has been challenged, presenting a high risk of recurrent stroke with a near-occlusion with full collapse[24], exemplifying the need of refining the diagnostics of the unstable stenosis.

Asymptomatic carotid stenosis

Not all carotid stenoses give rise to neurological symptoms and are often found by chance via clinical examination (neck bruit) or radiology (ultrasound, computed tomography (CT), magnetic resonance imaging (MRI)). These asymptomatic patients should also be treated medically as mentioned above since they are at an annual risk of 2% stroke or myocardial infarction[25]. However, whether surgical intervention of these asymptomatic patients is indicated or not is debated and in the 1990s two large multi-centre randomized trials were performed: ACAS and ACST-1. The ACST-1 showed a benefit of CEA in patients <75 year of age and with a stenosis of >60% with a reduced stroke risk in 10 years[26]. In ACAS, the result was similar in the same patient group, with a stroke risk reduction in 5 years, for patients who received immediate CEA[27]. After these studies

were performed, the medical treatment has been vastly improved and the results of the studies have also been questioned as medication was not standardised and not followed up to ensure adequate blood pressure and plasma-lipid levels. The efficacy was also poor and according to a meta-analysis the NNT for asymptomatic patients approached 50 to avoid one stroke within 3 years[6].

Hence, the need of more accurate diagnostics than just symptomatology and degree of stenosis are highly called for in order to improve patient selection for intervention. More modern RCTs comparing CEA, CAS and optimal medical treatment in asymptomatic patients are ongoing such as ACST-2, CREST-2 and ESCT-2 where the results are eagerly awaited. But increasing attention is at the same time given to improve assessment of plaque phenotype, its' composition and structural characteristics to replace the poor predictive power of stenosis grading with risk prediction actually based on the biology of the disease. The research field of plaque imaging for prediction of plaque vulnerability is currently exploding aiming towards future individualized patient-tailored therapy.

Atherosclerosis

Atherosclerosis is a systemic disease of large and medium-sized arteries featured by accumulation of lipids and fibrous elements together with contributing immune cells, smooth muscle cells (SMC) and endothelial cells, in the arterial wall, forming plaques and eventually sometimes stenosis in the arteries.

The initiation of the process is not fully understood but is most likely the result of an interplay between several different processes such as plasma lipid-levels, blood flow mechanics (shear stress and turbulent flow) and genetic predispositions. The subsequent progression of the disease includes lipoprotein retention, recruitment of inflammatory cell, formation of foam cells, apoptosis and necrosis, migration of smooth muscle cells (SMCs) and their proliferation and secretion of extracellular matrix (ECM) components (such as collagen, elastin and proteoglycans), calcification, neovessel formation, arterial remodeling, rupture of the fibrous cap and thrombosis[28–30]. The Stary descriptions of the plaque types histology are some of the most extensive[31] and lay as a basis for one of the standard classifications of American Heart Association (AHA types I-VIII). Virmani et al. has since suggested a classification linking the plaque morphology and clinical disease[32], and further on, this overview will refer to this particular classification, (figure 3).

Pathophysiology of atherosclerosis

Where and how the atherogenesis begins is most probably a combination of different factors, see (figure 2). The most inner layer of the arterial wall consists of a monolayer of endothelial cells. These cells control permeability and vascular tone. Normally the blood flows laminarly past these cells and creates so called shear stress on the vessel wall, which will prevent the blood cells to adhere. However, when the flow is turbulent or oscillatory the endothelial cells start to express adhesion molecules, for instance vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). When studied in animals, low shear stress or oscillatory, disturbed flow induces atherosclerosis[33]. This is seen in bifurcations or inner curvatures, explaining why some parts of the vascular tree is affected and not others even though exposed for the same risk factors. Other factors that affect the endothelial function is nitric oxide [34] which inhibits VCAM expression, and subendothelial dendritic cells which seems to upregulate adhesion molecules [35]. Monocytes attach to adhesion molecules and transmigrate through the endothelial layer into the intima, with help of chemoattractant cytokines, e.g. monocyte chemo-attractant protein-1 (MCP-1) or macro-phage colony stimulating factor (MCSF-1) and subsequently diff-erentiate into macrophages[36].

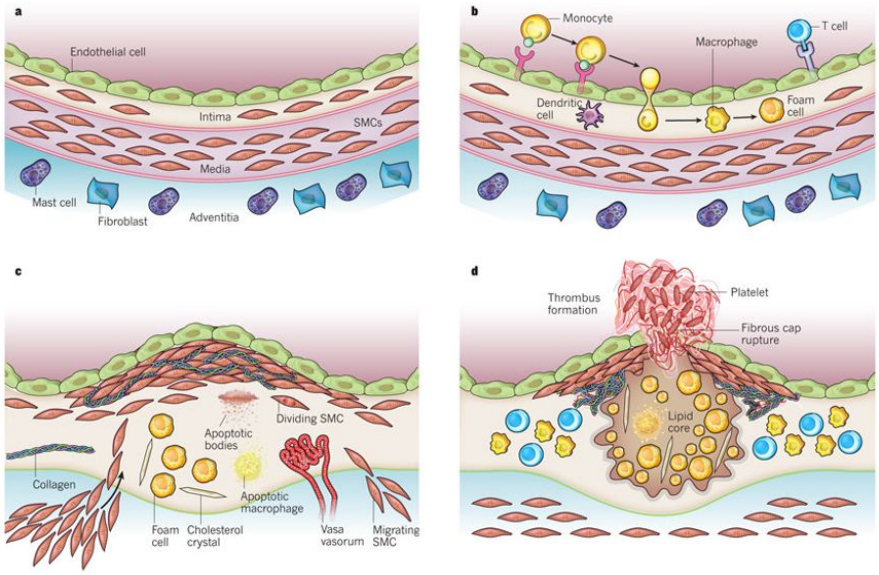


Figure 2. The process of atherogenesis with adhesion of leukocytes on the endothelium, invasion of the monocytes and T-lymphocytes into the intima where the inflammation of the atherosclerosis continues with oxLDL, chemokines, cholesterol crystals, apoptotic cells and bodies serves as pro-inflammatory stimuli, attracting SMC migrating into intima, creating fibrous cap with extensive collagen synthesis and proliferation. The lipid core is formed of oxLDL, debris, foam cells, cholesterol crystals, and is lacking structural collagen making it to a fragile plaque component especially if hidden under a thin fibrous cap which may rupture and expose its highly pro-thrombotic elements to the circulating blood in the lumen. Libby P et al. Nature vol 473, pages317–325 (19 May 2011)

In parallel with endothelial dysfunction, Low Density Lipoprotein (LDL) particles circulating in the blood stream in a high concentration infiltrates the arterial wall and is modified by oxidation or by enzymes in the subendothelial layer[37]. Oxidized LDL particles (oxLDL) induce leukocyte adhesion on the endothelium[38]. The modified LDL particles are recognized by macrophages via scavenger receptors, and are engulfed. Lipid-filled macrophages are called foam cells[39] and forms a precursor stage of an atherosclerotic plaque. When these macrophages are accumulating with intimal proteoglycans this process can be seen in histopathological analysis and is called fatty streak lesion or xanthomas[28,31,40]. The macrophages secrete pro-inflammatory cytokines and reactive oxygen species that promote retention of LDL and release of matrix metalloproteinases (MMPs)

and cathepsins, among others[41]. The migrated monocytes can moreover differentiate into dendritic cells operating as antigen-presenting cells, contributing to the inflammation in the plaque[42].

Lymphocytes, representing the adaptive immune system circulating in the blood, are also triggered by oxLDL, adhere to and roll on activated endothelial surface and transmigrate into the intima[43]. Besides oxLDL, other triggers are antigens in the atherosclerotic process as well as from the above-mentioned dendritic cells and the intimal macrophages expressing CD68 and or CD36. The lymphocytes are mostly T helper 1 cells which accentuates inflammation by secreting interferon-γ and tumour necrosis factor-α[40]. Regulatory T cells and also B cells are involved in amending the inflammation[44].

Subsequently, SMCs in the media migrates into the intima in response to growth factors, growing levels of oxLDL and different cytokines[45]. SMCs go through a phenotypic switch from the non-proliferative contractile state typically expressing genes such as calponin 1 (CNN1), actin alpha 2 (ACTA2), myocardin (MYOCD) to the non-contractile synthetic phenotype, which is characterised by proliferation and production of extracellular matrix such as collagens (e.g. COL2A1), elastin (ELN) and proteoglycans (e.g. ACAN or HSPG2), contributing to the fibrous component of the plaque as well as plaque growth[46]. In a recent study from our group, new sensitive markers such as PDLIM7 and LMOD for SMCs early phenotypic modulation were identified[47]. In addition, the SMC also can take up lipids in the atherosclerotic process[48]. There have also been studies showing that SMC may turn into a proinflammatory phenotype where cytokines like IL-8 and IL-6 are secreted and where cell adhesion molecules are expressed, which interacts with the monocyte and macrophage adhesion within the process of atherosclerosis[49].

The advanced atherosclerotic lesion

In progression of the plaque the lipid accumulation increases together with macrophages, foam cells and T cells activities leading to accentuating the inflammation and also apoptosis of SMC and macrophages[50] which eventually forms a *necrotic core*. It consists of acellular, lipid-rich material, cholesterol crystals, and the presence of it classifies the plaque as a fibroatheroma, (figure 3D). The apoptosis of SMC and macrophages and its' debris in advanced lesions increases the formation of necrotic core and inflammation of the plaque[51,52]. In the *lipid rich necrotic core* (LRNC) the collagen is degraded by matrix metalloproteinases (MMPs).

In the advanced atheroma, neovessels are formed from the adventitial vasa vasorum layer growing into the plaque. These vessels are fragile and leaky and can easily break causing *intraplaque haemorrhage* (IPH) which contributes further to the necrotic core and also to inflammation[53]. IPH contributes to enlargement of LRNC as well as plaque progression[54], and has been linked to plaque vulnerability. IPH can also occur in the case of plaque rupture when the fibrous cap ruptures and the haemorrhage origins from the luminal side[55]. *Calcification* is a common feature of the atherosclerotic plaque. It is increasingly defined as either micro-(0.5 μm, <15 μm in diameter), or macro-calcification (>2mm in sheet-like formations) in the literature. Microcalcification (spotty or

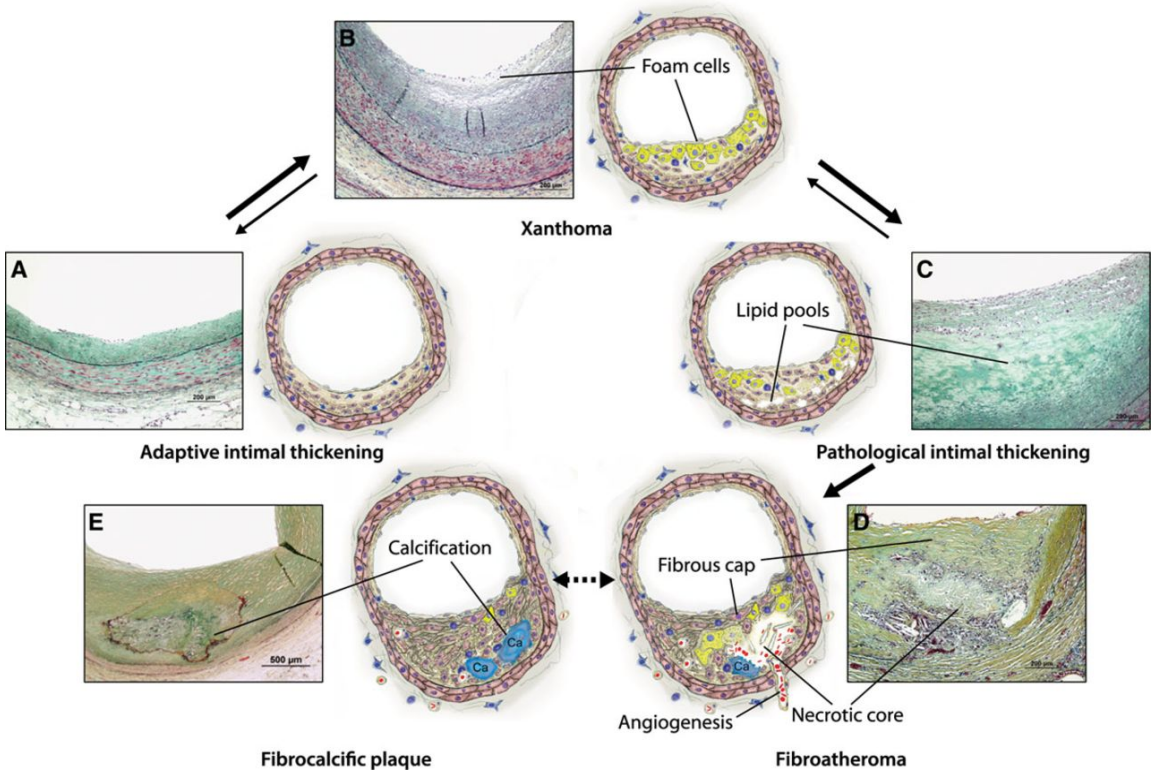


Figure 3 Classification an atherosclerosis progression according to Bentzon et al 2014. Stage A-C are most often asymptomatic while D and E can lead to plaque rupture or erosion and subsequently thrombosis.

granular) can be present already in the early lesion, (figure 3C), but also in the end-stage atheroma, (figure 3E), as a part of the lipid rich necrotic core[56]. The macro-calcification is a feature of the end-stage atheroma and is by some researchers believed to be the continuation of the lipid rich necrotic core that has calcified[57]. The mechanism of calcification in the human arterial intima shares many features of the skeletal ossification[58], however the process is not fully understood. Many studies have shown that apoptosis of SMCs and macrophages with release of matrix vesicles seems to generate nidus for microcalcification in the presence of free calcium and phosphate[56,59,60]. Many of the SMCs also seem to transdifferentiate into an osteogenic state not very different from the osteoblast, also leading to calcification. The role of calcification will be further discussed below.

Plaque vulnerability

"The major clinical consequences of atherosclerosis such as myocardial infarction or stroke are not a function of gradual narrowing of the lumen, but rather due to thrombotic events associated with acute rupture or erosion of an unstable plaque". (Bennett et al. 2016)

In 2003 Naghavi and Libby with 56 colleagues proposed a definition of the vulnerable plaque as "thrombosis-prone plaques and plaques with a high probability of undergoing rapid progression"[61]. Furthermore, the vulnerable patient was also defined as "subject susceptible to an acute coronary syndrome or sudden cardiac death based on plaque, blood, or myocardial vulnerability" emphasising the systemically multifactorial complexity that leads to the clinical outcome. These definitions have since then been widely used.

In thromboembolic events such as myocardial infarction or stroke, lumen is obstructed or occluded by a thrombus generated on top of an atherosclerotic plaque. In histopathology studies from autopsies after coronary acute syndrome three different plaque pathologies are defined as underlying mechanism: plaque rupture, plaque erosion or calcified nodule[62] protruding from the plaque into the lumen, where that latter are rare with only prevalence of 4-14% in carotid plaques[63]. Plaque rupture is the most common cause of thromboembolic event[62] and is characterised by a thin fibrous cap poor in collagen, a large lipid core and many macrophages. Eroded plaques are rich in proteo- and glycosaminoglycans, little or no lipid core, neutrophils and plenty of SMC[64].

Plaque inflammation, angiogenesis with IPH, activity of MMPs as well as endothelial denudation and platelet aggregation are activities in the plaque which make it prone to rupture. Plaques that have the same degree of stenosis might have very different content, i.e. an activated inflamed plaque with large LRNC thin fibrous cap can have the same stenosis degree as a completely fibrous or highly calcified plaque with little or no inflammation on-going, which therefore makes the simple stenosis degree into a rudimentary tool to diagnose the vulnerable plaque[61,65].

Morphological features

Morphological characteristics of the vulnerable plaque are defined as thin fibrous cap, large lipid core size, high grade luminal stenosis, remodeling, lesser collagen content *vs* more lipid content (mechanical instability), large calcification burden and pattern (nodule *vs* scattered, superficial *vs* deep etc)[61,62].

Also traits like IPH, carotid plaque thickness, surface morphology and carotid plaque volume possible to image through modern imaging techniques are known to affect the vulnerability of the plaque[29,65–67], (figure 4).

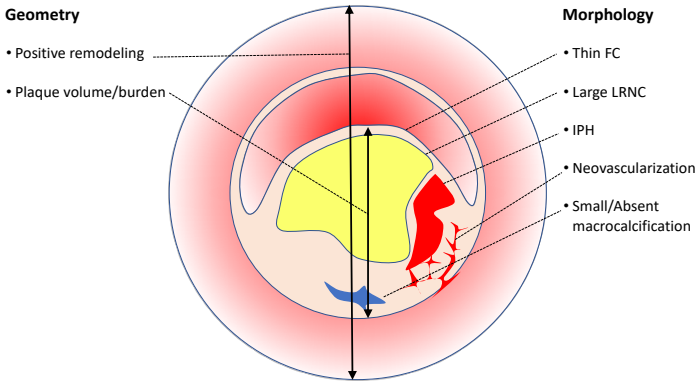


Figure 4 Schematic image of an unstable plaque rich of LRNC, thin fibrous cap, presence of IPH, and small calcification, large plaque burden.

Calcification

The difference between micro- and macrocalcification seems to partially determine the fate of the plaque. Microcalcification is formed initially as calcium deposits within apoptotic bodies or matrix vesicles released from macrophages or SMCs as a response to inflammatory stimuli[68]. This promotes additional inflammatory response and calcium deposits, causing further damage and disturbed efforts of healing[69]. This leads into a vicious cycle that eventually leads to thinning of the fibrous cap and higher risk of plaque rupture[70]. Nevertheless, if an adaptive response succeeds with a shift of the T lymphocyte and the macrophages from Th1 to Th2 and from M1 to M2 respectively, the inflammation is decreased[60]. Subsequently SMCs survive and generate fibrosis and stabilization of the plaque. If the pro-osteogenic conditions continue, SMCs transdifferentiate into osteoblast-like phenotype expressing markers like RUNX2, BMP2, OPN, ALP, Sox9 etc, creating

formations of macrocalcification which functions as a barrier against spreading the inflammation but also as a mechanical stabilisation[69], (figure 5).

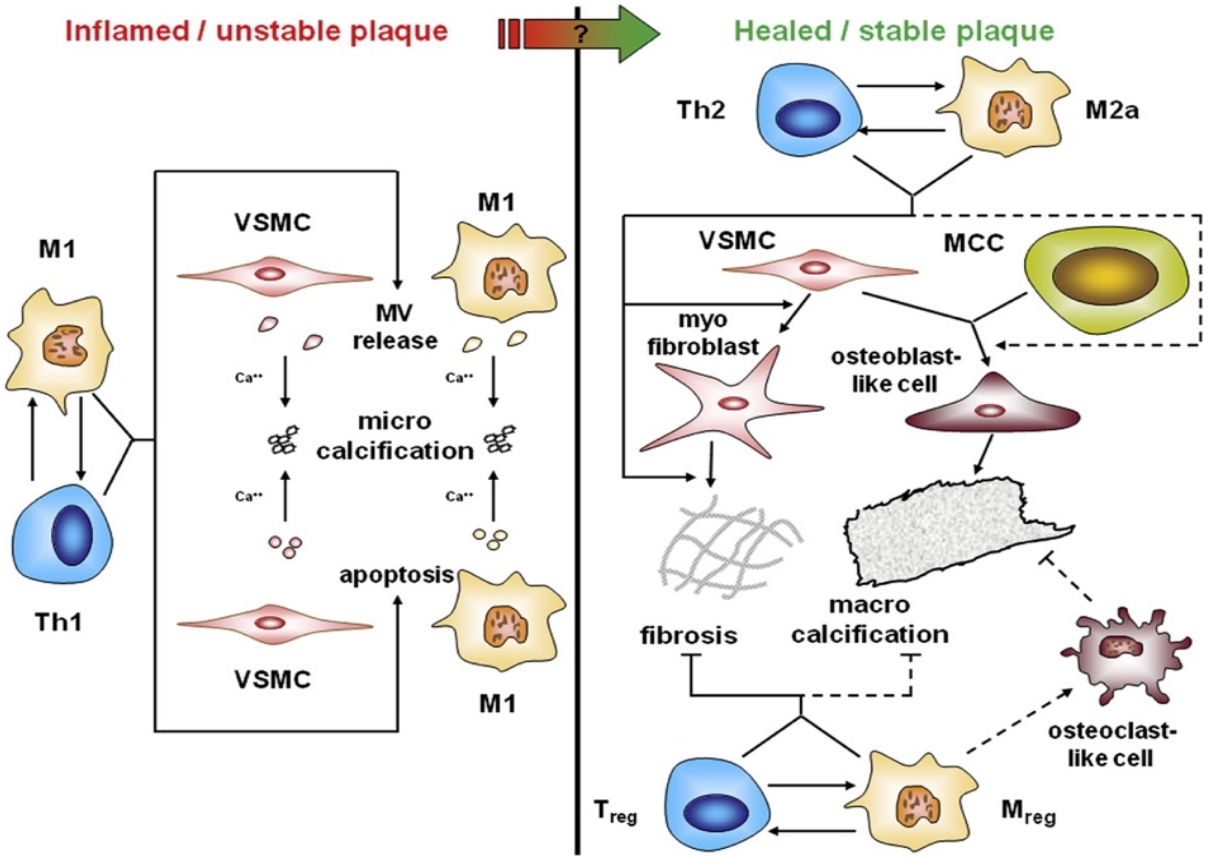


Figure 5 Relationship of calcification and inflammation suggested by Pugliese. The pro-inflammatory M1 and Th1 cells induces VSMC and macrophage (M1) apoptosis and matrix vesicle release which in presence of Ca2+ is forming microcalcification. In the stable plaque the anti-inflammatory cells Th2 and M2a are promoting the VSMC and Myeloid Calcifying Cells (MCC) into osteoblast-like cell and myofibroblast state which creates macrocalcification and fibrosis respectively. The regulatory Treg and Mreg cells might be ameliorating the processes of fibrosis and macrocalcification. The mechanism of transformation from inflamed to healed plaque is poorly understood. Pugliese et al. Atherosclerosis. 2015 Feb;238(2):220-30

In a clinical setting, the presence of calcification is seemingly contradictory. When assessing coronary arteries, calcification is known to be strongly associated with high cardiovascular disease risk in the terms of coronary artery calcification (CAC). CAC score is a scoring system developed by Arthur Agatston weighing the density of the calcifications, providing a score of Agatston units, AU. In a large population-based study the risk of coronary event increased by factor 7.73 with CAC score 101-300 AU and by factor 9.67 with CAC >300AU[71]. Several studies have confirmed the predictive value of CAC score[71,72], superseding other predictive scores such as Framingham risk score[73], leading to recommendation in the guidelines of American College of Cardiology Foundation/American Heart Association of measuring CAC score in individuals at risk[74]. In other studies, high CAC score has showed a stronger association of chronic coronary heart disease such as stable angina pectoris with macrocalcification[75,76].

Interestingly, in a double blind randomised controlled trial, statin use was shown to decrease the systemic inflammation as well as the LDL- cholesterol levels in serum but not to halt the

progression of CAC-score[77]. The progression of calcification with statin use was confirmed in another longitudinal study on carotid lesions[78].

When mapping the pattern of calcification with IVUS (Intra Vascular UltraSound), the spotty pattern with a fibro-atheromatous lesion and positive remodeling associated with acute myocardial infarction, lesions with no calcification associated with negative remodeling and with unstable angina pectoris and stable angina pectoris were strongly associated with extensive calcification[75]. When examining lesions with IVUS in longitudinal studies, correlation between large plaque burden, less dense calcium and larger non-calcified plaque, associated with events[79], (figure 6).

In carotid lesions similar results were found, showing that large calcification and osteoid metaplasia associated with asymptomatic lesion[80,81]. In comparison, in non-calcified plaques it has been suggested that the fibrous cap contains higher degree of inflammatory processes contributing to vulnerability, and therefore indicating that calcification is associated with plaque stability[82,83]. This was confirmed in a systematic review of calcification of carotid lesions and symptomatology where both calcification value or weight as well as percentage showed to be a strong prognostic parameter for plaque stability, where the percentage was the strongest parameter[84]. So far, partial explanation to this was suggested as plaques having a large calcification volume have a smaller LRNC making them more stable[84].

The location of the calcification seems to matter in assessing vulnerability, as stated by Virmani et al. showing rare cases of calcification nodules as cause of plaque rupture[62]. Abedin et al. hypothesized that the interface in between hard calcification and softer components could lead to mechanical instability explaining why mild-moderate calcification degree associated with symptoms[85]. This was further studied in a simulator computational environment, showing plaques more prone to rupture when calcifications were situated close to a thin fibrous cap, while calcifications deeper in the plaque had no impact on plaque stress[86]. Another feature of calcification location in the plaque that has been studied increasingly is the peripheral calcification or the so called 'adventitial calcification' in line with radiology appearance, also called the 'rim sign'. The rim sign in combination with a large soft plaque has shown to have association with IPH and therefore plaque vulnerability[87].

Lipid rich necrotic core (LRNC) and fibrous cap

The proportional size of LRNC is clearly correlated with plaque vulnerability[53]. A large LRNC, has been shown to be a strong predictor of both fibrous cap thickness and disruption[88,89]. Higher risk of plaque rupture has also been seen in plaques with percent of LRNC area exceeding 40% of the vessel wall area, as opposed to stenosis alone where no correlation could be seen with plaque rupture[88]. Furthermore, in a meta-analysis it has been shown that a large LRNC, thin fibrous cap and IPH, each are predictive of stroke[90] but also in single centre studies following patients over time this association has been confirmed[91,92].

Intraplaque haemorrhage (IPH)

IPH is considered one of the key features of vulnerable plaques[93]. In single-centre studies, a clear implication with increased risk of ischemic stroke has been elucidated[91,92,94]. In histopathological

studies, IPH contained components like intraplaque iron and fibrin which co-existed with extravasated erythrocytes, as well as other blood components like platelets but also plasma components[95]. In a large longitudinal study, presence of IPH and neovessels associated with symptomatology individually, but also suggested that the grade of plaque neovessels and haemorrhage in one site may mirror the atherosclerotic status in other vascular beds[96].

Carotid plaque remodeling

The remodeling can be either positive (outward expansion of the vessel wall) or negative (inward into the lumen) causing stenosis. Positive remodeling occurs initially as an attempt to maintain the luminal area for the bulky atheroma, but as the atherosclerosis progresses the negative remodeling interferes into the lumen[97]. The hemodynamic effects surrounding a negatively remodelled plaque are altered, which has been correlated with increased risk of stroke[98]. The positive remodeling has been indicated as associated with risk of stroke[99] and is synonymous with following section of plaque volume, thickness and burden. This was further confirmed in studies based on CT assessment where positive remodeling was significantly larger in symptomatic patients than in asymptomatic patients[100,101]

Carotid plaque volume / thickness / burden

Plaque volume has been studied and its role in vulnerability has been discussed extensively. Several studies have conferred plaque volume being involved in determining plaque vulnerability and risk for cardiovascular events[99,102,103]. It has been shown to be associated with plaque vulnerability and stroke[78]. *Progression* of total plaque volume is a significant predictor of cardiovascular events or cardiovascular risk factors[104], confirmed in a prospective study as predictor individually and also together with IPH and fibrous cap rupture[105]. However, another study has exposed no clear correlation of plaque volume with symptomatology, though a negative correlation between proportion of calcification and symptoms could be stated[106]. Some researchers claim the idea that the volume of the plaque could be a superior marker of the severity of the systemic atherosclerotic disease than the stenosis degree only[88]. Carotid plaque burden has been demonstrated to be closely associated with CAC score[107] and is currently investigated to be clinically utilised as a predictor of future cardiovascular events in the large prospective BioImage study (ongoing)[108].

Imaging modalities

Historically, selection of proper treatment for carotid stenosis was based on stenosis degree, decided by either invasive, intra-arterial angiography or by ultrasound. With modern imaging, the focus has shifted from the lumen's diameter to the wall's morphology where plaque vulnerability can be predicted via imaging biomarkers, looking beyond the stenosis degree[65]. Here follows a brief summary of modalities that are implemented in imaging of the carotid artery.

Angiography

Conventional angiography was for many years considered the gold standard of assessment of carotid stenosis. The constitutive studies ECST, NASCET and ACAS were all based on this method. However, this invasive method shows the lumen and excludes the wall, focusing on the stenosis degree. As other modalities developed and as both clinical and research focus has shifted

towards the plaque morphology, in addition to its' invasiveness, angiography has now essentially become reserved for endovascular intervention.

Ultrasound (US)

Ultrasound is often used as the first line diagnostics of carotid stenosis degree and has become the new gold standard diagnosing carotid disease with its low-cost and availability with low-risk. The technique has also vastly improved in characterising the plaque, and has shown satisfactory results in identifying high-risk plaques[109]. The method has additionally improved with the use of microbubble contrast which can visualize and identify important plaque characteristics, such as neovessel formation or the absence of it[110,111]. Additionally, the novel techniques of volume rendering are adding preciseness to the assessment[112]. In addition to the well-known constraint of operator variability, US has its' limitations of difficulties visualising the carotid bifurcation and wall in some cases, such as muscular short necks, obese patients or patient with previous radiation therapy against neck[113]. To detect an eventual tandem lesion can also be omitted since the distal proportion of the internal carotid artery is hard to reach underneath the mandibular bone. The morphology characteristics best visualised by US are plaque ulceration[114], and according to some studies neo-vascularisation[110,111] but the reproducibility has been hard to prove. The high echodensity of calcification is causing shadows which makes that component difficult to visualize. Studies diagnosing IPH and fibrous cap with US have shown suboptimal results[115,116]. The invasive type of ultrasound, intravascular ultrasound, (IVUS), has added the advantage of increasing spatial resolution and can visualise the plaque well, but due to its' invasiveness and risk of embolization only rare carotid cases with stenting have been studied. Vazques-Figueroa et al followed patients prospectively after assessing their coronary arteries with IVUS, identifying plaque burden, fibrofatty volume and proportion associated positively and calcium negatively with Major Adverse Cardiovascular Events (MACE)[79], (figure 6).

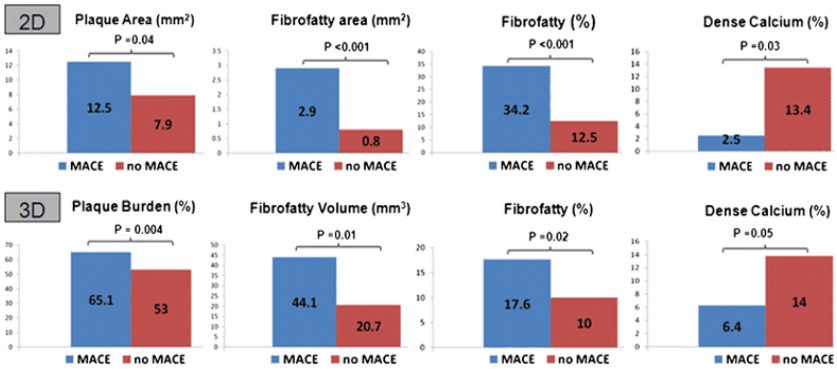


Figure 6 Prospective assessment of coronary plaques with IVUS of 60 patients during 12 months, the plaque characteristics association with Major Adverse Cardiovascular Event (MACE) Vazques-Figueroa et al 2013

Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) has shown great potential in atherosclerotic imaging with its ability to detect soft-tissue, great resolution and high reproducibility. The soft tissue features like LRNC, fibrous tissue and IPH can be detected with high accuracy without administration of contrast agents, though detection of neovessels requires contrast, (figure 7), [117,118]. Detection of calcification with MR is more debated but a few studies have shown decent results[119,120]. Disadvantages with MRI are low availability, long procedure time, sensitivity to patients' movement in addition to that the required scanner (≥3 Tesla) for detecting the above-mentioned components is rarely accessible for the daily work in the clinic[116].

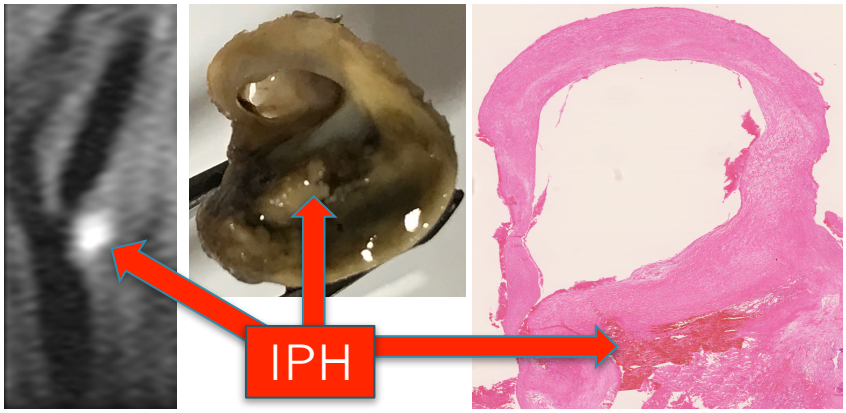


Figure 7 Intraplaque haemorrhage (IPH) visualized in a patient with asymptomatic carotid stenosis by 3T MRI(left), in the endarterectomy specimen (middle) and in histological staining (right)

Computed Tomography (CT)

Computed Tomography (CT) is a form of x-ray where many measurements of the x-rays are made from different angles and produce cross-sectional images, "slices" of the body. The CT can be performed with contrast enhancement (Computed Tomography Angiography, CTA) often used when visualizing blood vessels. The images are computer processed into a three-dimensional image and render reconstructions greatly useful in the clinical praxis.

The fundamental principle of CT is based on the difference of the x-rays emitted by the energy source that is absorbed by the body and that is transmitted through the body. This is called attenuation, which is determined by the density of the specific tissue. For example, high density tissue (such as cortical bone) absorbs much of the radiation and less x-rays are reached by the detector, leading to high attenuation. On the opposite side of the scale there is low density tissue (such as lungs filled with air) where more x-rays reach the detector, rendering low attenuation.

The CT image consists of a number of picture elements called pixels. When the pixels are combined with the slice thickness into a volume unit, this is called a voxel. Each pixel or voxel has a mean of attenuation of the tissue in radiodensity. This can be represented by a number called

Hounsfield[1] Units (HU). HU can range from +1000- (e.g. cortical bone) to -1000 (e.g. air) on the Hounsfield scale where water has attenuation of 0, (table 1).

Substance	Hounsfield Units (HU)
Air	-1000
Fat	-100
Water	0
Muscle/soft tissue	+40
Contrast	+130
Bone	+1000

Table 1 Typical HU for different tissues

CT machines are built with an energy source which sends out the x-rays through the patient to a detector in the scanning gantry. The early models of CT scanners used one energy source and one detector, where a single axial image was taken at a time while the patient was asked to hold the breath, leading to long acquisition times and risk of missing small pathologies as the patient could not hold the breath in the same way each time. Current scanners are built as a spiral where the patient is moved continuously through the scanning gantry while holding one breath, while the energy source and detector rotates around the patient. Further development of the machines has expanded the number of energy sources and also detectors, increasing both the temporal and the spatial resolution. These techniques are named multi*energy* CT or Dual-Source Computed Tomography (DSCT) and multi*detector* CT (MDCT). DSCT is using two different x-ray sources, permitting different x-ray energies (80 and 120 kV) which renders different HU in the tissue. This improves the resolution and has shown promising results of plaque imaging in complex vasculature, including both coronary and carotid arteries[121,122]. The introduction of MDCT (modern scanners with up to 32 and 64 detectors) with has led to an enormous increase in imaging acquisition speed, more coverage of the patient and high spatial resolution[123].

CTA is a widely used diagnostic tool with high availability, and may provide a rapid high-resolution imaging of the carotid wall and lumen. CTA is outstanding in diagnosing calcification of the plaque using the density, measured in HU of the pixels in the image, where thresholds have been proposed and set with validation to histology[124,125]. These thresholds were proposed as lipid rich plaque <60HU, mixed attenuation values 60-130HU, and calcification >130HU and have been tested in association with clinical symptoms showing that the calcified plaque was 21 times less likely to be symptomatic[106] while fatty plaques were associated with high risk of rupture[126]. Luminal morphology and ulceration are well depicted, better than in US[127] even though small ulceration can be omitted by halo edge blur. Visualising the fibrous cap is at present challenging and it is debated whether IPH can be detected in comparison to MRI[128,129].

The main disadvantages of CTA consist of the radiation dose for the patient, especially in longitudinal studies. Secondly, the necessary intravenous iodine contrast which may lead to contrast-induced nephropathy or anaphylactic reaction. And finally, high-density areas such as calcifications are overestimated in volume, i.e. the "blooming effect". This is an effect of the point spread function which is a phenomenon that can be defined as "diffraction of light, which determines the image system's resolution limit, blurs out any point-like object to a certain minimal size and shape", *Zeiss microscopy, bitesizebio.com*. Additional published data of reliability of the

[1] Sir Godfrey N. Hounsfield was an electrical engineer who won the Nobel Prize 1979 for development of the CT in the 1960s. Up until 1967 he had no experience in medical field but had only worked with developing computers and radar in the EMI (Electric and Musical Industries).

advanced, constantly developing, CT techniques and its plaque feature assessment associated with stroke risk is highly warranted.

The CT technique has evolved immensely with the multi-energy technology, together with developing detectors and advances in high spatial resolution, contributing to making CTA a rapid, trustworthy method to characterise plaque morphology, visualising location and extent of the plaque is also highly important before surgery. Another important factor in improving the reliability of CTA detection is the advances in software development, reconstructing and analysing the CT image, rendering new possibilities for analysing plaque structural characteristics and components.

Miscellaneous imaging methods

18-Fluorodeoxyglucose ([18F] FDG) positron emission tomography (PET) is a functional imaging method that traces the macrophages uptake of glucose analogue FDG. Activated macrophages have increased glycolysis, representing inflammation. The FDG is competing with glucose in the uptake but instead of getting metabolised, it is accumulating in the activated macrophages[130]. Several studies have presented the potential of [18F] FDG PET to detect plaque inflammation[131,132] and neovascularisation[133]. In a clinical setting it has been shown that an increased uptake of FDG associates with early recurrent stroke independent of stenosis degree[134] and that echo-rich plaques in US associates with decreased uptake[135]. This method is however unlikely to become a routinely practised imaging modality due to its limited accessibility and cost, but is nevertheless of high interest to evaluate other imaging modalities mentioned above[136].

Optimal Coherence Tomography (OCT) is an invasive optical technique using similar imaging performance as a microscope combined with principle of ultrasound but with near-infrared light which is reflected, though with a significantly higher spatial resolution than any ultrasound technique[137]. OCT imaging of plaque components has shown good correlation to histology in coronary plaques, especially with thin-cap fibroatheroma[138], but also fibrous, lipid rich and fibrocalcific plaques[139,140]. The related method of near-infrared spectroscopy (NIRS), results have been promising in carotid plaque vulnerability assessment[141]. However, the invasiveness of these imaging modality is limiting its application on carotid arteries, though attempts on performing OCT externally on the carotid artery has been done, where the results of the technique were not satisfactory in the current form[142].

Image analysis software

TeraRecon

TeraRecon (Aquarius, iNtuition, TeraRecon, Foster City, CA, USA) is one of the largest providers of advanced visualisation and interpretations software which can be used in a wide range of parts of the body as implementation of oncology and perfusion. Clinical applications contain advanced image processing and 3-D visualisation for CT, MRI and PET. The function *plaque analysis* in CT, renders the area and volume of the plaque, defined by thresholds of HU set according to individual preferences. This function was earlier available for carotid artery analysis, but is today applied in coronary artery plaque assessment.

vascuCAP

vascuCAP software analysis (Elucid Bioimaging, Boston, MA, USA) is a histology-validated tool for computer-aided phenotyping of vasculopathy[143–148]. Measures made only on Hounsfield densities may be influenced by errors like blurring caused by the imaging system point spread function (PSF), when comparing with histology. For example, calcification can be overestimated and in some cases adjacent necrotic core can be underestimated caused by a "blooming" calcification[125]. vascuCAP creates 3-dimensional segmentations of lumen, wall, and each tissue type at an effective resolution with improved soft tissue plaque component differentiation relative to manual inspection[143]. Algorithms included in the software decrease blur caused by image formation in the scanner, where the determination of the patient-specific PSF is addressed so that image intensities are restored to more closely represent the original materials imaged. This mitigates artefacts such as calcium blooming and enables discrimination of less prominent tissue types. By mimic expert annotation at microscopy, the software makes mathematical judgements to interpret the HU of adjacent voxels, simultaneously mitigating variation between scanners, reconstruction kernels, and contrast levels. In this way, subjectivity inherent to other analysis methods is fundamentally addressed.

Different components of the plaque can be defined: calcium (CALC), lipid rich necrotic core (LRNC), intraplaque haemorrhage (IPH) and matrix (MATX, tissue not defined as any other tissue) as well as different structural characteristics like stenosis, plaque burden, minimum cap thickness and remodeling.

Risk scores for stroke, ABCD2 and CAR

Clinical risk scores are popular, easily accessible methods to acquire a prediction of a preventable medical incident. One the of most established clinical risk scores is the Framingham risk score which estimates the risk of cardiovascular disease within ten years and is intended for patients in primary health care[15]. More specifically for stroke risk, the ABCD2 and CAR score have been suggested as complement to the clinical patient management. The ABCD2 (Age, Blood pressure, Clinical Features, Duration of TIA, and presence or absence of Diabetes) is developed as estimating the risk of stroke within 2, 7 and 90 days after a TIA.[149]. The factors that are included in the risk scoring in ABCD2 is age, blood pressure, duration and clinical features of the TIA, history of diabetes, where the points range from 0 to 7. The resulting points are stratified into low (0-3 points), intermediate (4-5 points) or high risk (6-7 points). Neither presence of carotid stenosis nor atrial fibrillation are considered in this score. ABCD2 was developed as a guide for clinicians' decision of work-up urgency for patients with TIA being evaluated in the primary health care centre or in the emergency room. However, the applicability of the ABCD2 score has been questioned, especially after that Wardlaw *et al*, concluded in a large meta-analysis that high and low score of ABCD2 cannot discriminate the early risk of stroke, but in dichotomising the score ≥4 the score had a reasonable high sensitivity (87%) but low specificity (35%) in the stroke risk within 7 days[150]. ABCD2 score has been further developed to ABCD2-I including the factor of cerebral infarction in CT/MRI and additionally in ABCD3, (max score of 9) including factors of dual TIA, carotid stenosis (50-99%) approaching the CAR score described below. In studies evaluating

ABCD2 and ABCD3, the latter performed better in predicting stroke[151], a score of ≥4 could aid triaging patients with TIA in a clinical setting to acute carotid imaging[152].

Carotid Artery Risk (CAR) score estimates the risk of ipsilateral ischemic stroke within 5 years for symptomatic patients with carotid stenosis of >50%, treated with best medical treatment. The CAR score takes into account following parameters: sex, age, degree of carotid stenosis according to NASCET, near occlusion or not (defined as severe stenosis with distal collapse of the artery), number of days from event to CEA, most severe ipsilateral event, diabetes, myocardial infarction, peripheral vascular disease, hypertension, presence of plaque ulceration or not. The CAR score results from the ECST-I, the algorithm is based on the carotid stenosis risk prediction tool[153,154]. The CAR score is awaiting its validation through the on-going study of ECST-II [3,155].

Transcriptome profiling by microarray

Microarray profiling is a well-established large-scale technique to globally determine which genes are actively expressed in one tissue compared to another tissue. mRNA extracted from a tissue, after controlling quality, is converted to complementary DNA (cDNA) and then marked with a fluorescence probe. The cDNA is placed on a chip with a large number of probes, where several probes are representing one gene in order to gain specificity and quantity of detection. The cDNA under investigation bounds to the matching probes if they exist in the specimen, (figure 8). In this way a large number of activated genes can be detected and also relatively quantified in terms of fold changes and significance in upregulation *vs* downregulation.

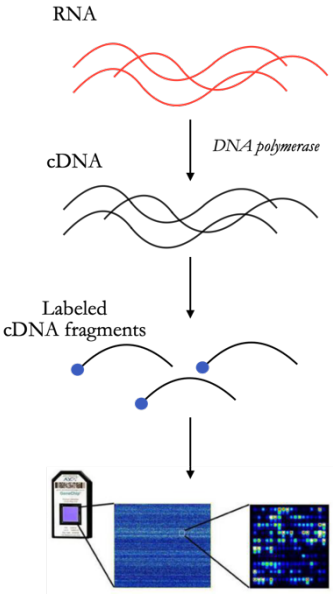


Figure 8 Schematic figure of the process for Affymetrix microarray

Bioinformatics

The large data set of genes that is obtained from the microarray analysis is processed in bioinformatic analysis. One approach is to compare two groups of microarray data with statistical tests (e.g. ANOVA or Student's t-test) which render both fold change and significance (p-value) One inherent problem with such large data sets (approximately 25-50 000 genes) is the possibility of reaching significant p-value of <0.05 without it being truly significant. To address this problem, the Bonferroni correction for multiple comparisons can be applied. Another solution to address the multiple genes comparison problem is the false discovery rate (FDR) calculation to adjust p-values in proportion to the number of parallel tests involved. One risk with these adjustments is that they may reduce the number of significant genes to zero although there is in fact a differential expression.

An alternative analysis method to the above-mentioned statistics, is the Transcriptomic Analysis Console (TAC) for Affymetrix Microarrays, provided by Thermo Fisher scientific. In the TAC program the array data is analysed with the LIMMA Bioconductor package which provides Linear Model-based analysis of the MicroArray data. LIMMA is one of the most commonly used, statistically rigorous methods of analysis for differential expression and is well validated[156]. The default method in LIMMA uses an empirical Bayes estimate to "moderate" the standard deviation

in the *t*-test denominator using the distribution of all the standard deviations, which is especially valuable in small number samples. It is especially well fit for studies with complex experimental design such as comparing across multiple attributes. The TAC program visualises the data in a variant of graphical forms, such as PCA-plots, scatter and volcano plots, hierarchial clustering and is rendering ranked list of genes, sorted on optional value, e.g. fold change or p-value.

GSEA

Gene Set Enrichment Analysis (GSEA) is a computational method that evaluates microarray data at the level of gene sets instead of individual genes. The gene sets are defined based on biological knowledge through published information about expression patterns across tissues, biochemical pathways, interacting networks or co-expression in previous experiments and is grouped together by their involvement in the same biological pathway or by location. It was developed by Broad Institute, MIT, MA, USA[157]. In addition to the above-mentioned problem with the adjustment for multiple comparisons of single gene expression analysis, the single-gene analysis may miss important effect on pathways, since sets of genes often act conjointly to affect cellular processes. For example, 10% up-regulation of a whole set of genes involved in a cellular process is likely more important than a 10-fold up-regulation of a single gene. The GSEA determines whether a set of genes is randomly distributed throughout a list of differentially expressed genes or is overrepresented at the top or bottom of the list, rendering an enrichment score (ES) and a normalised ES (NES) with an estimate of statistical significance of the ES including adjustment for multiple hypothesis testing. In this way results of significantly up- and down-regulated gene sets associated with specified biological processes, can be defined in comparison of the two different phenotypes. The collection of annotated gene sets are accessible via various public databases, e.g. at the Molecular Signatures Data Base (MSigDB)[158].

Machine learning

Machine learning can be defined as "the computer science that gives computers the ability to learn without being explicitly programmed". One of the pioneers to coin and define the machine learning concept was Arthur Samuel in 1959 in *IBM Journal of Research and Development* in an example of machine learning applied in a game of checkers "to verify the fact that a computer can be programmed so that it will learn to play a better game of checkers than can be played by the person who wrote the program"[159]. The concept of self-learning is the main feature of machine learning and refers to the application of statistical modeling to detect patterns and improve performance based on data and empirical information, but without direct programming commands. One could say that machine learning uses data as input, not a command, to build a decision model. The basic idea is to divide the input data into a training data set on which the models are initially developed. The models (for example Support Vector Machines, Artificial Neural Networks or Decision Trees) are then tuned and adjusted, re-run, re-adjusted, iteratively. When the models are at a satisfactory prediction performance level, they are tested on the remaining data, the test data set. If or when the model's performance is of satisfaction, it is ready to use.

Machine learning can be divided into three main categories: 1) supervised learning when both the input and output data is known; 2) unsupervised learning when only the input data is known,

analysing inputs to generate an output; and 3) reinforcement learning where only output data is known and a large number of variables are randomly trialed. In this thesis (in study IV) supervised and unsupervised learning were applied.

3 RESEARCH AIMS

Our research group has previously examined how the patient phenotype correlates with plaque phenotype by comparing plaques from symptomatic patients to those from asymptomatic ones[160]. An outstanding clinical need in identifying the vulnerable plaque is the association of plaque phenotype to patient imaging, which has the potential to considerably improve the diagnostic accuracy, and as a result lowering the NNT for carotid surgery which was one of the main aims of this thesis. The second main aim was to explore the possibility to predict the plaque biology from CTA data and through this creating an individual plaque biology profile possible to treat patient specifically.

The specific aims were:

I. To relate **calcification** grade via CT imaging to the overall gene expression profiles in carotid plaques.

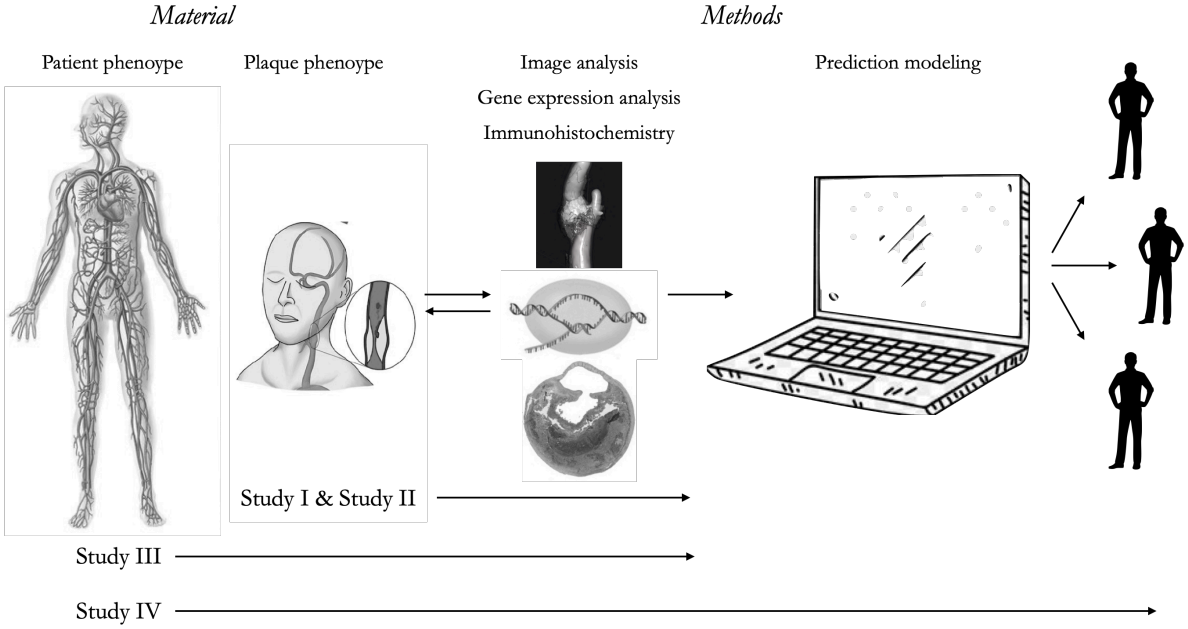
II. To relate **various components and structural features** of the carotid plaque via CT imaging to the overall gene expression profiles and patient clinical parameters.

III. To relate **clinical stroke risk scoring of patients** with plaque components via CT imaging, gene expression profiling in plaques and periperal blood.

IV. To decode the atherosclerotic plaque molecular phenotype non-invasively via CT imaging, by generating a novel **predictive model for gene expression**, refered to as virtual transcriptomics.

4 MATERIALS AND METHODS

Overview of material and methods used in studies I-IV:



Study I: Comparison of global transcriptome of high *vs* low calcified carotid plaques in pre-operative CTA of both symptomatic and asymptomatic patients. Transcriptomic microarray data was analysed for differentially expressed genes and gene ontologies with gene set enrichments. Validations with histochemistry, immunohistochemistry staining and qPCR were performed. Furthermore, bioinformatic subgroup analysis of only symptomatic patients with high *vs* low calcification was performed. The top up-regulated gene in highly calcified lesions, PRG4, not previously described in atherosclerosis, was validated with more detailed studies by targeted bioinformatic analyses, immunohistochemistry and immunofluorescence with cell specific markers.

Study II: Comparison of carotid plaque microarray transcriptome of high *vs* low volume of different components such as lipid rich necrotic core, intraplaque haemorrhage, matrix, plaque burden and calcification, and their association to symptomatology in univariate and multivariable analysis. Transcriptomes were evaluated for differentially expressed genes and gene set enrichments. CTA morphology parameters were compared with clinical data and stenosis degree, and were also tested as predictors for symptomatology in a holdout set of patients in a prediction modeling approach.

Study III: 101 symptomatic patients were scored according to clinical risk scores for stroke ABCD2 and CAR, and subsequently stratified into groups of low, intermediate and high risk patients. The high *vs* low risk groups were compared in regards to clinical features, microarray transcriptomes from carotid plaques but also from peripheral blood monocytes (PBMCs) and imaging data from CTAs. Transcriptomes were evaluated for differentially expressed genes and gene set enrichment.

The top gene ABCB5 in high risk patients was further evaluated with immunohistochemistry in vascular tissues and targeted bioinformatic analyses.

Study IV: Using machine intelligence approaches, prediction models were created based on the microarray transcriptomics from the cohort of 40 plaques paired with the data from CTAs. These trained models were then validated on additional four sequestered unseen patients, testing the models for comparing the predicted transcript expression with the true expression.

Patients and the Biobank (BiKE)

All studies are based on patients undergoing carotid endarterectomy for high degree carotid stenosis who were enrolled to the BiKE biobank after informed consent.

The Biobank of Karolinska Endarterectomy (BiKE) was established 2002 for prospective collection of atherosclerotic plaque tissue and blood from patients. The biobank currently consists of plaques and blood samples from >1500 patients, with a database of clinical parameters including risk factors, medication, symptoms, time of surgery, preoperative imaging and laboratory measurements. Transcriptomic and genomic profiles have been generated by microarray and genotyping chips, enabling multivariate analysis of gene expression patterns in relation to clinical parameters and patient phenotype. Neurological symptoms from cerebral embolism originating from the carotid stenosis were defined as transitory ischemic attack (TIA), minor stroke and amaurosis fugax (retinal TIA) Asymptomatic patients were defined as free from neurological symptoms 3 months prior to surgery, where indication for CEA was based on same criteria from the Asymptomatic Carotid Surgery Trial (ACST)[26].

Plaques were just after collection at surgery, divided transversally at the most stenotic part, the proximal half was used for RNA preparation and microarray and the distal half was fixed in formaldehyde for histology.

Study I, II and IV were based on one cohort selected out of either presence or absence of macrocalcification, n=40, 20 highly calcified and 20 with low calcification grade, undergoing surgery from 2008-2013. In study II additional consecutive 58 patients, undergoing surgery from 2006-2015, were included in image analysis; however, 5 patients were excluded due to poor image quality, rendering n=93 in total. In study IV an additional four patients, undergoing surgery from 2006-2007, was included as a test cohort to validate the prediction models developed in the starting cohort. See table 2.

In study III, 101 symptomatic patients from BiKE were included based on microarray data available, undergoing surgery from 2002-2011, out of these, 50 patients had also undergone CTA of the neck vessels. Gene expression in peripheral blood mononuclear cells (PBMCs) were also analysed with microarrays. Finally, the BiKE data base was merged with the Swedish Hospital Discharge Register and Swedish Cause of Death Register for follow-up of major adverse cardio- and cerebrovascular events for study III. See table 2.

In study I, the atherosclerotic calcified plaques were compared with a cohort with another type of calcification, the Mönckeberg calcification, which occurs systemically in the media layer of the

arteries in patients with for example advanced chronic kidney disease, diabetes mellitus, systemic lupus erythematosus, or other chronic inflammatory conditions. This cohort of Mönckeberg calcification, called Kärl TX, consists of biopsies of the epigastric artery in patients undergoing kidney transplant surgery at the Karolinska Hospital, in addition to patient clinical data.

	I	**II**	**III**	**IV**
n	40*†	40*† + 53*	101‡ (81†↔ 53*)	40*† + 4*†
Years	2008-2015	2008-2015 + 2006-2015	2002-2011 ↔ 2006-2015	2008-2015 + 2006-2007

Table 2. Basic overview of the cohorts for the studies
* = *undergone pre-operative CTA of the neck vessels*
† = *undergone transcriptional profiling of carotid plaque*
‡ = *undergone scoring in CAR and ABCD2*
↔ = *overlapping cohort*

Computed Tomography Angiography (CTA)

The CTA exams of the neck vessels included in all studies were performed as part of the routine pre-operative health care from 2005 to 2015. The CTA exams were performed all with 100 or 120 kVp at the admitting hospital in the great Stockholm area. Contrast was injected intravenously and the scanning was performed in a caudo-cranial direction from the aortic arch to vertex, reconstructing axial images with a thickness of ≤1 mm, mainly 0.625 mm. For further background of the CTA method, please see under Computed Tomography in Background.

CT analysis software

Vessel segmentation

The series of axial image reconstructions of 0.625 mm were obtained and transferred into a digital workstation for vascular CT-scan image. For both imaging software analysis, the initial process was the same: a centreline of the vessel was semi-automatically placed from the common to the internal carotid artery. The area of the atherosclerotic plaque was manually selected using the common carotid bifurcation as a reference point, marking the lesion going from normal vessel wall proximally to normal vessel wall distally from the lesion. The outer and inner borders of the artery wall were automatically defined, adjusted manually, the lumen was excluded from the analysis (in TeraRecon analysis, but included in some of the vascuCAP analyses, see below) and artery wall volumes automatically calculated.

TeraRecon

In study I the software of TeraRecon (iNtuition, TeraRecon, Foster City, CA, USA, 2015) was utilised for plaque morphology analysis. TeraRecon is an imaging program with many applications, one specifically developed for plaque analysis. Levels of Hounsfield Units (HU) can be set to the

examiner's own discretion, using these thresholds for quantifying areas of different attenuation. In study I, where calcification was the object of study, the threshold was set to >400 HU to mark this component. In this way the volume of calcification (Vcalc) was acquired and a ratio of this and the total volume of the vessel wall (Vtot) in the same region was calculated to obtain the calcification degree: Vcalc/Vtot=calcification degree. The marked lesion was restricted to the most proximal half of the plaque in order to correlate the CTA measurements to the corresponding part of the plaque used in the microarray analysis, (figure 9).

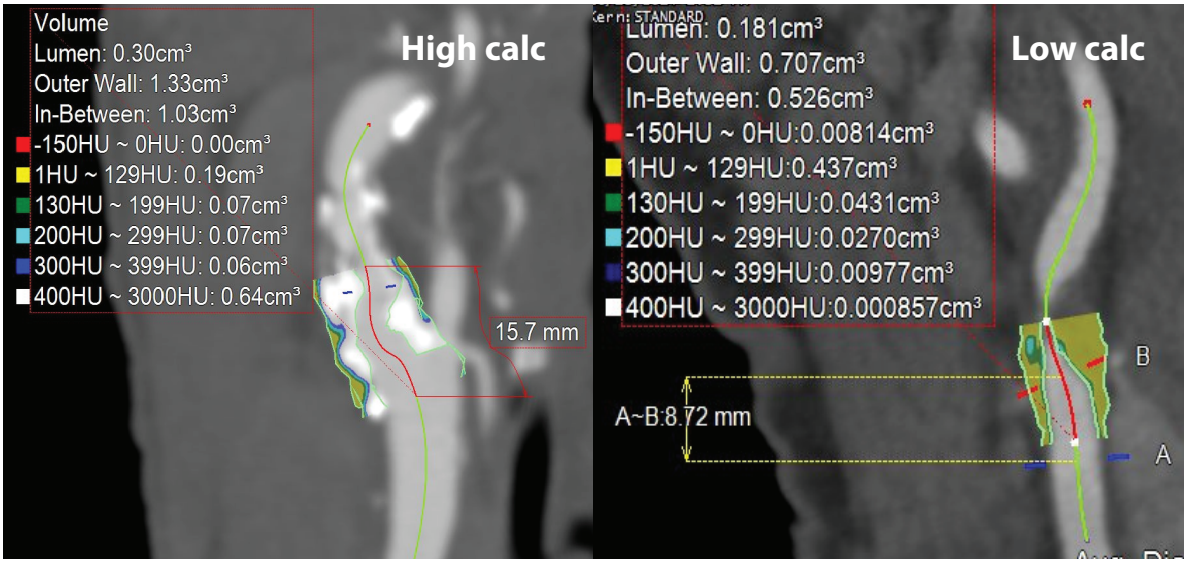


Figure 9 Examples of high calcified plaque (left) and low calcified plaque (right) analysed with TeraRecon iNuition, cases from study I.

vascuCAP

In the software vascuCAP many other measurands of the plaque can be obtained, both componential and structural characteristics. After the initial segmentation and manual adjustment, the plaque tissue composition analysis was performed which included additional components definition beyond calcification (CALC), namely lipid rich necrotic core (LRNC), intraplaque haemorrhage (IPH) and tissue not defined as any of the other, matrix (MATX), their volume and volume proportion. Furthermore, structural measurands such as plaque burden volume (ratio of the plaque volume of the total vessel volume), and minimum cap thickness (the shortest distance from LRNC to the lumen in the lesion) were obtained, (figure 10). When comparing the components with the gene expression in the matching plaque, the marked lesion was restricted to the most proximal half of the plaque in order to correlate the CTA measurements to the corresponding part of the plaque used in the microarray analysis. The correlation analysis between plaque morphology and clinical parameters such as diabetes, smoking, sex and laboratory results,

was done with the whole lesion included for calculations, considering the general/systemic nature of the clinical data.

One major difference between the two software is that TeraRecon holds a level of subjectivity due to only one evaluator, while in the histology validation of vascuCAP's plaque component analysis implicates a more objective analysis, even if the evaluator was the same.

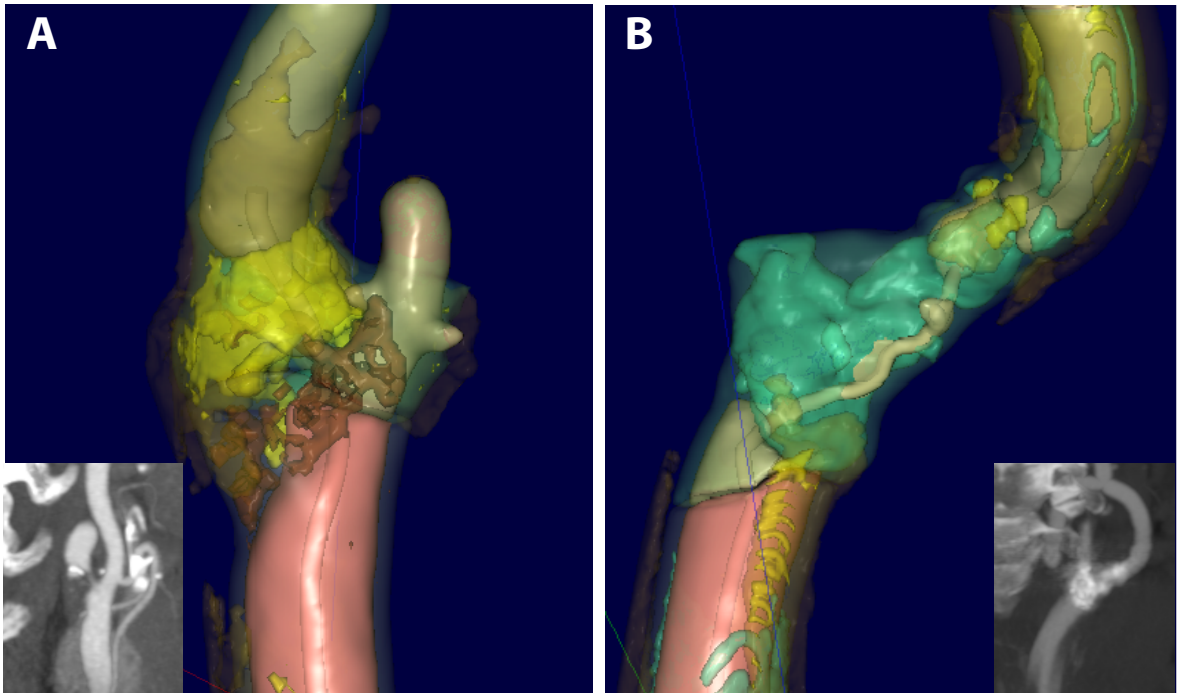


Figure 10 Examples of carotid plaque components measured with vascuCAP. One plaque with a large LRNC (yellow) and IPH (red) (A). One plaque with large calcifications (turquoise)(B). Same plaques in plain CTA in the recessed images.

Risk scoring of patients

In study III, evaluation of clinical risk scores was performed for each patient with a smartphone applications CAR and MDCalc (ABCD2). Neurological symptoms were classified as mentioned above. Both types of risk scores were stratified into low, intermediate and high risk. For ABCD2 this was 0-3 points low, 4-5 points intermediate and 6-7 points high risk. For CAR 5-10% low, 11-13% intermediate, 14-36% were considered as high risk of stroke.

Microarray profiling

The proximal half of the carotid plaque was sent for microarray profiling. RNA extracted from the tissue, was first controlled for suitable quality with respect to purity and integrity before it was sent to the core facility of Karolinska Institutet, BEA (Bioinformatics and Expression Analysis core facility), where the microarray profiling was performed, using the Thermo Fisher/Affymetrix, Human Transcriptome Array (HTA) 2.0 chip.

Bioinformatics and Statistical analysis

In order to investigate the transcriptome of the different plaque components in studies I and II, the quantification of each components was first performed and stratified into top and bottom.

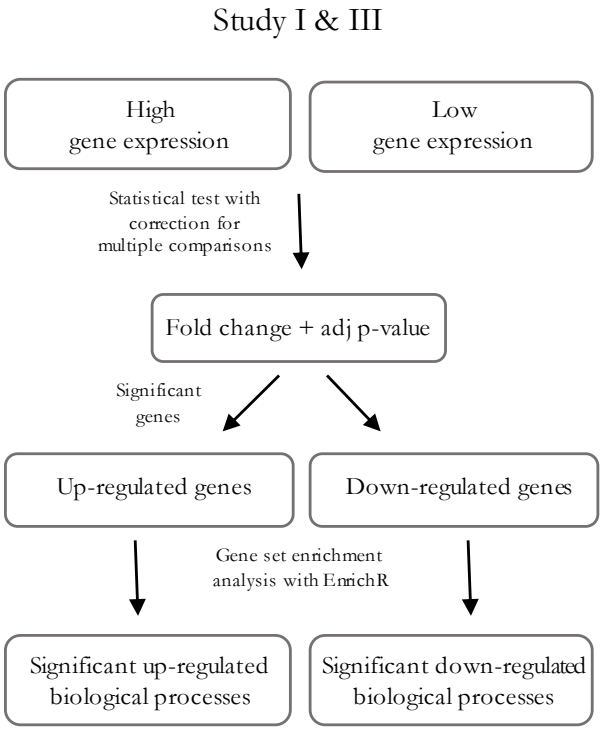


Figure 11 Bioinformatic and statistic workflow for study I & III.

With this approach, top *vs* bottom (10 *vs* 10 or 5 *vs* 5) patients whose plaques contained certain components were compared to each other.

In study I multiple two-sided unpaired Student's t-tests using the statistical software GraphPad Prism v.6 with correction for multiple comparisons according to Bonferroni rendered fold change and significance for each of the genes. The most differentially expressed genes were ranked in sorted lists according to fold change and the most up- and down-regulated genes were noted. Thereafter, the lists with differentially expressed genes were entered sequentially in a web based online tool (EnrichR), which assigned gene ontologies to each of the differentially expressed genes, and finally sorted them into enriched biological processes and pathways according to the lowest p-value. Overlapping ontologies and processes with p-value >0.05 were excluded using postprocessing in the Revigo public software. The bioinformatic analysis of study III was performed based on the same principle as in study I, comparing the microarray transcriptome of the high risk patients with the low risk patients of both ABCD2 and CAR. Workflow is depicted in (figure 11).

In study II the number of components were extended to LRNC, MATX, IPH in addition to CALC which was used in study I. Structural characteristics were also included such as Plaque Burden volume. In the same manner as described above the top ten were compared to the bottom ten of the quantification of each component. The array data of these two different phenotypes for comparison was fed into the Transcriptomics Analysis Console (Thermo Fischer Scientific) program rendering a list of genes that was filtered only to coding mRNA, excluding the X and Y chromosome bound genes since the groups were not matched for sex. The filtered genes were ordered in a ranked list, according to their differential expression between the phenotypes. This list was exported to the web-based analysis program Gene Set Enrichment Analysis (GSEA).

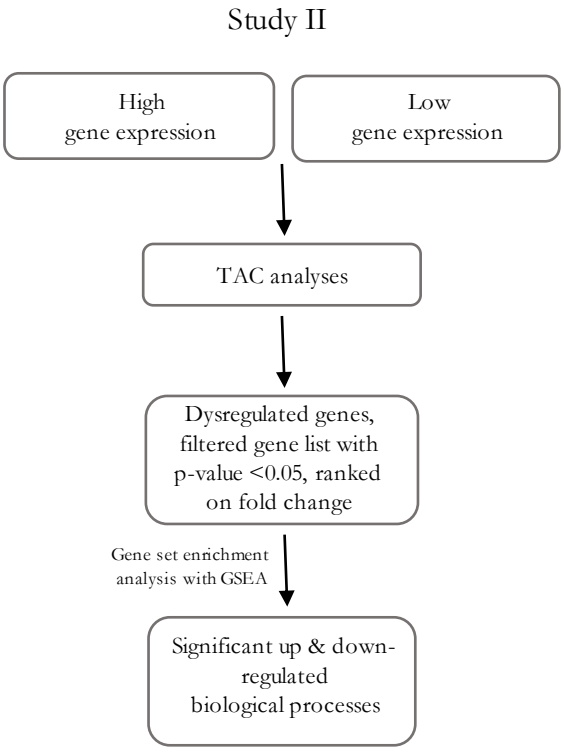


Figure 12 Bioinformatic and statistical workflow for study II.

GSEA rendered an enrichment score (ES) and a normalised ES (NES) with an estimate of statistical significance of the ES including adjustment for multiple hypothesis testing. In this way results of significantly up- and down-regulated gene sets associated with specified biological processes, were defined in comparison of the two different phenotypes.

To visualise the data comprehensively the lists of enriched gene sets were exported to the program Cytoscape with the plug-in application Enrichment Map (EM). EM allows visualisation of the results in clusters where the interacting biological processes are visualised in nodes and edges (lines). Nodes representing a pathway or biological process and edges the overlapping genes between the pathways. The size of the nodes correlates with the number of genes in that pathway, i.e. a larger gene set results in a larger node in the image. The thickness of edges (lines) associates with the number of overlapping genes. The cut-off FDR q-value for the nodes was set to <0.005. The Cytoscape function AutoAnnotate with WordCloud labelled the clusters in standardised way, using algorithms to use the most common words in the involved nodes, though often necessary to edit manually into a more comprehensive context. This method of GSEA analysis with subsequent Cytoscape visualisation is described in detail in Nature Protocols by Reimand el al [161]. Workflow of study II is depicted in (figure 12).

Prediction modeling

In study IV, principally, the transcriptomics from the BiKE subcohort of 40 plaques were paired with the vascuCAP-data from CTAs as 'ground truth' for which prediction models were created, via machine intelligence. These trained models were then validated on additional four sequestered unseen patients, for the purpose of testing the models for comparing the predicted transcript expression with true expression.

From study I, 3387 differentially expressed genes associated with calcification, were selected together with additional 91 genes selected from previous studies[162,163] related to plaque instability and to atherosclerosis in general. All models were built with three levels of variation: (1) differing sets of morphological measurements according to *hypothesized physiological rationale*, meaning considering predictors for which a transcript may be reasonably related to avoid coincidental spurious associations(on all 3478 transcripts); (2) automated optimization using 10-fold cross validation while simultaneously varying tuning parameter values (on all 3478 transcripts); and (3) data was partitioned such that a training set on which the cross-validation was performed was strictly separated from a sequestered validation data set to test performance using locked-down models. Both supervised and unsupervised methods were used. Initially supervised models were created as mentioned above for each transcript where the model quality (MQ) was computed using concordance correlation coefficient and regression slope of predicted *vs* observed continuous value estimation, and for dichotomised categoric prediction models the MQ was computed as the product of the area under the receiver characteristic curve times Kappa. The cut-off for robustly predicted transcripts were set to MQ >0.15 (e.g. AUC 0.75 and Kappa 0.2). The AUC states how much the model is capable of distinguishing between classes, but leaves out information of well it predicts for different types of outcomes. The Kappa value picks up on that and leaves information of how well the majority and minority class agreement is, where a high Kappa value excludes skewness (i.e. the ability to predict both outcomes with same exactness even of one of them has a

scarcer outcome). Those transcripts that exceeded 0.4 were classified as particularly robustly predicted and were further analysed with GSEA via EnrichR for gene ontology biological processes with adjusted $p<0.05$. Subsequently, unsupervised models of clustering were created to get a grasp of the associations between plaque morphology and expression levels of the transcripts, creating heatmaps including genes with highest and lowest expression. The models were then locked down and tested on four previous unseen patients, to validate the performance. Finally, the transcripts robustly predicted from the CTA-analysis in the test cohort were analysed with GSEA to reach a patient-specific profile of the on-going biological processes in the carotid plaque at the time of the CEA.

Immunohistochemistry and histology

In studies I and IV immunohistochemistry on carotid plaques was performed as to validate and locate the novel genes discovered in the context of atherosclerosis, PRG4 and ABCB5. Antibodies for these two genes were used in respective studies together with well-established markers for, e.g. smooth muscle cells (SMA, PDLIM7, LMOD1), macrophages/inflammation (CD68, tryptase), osteoclasts (TRAP), endothelial cells (vWF), in addition to other histological stainings for elastin (Weigert), collagen (Masson Trichrome) and proteoglycan/collagen (Movat pentachrome) in order to map the co-localisation.

In study II, histological stainings were performed to exemplify the typical type of plaque, e.g. calcified, lipid rich and containing haemorrhage, which were identified with Alizarin Red, Oil-Red-O and Perl's respectively to mark their specific traits.

Ethical considerations

Informed consent regarding all material and data was collected from all patients, organ donors or their guardians included in the BiKE biobank. The material and data from the patients were anonymised, were assigned an ID-number where the key for the personal information was securely stored, locked with password, only accessible by a couple of persons in our group. All studies were approved by the Research Ethics Committee at the Karolinska Institutet, Stockholm, Sweden.

Studies are performed with the following ethical permit numbers: BiKE EPN DNr: 95-277, 95-276, 01-199, 02-146, 02-147, 2009/295-31/2, 2011/950-32, 2013/2137-32, 2017/508-32, 2018/954-32, 2020-00274. Kärl TX: 2008/1748-31/2. Permit for Prospective Tissue collection Karolinska (PPK): 2009/512-31/2.

Tissue and blood sampling are conducted as part of the ordinary medical and surgical procedures and do not put the patients at unnecessary risk.

5 RESULTS AND DISCUSSION

Atherosclerotic cardiovascular disease, i.e. stroke and myocardial infarction, is increasing and is causing major morbidity and mortality worldwide. Carotid stenosis is a common and preventable cause of ischemic stroke, where the paucity of diagnostic methods of identifying the unstable atherosclerotic plaque is a desirable gap to fill. Only taking stenosis degree and symptom into consideration in assessment of patients with carotid stenosis have been partially successful, but the imaging technology accessible today can improve the accuracy considerably not the least for patients without symptoms or with non-significant stenosis where the recommendations from the large ECST and NASCET studies do not apply. In this PhD project we aimed to improve the accuracy of diagnosing the vulnerable atherosclerotic plaque through shifting focus from luminal stenosis degree to the on-going biology in plaque, correlating this to the CTA images, i.e. plaque morphology. In study I and II, CTA imaging data were correlated to the transcriptomes of the carotid plaques (the plaque phenotype). In study III the patient clinical history i.e. patient phenotype, was correlated to the transcriptomes and to CTA imaging data. In study IV, prediction models were created with machine learning which can link the transcriptome with the plaque morphology in order to predict the on-going biological processes on a patient specific level from the CTA analysis.

Plaque morphology

In both study I and II, our starting point was the plaque morphology as assessed by analysis of pre-operative CTA, linked with the transcriptome of the corresponding CEA specimen; first with calcification in focus and subsequently how the other plaque components and structural traits of the plaque associated with the plaque phenotype.

Calcification – a plaque stabilising factor

As a first step we wanted to investigate what calcification in atherosclerotic plaques assessed on pre-operative CTAs represent as for the phenotype of the plaque. In study I, in global gene expression analysis comparing high *vs* low calcified plaques we found a large number of differentially expressed genes. The up-regulated genes were interestingly clearly dominated by genes typical for smooth muscle cell contractility such as CNN1 (calponin1), ACTA2 (Actin Alpha 2) and MYOCD (myocardin) together with genes typical for extracellular matrix such as ELN (elastin), collagen, integrins and proteoglycans, where PRG4 was the top upregulated gene in the most calcified plaques. The most down-regulated genes were dominantly macrophages markers such as CD68, CD36, markers for lipid metabolism e.g. LPL, APOC1, PLIN1, degradation of extracellular matrix e.g. MMPs (no 7, 8, 9, 12) and chemokines such as CXCR4, IL8 (figure 13).

Both MMP 8, 9 and has in previous studies shown association with plaque vulnerability[164] as well as CXCR4 and IL8[165]

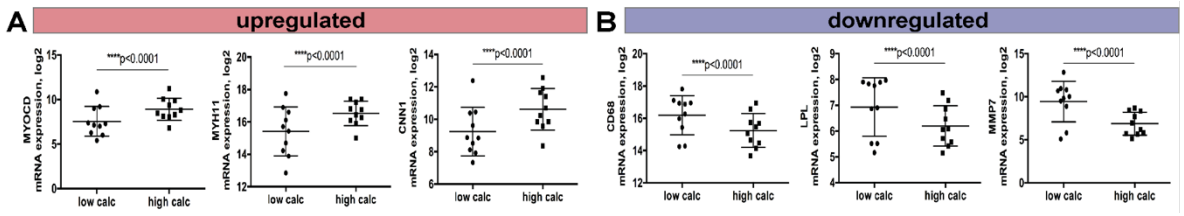


Figure 13 Up-regulated (A) and down-regulated genes (B) in highly calcified plaques compared to low.

Moving on the biological processes same patterns was seen with upregulated processes of smooth muscle cell contraction, Ca-signaling and calcification, osteoblasts and chondrocytes, (figure 14). As opposed to the repressed biological pathways which consisted of inflammation, ECM degradation, cholesterol metabolism and cytokines response where the most repressed processes were response to TNF, IL1, IFNg, phagocytosis and chemokine-mediated signaling.

In a clinical setting, elastin content of the carotid plaque has been proven to be important where a decreased level of elastin was associated with ipsilateral stroke and consequently plaque instability[166], same relation of decreased elastin content was seen when comparing symptomatic patients *vs* asymptomatic[167]. The repressed inflammatory profile of the calcified plaque also suggested a stabile phenotype.

Figure 14 Biological up-regulated processes in high calcified carotid plaques compared to low calcified, size of the piece of the pie indicates the enrichment

Overall, the calcified plaque emerged as a strong phenotype where high and low calcification could be 100% separated by a panel of only 20 genes in a prediction model. As a context, our research group had previously shown that a panel of 30 genes could separate symptomatic from asymptomatic with 78% precision[160]. This could conclude that the presence of macro-calcification on CTA could improve the prediction of stroke risk in patients with carotid stenosis[84]. Microcalcification, which is according to previous studies associated with unstable atherosclerosis[60,76], is not studied within this research project. Another carotid plaque trait that has gained attention recent years is the adventitial calcification with internal soft plaque, or the 'rim sign'. It has shown close association with IPH and therefore plaque instability[87]. Furthermore, several studies have shown that small calcifications, especially close to the lumen, could increase circumferential stress and contribute to plaque rupture[86,168,169] The location of calcification was not objectively and systemically registered in study I and II, partly because of the challenges of stratifying it in a standardised repeatable approach. However, the localisation of the calcification

in the plaque would certainly be of high interest as an extension for future projects, especially if it could be internalised in the semiautomated segmentation and analysis of the software.

When applying the same analyses onto the subgroup of only symptomatic patients a more heterogenous profile was revealed with signs of upregulation of both intraplaque haemorrhage and enrichment of ECM organisation and elastic fibre formation. However, inflammation was repressed in the same plaques confirming the stabilising phenotype that calcification most probably infer. These are intriguing findings, with processes associated with both stabilisation and destabilisation. One reasonable answer could be the fact how the cohort was selected, with the high calcification grade in focus with both symptomatic and asymptomatic patients not embodying a representative sample of the population, a typical atherosclerotic plaque is likely to be compounded with a large diversity of components[170]. In addition, this subcohort of high calcification was small, comparing 7 symptomatic vs 7 asymptomatic patients, most probably contributing to the somewhat incongruous results.

To evaluate the association of calcification and smooth muscle cell differentiation, we investigated the association between of ACTA2 and calcification which was positive, ACTA2 being an established marker for smooth muscle cells in the contractility phase. SMCs have in previous reports shown to transit from a contractile phase expressing the typical markers of ACTA2, MYH11 or CNN1 to other lineages of cells such as chondrocytes and osteoblast-like and adipocyte-like cells in atherosclerotic disease, resulting in production of calcification, matrix proteins or lipid accumulation losing the differentiated function of a contractile SMCs[171]. In our study, we could confirm that SMCs in a contractile state co-existed with macro-calcification, suggesting a transit back to the differentiated phase expressing not only ACTA2 but also the more sensitive markers of differentiated SMCs: PDLIM7 and LMOD1[47], (figure 14). In addition, we found a strong association between the genes ELN (elastin), COL1A1 (collagen) with ACTA2 (actin alpha 2, smooth muscle) also suggesting a contractile state of SMC and therefore also stability. This relationship was validated in tissue micro arrays where a positive association was shown between collagen and calcification as well as between elastin, collagen and SMA content. Furthermore, the same association was confirmed in medial calcification in biopsies from the epigastric artery from chronic kidney disease patients suffering from the Mönckebergs sclerosis. These findings could also be confirmed in histology stainings (see figure 15). To the best of my

knowledge, this is the first study to show that SMCs can return to or exist in a differentiated state in advanced atherosclerotic calcified lesions in humans.

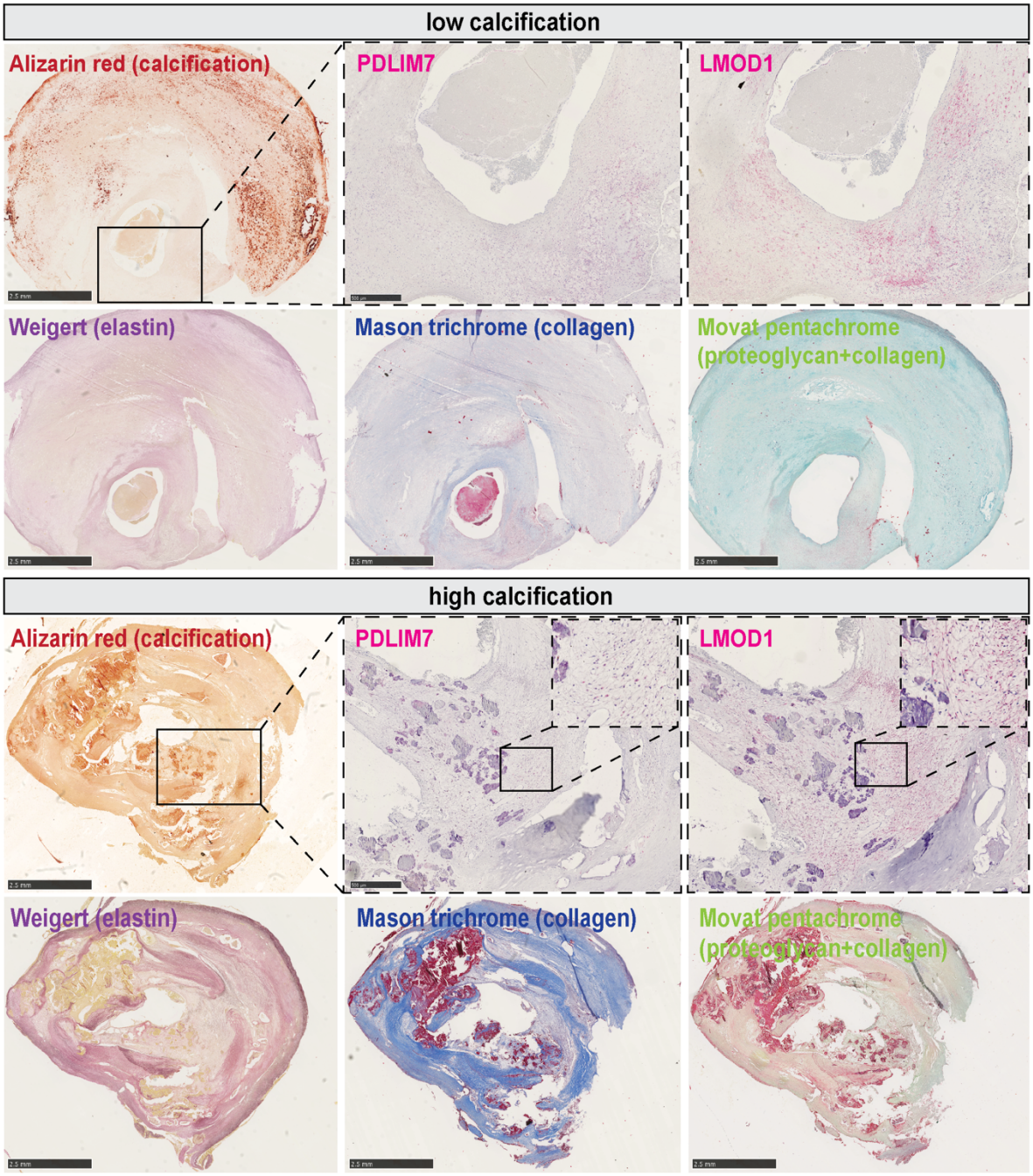


Figure 15 Histological stainings showed abundant signal for SMC sensitive markers (PDLIM7, LMOD1) in high calcified plaques. Calcification (red) elastin (dark purple), collagen (blue), proteoglycan + collagen (green).

Proteoglycan 4 (PRG4) was discovered in study I as the most up-regulated gene in the highly calcified plaques. This gene has not earlier been demonstrated in atherosclerotic disease. The gene PRG4 is coding for a glycoprotein produced by chondrocytes and synovial fibroblasts primarily in joint surfaces and functioning as a lubricant. It has also been shown to inhibit inflammation[172–174], fibrosis[175] and possibly a positive factor in patency of venous grafts via inhibiting migration of venous SMCs in the media layer of the vessel[176]. In study I, we found that PRG4 co-localised in the ECM with osteopontin (an ECM-protein in bone produced by osteoblasts), and osteocalcin,

(a marker for bone formation and osteoblasts). In immunohistochemistry stainings we saw PRG4 localised with CD68(macrophage marker)+ and TRAP(osteoclast marker)+ cells surrounding calcification nodules in the intimal calcification suggesting it being a part of the calcification process not unlike bone formation. Subsequently, we studied how PRG4 co-existed with SOX9 and RUNX2 (both markers for chondrocytes) and found a positive correlation suggesting a connection with chondrocytes producing proteoglycan-rich collagenous matrix, these chondrocyte-like cells most likely originated from SMCs undergone transdifferentiation[171]. However, PRG4 could not be seen in medial calcification.

Calcification of carotid plaques and its transcriptome was also studied in study II where the quantification was performed with another method, the result was similar in regards to gene expression profile association to stabilisation processes.

LRNC, IPH & Plaque Burden –an orchestration in plaque destabilisation

After mapping the transcriptome of calcification of atherosclerotic plaques, our curiosity of how other components' transcript could be mapped was awakened. With the software vascuCAP, other constituents of the plaques could be investigated. In study II, we stratified a cohort of 93 patients into subcohorts of either high (n=10 or 5) or low (n=10 or 5) content of other components, i.e. Lipid Rich Necrotic Core (LRNC), Intra Plaque Haemorrhage (IPH), calcification (CALC) matrix (MATX), and the structural feature Plaque Burden volume ratio as well as minimum Cap Thickness, (figure 16).

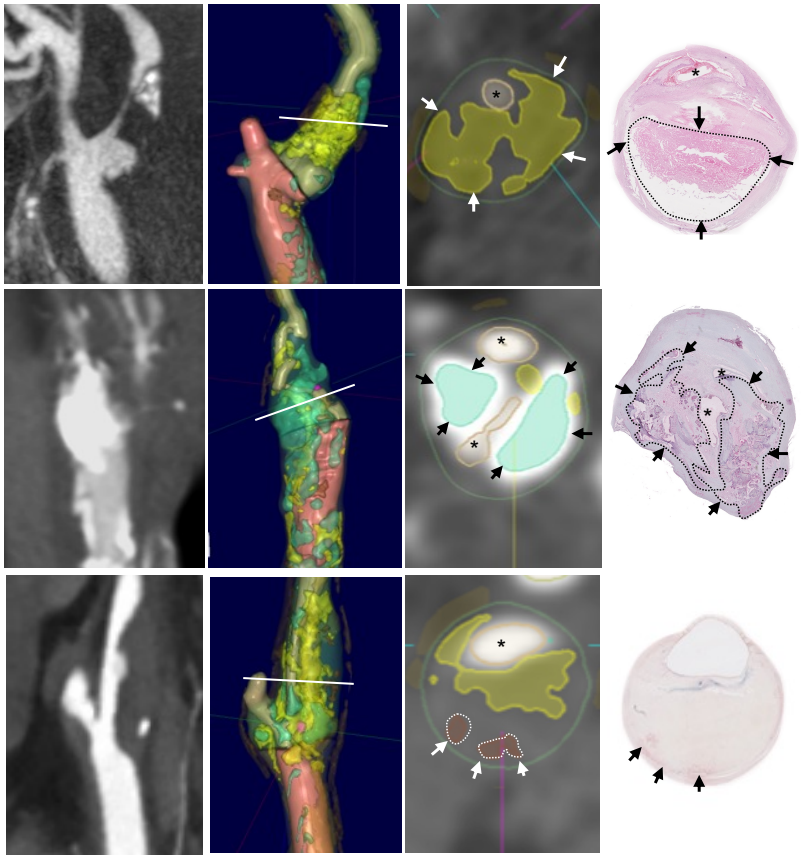


Figure 16 Examples of images from vascuCAP analysis, rows from top to bottom: LRNC, CALC, IPH. Columns from left to right plain CT, 3D images vascuCAP, axial views vascuCAP, corresponding histology slides.

Univariate correlation analyses of the components with clinical variables such as risk factors e.g. male sex, smoking, hypertension, use of statins, previous myocardial infarction, BMI, CRP or serum-creatinine did not show any relevant significant associations with the exception of that calcification volume and proportional volume significantly correlated positively with diabetes. This finding is in line with what is previously known; that patients with diabetes are predisposed to develop vascular calcification, most often medial calcification but

also as a part of a late stage atherosclerosis[177,178].

Global gene expression analysis was performed on forty of the patients. The transcriptomes of plaques rich of LRNC showed up-regulation of genes related to inflammation such as T-cells markers (CLECL1, CD28, TRGV5, 4 and 3), complement activation (C3), macrophages (CXADR, CLDN1) and pro-inflammatory mediators (S100A11), (figure 17). In line with other studies several of these genes have been related to inflammation in atheromatous plaques; C3[179], CXADR[180], S100A11[181] and T-cell associated genes[182]. Also, in the GSEA-analysis the enriched biological processes in the LRNC plaques were dominated with inflammation, cholesterol metabolism, ECM disassembly and resorption of bone, all of which contributing to destabilise the tissue more prone to rupture or erode with thromboembolus as a consequence. The most down-regulated processes were associated to calcification and cell proliferation. This finding could partly be explained by the inverse correlation between LRNC and CALC we saw. Nevertheless, it strengthens our findings from study I claiming macro-calcification as a stabilising phenotype, possibly due to inversely less volume LRNC. Moreover, these findings were strengthened by the outcome in univariate correlation analysis where LRNC volume associated significantly with neurological symptoms studying the whole cohort of 93 patients. In addition, volume of LRNC was one of the main predictors in the model of multivariables predicting neurological symptomatology. LRNC has previously been debated whether it can be detected in CTA[147,183] in the same way it has been validated in MRI[90] or as echolucency in ultrasound[184,185] and associated with increased stroke risk. In this study, LRNC detected on CTA analysed with histology validated software, was clearly connected with a genetic profile associated with inflammation and ECM degradation suggesting that CTA can be used in plaque vulnerability assessment.

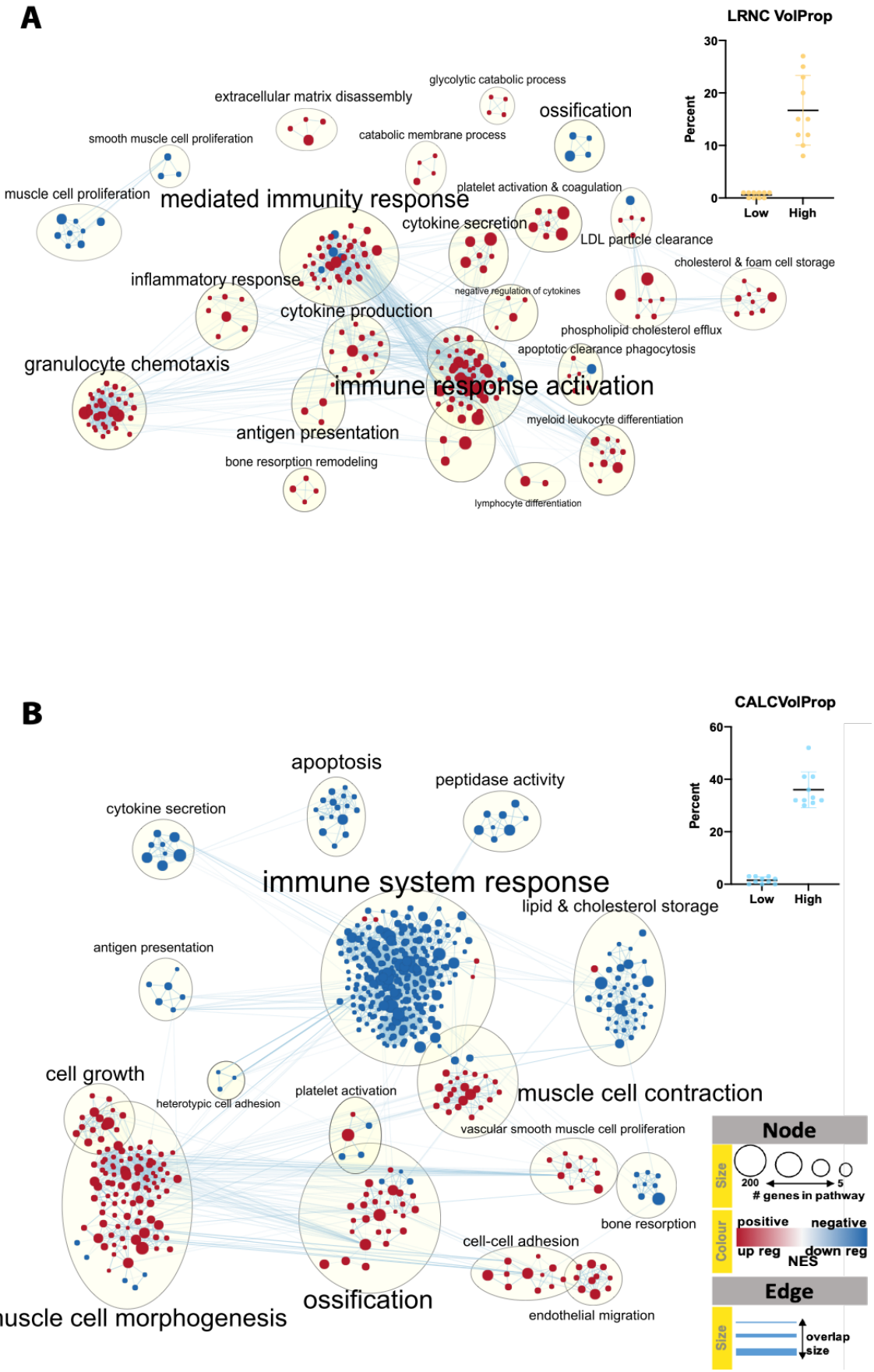


Figure 17 Gene Set Enrichment Analysis of gene ontologies and pathways of carotid plaques differences of high vs low LRNC (A) and CALC (B). Red nodes representing up-regulated and blue nodes down-regulated ontologies or pathways.

Another interesting component that has an important clinical interest is IPH. It is a well-known feature of plaque instability in clinical studies of relationship between plaque histology and symptoms[95,186] but has previously been difficult to identify on CTA due to overlapping HU with lipids and fibrous tissue. In our study we found that plaques analysed with high IPH proportional volume and volume also had a high presence of processes of inflammation, angiogenesis with genes related to platelets, immunoglobins, endothelial cells together with repression of ECM organisation and SMC migration and contraction. One interesting difference between the plaques with high LRNC compared with high IPH was Selectin E (SELE) which was one of the top up-regulated genes in the group of high IPH but one of the most down-regulated genes in the plaques with highest LRNC. SELE is a marker for endothelial cells, more specifically an adhesion molecule involved in the interaction of leukocytes and endothelium, known in atherosclerosis. One possible reason for this distinct difference between the two components could be the presence of endothelium in neo-angiogenesis, a factor that has been suggested a contributor to intraplaque haemorrhage[187,188]. LRNC however, consisting of necrotic debris from inflammation and apoptosis with no active endothelial cells had according to our findings a clear down-regulation of SELE. IPH had in this small study no significant correlation with symptomatology in the univariate analysis, but was together with LRNC and MATX one of the heaviest weighted predictors in the multivariate predictor modeling for symptomatology.

In addition to LRNC and IPH, plaque burden was another centrally interesting trait of the carotid plaque. Plaque burden and its volume has increasingly been discussed as a biomarker for plaque vulnerability[103,189,190]. It is important to understand that the degree of luminal stenosis not necessarily correlates to the plaque volume[191,192], explained with the positive remodeling the artery often undergo in the atherosclerotic transformation of the vessel wall. Plaque disruption followed by healing has been shown as a mechanism of increasing plaque burden in coronary arteries in clinical and histological studies[193–195]. In a clinical setting, in a prospective longitudinal study, progress of plaques volume independently associated with ipsilateral ischemic stroke[196]. But the biology behind this association is to our knowledge previously unknown. In study II, we found a slight correlation of plaque burden and calcification suggesting that calcified plaques often are bulky and represent a relatively large plaque burden. However, our results also showed that the gene expression profile of large plaque burden was mainly characterised by a clearly inflammatory with upregulation of MMPs, lipid and haemoglobin metabolism. The gene heme oxygenase 1 (HMOX1) was greatly up-regulated in the plaques with large burden with a fold change of nine. HMOX1 is coding for the protein heme oxygenase which is centrally involved in heme catabolism resulting in biliverdin as a metabolic product. A recent study from our group showed a link between HMOX1 and Biliverdin Reductase B (BLVRB) where it was identified as a biomarker of plaque instability. This was further validated in population samples with increased risk of future cardiovascular events [162]. Furthermore, processes related to SMCs and ossification were down-regulated in plaques with large burden. This genetic profile was similar to the plaques with most LRNC and distinctly different from the highly calcified, suggesting that plaque volume alone is not sufficient for assessment of vulnerability, but seems to require additional details of plaque components. Additionally, confirming plaque burden's central role of plaque vulnerability

assessment, we found a significant association between a large plaque burden with symptomatology in univariate correlation analysis.

When using plaque morphology data from CTAs in creating a prediction model for symptomatology, these data alone performed better than involving clinical variables and significantly better than stenosis degree alone. The model was also tested on thirty unseen patients where it performed reasonably well with AUROC 0.68 and Cohen's Kappa 0.37, which further reinforce the central position of plaque morphology itself in stroke risk assessment rather than simply focusing on the luminal stenosis.

Patient phenotype

How do clinical risk scores compare in plaque vulnerability assessment?

After mapping plaque phenotype in relation to plaque morphology, next step was to explore how clinical tools already existing in the clinical work today i.e. patient phenotype, associate with the plaque phenotype and its morphology in CTAs. The clinical risk scores ABCD2 respective CAR both gives an estimate of stroke risk within days to months respective 5 years. In study III, we correlated the scores to the on-going biology in the plaques but also in circulating PBMCs, as well as to the CTA images. 101 symptomatic patients were scored for both scoring systems, where the highest scored patients were compared with the lowest scored, resulting in 29 (5-10%) *vs* 29 (14-36%) for CAR score and 15 (0-3 points) *vs* 15 (6-7 points) for ABCD2 score, intermediate scored patients were excluded from the bioinformatic analysis, (figure 18).

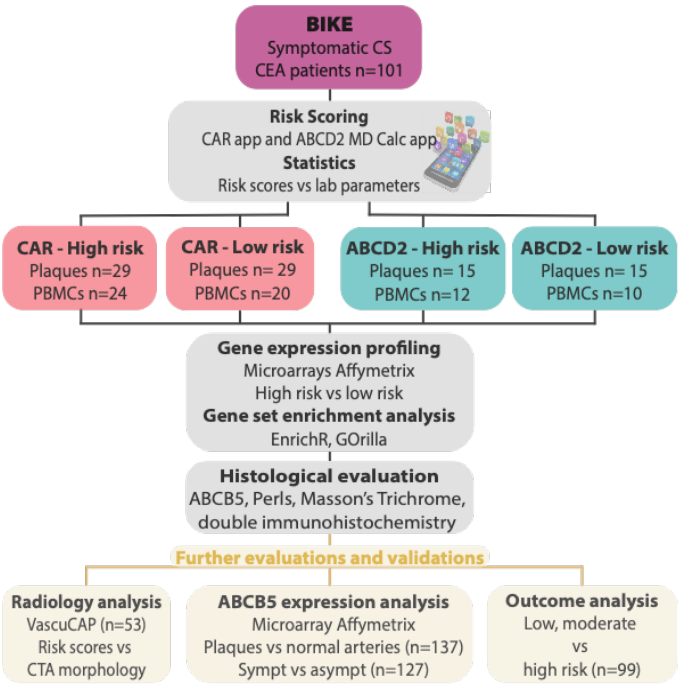


Figure 18 Work flow chart of study III. As indicated, the yellow boxes represent evaluations of the basic results of the study

The two scoring systems correlated reasonably well with each other with Pearson's r coefficient of 0.52, p <0.0001. This is quite surprising since the ABCD2 is developed for an outpatient clinic non-emergent situation predicting the stroke risk for patients' with TIAs with only five factors input, not specific for carotid stenosis. CAR on the other hand is more specific for predicting stroke risk for patients with carotid stenosis in a more emergent situation, using eleven factors input including the important factor of plaque ulceration. This correlation speaks in favour for the traditional idea of how important patient phenotype is in assessing stroke risk.

In global gene expression analysis with microarray comparing the high *vs* low risk score in CAR, the upregulated genes in the high scored plaques consisted of ABCB5 ion transporter as the top up-regulated gene but also genes coding for cytokines such as IL-8, scavenger receptor CD36 and

matrix metalloproteases MMP7 and MMP8 were among the most up-regulate genes. As examples, IL-8 is a proinflammatory protein which is known to be involved in cardiovascular disease and endothelial dysfunction together with angiogenesis, IL-8 being a potent angiogenic factor[197,198]. MMP8 in CEA specimens have been shown to have associations to vulnerability in plaques and cardiovascular outcome[164,199]. Among the downregulated genes we found actin cytoskeleton and SMC associated markers such as PDLIM7 known from our previous studies[47]. In the GSEA analysis, the up-regulated biological processes consisted of inflammation, foam cell differentiation and lipid transport, SMC and endothelial cell migration, all of which are known processes in more or less advanced atherosclerosis in general. Additionally, angiogenesis and coagulation, iron homeostasis and wound healing were up-regulated which represent processes associated with neovessel formation and IPH in particular[188]. The repressed biological processes were for example ECM organisation and progenitor cell differentiation. This kind of phenotype was recognisable from study II where plaques high of LRNC and IPH contained similar biological processes. When performing GSEA analyses of the CAR cohort on the PBMCs the induced processes were represented by inflammatory responses, cytokine mediated signaling, platelet activation, aggregation and degranulation. The gene expression profile of the PBMCs which seemed to reflect the biology of the plaques could be a hopeful indication that a serum biomarker for plaque vulnerability could become within reach.

In the same manner, analyses were performed on the ABCD2 cohort, where ABCB5 ion transporter interestingly again was among the top up-regulated genes together with bone matrix protein, leukocyte activating proteins, lipid metabolism associated genes. And down-regulated genes were associated with SMC; such as MYOCD, MYH10, and SOST where the latter is involved in inhibition of calcification. In the ABCD2 group, the top upregulated pathways in the GSEA analysis were neutrophil mediated immunity, foam cell differentiation, cholesterol transport and coagulation. The repressed processes consisted of ossification, chondrocyte differentiation, SMC migration and ECM organisation. In the PBMCs the pattern of increased inflammatory processes was consistent except that IL-6 and VEGF signaling were repressed. To summarise, the findings of the associations between an unstable plaque and blood phenotype and high risk scores/unstable patient phenotype are new to the field, but also confirms the results from study I and II.

The gene of ATP binding cassette subfamily B member 5 (ABCB5) was one of the top up-regulated genes in both scoring systems. It has never been associated previously with atherosclerosis or cardiovascular disease, but with melanoma, the protein product is involved in transport of e.g. glucose, bile salts, metal ions over the cell membrane. In study III, ABCB5 was further evaluated in extended cohorts comparing the expression within different phenotypes, where it was significantly upregulated in plaques compared to normal arterial wall as well as in symptomatic compared to asymptomatic patients. It also correlated positively with markers previously related to IPH, such as BLVRB and HMOX which previously has been studied in our research group[162]. In immunohistochemistry, the protein product of ABCB5 was localised in the necrotic core of the plaque, especially close to IPH areas and in neovessels stained with Perl's blue stain, co-localised to CD68+ macrophages. Since ABCB5 was one of the top genes in both of the

risk scoring groups, it is an interesting candidate gene to explore further as a biomarker for unstable atherosclerotic disease.

Comparing the risk scores to the plaque morphology in the CTA images analysed with vascuCAP we found a positive correlation between the component MATX and high risk score of both CAR and ABCD2. Consistently with this, the component of MATX showed a quite inflammatory profile with leukocyte activation and lipid metabolism and a repression of SMC proliferation and muscle contraction, in the bioinformatic analysis done in study II. Furthermore, we saw also a slight negative correlation with the proportional volume of calcification and CAR and with ABCD2 implicating the low risk patients had higher calcifications degree, which supports the findings in study I and II inferring macro calcification as a stabilising trait of the plaque.

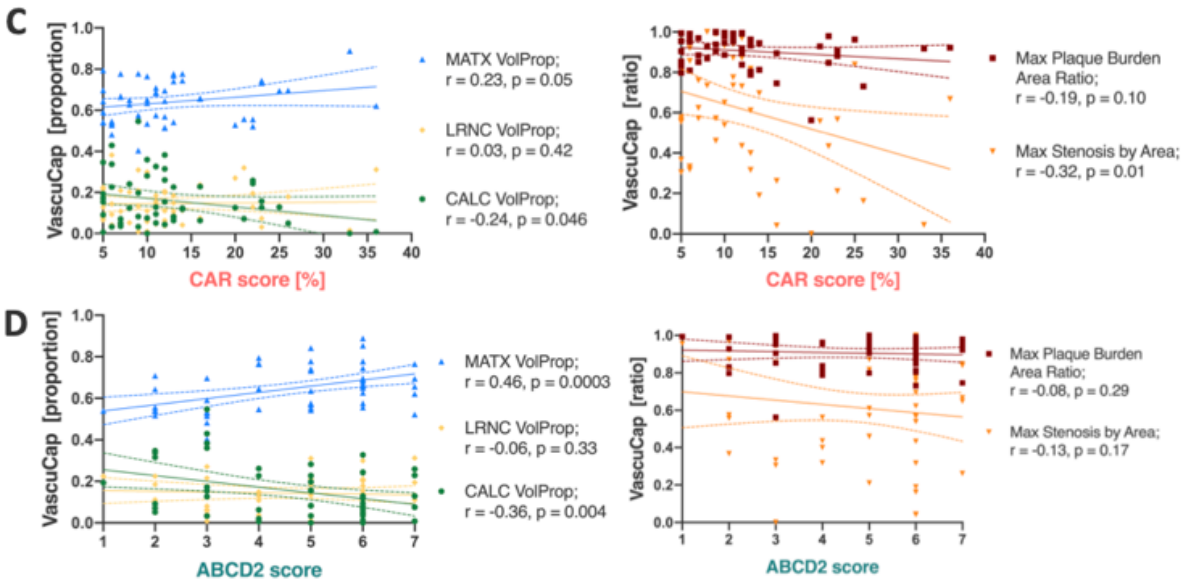


Figure 19 Scatter plots with correlations of plaque characteristics (components and structure) with CAR and ABCB2.

No significant correlations were seen between LRNC and neither of the risk scores, (figure 19). One reason to this quite surprising finding could be that this cohort consisted of only symptomatic patients with presumably unstable LRNC-containing plaques, in which the presence or absence of stabilising macrocalcification is crucial in defining the score; e.g. the more calcification the lower risk score confirming our hypothesis of macrocalcification being a stabilising factor. The stenosis degree, which is the main parameter used as per today in the daily clinical praxis, correlated to CAR but surprisingly in a negative fashion. Plaque burden did not correlate to any of the risk scores, even though it has been shown to have associations to stroke risk[79,103] as previously mentioned in study II. Three of the high risk plaque were studied in histology slides, contained no calcification or lipid content, but neovessels and iron deposits were plentiful confirming both the imaging and the bioinformatic findings.

Even if this study showed that symptomatic patients scored with high stroke risk correlated to plaque biology signifying more unstable lesions, and also associated with clinical features indicating increased risk, the clinical applicability of these findings are questionable. Given our results in study I, II and IV at our hands and the questioned utility of scoring systems for stroke such as ABCD2[150,200], imaging to assess plaque morphology seems far more relevant for vascular surgery

practise. Nevertheless, these risk scores still aid in stratification of symptomatic patients and are clearly useful in research, as demonstrated in our study for the identification of novel molecular targets such as ABCB5.

Personalised medicine

Machine learning for predicting plaque phenotype – the clinical tool we need in the future?

After exploring plaque and patient phenotypes in relation to plaque morphology we moved on to the quest of exploring whether the biological profile of the plaque can be predicted. The idea was to pair data from CTAs with the transcriptomes of the corresponding specimens and via help from machine learning create prediction models which extendedly could be used as a clinical tool to assess the plaque biology on a more patient-specific level. This was executed with help from previous studies (partly study I)[162,163] where 3478 genes related to calcification, plaque instability or atherosclerosis in general were selected as a base for the analysis. First, models were created from CTA data with output of each one of these transcripts. The models were considered robustly predicted when the model quality exceeded >0.4. This requirement was met for 414 transcripts, using both continuous and dichotomized inputs. In some cases, morphology alone generated the highest value in model quality and in some models adding the clinical features increased the performance. For instance, two transcripts were especially highly rated for model quality using morphology and clinical variables: interleukin-1 receptor 1 (IL1R1) associating with LRNC and transforming growth factor-β receptor type 2 (TGFBR2) linked with calcified lesions. These findings were validated in immunohistochemistry stainings of CEA specimens, (figure 20).

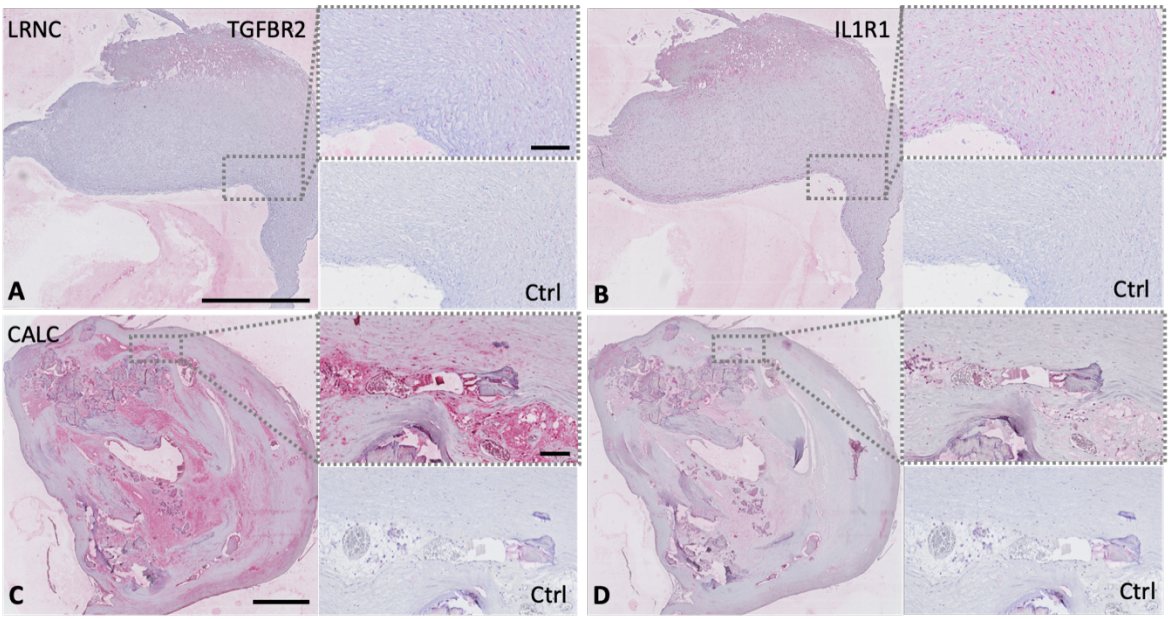


Figure 20 Immunohistochemistry of a LRNC rich plaque with TGFBR2 (A) and IL1R1 (B) and a plaque rich of calcification (CALC) with TGFBR2 (C) and IL1R1 (D)

The gene IL1R1 is coding for the cytokine interleukin-1 receptor IL1R1 and is an essential mediator of many immune and inflammatory cytokine-driven responses. Inhibiting interleukin-1B has been coupled to anti-inflammatory effect in atherosclerotic vascular disease[201] and has in animal studies shown a decrease in plaque progression[202]. Inhibiting IL-1B with antibodies (canakinumab)

has been subject of both preclinical and clinical trials investigating it as therapeutic strategy in patients different inflammatory disorders[203,204]. One large clinical randomised trial (CANTOS) of more than 10 000 high-risk patients with prior myocardial infarction and persistent inflammation were randomised to one of three doses of canakinumab or placebo. A dose of 150 mg canakinumab every 3rd months during two years resulted in a reduction of cardiovascular events without reduction of serum-lipids proving the inflammatory pathogenesis of atherosclerosis[205]. However, in a subsequent randomised trial of methotrexate *vs* placebo did not show any impact of methotrexate on levels of IL-1B, IL-6 or hs-CRP nor did it differ in the end points being nonfatal MI, stroke or cardiovascular death[206].

The TGFBR2 gene is coding for the receptor of TGFB which is an important mediator in cell proliferation, wound healing, cell cycle arrest and immunosuppression. It plays a significant role in cell integrity in the vessel wall and mutations in this gene associates with Marfan's syndrome, Loeys-Dietz syndrome 2 (both related to aortic aneurysm) and various tumour diseases. TGFB is considered as one of the most central regulators of ECM, with diminished fibrosis as a result of blocking it[207]. These functions of TGFB are well in line with our findings of the stabilising processes in the high calcified plaques from study I and II, rendering suggesting TGFB and its receptors as stabilising markers possible to robustly predict with CTA.

Out of the 414 robustly predicted transcripts, 237 were categorised as particularly robustly predicted transcripts and were further analysed with GSEA to clarify the ongoing biological processes. This analysis showed several fundamental biological processes related to atherosclerosis, such as collagen degradation, SMC proliferation, ECM organisation, apoptosis, phospholipid and cholesterol efflux and neutrophil mediated immune response. More specifically, for example LRNC was coupled to biological processes associated with inflammation and ECM degradation, confirming the results in study II.

Subsequently, unsupervised clustering of CTA measures and these 414 transcripts was performed to get an understanding of the relationship between the morphology and expression levels, creating heatmaps for each type of the components, (figure 21). In this analysis, the proportional and absolute volume of different components were coupled to the 414 transcripts where the association between high CALC and PRG4 again was confirmed as from study I and II, high LRNC was related to MMP12 and IPH was coupled to BLVRB (Biliverdin reductase B) previously studied by our group as a

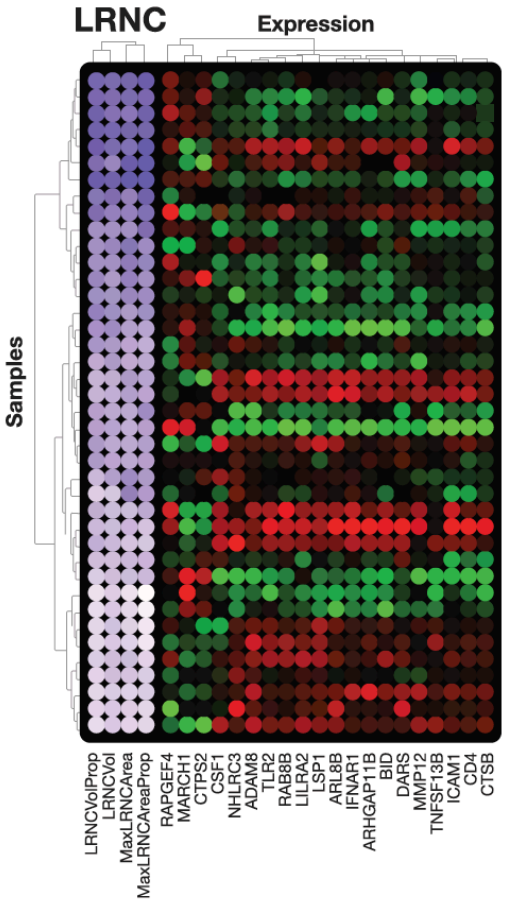


Figure 21 Example of the unsupervised clustering analysis here showing the 20 of the most up-(green) and down(red)-regulatea (black indicates intermediate) genes of the component LRNC, volume, maximum area, proportional volume and area of the component LRNC are depicted in purple.

biomarker for IPH[162] (as discussed above under plaque morphology). MATX was not as clear in the associations with a specific profile, but more nuanced possibly depending on a more heterogenic tissue type.

The predictive ability of for example LRNC and IPH as morphological biomarkers to predict transcripts known to have associations to plaque vulnerability such as MMP12[208,209], MMP8[199], IL1B[201] and BLVRB[162] is of high interest both in diagnostics of the vulnerable plaque but also in drug development.

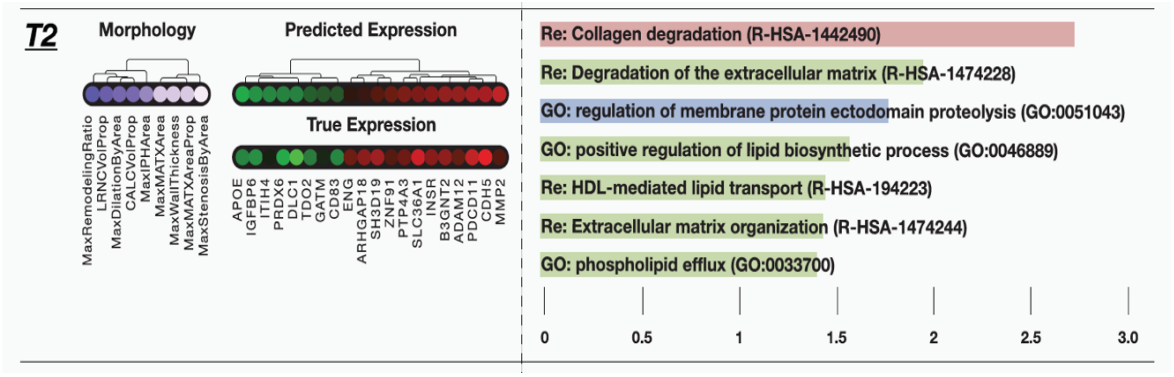


Figure 22 Performance using locked-down models on (unseen) test patient, here exemplified by test patient T2. Heatmaps for the test patient representing: morphology (the most important morphology variables) in purple, the predicted gene expression of the 20 most significant genes according to the best performing prediction models and the true gene expression according to the microarray results. To the right is the GSEA analysis of the predicted expressions making the results more accessible and possible to adapt the treatment in a patient specific approach.

Finally, the predictive models were tested on four previously unseen patients where the level of transcripts of the predictive models were compared to the true expression, where the most significantly dysregulated transcripts were analysed with GSEA and showed a unique biological profile. In figure 22, one example of predicted transcript made from the plaque morphology is shown. This plaque was high in LRNC, low MATX and intermediate levels of CALC and IPH, resulting in gene profile of seven significant processes: e.g. degradation of collagen and ECM, phospholipid efflux and HDL-mediated lipid transport. These kinds of results, when validated in larger studies, can contribute developing precision medicine with patient-tailored therapy which infers a great improvement in comparison to therapies of today where indications of treatments are based on principles of large groups taking little or no notice of the individual patient and plaque phenotype. Prediction models that can link the transcriptome with the plaque morphology in order to predict the on-going biological processes on a patient specific level from the CTA analysis, could have the capacity of tailoring the best therapy for an individual patient e.g. surgery, ASA, statins, PCSK9 inhibitors and/or canakinumab.

6 CONCLUSIONS

The results of the studies in this PhD project show that biological processes in carotid plaques associated to vulnerability, can be linked to plaque morphology analysed with CTA image analysis. Patient phenotype classified with clinical risk scores associates to plaque phenotype and morphology in CTA. The biological processes in the atherosclerotic plaque can even be predicted with plaque morphology CTA analysis in this small pilot study, providing a possibility to precision medicine after validation in larger scale studies. Clinical implementation of these methods and results could lead to improving the diagnostic accuracy of the vulnerable carotid plaque, consequently decreasing NNT for carotid surgery and also improving medical therapy for the individual patient.

I. Calcification in carotid plaques associates with a lesion-stabilizing transcriptome and smooth muscle cell function.
II. Plaque morphology in CTA can help assess plaque molecular phenotype and reflect e.g. inflammatory, cholesterol and tissue degradation processes but also smooth muscle cell function.
III. Assessment of plaque morphology by CTA is suggested as superior in prediction of stroke risk compared to other clinically accepted parameters (e.g. stenosis degree).
IV. High clinical risk scores are related to molecular processes previously associated with plaque vulnerability.
V. High clinical risk scores are associated with certain components of the plaque assessed with CTA.
VI. Genes, not previously known associated with atherosclerosis were identified having a association with atherosclerosis; PRG4 and ABCB5.
VII. Phenotyping atherosclerotic plaques by CTA imaging can clarify the molecular signature of atherosclerotic lesions in a multi-scale setting, which could serve as a base for optimized personalised therapy in the prevention of myocardial infarction and ischemic stroke.

7 POINTS OF PERSPECTIVE

Modern management of carotid stenosis

Routinely, imaging of carotid disease in Sweden is currently consisting mainly of US and CTA. In most centres the indication for CEA is still depending on a stenosis degree above 50-70% (NASCET) and the occurrence of neurological symptoms, but assessing the plaque morphology has increased in interest, especially after recommendations in the ESVS guidelines 2017 which brings up certain imaging features associated with an increased risk of stroke: large plaque area, large juxta-luminal black area in US, plaque echolucency in US, IPH in MRI[210]. The guidelines also recommend that patients with <50% stenosis could possibly be considered for re-vascularisation if a plaque is causing iterative strokes, is visualised with a large LRNC (US, MR, possibly CTA), have ulceration (US, CTA, MRI) or intraplaque haemorrhage (MRI) as practiced in some centres.

However, in order to accommodate guidelines with respect to these plaque features, associations to future risk of stroke or myocardial infarction has to be established and large prospective studies are ongoing to examine the predictive value of these features: PARISK (Plaque At Risk), ARIC (Atherosclerosis Risk in Communities), CARE-II (Chinese Atherosclerosis Risk Evaluation-Phase II), CAPIAS (Carotid Plaque Imaging in Acute Stroke) and CAIN (Canadian Atherosclerosis Imaging Network)[211–215]. The outcome of these studies will most probably change the management of carotid disease and lead to inclusion of assessment of plaque morphology in clinical practise.

Meanwhile, it is important to further explore and validate the potential of the diagnostic tools we have available today. The development of multi-energy CT, which is increasingly accessible, is generating large amount of data possible to use in analytical software using algorithms and machine learning capabilities after semi-automated segmentation of the vessel. The methodology explored in this PhD project, rendered interesting results, is promptly applicable in the clinical praxis as a guiding tool as of today. However, validation of the methodology in a clinical setting where its predictive power can be established in large, prospective clinical studies is highly warranted.

Strengths and weaknesses

The studies of this PhD project are methodologically unique in correlating the CTA plaque morphology to the global gene expression signatures of the ongoing biology in the plaque. Several studies have connected plaque morphology to stroke risk in the recent years, but linking plaque morphology to the global gene expression profile and the ongoing biological processes has never been done before, and throughout this thesis, add support for the ability of detailed plaque morphology to capture biological processes relevant for assessment of plaque (in)stability. The findings of these studies could readily be applied to atherosclerosis in other vascular beds such as the coronary arteries, where actual plaque specimens are not available as coronary plaques rarely get excised, but instead are stented or by-passed. In this way, the carotid disease has a special value since it provides opportunities to link imaging to the disease on a molecular and cellular level.

However, our studies have limitations. First of all, the transcriptomic cohort was selected from a calcification perspective, selecting the high and the low calcified plaques from patients undergoing

CEA at our unit, primarily selected for study I. This could skew the findings as the cohort was more representative of calcified plaques than other components, and fewer plaques were left to study the other components rendering a rather small sample size. This size was probably the reason for the lack of significantly differentially expressed single genes in study II for components of LRNC, MATX, IPH, but rendered a substantial number of significantly differentially expressed genes studying calcification. We believe that calcification is a relatively strong phenotype, when comparing it with, for instance, a group of plaques from of symptomatic *vs* asymptomatic patients. However, the sample size was larger when studying the correlation between clinical variables and morphology in study II, for instance the univariate correlation to symptomatology. In study III a rather large cohort was scored (n=101) but only the high and low risk patients were selected for further analysis, also losing power in significant correlations.

Another limitation is that there was only one reader of the CTAs and vessel segmentations in all of the studies without traditional intra- or interobserver validation. Conversely, both of the analysis software are semi-automated programs made for, and validated for, different users, where vascuCAP is histology-validated, expectantly mitigating the possible subjectivity. In the preparation of the analysis with vascuCAP, a quality assurance protocol was followed to set standard quality of the analyses, excluding the cases that did not meet the quality criteria, e.g. movement- and dental artefacts etc.

An additional weakness is how we limited the marked lesion of interest in the CTA analysis program to its proximal half, in order to match measurements to the corresponding tissue used in the microarray data analysis. This issue was dealt with in a standardised way, always starting the analyses proximally by the border between healthy and diseased vessel, going up until the culprit point of the plaque or stenosis. The whole lesion (from healthy to healthy vessel wall) was used in analysis of clinical variables and plaque morphology. Also, only CTA with contrast administration and no native series was performed, which in theory could interfere with calcification in the plaque. The border between these two similar attenuations could however be visualised by changing the windows width and level and edited manually.

In study III, only fifty of the scored patients had a CTA done, presumably mitigating possible correlations between risk scores and plaque morphology, probably requiring more power to detect significance in trends.

Future applications

Up until now, most patients with carotid stenosis are assessed by simply measuring the degree of luminal stenosis and symptomatology. But a paradigm shift is emerging with a focus towards the actual underlying disease, the unstable lesion and applying quickly developing imaging technology together with known features of plaque instability. In this PhD project, all results point to the fact that the degree of stenosis is blunt and unprecise for risk prediction and inferior to assessment of plaque morphology and biology, but also to risk scoring. The gene expression profile of the tightest stenosis did not show any convincing association to enrichment of uniform biological processes in study II. In addition, the stenosis degree was actually negatively correlated to high risk score (CAR). Finally, in study IV, the created prediction models performed better when using the input

from plaque morphology data sometimes together with the clinical variables but always superior to stenosis degree alone.

In order to launch the method of plaque morphology analysis as a part of diagnosing the vulnerable plaque in a clinical setting, a larger scale longitudinal study is necessary where asymptomatic patients not undergoing surgery would be assessed by CTA plaque analysis and subsequently followed prospectively in regards to end-points such as ischemic ipsilateral ischemic stroke, and at the same time compared with other risk predictors such as the degree of stenosis.

Another development of the method would be to evaluate how the position of macro calcification in the plaque influence vulnerability and risk, both in regards to adventitial calcification (the 'rim sign') or calcification in proximity to the lumen which both could implicate plaque vulnerability. CTA is undoubtedly superior to ultrasound in this regard, rendering spatial information (location and extent) of the plaque and its' calcifications.

In an ideal future, I foresee that patients with a vulnerable plaque will be detected *before* they become symptomatic, and suffer either a stroke or a myocardial infarction. I envision that this could be achieved as outlined in (figure 23). A patient with cardiovascular risk factors will get a work-up done with blood samples for detection of serum biomarkers capable of determining plaque vulnerability, together with a CT scan of neck vessels or coronaries, which, in case atherosclerotic plaque are found, go through plaque analysis with machine learning. Biomarkers and virtual transcriptomics from plaque imaging, can be used for a qualified estimation of the plaque biology and its' vulnerability. This estimation would be an advanced guide for the clinician in deciding what is the most adequate type of treatment for this particular patient, i.e., patient-specific medication(s) with or without surgery. Through this improvement of stroke risk prediction and individualised treatment of patients with carotid atherosclerosis the two main aims of this thesis would be reached, explicitly: 1) lowering the NNT of carotid surgery closer to one; 2) adapting the required medications to that patients' particular biology.

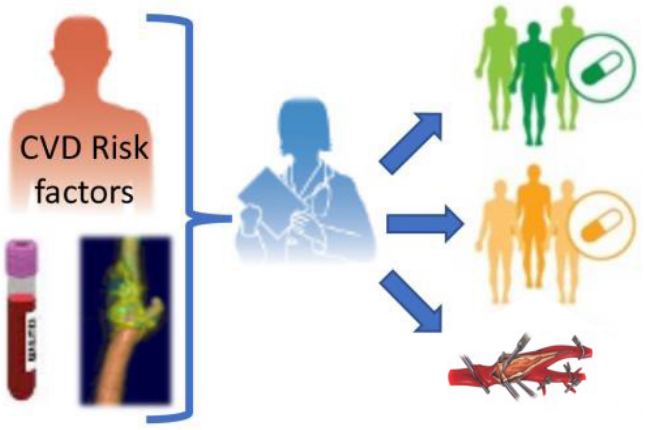


Figure 23 Idea of how future assessment of carotid atherosclerosis can develop, improving accuracy of both diagnostics and treatment

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* *borrowed from Winston Churchill, 1942*

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