Ultrasound assessment of carotid atherosclerosis focusing on plaque characteristics and changes over time

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Cover image: Adapted from an illustration from www.adam.com combined with an ultrasound image from the DIWA study.

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I would like to dedicate this thesis to my extraordinary mother, Irene.

No matter the situation she always finds a quote (and a way).

Sapias, vina liques et spatio brevi spem longam reseces.

Dum loquimur, fugerit invida aetas:

***CARPE DIEM**, quam minimum credula postero.*

Be wise, strain the wine, and scale back your long hopes to a short period.

While we speak, envious time will have already fled.

***SEIZE THE DAY**, trusting as little as possible in the future.*

Horace, 23 BC

In the words of the character John Keating, from the movie Dead Poets Society:

"Carpe diem… Make your lives extraordinary."

Tack Mamma!

ABSTRACT

Background and objective: In a clinical perspective better methods to identify subjects at increased cardiovascular risk are needed. Ultrasound-assessed measures of atherosclerotic plaques in the carotid arteries have previously been studied both with regard to occurrence, size and morphology. One promising plaque feature is echogenicity, as low plaque echogenicity has in several studies been related to future clinical events. However, better methods to assess plaque characteristics are needed as well as more information on the variability and change over time of echogenicity in relation to occurrence and total area of non-stenotic carotid plaques. Accordingly the aims were to develop a new method for plaque assessment, study variability over time and to examine plaque characteristics in relation to diabetes mellitus and hsCRP as a novel inflammatory risk marker of cardiovascular disease.

Methods: A population sample of 64-year-old Caucasian women (n=638) with varying degrees of glucose tolerance underwent assessment of cardiovascular risk factors and bilateral ultrasound of the carotid arteries for measurement of intima-media thickness (IMT), plaque burden and plaque echogenicity, at baseline and at 6 year follow-up. A semi-automated method to evaluate echogenicity (SAMEE) and its main feature, *Percentage White* (PW) were developed with the visual Gray-Weale classification as reference method. Validation was performed and PW was compared with the established Gray Scale Median (GSM) method. PW was then also analysed in images from the follow-up examination.

Results: PW was valid and highly reproducible, and correlated numerically to a higher extent than GSM with cardiovascular risk factors. Increasing number of intra-individual plaques was associated with an increase in average echogenicity as well as increasing variability of echogenicity. There was a rapid increase in plaque occurrence from 38% to 71% after 6 years. Although mean number of plaques per subject and total plaque area increased significantly at follow-up no significant differences in echogenicity were shown. In comparison with women with no diabetes, those with diabetes had more often plaque and lower echogenicity at baseline, but no difference in echogenicity at follow-up. The explanation may be concomitant treatment and improvement in life style leading to a favorable change in several risk factors. hsCRP≥2mg/ml was associated with an increase in maximum carotid bulb IMT at baseline, independently of other cardiovascular risk factors compared with those having low hsCRP. hsCRP was not associated with plaque echolucency or plaque occurrence but with total plaque area among women having carotid plaques.

Conclusion: The SAMEE program and its main feature, *Percentage White* (PW), was constructed and validated to handle different technical and artifact-related sources of variability. We showed that PW is highly reproducible and correlates to a higher extent than GSM with a number of cardiovascular risk factors. Our results suggest that the problem of multiple plaques in individual subjects in our data set is best managed by measuring the average PW of all plaques. Plaque area increased as expected during 6 years of follow-up, but this was not accompanied by a change in echogenicity. Diabetes was associated with increased plaque burden and plaque echolucency at baseline. Risk factor intervention and new medication may have impacted the findings at follow-up. hsCRP≥2mg/ml as a risk marker of future cardiovascular disease was associated with carotid bulb IMT and total plaque area among women with carotid plaques. Taken together, the SAMEE program and measurement of PW may be potentially valuable tools in the identification of subjects at increased cardiovascular risk. This has to be investigated in future studies.

Key words: Ultrasound, Carotid artery, Women, Plaques, Echogenicity, Atherosclerosis, hsCRP, Semi-Automated Method to Evaluate Echogenicity

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärtkärlsjukdomar till följd av åderförfettning är en av de vanligaste dödsorsakerna i västvärlden. Fortfarande är det svårt att med hög säkerhet förutsäga vilka personer som kommer att drabbas av sådana hjärtkärlsjukdomar. Mycket arbete pågår därför för att hitta metoder som kan identifiera personer med ökad risk innan de insjuknar. Åderförfettning, eller åderförkalkning som är ett annat namn på sjukdomen när den varat under lång tid, yttrar sig som en lokal förtjockning i pulsåderväggen. Denna förtjockning kallas plack och kan ha ett varierande innehåll av fett, bindväv och inflammation. Plack som innehåller en stor kärna med mycket fett och inflammation har ökad risk för att brista och därmed orsaka blodpropp som kan leda till sjukdomar som stroke och hjärtinfarkt. Med ultraljud går det att undersöka inte bara förkomst av plack i halspulsådror, antal och storlek, utan också plackets karaktär. Det har visat sig att plack som ger litet eko vid undersökningen har ett innehåll av mycket fett och andra komponenter som kännetecknar plack med risk för att brista. Nuvarande ultraljudsmetod för att undersöka plackets karaktär har flera begränsningar, framför allt för att den är subjektiv och kräver stor vana av undersökaren, vilket i sin tur leder till problem vid uppföljning och jämförelse mellan olika undersökare.

Syftet med avhandlingen var att utveckla och testa en halvautomatiserad mätmetod för att på ett säkert och reproducerbart sätt kunna utvärdera plackets karaktär. Undersökningarna gjordes på 638 kvinnor med och utan diabetes. Den första undersökningen gjordes när de var 64 år gamla och den andra undersökningen nästan 6 år senare.

Resultaten visade att det gick bra att utveckla en ny halvautomatisk tillförlitlig metod med en förbättrad metod för bedömning av plackkaraktär. Ett tidigare föga beaktat problem är att det är ganska vanligt med många plack hos samma individ. Studien kunde visa att med ökat antal

plack blir det allt större variation i plackens karaktär, samtidigt som placken blir mer ekotäta. Resultaten ger anvisning om hur mätresultaten ska räknas fram för den enskilda individen. Kvinnor med diabetes hade i jämförelse med dem utan sjukdomen oftare plack, och dessa plack hade oftare en karaktär som är förknippad med ökad risk för hjärtkärlsjukdom. Under uppföljningen på 6 år ökade förekomsten av plack kraftigt i hela den studerade gruppen. Med ett blodprov (CRP eller s.k. mikrosänka) kan graden av inflammation i kroppen mätas. Det har visat sig att en mycket lätt CRP-stegring är förenad med ökad risk för hjärtkärlsjukdom. I denna studie visade det sig att kvinnor med lätt CRP-stegring hade tjockare väggar och större plack i halspulsådrorna jämfört med kvinnor med normalt CRP.

Sammanfattningsvis kan konstateras att den nya metod som tagits fram för bedömning av åderförfettningsplack visar lovande resultat och kan bli ett viktigt verktyg i kommande studier för att utvärdera både risken för hjärtkärlsjukdom och effekter av läkemedelsbehandling på åderförfettningsplack.

LIST OF PUBLICATIONS

This thesis is based upon the following papers, referred to in the text by their roman numerals:

I. Percentage white: a new feature for ultrasound classification of plaque echogenicity in carotid artery atherosclerosis.

Prahl U, Holdfeldt P, Bergström G, Fagerberg B, Hulthe J, Gustavsson T.

Ultrasound Med Biol. 2010 Feb;36(2):218-26. Epub 2009 Dec 16.

II. Slightly elevated high-sensitivity C-reactive protein (hsCRP) concentrations are associated with carotid atherosclerosis in women with varying degrees of glucose tolerance.

Prahl U, Wikstrand J, Bergström GM, Behre CJ, Hulthe J, Fagerberg B.

Angiology. 2010 Nov;61(8):793-801. Epub 2010 Jun 13.

III. Carotid plaque burden and echogenicity in a prospective study of 64-year-old women

Prahl U, Bergström GM, Fagerberg B, Hulthe J

In manuscript

LIST OF ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
ANOVA	ANalysis Of Variance
Apo	Apolipoprotein
B-glucose	Blood glucose
BMI	Body Mass Index
BP	Blood Pressure
CCA	Common Carotid Artery
CEUS	Contrast Enhanced UltraSound
CT	Computed Tomography
CVD	Cardio-Vascular Disease
DIWA	the Diabetes and Impaired glucose tolerance in Women and Atherosclerosis
DM	Diabetes Mellitus
ECG	ElectroCardioGram
GSM	Gray Scale Median
HbAIc	glycated haemoglobin
HDL	High-Density Lipoprotein
hsCRP	high-sensitivity C-Reactive Protein
HR	Heart Rate
IGT	Impaired Glucose Tolerance
IMT	Intima Media Thickness
IVUS	IntraVascular UltraSound
JUPITER	the Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin
LDL	Low-Density Lipoprotein
Lp(a)	Lipoprotein a
MRI	Magnetic Resonance Imaging
NGT	Normal Glucose Tolerance
OGTT	Oral Glucose Tolerance Test
PET	Positron Emision Tomography
PW	Percentage White
SAMEE	Semi-Automated Method to Evaluate Echogenicity
SD	Standard Deviation
TG	TriGlycerides
WHO	World Health Organization
WHR	Waist Hip Ratio

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INTRODUCTION

ATHEROSCLEROSIS AND STROKE

The World Health Organization estimated in 2004 that 15 million people suffer stroke every year worldwide, of these 5.5 million die and another 5 million become permanently disable [1]. A closer examination of the number of deaths in stroke, show that women more often than men die from stroke (11 percent vs 8.4 percent) [1].

Many ischemic strokes appear to be the result from an embolization from an atherosclerotic plaque or an acute occlusion of the carotid artery and propagation of thrombus distally [2]. Obstructive carotid atherosclerosis with 70% stenosis is a well-known risk factor for stroke that is treated with endarterectomy. However, also smaller plaques are associated with increased risk of stroke [3]. Since atherosclerosis, as an important underlying disease of stroke can remain silent for several decades it is important not just focus on the stenotic plaques but rather on the subclinical stages to acquire a better knowledge of the disease progression. As stated by Ross already in 1993, data both from humans and animal models show that the initial lesions of atherosclerosis may progress over time to become advanced, occlusive lesions although in some instances lesions may lie dormant or even regress [4]. According to Falk *et al* cardiovascular risk factors, such as hypertension, diabetes, smoking, male gender, and possibly inflammatory markers (*e.g.*, C- reactive protein, cytokines,etc), all seem to accelerate the disease driven by atherogenic lipoproteins, the most prominent being low-density lipoprotein (LDL) [5]. Development of methods to identify atherosclerotic plaques, which later transform into complicated lesions leading to clinical events would open up for possibilities to improve prevention of stroke diseases.

ATHEROSCLEROSIS AND DIABETES

Diabetes affects over 100 million people worldwide [6] and it is estimated that the prevalence will rise to cover more than 5 % of the world population in 2025 [7]. Metabolic syndrome, insulin resistance, so called prediabetes (impaired glucose tolerance and impaired fasting glucose) and overt type 2 diabetes are all associated to a more extensive and even premature atherosclerosis both in the coronary and the carotid arteries [8-14]. Type 2 diabetes is often accompanied by hypertension and dyslipidemia which are major risk factors for cardiovascular disease (CVD). However, after adjustment for established such risk factors there is a remaining 2-3 fold increase in risk of CVD [15]. Hyperglycemia and the duration of diabetes seem to be other important contributors to increased risk [15-19]. However, it is still unclear why type 2 diabetes is associated with increased risk of atherosclerotic disease, beyond that which can be related to established cardiovascular risk factors. One approach to investigate that further is to study subclinical atherosclerosis by using non-invasive methods in population-representative samples of subjects with and without diabetes.

ATHEROSCLEROSIS AND INFLAMMATION

Inflammation plays a central role in all the phases of the atherosclerotic process and several soluble markers of inflammation have been associated with the progression of atherosclerosis. The acute-phase C-reactive protein (CRP) has surfaced as a major predictor of cardiovascular disease [20-23]. Another important element as mentioned above for cardiovascular disease is the metabolic syndrome and a well-known association exists between serum CRP levels and components of the metabolic syndrome such as central obesity, insulin resistance, impaired glucose tolerance, and hyperlipidemia [24-26]. An increased intima media thickness in the carotid arteries is associated with hsCRP, although not a consistent finding throughout all studies [27, 28]. Furthermore some studies have reported an association between hsCRP and

occurrence of plaque in the carotid arteries [29-31],but most studies have not shown such an association [32-38]. In observational studies a suggested cut-off level such as hsCRP ≥ 2.0 mg/mL, have indicated an increased risk for CVD [39, 40]. By using this hsCRP cut-off level for identification of subjects at risk, rosuvastatin treatment in the JUPITER trial (the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin) lowered both the concentrations of hsCRP and gave a 44% relative reduction in primary end point [41]. A logical question related to these studies is whether hsCRP ≥2.0 mg/mL also is associated with an increased prevalence of carotid plaques compared with lower hsCRP concentrations in serum?

NON-INVASIVE MEASUREMENTS OF ATHEROSCLEROSIS

There are many various imaging modalities in use to evaluate and quantify atherosclerosis. Among the non-invasive methods are: magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and ultrasound. Every technique has both strengths and drawbacks, but we believe that ultrasound have the benefit of being non-invasive (valid for most ultrasound methods), non-expensive as well as having easy accessibility. Ultrasound can be separated into two dimensional (2D), three dimensional (3D), contrast enhanced ultrasound (CEUS) and intravascular ultrasound (IVUS). The 2D B-mode ultrasound imaging of the carotid arteries offers an efficient and cost-effective diagnostic tool for early detection and risk assessment of atherosclerotic disease even in the earlier clinically "silent" stages whether it is measuring the intima media thickness (thickness, rate of thickening) or identification, classification and measurement of plaques [3, 42-44].

Intima media thickness

The wall thickness of the carotid artery, measured as intima media thickness (IMT) is an indicator for early carotid atherosclerosis. We and others have previously shown that an increased common carotid artery (CCA) IMT is associated with the established risk factors for cardiovascular disease, coronary atherosclerosis, and cardiovascular morbidity [42, 45-52]. Moreover the Rotterdam Study and the Cardiovascular Health Study showed that each 1 SD change in CCA IMT both increased the risk for stroke by 34 % over a period of 2.7 years, and increased the yearly incidence of stroke by 28 % in asymptomatic subjects independently of other cardiovascular risk factors [51, 53]. As shown by O'Leary *et al* [54] the thickening of the carotid wall may progress at various rates for different areas in the carotid artery. Because of the association between an increased IMT and the presence of plaque elsewhere in the carotid arteries and in the coronary arteries [55, 56] IMT of the CCA appears to reflect systemic atherosclerosis although Ebrahim *et al* have shown that focal carotid plaque are more strongly associated with cardiovascular risk than a diffuse increase in IMT [57]. Johnsen *et al* concluded that plaque measured in the carotid bulb or internal carotid artery is stronger related to hyperlipidemia and smoking and is a stronger predictor for MI, whereas CCA-IMT is stronger related to hypertension and ischemic stroke [58]. The carotid bulb (or internal carotid artery) might be a marker better exhibiting early changes in the intima-media complex, for example previous studies have shown that plaques tend to form near areas of hemodynamic stress [54, 59]. There has also been suggested that the CCA IMT might be a medial thickening rather than an intimal thickening thus differing the diffuse predilection from the more focal stenotic disease [59, 60].The diffuse predilection has been correlated to procoagulant factors for example fibrogen rather than inflammatory markers such as hsCRP [59].

Plaques

The atherosclerotic disease progresses in several stages via fatty streak to fibrotic plaque that may develop into a vulnerable plaque which is prone to rupture, leading to a complicated lesion that may develop into a cardiovascular event [61]. In clinical practice the degree of carotid artery stenosis is used as an indicator of high cerebrovascular risk in some patients but it remains unknown why some stenotic atherosclerotic lesions lead to cerebrovascular disease whereas other lesions with an equal degree of stenosis do not [2, 62]. Moreover results from the Asymptomatic Carotid Surgery Trial show that in the group of subjects under medical treatment the incidence for stroke was < 10 %, further underlining that other factors than the degree of stenosis play important parts [63]. Plaque morphology has emerged in recent years as an important contributory factor in cardiovascular risk. The development of vulnerable plaques and their causal relation to clinical disease was first recognized for coronary artery disease, but is now also identified as valid for symptomatic carotid atherosclerosis [64]. Striking features of the vulnerable plaque are a large necrotic core, thin fibrotic cap, and inflammation [65]. Furthermore the inflammatory response mainly in the thin fibrotic cap is a major mechanism in plaque vulnerability [66], and the risk for plaque rupture depends more on the composition of the lesion than on the actual degree of stenosis [61, 67, 68]. Furthermore as shown by Wahlgren *et al*, a fibrous cap inflammation is more frequent in non-calcified plaques, suggesting that plaque calcification actually is a marker for plaque stability [69].

Even the occurrence of small, non-stenotic plaques is associated with an elevated risk for future cardiovascular disease [3] and the ultrasound technique makes it possible to identify and classify those. However, one should keep in mind that the development of carotid plaques is related to aging, and plaques are frequently occurring in individuals over the age of 60 [70]. Among several ultrasound-derived plaque features echogenicity has been the focus of many

research-groups, whether they classify the plaques subjectively or objectively [71-75]. The value of plaque echogenicity for predicting cardiovascular disease have also been discussed in several studies[76-78]. Even if the ultrasound method for assessing carotid plaque echogenicity may vary, comparing plaque echogenicity with histological findings correlates well [79-82]. It has been shown that echogenic plaques are rich in calcium and fibrous tissue, wheras echolucent plaque contains more elastin, lipids and hemorrhage [62, 75, 83] *i.e.,* features related to plaque vulnerability. Furthermore, both large plaque volume and low plaque density (measured by gray scale median [GSM] or visually evaluated) are associated with increased cardiovascular risk[3, 79].

If one would draw parallels with the coronary circulation, where plaque instability is not just a local phenomenon and fatal AMI is associated to a diffuse coronary instability [84, 85]the method of letting one plaque of the carotid arteries be equivalent to the plaque burden of a patient might be a reasonable approach unless there is a great heterogeneity within subjects.

Manual classification of plaques characteristics

The assessment of plaque echogenicity can be separated into subjective and objective (*i.e.*, computer assisted) methods. There are several subjective methods, but most are focused on visually estimating the pattern and appearance in gray scale of the ultrasound image of the plaque. As stated previously, a relation between carotid plaque echogenicity assessed subjectively, and cerebrovascular disease clearly exists [77, 86-88]. But even if the subjective method to classify plaque seem to be correlated to histopathological findings, there is still a considerable variation in observer reproducibility showing only fair to good κ values [73, 89, 90]. Among the suggested subjective plaque classification is the Gray-Weale method, which classifies plaques into four types [71]. The Gray-Weale method have been used in several

studies and has been tested for its predictive value in prospective studies of cerebrovascular and cardiovascular events [77, 91].

Several studies have emphasized the need of a more standardized and quantitative method of plaque characterization [72, 88, 89]. One reason for the lack of conformity in the data might be because that the plaque appearance is visually judged by an examiner. The result is a subjective and highly user-dependant description of different aspects of plaque morphology that is difficult to reproduce. However, if automatic image analysis tools would be applied the features can be standardized and used by other laboratories in clinical trials and possibly implemented in clinical work flow. In addition an automatic image analysis tool should be design to also handle and reduce different technical and artifact-related sources of variability. If the image analysis would be performed automatically it is likely that more objective and user-independent information would be extracted. Another reason for discrepancies is the choice of which plaque to use for the characterization. While many studies use stenotic plaques [73, 92-94],studies of non-stenotic plaques choose either the biggest plaque per patient or the plaque with the most echolucent appearance [91, 95, 96] even if the Tromso study used weighted means of GSM of all recorded plaques [97]. To our knowledge no systematic approach has been used to estimate plaque echogenicity on the individual level in subjects with multiple non-stenotic plaques. Hence, it is important, both for research and clinical use, to establish tools to objectively characterize and stratify the cardiovascular risk of carotid plaques from a population perspective.

Automated classification of plaque echogenicity[1]

Although there is a consensus on how to differ a intima media thickness from the focal plaque [98], an automatic procedure to identify plaques within the carotid artery ultrasound image is clearly a methodological challenge. Automatic procedures could reduce the evaluation time, reduce the subjectivity in plaque delinearization and plaque characterization as well as allow less experienced readers to evaluate images with good results. At present, mainly semi-automatic solutions are available and seem to be more or less successful in estimating the composition of the ultrasound image from plaque tissue [74, 99-105].

A procedure used to automatically assign objects into different classes is called a classifier. There are a number of technical aspects that needs to be considered to develop an automated procedure for image classification. Briefly, it can be described in three major steps: training, validation and classification.

The training of the classifier is done with the help of a training dataset using pre-classified samples and with the purpose to find patterns in the data that separate the different classes from each other. In image analysis, however possible to consider all the individual pixel values and their information it is usually better to aggregate this detailed information into one or more groups so-called features for example the gray-scale mean and variance, features based on gray level co-occurrence matrices (also called "gray-tone spatial-dependence matrices") [106], and features based on frequencies in the image *i.e.,* its Fourier spectrum.

Once the training process has yielded an adequate result, the classifier needs to be validated using a validation dataset with images that have not yet been tested. Besides selecting the feature set, the decision on which classification technique to use is crucial. A number of

[1] Parts of text also featured in a forth-coming textbook (*Carotid Atherosclerosis and Ultrasound, chapter: Automated Classification of Plaques. Bergström G, Prahl U, Holdfeldt P)*

different classifier techniques exist, some of the most common are: Bayesian classifiers, which try to find the most probable class for an object by using feature statistics (mean vectors and covariance matrices); K-Nearest-Neighbors (k-NN) classifiers classify an object by looking at the k nearest samples in the training set (most similar feature values); Neural networks are classifiers that are inspired by modern brain research. The computations are done by units called "neurons" that are organized in a network. More information about classification methodology can be found in the book by Duda *et al* [107].

Images are often pre-processed to facilitate the work of the classifier. Different ultrasound equipment, user settings and differences in the patients' ultrasound characteristics may result in differences in the images that are not related to the actual plaque tissue. Rescaling the images which will normalize the image gray-scale appearance in relation to different ultrasound equipment, different user settings and differences in patient ultrasound characteristics is a usual technique. A way of reducing this problem is to normalize the images by linear rescaling of their intensity (gray scale) values [108].

In both research and clinical work, the boundary outlining of the plaque from the 2D ultrasound image of the carotid artery is mainly done manually by experienced sonographers with reasonably high inter- and intra-observer reproducibility [109-111].

Last but not least, the 2D representation of a plaque is an over-simplification that does not necessarily reflect the true 3D appearance. Attempts have been made to reconstruct 3D-volumes of plaques that could possibly have higher predictive value for future clinical events than 2D-areas [112, 113].

The real challenge is to analyze the properties of the back-scattered ultrasound from the plaque in a biologically meaningful way. The goal would be to find features that can predict if the imaged plaque will eventually cause clinical events. So far the ultrasound image features that have been tested for their predictive value can basically be divided into two groups: (i)

features related to the overall echogenicity of the plaque, which are dominating [103, 114-116]; and (ii) features related to the texture of the plaque [104, 105, 117]. The texture of the plaque is often referred to as either homogenous (equal distribution of echogenicity) or heterogenous (unequal distribution of echogenicity). The overall echogenicity of two plaques can thus be similar but the texture differs.

Several publications show that both overall plaque echogenicity and plaque texture carry information on future risk for stroke as well as risk of other cardiovascular events [77, 118-121].

To summarize: there are basically three aspects of carotid plaque ultrasound appearance that needs to be dealt with to stratify atherosclerotic carotid disease in different subtypes: i/ plaque presence (yes/no); ii/ plaque size; and iii/ the properties of the reflected ultrasound from plaque tissue.

HYPOTHESIS

We suggest that it is possible to improve the characterization of non-stenotic carotid artery plaques regarding morphology and risk for future cardiovascular disease by using the most modern ultrasound technology and develop computerized image analysis of the ultrasound data.

Comment: There is a great interest in identifying asymptomatic individuals at risk, who would possible be candidates for intensive medical interventions aimed at preventing death and disability from coronary heart disease and stroke. Since echolucent carotid plaques are associated with a higher risk for ischaemic stroke, as well as as a higher risk for restenosis

after endarterectomy and myocardial infarction [90] risk intervention might be more beneficial for patients with echolucent plaques. For the possible beneficial preventive treatment for such patients to be evaluated properly, the method on how to characterize rupture-prone echolucent plaques must become more standardized and objective and validated in prospective cohort studies as well as in randomized clinical trials. The underlying concept is that the echolucent vulnerable plaque has properties which are possible to identify by ultrasound technique However, no previous study have assessed all present plaques for echogenicity determination.

AIMS OF THE THESIS

The aims of the current thesis were to: I) develop a new method for semi-automated ultrasound image analysis to classify non-stenotic carotid plaques, evaluate cases with multiple plaques, and examine the association between a new image analysis feature of echogenicity and predictors of cardiovascular disease; II) examine if the cut-off value of hsCRP $\geq$ 2mg/L is associated with increase in carotid IMT independently of common cardiovascular risk factors, and also with increased plaque burden in the carotid arteries, and increased occurrence of echolucent plaques, and III) assess the variability in echogenicity between plaques in the same individual as well as echogenicity in relation to number of plaques, and furthermore to explore the change in plaque burden and echogenicity at 6 years of follow-up.

METHODS AND STUDY POPULATION

OVERVIEW OF STUDY DESIGN

This project is based on a population sample of originally 64-year-old women undergoing a baseline examination and a follow-up examination as shown in Figure 1. Paper I and II were based on the cross-sectional examination at baseline whereas paper III included both the baseline examination and the follow-up examination (Figure 1).

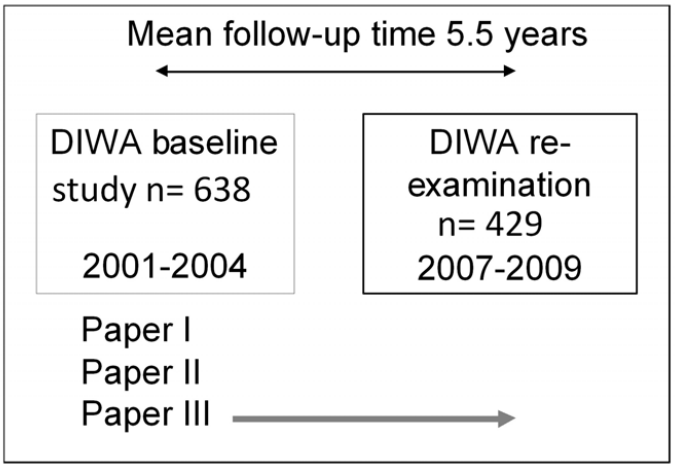

Figure 1. Overview of study design DIWA and relation to paper in this thesis.

Paper I

The first paper focus on developing a semi-automated software program for plaque classification **[**Semi-Automated Method to Evaluate Echogenicity (SAMEE)]. The development of this program used as a reference method the established visual Gray-Weale classification system that has been validated against histological plaque characterization [71]. The software should also give quantitative values for echogenicity that was assessed as Gray Scale Median and as the new measure Percentage White (see below). The different measures of echgenicity were also compared as regards associations with predictors of cardiovascular disease. These predictors included smoking, waist circumference, systolic blood pressure and serum concentrations of low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, apolipoproteins A-I and B, lipoprotein(a), blood glucose, HbA1c and adiponectin.

Paper II

The association between serum hsCRP $\geq$ 2mg/L and carotid atherosclerosis was examined in paper II. The predictors of cardiovascular disease that were included included were, apart from hsCRP, smoking (assessed as cigarette years and smoking status), anthropometric data, serum concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, apolipoproteins A-I (Apo A-I) and B (Apo B),Apo B/Apo A-I ratio, lipoprotein (a) (Lp(a)), blood glucose, HbA1c, blood pressure, and heart rate. Systolic and diastolic blood pressures were assessed in supine patients at rest. Heart rate was assessed using electrocardiogram (ECG).

Paper III

The third paper systematically examined the relation between echogenicity and the number of plaque in the same individual, explored the change of plaque burden and plaque echogenicity at 6 years follow-up, and furthermore investigated the relationship between diabetes and plaque burden and plaque echogenicity both at baseline and at 6 years of follow- up. In paper III, the predictors of cardiovascular disease included smoking (smoking status), diabetes, anthropometric data, serum concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, Apo B/Apo A-I ratio, HbA1c, hsCRP, blood pressure, and heart rate. Heart rate was assessed using electrocardiogram (ECG).

POPULATION SAMPLE

The Diabetes and Impaired glucose tolerance in Women and Atherosclerosis (DIWA) study is based on screening of 64-year-old women in Gothenburg, Sweden to identify those with diabetes, impaired glucose tolerance and normal glucose tolerance [122]. The study was approved by the regional ethics committee and all participating subjects gave informed consent. From the screened cohort of 2295 women, a stratified sample of 638 women underwent ultrasound examination.

The numbers of women participating in the analyses and reasons of non-participation were as follows: In paper I there were 264 women who had carotid plaques, the other were not included in this analysis. In paper II 635 women were included as hs-CRP values were missing in 3 women but for the ultrasound examination 559 women had IMT measurements. Paper III was based on the fact that from the 638 women that were included in the ultrasound examination at baseline, 588 women were found to have complete sets of ultrasound images

for interpretation. At the 6 years follow-up, 429 women were found to have complete set of ultrasound images comparable with the baseline examination. The reasons for not participating were:(i) no ultrasound images (n=11), (ii) incomplete ultrasound interpretation at baseline (n=39),(iii) no follow-up exam (n=141), and (iv) incomplete ultrasound interpretation at follow-up (n=18), see Figure 2.

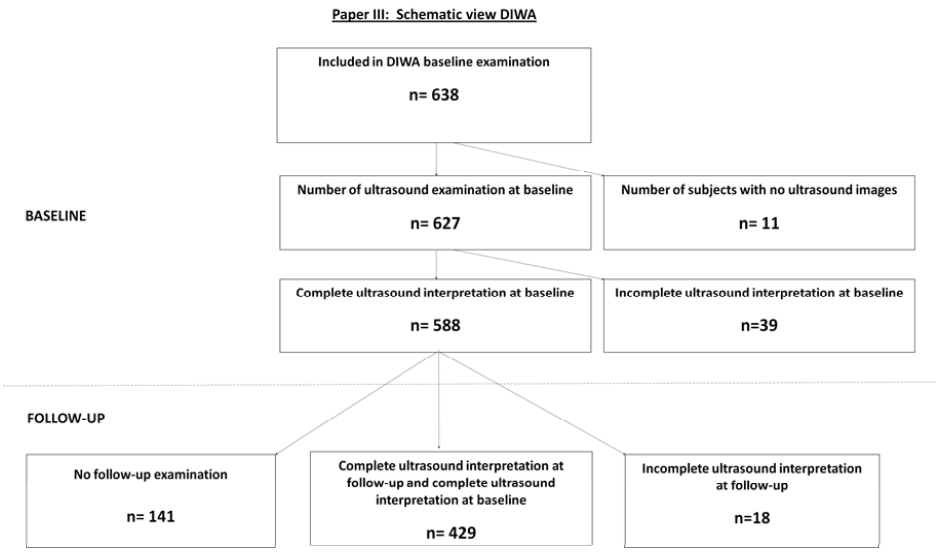

Figure 2. Schematic view for ultrasound examinations in paper III. Adapted from paper III.

EXAMINATIONS OTHER THAN ULTRASOUND

Both the examinations at baseline and follow-up included completion of questionnaires, anthropometric measures, measurement of blood pressure, recording of ECG, blood samples for biochemical analyses and ultrasound examinations of the carotid arteries. The blood samples were drawn in the morning when the subjects had been fasting overnight.

Questionnaires

Self-administered questionnaires were used to obtain information on previous and present disease, current medication, smoking habits and family history of diabetes as previously described [122].

Anthropometry

Body weight was measured in underwear on a balance scale to the nearest 0.1 kg and height to the nearest 1.0 cm. Waist and hip circumference were performed with the patient standing and in accordance with current guidelines. Waist-hip-ratio (WHR) and body mass index (BMI) were calculated. BMI was defined as weight in kilograms divided by the squared height in meters.

Blood pressure

Blood pressure was measured in the right arm with the patient in supine position, using a cuff of appropriate size after at least 5 minutes of rest. The mean of two recordings was used.

Oral glucose tolerance test (OGTT)

At the baseline examination a 75g OGTT was performed in the morning (before 11 a.m.), fasting- and 2-h post load capillary blood glucose were measured. The participants had been asked to fast overnight, to avoid heavy physical activity during the previous day and to avoid smoking in the morning before the test. Women who reported a current infection had the examination postponed two weeks. Women fulfilling the criteria for DM or IGT were re-

examined within 2 weeks with a repeated OGTT. If fasting glucose was in the diabetic range at the second examination, OGTT was not performed.

At the re-examination fasting plasma glucose was measured and repeated if elevated. The WHO-definition was used in the classification of diabetes mellitus [123].

Biochemical measurements

Blood samples for biochemical analysis were collected and serum and plasma were frozen in aliquots at -70ºC within 4 hours.

The cholesterol and triglyceride levels were determined by fully enzymatic techniques (Thermo Clinical Labsystems, Espoo, Finland). All analyses were performed on a Konelab 20 autoanalyser (Thermo Clinical Labsystems) at the Wallenberg Laboratory. High-density lipoprotein (HDL) was determined after precipitation of apolipoprotein (apo) B-containing lipoproteins with magnesium sulfate and dextran sulfate (Thermo Clinical Labsystems). Low-density lipoprotein (LDL) was calculated as described by Friedewald *et al*. [124].

HbA1c was determined with high pressure liquid chromatography on a Mono S HR 5/5 column (Amersham Biosciences, Piscataway, N.J., USA and Pharmacia, Uppsala, Sweden) [125]. Fasting capillary blood glucose was measured immediately with the modified glucose dehydrogenase reaction (Hemocue AB, Ängelholm, Sweden). Serum levels of adiponectin was determined by a sandwich ELISA kit (R&D Systems Europe, Abingdon, UK). High sensitive CRP (hsCRP) was measured by an ultra sensitive method using particle enhanced immunoturbidimetri (Orion Diagnostica, Espoo, Finland) and the coefficient of variation was 3.4%. Lipoprotein (a) was analysed by an immunoturbidimetric method (Kamiya Biomedical Company, Seattle, USA). All analyses were performed on a Konelab 20 autoanalyser (Thermo Fisher Scientific, Vantaa, Finland). Interassay coefficient of variation was for all Konelab analyses below 5%.

ULTRASOUND

For papers I, II and baseline measurements in paper III, ultrasound examinations were performed with an ultrasound scanner equipped with a linear 8L5-MHz transducer (Sequoia 512,Siemens, Mountain View, California). For follow-up measurements in paper III, the ultrasound examination was performed using an ultrasound scanner equipped with a VF10-5 MHz transducer (Antares Sonoline, Siemens, MountainView,CA). The methods to obtain the ultrasound data was the same for all three papers. In brief: to minimize variability during the cardiac cycle an ECG signal (lead II) was simultaneously recorded to synchronize image capture to the peak of the R wave. The left and right carotid arteries were scanned at the level of the bifurcation, and the images used to measure IMT (paper II) were recorded from the far wall in the common carotid artery and the carotid bulb from the real-motion image loop (real-time images). To identify and record the occurrence of atherosclerotic plaques (papers I, II and III), carotid arteries were scanned from the distal part of the CCA to 10 mm into the external and internal carotid arteries. A sequence of real-time images was captured and saved digitally from the position yielding the best visibility of the plaque *(i.e.,* the largest cross sectional area in a longitudinal transaxial view, as judged visually). We have previously good reproducibility of ultrasound measurements in our laboratory, intraobserver variability, IMT mean CV= 5.3 % [126] and plaque echogenicity (PW) CV=9.85% [127].

Paper I [*Semi-Automated Method to Evaluate Echogenicity (SAMEE)]*

On the bases of the success of GSM for image classifications we were encouraged to further develop this concept by incorporating some other aspects of image information. Our aim was to develop an automatic software solution to classify plaques into high echogenicity and low echogenicity plaques according to the visual and subjective Gray-Weale classification [71] The objective was also that the software better should mimic the human eyes possibility to

incorporate information on general image noise and overall image echogenicity in the automatic procedure. The software was thus trained to correctly classify and separate a set of high echogenicity plaques from a set of low echogenicity plaques obtained from one of our population-based studies [122]. We used the visual Gray-Weale [71] classification as our gold standard. The scale was slightly modified because we grouped dominantly echolucent and substantially echolucent plaques into one group called "echolucent"[2] and dominantly echogenic and uniformly echogenic plaques into another group called "echogenic"[2] (Figure 3). Our intention was to develop a classifier that in a similar way to that of an expert classifies the overall echogenicity of a plaque *i.e.,* it estimates the relative occurrences of echolucent versus echogenic regions inside the plaque. Three readability criteria were used for the plaques: (i) clearly visible delineation of the plaque, (ii) <50% echo loss and/or shadowing of the plaque, and (iii) no need for additional information from a real-motion image loop. The majority of these plaques were located in the far wall (73%).

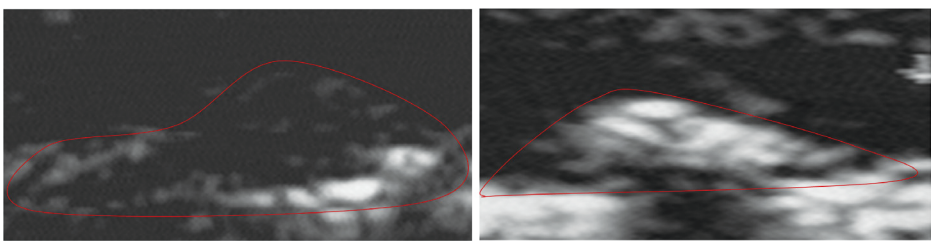

Figure 3. Echolucent plaque to the left, echogenic plaque to the right

SAMEE is based on a single feature, percentage white (PW). For each image, two reference values, black (*i.e*., the most echolucent pixel) and white (*i.e*., the most echogenic pixel) were

[2] The terms hypoechoic and hyperechoic are sometimes used instead of echolucent and echogenic.

selected and used to normalize the image by linear rescaling (0 to 255). Briefly described the classification is divided into several steps with the goal to create an intensity threshold (I_T) which can determine if a pixel is echogenic or not: (i) four different regions are first defined as described in Figure 4: Image Region, Plaque Region, Extended Plaque Region and Noise Reference Region. The Extended Plaque Region gives the value for the tissue echogenicity (I_E), whereas the Noise Reference Region gives the value for the noise in the image (I_N); (ii) taken together with the weighted constants (found with grid search and differing between far and near wall) an intensity threshold is formed using eqn (1):

$$I_T = w_E I_E + w_N I_N + w_0 \quad (1)$$

If the echoes inside the adventitia are weak, then the medium intensity ("gray") pixels inside the Plaque Region are more likely to belong to "real" echoes. Consequently, a decrease in the Extended Plaque Region intensity I_E corresponds to a decrease in intensity threshold I_T. If there is a considerable amount of noise inside the Noise Reference Region, then the probabilty of noise inside the Plaque Region is high. In that case the "gray" pixels are more likely to be "false" echoes, increasing the Noise Reference intensity I_N increases the intensity threshold I_T. The value of the intensity threshold I_T can be viewed as an adaptive threshold that takes into account echogenicity and noise. Furthermore, SAMEE also provides values for plaque size (height, width and area), gray scale mean and gray scale median (GSM).

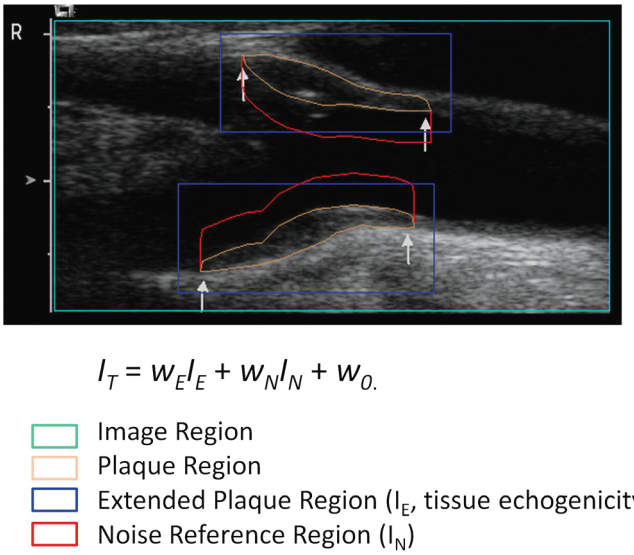

Figure 4. Different regions used for classification in SAMEE. Adapted from paper I.

Procedure to develop and validate SAMEE

To develop and validate SAMEE three steps where performed much like previously described ("Automated classification of plaques"): (i) a training dataset was used in the development of the method with the visual classification according to Gray-Weale [71] as a reference for plaque classification,(ii) SAMEE was then subsequently tested in a new data set for validation, and (iii) PW was related to circulating predictors of cardiovascular disease, also with the aim to further explore cut-off values. In order to compare the methods when choosing the biggest plaque, the most echolucent plaque or as we suggest all plaques possible for interpretation PW measurements were performed to determine: (i) average PW, the average value of PW of all plaques in each subject; (ii) biggest plaque PW, the PW from the

plaque with the biggest area in each subject; and (iii) worst case PW, the PW from the plaque with the lowest PW-value in each subject(*i.e.*, the least echogenic plaque). Reproducibility tests for visual and automatic classification, as well as comparison between PW and GSM were performed.

Paper II

Measurements of IMT and plaque characteristics were done according to the definitions previously used [128], which supports the American Society of Echocardiography (ASE) consensus [129]. A composite measure of IMT was calculated as the mean IMT of the CCA and carotid artery bulb. Cross sectional area for the CCA was calculated as the difference between the total area inside the adventitia and the lumen area: $\pi(LD_{mean}/2+ IMT_{mean})2 - \pi (LD_{mean}/2)$. Plaque echogenicity was assessed by (1) visual classification, using the Gray-Weale method[71] and (2) by using a new software, Semi-Automatic Method to Evaluate Echogenicity (SAMEE) described previously in this thesis ("Ultrasound": paper I). SAMEE presents values for GSM and percentage white (PW). For participants with multiple plaques, we calculated the average GSM and average PW (the average of GSM and PW values from all plaques in each participant, respectively ("Methods and study population": paper I).

Paper III

Definitions used for plaque characteristics were the same as those described for Paper II. Plaque echogenicity was assessed as PW and GSM by using SAMEE as described above. Low values indicate echolucency. For participants with multiple plaques, the average GSM and average PW were calculated. The variability in plaque echogenicity was examined by calculating the difference between the plaque with the highest and lowest echogenicity (PW,

GSM) for each subject with multiple plaques. Furthermore, the percentage of subjects with an average echogenicity below a low echogenic cut-off (PW below 28.9) was calculated.

STATISTIC ANALYSES

Paper I

Statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Values are given as median (interquartile range) and numbers (%). Sensitivity and specificity for binary classification tests were calculated. Cohen's kappa coefficient (κ) was calculated to assess the reproducibility of classifications [130]: $\kappa = 0.00–0.20$ indicates zero to slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61- 0.80, substantial agreement; whereas ≥ 0.81 is regarded as almost perfect agreement. The measurement of variation(s) was defined as $SD/\sqrt{2}$ and the coefficient of variation was calculated as $CV (\%) = s * 100/\mu$, where μ represents mean of the population. Non-normally distributed variables such as the predictors of cardiovascular disease were correlated to average PW, biggest plaque PW, worst-case PW and average GSM, using Spearman's rank correlation coefficient. Furthermore, average PW, biggest plaque PW, worst-case PW and average GSM were divided in tertiles, and Mann-Whitney's U test was used to compare the levels of predictors between tertile 1 and 3. A p-value less than 0.05 (two-sided) was considered statistically significant. For the comparisons between PW and GSM, Pearson product-moment correlation coefficients were calculated**.**

Paper II

The cohort was divided into 2 groups, hsCRP <2.0 mg/L and hsCRP ≥2.0 mg/L for statistical analyses. Statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results are presented as means ± standard deviation or number and percentage unless otherwise indicated. Categorical data were analyzed by Fishers exact test and chi-square test. Mann-Whitney U tests were used for comparison of continuous variables. A well-known problem is the co-variability between risk factors related to hsCRP and carotid IMT. To reduce the number of variables included in the multiple regression analysis, a correlation matrix was used to select the most representative variable, that is, the variable with the highest correlation coefficient with both carotid IMT and hsCRP for each of the risk factor clusters representing obesity and glucose/insulin metabolism, respectively. Regarding lipoproteins, Apo B/Apo A-I ratio was included in the multivariate analysis because it is known to mirror both proatherogenic and antiatherogenic lipoproteins in the circulation. For the regression analyses, skewed variables were log transformed. However, to obtain measured values, additional analyses with the same adjustment was performed not log transforming the IMT variable. Two-tailed $p < 0.05$ was considered significant.

Paper III

Statistical analyses were performed with SPSS18.0 (SPSS Inc., Chicago, IL, USA). Results are presented as means ± standard deviation (normally distributed data), medians with interquartile range (non-normally distributed data) or number and percent. Categorical data were analyzed with chi-square test. For non-normally distributed variables, Mann-Whitney U or Kruskal-Wallis one-way analysis of variance by ranks tests (depending on the number of variables) was used for comparison of continuous variables. To evaluate the variability in echogenicity, the ANOVA test for linearity to compare means in-between groups were used on log-transformed values. For the longitudinal comparison between baseline and follow-up values the paired sample t-test was used for normally distributed data, and the Wilcoxon

matched–pair signed-rank test was used for non-normally distributed variables. Two-tailed $p<0.05$ was considered as statistical significance.

SUMMARY OF RESULTS AND DISCUSSION

PAPER I

The gold standard for ultrasound assessment of plaque echogenicity have yet to be established and several methods exists, both subjective and objective [71, 73, 74, 99]. The manual classification according to Gray-Weale [71] have several benefits: (i) it is well established and frequently used in clinical settings, (ii) have been tested in prospective studies for predictive value for cardiovascular events [77, 88, 91], and (iii) uses the superior advantage of the human possibility to "process" images, both the integrative image processing of the human eye and the possibility for us to discriminate information not needed in the image. However, the method's benefits can also be its downfall because the result is highly user-dependant which makes standardization and comparison with other laboratories, for example in clinical trials difficult. We used the Gray-Weale classification as a reference to create a new automated, objective method with the aim to include the benefits of the subjective visual classification to create a standardized, quantitative classification.

Training results

To gain optimal values for the intensity threshold a training session with calibration against the well established visual Gray-Weale classification was performed. Reproducibility tests during the training session (far-wall plaques) showed a very good agreement in the visual

intraobserver variability (κ = 0.88), further improved in SAMEE (κ = 0.97). Comparing visual classification by Gray-Weale to objective classification by SAMEE showed a substantial agreement in inter-methodological variability (far-wall plaques, κ = 0.78; near-wall plaques, κ = 0.63). Sensitivity and specificity showed slightly lower values for sensitivity than specificity throughout all the tests. Using the visual Gray-Weale as the gold standard proved SAMEE to be good at correctly identify both the actual positives and the actual negatives.

Validation data

Validation of SAMEE made in a different data-set (both far- and near-wall plaques) gave similar results (κ=0.77), and the intra-observer variability of SAMEE classification showed almost perfect agreement (κ= 0.90) in the hands of an experienced technician. The reproducibility test for the new feature, PW revealed a high correlation between the two measurements ($r=0.96$, $p<0.0001$) and CV=9.85%.

Echogenicity in multiple plaques vs predictors of cardiovascular disease

To our knowledge, no standardized approach exists to estimate plaque echogenicity on the individual level for non-stenotic multiple plaques. Furthermore, the relationship between risk factors or outcomes and plaque echogenicity has not previously been studied in subjects with multiple plaques. In our population sample more than 40% of the women had multiple plaques, and it is well established that with increasing age more plaques seems to develop [70]. Previous studies by us and others have used biggest plaque per subject [82, 95, 96] , or so called the “worst case” per subject *i.e.*, the most echolucent plaque per subject [91]. We evaluated whether there was any difference in the relation to risk factors when using the previously most common approach (one plaque per patient) or the new approach, average PW. We found that average PW was the variable with numerically most significant

associations with risk predictors for cardiovascular disease as compared to PW measurements on one plaque only (biggest plaque or plaque with lowest PW). Well-established factors or markers of cardiovascular risk that were related to PW were ApoA-I [131], ApoB/ApoA-I [132], HbA1c [133] and blood glucose [134], Lp(a)[135, 136] and adiponectin. Adiponectin, a molecule characterized by anti-inflammatory and antiatherosclerotic properties [137] proved to be lowest in the group of women with the most echolucent plaques. Further comparing tertile groups 1 and 3 of all three methods of measure, one might speculate that the biggest plaque, having correlations with blood glucose and HbA1c is more related to glucose intolerance and as such associated to a more extensive and fibrous atherosclerosis both in the coronary and the carotid arteries [8-14]. Moreover, the worst case plaque was related to Apo B/ApoA-I which mirrors the relation between pro-atherogenic lipoprotein (LDL) and anti-atherogenic lipoprotein(HDL) [132], and plaques with low echogenicity have been showed to be characterized by a high lipid content and hemorrhage [138]. However, the average PW showed significant associations with both the risk factors related to glucose intolerance and lipid profile. All three methods were associated to Lp(a), a risk marker for cardiovascular events [135, 139]. However, given the cross sectional nature of those associations one should be careful in drawing to many conclusions from those observations.

To have a cut-off for vulnerable symptomatic plaques might help improving preventive treatment of atherosclerosis, and have been the focus of many authors[74, 78, 82, 140]. Our study indicates that regardless of method of choice (biggest, most echolucent or all plaque) a PW cut-off level of less than 30% may characterize plaques at higher risk. This cut-off value for the average PW of all plaques was 28.9% (Table 1).

Risk Factor	Average PW			P1 vs P3
Percentile Group	**1**	**2**	**3**	***p***
PW min-max (%)	0.9-28.93	28.93-41.77	41.92-80.12	
HbA1c (%)	4.9(0.8)	4.7(1.1)	4.7(0.8)	**0.017**
Fasting blood glucose (g/l)	5.75(1.76)	5.33(1.58)	5.23(1.63)	**0.039**
Lp(a) (g/l)	0.304(0.421)	0.271(0.425)	0.221(0.257)	**0.005**
Adiponectin (mg/ml)	12.31(10.16)	14.37(9.89)	15.41(9.84)	**0.037**
ApoA (g/l)	1.52(0.26)	1.52(0.36)	1.63(0.36)	**0.026**
ApoB/ApoA1	0.76(0.32)	0.70(0.30)	0.72(0.30)	**0.050**

Skewed variables. Median±IQR is shown.

Table 1. Average PW by tertiles, showing the cut-off for the most echolucent tertile. Table adapted from paper I.

GSM vs PW

GSM has been developed as a computer-assisted measure of plaque morphology [79] and related to clinical events in several studies [75, 141, 142]. Although Fosse *et al*. [143] demonstrated this method's excellent reproducibility there was still a substantial measurement error related to the choice of standardization reference points. PW and GSM seem to give complementary information on the plaque.

PAPER II

In paper II we examined if hsCRP levels ≥ 2.0 mg/L was related to subclinical ultrasound-assessed atherosclerosis.

hsCRP ≥2.0 mg/L and carotid bulb IMT

We found that hsCRP ≥2.0 mg/L was significantly associated with a larger IMT of the carotid bulb independent of other cardiovascular risk factors. Moreover, the maximum carotid bulb IMT was significantly larger in the high hsCRP group even after adjustment for risk factors associated with both IMT and hsCRP. No such association was seen for the common carotid artery. The carotid bulb is known to be the predilection site for development of atherosclerosis, as a reply to the flow conditions in the carotid bifurcation [54, 59, 144]. The consensus statement from the American Society of Echocardiography (ASE) provides general guidelines which our laboratory supports. The consensus also states that mean-maximum values are more sensitive to change but might be less reproducible [129]. Baldassarre *et al* examined the association between serum hsCRP levels and carotid IMT in a review from 2008 [28]. The review included studies that represented healthy participants, population samples, patients with vascular risk factors, and those with overt cardiovascular disease. In about a third of the groups C-reactive protein was independently associated with carotid IMT, after adjustment for factors that covariate. We found significant univariate correlations between all measurements of IMT (composite, bulb and cca mean and max) and hsCRP, which remained significant for maximum carotid bulb, mean carotid bulb, and maximum composite carotid IMT even after adjusting for glucose tolerance group, waist circumference, plasma insulin, and Apo B/Apo A-I ratio (Table 2). In the above mentioned review the authors found that significant positive associations between hsCRP and carotid IMT were more common among men than among women.

Log values	Differences between the groups with hsCRP ≥2.0 mg/L, and hsCRP <2.0.mg/L	
	Unadjusted (mean [95 % confidence interval])	Adjusted[1] (mean [95 % confidence interval])
Carotid bulb IMT_{mean}	0.039 (0.019 to 0.059) ***	0.029 (0.007-0.052)*
Carotid bulb IMT_{max}	0.045 (0.022 to 0.067)***	0.037 (0.011-0.062)**
CCA IMT_{mean}	0.014 (0.001 to 0.027)*	0.00 (-0.014 to 0.015)
CCA IMT_{max}	0.016 (0.003 to 0.030)*	0.00 (-0.013 to 0.017)
Carotid $composite_{mean}$	0.026 (0.011 to 0.042)**	0.015 (-0.002-0.032)
Carotid $composite_{max}$	0.032 (0.015 to 0.049)***	0.022 (0.003 to 0.041)*

[1]Adjusted for glucose tolerance group (diabetes, impaired and normal glucose tolerance), log waist circumference, serum apolipoprotein B/A-I and log plasma insulin. *p<0.05, **p<0.01, ***p<0.001
Abbreviations: CCA = Common Carotid Artery, IMT = Intima Media Thickness.

Table 2. Univariate correlations for IMT and hsCRP. Table adapted from paper II.

hsCRP ≥2.0 mg/L and carotid plaques

In contrast, even if we found an association between hsCRP ≥2.0 mg/L with increased carotid bulb IMT no such association was found when examining plaque burden in the entire diabetic cohort. Comparisons between the low and high hsCRP groups did not reveal any differences in the number of women with or without plaques (158[41%] vs. 105[42%], respectively, n.s.), nor in the mean number of plaques observed (0.64 ± 0.94 vs. 0.71±1.0, respectively, n.s.), nor mean plaque area (10.9 ± 19.6 vs. 14.4 ± 25.5 mm^2, respectively, n.s.). However, a closer analysis of the women with plaques showed that women in the high hsCRP group had a larger mean plaque area than women in the low hsCRP group with plaque (Table 3). Interestingly, further examination of the high and low hsCRP groups with plaques showed that there was

also a significant difference in waist circumference, plasma insulin and apolipoprotein B/A-I ratio (Table 3). No other variable showed a significant difference (data not shown).

Variable	hsCRP <2.0 mg/L n = 158	hsCRP ≥2.0 mg/L n = 105	p
hsCRP (mg/L)	0.86(0.52-1.28)	4.10(2.71-5.90)	**n.a.**
Waist circumference[1], (cm), mean (SD)	89(±10)	99(±12)	**<0.001**
Plasma insulin (pmol/L) median (IQR)	40.0(26.1-57.4)	54.9(34.5-91.7)	**<0.001**
ApoB/ApoA-I, mean (SD)	0.70(±0.18)	0.81(±0.23)	**<0.001**
Total plaque area/subject (subjects with plaque), mm^2, median (IQR)	19.4(10.7-34.6)	25.8(13.8-42.9)	**0.014**
Percent echolucent plaque/subject (Gray-Weale), mean (SD)	88(±24)	91(±20)	**n.s.**
Average Gray Scale Median, median (IQR)	52.8(40.4-62.4)	49.0(38.0-59.0)	**n.s.**
Average Percentage White, median (IQR)	37.6(24.0-47.4)	34.3(24.2-45.5)	**n.s.**

[1] Mean value of screening and ultrasound measurements.
Abbreviations: PW = Percentage White, GSM = Grey Scale Median, IQR = Inter Quartile Range (shown as percentile 25-75).
n.a.= not analyzed, n.s.= non significant.

Table 3. Characteristics of women with carotid plaques in the high and low hsCRP group. Adapted from paper II.

After adjustment for waist circumference, log plasma insulin, and apolipoprotein B/A-I, hsCRP levels ≥ 2.0 mg/L still remained significantly associated with a larger log median total

plaque area. When median total plaque area was not log transformed, and after the same adjustment described previously, median total plaque area was 7.73 mm^2 larger in the high hsCRP group compared with the low hsCRP group (95% CI 0.50 to 14.95 mm^2, p:0.04).

Hence, there is a discrepancy between our findings that, on one hand hsCRP ≥2.0 mg/L was associated with increased carotid bulb IMT but not in plaque burden measured as plaque occurrence or total plaque area. On the other hand, among women with plaques, those with hsCRP ≥2.0 mg/L had significantly larger total plaque area independent of other cardiovascular risk factors compared to those with low hsCRP levels .This discrepancy is probably explained by the fact that plaque occurrence may be less precise than IMT measurements, which constitute a continuous variable not including the zero level. Previously published studies that have examined associations between hsCRP and carotid plaque occurrence show inconsistent results. Hence, while some studies confirms a relationship between plaque formation and elevated levels of hsCRP in men [35, 36, 145], others like the Tromsø cohort study did not show any such association (Halvorsen2009). Furthermore, previous studies performed in our group did not show a significant correlation between hsCRP and subclinical atherosclerosis in carotid arteries of healthy males [33].

hsCRP ≥2.0 mg/L and plaque echogenicity

Although we and others have presented data indicating that echolucent carotid plaques are associated with increased risk of cardiovascular disease [79, 91]the present study did not show any significant relationship between hsCRP and plaque echogenicity. The relationship between circulating biomarkers and echogenicity is not fully understood. Findings in the literature shows that the acute-phase reactant orosmucoid is associated with echolucent carotid plaques while fibrosis, assessed by histopathology shows an inverse correlation with

GSM [29]. Although observational studies have indicated that hsCRP levels ≥2.0 mg/L is associated with medium- and high-risk cardiovascular disease [40, 146], no studies have showed an association between carotid plaque echogenicity and hsCRP concentrations [38, 147]. To this day it remains unknown whether echolucency (low echogenicity) in asymptomatic plaques is related to atherogenic properties such as rate of plaque development, size, or persistence over time. However, in the cardiovascular health study (CHS), CRP correlated weakly with carotid IMT and carotid plaque severity [148, 149]. Comparing women in the high and low hsCRP groups no difference in statin treatment were found, but there was a significant difference in oral antidiabetic treatment (p= 0.005).

PAPER III

The aims of this paper where to explore variability in echogenicity as well as average echogenicity in relation to numbers of plaques in subjects with multiple plaques We also examined plaque occurrence, plaque area and plaque echogenicity at baseline and at 6 years of follow-up, and in relation to diabetes mellitus.

Echogenicity and multiple plaques at baseline

Both the intra-individual variability in echogenicity, and the average echogenicity increased by number of plaques at baseline. Among all women who were examined at baseline, 353 women had no plaque and 140, 63, 20 and 12 had 1, 2, 3 and ≥4 plaques, respectively.

Only women with more than one plaque at baseline were included in the analysis for intra-individual variability (n=95). The difference between the plaque with the highest and lowest echogenicity (PW, GSM) for each subject was used as a measure of variability in plaque echogenicity. As shown in Figure 5 (upper panel) this variability within subjects showed a significant increase for PW as the number of plaques increased ($p<0.001$).

All women with plaques at baseline (n= 235) was used examining the relation between average echogenicity and numbers of plaque. The ANOVA test for linearity was significant for PW in between the groups separated by the number of plaque (number of plaques, 1, 2, 3, ≥4), (p=0.042) and the data showed higher values for echogenicity in all groups with more than 1 plaque (Figure 5, lower panel). Both for intra-individual variability and average echogenicity similar values were found for GSM. Furthermore median values for PW and GSM also showed a lower value for women with one plaque than women with more than one plaque (PW: 31.1 IQR 24.6 vs 39.3 IQR 17.9, p =0.002; GSM:44 IQR 28 vs 54 IQR 19, p=0.001).

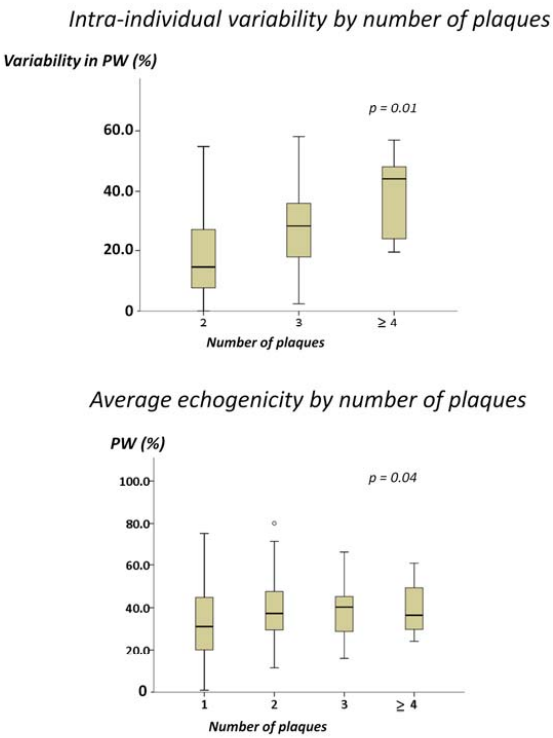

Figure 5. Echogenicity and multiple plaques. Figure adapted from paper III.

Taken together the results showed that with increasing numbers of intra-individual plaques there was an increase in average echogenicity as well as an increase in the variability of plaque echogenicity in this population. This observation that the variability of plaque echogenicity increases by number of plaques is not unexpected, especially in relation to the knowledge that wall shear stress differs in different sections of the carotid artery [150, 151].Hence, this may result in plaques at different sites having different morphology. To our knowledge no one have previously examined this in a systematic way besides our previous publication, which examined how echogenicity values from one plaque (biggest or most echolucent plaque) relates to known cardiovascular risk factors compared to the average echogenicity value from all identifiable plaques in the individual [127]. In that paper (paper I in this thesis) we showed that the average value of plaque echogenicity, taking all plaques into consideration, is associated with numerically more risk for cardiovascular disease[127] than using only one plaque for the analysis.

Those findings need to be corroborated by others, but looking at the substantial variability within individuals found in this paper this clearly will have an impact on how to assess plaque echogenicity in non-stenotic plaques. The previously most common approach to assess only one plaque per patient independent of how many plaques present may lead to increased measurement error.

Prospective changes in plaque status

We compared plaque status at baseline and after 6 years follow-up in all women from whom we had obtained complete ultrasounds examinations at both time points (n=429). The prevalence of subjects with plaques increased significantly over the 6 years (Table 4). Furthermore both mean number of plaques per subject and total plaque area increased. However, no significant differences in echogenicity were shown (Table 4). During follow-up,

the prevalence of diabetes increased, together with increases in BMI, WHR, blood pressure, HbA1c and hsCRP. However, total cholesterol, LDL and triglycerides decreased, whereas HDL cholesterol increased. Fewer women smoked at the 6 year follow-up. Treatment with statins increased during follow-up.

Variabel	**Baseline** *Women with comparable ultrasound images*	**Follow-up** *Women with comparable ultrasound images*	***p***
N	429	429	**na**
Subjects with plaque, n(%)	161(38)	304(71)	**<0.001**
Mean no plq/subject	1.57 ± 0.88	2.36 ± 1.45	**<0.001**
Total Plaque Area, mm^2	19.0 IQR 24.4	26.1 IQR 35.8	**<0.001**
PW, %	31.6 IQR 21.3	30.8 IQR 23.1	**0.288**
GSM	48 IQR 24	51 IQR 24	**0.246**

Abbreviations: plq, plaques; PW, Percentage White, GSM, Gray Scale Median; na, not analyzed; IQR, interquartile range.

Table 4. Plaque variables for baseline and follow-up. Table adapted from paper III.

Moreover we prospectively followed the change in plaque status over time with specific focus on women with type 2 diabetes. In the present cohort the prevalence of diabetes increased from 29 to 41 %. The women were divided into two groups according to whether they had diabetes mellitus (DM) or not (non-DM) at the specific time of examination. We showed that there was a higher prevalence of women with plaques in the DM-group at baseline and follow-up (46% and 80%, respectively) compared with those without diabetes (22% at

baseline and 65% at follow-up, respectively). The plaques in the DM-group at baseline were also characterized by having significantly lower PW and GSM (p= 0.003 and p= 0.012, respectively). Furthermore, the DM-group at baseline also showed a higher prevalence of subjects below our previously suggested cut-off for risk-prone plaque [127] (53% and 38 % respectively, p=0.031) (Table 5).

At the 6 year follow-up women with diabetes still had a higher prevalence for plaques but no significant difference in echogenicity was seen (Table 5). This finding may be explained by concomitant changes in life style and medical treatment such as statin therapy.

Variable	Baseline			Follow-up		
	DM	*Non-DM*	***p***	*DM*	*Non-DM*	***p***
Subjects with plaque, n(%)	57 (46)	66 (22)	**0.017**	141(80)	162 (65)	**0.001**
Mean no plq/subject, n	1.70 ± 1.09	1.50 ± 0.74	**0.577**	2.38 ± 1.56	2.35 ± 1.35	**0.646**
Total Plaque Area, median	20.5 IQR 32.2	18.8 IQR 21.5	**0.408**	24.9 IQR 44.1	28.4 IQR 33.5	**0.606**
PW, median	27.8 IQR 26.9	36.0 IQR 19.0	**0.003**	31.0 IQR 22.4	30.3 IQR 23.0	**0.645**
GSM, median	42 IQR 27	50 IQR 22	**0.012**	50 IQR 22	52 IQR 27	**0.454**
Cut-off < p1 (PW<28.9) n (%)	32 (56)	40 (38)	**0.031**	64 (45)	78 (48)	**0.631**

Abbreviations: DM, Diabetes Mellitus; plq, plaques; PW, Percentage White; GSM, Gray Scale Median; p1, percentile group 1; IQR, interquartile range.

Table 5. Plaque characteristics for women with and without diabetes mellitus. Table adapted from paper III.

Risk factors for plaque echogenicity and changes over time

In the present study plaque echolucency (low echogenicity) was associated with diabetes but did not change overall during follow-up although the mean number of plaques per subject as well as total plaque area per subject increased. Ostling *et al.* showed an increased echolucency of carotid plaques in patients with type 2 diabetes [95]. In a cross-sectional study of elderly men carotid plaque echolucency was associated with increased Framingham risk score, systolic blood pressure, higher BMI and decreased HDL and reversely to smoking, [152]. However, the observations in the two mentioned studies were made on the largest visible plaque and therefore may not be fully translated to our findings. In the Tromso study a cross-sectional analysis in subjects with stenotic carotid plaques, plaque echolucency (low echogenicity) was associated with low HDL cholesterol levels, lower age and higher degree of stenosis [77]. From the Tromso study it is also known that plaque echolucency is less frequent among women compared with men [97]. The same study has also shown that HbA1c is strongly related to carotid plaques with high degree of echogenicity in non-diabetic subjects [153].

The Tromso study examined changes in carotid plaque growth and echogenicity with a follow-up time of 7 years in a large cohort of middle-aged men and women which, however, included very few subjects with diabetes [97]. Echogenicity was measured as average GSM in all detected plaques in each subject. In that study men had somewhat lower echogenicity at baseline and echogenicity increased during follow-up in approximately 60% of the cases, with no difference between sexes. BMI, systolic blood pressure increased whereas HDL cholesterol and proportion of current smokers decreased [97].

In the present longitudinal study, we also observed that BMI, blood pressure increased and smoking rates declined but, in contrast LDL cholesterol decreased and HDL increased, as did hsCRP. This may be explained by the fact that women included in the study were given information on how to improve their health status by for example changes in life style such as: less smoking, increase in physical exercise and in cases needed treatment with medication. The combination of less smoking, physical activity and medication have previously been proven to significantly improve the lipid profile [154, 155]. Statin treatment also increased, especially among women with diabetes and such treatment is known to decrease the fat content in carotid plaques and have also been shown to reduce plaque echolucency [156]. These changes in life style and medication in the present study may have affected plaque echogenicity in contrary directions. Given changes in life style and effects of medication it is not possible to study the natural history of changes in plaque echogenicity. A further aspect is that the echogenicity of plaques may increase over time and in relation to the severity of atherosclerosis. This suggestion is supported by two observations in the present study. First, the women who did not participate in the follow-up examination had at baseline larger total plaque area and higher plaque echogenicity, than women included in the comparison (regarding plaque data between baseline and follow-up). Second, with increasing intra-individual numbers of plaques there was a parallel increase in PW and GSM indicating higher echogenicity. The Tromso study also observed an inverse association between age and echolucency [97, 157]. However, a histopathology study of carotid endarterectomies indicates that, if anything, plaques get less fibrous and more atheromatous with increasing age [158]which would render the plaques more echolucent *i.e.*, lower values in echogenicity, but this was not a population based study.

Plaque burden and change over time

As expected we observed that plaque prevalence increased from 38% to 71% during 5.5 years of follow-up. This finding can be compared with results from previously published observational data from the Tromso study showing prevalences of about 30% and 45% in women at corresponding ages [70]. The difference in prevalence is probably explained by the enrichment of women with diabetes in the present study with an increased risk of subclinical atherosclerosis that is added on the well known increase in plaque prevalence by raising age [159]. In the diabetes group plaque occurrence increased by 34% and in the group without diabetes by 43%. This more pronounced increase in plaque prevalence among those with no diabetes may be partially explained by a more vigorous treatment of women with diabetes. For instance, statin treatment was more common among women with diabetes, In the Tromso study low HDL cholesterol, increasing age, systolic blood pressure and smoking were independent predictors of carotid plaque growth [97]. It was also observed that plaques remaining echolucent over time had a higher growth potential than those remaining echogenic. The present study did not aim to examine the risk factor pattern for development and growth of carotid plaques.

LIMITATIONS

We only examined women at a specific age, screened for diabetes and impaired glucose tolerance. However, this can also be viewed as strength, reducing confounding factors such as age and sex. Women with impaired glucose tolerance and diabetes are of particular interest, as the incidence of diabetes increases steeply in this age category and these phenotypes are

typically accompanied by a very strong relative increase in cardiovascular risk in women [15]. In the DIWA-cohort 4% of the participants had type 1 diabetes. There were no indications that these subjects affected the results.

In paper I, the new measure of plaque echogenicity should have been tested to histopathology and clinical events during follow-up. We have not had the opportunity to perform such investigations. We used a population-representative stratified sample of women with non-stenotic asymptomatic plaques and validated the measures against the visual Gray-Weale classification. Furthermore, the value of juxtaluminally located echolucencies could not be validated because the majority of the plaques included were small, making it very challenging technically to develop a method for evaluation of juxtaluminal echolucency location. In paper II, the relation between the high hsCRP group and events and outcome of cardiovascular disease should have been tested to further evaluate but at the time these results were not available. In paper III, the number of women lost to follow-up were considerable and their characteristics obtained at the baseline examination indicate that they are associated with a higher risk of cardiovascular disease compared with the participatin women. Consequently, the results at follow-up will be biased by the loss of extreme cases. However, when events and outcome of cardiovascular disease are available these can be related to the baseline data for this subgroup and add further information. Finally, it should be kept in mind that women differ from men in several of the manifestations of atherosclerosis which have been investigated in the present study. Plaque occur less often in women than in men, and echolucent plaques are also more common among men [70, 160]. Carotid atherosclerosis was a stronger risk factor for MI in women than in men, and the risk of myocardial infarction increased with plaque echolucency [161].

CONCLUSION AND FUTURE PERSPECTIVE

Manifestations of atherosclerotic disease are still a main contributor to death in industrialized countries. To be able to reach the goal to reduce morbidity and mortality of atherosclerosis we have to improve our methods to study early stages of the disease. Hence, dedicated imaging methods and biomarkers to identify individuals at risk before an event has occurred are highly warranted to guide life style modifications and treatment.

Plaque occurrence and plaque morphology are both important predictors of elevated cardiovascular risk. The benefits of identifying risk-prone plaques in a patient before any major or minor event are several, ranging from prolonged survival to improvement of patient quality of life and reduction of health costs. Traditionally a composite of risk markers are being used (*e.g.,* the Framingham risk score [162]). However, so far validated and qualified biomarkers assessing arterial health are lacking. As discussed above even the occurrence of small subclinical changes such as an enlarged carotid IMT or non-stenotic plaques is associated with an elevated risk for future cardiovascular disease and the ultrasound technique makes it possible to determine and classify these changes. In the literature both prospective and histopathological studies show associations between the subjective plaque classification method according to Gray-Weale (a method which classifies plaques into four types according to visual gray scale appearance) and clinical out-comes. Even if the method is frequently used it remains subjective, user dependant and with a high degree of variability which makes it limited for use in multicenter studies and prospective studies over many years (different ultrasound equipment, several sonographers). The objective GSM method, developed by El-barghouty *et al* [74] is frequently used but has some drawbacks and has not reached use in everyday clinical practice. In an attempt to improve this technology the

SAMEE program and its main feature, *Percentage White* (PW), have been constructed to handle different technical and artifact-related sources of variability. We have now shown that it compares well with the visual Gray-Weale classification and is highly reproducible. PW also correlates to a higher extent than GSM with a number of factors known to be associated with cardiovascular diseases. In paper III we followed the development of subclinical atherosclerosis in the same cohort of women with varying degrees of glucose intolerance. We thoroughly examined intra-individual variability as well as average PW in individuals with multiple non-stenotic plaques and found evidence that with increasing number of plaque both the intra-individual variability and average value of PW increased. This result further underlines our conclusion from paper I, that the problem of multiple plaques in individual subjects is best managed by measuring the average PW of all plaques. These results need to be validated in larger studies, different populations, and evaluated against cardiovascular events and outcome with an additional aim to examine whether PW can be used for better risk stratification of patients with high-degree carotid stenoses.

Furthermore, ultrasound data from the follow-up at 6 year indicates that even if plaque burden as measured by prevalence of women with plaque, mean number of plaques per individual and total plaque area increased significantly there was no significant difference in echogenicity between baseline and follow-up. Possible explanations might be change in risk - profile since HDL increased and prevalence of smoking decreased in the cohort. At baseline, we unintentionally interfered with the natural course of development since the women were given advice on beneficial lifestyle changes and prescribed medication if needed. Statin treatment and additional medication might have affected plaque echogenicity and made it difficult to study the natural history of changes in plaque echogenicity. Furthermore, we compared women with diabetes mellitus to those without, both at baseline and follow-up. As

expected there was a higher prevalence of diabetes at follow-up. The plaque variables showed a higher prevalence of subjects with plaque in the DM-group both at baseline and at follow-up although the significant difference in echogenicity at baseline (lower values in the DM group) where not seen at the follow up. Apart from the fact that statin treatment were more common in women with compared with those without diabetes, there might be several answers to why the plaque variables do not differ; the echogenicity might increase over time, the diabetes treatment might affect the echogenicity, or the women lost to follow- up would have change the overall result. At baseline the women lost to follow-up had a larger plaque area but in contrast a higher echogenicity. Future studies should include interventional studies regarding medication (statins, betablockers, diabetes treatment) and their effect on plaque burden measured as plaque occurrence, size and echogenicity. For the group of women lost to follow-up, future information on clinical events in relation to baseline plaque variables might add further information to how plaque echogenicity relates to cardiovascular disease.

The results from paper II validates the relation between low-grade inflammation as measured by hsCRP ≥2.0 mg/L, and subclinical stages of atherosclerosis assessed as IMT of the carotid bulb. Furthermore, women with plaques and hsCRP levels above 2.0 mg/L had a larger plaque area independent of other cardiovascular risk factors however no difference was found in echogenicity. We found no significant difference in statin treatment, which have been shown to give an increase in echogenicity but oral antidiabetic treatment did differ between the hsCRP groups. However, little is known of the effects of antidiabetic treatment and plaque echogenicity. The level of hsCRP at 2.0 mg/L might be a to small elevation to yield different results in echogenicity and future studies should examine this cut-off in the relation to outcome and events of cardiovascular disease in this cohort of women with varying degrees of glucose intolerance.

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To accomplish great things, we must not only act, but also dream; not only plan, but also believe.

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