

Carotid atherosclerosis: imaging and indicators of plaque instability

Thesis for the degree of philosophiae doctor (Ph.D.)

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TABLE OF CONTENTS

1. PREFACE

- 1.1 Acknowledgements
- 1.2 Abbreviations
- 1.3 List of papers

2. GENERAL INTRODUCTION -Atherosclerosis

- 2.1 The epidemiology of atherosclerosis and cardiovascular disease
- 2.2 The concept of carotid atherosclerosis: from a passive cholesterol storage-disease to an active generalized inflammatory disorder
- 2.3 The pathophysiology of atherosclerosis
- 2.4 Imaging of carotid atherosclerosis and review of current literature
- 2.5 Carotid atherosclerosis and the assessment of the unstable carotid artery plaque/stroke risk in current clinical practice

3. AIMS OF THE PRESENT STUDY

Research questions (1-4)

4. METHODS AND SUBJECTS

- 4.1 The unstable carotid artery plaque study- Papers 1-4
- 4.2 Statistical methods
- 4.3 Ethical issues

5. SUMMARY OF PAPERS

- 5.1 Paper 1
- 5.2 Paper 2
- 5.3 Paper 3
- 5.4 Paper 4

6. GENERAL DISCUSSION

- 6.1 Ultrasound imaging of carotid atherosclerosis

- 6.2 PET imaging of carotid atherosclerosis
- 6.3 MRI of carotid atherosclerosis
- 6.4 Carotid atherosclerosis and cardiovascular risk beyond traditional risk factors
- 6.5 Research questions and findings
- 6.6 Methodological strengths, limitations and considerations
- 6.7 Clinical implications

7. MAIN CONCLUSIONS

8. FUTURE PERSPECTIVES

9. REFERENCE LIST

10. PAPERS 1-4

PREFACE

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1.2 Abbreviations

γ BB	gamma-butyrobetaine
CCA	Common carotid artery
CEA	Carotid endarterectomy
CRP	C-reactive protein
CT	Computed tomography
CTA	Computed tomography angiography
CVD	Cardiovascular disease
EDV	End diastolic volume
ECST	European carotid surgery trial
^{18}F -FDG	2-deoxy-2 [^{18}F] fluoro-D-glucose
FMOs	Flavin-containing monooxygenases
FWHM	Filter with full-width at half maximum
GSM	Gray-scale median
HDL	High-density lipoprotein
ICA	Internal carotid artery
INF- γ	Interferon-gamma
IMT	Intima-media thickness
IPH	Intraplaque hemorrhage
LDL	Low-density lipoprotein
LRNC	Lipid-rich necrotic core
M-CSF	Macrophage colony-stimulating factors
Mbq	Megabecquerel
MMP	Matrix metalloproteinase
MCP-1	Monocyte chemo attractant protein-1
MRI	Magnetic resonance imaging
NASCET	North American symptomatic carotid endarterectomy trial
ox-LDL	oxidized low-density lipoprotein
PSV	Peak systolic velocity
PET	Positron emission tomography
ROI	Region of interest

SMC	Smooth muscle cell
SUV	Standardized uptake value
TIA	Transient ischemic attack
TBR	Target-to-background ratio
TMAO	Trimethylamine-N-oxide
TML	Trimethylamine
TRFC	Thin ruptured fibrous cap
TGF- β	Transforming growth factor- β
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organization

1.3 List of papers

Paper 1

Skagen K, Johnsrud K, Evensen K, Scott H, Krohg-Sørensen K, Reier-Nilsen F, Revheim ME, Fjeld JG, Skjelland M, Russell D. Carotid plaque inflammation assessed with ^{18}F -FDG PET/CT is higher in symptomatic compared with asymptomatic patients.

Int J Stroke 2015; 5:730-736.

Paper 2

Johnsrud K, Skagen K, Seierstad T, Skjelland M, Revheim ME, Russell D. Methods for the quantification of carotid artery plaque inflammation with ^{18}F -FDG PET/CT.

Submitted for publication.

Paper 3

Skagen K, Evensen K, Scott H, Krohg-Sørensen K, Vatnehol SA, Hol PK, Skjelland M, Russell D. Semi-automated MRI assessment of carotid plaque lipid content.

Accepted for publication in Journal of Stroke and Cerebrovascular Disease.

Paper 4

K Skagen, M Troseid, T Ueland, S Holm, A Abbas, I Gregersen, M Kummen, V Bjerkeli, F Reier-Nilsen, D Russell, A Svardal, RK Berge, T Hemming-Karlsen, PAukrust, JER Hov, B Halvorsen, M Skjelland. The carnitine-butyrobetaine-TMAO pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis.

Accepted for publication in Atherosclerosis.

2. GENERAL INTRODUCTION

2.1 The epidemiology of atherosclerosis and cardiovascular disease

Atherosclerosis is the main cause of cardiovascular disease (CVD), which includes cerebrovascular disease, coronary artery disease, and peripheral artery disease. The origin of the current epidemic of atherosclerotic cardiovascular disease is commonly traced back to modern times and the industrial revolution in the 1700s with decreased physical activity, tobacco use and increased intake of dietary sugar, carbohydrates and fat. After the Framingham study (commenced in 1946) identified modifiable risk factors for CVD (1), both the US and the British governments, launched major health promotion campaigns aimed at reducing the prevalence of the identified risk factors. As a consequence, after having peaked in the western world in the mid-1960s, the number of deaths per 100,000 population attributable to CVD started to decline (2). In fact, the CVD rates and case-fatality rates have fallen considerably over the last two decades in industrialized countries. Furthermore, this reduction has occurred despite a quadrupling of the proportion of the population aged over 65 years of age. This decline in death rate from CVD slowed in the 1990s, for which the large increase in obesity and type 2 diabetes mellitus as well as an increase in cigarette smoking has been given responsibility (3).

The consequences of atherosclerosis on health remain a massive public health challenge. The World Health organization (WHO) estimates that cerebrovascular disease is the leading cause of death globally, causing over 17 million deaths in 2012 (31 % of all global deaths). Of these, 7.4 million were due to coronary artery disease and 6.7 million were due to stroke. Substantial increases in the absolute numbers of individuals affected by stroke are projected to increase. Due to the increasing population life expectancy, this increase is expected even if stroke incidence rates are reduced or maintained at current levels (4). In Norway there are approximately 16,000 strokes each year and 25 % of men and 20 % of women in this country can expect to experience a stroke if they live to be 85 years old (5). Cerebrovascular disease is the leading cause of long-term disability in older adults (6), and is a substantial contributor to healthcare costs. The American Heart Association estimated that the annual total (direct and indirect) costs of stroke were \$320 billion in 2011 (6). The total yearly cost of stroke in Norway was in 2006 estimated to be approximately 7-8 billion NOK (7).

The Tromsø Study estimated the prevalence of asymptomatic internal carotid stenosis of 50 % or greater in the Norwegian population aged 50 or older to be approximately 2 %, and the prevalence of high-grade (>70%) internal carotid stenosis to be just under 1% (13040 people in 2002) (8, 9). International population studies including patients over 65 years of age have reported the prevalence of asymptomatic carotid stenosis >50% to be higher at 5-10 % (10, 11). Ipsilateral carotid stenosis (defined as carotid atherosclerosis causing an arterial lumen narrowing of $\geq 50\%$) is responsible for 9-18 % of all ischemic strokes in the anterior circulation (12, 13).

2.2 The concept of carotid atherosclerosis: from a passive cholesterol storage-disease to an active generalized inflammatory disorder

Traditionally, atherosclerosis has been viewed as a slowly progressive, cholesterol storage disease involving the passive accumulation of cholesterol debris in the arterial wall. Identification of asymptomatic patients who have a high risk of stroke has been challenging, and consequently there has been little emphasis on the diagnosis and treatment of sub-clinical carotid atherosclerosis. Disease management has been dominated by interventional re-vascularization approaches, targeting the most severe, symptomatic stenosis with endarterectomy or angioplasty. In recent years, however, atherosclerosis has been recognized as a multifactorial, progressive and chronic inflammatory disease. In fact, inflammation plays an important pathogenic role in all stages of atherosclerosis including the destabilization of atherosclerotic plaques (14-16). Plaque instability is recognized, not as a localized vascular event, but as a systemic, inflammatory condition, often with unstable plaques present at multiple different vascular sites simultaneously (17-19). An unstable/symptomatic carotid plaque therefore reflects a generalized activation of the atherosclerotic process and unstable carotid plaques have been found to be associated with unstable plaques in the contralateral carotid and coronary arteries, as well as a higher risk of future cerebrovascular events (17, 20).

A common inflammatory pathogenic mechanism (common inflammatory link) has been suggested in patients who have both carotid and coronary plaque instability. This is supported by the presence of common, traditional risk factors (hypertension, hypercholesterolemia, smoking and diabetes) as well as increased levels of inflammatory markers such as C-reactive

protein (CRP), white blood cells and fibrinogen (20-22). Furthermore, inflammation is the presumed cause of accelerated atherosclerosis and increased risk of CVD in patients with chronic inflammatory diseases like rheumatoid arthritis and systemic lupus erythematosus (23, 24). Inflammatory cytokines move from the primary, disease-specific site of local inflammation into the systemic circulation where they start a systemic inflammatory response. The resulting state of systemic inflammation can cause dysfunction of the vascular endothelium (25), which is thought to be the initiating mechanism of atherosclerosis. Consequently, the focus of atherosclerosis research has broadened to include not only the study of a single atherosclerotic carotid plaque, but also the identification and assessment of generalized inflammation (with Positron Emission Tomography (PET) /Computed Tomography (CT) imaging and serum markers of inflammation), as well as the association between systemic inflammation and cardiovascular risk. A new, rapidly expanding dimension in atherosclerosis research is the role of the gut microbiota, i.e. the microbial inhabitants of the gastrointestinal tract, which have been suggested as a novel determinant of CVD risk (26). Traditional risk factors including CRP explain only half of the carotid atherosclerotic burden (27, 28). Cardiovascular disease in the absence of these risk factors is thought to be due to genetic predisposition. However, large-scale, genome-wide association studies only explain approximately 10 % of CVD heritability (29), suggesting that other important unrecognized pathophysiological mechanisms and risk factors are involved. Recently, gut microbiota, have been linked to modifiable risk factors for CVD as well as related metabolic diseases such as diabetes (30), hyperlipidemia (31) and obesity (32).

The influence of diet on the development of atherosclerosis and cardiovascular risk is well established, and a healthy diet is considered key in the promotion of cardiovascular health. Diet, however, is not an independent risk factor for atherosclerosis and its effect on stroke risk in patients who make dietary changes is variable. The role of intestinal microorganisms in the metabolism of food, and their potential consequences for host metabolism and disease pathogenesis has been neglected until recently. Gut flora can significantly influence the bioavailability of dietary constituents and their metabolism. The gut microbiota have not only been found to be associated with the inflammatory status of patients, but it has also been shown that patients with symptomatic atherosclerosis harbor characteristic changes in the gut metagenome (33). Recent studies have also shown a mechanistic link between the intestinal microbiota metabolism of dietary carnitine and choline, and coronary artery disease through

the production of the pro-atherosclerotic metabolite trimethylamine-N-oxide (TMAO) (34). TMAO promotes atherosclerosis in part by enhancing the accumulation of cholesterol in foam cells. Elevated TMAO levels can predict myocardial infarction and stroke (35, 36). TMAO has been proposed to be produced through dietary intake of carnitine (red meat) or phosphatidylcholine (egg yolk, soya beans) which is converted to trimethylamine (TMA) by the intestinal microbiota and further oxidized by flavin-containing monooxygenases (FMOs) in the liver to TMAO (37). An alternative pathway of TMAO formation from carnitine, via the microbiota-dependent intermediate metabolite γ -butyrobetain (γ BB) was also recently reported (26).

2.3 The pathophysiology of atherosclerosis

The term “atherosclerosis” describes the two main components of the atherosclerotic lesion; “athero” (the Greek word for gruel), corresponding to the necrotic area at the base of the atherosclerotic plaque, and “sclerosis” (from Greek for hardening) describes the fibrotic cap and the calcified portions of the plaque.

Atherosclerotic lesions develop slowly over many years, passing through several stages. Traditional risk factors such as hypertension, diabetes, smoking, genetic disposition, infection, elevated homocysteine and oxidized low-density lipoprotein (ox-LDL, due to hypercholesterolemia) can activate the anti-inflammatory and anti-thrombotic responses which induce endothelial dysfunction (17). These dysfunctional endothelial cells then express adhesion molecules (vascular cell adhesion molecule-1, VCAM-1 and p-selectin) allowing the adherence of monocytes, T-lymphocytes and platelets to the endothelium, which then become pro-inflammatory and pro-thrombotic (38). Monocytes are recruited into the intima (by monocyte chemo attractant protein-1 (MCP-1) and differentiate into macrophages in response to macrophage colony-stimulating factors (M-CSF) and other stimuli. Macrophages ingest oxLDL by receptor-mediated phagocytosis, which promotes the transformation of macrophages into lipid-laden foam cells. Foam cells form “fatty streaks” (sub-endothelial accumulation of lipid-laden macrophage foam cells and associated T-lymphocytes) that are histologically the earliest sign of atherosclerosis. These fatty-streaks are asymptomatic and non-stenotic. Post mortem studies have shown that they are present in the aorta at the end of the first decade of life, in the coronary arteries by the second and begin to appear in the

cerebral circulation by the third decade (39). Macrophages release cytokines, chemokines and growth factor from these fatty streaks, which mediate further leukocyte recruitment and activation. This continues the pro-inflammatory cascade with activation of T-cells that secrete cytokines leading to further inflammation. Migration of smooth muscle cells (SMCs) from the intima and the proliferation of SMCs lead to further plaque expansion and the development of the fibrous cap. The apoptosis of SMCs and macrophages is mainly regulated by cytotoxic T-cells, which leads to the formation of a lipid-rich necrotic core and increased instability.

This lesion progresses and the plaque core becomes necrotic, containing cellular debris, crystalline cholesterol and inflammatory cells, especially macrophage foam cells. The necrotic core becomes covered by an endothelialized fibrous cap, which consists of vascular smooth muscle cells and collagen matrix. The fibrous cap also contains inflammatory cells mainly in the “shoulder” region (the portion of the plaque lateral to the lipid core) where T-cells, mast cells and especially macrophages have a tendency to accumulate (40, 41). Advanced plaque lesions become increasingly complex with calcification, new vessel formation, thinning of the fibrous cap and eventually plaque rupture. The strength of the fibrous cap, which is very important for plaque stability, is determined by the activity of the different inflammatory mediators within the plaque. This determines the balance between production (transforming growth factor- β (TGF- β)) and breakdown (matrix metalloproteinase, MMPs and interferon- γ (IFN- γ)) of collagen.

In the event of plaque rupture the plaque content including the thrombogenic lipid core is exposed to blood with a high risk of thrombus formation. Cerebrovascular symptoms are the result of these thrombi causing vessel occlusion at the plaque location and/or emboli that are carried in the blood stream to brain where they occlude cerebral blood vessels.

2.4 Imaging of carotid atherosclerosis and review of current literature

Doppler Ultrasound

Carotid Doppler ultrasound of the carotid arteries is currently the first line investigation for evaluating suspected carotid artery disease. This technique is non-invasive, relatively inexpensive and widely available for the assessment of both luminal stenosis and plaque morphology (42, 43).

Basic Principles: The apparent frequency of ultrasound changes when it is reflected from red blood cells that are moving relative to the ultrasound source (transducer or probe). If the blood cells are moving toward the source, the frequency of the reflected sound will be increased, whereas if the cells are moving away from the source, the frequency will decrease. The Doppler shift frequency, first proposed by Christian Doppler in 1842 is the difference between the frequency of the transmitted ultrasound and the frequency of the reflected ultrasound. The change in ultrasound frequency correlates directly to the speed of the blood cells and this ultrasound frequency shift which lies within the audible range can be expressed as:

$$F = 2 \times F_o \times V \times \cos \alpha / c$$

where F is the Doppler shift (Hz), F_o is the mean emitted frequency, V is blood flow velocity, α is the angle between the emitted sound and the blood flow direction, and c is velocity of sound in the tissue.

This Doppler shift frequency (peak systolic frequency) occurring during systole is one of the most important parameters in the assessment of carotid stenosis (see criteria for assessment of artery stenosis in the methods section). Color duplex ultrasound examination combines grey-scale and B-mode tissue imaging for the assessment of plaque echogenicity with Doppler measurement of blood flow velocity to measure the degree of the stenosis.

Plaque echogenicity, total plaque area and plaque surface irregularities have all been suggested as ultrasound markers of unstable plaques and increased stroke risk (44-47). In this thesis, the degree of artery stenosis, and plaque echolucency, are used in the assessment of plaque stability (further described in methods section).

Intima media thickness (IMT):

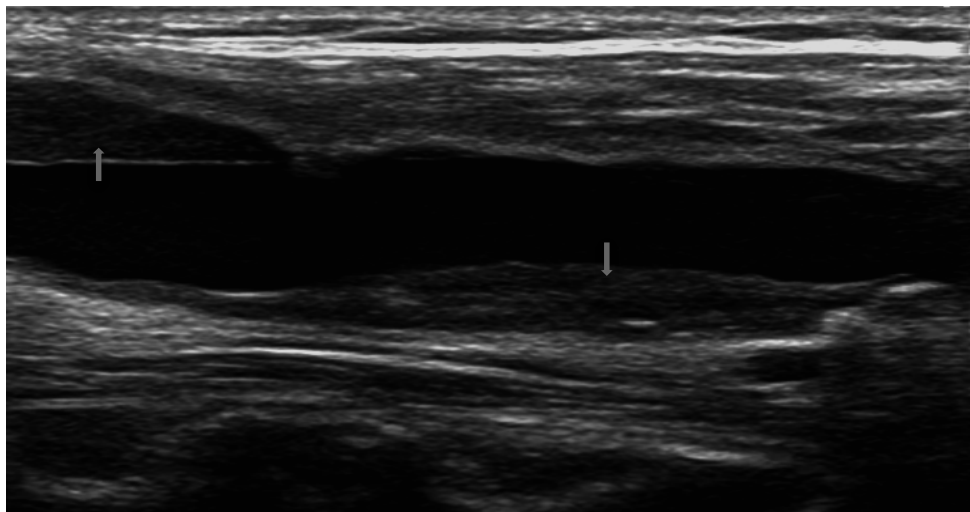
IMT is the measurement of the thickness of tunica intima and the innermost two layers of the wall of an artery made by carotid ultrasound. Increased carotid IMT in patients over the age of 50 has been shown to be predictive of future cardiovascular events (48). However, the clinical usefulness of measuring the progression or potential decrease in the IMT as a surrogate endpoint to assess drug efficacy in clinical trials or in clinical management in cardiovascular disease is unclear (49, 50).

Plaque echogenicity:

Echogenicity assessed with ultrasound reflects plaque content. Areas of low-level echogenicity (echolucency) are associated with the presence of lipids and hemorrhage, whereas areas of high-level echogenicity (echogenic) suggest underlying fibrosis or calcification (51, 52). Ultrasound plaque appearance or echogenicity can be classified into plaques with low-level echogenicity (echolucent plaques), and plaques with high-level echogenicity (echogenic plaques). These two categories can be further subdivided into predominantly echolucent and predominantly echogenic (see methods section for further description).

The association between echolucent plaques, plaque instability and cerebrovascular events is not fully understood. There is, however, a probable association between echolucency and inflammatory burden (53, 54), with macrophages contributing to plaque destabilization in echolucent lesions (55). Echolucent carotid artery plaques (lipid-rich) are associated with increased risk of stroke (44, 56), independent of the degree of artery stenosis (57, 58).

Figure 1. Carotid ultrasound showing echolucent plaques.



Plaque in the far wall of the common carotid artery (arrow pointing down) and in the near wall at the carotid bifurcation (arrow pointing up).

Computed tomography (CT)

CT angiography (CTA) is used in clinical practice for the assessment of plaque and degree of artery stenosis. Studies have found that the overall agreement on the estimation of the degree of artery stenosis between carotid Doppler ultrasound and CTA is good at 79.1% (95% CI 0.72-0.83). However, CTA is unable to reliably distinguish between moderate (50%–69%) and severe (70%–99%) stenosis, which is important for clinical management and a limitation of this investigation (59, 60).

PET

There has, in recent years, been growing interest in the ability of PET to assess plaque inflammatory content. Due to the infiltration and retention of oxidized lipids in the arterial wall, vulnerable plaques contain a greater density of macrophages compared to asymptomatic plaques (61). Activated macrophages have a significantly increased metabolic rate and therefore increased 2-deoxy-2- [^{18}F] fluoro-D-glucose (^{18}F -FDG) uptake. Rudd et al. found

increased ^{18}F -FDG in macrophage-rich regions of carotid plaques, removed at endarterectomy, in eight symptomatic patients compared to contralateral asymptomatic plaques in the same patients (62). Tawakol et al. demonstrated that in vivo ^{18}F -FDG uptake correlated with the degree of carotid plaque inflammation in 17 patients when macrophage staining was assessed histologically (63).

^{18}F -FDG uptake has also been shown to correlate with other factors that are associated with plaque instability. Carotid plaques with decreased ultrasound echogenicity and patients with increased serum lipids have been found to have higher degrees of ^{18}F -FDG uptake on PET (64, 65). Evidence from longitudinal studies also suggests that arterial ^{18}F -FDG uptake may be related to patient outcome. Figueroa et al. followed 513 patients without symptomatic cardiovascular disease for a mean of 4.2 years. They found that ^{18}F -FDG uptake in the wall of the ascending aorta was an independent predictor of future cardiovascular events (66). Results from the Dublin Carotid Atherosclerosis Stroke Study showed, in 67 patients with a recent ischemic event (Transient ischemic attack (TIA) or stroke), that carotid plaque inflammation, measured by ^{18}F -FDG PET, was associated with a high risk of early stroke recurrence, independent of the degree of stenosis (67).

These previous studies have, however, been limited by relatively small sample sizes and time delays of weeks or months from symptoms to ^{18}F -FDG PET imaging and histology following endarterectomy. There is therefore a possibility that plaque inflammation may have been modified by medications and life-style changes during these time-delays from symptoms to imaging and histological assessments (68, 69). Increased ^{18}F -FDG uptake must be closely correlated in time to ipsilateral ischemic cerebral events and higher in symptomatic compared to asymptomatic patients if this method is to be of value in the clinical management of patients.

MRI

Histopathological studies have demonstrated that histological markers of plaque instability are a lipid rich necrotic core (LRNC), intraplaque hemorrhage (IPH) and a thin, ruptured fibrous cap (TRFC). Larger amounts of calcification and fibrous tissue have been found to be associated with stable plaques (70-74). These plaque characteristics are related to thromboembolic risk independent of arterial narrowing, and are more likely to cause symptoms, making them hallmarks of unstable plaques.

Although not in routine clinical use, recent developments in Magnetic resonance imaging (MRI) technology have shown promise allowing the identification of high-risk plaque characteristics and the accurate discrimination between the specific histological subtypes of carotid plaques as proposed by the American Heart Association (75). Gupta et al. did a systematic review, which included 9 MRI studies with a total of 779 subjects where they found that carotid plaques with LRNC, IPH and TRFC were significantly more likely to result in ipsilateral ischemic events (76). Four of the 7 studies examined more than one plaque element, but a multiparametric testing approach addressing the significance of each plaque component was not performed in any of the studies. The authors concluded that MRI characterization of specific plaque elements (LRNC, IPH and TRFC) could provide additional measures of stroke risk not provided by simple measurements of luminal stenosis. The majority of current imaging studies of atherosclerotic plaques rely on a human observer's interpretation of the MRI findings with different contrast weighting, producing measurements that have been compared with histological assessments. This manual plaque segmentation requires expertise, is time consuming, and produces results that are subject to interobserver variability (77). Contrast automated classification may provide more objective and reliable assessments of plaque composition (78, 79). Van't Klooster et al. imaged 40 patients who had carotid plaques with MRI and found good agreement between automatic and visual identification of plaque components. They found that the volumes of hemorrhage and lipids assessed by visual and automatic assessments were reasonably consistent but not for calcium (80).

MRI of plaque composition is a relatively new technique and studies have shown a high diversity in findings using different MRI protocols and different techniques in the histological assessments (76, 81). It is therefore difficult to draw definite conclusions regarding the value of carotid plaque MRI characterization. Literature reviews often included studies using different MRI techniques making it difficult to compare results. In addition they included patients studied after a long time interval from symptoms and the possibility that the plaque components may change over time was therefore not taken into account.

2.5 Carotid atherosclerosis and the assessment of the unstable carotid plaque/stroke risk in current clinical practice

At least 20 % of strokes are thromboembolic being caused by thromboembolism from an atherosclerotic plaque at the carotid bifurcation or internal carotid artery (82). Such strokes have been shown to be preventable by surgical removal of the plaque with carotid endarterectomy (CEA) or stenting (83, 84). In current clinical practice both risk stratification and patient selection for revascularization primarily involves assessment of the severity of artery lumen stenosis using carotid ultrasound and CT angiography.

Along with the acceptance of atherosclerosis as a generalized disease has come the realization that the degree of luminal stenosis in the carotid artery alone may not be the best predictor of stroke risk. This is apparent when considering the strokes that occur due to non-stenotic carotid disease, and conversely, the non-negligible proportion of patients with significant carotid stenosis who remain completely asymptomatic throughout their lifetime. Vessel lumen narrowing alone is not a good estimator of plaque size and probably underestimates the atherosclerotic burden. This was demonstrated by Glagov et al (85) who showed that, vessels can have a large increase in atherosclerotic plaque volume without luminal narrowing due to remodeling with compensatory enlargement of the adventitial boundary. The composition and degree of inflammation in an atherosclerotic plaque may therefore be more important than degree of stenosis for stroke risk.

3. AIMS OF THE PRESENT THESIS

The burden of cardiovascular disease remains high, with atherosclerotic carotid stenosis as a leading cause of ischemic stroke, demonstrating the demand for better strategies for the prevention and treatment of this disease. The assessment of stroke risk for patients with carotid atherosclerosis in current clinical practice is based on studies more than 15 years old (NASCET 1999, ESCT 1998) in which the degree of stenosis was used to predict stroke risk and also the main criteria for selecting patients for endarterectomy (83, 84). New methods to assess carotid atherosclerosis should take into account the acquired knowledge on the inflammatory and generalized nature of this disease. Additionally, in view of the large proportion of carotid atherosclerosis not explained by traditional risk factors or heritability it is important to look beyond the traditional school of thought, and explore new potential causal associations involved in the progression of this disease.

Methods able to identify features associated with increased cardiovascular risk in patients with carotid atherosclerosis would allow for a better estimation of stroke risk for the individual patient. This, in turn would improve selection of patients at risk of stroke for surgery and medical treatment as appropriate with resulting prevention of ischemic stroke. The main aim of this thesis was therefore to evaluate different methods for imaging carotid atherosclerosis. We included patients with known carotid atherosclerosis and examined them with carotid Doppler ultrasound, PET/CT and carotid MRI to assess whether these methods could identify features of carotid atherosclerosis known to be associated with increased risk of cardiovascular disease.

In addition, using the same patient population, we used a translational approach combining clinical and radiological findings with measurements of serum gut microbiota metabolites, exploring the association with cardiovascular outcome.

The specific research questions were:

- (i) Does carotid plaque ^{18}F -FDG uptake on PET/CT correlate with histological plaque assessments of the degree of inflammation, ultrasound plaque echogenicity, and the risk of ischemic stroke?
- (ii) Which method is best for quantifying ^{18}F -FDG uptake in the assessment of plaque inflammation?
- (iii) Can semi-automated MRI assess features of carotid plaque instability (necrotic core size)?
- (iv) Can increased levels of carnitine-related metabolites measured in plasma predict outcome in patients with carotid atherosclerosis?

4. SUBJECTS AND METHODS

The present thesis is based on the study population from "The Unstable Carotid Artery Plaque Study" established at the Department of Neurology, Oslo University Hospital, Rikshospitalet.

4.1 "The unstable carotid plaque"-paper 1-4

Study design and patient selection

This was a prospective study, which included consecutive symptomatic and asymptomatic patients with ≥ 70 % carotid artery stenosis. All patients underwent a clinical neurological examination and registration of the following cardiovascular risk factors: hypercholesterolemia, hypertension, coronary artery disease, diabetes, smoking history and weight. Exclusion criteria were prior CEA, stenting, carotid occlusion, vasculitis, malignancy, prior radiation therapy to the neck, treatment with immunomodulating drugs or oncological disease. All included patients had a carotid ultrasound examination and venous blood sampling. Some patients were scheduled for carotid endarterectomy and the plaques from these patients were collected after surgery and assessed histologically. For patients who gave consent, carotid PET, and/or MRI examinations were also performed before endarterectomy.

Carotid Ultrasound

Colour duplex ultrasound was performed with a General Electric Vivid 7 (General Electric, Horten, Norway) using a M12L probe (14 MHz) on both carotid arteries.

The degree of stenosis was determined using grey-scale and increased peak systolic velocities (PSV) in the internal carotid artery (ICA), or common carotid artery (CCA) according to consensus criteria of the Society of Radiologists in Ultrasound (86). The area with the most severe stenosis was located by colour Doppler ultrasound and PSVs were measured with a Doppler angle of approximately 45°. Stenoses were classified into 5 groups according to ultrasound findings based on the following criteria:

<u>Degree of stenosis</u>	<u>Parameter</u>
- 0-49 %	PSV <125 cm/sec
- 50-69%	PSV 125-230 cm/sec

- 70-89%	PSV >230 cm/sec
- Near occlusion	PSV >230 cm/sec and marked narrow lumen
- Occlusion	No detectable flow

Two additional parameters were used if the ICA PSVs were believed not to be representative of the extent of disease (in the presence of tandem lesions, contralateral high-grade stenosis, discrepancy between visual assessment of plaque and ICA PSV, elevated CCA velocity, hyperdynamic cardiac state (increased circulatory volume), or low cardiac output). These two parameters were: 1) ICA-to-CCA PSV ratio, where PSV in the stenotic ICA was compared to the PSV in the distal (normal) CCA to give the ratio of PSV ICA/CCA, and 2) End diastolic volume (EDV) in the stenotic ICA. The following criteria were used:

<u>Degree of stenosis</u>	<u>ICA/CCA PSV ratio</u>	<u>ICA EDV (cm/sec)</u>
Normal	<2.0	<40
<50	<2.0	<40
50-69	2.0-4.0	40-100
≥70	4.0	100
Near occlusion	variable	variable

Plaque echogenicity was assessed with the vessel lumen as the reference structure for defining echolucency, and the bright echo zone produced by the media-adventitia interface as the reference for defining echogenicity (57). Echogenicity was graded from 1 to 4 in a modified version of the classification proposed by Gray-Weale (51) as follows:

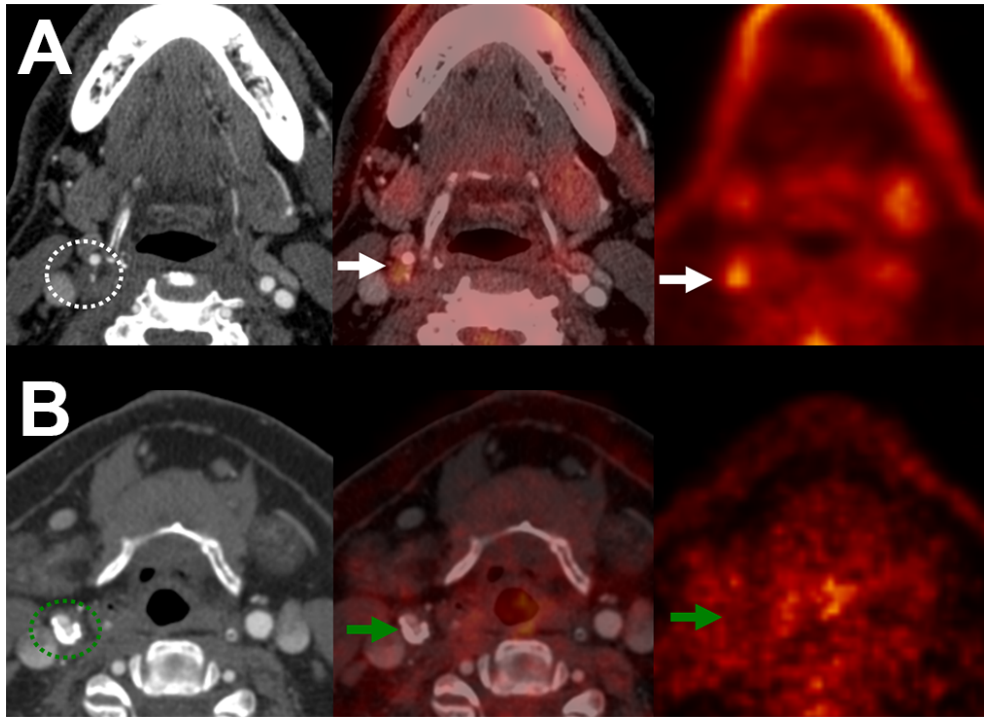
- Echolucent (as vessel lumen)
- Predominantly echolucent, >50 % of plaque area is echolucent
- Predominantly echogenic, >50% of plaque area is echogenic
- Echogenic (similar to the media-adventitia interface)

The plaque was classified as echolucent or echogenic if > 80% was homogenously low or high in echolucency (87). The classification was assessed visually in high-resolution B-mode ultrasound grey-scale pictures.

¹⁸F-FDG PET/ CT

Patients were examined with a hybrid PET/CT scanner (Siemens Biograph 64, Siemens Medical Systems, Erlangen, Germany). After an overnight fast (minimum 6 hours), an ¹⁸F-FDG PET/CT was performed from the base of the skull to the aortic arch, approximately 90 min after the injection of 5 Mbq/kg ¹⁸F-FDG. Blood glucose levels were measured. The PET data were reconstructed to 2 mm thick slices with a matrix size of 256 x 256 pixels (pixel size 2.67mm) using the OSEM 2D algorithm with four iterations (i), eight subsets (s) (4i/8s), and Gaussian post-reconstruction filter with full-width at half maximum (FWHM) of 3.5 mm (88). A CT without contrast was performed immediately before the PET scan with the patient in the same position. The contrast enhanced CT was used for localizing the carotid artery plaque. A specialist in nuclear medicine blinded for patient data placed the regions of interest (ROI). The contrast enhanced CT angiography was used as a guide for drawing the ROIs on the PET slice (fused with non-contrast CT). ROIs covering the whole plaque including vessel wall thickening and the lumen contrast-filling defect were drawn on each axial slice from the most cranial to the most caudal slice of the plaque (89). The ROIs were minimized to only cover parts of the plaque uptake when nearby ¹⁸F-FDG activity, e.g. lymph nodes, paravertebral muscles or salivary glands could have influenced the measured ¹⁸F-FDG activity in the ROIs. Four regions of interest were placed in the lumen of the jugular vein close to the plaque for calculation of the target-to- background ratio (TBR). Maximum standardized uptake values (SUV_{max}); the highest activity concentration per injected dose per lean body mass (lbm - a factor derived from each patients height, weight and gender) after correction for decay in each ROI were measured. SUV normalized to lbm is an established parameter for the quantification of ¹⁸F-FDG uptake (90). The following uptake parameters were used for the statistical analysis for each plaque: 1) SUV_{max}= the single highest SUV_{max} value 2) Mean SUV_{max}= mean of all plaque SUV_{max} values, 3) TBR = mean SUV_{max} divided by mean SUV_{mean} in the four venous regions.

Figure 2. ^{18}F -FDG PET/CT images of carotid artery plaques from a symptomatic (A) and an asymptomatic (B) patient.



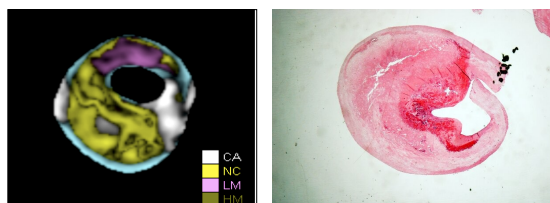
From left to right the images shown are contrast enhanced CT, co-registered PET/CT and PET. Patient A has a non-calcified plaque with a high degree stenosis ($> 70\%$) in the right internal carotid artery (white circle). Patient B has a calcified plaque in the right bifurcation (green circle) with no focal ^{18}F -FDG uptake.

MRI

All carotid arteries were imaged using a 3T whole-body scanner (Achieva, Philips Healthcare, Best, The Netherlands) equipped with an 8 channel carotid coil (Philips/Shanghai Chenguang Medical Technologies, Shanghai China). For each scan, the location of the carotid bifurcation was located using a 3D time-of-flight angiographic sequence, followed by 8 continuous slices using proton density, high resolution 3D time-of-flight T2 weighted and T1 weighted images. The T1 weighted images were obtained before and after the injection of 0.2 ml /kg contrast

agent (Dotarem, Guerbet; Paris, France). The imaging parameters were: field of view 160x160 mm, matrix 268x260, image resolution 0.6x0.6, 2 mm slice thickness and time to repetition (TR) /time to echo (TE) of 850/10, 3584/50 and 4600/20 ms for T1, T2 and proton density weighted sequences, respectively. Total scan time per patient was 17-20 minutes. Custom software (VP Diagnostics, Seattle, USA) was used for the automatic analyses of the MRI findings. The boundaries of the lumen and the outer vessel wall were detected by the software, followed by alignment of the other contrast weightings to the T1TW images. Manual corrections were applied by a reader blinded for the results of other imaging modalities and clinical information. The software then automatically calculated the absolute (in mm³) and relative (in percentage) volumes of the different plaque components such as the necrotic core, calcification, haemorrhage and fibrous tissue in each slice and summarized for total plaque component volumes. The percentage plaque lipid content (lipid rich necrotic core), plaque calcification content and IPH were included.

Figure 3. Semi-automated MRI (left) and histology slice (right) showing carotid plaque components.



CA=calcium, NC=necrotic core, LM=loose matrix, HM=haemorrhage.

Tissue processing and histological Analysis

The plaques were removed en bloc (intact) at carotid endarterectomy, fixed in 4% formaldehyde, decalcified in EDTA, and cut into 2-3mm slices. After dehydration the slices were embedded in paraffin and histological sections were cut at 5 µm and stained with hematoxylin and eosin. Plaques were assessed by a research physician and a pathologist who was blinded for clinical patient information and ¹⁸F-FDG PET findings. A section from each slice was evaluated with 120 times magnification. The amount of inflammation per plaque was calculated as the sum of all areas with inflammatory activity (macrophages and leukocytes) divided by the total area of the sections to give a percentage of inflammatory cells per plaque. This method for evaluating and grading inflammation has been shown to have a good to excellent intra- and inter-rater variability (91). Histological assessments were made on eleven slices from 3 plaques on two occasions more than 2 months apart to assess the reproducibility of the findings. For this analysis the percentage inflammatory cells per slice was classified into the following categories: 0-5%, 5-10%, 10-15% and 15-20% and the results assessed using Kappa statistics.

Blood sampling

Venepuncture of a forearm vein was performed with the patient in a non-fasting state at the day of study inclusion. Blood was drawn into pyrogen-free tubes without any additives and allowed to clot at room temperature (within 1 hour) before centrifugation at 2500g for 20 minutes. Serum samples were stored at -80 °C and thawed less than three times.

Measurement of the metabolites carnitine, γBB, TML and TMAO: Serum levels of carnitine, γBB, TML and TMAO were quantified using high performance liquid chromatography as described previously (92).

4.2 Statistical analysis

SPSS for Windows statistical software (version 18.0; SPSS Inc., Chicago, III) was used for data analysis. Continuous, non-parametric variables were compared with Mann-Whitney U test (two groups) and the Kruskal-Wallis comparison test (three groups). Student's t-test was used for comparison of normally distributed data. The Chi-square test was used for analyzing categorical data. Kaplan-Meier curves and the log rank test were performed to analyze survival. In paper 4 the importance of the metabolites in relation to outcome was investigated

by multivariable Cox regression including traditional risk factors associated with outcome. Variables were log transformed and expressed per SD for regression. Probability values (2-sided) were considered significant at $P < 0.05$.

4.3 Ethical issues

All studies included in this thesis were approved by the Regional Committee for Medical and Health research Ethics; 2009/282a THE UNSTABLE PLAQUE (S-09092a), biobank no. 2733. All patients gave informed, written consent prior to study participation. The studies conformed to the principles outlined in the Declaration of Helsinki for use of human tissue or subjects.

Both MRI and PET/CT scanning involve intravenous puncture and the injection of contrast and for PET the nuclear tracer 18-FDG. These investigations also require the patient to lie still while inside the scanner, which can be associated with some discomfort.

The majority of the carotid ultrasounds were performed as part of routine practice and therefore did not involve additional discomfort or risk to the patient.

Venepuncture, both for taking blood and introducing contrast, introduces a risk of infection and phlebitis for the patient. All patients were informed of these risks and discomforts prior to consenting to participate in the study. They were also informed of their possibility to cancel the examinations at any time should they wish to do so.

5. SUMMARY OF PAPERS

5.1 Paper 1

Skagen K, Johnsrud K, Evensen K, Scott H, Krohg-Sørensen K, Reier-Nilsen F, Revheim ME, Fjeld JG, Skjelland M, Russell D. Carotid plaque inflammation assessed with ^{18}F -FDG PET/CT is higher in symptomatic compared with asymptomatic patients. *Int J Stroke* 2015; 5:730-736.

Aim: Carotid artery plaque inflammation is thought to be an important marker of plaque vulnerability and increased stroke risk. The main aim of this study was to assess the level of agreement between 2-deoxy-2- ^{18}F FDG] fluoro-D-glucose (^{18}F -FDG) uptake on PET (positron emission tomography) imaging in carotid artery plaques, with cerebrovascular symptoms, carotid plaque ultrasound echogenicity and histological assessments of plaque inflammation.

Results: The amount of ^{18}F FDG uptake in plaques and the amount of inflammation on histological assessment were significantly correlated ($r=0.521$, $P=0.003$). ^{18}F FDG uptake was significantly higher in symptomatic plaques with median SUV_{max} 1.75 (1.26-2.04) in symptomatic and 1.43 (1.15-2.28) in asymptomatic patients ($P=0.03$). ^{18}F FDG uptake was also positively correlated with echolucency on Doppler ultrasound ($P=0.03$).

Conclusion: ^{18}F FDG uptake assessed by PET/CT correlated with histological assessments of inflammation and was higher in patients with symptomatic compared to asymptomatic carotid artery plaques. These results support the use of ^{18}F -FDG PET/CT in the detection of inflammation in carotid atherosclerosis, which may help in the detection of unstable carotid artery plaques.

5.2 Paper 2

Johnsrud K, Skagen K, Seierstad T, Skjelland M, Revheim ME, Russell D. Methods for the quantification of carotid artery plaque inflammation with ^{18}F -FDG PET/CT. *Submitted for publication*.

Aim: Positron emission tomography (PET) imaging, with 2-deoxy-2- (^{18}F) fluoro-D-glucose (^{18}F -FDG), of inflammation in atherosclerotic carotid artery plaques is increasingly used in research on plaque stability. Several different methods for quantifying FDG uptake have been applied. The aim of this study was to compare the different methods used to quantify of ^{18}F -FDG uptake in carotid artery plaques.

Results: The study showed a high degree of correlation between the different methods used for quantification of ^{18}F -FDG uptake in carotid plaques. The quantification methods included in this study showed a similar correlation to histology, but only the quantification methods without blood background correction showed a significant difference in ^{18}F -FDG uptake in symptomatic compared to asymptomatic plaques.

Conclusion: The results from this study suggest that plaque max SUV_{max} may be the most reliable method for quantifying ^{18}F -FDG uptake in carotid plaques, both in clinical practice and when comparing results from future clinical studies.

5.3 Paper 3

Skagen K, Evensen K, Scott H, Krohg-Sørensen K, Vatnehol SA, Hol PK, Skjelland M, Russell D.

Semi-automated MRI assessment of carotid plaque lipid content. *Accepted for publication in Journal of Stroke and Cerebrovascular Disease.*

Aim: The composition of a carotid plaque is important for plaque stability and stroke risk. The main aim of this study was to assess the level of agreement between semi-automated MRI assessments of plaque components (LRNC, calcification and IPH) and histological assessments of plaques removed at carotid endarterectomy.

Results: The size of the LRNC on MRI was significantly correlated to the percentage amount of lipid per plaque on histological assessment ($p=0.010$), and to echogenicity on ultrasound with echolucent plaques having larger LRNC compared to echogenic plaques ($p=0.001$). The correlation between the amount of calcification on MRI and histological assessments was not significant. Percentage of IPH was not assessed on histological specimens.

Conclusion: This study provides further evidence that semi-automated carotid MRI is of value in the assessment of carotid plaque components known to be important for stroke risk, including LRNC. However, additional large-scale studies on semi-automated MRI carotid plaque assessments are required to more accurately define the unstable plaque and stroke risk for the individual patient.

5.4 Paper 4

K Skagen, M Troseid, T Ueland, S Holm, A Abbas, I Gregersen, M Kummen, V Bjerkeli, F Reier-Nilsen, D Russell, A Svardal, RK Berge, T Hemming-Karlsen, PAukrust, JER Hov, B Halvorsen, M Skjelland. The carnitine-butyrobetaine-TMAO pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis. *Accepted for publication in Atherosclerosis*.

Aim: γ -butyrobetaine is a metabolite from dietary carnitine, and involved in the gut microbiota-dependent conversion from carnitine to the pro-atherogenic metabolite trimethylamine-N-oxide (TMAO). Orally ingested γ BB has been shown to have a pro-atherogenic effect in studies on mice. Oral γ BB or pre-carnitine are used as over-the-counter dietary supplements, but γ BB has not been studied in relation to atherosclerosis in humans. The aim of this study was to investigate the potential effects of serum levels of γ BB, TMAO and their common precursors carnitine and trimethyllysine (TML) on carotid atherosclerosis and cardiovascular mortality.

Results: Serum levels of γ BB ($p=0.004$) and carnitine ($p=0.001$) but not TMAO or TML were increased in patients with carotid atherosclerosis compared to controls. TMAO and TML were significantly associated with non-vascular mortality, whereas only γ BB and TML were associated with cardiovascular death after adjustment for traditional risk factors (adjusted HR 3.3 [95% CI 1.9-9.1] and HR 6.0 [1.8-20.34] respectively).

Conclusion: Patients with carotid atherosclerosis had significantly raised plasma levels of γ BB when compared to healthy controls. Elevated levels of γ BB and its precursor TML predicted cardiovascular mortality. Long-term clinical studies of γ BB as a cardiovascular risk marker and safety studies regarding supplementation of γ BB are therefore warranted.

6. GENERAL DISCUSSION

6.1 Ultrasound imaging of carotid atherosclerosis

In this thesis, ultrasound carotid plaque echolucency was found to correlate to ^{18}F -FDG uptake on PET in study 1, and to the size of the lipid-rich necrotic core on MRI in study 3. This correlation of echolucency to plaque components known to be associated with increased risk of stroke, further demonstrates the potential role of ultrasound as a valuable tool in the risk assessment of carotid atherosclerosis. In study 1 plaque echolucency was also correlated to symptoms, further supporting this association.

6.2 PET imaging of carotid atherosclerosis

By correlating PET/ CT imaging to histological assessment as well as ultrasound and clinical findings in study 1, it was demonstrated that ^{18}F -FDG uptake can quantify inflammation in carotid artery plaques, and that this uptake is higher in symptomatic compared to asymptomatic plaques. Whilst these findings need confirmed in large-scale studies, they show the potential of PET/CT as an important method in the assessment of carotid atherosclerosis and stroke risk.

A wide variation of methods for quantifying ^{18}F -FDG uptake are in current use, we therefore moved on from the findings in study 1, to study 2 where we investigated which is the most accurate method for quantifying ^{18}F -FDG uptake when compared to histological assessments. We found equally good correlation to histology with- and without blood-background correction. However, only values not corrected for blood-background were able to distinguish between symptomatic and asymptomatic patients. In addition we found that quantification methods using fewer uptake values (single SUVmax or Most Diseased Segment) performed as well as findings that included several measurements, i.e. using mean SUVmax where ^{18}F -FDG uptake from the whole length of the plaque is included.

Despite the demonstration of higher ^{18}F -FDG uptake in symptomatic compared to asymptomatic plaques in study 1, the confidence intervals of ^{18}F -FDG uptake in these two patient groups overlapped. There are several possible explanations for this. Firstly, it may be due to the PET scanners limited spatial resolution, which could lead to an underestimation of

the true metabolic activity in small lesions as demonstrated by Huet et al (93). Future PET scanners with better resolution are anticipated to minimize this effect. Secondly, the definition of symptomatic only allowed the inclusion of patients with plaques that had caused clinical symptoms within 30 days prior to study inclusion. This could potentially label plaques with on-going inflammation as asymptomatic. Histological studies have found that the amount of macrophage content varies, being highest in patients with stroke, less with TIAs and least in patients who have had ocular events (amaurosis fugax) (94, 95). The amount of macrophage content also decreases in the first days/weeks after symptoms before stabilizing (95, 96). Thirdly, a significant proportion of infarcts are clinically silent and some patients may therefore have unstable plaques with high inflammatory content despite being defined as asymptomatic. Future studies should therefore include cerebral MRI examinations for the assessment of clinically silent cerebral infarcts.

The fact that ^{18}F -FDG is not macrophage-specific means that it is taken up in all tissues with active glucose metabolism, not only macrophages, but all inflammatory cells and also other cells in the plaque. The histological method applied in this thesis was designed to take account for this as we included all inflammatory cells (leukocytes) and not only macrophages. This may explain the good correlation between ^{18}F -FDG uptake on PET and histological findings. However, ^{18}F -FDG uptake is not specific enough to distinguish between symptomatic and asymptomatic plaques for the individual patient. New macrophage specific tracers, which are presumed to increase specificity, are currently being assessed (97, 98).

The results from study 2 raise the possibility that ^{18}F -FDG PET using single uptake values without blood background correction can identify plaque instability and thereby be used to predict outcome in atherosclerotic carotid artery disease, especially in asymptomatic subjects and for patients with less severe stenosis. The use of single uptake values has the potential advantage over correction for blood-background by eliminating the possible effect of variations in blood glucose.

6.3 MRI of carotid atherosclerosis

The potential of visual assessment MRI for the assessment of carotid plaque stability has been illustrated by several studies documenting its ability to identify carotid plaque tissue components. If semi-automated MRI is to be of value the ability of this imaging method to

identify features of carotid plaque instability must be demonstrated. Study 3 provides further evidence that semi-automated assessments of carotid MRI can accurately assess LRNC size. The ability of semi-automated carotid MRI to identify additional known plaque components of instability, i.e. IPH, neovascularization and the fibrous cap would further improve the value of this imaging modality for both research and clinical purposes.

In the semi-automated MRI assessments in study 3 the range of LRNC size overlapped for symptomatic and asymptomatic patients. As discussed in study 1 this could be explained by the definition of symptomatic and clinically silent infarcts (see earlier discussion).

Semi-automated MRI of atherosclerotic plaques can significantly reduce processing time as well as eliminating inter-observer variability, and provide a quantitative measure of the size of the lipid-rich necrotic core and other markers of plaque instability. Automated MRI analysis may therefore become a valuable tool for the pre therapeutic assessment of atherosclerotic artery stenosis and carotid plaque stability.

6.4 Carotid atherosclerosis and cardiovascular risk beyond traditional risk factors

The findings in study 4 suggest an association between carnitine-related metabolites and carotid atherosclerosis, potentially involving a microbiota dependent mechanism. Elevated levels of γ BB and its precursor TML were found to be predictive of cardiovascular mortality. This association was not found for TMAO for which there are different potential explanations. Firstly, γ BB is partly converted to TMA in the colon, suggesting that γ BB could mediate some of its effects through TMA and subsequently TMAO. Secondly, in addition to microbiota dependent metabolism from carnitine γ BB is also produced endogenously from TML. The finding in paper IV that both γ BB and TML, but not TMAO were independently associated with cardiovascular mortality, may support an association between carnitine related pathways and atherosclerosis, at least partly independent of TMAO formation.

Increased levels of carnitine-related metabolites were, however, not associated with plaque echogenicity on carotid ultrasound. There are several possible explanations for this; firstly, this could be due to the classification of echolucent in this study into 4 categories as described. The use of a grey-scale median in the assessment of echogenicity may have given more accurate results (see later discussion on visual vs GSM assessment of ultrasound

echogenicity). Secondly, for some patients the blood test and ultrasound examinations may have been taken at different stages in the plaque development with plaque echolucency having changed in the time between the two investigations. Thirdly, there is limited knowledge on the pathophysiological mechanisms regarding microbiota and stroke risk, and these may not include increased plaque lipid content.

The results from study 4 support an association between carnitine-related pathways and carotid atherosclerosis. This association could potentially include both microbiota-dependent and endogenous pathways, where γ BB appears to be the more sensitive marker of pathway activity associated with carotid atherosclerosis. Long-term clinical studies and further in-vitro studies of γ BB as a cardiovascular risk marker and its role in the partly microbiota-dependent carnitine-TMA-TMAO pathway are therefore warranted.

6.5 Research questions and findings

Conclusions made from research findings in response to the research questions included in this thesis showed that both carotid ultrasound, PET and MRI are of use in the assessment of carotid artery plaques by providing measures of carotid plaque instability with echogenicity, ^{18}F -FDG uptake and LRNC size respectively. Additionally, the results from study 4 support an association between carnitine-related pathways and carotid atherosclerosis, potentially include both microbiota-dependent and endogenous pathways, where γ BB appears to be the more sensitive marker of pathway activity associated with carotid atherosclerosis, hypothesizing the potential of γ BB as a cardiovascular risk marker.

Taken collectively, these findings include new scientific knowledge for the assessment of carotid artery plaques with carotid ultrasound, PET, MRI, and potentially also plasma markers able to give information on plaque stability and stroke risk. This can contribute to better assessment of carotid plaque instability aiding stroke risk prediction, enabling preventative treatment measures for patients with resultant reduction of stroke incidence. The inclusion of these methods in further research is therefore warranted. Carotid ultrasound is already a valuable tool in the clinical assessment of carotid atherosclerosis. However, to enable implementation of PET, MRI and carnitine-related metabolites as biomarkers in clinical practice further and larger studies are needed. The identification of which cut-off values for

¹⁸F-FDG uptake and LRNC size are associated with higher risk of developing symptoms for the individual patient is necessary if these investigations are to be of value for individual patients. Long-term clinical studies and further in-vitro studies of γ BB and its role in the partly microbiota-dependent carnitine-TMA-TMAO pathway is necessary to further investigate its potential as a biomarker for cardiovascular risk.

6.6 Methodological strengths, limitations and considerations

The major methodological strength of study 1-3 is the collection of all data within 48 hours including blood tests, PET, ultrasound and plaque following endarterectomy. The majority of previous studies have had long time-intervals between imaging and endarterectomy with the potential of plaques stabilizing in the time between symptoms, imaging and endarterectomy (histological analysis).

The definition of symptomatic/asymptomatic: The predefined cut-off for symptomatic patients in this study was 30 days, which may have limited the positive findings in study 1 and 3 as discussed earlier. There is no consensus on the time frame for study inclusion of patients with symptomatic carotid artery stenosis. The previously agreed threshold for surgical treatment with carotid endarterectomy was 6 months because this was an inclusion criterion for randomizing patients in the ECST and NASCET trials. There is now compelling evidence that the highest risk period for suffering a stroke after a TIA is the first two weeks, and the trend in research and in clinical practice is to include and treat patients as soon as possible after symptoms (guideline recommendation is TEA within 2 weeks of symptoms (99, 100)). In this thesis the definition of symptomatic was based on patient history with the possibility of missing silent brain infarcts. Further studies should therefore include cerebral MRI.

Plaque morphology classification: In the papers included in this thesis echogenicity was classified visually as described. Computer assisted classification of echogenicity with grey-scale median (GSM) commonly used for research purposes for the assessment of echogenicity has in some studies been shown to have better reproducibility compared to visual classification (101, 102). GSM echogenicity is expressed as a continuum on a grey-scale, which is averaged for all pixels in the outline of the plaque (plaque area), using the same reference structures of echogenicity (vessel lumen and adventitia). This computer-assisted

characterization gives a more objective assessment of plaque echogenicity and is less operator-dependent. However, GSM is also influenced by measurement errors when outlining the plaque (especially large echolucent plaques) and in the standardization procedures using the selected reference areas.

While some studies, as mentioned previously, have found GSM to have better reproducibility than the visual assessment of plaque echogenicity other studies comparing these two methods have found good agreement between GSM values and visual classification (103-105).

Histological assessments: The histological assessments of carotid plaques were by made by visual identification of inflammatory cells and the estimation of percentage inflammatory content per plaque. This method allows the assessment of the total amount of inflammatory cells in the plaque, including macrophages and leucocytes and has been shown to have a good to excellent intra- and inter-rater variability. Several methods have previously been described for estimating plaque inflammation histologically. These have included counting labeled macrophages. Leukocytes are also hypermetabolic cells with increased ^{18}F -FDG uptake. We therefore assessed both macrophage and leukocyte activity, based on the rationale that this would be more accurate when comparing histological evidence of inflammation with ^{18}F -FDG uptake. Slicing of the plaque which is necessary for histological analysis invariably leads to some cell nuclei being divided with parts of the same nuclei in different slices. In automated registration of labeled macrophages, depending on the predefinition of a cell (i.e. whether a complete nucleus is required in the definition), this can lead to cells not being counted or counted twice resulting in an under- or overestimation of inflammatory content. All methods used to correlate histological findings with FDG uptake on PET/CT imaging have potential weaknesses. Despite the careful removal of the plaque at endarterectomy, fragmentation of the plaque is sometimes unavoidable resulting in a reduction in the observed amount of inflammation on histology. The analysis of reproducibility for histological estimation of inflammation in our studies showed good agreement between assessments done two months apart.

Patient data in paper IV

Paper IV has some limitations due to limited patient data. Firstly, although blood samples were taken before lunch, reducing the likelihood of a large meal intake containing substantial amount of carnitine, the use of non-fasting samples as well as lack of information on vegetarians versus omnivores is a limitation of the analysis. Secondly, the controls were not investigated with carotid ultrasound and we cannot exclude the possibility that some of the controls also had carotid atherosclerosis. Thirdly, we did not have information on antibiotic treatment for each patient. These are all factors that potentially can affect gut microbiota and consequently blood levels of the carnitine-related metabolites. Future studies should therefore include data on these factors.

6.7 Clinical implications

Carotid Ultrasound, PET and MRI can accurately measure features of plaque instability and can be applied in research. Neither imaging modality has an established cut-off for the applied marker of instability (^{18}F -FDG uptake and necrotic core size respectively), which at present prevents their routine clinical use for the assessment of plaque instability. More accurate methods for identifying carotid plaque instability are very important if the prediction of stroke risk and treatment for patients with carotid atherosclerosis is to be improved. Long-term clinical studies of γBB as a cardiovascular risk marker and its role in the microbiota-dependent carnitine-TMA-TMAO pathway is necessary to further explore its potential as a biomarker of use in clinical practice.

7. MAIN CONCLUSIONS

The results of this thesis have shown that carotid ultrasound, PET and MRI may be used to identify components of carotid atherosclerosis known to be associated with increased cardiovascular risk. The following conclusions may be drawn on the specific research questions:

- (i) Carotid plaque ^{18}F -FDG uptake on PET/CT significantly correlates with degree of inflammation on histological assessments, and is associated with ultrasound plaque echogenicity and the risk of ischemic stroke.
- (ii) ^{18}F -FDG uptake on PET/CT is accurately measured using fewer/single measures of uptake such as plaque max SUV_{max} .
- (iii) Semi-automated carotid MRI can assess carotid plaque lipid core size in carotid artery plaques.
- (iv) Carnitine-related metabolites measured in plasma are linked to the prevalence of atherosclerosis, and may predict cardiovascular death.

8. FUTURE PERSPECTIVES

Improvements in new-generation PET scanners are believed to provide better resolution and thereby better imaging of small structures such as carotid plaques. The use of macrophage specific tracers, ^{64}Cu DOTATAE, ^{11}C -PK11195, are being evaluated with the hope of improving the plaque /background ratio of glucose (98). In addition to improving the assessment of inflammation in carotid plaques, these tracers may eventually allow the imaging of atherosclerosis in arteries where background uptake of ^{18}F -FDG would be prohibitively high, such as those in the cerebral and coronary circulation.

Combined MRI and PET can be expected to have an additional value over PET/CT in non-invasive imaging of atherosclerosis since CT does not visualize the vessel wall but primarily the lumen. Despite the limited spatial resolution of PET, CT can give the anatomical localization of the ^{18}F -FDG signal with regard to individual atherosclerotic lesions. CT angiography, however, cannot accurately measure plaque volume (because remodeling can accommodate large plaques with little impact on lumen diameter). A whole-body PET/MR imager allowing simultaneous MR and PET imaging has therefore been developed. The main advantage of this method is the perfect alignment between PET and MRI that allows for a precise delineation of the vessel wall, or plaque, and characterization of plaque components (e.g. the necrotic core). In addition it requires less examination time compared to sequential MRI and PET. In comparison with PET/CT the advantages of MRI are decreased radiation and superior visualization of soft-tissue plaque components.

Further studies, investigating the role of butyrobetain and other carnitine-related metabolites, and the association with atherosclerosis are warranted. Prospective studies including larger study populations with longer follow-ups, 5-10 years, including diet and stool analysis with assessment of cardiovascular outcome could give knowledge on the role of microbiota and carnitine- related metabolites for cardiovascular risk.

Carotid ultrasound is an excellent method for assessment of the carotid plaque. New ultrasound techniques are currently being investigated to increase the performance of this technique. These include the use of contrast and new software, which provide superior

visualization of the carotid artery plaque including neovascularization and elastography (106). This will potentially provide new important information that may be useful for the assessment of plaque instability.

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