CLINICAL EVALUATION OF CONTRAST-ENHANCED CAROTID ULTRASOUND IMAGING

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Voor mijn familie

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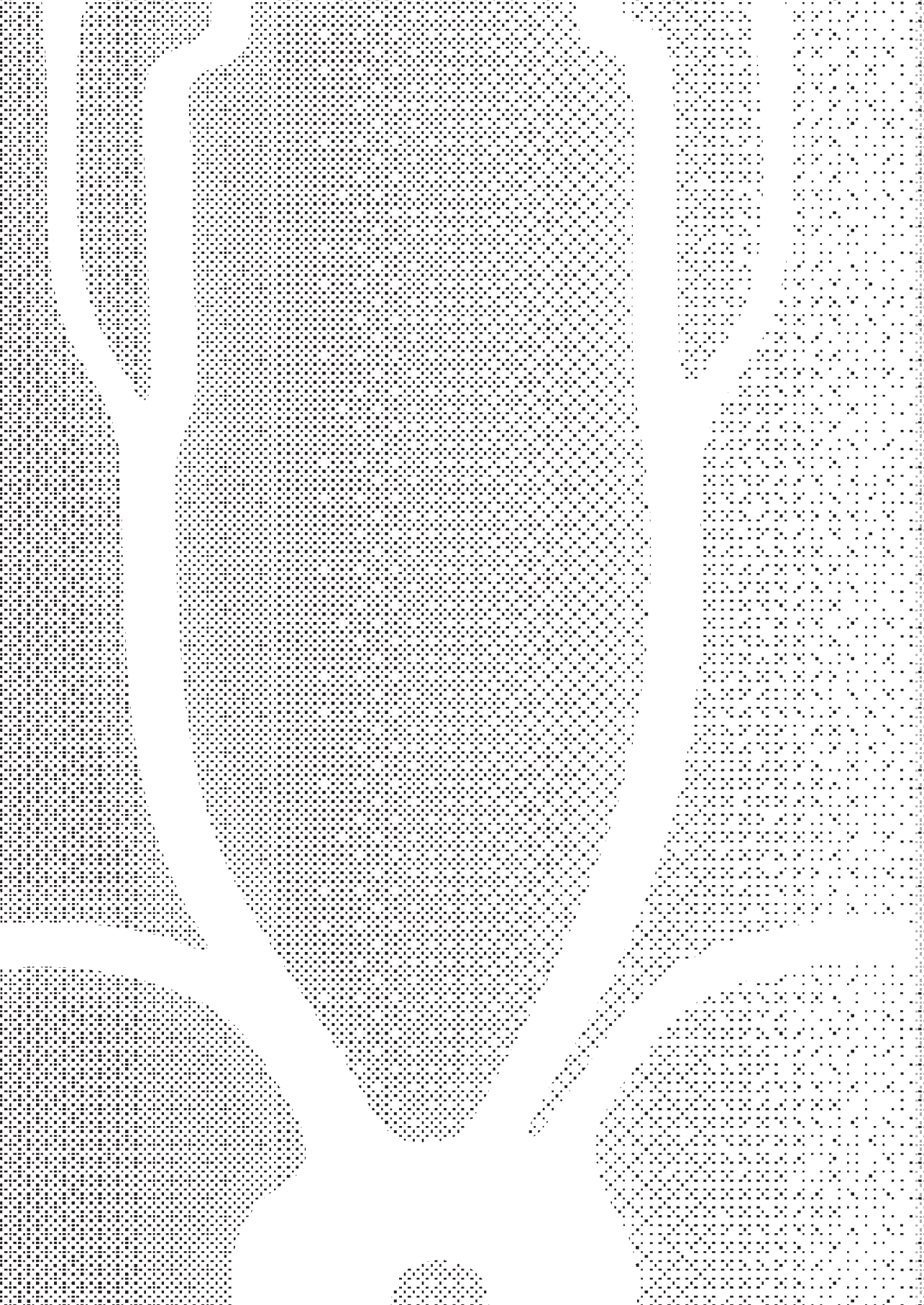
PART I



CHAPTER 1

GENERAL INTRODUCTION

Impact of cardiovascular diseases

Cardiovascular diseases (CVD) are an important cause of mortality. An estimated 17 million deaths are caused by CVD, representing 30% of all-cause mortality [1]. CVD are all disorders of the heart and blood vessels. It includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis or pulmonary embolism. The main part of deaths due to CVD worldwide are caused by coronary heart disease (an estimated 7.3 million deaths) and due to stroke (an estimated 6.2 million deaths) [1].Compared to any other cause of death, CVD are the most important cause of death. For example, the number of annual deaths due to cancer is estimated on 7.6 million [2]. Trends show that CVD remain the leading cause of death the upcoming years, leading to an increase in deaths caused by CVD to 23.3 million in 2030 [3].

Besides the contribution of CVD to the global death rates, CVD affect the global health as well [1]. Non-fatal cardiovascular events (CVE) may lead to disability and morbidities [4]. This may result in a decreased quality of life for survivors. In addition the ongoing developments in the field of CVD, influencing both preventive and therapeutic strategies, increase the chances of survival after a cardiovascular event. This leads to an increased number of survivors who increasingly need healthcare, resulting in rising future healthcare costs for CVD [5].

Due to the increasing mortality and morbidity rates as a result of CVD and the increasing burden of CVD on global healthcare, the World Health Organization aims to develop prevention programs to decrease the number of people at risk for the development of CVD. These programs aim to alter an individual's cardiovascular risk by controlling cardiovascular risk factors such as smoking, diabetes mellitus and hypertension. Despite advances in preventive medicine over the last decades, CVD remains the leading cause of morbidity and mortality [4,5].

Atherosclerosis and vulnerable atherosclerotic plaques

Atherosclerosis is a systemic disease of the arteries and is the main underlying cause of the two most important contributors of CVD (i.e. coronary heart disease and stroke) [6,7]. Atherosclerosis is the formation of atheromatous plaques in the arterial vessel walls. The formation of plaques is characterized by infiltration of the vessel wall by inflammatory cells (figure 1, panel A) together with the accumulation of cholesterol particles (figure 1, panel B), migration of vascular smooth muscle cells into the lesion and the formation of a fibrous cap which consists of collagen (figure 1, panel C). These atheromatous plaques grow and may destabilize, which increases the risk of atherosclerotic plaque rupture. A rupture of the atherosclerotic plaque leads to a thrombotic response which increases the risk for a thrombo-embolic event (figure 1, panel D) [8,9]. Thrombo-embolic events are caused by free floating thrombus particles or parts of debris from the ath-

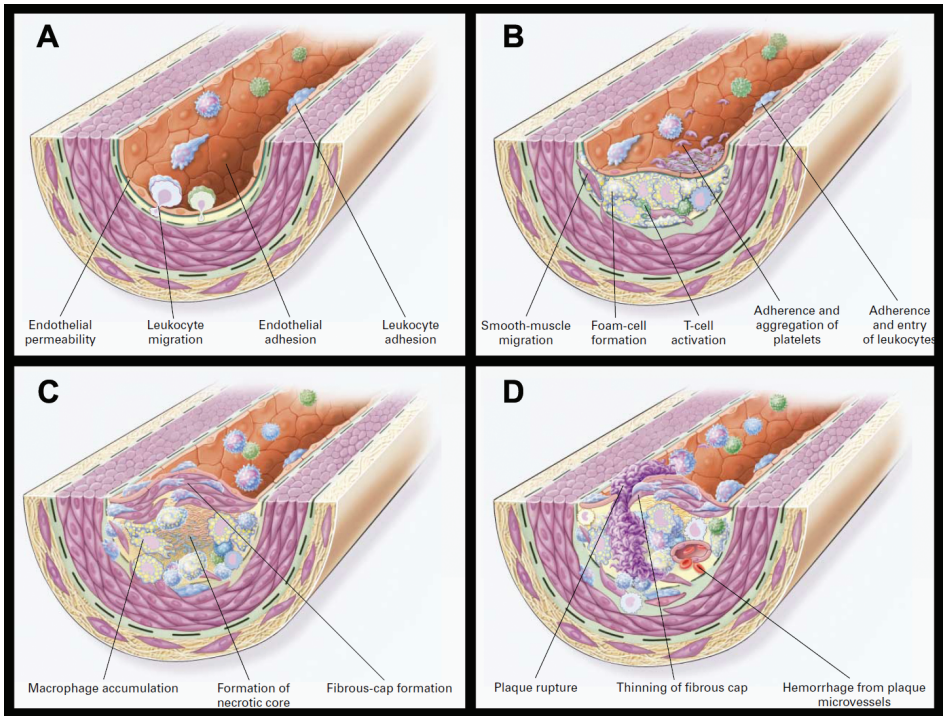


Figure 1
Simplified representation of atherogenesis. Panel A: Endothelial dysfunction leading to an increased permeability of the endothelial layer to lipoproteins and other plasma constituents. Panel B: formation of fatty streaks which consist of foam cells (lipid-laden monocytes and macrophages) and other immune mediated cells. Panel C: progression of fatty streaks into more advanced lesions by formation of a fibrous cap which forms the boundary between the arterial lumen and the atherosclerotic plaque. In this phase the atherosclerotic plaque core may get necrotic. Panel D: destabilization of the atherosclerotic plaque leads to thinning of the fibrous cap. In this phase the atherosclerotic plaque has its own blood supply by a network of intraplaque neovessels. These vessels are weak and immature which increases the risk of rupture and consequently intraplaque hemorrhage. Rupture of the thin fibrous cap may lead to a thrombo-embolic response. Reproduced with permission from [8], Copyright Massachusetts Medical Society.

erosclerotic plaque. Free-floating particles will get stuck in the flow-area distal from the atherosclerotic plaque rupture. Occlusion of the arteries distal from the plaque will lead to ischemia of the tissue which is perfused by that particular flow-area. In coronary arteries, this may lead to a myocardial infarction. In carotid arteries it may lead to an ischemic cerebrovascular event.

Although the etiology of destabilization of atherosclerotic plaques it is not completely known yet, destabilization of the atherosclerotic plaques is dependent on multiple factors. These factors are widely investigated, and the search is ongoing to find factors to characterize atherosclerotic plaques [10]. This is point of an attempt to develop risk prediction models that are able to distinguish between stable atherosclerotic plaques (i.e.

plaques that have a low risk to destabilize) and vulnerable atherosclerotic plaques (i.e. plaques with an increased risk to destabilize, rupture and cause a CVE). So far, studies have shown that major components of the vulnerable atherosclerotic plaque are 1. a lipid rich, atheromatous core and 2. a thin fibrous cap with infiltration of macrophages and lymphocytes and a decreased smooth muscle cell content [11].

Other components that could play an important role in destabilization of the atherosclerotic plaques are intraplaque neovascularization (IPN) and the presence of intraplaque hemorrhage. Hellings et al recently investigated the histological components of carotid specimens of 818 patients undergoing carotid endarterectomy [12]. A prospective follow-up of all patients was performed with a primary end-point of any CVE or vascular intervention. After follow up, the density of intraplaque neovessels and the presence of intraplaque hemorrhage were significantly associated with the primary endpoint. Other components such as the presence of calcification or the amount of lipids in the plaque core were not associated with the primary endpoint. This suggests an important role for both the density of IPN and the presence of intraplaque hemorrhage in predicting future cardiovascular events.

Although the vessel walls of healthy arteries are perfused and nourished by physiological adventitial vasa vasorum, the presence of IPN is a strict pathological characteristic. Under influence of several proangiogenic factors (e.g. hypoxia induced factors, hypertension, nicotine), the physiological vasa vasorum will sprout through the media into the atherosclerotic plaque [13,14]. Extension of the vasa vasorum to the atherosclerotic plaque leads to a disorganized and immature network of IPN. This network foresees the atherosclerotic plaque in its metabolic demands, it delivers inflammatory cells and mediators to the plaque and due to its increased permeability it delivers lipids to the atherosclerotic plaque. The intraplaque neovessels are leaky due to their relatively large diameter, the lack of smooth muscle cells or pericytes in the vessel wall and a structural weak extracellular matrix in the atherosclerotic plaque. This may eventually lead to rupture of the intraplaque neovessels resulting in intraplaque hemorrhage. Intraplaque hemorrhage may lead to critical progression of the atherosclerotic plaque due to rapid accumulation of erythrocyte membranes and macrophage infiltration. This leads to further destabilization of the atherosclerotic plaque with an increased risk of plaque rupture [15].

Cardiovascular risk prediction

Current cardiovascular risk prediction models are developed to stratify patients into a low, intermediate or high cardiovascular risk. The stratification of asymptomatic patients is based on data from large epidemiological studies. Those models are based on several traditional cardiovascular risk factors (e.g. age, hypertension, diabetes mellitus, smoking habits and family history). Examples of such risk prediction models are

"The Framingham Risk Score", "PROCAM" and "SCORE" [16-18]. The Framingham risk score estimates the 10-year risk of developing coronary heart disease based on gender, age, cholesterol levels, smoking status and systolic blood pressure. The PROCAM risk score, which calculates the 10-year risk for developing a major coronary event, is based on models including gender, age, cholesterol levels, smoking status, glucose levels or the presence of diabetes mellitus and family history. The "SCORE" risk score estimates the 10 year risk to suffer of fatal CVD based on gender, ages, total cholesterol, smoking status and systolic blood pressure.

The performance of those models is limited [19]. Although the models fit in a general population, it remains impossible to reliably distinguish individuals at low risk to suffer a CVE from those at increased risk to suffer a CVE. Therefore, new strategies are needed to further improve current cardiovascular risk stratification, leading to accurate identification of patients at severely increased risk before the occurrence of a CVE [20]. Several methods have been proposed that may provide additional information to current cardiovascular risk stratification models. These methods include measuring biomarkers such as high-sensitivity CRP and imaging subclinical atherosclerosis in various vascular beds.

Imaging subclinical atherosclerosis

Imaging subclinical atherosclerosis provides an overview of cumulative exposure to known and unknown cardiovascular risk factors and risk modifiers [21]. The presence and amount of subclinical atherosclerosis may therefore provide valuable information on cardiovascular risk. Imaging of subclinical atherosclerosis can be performed using a diversity of invasive and non-invasive imaging techniques. Invasive techniques include intra-vascular ultrasound (IVUS), optical coherence tomography (OCT) and near infrared spectroscopy (NIRS) [22-24]. These techniques are able to detect plaques and to characterize the composition of subclinical atherosclerotic plaques. However, they share a major limitation: all require an invasive catheterization procedure. Each catheterization procedure carries a low but not negligible chance of causing adverse events. Therefore, invasive imaging techniques are less suitable for the improvement of cardiovascular risk stratification models. Non-invasive imaging techniques for the detection of subclinical atherosclerosis include ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) [25-27]. These techniques are able to detect subclinical atherosclerosis in multiple vascular beds and all have their specific advantages and disadvantages.

The MRI technique is a safe and non-invasive method to assess subclinical atherosclerosis without involvement of ionizing radiation. Due to the absence of ionizing radiation, MRI is eligible for monitoring changes in atherosclerotic disease burden over time. In addition, using combined analysis of different tissue signal intensities it is possible to distinguish between different tissue types. Therefore MRI is also suitable to obtain

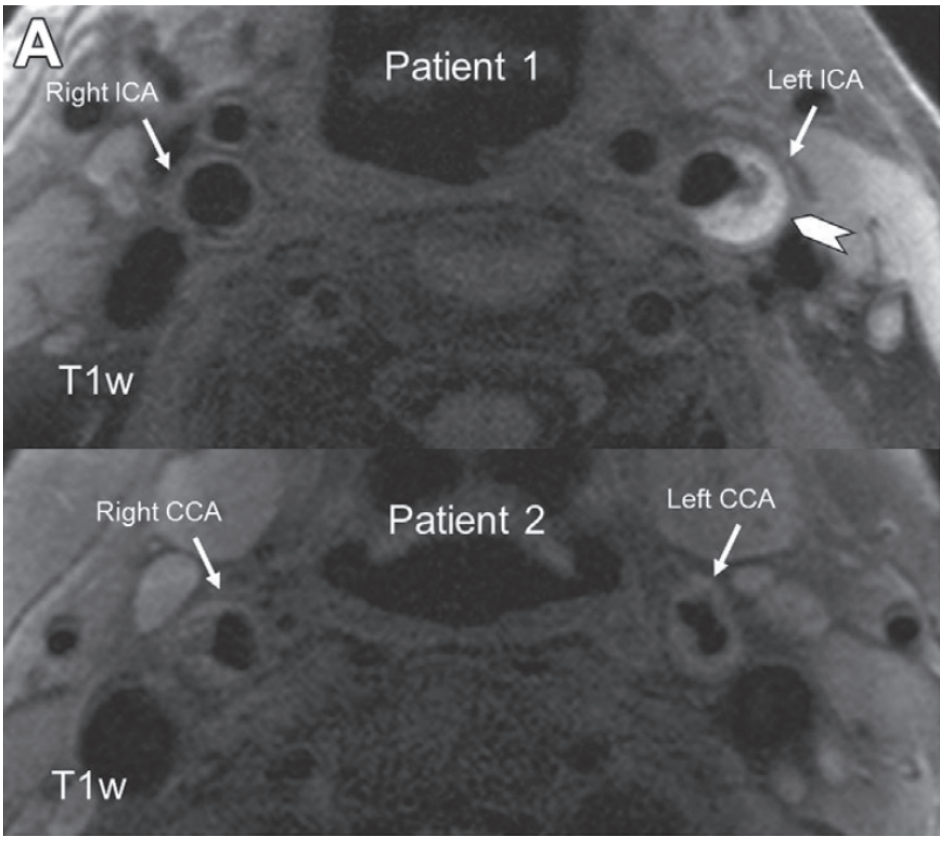


Figure 2.
Upper panel: T1-weighted (T1w) fat-suppressed MR images of an 86-year-old patient with acute ischemic stroke ipsilateral to a large atherosclerotic plaque with intraplaque hemorrhage (IPH) (chevron) in the left ICA; lower panel: T1w fat-suppressed MR images of a 68-year-old asymptomatic patient with long-lasting hypercholesterolemia and large atherosclerotic plaques without intraplaque hemorrhage in both carotid arteries. Reprinted from [52], with permission from Elsevier.

plaque composition characteristics [27,28] (Figure 2). Although MRI has an excellent spatial resolution, the technique is limited by a relatively low temporal resolution. Due to the relatively low temporal resolution, the applicability of MRI in imaging atherosclerosis in vessels with relatively large movement (e.g. the coronary arteries) is limited. Another limitation of MRI is the lack of generally accepted protocols for vascular imaging [27].

The CT technique is also shown safe and non-invasive and has a better temporal resolution compared to MRI. This allows CT to detect and quantify atherosclerosis in the coronary arteries. CT is mainly used to quantify the amount of calcification in vascular beds (calcium scoring) and to determine atherosclerotic plaque burden and degree of stenosis (CT-angiography) (Figure 3) [26]. Due to improvements of CT it is possible to detect several plaque characteristics such a large lipid pool plaque cores and plaque

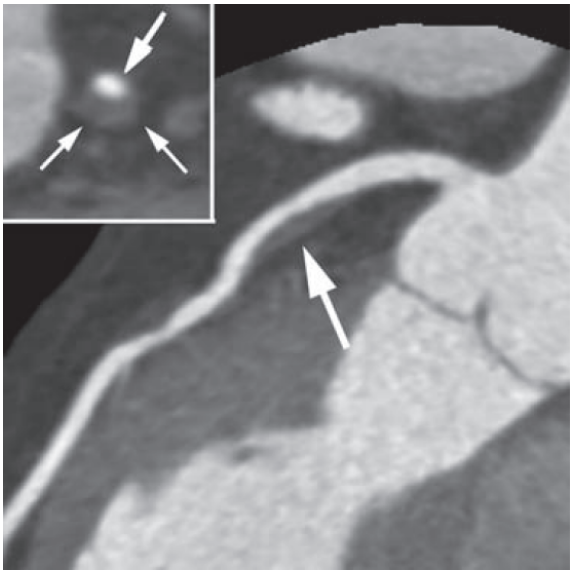


Figure 3.
Longitudinal reconstruction of the proximal left anterior descending coronary artery. A plaque with pronounced positive remodeling is visualized by computed tomography angiography (arrow). The total vessel area (lumen plus plaque) is substantially larger than for normal vessel segments proximal or distal to the lesion. The insert shows a cross-section of the plaque. The large arrow indicates the contrast-enhanced lumen, whereas the small arrows indicate the eccentric non-calcified plaque. Reproduced from [26], by permission from Oxford University Press.

ulcerations [29,30]. However, the spatial resolution of CT is still inferior to MRI for imaging morphological plaque components. In addition, CT requires the use of ionizing radiation which limits the use of CT for monitoring changes in atherosclerosis over time. Nevertheless, improvements of CT and development of new imaging protocols have substantially reduced the levels of radiation needed for imaging atherosclerosis [31,32].

Vascular ultrasound is a relatively low-cost, safe and non-invasive method to detect atherosclerosis without using ionizing radiation. Ultrasound benefits a relatively high spatial and high temporal resolution, especially in systems using high frequency pulses [33]. With increasing frequencies, penetration depth of ultrasound decreases. Therefore, vascular ultrasound is in particular useful for detecting subclinical atherosclerosis in superficial arteries such as the carotid arteries and the femoral arteries [33]. Unfortunately, vascular ultrasound is not feasible for assessment of atherosclerosis in the coronary arteries due to the anatomical position. In more superficial arteries, the vessel wall thickness (i.e. intima-media thickness) can be measured and the presence of plaques can be assessed as markers for subclinical atherosclerosis (Figure 4) [34-37]. It is also possible to characterize plaque tissue and classify into soft (i.e. lipids or intraplaque hemorrhage) or

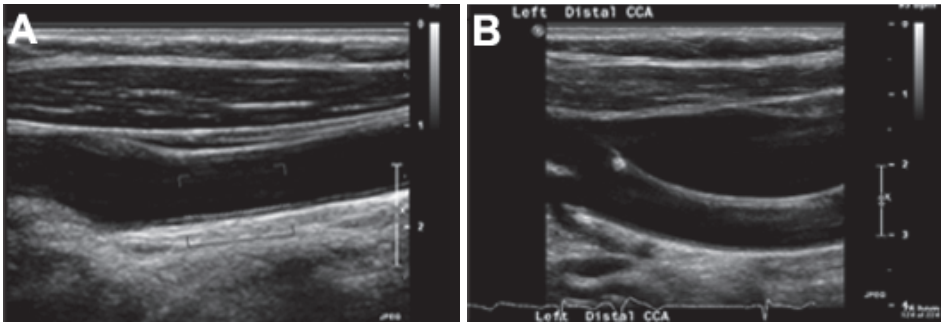


Figure 4.
Left panel: semi-automated measurement of the carotid intima-media thickness. Right panel: atherosclerotic plaque detection using carotid ultrasound. An atherosclerotic plaque is visible in the distal common carotid artery and carotid bulb.

hard plaques (i.e. calcification or fibrous tissue) based on echogenicity [38,39]. In addition, the wall of the carotid plaque can further be classified as regular, irregular or ulcerated [40].

Although vascular ultrasound is able to detect some plaque characteristics, contrast-enhanced ultrasound (CEUS) may provide additional information on the presence and composition of atherosclerosis [41,42]. CEUS is a technique in which tissue is suppressed and blood is enhanced using microbubble contrast agents (Figure 5). Studies have shown that this results in an improved visualization of the carotid lumen [43]. The contrast agents consist of lipid coated microspheres filled with an inert gas. Microbubbles have the size of red blood-cells resulting in their capability of trans-capillary passage [44]. The ultrasound contrast agent acts as a strict intravascular tracer and physical characteristics allows detection of small amounts of microbubbles [45]. Therefore, using CEUS it is also possible to detect microvessels in tumors or atherosclerotic plaques. Detection of intra-plaque microvessels (i.e. intraplaque neovascularization, IPN) using contrast-enhanced ultrasound may provide additional information on plaque vulnerability. The number of studies evaluating the use of carotid CEUS for the detection of IPN is increasing over the last decade [46-51]. The amount of IPN detected using carotid CEUS has already been correlated to the cardiovascular history of patient [47]. Nevertheless, it lacks standardized ways of scoring the presence and amount of IPN on carotid CEUS. In addition, no data is available on the amount of IPN in asymptomatic patients.

The aims of this thesis are to develop and test semi-automated quantifications methods for the presence of IPN on carotid CEUS and to assess the applicability of that custom developed software in clinical studies investigating the amount of IPN in asymptomatic individuals at increased risk for development of atherosclerosis or individuals with other forms of vasculopathy.

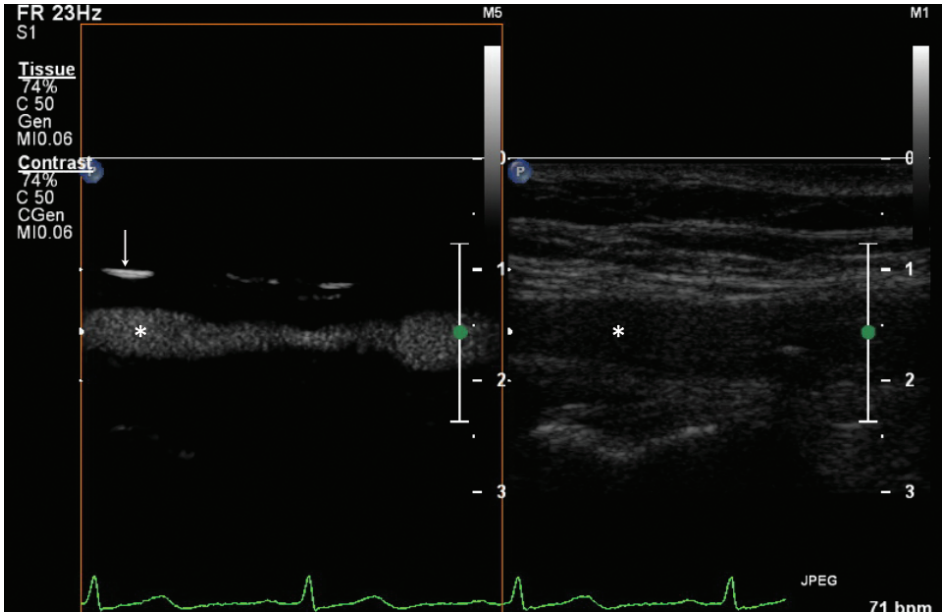


Figure 5.
Example of a side-by-side carotid CEUS clip. The clip simultaneously shows a B-mode image of the carotid artery (right panel) and a CEUS image of the exact same part of the carotid artery (left panel). The frame is taken right after inflow of contrast in the carotid lumen. The luminal narrowing in the bifurcation indicates the presence of an atherosclerotic plaque. There is no contrast-enhancement in the atherosclerotic plaque. * = carotid lumen, Arrow = saturation artifact.

Outline of the thesis

I – Introduction:
The additional value of carotid intima-media thickness (CIMT, a surrogate marker of subclinical atherosclerosis) to existing cardiovascular risk prediction models is assessed in a systematic review of the literature and a meta-analysis. Furthermore, the current status of carotid CEUS is reviewed and the current role of CEUS in detecting IPN is discussed.

II – Detection of subclinical atherosclerosis using carotid CEUS:
The additional value of CEUS for the detection of subclinical atherosclerotic plaques in asymptomatic patients is reported. In addition, the effect of carotid plaque screening using CEUS on cardiovascular risk stratification according to current guidelines is reported.

III – Quantification of intraplaque neovascularization:
Accurate assessment of IPN requires the development of standardized and automated methods for quantification of IPN detected using carotid CEUS. Automated software is developed and the performance will be tested in patients with symptomatic carotid atherosclerosis. Furthermore, the results of a histological validation study of the new methods are reported.

IV – Implementation of carotid CEUS in clinical studies:
The use of CEUS in asymptomatic patient populations is reported. The amount of IPN in specific patient populations is compared to other plaque characteristics and comorbidities. The amount of IPN was evaluated in patients with diabetes and patients with familial hypercholesterolemia. Both patients with diabetes mellitus and patients with familial hypercholesterolemia are at severely increased risk for development of atherosclerosis. Characterization of subclinical atherosclerotic plaques using CEUS could eventually lead to better risk stratification in these patient groups. Furthermore, the impact of gender on the amount of IPN is reported and the use of carotid CEUS in the evaluation of vasculitides is illuminated.

V – Summary and Discussion:
The main findings of this thesis are summarized and discussed, taking into consideration the limitations of the performed studies. The clinical implications of the results in this thesis are discussed and future directions for research on carotid CEUS are suggested.

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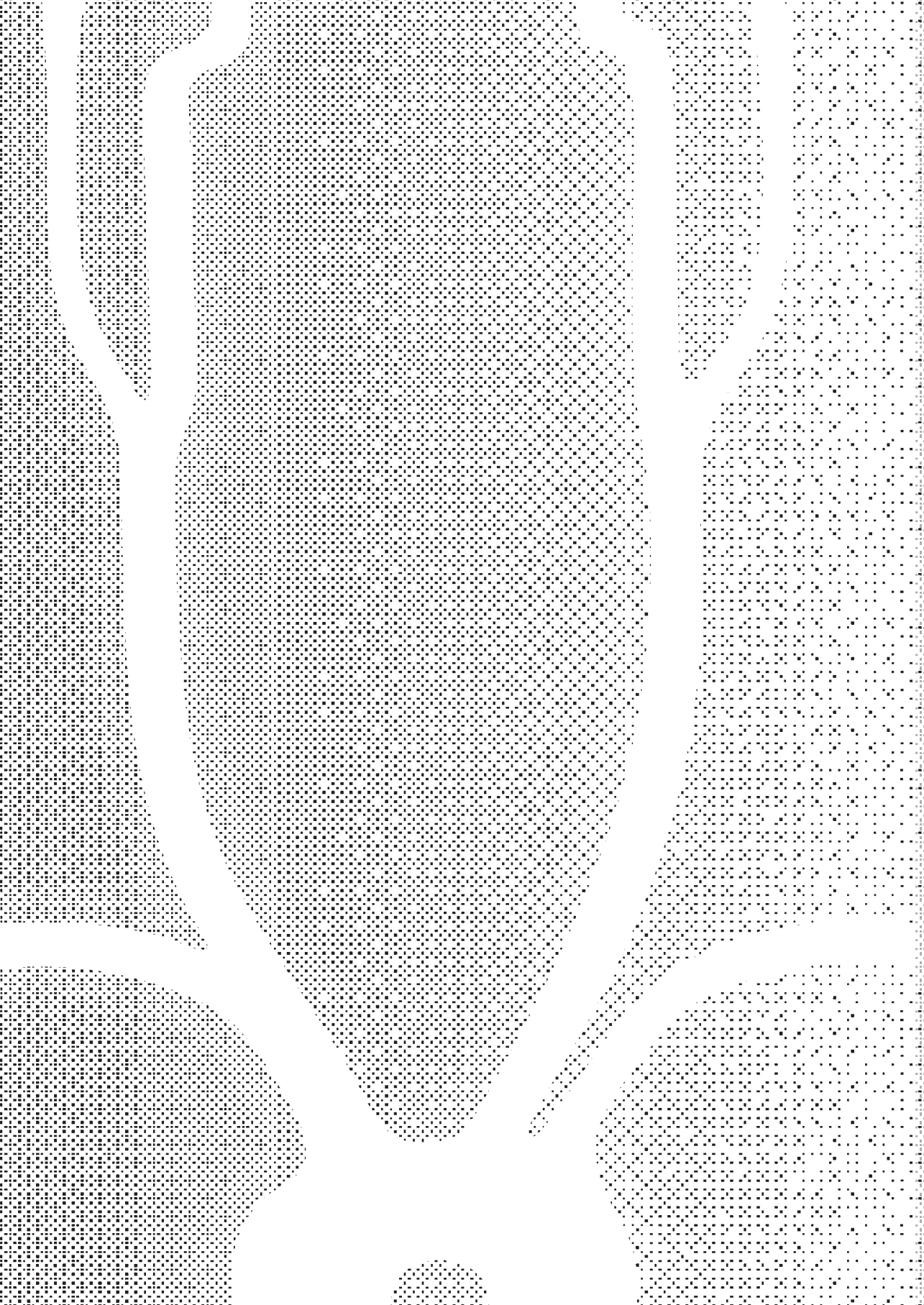

CHAPTER 2

CAROTID INTIMA-MEDIA THICKNESS FOR CARDIOVASCULAR RISK ASSESSMENT: SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

Objective: B-mode ultrasound measurement of the carotid intima-media thickness (CIMT) is a widely used marker for atherosclerosis and is associated with future cardiovascular events. This article provides a review and meta-analysis of the published evidence on the association of CIMT with future cardiovascular events and its additional value to traditional cardiovascular risk prediction models.

Methods: A systematic review and meta-analysis of the evidence on the association of CIMT with future cardiovascular events and the additional value of CIMT to traditional cardiovascular risk prediction models was conducted. The association of CIMT with future cardiovascular events and the additional value of CIMT were calculated using random effects analysis.

Results: The literature search yielded 1196 articles of which 15 articles provided sufficient data for the meta-analysis. A 1 SD increase in CIMT was predictive for myocardial infarction (HR 1.26, 95% CI 1.20-1.31) and for stroke (HR 1.31, 95% CI 1.26-1.36). A 0.1 mm increase in CIMT was predictive for myocardial infarction (HR 1.15, 95% CI 1.12-1.18) and for stroke (HR 1.17, 95% CI 1.15e1.21). The overall performance of risk prediction models did not significantly increase after addition of CIMT data. The areas under the curve increased from 0.726 to 0.729 ($p = 0.8$).

Conclusions: CIMT as measured by B-mode ultrasound is associated with future cardiovascular events. However, the addition of CIMT to traditional cardiovascular risk prediction models does not lead to a statistical significantly increase in performance of those models.

INTRODUCTION

Data from the European Society of Cardiology demonstrates that cardiovascular disease accounts for 38–42% of the mortality in Europe [1]. Atherosclerosis is the underlying cause of the majority of clinical cardiovascular events. This generalized inflammatory disease is characterized by an accumulation of lipids, inflammatory cells, and development of scar tissue covered by a fibrous cap build within the walls of large and medium-sized arteries [2].

Individuals with subclinical atherosclerosis should preferably be identified at an early stage, so that primary preventive measures can be initiated [3]. The development of atherosclerosis usually takes decades, and one of the first detectable stages of atherosclerosis is thickening of the arterial wall. Carotid ultrasound is a widely used imaging modality for the detection of subclinical atherosclerosis [4]. B-mode ultrasound measurement of carotid intima-media thickness (CIMT) is frequently used for non-invasive evaluation of subjects at risk of atherosclerosis. Still, the exact risk of cardiovascular events associated with an increased CIMT in general populations is not entirely clear. More importantly, the additional value of CIMT measurement on top of currently used risk prediction models is not entirely clear as well.

Therefore the goals of this review and meta-analysis were 1. to provide a short historical perspective on CIMT measurement using ultrasound, 2. to discuss current CIMT acquisition and measurement methods, 3. to assess the association of CIMT with future cardiovascular events using up-to-date data from cohort studies, 4. to determine the additional value of CIMT measurement in cardiovascular-risk prediction and 5. to provide recommendations for future studies on the role of CIMT for cardiovascular risk assessment.

Historical perspective on carotid intima-media thickness measurement

In 1986, Pignoli et al. reported the first in vitro results of a study investigating the arterial wall thickness with real-time B-mode ultrasound [5]. In that initial study, the distance between two parallel echogenic lines correlated well with the intima-media thickness (IMT) measured on pathologic examination. The authors concluded that B-mode ultrasound represented a useful tool for the measurement of IMT of human arteries in vivo. Subsequently, Persson et al. demonstrated in an in vivo study that IMT measurement with B-mode ultrasound was highly reproducible [6]. Partly because of the easily accessible anatomical position of the carotid arteries, the carotid CIMT has become a frequently used measurement in clinical practice and scientific studies.

CIMT is a well-studied phenotype of atherosclerosis. Using B-mode ultrasound CIMT can be assessed quickly, non-invasively at relatively low cost [7,8]. The measurement of CIMT is widely used as a non-invasive indicator of (subclinical) atherosclerosis and is

both used in prospective follow-up studies to investigate the relationship of CIMT with future cardiovascular events and in clinical intervention trials to monitor changes of any given treatment [8,9]. Based on the assumption that change in CIMT is directly correlated with change in cardiovascular risk, CIMT is a frequently used surrogate endpoint in clinical trials [10-13].

How to measure CIMT?

In 2008 the American Society of Echocardiography has published a consensus statement, which provides a protocol for CIMT measurement [14]. CIMT measurement should be obtained in both the left and right carotid artery. Images of the far wall should be made of the distal 1 cm of the common carotid artery in 3 angles. The CIMT is calculated in R-wave gated still frames. Mean CIMT is calculated as the mean of the left and the right measurements. Semi-automated CIMT measurement tools have proven to provide a reliable, accurate and reproducible measurement of the CIMT [15]. Nowadays, most vascular ultrasound systems are provided with such semi-automated measurement tools. The American Society of Echocardiography recommends using their protocol in combination with a semi-automated measurement tool for obtaining a reliable and reproducible CIMT [14]. This is supported by a recent consensus statement from the European Stroke Conferences [16].

Multiple factors may affect the reliability and reproducibility of CIMT measurement. The reader's personal interpretation of the CIMT and ultrasound settings may affect the measurement [17,18]. Due to a lack of a standardized method for image acquisition and CIMT measurement, previous studies have used dissimilar ultrasound protocols to assess the CIMT. The angle of insonation of the near and/or the far wall along different segments of the carotid artery (common carotid artery (CCA), carotid bifurcation (BIF), internal carotid artery (ICA)) may lead to variability in CIMT measurements [10,11]. The development of ultrasound probes with higher frequencies has led to a better visualization of CIMT. Manual CIMT measurement can be relatively time consuming. Semi-automated assessment of the CIMT measurement at multiple sequential locations has been introduced to overcome inter and intra-reader variability. Several studies have demonstrated that inter-reader variability is reduced in semi-automated CIMT measurements [7,10,11].

CAROTID INTIMA-MEDIA THICKNESS AND CARDIOVASCULAR RISK PREDICTION; A META-ANALYSIS

METHODS

Search strategy and data extraction

A systematic search of the literature published in the MEDLINE database was performed using PubMed on the 10th of January 2012 to find: 1. articles on the association of CIMT with future cardiovascular events and 2. articles on the additional value of CIMT to existing cardiovascular risk assessment tools. Search terms were 'Carotid intima-media thickness', 'Prognosis', 'Outcome', 'Prediction', and 'Association'. The MOOSE guidelines for meta-analysis of observational studies were followed [19]. No time restriction for publication dates was used. Meta-analyses, reviews, animal studies and articles not in the English language were excluded. Abstracts and unpublished articles were not considered for inclusion. Two independent reviewers (SCHvdO & AFLS) evaluated all titles and abstracts of the articles. There was no assessors blinding involved in the review process. Articles with data on the association of CIMT with future cardiovascular events and articles with data on the additional value of CIMT to existing cardiovascular risk assessment tools were selected. Studies not focusing on a general population sample were excluded. A manual review of the reference lists of the selected articles was performed to find additional relevant articles. The literature search yielded 1196 articles (Fig. 1). A total of 30 articles were identified to report data on the association of CIMT with future cardiovascular events or data on the additional value of CIMT for cardiovascular risk prediction [7,20-48](Addendum 1). General study characteristic as well as CIMT definition, study end-points, hazard ratios (HRs) of the association of CIMT with future cardiovascular events and data on the additional value of CIMT to cardiovascular risk prediction models based on traditional risk factors (TRF) were extracted by 2 reviewers (SCHvdO & AFLS). Discrepancies in the extracted data were resolved with a coupled review of the articles and consensus. If the extracted data was incomplete, authors of the concerning articles were contacted and asked to provide additional data. Assessment of study quality was based on the availability of 1. sufficient information on the number of patients and their characteristics, 2. information on CIMT measurement methods, 3. definition(s) of endpoint(s) and 4. information on the relation between CIMT and outcome. To avoid publication overlap in cohort studies with multiple publications, only the most recent publication of each cohort was included in the meta-analysis. For each study that was included in the analysis, methodological data regarding CIMT measurements were extracted (i.e. measurement sides, walls included in CIMT measurement, anatomical segments included in measurements, CIMT reporting method, definition of atherosclerotic plaque).

Figure 1
Flowchart of the literature search strategy

Statistical analysis

The statistical analyses were performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond Wash, USA) and MedCalc Software (version 12.2.1.0, MedCalc Software, Mariakerke, Belgium). Fixed and random effects models were used to calculate summary estimates and to construct forest plots. Heterogeneity among the studies was assessed using the Q test and I^2 index. For the fixed effects model there was substantial heterogeneity among the studies, therefore only data from the random effects model are reported. A meta-analysis was performed on HRs for myocardial infarction, stroke and a combination of these endpoints using the method of Neyeloff [49]. A comparison of the age and sex adjusted HRs and confidence intervals (CI) per SD increment and per 0.1 millimeter (mm) increment was performed. Therefore, the standard errors of the HRs were recalculated by log transforming the CI points and divide the symmetrical confidence intervals by 2*1.96 [50]. Overall HRs were plotted in forest plots. For each carotid segment, an overall HR was calculated if ≥ 2 studies reported sufficient data. The predictive value of mean and maximum CIMT was analyzed if ≥ 2 studies reported sufficient data. To investigate the additional value of CIMT to cardiovascular risk prediction models, the

overall AUC was calculated. For calculation of the overall AUC, data from eligible studies was analyzed using a random effects analysis. If the standard error was not available in the eligible studies, we estimated the standard error according to the method proposed by Hanley and McNeil [51]. In short, the standard error was estimated by the square root of ((A(1 − A) + (Na − 1)*(Q1 − A2) + (Nn − 1)*(Q2 − A2))/(Na*Nn)), where A is the area under the curve, Na and Nn are the number of abnormal and normal respectively, and Q1 and Q2 are estimated by: Q1 = A/(2 − A) and Q2 = 2A2/(1 + A). The difference between the overall AUC of the TRF model without CIMT and the overall AUC of the TRF model with CIMT was tested according to the method of Hanley and McNeil [52].

RESULTS

From the 30 articles found in the literature search, 17 articles were excluded from the meta-analysis of the association of CIMT with future cardiovascular events because they did not report sufficient data [7,20,21,24-28,33-35,38,40-42,45,47]. An overview of the remaining articles is provided in Table 1. Eventually 7 articles reported an association of CIMT with future myocardial infarction [22,29,31,32,37,39,44]. Eight articles reported an association of CIMT with future stroke [23,30-32,36,39,43,44]. Five articles reported an association of CIMT with future stroke or myocardial infarction [32,39,44,46,48]. Additional data was requested for 4 articles, and 3 authors provided the additional requested data. Finally, 2 articles were excluded because an overlapping publication with longer follow up of the same cohort was available [29,30] and 2 articles were excluded because data from a previous report were used [22,23]. Three studies used unilateral CIMT measurements in their analysis [36,37,43] and 10 studies used bilateral CIMT measurements [22,23,29-32,39,44,46,48]. Four studies reported associations using the maximum CIMT value [29,32,44,46] and 9 studies reported associations using a mean CIMT value [22,23,30,31,36,37,39,43,48]. The definition of atherosclerotic plaque, and its inclusion or exclusion from CIMT measurements, could be retrieved in 5 studies [29,43,44,46,48]. In the remaining 8 studies, no clear definition of atherosclerotic plaque was available [22,23,30-32,36,37,39]. Meta-analysis of the data showed that the association of CCA–CIMT with future stroke (HR 1.31, 95% CI 1.26–1.36 per 1-SD increase in CIMT, HR 1.17, 95% CI 1.15–1.21 per 0.1 mm increase in CIMT) was stronger than the association of CCA–CIMT with future myocardial infarction (HR 1.26, 95% CI 1.20–1.31 per 1-SD increase in CIMT, HR 1.15 95% CI 1.12–1.18 per 0.1 mm increase in CIMT) (Fig. 2 and Fig. 3). The association of CCA–CIMT with the combined endpoint future stroke or future myocardial infarction was summarized in Fig. 4 (HR 1.26 95% CI 1.17–1.36 per 1 SD increase in CIMT, HR 1.17 95% CI 1.05–1.30 per 0.1 mm increase in CIMT). The heterogeneity in the random effects models was calculated with I^2 and varied between 0% and 37%. This indicates that the heterogeneity between studies used in the overall estimates was small to moderate.

Table 1. Summary of the studies used in the meta-analysis, reporting the association of CIMT with future cardiovascular events.

Cohort	Region	Author	Year	Population	Sample size	Age (years)	% ♀	Carotid segments	IMT definition	Endpoints	Follow-up (years)	Adjusted variables
ARIC (Atherosclerosis Risk in Communities) Study	USA	Chambless [22]	1997	Householdmembers aged 45-64 years, without previous CHD	12841	NR	57	CCA, BIF, ICA	Mean far wall IMT	Myocardial infarction, CHD death	5.2	Age, race, diabetes, LDL, HDL, Hypertension, smoking status
		Chambless [23]	2000	Householdmembers aged 45-64 years, without previous stroke	14214	NR	55	CCA, BIF, ICA	Mean far wall IMT	Stroke	7.2	Age, race, LDL HDL smoking status, hypertension, BMI, waist:hip ratio, diabetes, fibrinogen level, LVH, white bloodcell count
Rotterdam Study	Netherlands	Iglesias Del Sol [29]	2002	Elderly ≥55 years living in the suburb of Ommoord in Rotterdam, history of MI or stroke was no exclusion criteria	2267	70.2	59	CCA, BIF, ICA	Mean maximum far & near wall IMT	Myocardial infarction	4.6	Age, sex, BMI, SBP DBP, total cholesterol, HDL, smoking status, diabetes
		Hollander [30]	2003	Elderly ≥55 years living in the suburb of Ommoord in Rotterdam, without previous stroke	5479	NR	NR	CCA	Mean far & near wall IMT	(non-)fatal stroke	6.1	Age, sex, diabetes, smoking status, SBP, DBP, cholesterol levels, HDL, history of cardiovascular disease
		Elias-Smale [31]	2011	Elderly aged 55-75 years living in the suburb of Ommoord in Rotterdam, without previous stroke and / or CHD	3580	64.7	61	CCA	Mean far & near wall IMT	Myocardial infarction, stroke or mortality caused by stroke or CHD	12.2	Age, sex, smoking status, total cholesterol, HDL, SBP, hypertension medication, atrial fibrillation (stroke)
CHS (Cardiovascular Health Study)	USA	O'Leary [32]	1999	Participants were aged ≥ 65 years, without previous stroke and or CHD	4476	72.5	61	CCA, ICA	Maximal far & near wall IMT	Myocardial infarction, stroke or cardiovascular death	6.2	Age, sex, SBP, DBP, diabetes, smoking status
MDCS (Malmö Diet and Cancer Study)	Sweden	Rosvall [36]	2005	Participants were aged 46-68 years, without known CVD	5163	57.5	60	right CCA	Mean far wall IMT	(non-)fatal stroke	7	Age, sex, low physical activity, smoking status, SBP, hypertension medication, diabetes, LDL, HDL, Triglycerides, waist circumference

Table 1. Summary of the studies used in the meta-analysis, reporting the association of CIMT with future cardiovascular events. (continued)

Cohort	Region	Author	Year	Population	Sample size	Age (years)	% ♀	Carotid segments	IMT definition	Endpoints	Follow-up (years)	Adjusted variables
		Rosvall [37]	2005	Participants were aged 46-68 years, without known CVD	5163	57.5	60	right CCA	Mean far wall IMT	(non-)fatal myocardial infarction or death from CHD	7	Age, sex, low physical activity, smoking status, hypertension, diabetes, LDL, HDL, waist circumference
CAPS (Carotid Atherosclerosis Progression Study)	Germany	Lorenz [39]	2006	Members of a German primary healthcare scheme aged 19-90 years, 1.9% history of stroke, 2.1% history of myocardial infarction	5056	50.1	51	CCA, BIF, ICA	Mean far wall IMT	Myocardial infarction, or stroke, or death.	4.2	Age, sex, BMI, SBP, DBP, hypertension medication, LDL, lipid lowering medication, smoking status, diabetes
Tromsø Study	Norway	Mathiesen [43]	2011	Participants aged 25-84 years without previous stroke	6584	60.2	51	Right CCA, Bulb	Mean far and near wall IMT	(non) fatal stroke	9.6	Age, sex, total cholesterol, HDL, SBP, hypertension medication, smoking status, diabetes, CHD
MESA (Multi-Ethnic Study of Atherosclerosis)	USA	Folsom [44]	2008	Participants aged 45-84 years without previous CVD	6698	NR	53	CCA, ICA	Mean far and near wall IMT	(non) fatal myocardial infarction, (non) fatal stroke, other cardiovascular death	3.9	Age, race, sex, smoking status, diabetes, blood pressure, HDL, total cholesterol, lipid lowering medication
Framingham Offspring Study	UK	Polak [46]	2011	Participants were non-Hispanic whites, without current disease	2946	58	55	CCA, ICA	Mean and max far wall IMT	(non) fatal myocardial infarction, (non) fatal stroke, peripheral arterial disease, heart failure.	7.2	Age, sex, SBP, total cholesterol, HDL, diabetes, smoking status, hypertension medication
PRC-USA study	China	Xie [48]	2011	Participants living in rural Beijing aged 43-79 years, without previous myocardial infarction or previous stroke	1734	NR	64	CCA, BIF	Mean far wall IMT	(non) fatal myocardialinfarction, (non) fatal stroke	4	Age, sex, diabetes, hypertension, BMI, smoking status, total cholesterol, HDL

BIF = carotid bifurcation, BMI = body mass index, CCA = common carotid artery, CHD = coronary heart disease, CVD = cardiovascular disease, DBP = diastolic blood pressure, HDL = high density lipoprotein, ICA = internal carotid artery, IMT = intima-media thickness, LDL = low density lipoprotein, LVH = left ventricular hypertrophy, NR = not reported, SBP = systolic blood pressure.

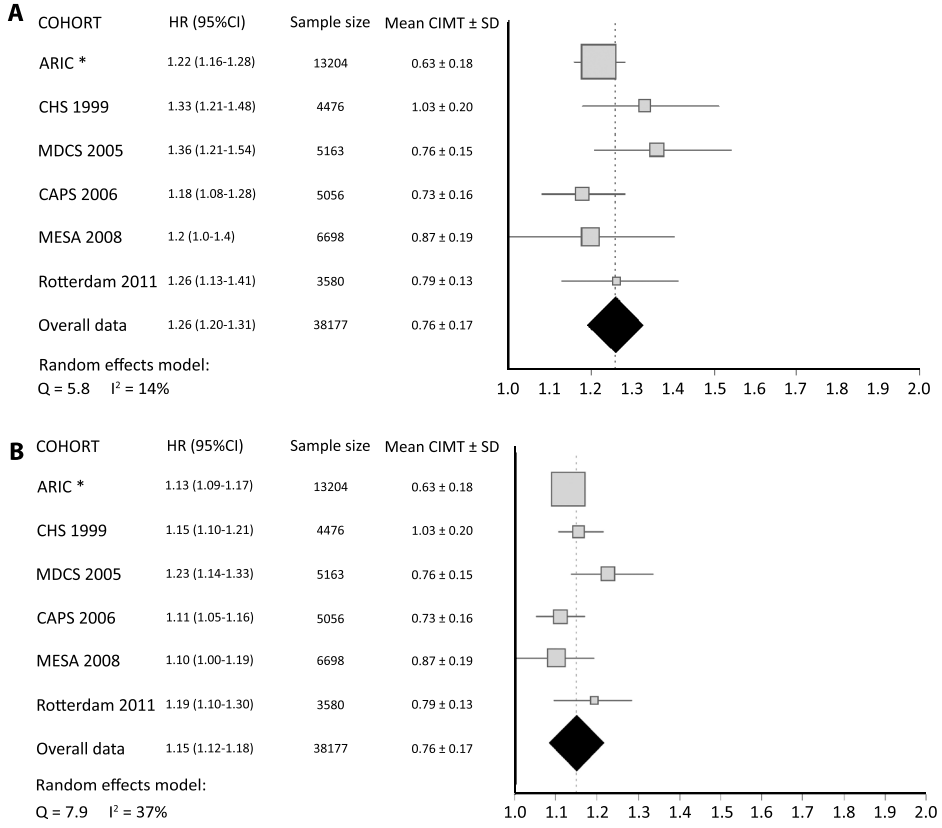

Figure 2

Forest plots of hazard ratios for myocardial infarction. 2A Hazard ratios were calculated per SD increment in CCA-CIMT, adjusted for age and sex. 2B Hazard ratios were calculated per 0.1 mm increment in CCA-CIMT, adjusted for age and sex. * = Additional data was obtained by personal communication with the author.

The same analyses were performed to assess the predictive value of ICA–CIMT. A 1 SD increase in ICA–CIMT was predictive for future stroke (HR 1.25, 95% CI 1.09–1.41) and for the combined endpoint (HR 1.24, 95% CI 1.11–1.37). For future myocardial infarction, a 1 SD increase in ICA–CIMT was no statistically significant predictor (HR 1.27, 95% CI 0.96–1.58). Only 1 study provided sufficient data on the association of BIF–CIMT with future stroke and myocardial infarction [39]. Therefore a meta-analysis was not performed. Meta-analysis of the predictive value of maximum CIMT and mean CIMT demonstrated that both maximum and mean CCA–CIMT predicted stroke and myocardial infarction (Table 2).

A total of 13 articles were identified to report data on the additional value of CIMT to existing cardiovascular risk prediction scores [7,24-26,28,31,33,40,41,44-47](Table 3). Four articles

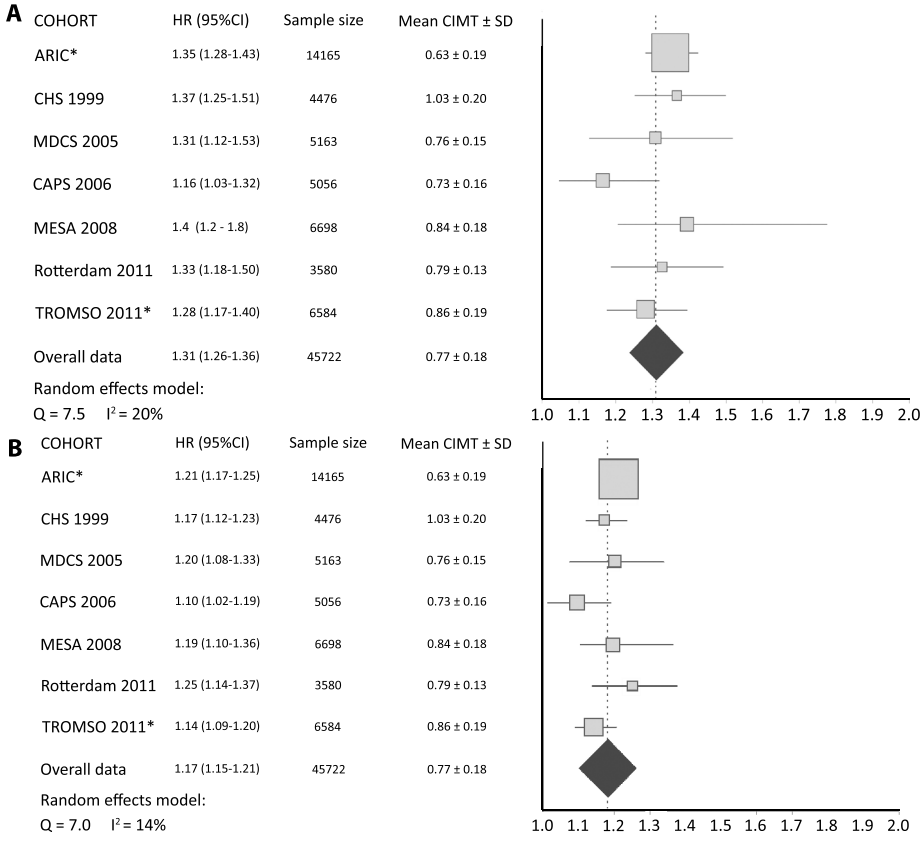

Figure 3

Forest plots of hazard ratios for stroke. 3A Hazard ratios were calculated per SD increment in CCA-CIMT, adjusted for age and sex. 3B Hazard ratios were calculated per 0.1 mm increment in CCA-CIMT, adjusted for age and sex. * = Additional data was obtained by personal communication with the author.

were excluded because they reported the additional value of CIMT in combination with another parameter (e.g. CRP or carotid atherosclerotic plaque) or they did not report an eligible AUC, ROC or c-statistic and thus were not eligible for calculating an overall AUC [33,40,45,47]. Finally, 3 articles were excluded because an overlapping publication with longer follow up of the same cohort was available [24,25,28]. The overall AUC analysis included 32,299 individual patients from 6 cohort studies. The overall AUC of the TRF only models was 0.726 (95% CI 0.700–0.753, I2 = 50%). The overall AUC of the TRF + CIMT model was 0.729 (95% CI 0.700–0.758, I2 = 49%). There was a small increase in the overall AUC when CIMT was implemented in traditional cardiovascular risk prediction models, but this increase was statistically not significant (p = 0.8) (Table 3).

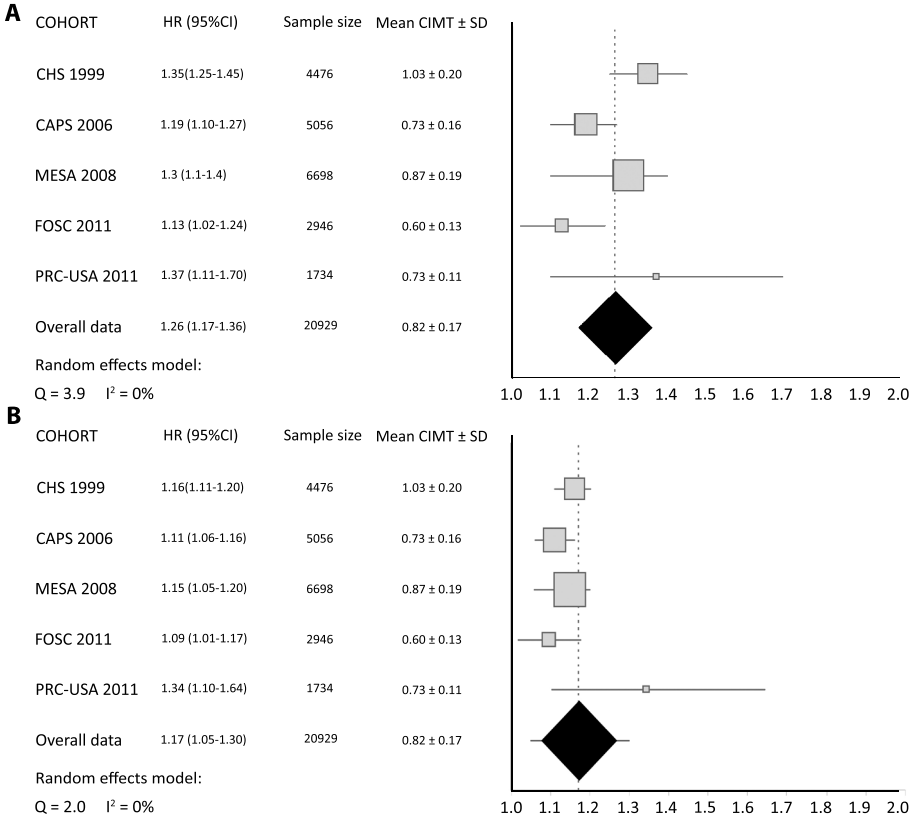

Figure 4

Forest plots of hazard ratios for the combined endpoint myocardial infarction and stroke. 4A Hazard ratios were calculated per SD increment in CCA-CIMT, adjusted for age and sex. 4B Hazard ratios were calculated per 0.1 mm increment in CCA-CIMT, adjusted for age and sex.

Table 2. Summary of the predictive value of maximum CCA-CIMT and mean CCA-CIMT.

	Overall HR maximum CCA-CIMT (95% CI)		Overall HR mean CCA-CIMT (95% CI)	
	Per SD increment	Per 0.1 mm increment	Per SD increment	Per 0.1 mm increment
Future stroke [31, 32, 36, 39, 43, 44]	1.38 (1.34-1.41)	1.18 (1.16-1.20)	1.29 (1.22-1.35)	1.18 (1.14-1.22)
Future myocardial infarction [31, 32, 37, 39, 44]	1.27 (1.14-1.40)	1.13 (1.08-1.18)	1.25 (1.19-1.31)	1.17 (1.12-1.21)
Combined endpoint [32, 39, 44, 46, 48]	1.26 (1.12-1.40)	1.14 (1.11-1.17)	1.27 (1.10-1.45)	1.22 (1.00-1.45)

CCA = common carotid artery, CI = confidence interval, CIMT = carotid intima-media thickness, HR = hazard ratio.

Table 3. Summary of the studies used for the overall AUC on the additional value of CIMT measurement for the prediction of cardiovascular events in traditional cardiovascular risk models.

Cohort	Author	Year	Sample size	AUC TRF-model	AUC TRF-model + CIMT	NRI	P-value
EAS	Price [41]	2007	10070	0.614	0.620	NR	
MESA	Folsom [44]	2008	6698	0.772	0.782	NR	
ARIC	Nambi [26]	2010	13145	0.742	0.750	16.7 %	
CAPS	Lorenz [7]	2010	4904	0.729	0.732	-2.05%	
Rotterdam	Elias-Smale*	2011*	3580	0.700	0.706	2.5 %	
FOSC	Polak [46]	2011	2946	0.748	0.751	0.0%	
Summary value			32299	0.726	0.729	NA	p=0.8

Details of these studies are presented in Table 1 & Addendum 1. An overall AUC was calculated for the reported cohort studies. The increase in AUC between the TRF-model without CIMT and the TRF model with CIMT was tested according to the method of Hanley and McNeil. AUC = area under the curve, CIMT = carotid intima-media thickness, NA = not available, NR = not reported, NRI = net reclassification improvement, TRF = traditional risk factors. * = Additional data was obtained by personal communication with the author.

DISCUSSION

This systematic review and meta-analysis demonstrates a positive association between increasing CIMT and cardiovascular risk. With the overall estimates in this meta-analysis it has become clear that 1 SD increase of CCA–CIMT increases the risk for myocardial infarction by 26%. A 1 SD increase in CIMT increases the risk for stroke by 31%. The current meta-analysis differs from previously published reports because it provides the most recent follow-up results of multiple cohort studies [50]. Furthermore, this meta-analysis provides an overall analysis of the additional value of CIMT to existing cardiovascular risk prediction models. This information was not previously available and is particularly relevant for clinicians who are confronted with patients who require cardiovascular risk stratification and hence consider the use of CIMT measurement in daily clinical practice. The lack of additional value of CIMT to existing cardiovascular risk prediction models could be explained by at least two factors namely 1. the good performance of cardiovascular risk prediction models based on TRF only and 2. the known association of CIMT with those TRF.

Although there is a well-documented association between CIMT and future myocardial infarction and/or stroke, its additional value to existing cardiovascular risk prediction models has been questioned. For years, cardiovascular risk prediction methods were based on traditional cardiovascular risk factors such as increasing age, gender, smoking status, cholesterol levels, blood pressure and diabetes [53]. Addition of a surrogate marker for atherosclerosis to these models, such as CIMT could further improve the predictive power of such models [4]. Several studies investigated the performance of risk

prediction models with and without CIMT. This meta-analysis provided an overall AUC on follow-up data of 32,299 patients who underwent CIMT measurement. The increase in performance of the model with CIMT was 0.003. Since the absolute increase in performance was small and not statistically significant, the clinical relevance of this increase in prognostic performance has to be taken into consideration.

In 2008 Pencina et al. [54] questioned the use of only the AUC, ROC or C-statistic method, since adding a new biomarker to existing models would only lead to a small increase or decrease of performance. Therefore, in the setting of risk prediction, the proportion of patients reclassified correctly would seem to have more relevance [54-57]. The net reclassification improvement (NRI) and integrated discrimination improvement are measures which monitor the proportion of patients reclassified correctly. Four studies provided information on the NRI. However, they report conflicting results (Table 3) [7,26,31,46].

For future purposes, it's advisable to standardize the CIMT acquisition protocol and outcome measures. Studies investigating the performance of CIMT measurement as addition to traditional cardiovascular risk models should agree on a homogeneous way to report their results. A more homogeneous sample of publications on the additional value of CIMT to future cardiovascular prediction models could eventually provide enough data to answer the question whether the addition of CIMT to these models does have incremental value for cardiovascular risk assessment in specific subgroups (e.g. intermediate and/or high risk).

Despite limited evidence for a relevant increase in cardiovascular risk prediction, recent guidelines recommend to measure the CIMT in asymptomatic individuals at intermediate cardiovascular risk. In these patients, the CIMT value should help the clinicians in the decision making for aggressive or less aggressive medical therapy [3,14,58]. Stein et al. have reported that a CIMT value above the 75th percentile for the patient's age, sex, and race/ethnicity is indicative of increased cardiovascular risk and thus may indicate a more aggressive risk reducing therapy. Patients with a CIMT value below the 25th percentile are considered to be at lower cardiovascular risk [14]. However, it lacks prospective follow up data supporting interventions in medical therapy based on CIMT values.

CIMT is a well-accepted surrogate marker for subclinical atherosclerosis. However, thickening of the carotid intima-media complex does not necessarily represent subclinical atherosclerosis. It is suggested that other morphological imaging markers could play a more important role in subclinical atherosclerosis detection and cardiovascular risk prediction. In several articles the presence of atherosclerotic plaque on carotid B-mode ultrasound is a better individual predictor of future cardiovascular events than CIMT [26,59,60]. Combination of multiple imaging markers will likely further improve imaging based cardiovascular risk prediction [4] and [61]. Further research is needed to investigate whether CIMT solely, is a feasible imaging marker in particular groups such as young adults [62].

Limitations

This review and meta-analysis has some limitations. First, publication bias could have altered the results in this meta-analysis. Presence of publication bias could have resulted in an overestimation of the association of CIMT with future cardiovascular events, and an overestimation of the additional value of CIMT to existing cardiovascular risk prediction models. In addition, non-English language articles were excluded due to practical difficulties in extracting data from articles from other languages. Nevertheless, this meta-analysis included data from articles originating from many countries and different cohorts. Furthermore, the quality of the studies was assessed using a self-developed quality assessment tool. Since this tool was partly based on standard study characteristics, this could have led to overestimation of the quality of specific CIMT studies.

Another inherent limitation of meta-analyses is the heterogeneity of the source data. Each cohort study that was included in the forest plots was based on a general middle aged to old population. However, slight differences in inclusion criteria result in more dramatic differences for baseline population characteristics. Besides, not all cohorts used the same ultrasound scanning protocol. As a result, most studies used different CIMT measurement methods. These methods differed in anatomical side (i.e. left vs. right vs. combination), in carotid segment used for CIMT measurement (i.e. CCA vs. ICA vs. BIF vs. combination), in the definition of CIMT (i.e. including or excluding atherosclerotic plaque, far wall vs. near wall vs. combination) and in reporting methods (i.e. mean value, maximum value, mean maximum value). In addition, the studies investigating the additional value of CIMT to cardiovascular risk prediction models used different covariates in their risk prediction models. These inconsistencies between the cohort studies hinder comparison of these studies. To provide more homogeneous data, the cohort studies should consider to investigate the value of CIMT measurements by using

Table 4. Overview of the Mannheim consensus statement for assessment of CIMT and atherosclerotic plaque

Ultrasound equipment	High resolution B-mode ultrasound system with a linear ultrasound transducer > 7 MHz
Angle of insonation	Lateral probe position
Anatomical side	Left and right
Carotid segment	Distal CCA (5 mm before the carotid wall starts diverging)
Walls	Far wall only
Tools	Semi-automated edge detection
Reporting method	Mean CIMT (3 measurements left, 3 measurements right)
Atherosclerotic plaque	Exclusion from CIMT measurement, maximal plaque thickness should be assessed from two different angles of insonation in longitudinal and cross-sectional views, area/location and number of plaques should be recorded

Based on the Mannheim consensus [16]. CCA = common carotid artery, CIMT = carotid intima-media thickness.

Table 5. Recommendations for reporting the methodology of studies on the predictive value of CIMT

Study population	
Selection of participants	Population based
Inclusion and exclusion criteria	Age, symptoms, history of vascular disease
Ultrasound protocol and definition of CIMT	
Ultrasound equipment	Ultrasound system brand and type, ultrasound transducer details (linear/convex/phased array, frequency), standard ultrasound system settings (log-gain compensation, depth, frame-rate).
Angle of insonation	Anterior, lateral, posterior
Anatomical side	Left, right, combination
Carotid segment	CCA, BIF, ICA, combination
Walls used in CIMT measurement	Far wall, near wall, combination
Atherosclerotic plaque	Definition of plaque, inclusion or exclusion of plaque
Reporting method	Mean CIMT, mean maximum CIMT, maximum CIMT
Statistical analysis	
Associations	Hazard ratio, adjustable covariates
Addition of CIMT to TRF prediction model	Covariates in TRF prediction model, performance indicator (AUC, C statistic, ROC-curve), reclassification indicator (NRI)

AUC = area under the curve, BIF = carotid bifurcation, CCA = common carotid artery, CIMT = carotid intima-media thickness, ICA = internal carotid artery, NRI = net reclassification improvement, ROC-curve = receiver operating characteristic-curve, TRF = traditional risk factors.

Table 6. Recommendations for reporting the results of studies on the predictive value of CIMT

Study population	
Baseline characteristics	Standard characteristics including traditional cardiovascular risk factors and cardiovascular risk estimation (low, intermediate, high)
Follow-up	
Duration	Months, years (mean ± SD)
End points	Definition of myocardial infarction, definition of stroke, definition of a combined end point
Events	Number of events
CIMT	
General data	Mean CIMT (±SD)
	Presence of atherosclerotic plaque (%)
Associations	
Unadjusted	Hazard ratio (95% CI)
Adjusted for age and sex	Hazard ratio (95% CI)
Adjusted for multiple covariates	Hazard ratio (95% CI)
Additional value of CIMT to TRF prediction models	
Performance	AUC of model without CIMT, AUC of model with CIMT, statistical comparison of both models
Reclassification	NRI (95% CI, p-value)

AUC = area under the curve, CI = confidence interval, CIMT = carotid intima-media thickness, NRI = net reclassification improvement, SD = standard deviation

a standardized CIMT measurement protocol as suggested in the Mannheim consensus statement which is summarized in Table 4[16]. In addition, a standardized protocol for reporting methodology and results would help the reader to compare individual studies. Recommendations for such a protocol are provided in Table 5 and Table 6. Despite all inconsistencies, statistical tests were used in this review and meta-analysis for assessment of the heterogeneity between the included studies in the random effects model. These tests indicate that heterogeneity was limited between studies investigating the association of CIMT with future cardiovascular events. The heterogeneity was moderate in studies investigating the additional value of CIMT for cardiovascular risk stratification. Another limitation is the possibility to extrapolate data reported in this review and meta-analysis. Extrapolating data to specific populations, such as children, should be done with caution. Despite the population based cohort design of the studies included, the data are not valid for specific populations that are at extreme low risk or extreme high risk for cardiovascular diseases. Finally, the data reported in this meta-analysis reflects the situation in which CIMT is measured once, and cardiovascular risk prediction is (partly) based on this measurement. This meta-analysis takes not into account changes in CIMT that may occur over time, and that may influence cardiovascular risk.

CONCLUSION

Carotid intima-media thickness as measured by B-mode ultrasound is a predictor of future myocardial infarction and stroke. The addition of CIMT to cardiovascular risk prediction models based on traditional cardiovascular risk factors leads to a small increase in the performance of those models. Nevertheless this increment is not statistically significant. Thus, the addition of CIMT measurements to traditional cardiovascular risk prediction models has no clinical value so far.

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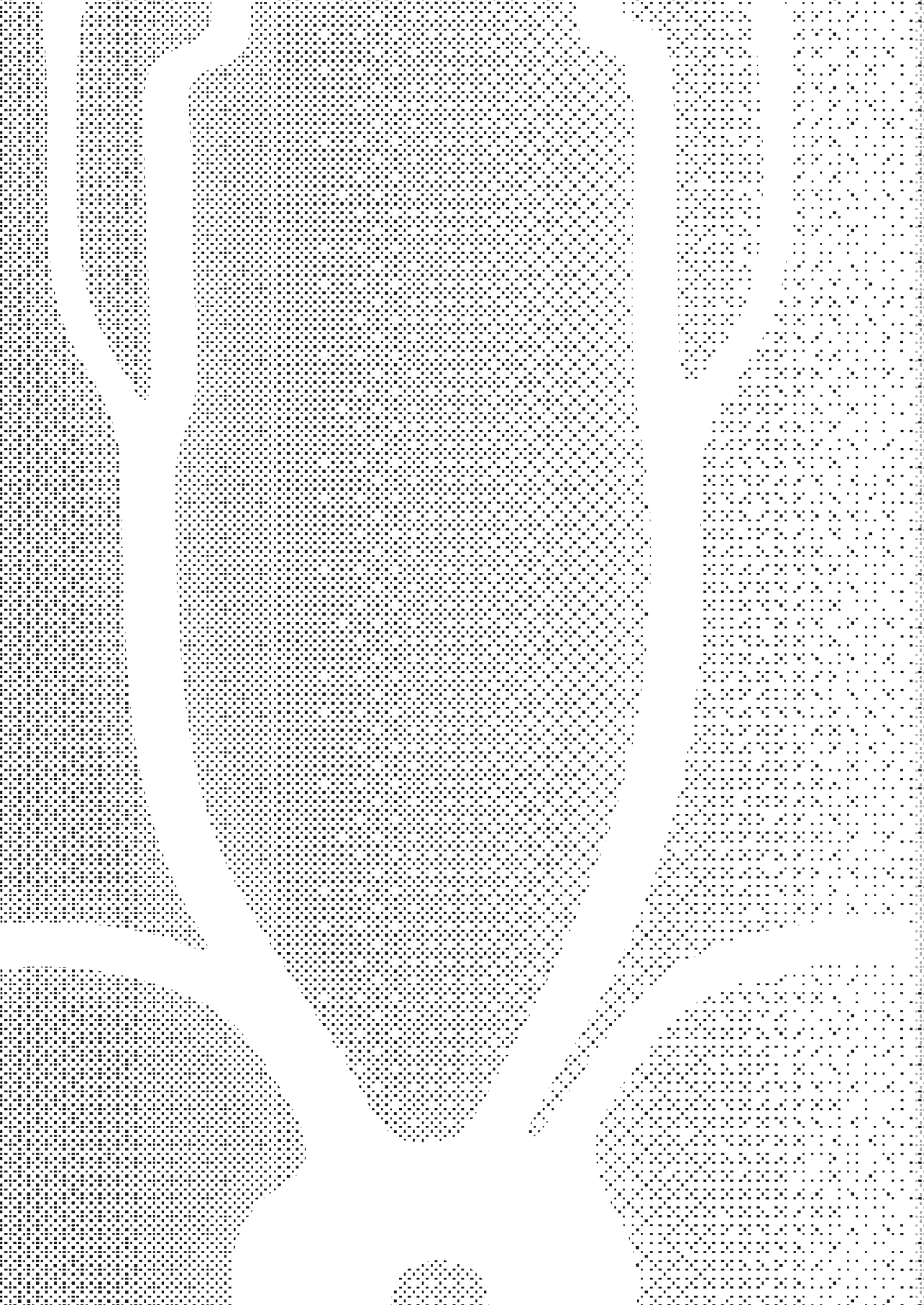

CHAPTER 3

CURRENT STATUS AND FUTURE DEVELOPMENTS OF CONTRAST-ENHANCED ULTRASOUND OF CAROTID ATHEROSCLEROSIS

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ABSTRACT

B-mode and Doppler ultrasound are commonly used for the evaluation of atherosclerosis in the carotid arteries. Recently, contrast-enhanced ultrasound (CEUS) has been introduced as a technique to improve the detection of carotid atherosclerosis and evaluate the presence of intraplaque neovascularization, which is considered a marker of plaque vulnerability. The present review focuses on the role of CEUS for the assessment of atherosclerosis and plaque instability. Currently available literature and future developments with CEUS are discussed.

INTRODUCTION

According to the current guidelines, carotid endarterectomy is highly beneficial in patients at average or low surgical risk who experience nondisabling ischemic stroke or transient ischemic cerebral symptoms and a >70% stenosis present in the ipsilateral internal carotid artery. Carotid endarterectomy in patients with a high-grade stenosis but asymptomatic for cerebrovascular disease and in those with a 50% to 69% stenosis may be beneficial, but the benefit-to-risk ratio decreases and more individualized management decisions must be made [1]. A risk stratification method distinguishing patients with stable carotid plaque from those with unstable plaque could improve selection of patients for carotid surgery.

The concept of the vulnerable atherosclerotic plaque was first described for coronary artery disease and relates to plaques with a thin fibrous cap covering a large lipid necrotic core, and active inflammation, characteristics that make the plaque at increased risk of rupture [2]. Intraplaque neovascularization is a novel marker of plaque vulnerability [2,3]. The intraplaque neovessels are leaky due to increased gap junctions, thus serving as a port of entry for inflammatory cells, lipids, and even red blood cells, which contributes to plaque growth. Moreover, the neovessels are at increased risk for rupture, causing intraplaque hemorrhage and subsequent rapid progression to symptomatic disease. The presence of neovascularization in carotid plaques has been associated with symptomatic cerebrovascular disease [4]

B-mode and Doppler ultrasound are widely available and are frequently used for evaluation of atherosclerosis in the carotid arteries.1 Recently, contrast-enhanced ultrasound (CEUS) has been introduced for the evaluation of carotid atherosclerosis and plaque vulnerability by identifying the presence of intraplaque neovascularization [5-19]. The present review provides a brief overview of the commonly used ultrasound techniques for the evaluation of carotid atherosclerosis and subsequently focuses on the role of CEUS for the assessment of carotid atherosclerosis and plaque instability. The promising results of the initial CEUS studies in humans are discussed. Additionally, quantification of CEUS images and future developments, including molecular imaging are discussed.

METHODS

Study design

This review included all available original studies reporting the use of transcutaneous CEUS for the evaluation of carotid atherosclerosis, in particular intraplaque neovascularization. Review articles and animal studies were excluded.

Literature search and data extraction

The online MEDLINE database was searched for literature in June 2012 using PubMed (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Md). The search strategy was "carotid," "contrast-enhanced," and "ultrasound." No time restriction for publication date was used. All titles and abstracts of the articles were evaluated. After exclusion based on the title and abstract, full articles were evaluated, and articles meeting the inclusion criteria were identified. In addition, a manual search of the reference lists of the identified studies was performed, and references were evaluated. Selected studies were reviewed and relevant patient characteristics and CEUS findings were registered. Extracted parameters were the number of included patients, age, gender, studied population, degree of stenosis, proportion of symptomatic patients, use of quantification of CEUS, and whether histology was used as a reference method.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Wash) and SPSS version 15.0 (SPSS, Chicago, Ill). Continuous variables were reported as mean. Categorical variables were summarized as percentages. From the pooled data, summary estimates were calculated.

RESULTS

B-mode ultrasound scan

Based on the B-mode images, an evaluation can be made whether the plaque is predominantly echolucent, echogenic, or mixed (heterogenic)[20]. Echolucent plaques are thought to consist of soft plaque components (lipids, intraplaque hemorrhage), whereas echogenic plaques contain more hard components (fibrous tissue, calcifications)[21]. Consequently, echolucent plaques are considered to be more at risk of rupture and thrombus formation. Hence, standard B-mode ultrasound scan measures can be used to improve prediction of cerebrovascular events and thus possibly refine the selection for carotid endarterectomy.

Contrast-enhanced ultrasound: Current status

CEUS is an enhanced form of ultrasound scan using intravenous administration of a microbubble contrast agent. The contrast agent is an intraluminal tracer and can be used to obtain angiography-like images of the carotid arteries. Ultrasound scan contrast agents were introduced in clinical practice in the early 1990s. The currently approved and used agents include SonoVue (Bracco SpA, Milan, Italy), Optison (GE Healthcare, Princeton, NJ), Definity (Lantheus Medical Imaging, N. Billerica, Mass), and Levovist (Schering AG, Berlin, Germany). The contrast agents consist of microbubbles (approximately 1-8 μm), generally filled with a perfluorinated gas that has a low solubility, and stabilized with a phospholipid or protein shell to improve circulation time. Microbubble contrast agents are intravascular tracers that, because of their size, cannot leave the intravascular compartment. The microbubble shell is eliminated from the body through the reticuloendothelial system when the gas is exhaled. Contraindications for microbubble contrast agents are unstable angina, acute cardiac failure, acute endocarditis, known right-to-left shunts, and known allergy for microbubble contrast agents. Microbubble contrast agents have been administrated in millions of patients and are safe; side effects are extremely rare [22].

For clinical application, microbubble contrast agents have been registered for tissue perfusion imaging and cardiac chamber border enhancement. None of the contrast agents has been registered for imaging of carotid intraplaque neovascularization. However, the 2011 updated guideline on nonhepatic applications of CEUS by the European Federation of Societies for Ultrasound in Medicine and Biology, recommends the use of CEUS for the differentiation between total carotid occlusion and residual flow in tight stenosis, the improvement of lumen delineation in technically difficult carotid arteries, and for the evaluation of carotid plaque neovascularization [23]. Previous studies by Ferrer et al [24] and Droste et al [25] have demonstrated that CEUS has a high diagnostic accuracy for the detection of carotid artery occlusion and assessment of carotid artery stenosis. These studies have demonstrated that CEUS findings were in good agreement with the results from digital subtraction angiography and/or surgery, and also in patients with poor nonenhanced examination conditions.

Most commercially available ultrasound systems provide specific pulse sequences (eg, amplitude modulation or pulse inversion) that retrieve nonlinearities at low acoustic power, which are only exhibited by microbubbles [26]. These techniques enable the suppression of tissue in the image and allow the specific identification of individual microbubbles. Microbubble contrast agents enhance the arterial lumen, improving the delineation of the lumen, and consequently the detection of atherosclerotic plaques and vessel wall irregularities (Fig 1) [18,27]. The capability of ultrasound scans to detect individual microbubbles passing though the capillary system allows direct visualization of intraplaque neovascularization. Because the microbubbles are strict intravascular

tracers, their presence in plaque represents the presence of an intraplaque neovessel (Fig 1 and Fig 2). The identification of individual neovessels cannot be matched by any of the currently available imaging modalities (eg, magnetic resonance imaging is capable of making estimations about the amount of intraplaque vascularization based on contrast diffusion [28]). This has been supported by in vivo studies in animal models of atherosclerosis, showing an increase in plaque echogeneity after contrast infusion and confirming the presence of neovascularization with histology[29].

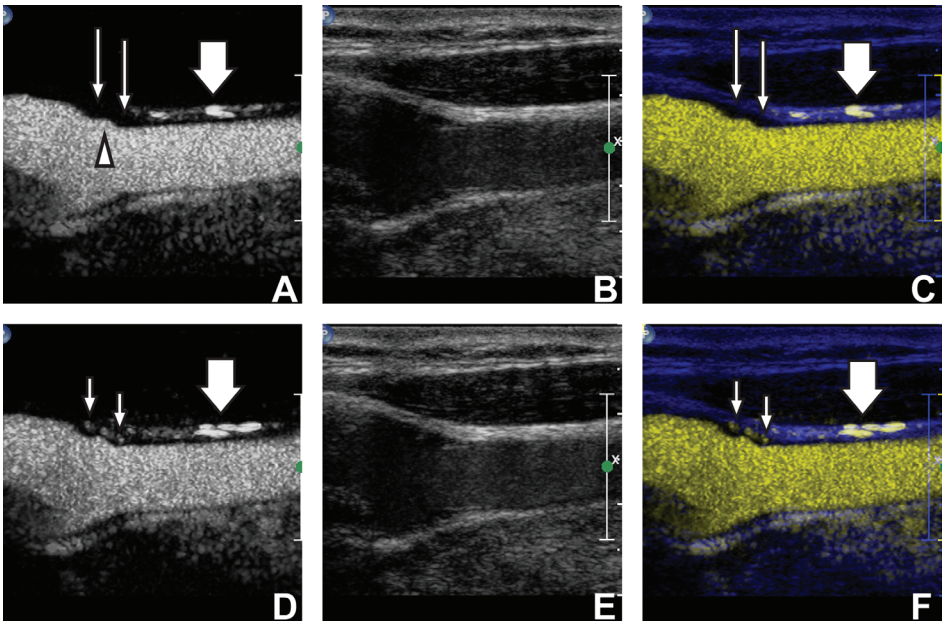

Figure 1
Evaluation of vascular wall irregularities and intraplaque neovascularization by contrast enhanced ultrasound.
Carotid artery ultrasound performed in the side-by-side contrast mode, acquiring the contrast only images (Panel A and D) and B-mode images (Panel B and E) at the same time. Panel C and F present an overlay image of both the contrast (yellow) and the B-mode (Blue) images, allowing for better delineation of the plaque.
Upper panels: In the contrast-enhanced image (Panel A) a small non calcified plaque of 1,5mm thick is present in the near wall, proximally to the carotid bifurcation (long thin arrows), which is barely noticeable on the B-mode image (Panel B). In the overlay image (Panel C) a void can be observed between the lumen contrast and vessel wall. The luminal plaque surface appears irregular and is probably ulcerated (arrow head). The bright regions outside the vessel lumen (thick arrows) are saturation artifacts, caused by high scattering regions and are constantly present during the examination.
Lower panels: approximately 10 seconds after contrast has appeared in the carotid lumen, the contrast microbubbles have entered the atherosclerotic plaque indicating intraplaque neovascularization (Panel D and F, small arrows). (Images acquired with a Philips iU22 ultrasound system equipped with a L9-3 probe, using amplitude modulation at low mechanical index (MI = 0.06). SonoVue contrast agent injected intravenously in repeated boluses of 1.0 ml).

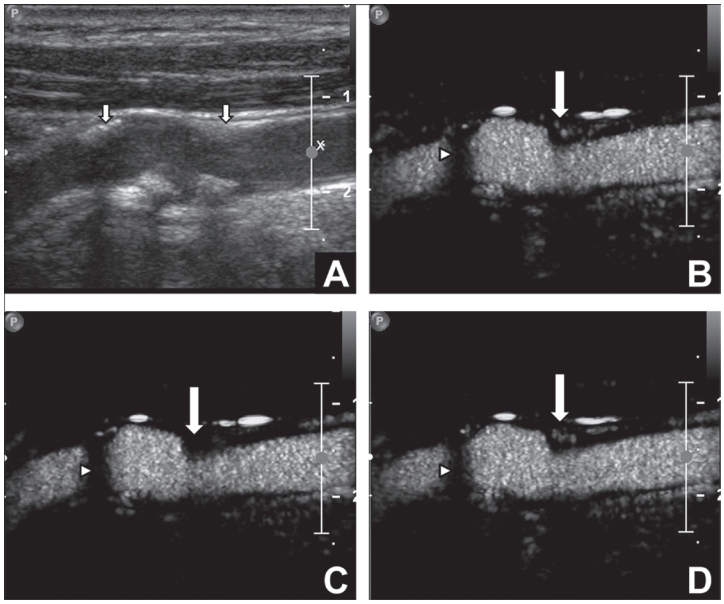

Figure 2

Identification of intraplaque neovascularization by contrast ultrasound.
Carotid artery B-Mode ultrasound (Panel A), and CEUS (Panels B, C, and D) acquired at three consecutive time points. Atherosclerotic plaques are present (small arrows) in the proximal part and distal part of the bulbus. The distal plaque contains a calcification, identifiable by the shadowing (Panel B, C and D: arrow heads). The highly enhanced regions in panel B,C and D on the left and right side of the arrow are saturation artifacts. Panel B was acquired 0.5 seconds before a high mechanical index flash to destroy the contrast is given. Contrast enhancement can be observed in the plaque (large arrow). Panel C was acquired 1.5 seconds after the flash, the intraluminal contrast has been replenished quickly, while the plaque enhancement has disappeared (large arrow). Panel D was acquired 6 seconds after the flash and shows the contrast microbubbles in the plaque (large arrow) indicating the presence of vasa vasorum. (Image acquisition described in Figure Legend 1).

How to perform CEUS

Carotid CEUS can be relatively straightforward, implemented in the routine carotid ultrasound scan acquisition protocol. After a patient has been referred for a clinically indicated carotid ultrasound examination, informed consent should be obtained for the off-label use of the microbubble contrast agent [30]. Subsequently, a peripheral venous access catheter is placed. After acquisition of the standard carotid ultrasound scan images, the contrast presets of the ultrasound system are selected. These contrast presets are available in nearly all currently used vascular ultrasound systems. The presets use an ultrasound pulse sequence with a low mechanical index to avoid destruction of the microbubbles. After injection of contrast agent followed by a saline flush, high quality CEUS images can be obtained. After a few minutes, the contrast is eliminated, the ultrasound signal intensity becomes weaker, and administration of the contrast agent can be repeated.

Assessment of intraplaque neovascularization with CEUS

The literature search yielded 163 articles (Fig 3). After review, exclusion, and cross-referencing, a total of 15 observational studies were included in the review (Table I). These 15 studies included a total of 802 patients (69% men, mean age 66 years). The majority of the patients were asymptomatic for transient ischemic attack (TIA) or stroke (88%). Feinstein [5] was the first to report on the role of CEUS of the carotid arteries in humans to identify the vasa vasorum in vivo. In his 2006 review on carotid ultrasound, a case report was presented demonstrating the identification of the vasa vasorum by CEUS and regression of the vasa vasorum 8 months after initiation of statin therapy. In this report, a visual qualitative evaluation of the vasa vasorum was provided and, although no histologic conformation was performed, this initial report led the way for others.

Six studies, including a total of 95 patients, provided histologic validation of the CEUS images by carotid endarterectomy; a summary of these studies is provided in Table II. It was shown that plaque with a higher amount of contrast enhancement had a significantly increased density of small diameter (20-30 μm) microvessels in the corresponding region on histology [8,13]. No association has been found between plaque echolucency and vessel density [8] Histologic staining for specific vascular (CD31, CD34, hemosiderin, and von Willebrand factor) and angiogenic markers (vascular endothelial growth factor) showed a correlation between intraplaque contrast enhancement and the amount of staining [7,13,19]. Thus, contrast enhancement was shown to correlate with the presence and degree of intraplaque neovascularization. A major limitation of the currently available studies (Table II) on the relation between CEUS findings and histologic examination of the plaques after surgery is the poor description of the diagnostic accuracy of CEUS for the assessment of intraplaque neovascularization. Authors of future studies on this topic are encouraged to report at least a cross tabulation of the CEUS findings and his-

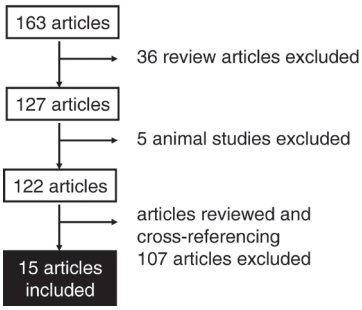

Figure 3

Flow chart of the literature search and study selection. The initial study search yielded 163 eligible studies, of which 36 review articles and 5 animal studies were excluded. The remaining 122 studies were evaluated, and after cross-referencing, a total of 15 articles were included in the review.

tology results, with the sensitivity and specificity of the test, and a measure of statistical uncertainty. Moreover, it should be clear how indeterminate, or missing results, and outliers were handled.

Several B-mode ultrasound scan-derived plaque characteristics have been investigated in association with contrast enhancement. The degree of stenosis and maximum plaque thickness have a weak correlation with the degree of contrast enhancement.16 Characterizing the plaques by their echolucency has shown that the majority of plaques characterized as soft or mixed plaques contained vascularization (94% and 73%, respectively), whereas hard and calcified plaques seem to lack vascularization.6, 9 and 18 Histologic studies are needed to confirm these findings, because calcification in hard plaques may hinder the CEUS evaluation of intraplaque neovascularization due to acoustic shadowing, leading to false-negative results. The echogenicity was shown to have an inverse but weak correlation to plaque vascularization ($P = -.199$, $P < .001$)[16]. Vicenzini et al identified plaque with an ulceration in the luminal border, and observed that every ulceration had an intraplaque vessel present underneath the ulceration. The clinical relevance of this observation deserves further investigation [6].

Magnoni et al [10] combined contrast ultrasound scan with B-flow imaging to evaluate the presence of adventitial vasa vasorum in atherosclerotic (stenosis >50%) and healthy (intima-media thickness <1.0 mm) vessels of patients matched for gender, age, and clinical features. B-flow imaging is a non-Doppler technique that provides information on blood flow during gray scale ultrasound scan by displaying flowing intravascular echo-signals. It differs from power Doppler imaging, a technique using the Doppler effect, which is a shift in the frequency of the reflected ultrasound from that of the initial ultrasound pulse. A peri-adventitial B-flow signal was detected in all patients. However, the thickness of the B-flow signal was significantly higher in patients with carotid atherosclerosis (1.10 ± 0.11 mm vs 0.80 ± 0.06 mm; $P < .0001$) [10].

Four studies evaluated the difference in contrast enhancement between patients symptomatic and asymptomatic for cardiovascular diseases. Patients with cerebrovascular symptoms (TIA or stroke) were shown to have significantly higher contrast enhancement compared to asymptomatic patients with a similar plaque thickness (13.9 ± 6.4 dB vs 8.8 ± 5.2 dB; $P < .001$)[12,13]. Staub et al [14] investigated the association between CEUS findings and cardiovascular disease (peripheral artery disease, coronary artery disease, myocardial infarction, or cerebrovascular disease, including TIA and stroke) and events (myocardial infarction, TIA, or stroke). It was shown that the presence of an intraluminal plaque was significantly associated with cardiovascular disease (odds ratio, 4.7; 95% confidence interval, 1.6-13.8), whereas intraplaque neovascularization was associated with a history of cardiovascular events (odds ratio, 4.0; 95% confidence interval, 1.3-12.6). These findings support the concept that intraplaque neovascularization is a sign

of plaque vulnerability [14]. Prospective follow-up studies have been initiated to further elucidate this issue.

Owen et al [15] investigated the in vivo value of late phase-contrast enhancement (6 minutes after contrast injection) as a marker for damaged endothelium and plaque inflammation. In vitro studies have demonstrated that untargeted microbubbles are both phagocytosed by monocytes and directly adhere to damaged endothelium [31,32]. The retention of microbubbles in a plaque might thus indicate the presence of inflammatory cells adhering to the vessel wall and endothelial damage. Owen et al [15] reported a significant increase in contrast enhancement 6 minutes after contrast injection in symptomatic patients, compared to asymptomatic patients. Hence, late phase-contrast enhancement might have the potential to identify plaque inflammation and endothelial damage in vivo. In a second study, it was shown that late phase enhancement correlates with biological features of inflammation and angiogenesis on histology [17].

Quantification

Quantification methods to analyze ultrasound images are under development to further improve the accuracy of interpretation and to decrease inter-reader variation. The majority of the previously discussed studies used a qualitative scale to visually score the presence and amount of intraplaque neovascularization [6-8,12-14]. These scales are easily implemented and already show significant differences in contrast enhancement between symptomatic and asymptomatic patient groups [12-14]. Quantitative measurement of the contrast enhancement should further improve the reproducibility and observer variability.

Most quantitative techniques currently used are based on the evaluation of signal intensity increase after contrast infusion and show significant differences between symptomatic and asymptomatic patient groups [11,12,15]. However, the accuracy and reproducibility of these methods need further evaluation. The use of B-flow imaging as proposed by Magnoni et al [10] is an interesting technique to quantitatively evaluate the adventitial vasa vasorum in the vessel wall. Histopathological studies have demonstrated that intraplaque neovascularization sprouts from these adventitial vasa vasorum. Clearly, further studies are desired to evaluate the exact role of B-flow imaging for this purpose.

Hoogi et al [19] showed promising results with a more sophisticated quantification technique. They calculated the ratio of the neovascularized plaque area to the total plaque area and reconstructed different routes microbubbles travel through the plaque with an automated algorithm. A highly significant relationship was found between the neovascularization to plaque area ratio on CEUS and histology ($R_2 = .7905$; $P < .01$), making this a promising tool for quantification of neovascularization.

Limitations of CEUS

The current commercially available ultrasound systems are equipped with 2D transducers for carotid imaging. The use of 2D data is subject to operator dependency and out of plane probe motion. Several producers of ultrasound equipment are currently developing vascular 3D matrix probes. The implementation of 3D CEUS will allow acquisition of full volume information of carotid lumen and plaque, including intraplaque neovascularization (Fig 4). The 3D carotid ultrasound scan will probably also improve interobserver and intraobserver variability.

A recently recognized limitation of CEUS is the pseudoenhancement artifact [33]. This artifact is caused by nonlinear propagation of ultrasound waves through the microbubble contrast agent, leading to pseudoenhancement visible in the far wall (the vessel wall furthest from the probe) at locations where no contrast agent is present. Misidentification of pseudoenhancement may lead to false-positive results. New ultrasound pulse

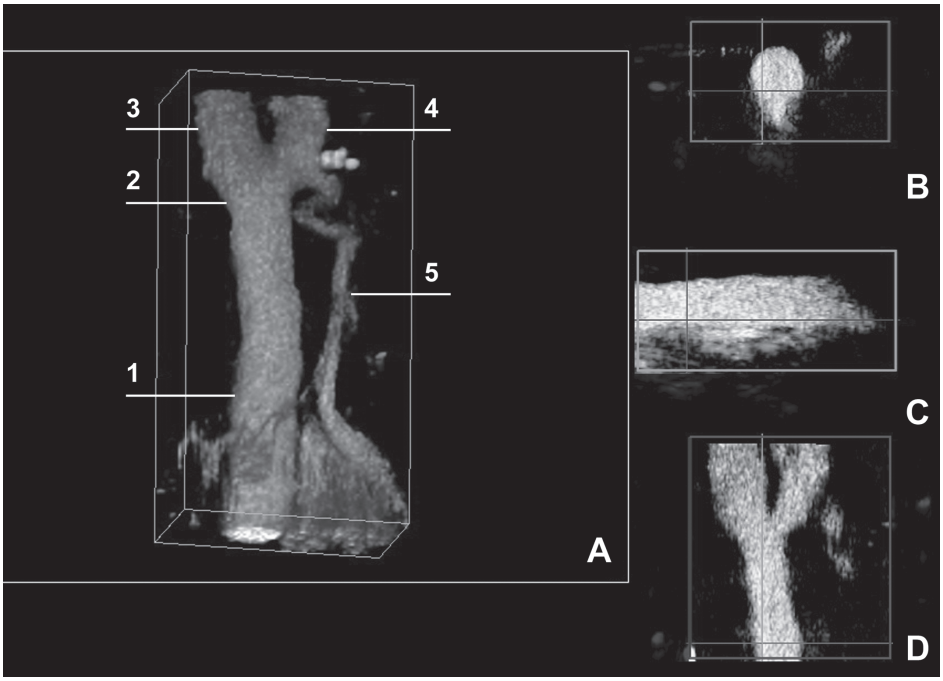

Figure 4

Contrast enhanced 3D ultrasound using volume rendering (A) and multi planar reconstruction demonstrating the transversal (B), sagittal (C), and coronal plane (D), reconstructed form a freehand sweep of the carotid artery (QLAB; Philips Healthcare, Best, the Netherlands). The volume rendering image shows the common carotid artery (1), carotid bifurcation (2), proximal internal (3), and external carotid artery (4). The superior thyroid artery (5) branches from the external carotid artery and descends to the thyroid gland.

sequences are being developed to suppress the pseudoenhancement artifact and are expected to become commercially available in the near future.

Future developments in CEUS

Developments in targeted contrast agents present a prospective for molecular CEUS imaging of intraplaque neovascularization. By using molecular imaging, one would be able to not only identify the presence of intraplaque neovascularization but also evaluate the angiogenic activity in the plaque. Studies using targeted microbubbles have shown the possibility to identify cellular adhesion molecules and receptors associated with angiogenesis in atherosclerotic animal models [34]. The first human study evaluating vascular endothelial growth factor receptor targeted microbubbles has recently been performed in patients with prostate cancer, showing promising results for identification of active angiogenesis (http://clinicaltrials.gov; study no: NCT01253213)[35].

CONCLUSIONS

CEUS can be relatively easily incorporated into a standard carotid ultrasound protocol to improve the evaluation of plaque morphology and assess intraplaque neovascularization. Identification of an unstable vascularized carotid plaque using CEUS could refine the selection of patients considered for carotid endarterectomy.

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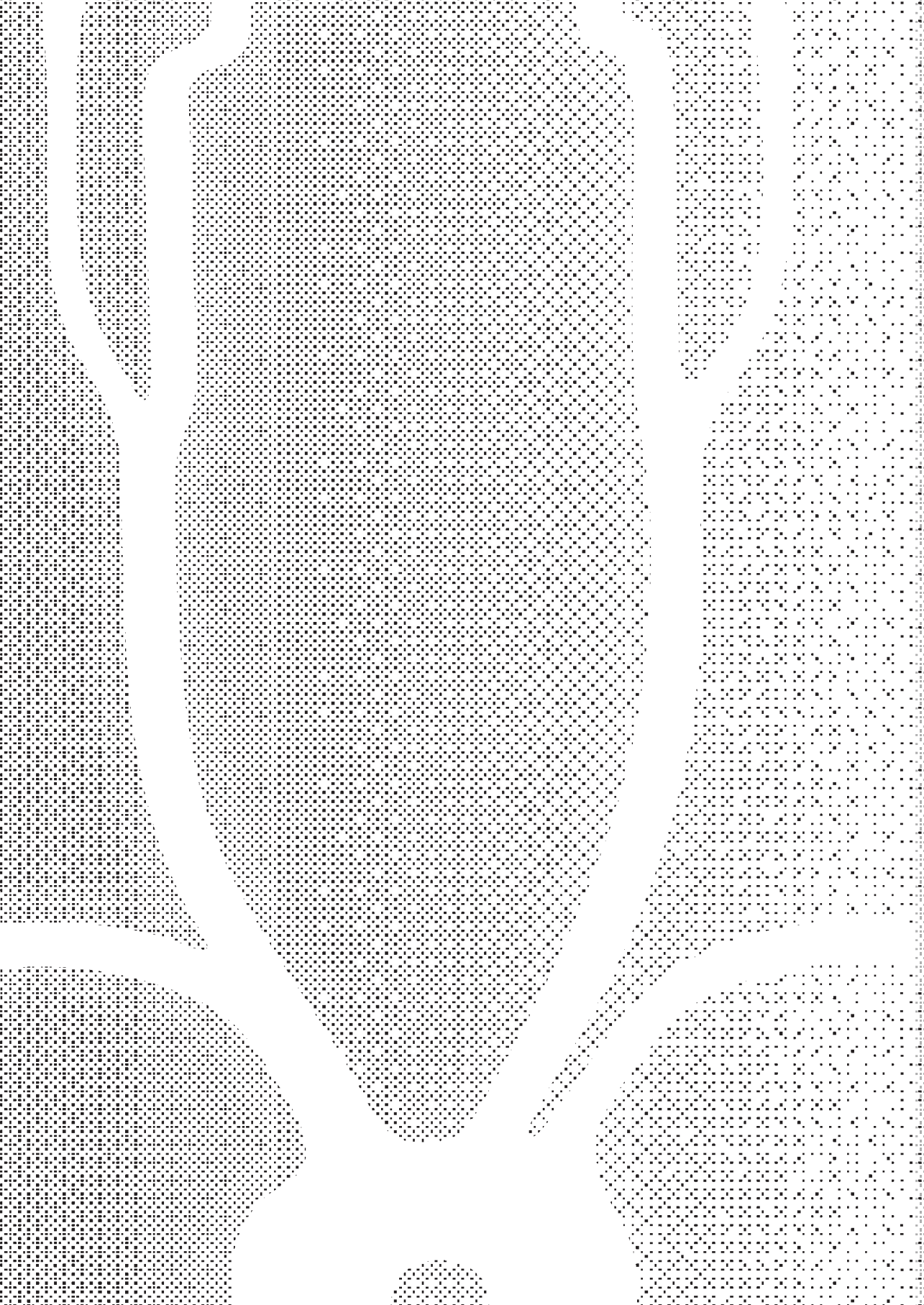
PART II



CHAPTER 4

ASSESSMENT OF SUBCLINICAL ATHEROSCLEROSIS USING CONTRAST-ENHANCED ULTRASOUND

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ABSTRACT

Aims: The sensitivity of standard carotid ultrasound and colour Doppler for the detection of subclinical atherosclerotic plaques is suboptimal. The aim of this study is to assess whether contrast-enhanced ultrasound (CEUS) added to standard carotid ultrasound improves the detection of subclinical atherosclerosis.

Methods and results: Carotid intima–media thickness (CIMT) measurement, standard carotid ultrasound including colour Doppler imaging, and CEUS were performed in 100 asymptomatic patients with one or more risk factors for atherosclerosis. CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). CIMT, standard ultrasound, colour Doppler, and CEUS were reviewed by two independent observers. Standard ultrasound, colour Doppler, and CEUS were scored for the presence of atherosclerotic plaques. Subclinical atherosclerosis was diagnosed if patients had a CIMT above their age-corrected threshold value or if atherosclerotic plaques were present on standard carotid ultrasound clips or CEUS clips. McNemar's test was performed to compare between groups. Twenty-one patients (21%) had a thickened CIMT value and were considered to have subclinical atherosclerosis. Standard carotid ultrasound including colour Doppler demonstrated atherosclerotic plaques in 77 patients (77%). The addition of CEUS to the standard ultrasound protocol demonstrated atherosclerotic plaques in 88 patients (88%). The incorporation of CEUS into the standard carotid ultrasound protocol resulted in a significantly improved detection of patients with subclinical atherosclerosis ($P < 0.01$).

Conclusion: CEUS has an incremental value for the detection of subclinical atherosclerosis in the carotid arteries. Atherosclerotic plaques which were only detected with CEUS and not with standard carotid ultrasound and colour Doppler imaging were predominantly hypoechoic.

INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in western countries [1]. It is by far the most frequent underlying cause of coronary artery disease, carotid artery disease, and peripheral arterial disease. Generally, the development of atherosclerosis starts at young age and progresses asymptomatically through adult life. The progression of atherosclerosis may result in atherosclerotic plaques prone to rupture. The rupture of these atherosclerotic plaques may cause acute thrombosis leading to clinical events [2]. The detection of atherosclerosis before it leads to clinical events may improve risk-reducing strategies.

Carotid ultrasound is a widely available and relatively inexpensive technique to detect subclinical atherosclerosis. Carotid intima–media thickness (CIMT), as measured by B-mode ultrasound, has frequently been used as a surrogate marker for atherosclerosis [3,4]. CIMT measurement is accurate, reproducible, and significantly related with clinical outcome. Still, there are indications that a systematic examination of the extracranial carotid arteries, including screening for the presence of plaques, may increase the sensitivity of ultrasound for identifying subclinical atherosclerosis. A recent meta-analysis demonstrated that the presence of plaques is a better predictor of clinical events when compared with CIMT measurement [5]. Nevertheless, the sensitivity of standard carotid ultrasound of the carotid arteries to detect atherosclerotic plaques is suboptimal [6].

Contrast-enhanced ultrasound (CEUS) provides a better delineation of the carotid arterial lumen than standard carotid ultrasound [7,8]. However, the incremental value of CEUS for the detection of subclinical atherosclerosis in the carotid arteries has not been tested. The purpose of the present study is to assess whether CEUS added to standard carotid ultrasound improves the detection of subclinical atherosclerosis.

METHODS

Patient population and study protocol

The study protocol was approved by the local Ethics Committee and all study participants provided informed consent prior to the ultrasound examination. Between April 2010 and February 2011, 100 consecutive patients (45 females) from the outpatient clinics of vascular medicine and cardiology were included in this prospective study. All patients had no known atherosclerosis but had an increased cardiovascular risk profile. Inclusion criteria were: one or more clinical risk factors for atherosclerosis and age ≥18 years. Risk factors were dyslipidaemia, hypertension, diabetes mellitus, smoking, and a familial history of cardiovascular disease. Exclusion criteria were contraindications for the use of ultrasound contrast agent, such as unstable angina, acute cardiac failure,

acute endocarditis, known right-to-left shunts, and known allergy for microbubble contrast agents. Patient characteristics were registered, and a structured clinical interview and physical examination were performed. All patients underwent CIMT measurement and a standard carotid ultrasound examination in conjunction with CEUS.

Carotid ultrasound acquisition

Standard carotid ultrasound including colour Doppler, and CEUS were performed with a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, WA, USA), equipped with an L9-3 transducer. Image acquisition was performed by a trained sonography technician using a standard scanning protocol according to the American Society of Echocardiography consensus statement [9]. In short, both left and right carotid arteries were examined with the patient in a supine position with the head supported at a 45° angle turned to the contralateral side. The left and the right common carotid artery (CCA), carotid bifurcation, internal carotid artery (ICA), external carotid artery (ECA), and vertebral arteries were imaged by B-mode ultrasound, colour Doppler, and pulse-wave Doppler. All anatomical sites were examined from different angles of view. Gain and imaging depth were adjusted per patient to obtain optimal ultrasound images. Each side was extensively evaluated for the presence of carotid plaques (Figure 1A). The degree of stenosis of the CCA, ICA, and ECA was assessed according to the current guidelines on the basis of spectral Doppler velocities [10].

After standard carotid ultrasound, CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). The contrast mode of the ultrasound system, using amplitude modulation and a mechanical index of 0.06–0.08, was used to optimize the CEUS examination. CEUS clips were recorded with a dual display mode for simultaneous standard B-mode ultrasound and CEUS view (Figure 1B). Before injection of the ultrasound contrast agent, the intravenous access was flushed with a 5.0 mL NaCl 0.9% solution bolus injection. The ultrasound contrast agent was injected in boluses of 0.5 mL. Each contrast agent bolus was followed by a saline flush using 2.0 mL NaCl 0.9% solution. After administration of contrast agent, high-quality contrast images could be obtained for ~1 min. Contrast administration was repeated when required up to a maximum total dose of 10.0 mL. Both carotid arteries were examined using a standard acquisition protocol. Still frames and cineclips were digitally stored for offline analysis.

Carotid ultrasound analysis

Standard ultrasound and CEUS were reviewed offline by two independent observers unaware of the clinical data. For each patient, the same analysis protocol was followed. First, CIMT was measured in the left CCA, followed by screening for the presence of atherosclerotic plaques in the left carotid artery using the standard ultrasound clips. This analysis was repeated for the right carotid artery. Finally, CEUS clips were analysed;

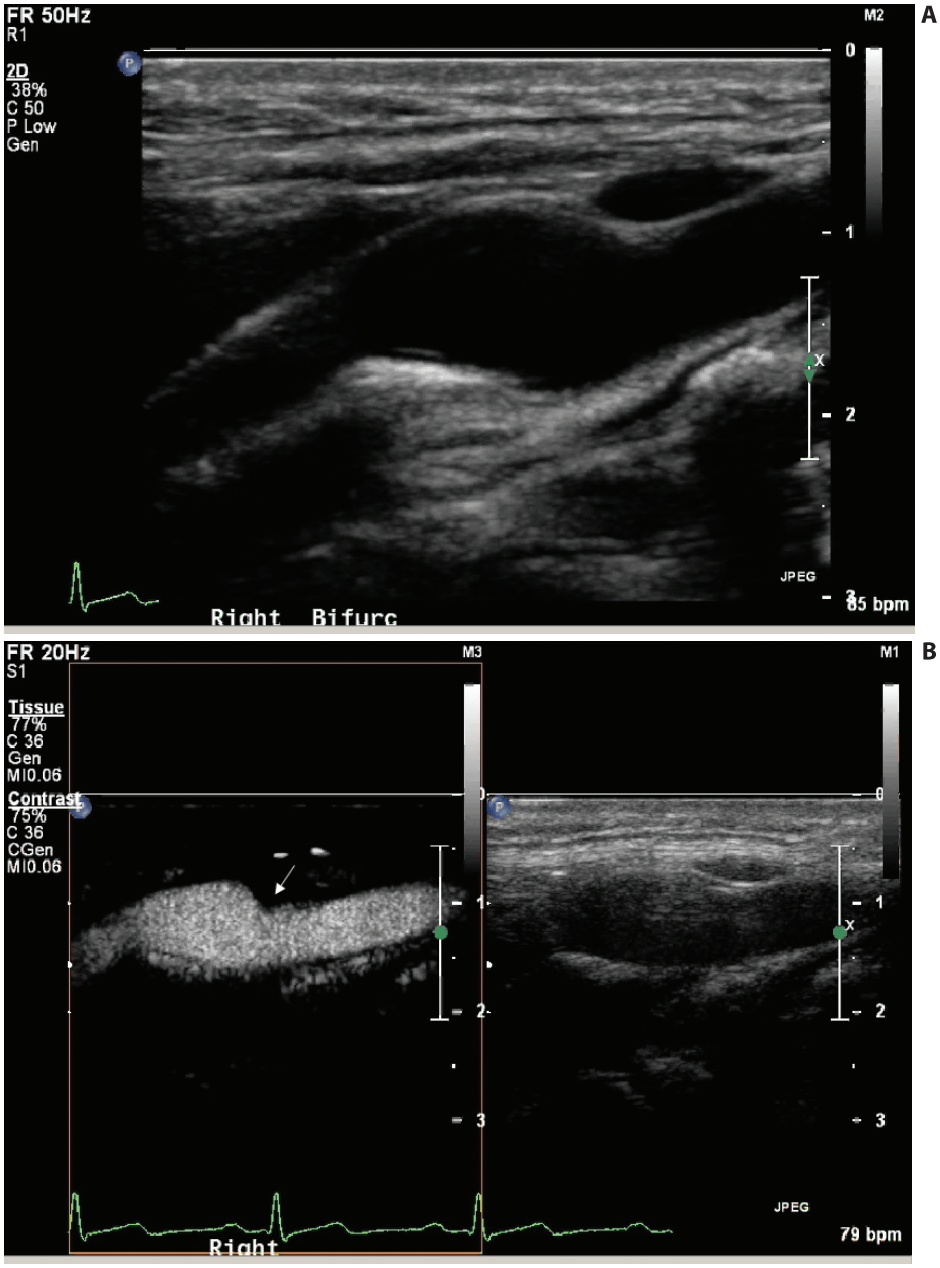

Figure 1

Assessment of subclinical atherosclerosis using standard carotid ultrasound and CEUS. (A) B-Mode still frame of the right carotid artery. On the image the distal common carotid artery, bifurcation and proximal internal carotid artery are visible (from right to left). No atherosclerotic plaques were detected. (B) Corresponding combined B-Mode and contrast-enhanced ultrasound still frame. An atherosclerotic plaque is visible on the contrast-enhanced still frame. It is located in the distal common carotid artery and bifurcation (white arrow).

first, the left carotid artery was screened for the presence of atherosclerotic plaques. This analysis was repeated for the right carotid artery. In accordance with previously published studies, the CIMT was measured in the far wall of the distal 1 cm of the CCA [11]. Semi-automated CIMT measurement was performed using Qlab quantification software (Philips Healthcare, Best, The Netherlands). For each side, the CIMT measurement was performed three times on selected still frames on different R-peaks of the ECG signal. The mean value of three measurements was used in further statistical analysis. The threshold for a high CIMT value was calculated for each patient individually according to the formula reported by Jäger and Staub (threshold value in mm = decade of life/10 + 0.2 mm) [12]. Patients were considered to have subclinical atherosclerosis if their mean CIMT was above this threshold value.

Carotid plaque screening was performed using the standard carotid ultrasound images and colour Doppler clips. Atherosclerotic plaque was defined as a focal structure encroaching into the lumen of at least 0.5 mm or 50% of the surrounding CIMT or demonstrates a thickness of >1.5 mm as measured from the media–adventitia interface to the intima–lumen interface [13]. The presence of plaques was recorded for each side.

Subsequently, carotid plaque screening was performed using the CEUS images. In the CEUS images, the carotid lumen is enhanced by the presence of contrast agent, resulting in enhanced visualization of plaque and luminal morphology. Typically, the lumen appears hyperechoic, plaques and intima–media complex are hypoechoic, and the adventitial layer is hyperechoic. The definition of atherosclerotic plaques was identical to that used for standard ultrasound [13]. The presence of plaques was recorded for each side.

If a new atherosclerotic plaque was found on CEUS, both standard carotid ultrasound and CEUS clips were reviewed in order to investigate why this plaque had been initially missed on standard carotid ultrasound alone. A systematic analysis of the standard carotid ultrasound clips of the region of the missed plaque was performed using the dual-display mode with simultaneous standard carotid ultrasound and CEUS. First, the image quality of the region of interest was scored based on a three-point scale as good-, moderate-, or poor-quality depending on artefacts or ultrasound shadowing. Secondly, echolucency was determined in the region of the missed plaque using the Gray-Weale score [14] as follows: 1, predominantly echolucent plaque; 2, substantially echolucent lesions with small areas of echogenicity; 3, predominantly echogenic lesions with small areas of echolucency; and 4, uniformly echogenic lesions.

Statistical analyses

Statistical analysis was performed using SPSS PASW software for Windows (Version 17.0.2, SPSS Inc., Chicago, IL, USA). Continuous data were expressed as mean ± standard deviation, and categorical variables were expressed as counts and percentages and/or median value. McNemar's test was used to compare between groups. Intra- and inter-

observer reproducibility for the assessment of CIMT and atherosclerotic plaque was calculated using Pearson's correlation and κ-statistics. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Clinical characteristics of the 100 patients (mean age 56 ± 9 years, 45% females) are summarized in Table 1. A total of 27 patients had one clinical risk factor for atherosclerosis, 34 patients had two clinical risk factors, 33 patients had three clinical risk factors, and 6 patients had four clinical risk factors. The standard carotid ultrasound and CEUS examinations were performed without adverse reactions in all patients.

Table 1 Clinical characteristics of the study population (N=100)

Characteristic	Data
Age (y)	56 ± 9
Female gender	45 (45)
Height (cm)	174 ± 10
Weight (kg)	83 ± 17
BMI (kg/m^2)	27 ± 5
Cardiovascular risk factors:	
Diabetes	22 (22)
Dyslipidemia	88 (88)
Familial history of CVD	51 (51)
Hypertension	33 (33)
Smoker (current)	24 (24)
Smoker (former)	23 (23)

BMI = Body mass index. CVD = Cardiovascular disease. Data are presented as numbers of patients (percentages) or as mean ± standard deviation.

Analysis of carotid arteries

A total of 200 carotid arteries were evaluated. The mean CIMT was 0.69 ± 0.16 mm. The standard ultrasound examination, including colour Doppler, revealed atherosclerotic plaques in 129 carotid arteries (65%), and in the remaining 71 carotid arteries (36%), no atherosclerotic plaques were detected. There were no haemodynamically significant carotid stenoses detected. The addition of CEUS to the standard ultrasound protocol demonstrated atherosclerotic plaques in 155 carotid arteries (78%). In 26 carotid arteries (13%), standard ultrasound demonstrated no atherosclerosis, whereas CEUS demon-

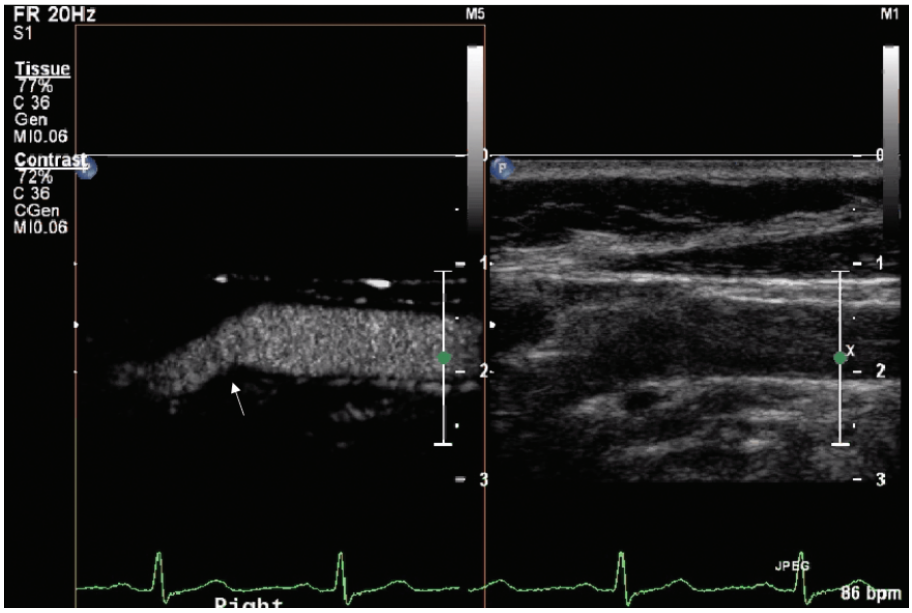

Figure 2
Detection of an atherosclerotic plaque using CEUS. Combined B-Mode and contrast enhanced ultrasound still frame of a right carotid artery. An atherosclerotic plaque is visible on the contrast enhanced still frame. It is located in the bulbus and proximal external carotid artery (white arrow).

strated atherosclerotic plaques (Figures 1 and 2). In another 26 carotid arteries (13%), atherosclerotic plaques were detected on standard carotid ultrasound, and additional atherosclerotic plaques were detected using CEUS. The incorporation of CEUS into the standard carotid ultrasound protocol resulted in a significantly improved detection of subclinical atherosclerotic plaques ($P < 0.001$).

The atherosclerotic plaques which were only detected with CEUS and had been missed using the standard carotid ultrasound examination including colour Doppler were predominantly hypoechoic, with a median Gray-Weale score of 1. A total of 39 plaque regions (70%) had Gray-Weale score 1; 13 plaque regions (23%) had Gray-Weale score 2; 4 plaque regions (7%) had Gray-Weale score 3; and 0 plaque region (0%) had Gray-Weale score 4. The image quality of standard carotid ultrasound in the regions with plaques that were detected using CEUS but missed using standard carotid ultrasound only was good in 32 regions (57%), moderate in 17 regions (30%), and poor in 7 regions (13%).

Reproducibility

Reproducibility of ultrasound-clip analysis was assessed using the complete ultrasound examinations of all 100 patients. The mean difference between inter-observer measure-

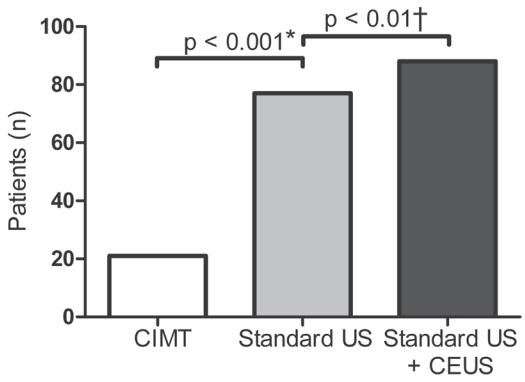

Figure 3
Identification of subclinical atherosclerosis by different imaging strategies. CIMT = Carotid intima-media thickness measurement, Standard US = standard carotid ultrasound, CEUS = Contrast-enhanced ultrasound. * = Standard ultrasound identified significantly more patients with subclinical atherosclerosis as compared to CIMT measurement. † = When standard carotid ultrasound was combined with CEUS, significantly more patients with subclinical atherosclerosis were detected than with standard carotid ultrasound only.

ments of the CIMT was 0.02 ± 0.03 mm. The correlation coefficient for inter-observer measurements of the CIMT was good (r = 0.97). The κ-statistics for the inter-observer agreement of atherosclerotic plaque detection with standard carotid ultrasound was 0.68. For combined standard carotid ultrasound and CEUS, the κ-statistics for inter-observer agreement of atherosclerotic plaque detection was 0.65, which suggests a substantial agreement.

DISCUSSION

The main finding of the current study is that CEUS has incremental value for the detection of subclinical atherosclerosis. According to a previously suggested age-specific threshold formula for CIMT,12 21% of the patients had subclinical atherosclerosis. Standard carotid ultrasound including colour Doppler images demonstrated atherosclerotic plaques in 77% of the patients. The addition of CEUS to the standard ultrasound protocol demonstrated atherosclerotic plaques in 88% of the study population. In 11% of the patients, atherosclerotic plaques were only detected by CEUS and not with standard ultrasound. These plaques were predominantly hypoechoic. The combination of standard carotid ultrasound and CEUS may thus be the preferred strategy for evaluation of subclinical atherosclerosis.

These results are in line with previous experimental studies. Sirlin et al. [8] reported that CEUS provides more accurate delineation of the arterial lumen than standard ultrasound. In an ex vivo human carotid artery model and an in vivo rabbit aorta model, CEUS enabled an improved depiction of wall abnormalities, while this study was performed without contrast-specific imaging settings. Kono et al. [7] demonstrated that phase-inversion harmonic imaging further increases the sensitivity of ultrasound to contrast agent, leading to an improved visualization of the vascular lumen and tissues. To the best of our knowledge, the current study is the first to demonstrate that CEUS has an additional value for the detection of subclinical atherosclerosis.

The atherosclerotic plaques that were only detected using CEUS and not with the standard carotid ultrasound examination were predominantly hypoechoic. A number of studies have demonstrated that hypoechoic plaques contain a large lipid pool, are related with intraplaque haemorrhage, and an increased incidence of cardiovascular events [15-17]. Large lipid pool and intraplaque haemorrhage are histopathological features of the vulnerable atherosclerotic plaque, which is at risk to rupture and may cause cardiovascular events [2]. The hypoechoic atherosclerotic plaques detected with CEUS may be of the vulnerable plaque type. A follow-up study is required to investigate whether the newly found hypoechoic atherosclerotic plaques are associated with an increased incidence of cardiovascular events.

The present study demonstrates that CEUS provides an incremental value for the detection of subclinical atherosclerosis, irrespective of the image quality of the standard carotid ultrasound images. The image quality of standard carotid ultrasound in the regions with plaques that were detected by CEUS but missed using standard ultrasound was good in 57% and impaired (moderate-or-poor image quality) in 43%.

The current findings may have implications for cardiovascular risk assessment. Carotid ultrasound has been extensively used for the non-invasive detection of subclinical atherosclerosis. Large prospective studies have demonstrated that an increased CIMT is associated with clinical events, including myocardial infarction and stroke [18-20]. CIMT measurement is an excellent research tool and numerous clinical trials have studied the effect of medical therapy on CIMT instead of hard cardiovascular endpoints. Still, an increased CIMT is associated with but not necessarily synonymous with atherosclerosis. Moreover, individual risk prediction using CIMT is challenging because well-established threshold values for CIMT do not exist. Carotid plaque screening has therefore been suggested as a better surrogate outcome for coronary artery disease [21]. Systematic examination of the extracranial carotid arteries, including screening for the presence of plaques, may increase the sensitivity of ultrasound for identifying subclinical atherosclerosis and improve cardiovascular risk stratification [5,22,23]. A recent meta-analysis demonstrated that carotid ultrasound screening for the presence of plaques is a better predictor of hard cardiovascular events when compared with CIMT measurement [5].

A limitation of this study is that well-established threshold values for CIMT do not exist. The formula used to calculate the threshold for abnormal CIMT only adjusts for age [12]. Previous studies have demonstrated that multiple factors including gender and ethnicity may influence CIMT. Therefore, in this study, CIMT measurement may have over- or underestimated the presence of subclinical atherosclerosis. Furthermore, the predictive value of the newly detected atherosclerotic plaques for cardiovascular risk assessment has not been investigated yet. Follow-up studies are required to determine the additional predictive value of the absence or presence of subclinical atherosclerosis detected with CEUS.

In conclusion, CEUS has an additional value for the detection of subclinical atherosclerosis in the carotid arteries. The atherosclerotic plaques that were detected by CEUS but missed using standard carotid ultrasound including colour Doppler were predominantly hypoechoic. These results suggest that the combination of standard carotid ultrasound and CEUS may thus be the preferred strategy for the evaluation of subclinical atherosclerosis.

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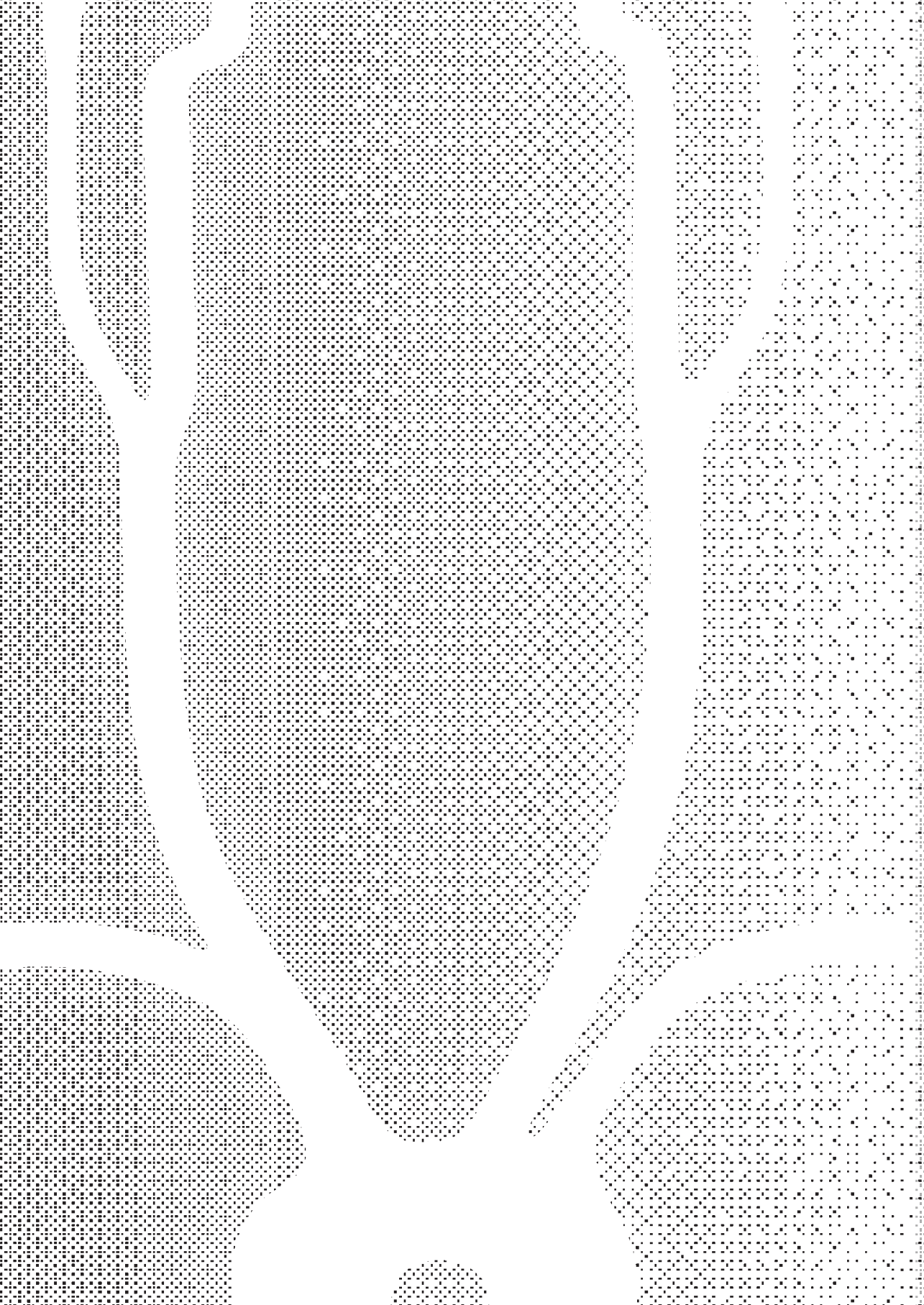

CHAPTER 5

EFFECT OF CAROTID PLAQUE SCREENING USING CONTRAST-ENHANCED ULTRASOUND ON CARDIOVASCULAR RISK STRATIFICATION

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ABSTRACT

Cardiovascular risk stratification of asymptomatic patients is based on the assessment of risk factors. Noninvasive imaging of subclinical atherosclerosis may improve cardiovascular risk stratification, especially in patients with co-morbidities. The aim of this study was to investigate the effect of contrast-enhanced ultrasound (CEUS) of the carotid arteries on cardiovascular risk assessment. The study population consisted of 100 consecutive asymptomatic patients with ≥1 clinical risk factor for atherosclerosis. Cardiovascular risk was estimated by calculating the Prospective Cardiovascular Münster Heart Study (PROCAM) risk score. This score was divided into 3 subgroups: low (≤5%), intermediate (6% to 19%), and high (≥20%). Subclinical carotid atherosclerosis was assessed using standard ultrasound for intima-media thickness and plaque screening and CEUS for additional plaque screening. CEUS was performed using SonoVue contrast agent. Patients with subclinical atherosclerosis were considered to be at high cardiovascular risk. McNemarəs test was used to compare PROCAM score to ultrasound findings. The mean PROCAM risk score was 9 ± 10; the PROCAM risk score was low in 72 patients (72%), intermediate in 17 patients (17%), and high in 11 patients (11%). A total of 21 patients (21%) had abnormal carotid intima-media thickness, 77% had plaques on conventional carotid ultrasound, and 88% had plaques on standard carotid ultrasound combined with CEUS. Detection of atherosclerosis led to the reclassification of 79 patients (79%) to high cardiovascular risk ($p < 0.001$). In conclusion, CEUS changes the risk category as estimated by a traditional risk stratification model in most asymptomatic patients. CEUS may thus be an additional method for cardiovascular risk prediction in patient groups with co-morbidities.

INTRODUCTION

Noninvasive imaging may substantially improve cardiovascular risk assessment [1]. The current guidelines recommend the use of ultrasound for the measurement of carotid intima-media thickness (CIMT) to evaluate cardiovascular risk in asymptomatic patients [2,3]. Recent data indicate that cardiovascular risk assessment by carotid ultrasound may be further refined by carotid plaque screening [4-7]. Still, the sensitivity and specificity of carotid ultrasound for the assessment of atherosclerotic plaque is suboptimal [8]. Contrast-enhanced ultrasound (CEUS) is an enhanced form of ultrasound imaging that has a favorable safety profile and may reduce the need for riskier and more expensive diagnostic testing. CEUS has been demonstrated to improve carotid plaque assessment, but information on the role of CEUS in cardiovascular risk evaluation is lacking [9-11]. Therefore, the aim of this study was to investigate the effect of carotid plaque screening using CEUS on the cardiovascular risk stratification of asymptomatic patients. The results were compared to traditional cardiovascular risk assessment, CIMT measurement, and carotid plaque screening using conventional B-mode ultrasound.

METHODS

The study protocol was approved by the local ethics committee, and all study participants provided informed consent before the ultrasound examinations. From April 2010 to February 2011, 100 consecutive patients (45 women) from the outpatient clinics of vascular medicine and cardiology at a university medical center were included in this prospective study. Patients were asked to participate in the study if they were asymptomatic, had no known cardiovascular disease, and had increased cardiovascular risk profiles. Inclusion criteria were ≥1 clinical risk factor for atherosclerosis and age ≥18 years. Risk factors were dyslipidemia (such as familial hypercholesterolemia), hypertension, type 2 diabetes mellitus, smoking, and a family history of cardiovascular disease. Dyslipidemia was defined as total serum cholesterol ≥200 mg/dl. Systemic hypertension was defined as blood pressure ≥140/90 mm Hg or treatment with antihypertensive medication [12]. Exclusion criteria were contraindications for the use of ultrasound contrast agent, such as hemodynamic unstable condition, known right-to-left shunts, and known allergy to microbubble contrast agents. A structured clinical interview and physical examination were performed. All patients underwent CIMT measurement and a standard carotid ultrasound examination in conjunction with CEUS.

Patients were risk stratified using the Prospective Cardiovascular Münster Heart Study (PROCAM) cardiovascular risk score [13]. This risk score is based on age, gender, history of diabetes, family history, smoking habit, systolic blood pressure, and lipid profile. The

PROCAM score gives an estimation of the global risk for a heart attack or dying from an acute coronary event within the next 10 years. For the PROCAM cardiovascular risk score, patients were stratified as low risk (PROCAM score ≤5%), intermediate risk (PROCAM score 6% to 19%), or high risk (PROCAM score ≥20%).

Standard carotid ultrasound, including color Doppler, and CEUS were performed using a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, Washington), equipped with an L9-3 transducer. Image acquisition was performed by a trained sonography technician using a standard scanning protocol according to the American Society of Echocardiography consensus statement [14]. In short, the left and right carotid arteries were examined with the patient in a supine position with the head supported at a 45° angle turned to the contralateral side. The left and the right common carotid artery, carotid bifurcation, internal carotid artery, external carotid artery, and vertebral arteries were imaged using B-mode ultrasound, color Doppler, and pulse-wave Doppler. All anatomic sites were examined from different angles of view, and each site was scanned in cross-sectional view and longitudinal view. Presets of the ultrasound system were as follows: gain 30%, compression 60, and imaging depth 3.0 cm. These settings were adjusted per patient to obtain optimal ultrasound images. Each side was extensively evaluated for the presence of carotid plaques (Figure 1 [15]).

After standard carotid ultrasound, CEUS was performed using intravenous administration of SonoVue contrast agent (Bracco S.p.A., Milan, Italy). The contrast mode of the ultrasound system, using amplitude modulation and a mechanical index of 0.06

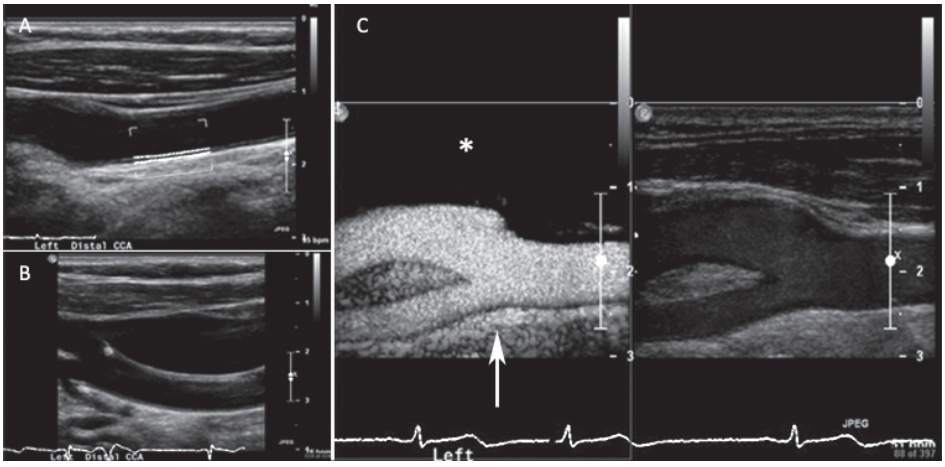

Figure 1

Panel A: Semi-automated carotid intima-media thickness measurement. Panel B: Atherosclerotic plaque detection using standard carotid ultrasound. Panel C: Atherosclerotic plaque detection using contrast-enhanced ultrasound of the carotid artery. * = soft tissue is suppressed using the contrast ultrasound mode based on amplitude modulation. Arrow = a pseudo-enhancement artifact seems to enhance the soft tissue behind the far wall. Note: the panels contain information of different patients.

to 0.08, was used to optimize the CEUS examination. CEUS clips were recorded with a dual display mode for simultaneous standard B-mode ultrasound and CEUS view. Before injection of the ultrasound contrast agent, the intravenous access was flushed with a 5.0-ml sodium chloride 0.9% solution bolus injection. The ultrasound contrast agent was injected in boluses of 0.5 ml. The 2 carotid arteries were examined using a standard acquisition protocol. Still frames and cine clips were digitally stored for off-line analysis.

Standard ultrasound and CEUS were reviewed off-line by 2 independent observers unaware of the clinical data. For each patient, the same analysis protocol was followed. First, CIMT was measured in the left common carotid artery, followed by screening for the presence of atherosclerotic plaques in the left carotid artery using the standard ultrasound clips. This analysis was repeated for the right carotid artery. Finally, CEUS clips were analyzed; first the left carotid artery was screened for the presence of atherosclerotic plaques. This analysis was repeated for the right carotid artery. In accordance with previously published studies, CIMT was measured in the far wall of the distal 1 cm of the common carotid artery [16]. Semiautomated CIMT measurement was performed using QLAB quantification software (Philips Medical Systems, Best, The Netherlands). For each side, the CIMT measurement was performed 3 times on selected still frames on different R-wave peaks of the electrocardiographic signal. The mean value of 3 measurements from the left and right carotid arteries was used in further statistical analysis. The threshold for a high CIMT value was calculated for each patient individually according to the formula reported by Jäger and Staub [17] (threshold value [mm] = decade of life/10 + 0.2 mm). Patients were considered to have subclinical atherosclerosis if their mean CIMT was higher than this threshold value. Carotid plaque screening was performed using the standard carotid ultrasound images and color Doppler clips. Atherosclerotic plaque was defined as a focal structure encroaching into the lumen of ≥0.5 mm or 50% of the surrounding CIMT or demonstrating a thickness >1.5 mm as measured from the media-adventitia interface to the intima-luminal interface [18]. The presence of plaques was recorded for each side. Subsequently, carotid plaque screening was performed using the CEUS images. In the CEUS images, the carotid lumen is enhanced by the presence of contrast agent, resulting in enhanced visualization of plaque and luminal morphology (Figure 2). The definition of atherosclerotic plaques was identical to that used for standard ultrasound [18]. The presence of plaques was recorded for each side. According to current guidelines, patients with atherosclerotic plaques present in the carotid arteries were considered to be at high risk for future cardiovascular events [2,3].

Statistical analysis was performed using PASW for Windows version 17.0.2 (SPSS, Inc., Chicago, Illinois). Continuous data are expressed as mean ± SD. Categorical variables are expressed as counts and percentages and/or median values. Cardiovascular risk stratification with PROCAM score and carotid ultrasound were compared using McNemar's test.

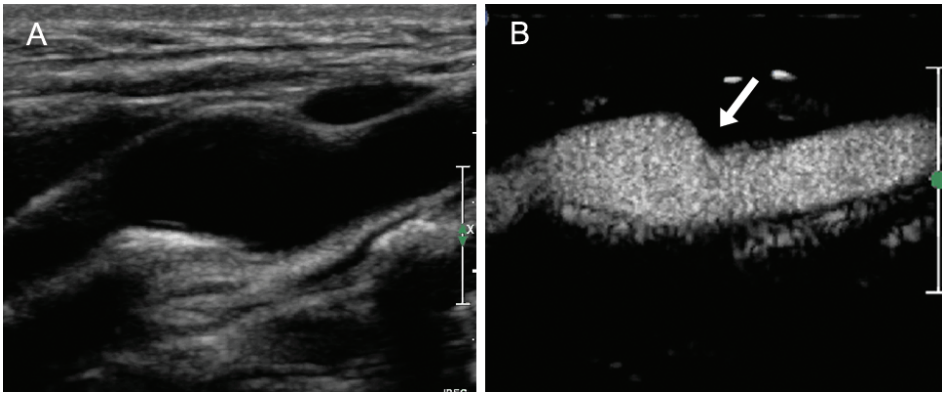

Figure 2
Example of an atherosclerotic plaque detected using CEUS. Using standard carotid ultrasound tools no subclinical atherosclerosis was detected in this patient. Panel A: B-mode still frame of the right carotid bulbus. Panel B: Corresponding CEUS still frame showing an atherosclerotic plaque (white arrow).

RESULTS

Characteristics of the study population are listed in Table 1. Standard carotid ultrasound examinations and CEUS examinations were performed without any complications in all patients. According to the PROCAM score, 72 patients were stratified as being at low risk, 17 at intermediate risk, and 11 patients at high risk for cardiovascular events.

The mean CIMT was 0.69 ± 0.16 mm. A total of 21 patients (21%) had CIMT values higher than their age-corrected thresholds and were considered to have subclinical atherosclerosis. Extensive screening for the presence of atherosclerotic plaques using the standard carotid ultrasound clips demonstrated atherosclerotic plaques in 77 patients (77%). Carotid CEUS demonstrated atherosclerotic plaques in an additional 11 patients (11%). Using the combination of standard carotid ultrasound tools (CIMT measurement and standard screening for the presence of plaques), 80 patients (80%) were considered to have subclinical atherosclerosis. The addition of CEUS led to the detection of atherosclerotic plaques in 9 patients (9%) who were not considered to have subclinical atherosclerosis using the standard ultrasound tools ($p < 0.01$).

The presence of subclinical atherosclerosis among the PROCAM risk categories was analyzed using CIMT measurement and detection of atherosclerotic plaques with standard carotid ultrasound and CEUS. Figure 3 demonstrates the prevalence of subclinical atherosclerosis according to conventional B-mode ultrasound and CEUS in patients with low-, intermediate-, and high-risk PROCAM scores. Carotid ultrasound substantially changed the classification of patients in different risk categories, as demonstrated in Figure 4. Standard carotid ultrasound and CEUS changed the risk category as estimated by the PROCAM risk stratification model in most asymptomatic patients ($p < 0.001$).

Table 1 Patient characteristics

Characteristic		Data (n=100)
Age (years)		56 ± 9
Women		45 (45%)
Height (cm)		174 ± 10
Weight (kg)		83 ± 17
Body masss index (kg/m^2)		27 ± 5
PROCAM risk sore (interquartile range)		5 (2-11)
Cardiovascular risk factors:		
	Diabetes mellitus	22 (22%)
	Dyslipidemia	88 (88%)
	Familial history of cardiovascular diseases	51 (51%)
	Hypertension	33 (33%)
	Smoker (current)	24 (24%)
	Smoker (former)	23 (23%)
Medication:		
	Statin	91 (91%)
	Beta-blocker	18 (18%)
	Angiotensin converting enzyme inhibitor	15 (15%)
	Angiotensin receptor blocker	14 (14%)
	Calcium antagonist	9 (9%)
	Diuretic	14 (14%)
	Oral anti-diabetic	13 (13%)
	Insulin	15 (15%)

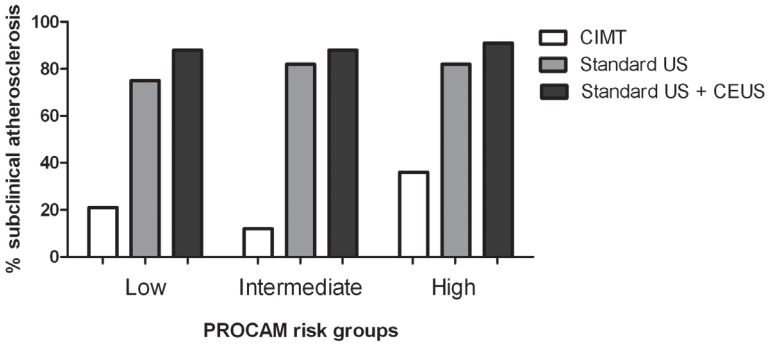

Figure 3
The prevalence of subclinical atherosclerosis among different PROCAM risk categories. Subclinical atherosclerosis was detected using intima-media thickness measurement, screening for the presence of atherosclerotic plaques using standard carotid ultrasound and screening for the presence of atherosclerotic plaques using contrast-enhanced ultrasound.

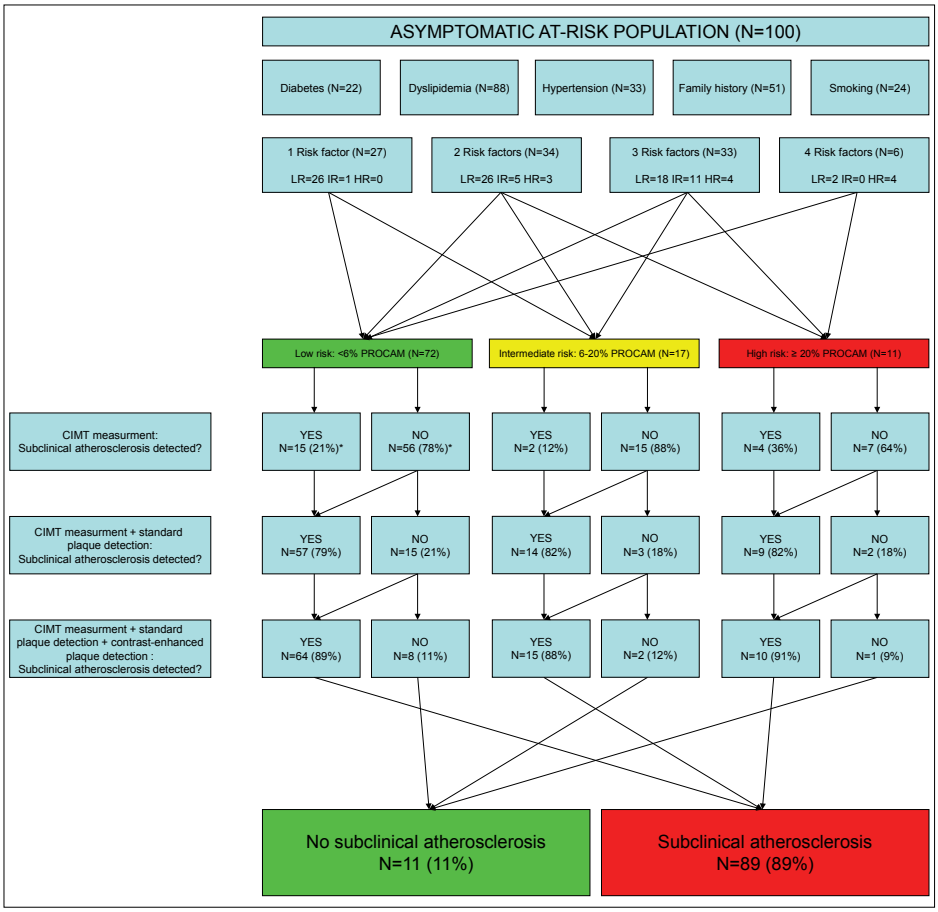

Figure 4
Risk flow chart. Patients were categorized according to their PROCAM risk score. Subclinical atherosclerosis detection was performed for all patients. The presence of subclinical atherosclerosis led to reclassification to high cardiovascular risk.

DISCUSSION

Asymptomatic patients may benefit from cardiovascular risk stratification to identify patients at increased risk for cardiovascular events and to initiate primary preventive measures and therapy at an early stage [19]. Traditional cardiovascular risk stratification models are well validated and widely used. However, these methods have some limitations. Therefore, various noninvasive imaging techniques have been used to assess the presence of subclinical atherosclerosis and to assess the 10-year risk for cardiovascular events. Carotid ultrasound is an attractive tool for cardiovascular risk stratification, because this technique is highly reproducible, is inexpensive, and lacks ionizing radia-

tion. Recently, CEUS has been demonstrated to further improve the accuracy of plaque assessment using carotid ultrasound [11].

The present study demonstrates that carotid ultrasound changed the risk category as estimated by traditional risk stratification in most patients. According to the guidelines, patients with abnormal imaging findings are considered to be at very high risk for cardiovascular events. In 18 patients, the PROCAM risk score was in line with the results of contrast-enhanced carotid ultrasound. PROCAM risk score underestimated cardiovascular risk in 64 patients and overestimated risk in 1 patient. These findings may have important implications for cardiovascular risk assessment. Carotid ultrasound may be a useful modality to refine risk assessment beyond traditional risk prediction models.

The discrepancies between traditional risk stratification and the carotid ultrasound findings in this study may have several causes. The PROCAM risk model was developed for a population of men and women aged 20 to 78 years. The age of the present study population was on average 55.3 years (range 39 to 76) in men and 57.5 years (range 42 to 78) in women. Previous studies have demonstrated that cardiovascular risk models may over- or underestimate risk in specific patients groups, including premenopausal women, patients with diabetes mellitus, and those with familial hypercholesterolemia [2,3]. Therefore the use of PROCAM risk stratification is not recommended in these patients. The present study population included 9 women in the reproductive age category (age <50 years), 22 patients with diabetes mellitus, and 63 patients with familial hypercholesterolemia. Additionally, 96% of the study population used medical therapy, including lipid-lowering and antihypertensive medications. Because the PROCAM score is based partially on the lipid profile and blood pressure, the use of medication in this study may have influenced risk estimation.

Multiple previous studies have evaluated the role of carotid ultrasound added to traditional risk prediction models for cardiovascular risk stratification. A meta-analysis demonstrated that CIMT is an independent predictor of cardiovascular events. A 0.1-mm increase in CIMT resulted in a 15% increase in the risk for nonfatal myocardial infarction or cardiac death [20]. Recent data indicate that plaque screening by carotid ultrasound may further improve cardiovascular risk prediction [7]. This study is the first to demonstrate that CEUS further improves carotid plaque assessment and substantially changes cardiovascular risk assessment. According to the results of this study, carotid plaque screening using CEUS may be particularly relevant in patients without plaques on standard carotid ultrasound and with normal CIMT. However, recent studies have shown that plaque screening compared to CIMT measurement is a better predictor of future cardiovascular events [7]. Therefore, plaque screening using CEUS may be considered in patients with normal and those with abnormal CIMT. In the present study, we used 2-dimensional carotid ultrasound; plaque assessment may be further improved by the implementation of 3-dimensional ultrasound techniques [21].

This study had some limitations. The number of patients included in this study was relatively small. The value of CIMT has been demonstrated in multiple studies, but well-validated cut-off values are lacking. In the present study, a CIMT cut-off value published by Jäger and Staub [17] was used. Using a different CIMT cutoff could have altered the CIMT-based cardiovascular risk stratification. In the present study, no follow-up data were available. Long-term follow-up studies are needed to confirm the independent predictive value of carotid CEUS compared to traditional cardiovascular risk stratification.

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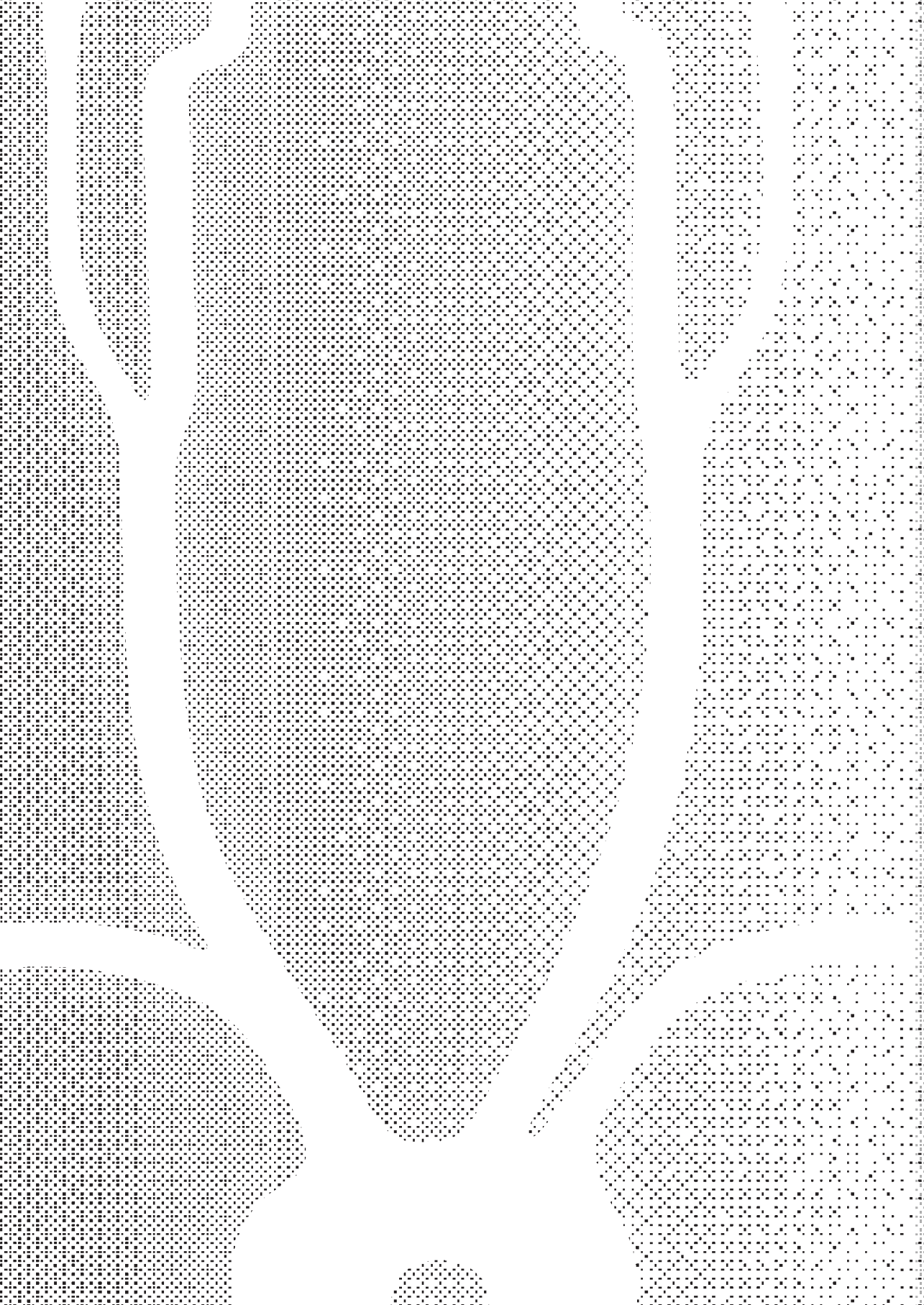
PART III



CHAPTER 6

QUANTITATIVE CONTRAST-ENHANCED ULTRASOUND OF INTRAPLAQUE NEOVASCULARIZATION IN PATIENTS WITH CAROTID ATHEROSCLEROSIS

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ABSTRACT

Aims: Intraplaque neovascularization (IPN) is an increasingly studied marker of the vulnerable atherosclerotic plaque, and contrast-enhanced ultrasound (CEUS) is an attractive in vivo imaging technique for assessment of IPN. The purpose of this study was to test novel quantification methods for the detection of carotid IPN using CEUS.

Methods and results: Twenty-five patients with severe carotid stenosis, underwent bilateral carotid CEUS using a Philips iU-22 ultrasound system with a L9-3 transducer. Visual scoring of IPN was performed using a 3 point score. Semi-automated quantification of IPN was performed using custom developed software. Regions-of-interest were drawn over the atherosclerotic plaques. After motion-compensation, several IPN features were calculated. Statistical analysis was performed using Spearman's rho and Kruskall Wallis testing. Forty-five carotid arteries were available for validation of the IPN features. Semi-automated motion-compensated quantification of IPN was feasible in all 45 carotid plaques. Semi-automated IPN area, IPN area ratio and neovessel count, had a good correlation with visual IPN score (respectively ρ=0.719, ρ=0.538, ρ=0.474 all $p<0.01$). The semi-automated IPN area score provided the best distinction between the visual IPN scoring groups ($p<0.01$). Exclusion of plaques with severe calcification led to further improvement of automated quantification of IPN (ρ=0.757, $p<0.001$).

Conclusions: Semi-automated motion-compensated quantification of carotid IPN on CEUS is feasible and provides multiple features on carotid IPN. Semi-automated IPN area, IPN area ratio and neovessel count correlate well with visual scoring of IPN. The semi-automated IPN area score provides the best distinction between the visual IPN scoring groups.

INTRODUCTION

Carotid atherosclerosis is an important cause of transient ischemic attack (TIA) and ischemic stroke, which may occur as a result of rupture of a vulnerable atherosclerotic plaque [1]. Vulnerable plaques are typically characterized by a large lipid pool, with a thin fibrous cap [2,3]. Recent data indicate that intraplaque neovascularization (IPN) is another marker of plaque instability [4,5]. Histological studies have demonstrated that IPN consists of immature neovessels with a poor structural integrity, which results in an increased risk for intraplaque hemorrhage and rupture. Clinical studies have confirmed that IPN is associated with future cerebrovascular and cardiovascular events [6-8].

Contrast-enhanced ultrasound (CEUS) improves the visualization of carotid atherosclerosis [9]. Moreover, CEUS can be used to visualize IPN in atherosclerotic plaques [4,10-15]. So far, validation studies showed a good correlation between the visual scoring of IPN and the number of intraplaque neovessels in histological samples [11,16]. However, visual analysis of carotid IPN on CEUS is subjective and may result in intrareader and interreader variability. Quantification software could improve assessment of carotid IPN by providing objective and reproducible information. The purpose of this study was to test novel custom developed quantification methods for the evaluation of IPN in carotid atherosclerotic plaques.

METHODS

Patient population and study protocol

The study protocol was approved by the ethical committee. All study participants provided informed consent. Between January 2011 and March 2012, 25 patients with symptomatic carotid atherosclerosis or high-grade carotid stenosis in ≥1 internal carotid artery were included in this prospective study.. The quality of prior imaging studies or information on plaque composition was not taken into consideration when including patients. Exclusion criteria were contraindications for the use of SonoVue ultrasound contrast agent (Bracco S.p.A., Milan, Italy). Clinical characteristics of the study population were recorded. All patients underwent a bilateral standard carotid ultrasound and CEUS examination.

Carotid ultrasound acquisition

The standard carotid ultrasound and CEUS examination were performed using a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, Washington), equipped with a L9-3 transducer. A standardized image acquisition protocol based on the American Society of Echocardiography consensus statement was used [17]. In short, the left and right

common carotid artery (CCA), carotid bulb, internal carotid artery (ICA) and external carotid artery (ECA) were evaluated using B-mode ultrasound, color Doppler and pulsed wave Doppler imaging.

For the CEUS examination, the contrast mode was selected, using amplitude modulation techniques and a mechanical index of 0.06-0.08 to optimize the CEUS images. Side-by-side display mode with a simultaneous B-mode and CEUS image was used for optimal coordination of the ultrasound examination. Other presets were as follows: gain 30%, compression 60, imaging depth 3.0 cm. These presets were adjusted per patient to obtain optimal quality of the ultrasound clips. CEUS was performed using intravenous administration of SonoVue™ ultrasound contrast agent (Bracco S.p.A., Milan, Italy). The ultrasound contrast-agent was injected in boluses of 0.5 ml, up to a total amount of 10.0 ml. Cineclips were recorded at the moment the concentration of microbubbles visually started to rise. To standardize the time-point of ultrasound analysis, a high mechanical index flash was applied to destroy microbubbles in the field of view right after starting the recording. This results in a clip with optimal information on replenishment. The carotid arteries were evaluated according to a predefined ultrasound protocol. The left and right CCA, carotid bulb, ICA and ECA were evaluated, with special emphasis for the present atherosclerotic plaques.

Ultrasound analysis

All standard carotid ultrasound clips and CEUS clips were reviewed offline by two observers using Xcelera PACS (V3.1, Philips Medical Systems Nederland B.V., Best, the Netherlands). Both observers were blinded from patient specific data. The analysis of the clips was performed in a predefined sequence. Standard carotid ultrasound clips were evaluated before the CEUS clips. Using the standard carotid ultrasound clips the CCA, carotid bulb, ICA and ECA were extensively evaluated for the presence of atherosclerotic plaques. The echogenicity of atherosclerotic plaques was graded according to the Gray-Weale classification as 1, predominantly echolucent; 2, substantially echolucent with spots of echogenicity; 3, predominantly echogenic with spots of echolucency; and 4, uniformly echogenic [18]. The maximum plaque thickness was measured perpendicular to the long axis of the carotid artery. The degree of stenosis was scored using peak systolic velocity (PSV) as follows: 1. <50% (PSV < 125 cm/s), 2. 50-69% (PSV = 125-230 cm/s), and 3. ≥70% (PSV > 230 cm/s); no detectable patent lumen, and no flow at color Doppler or pulsed wave Doppler, was considered to be total occlusion [19]. Using the CEUS clips, the atherosclerotic plaques were scored for the presence of IPN. For the left and right carotid artery independently, the CEUS clip with the highest intraplaque contrast-enhancement at visual evaluation was selected for further analysis to avoid underestimation of contrast-enhancement. Contrast enhancement was scored in the frames starting from the high mechanical index flash. Atherosclerotic plaques that could

have been affected by pseudo enhancement, an artifact that hinders the evaluation of IPN in plaques on the far wall or below contrast-pools, were excluded from analysis [20,21]. Dynamic contrast-enhancement in the atherosclerotic plaque was considered to represent IPN. The presence of blood flow activity was identified on the basis of the movement of the echogenic reflectors (microbubble contrast agent) observed in the microvessels in the carotid lesions. Fixed echogenic signals were considered to be tissue acoustic reflectors (i.e. saturation artifacts). These artifacts were neglected to avoid overestimation of contrast-enhancement. For each carotid artery the presence of IPN was graded as follows: Grade (0) no IPN, was used to indicate no appearance of microbubble contrast agent in the carotid lesion; Grade (1) limited or moderate IPN, was used to indicate limited to moderate visible appearance of moving microbubbles in the carotid lesion; or Grade (2) severe IPN, was used to indicate extensive vascularization of the atherosclerotic plaque with clear visible appearance of microbubbles [22].

Quantitative ultrasound analysis

Novel custom developed software was used for semi-automated quantification of IPN. A simple and user-friendly user interface was realized in the form of a wizard structure using MevisLab (MeVis Medical Solutions AG and Fraunhofer MEVIS, Bremen, Germany) (Fig. 1). This quantification software allows analysis of DICOM files of the ultrasound clips with side-by-side contrast mode and B-mode images. The DICOM files are based on logarithmic compressed data with the same compress setting. All analyses were performed according to a structured protocol. First, the time frame for the analysis was selected, starting after the high mechanical index flash to avoid overestimation of intraplaque contrast enhancement. In case of out-of-plane motion, the time frame that was used for analysis was shortened. Subsequently, 3 regions of interest (ROI) were drawn in the CEUS image: 1. atherosclerotic plaque, 2. carotid lumen (i.e. the luminal region with maximum contrast enhancement) and 3. background (i.e. a region which is visually not perfused by microbubbles). Drawing of plaque ROI may be hampered by saturation artifacts, which are caused by clipping of ultrasound signal and may lead to overestimation of IPN. Therefore, saturation artifacts were manually excluded from plaque ROI. Motion compensation of the plaque ROI was performed using an algorithm that calculated motion patterns found by dynamic programming combined with block matching [23]. After motion compensation, a maximum intensity projection (MIP) of the atherosclerotic plaque ROI was performed and time intensity curves were plotted. This data was used to calculate the different plaque perfusion features. Using the intensity of the lumen and background ROIs an adaptive threshold was calculated. This threshold adjusts for background noise and patient specific contrast enhancement characteristics which could lead to increased intensity in lumen or atherosclerotic plaque ROI. This threshold was applied to the MIP image of the atherosclerotic plaque, and IPN area and

Figure 1

Custom developed software. Panel a: In this panel the mandatory settings for analysis are shown. First, the time frame interval needs to be defined. Second, three ROI's are manually drawn for 1. atherosclerotic plaque, 2. lumen and 3. background. Third, the motion compensation is performed. Panel b: The combined CEUS and B-mode clip is loaded. Zoom function allows drawing accurate ROI's. Panel c: Numeric output data of several IPN features. Panel d: Graphical output of features. In this example the plaque intensity is plotted over time.

IPN area ratio were calculated. IPN area ratio was defined as the IPN area divided by the plaque ROI area. Furthermore, plaque mean intensity (PMI), mean plaque filling and calcification percentage were calculated (Fig. 2). The mean plaque filling was defined as the overall mean percentage of the plaque area which was perfused per individual frame included in the analysis. A fixed threshold (gray value ≥3) was used to eliminate noise in the CEUS clips. The percentage of calcification was estimated by calculating the shadow width below the plaque region relative to the atherosclerotic plaque width using a mean intensity profile. The shadow borders (start and end points) were determined if the contrast intensity below the atherosclerotic plaque dropped >70%. Plaques with presence of >50% of calcification were considered to be plaques with severe calcification. Finally, neovessel count was assessed using a microbubble tracking algorithm providing a neovascularization structure analysis of the atherosclerotic plaque [24].

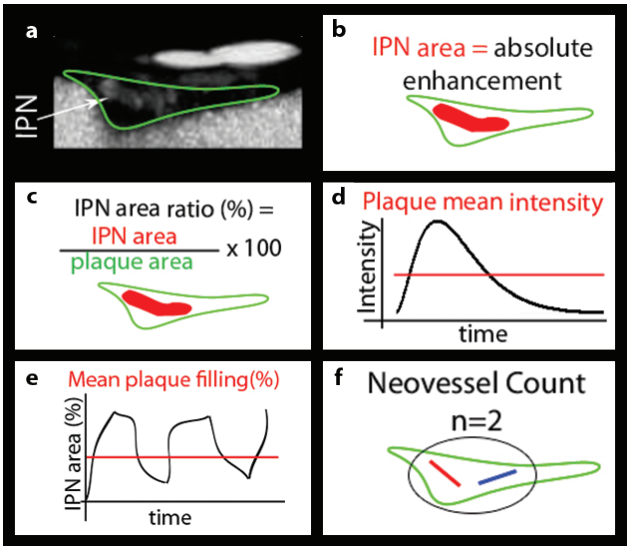

Figure 2
Intra-plaque neovascularization features. Panel a: Maximum intensity projection of an atherosclerotic plaque ROI. This projection is used for calculation of the features in panel b-c. Panel b: IPN area is calculated as the absolute enhancement of contrast in the atherosclerotic plaque ROI. Panel c: IPN area ratio is calculated as the absolute IPN area relative to the total atherosclerotic plaque ROI area. Panel d: Plaque mean intensity is calculated as the mean intensity value of the atherosclerotic plaque ROI over time. Panel e: Mean plaque filling is calculated as the mean IPN area ratio over time. Panel f: Neovessel count is calculated as the number of vessel paths that are detected using a microbubble tracking algorithm of Hoogi et al [24].

To assess intra-observer variability of the IPN features, plaque ROIs were redrawn at least six weeks after initial assessment of IPN features. To assess inter-observer reproducibility of the IPN features, plaque ROIs were redrawn by a second observer in a random sample of 15 carotid arteries.

Statistical analysis

Statistical analysis was performed using SPSS PASW software for Windows (Version 17.0.2, SPSS Inc., Chicago, Illinois). Continuous data were expressed as mean ± standard deviation or as median and interquartile range (IQR). Categorical variables were expressed as counts and percentages. Plaque perfusion features were log-transformed before statistical analysis. Spearman's rank correlation was used to test the association between the visual IPN score and the semi-automated scores. Calcification in atherosclerotic plaques may influence both visual and semi-automated scoring of IPN. Therefore, statistical analyses were performed twice; 1. on all carotid plaques, 2. after exclusion of plaques with severe calcification. Intra-observer agreement and inter-observer agreement were tested using intraclass-correlation coefficients (ICC) and Bland-Altman plots [25].

RESULTS

Patient characteristics

The clinical characteristics of the patients are summarized in Tab. 1. The mean age of the participants was 65 ± 9 years (84% males). The median number of cardiovascular risk factors per patient was 2 (IQR = 2). A total of 23 patients (92%) had ischemic symptoms of carotid atherosclerotic disease. No patients had contra-indications for the use of microbubble contrast agents and no complications occurred during the standard carotid ultrasound examinations and CEUS of the carotid arteries.

Table 1 Clinical characteristics of the study population (N=25)

Characteristic	Data
Age (y)	65 ± 9
Female gender	4 (16%)
Height (cm)	178 ± 9
Weight (kg)	90 ± 17
Body mass index (kg/m^2)	29 ± 6
Cardiovascular risk factors:	
Diabetes	6 (24%)
Dyslipidemia	9 (36%)
Hypertension	14 (56%)
Smoker (current)	11 (44%)
Smoker (former)	6 (24%)
Medication:	
Platelet aggregation inhibitor	24 (96%)
Beta-blocker	15 (60%)
Statin	23 (92%)
Oral antidiabetics	4 (16%)
Cerebrovascular history:	
Transient Ischemic Attack	13 (52%)
Stroke	8 (32%)
Ischemic ocular event	2 (8%)
Asymptomatic high grade stenosis	2 (8%)

Data are presented as number of patients (percentage) or as mean ± standard deviation

Carotid ultrasound findings

A total of 50 carotid arteries were analyzed for the presence of atherosclerotic plaques. Five carotid arteries were excluded from the analysis due to the absence of atherosclerotic plaques (n=2), due to a prior surgical intervention (n=1) or due to poor image quality (n=2). Forty-five arteries (90%) were included in the analysis. The average

maximum plaque thickness was 6.8 ± 1.2 mm. The atherosclerotic plaques in 11 carotid arteries were substantially echolucent but had spots of echogenicity, 26 plaques were predominantly echogenic but had spots of echolucency and 8 plaques were uniformly echogenic. In 12 carotid arteries the stenosis grade was <50%, in 13 carotid arteries the stenosis grade was 50-69% and in 20 carotid arteries the stenosis grade was ≥70%. The mean peak systolic velocity was 218 cm/s ± 140 cm/s. The mean end diastolic velocity was 76 cm/s ± 60 cm/s. A total of 5 plaques (11%) had visual IPN score 0, 29 plaques (64%) had IPN score 1 and 11 plaques (24%) had IPN score 2. The median IPN score per plaque was 1 ± 1.

Quantitative ultrasound findings

All 45 carotid arteries were evaluated using the custom developed software. Median number of frames in the selected time-frame was 315 (range: 90-445). Mean atherosclerotic plaque surface area was 11.6 ± 8.6 mm^2. The median output of IPN features and their correlations with visual IPN score are summarized in Tab. 2. The mean plaque filling (p=0.322) and the PMI (p=0.063) were not associated with the visual IPN score. IPN area (p<0.001), IPN area ratio (p<0.001) and the semi-automated neovessel count (p=0.001) were significantly associated with the different visual IPN scoring groups (Tab. 2). A total of 8 plaques (18%) were considered to have severe calcification. After exclusion of these plaques, the correlations of all IPN features improved (Tab. 2). IPN area, IPN area ratio and neovessel count provided a good distinction between the different visual IPN score groups (Fig. 3).

The intra-observer agreement of the different IPN features was excellent (ICC 0.84-0.98 p<0.001) (Fig. 4a). Calculations of mean differences for all IPN features showed no statistically significant intra-observer variability (p>0.05) (Fig. 4b). The inter-observer agreement was good to excellent (ICC 0.68-0.94 p<0.01) (Fig. 4c). The mean differences for all IPN features showed no statistically significant inter-observer variability (p>0.05) (Fig 4d).

Table 2 Comparison of quantitative and visual assessment of IPN

Quantitative features	Median output value (IQR)	Correlation with visual IPN score (ρ)	
		All atherosclerotic plaques (n=45)	Plaques without severe calcification (n=37)
IPN area (mm^2)	1.2 (1.1)	0.719 (p<0.001)	0.757 (p<0.001)
IPN area ratio (%)	3.5 (1.6)	0.538 (p<0.001)	0.574 (p<0.001)
Plaque mean intensity	1.3 (1.18)	0.277 (p=0.063)	0.343 (p=0.040)
Mean plaque filling (%)	12.3 (15.6)	0.151 (p=0.322)	0.219 (p=0.195)
Neovessel count	2 (4)	0.474 (p=0.001)	0.578 (p<0.001)

IPN = Intraplaque neovascularization. .

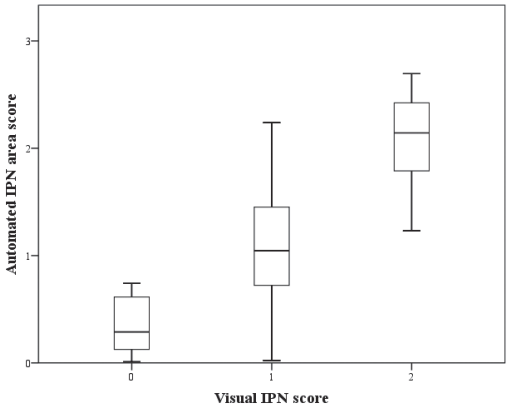

Figure 3
Association of IPN area score with visual IPN scoring.

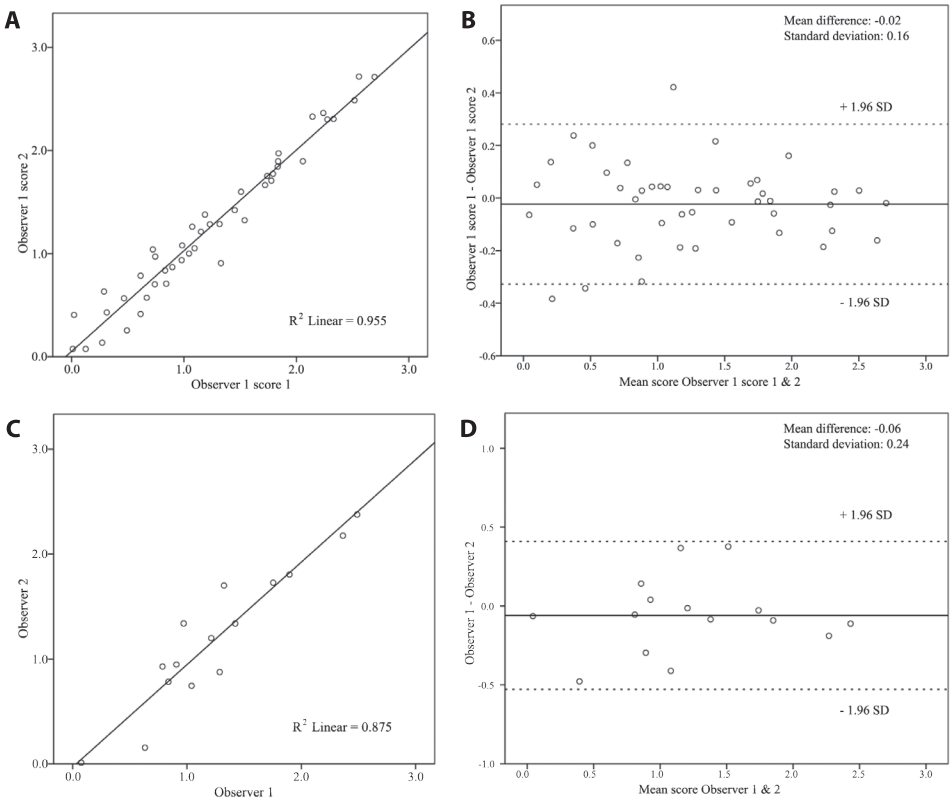

Figure 4
Intra-observer and inter-observer variability and agreement plots. Panel a: Correlation plot of intra-observer agreement of IPN area score. Panel b: Bland-Altman plot of intra-observer variability of IPN area score. Panel c: Correlation plot of inter-observer agreement of IPN area score. Panel d: Bland-Altman plot of inter-observer variability of IPN area score.

DISCUSSION

This study demonstrates that the amount of IPN in carotid atherosclerosis can be quantified using novel quantification software. This custom developed quantification method provided different features that were based on plaque perfusion characteristics and neovascularization structure. The area within atherosclerotic plaques that was perfused with microbubble contrast agents was significantly correlated with visual scoring of IPN. The quantification of IPN demonstrated a good to excellent intra-observer and inter-observer agreement. Therefore, these quantification methods of carotid IPN could be used to recognize and to monitor changes in IPN in vulnerable atherosclerotic plaques.

The presence and severity of IPN in atherosclerotic plaques has been hypothesized to be an important component of the vulnerable atherosclerotic plaque [26,27]. Recently, Hellings et al. studied the predictive value of IPN and intraplaque haemorrhage in 818 patients. During the follow up, these features were independent predictors of cardiovascular events [7]. These findings support the need for imaging studies investigating the possibilities of non-invasive assessment of those plaque components [28-30]. Validation studies have demonstrated a good correlation between visual assessment of IPN using CEUS and the amount of intraplaque neovessels in histological validation [11,16]. However, previous studies that evaluated IPN using CEUS relied on visual scoring. Information on quantitative assessment of IPN on carotid CEUS is scarce. In 2009, Xiong et al. described quantification of IPN in atherosclerotic plaques using peak-enhancement from time-intensity curves [31]. In that study, 104 patients with carotid atherosclerotic disease were included (mean age 63±9 years, 80% male, 34% symptoms of cerebrovascular disease). Symptomatic patients had a significantly increased amount of IPN compared to asymptomatic patients ($p<0.001$).

In the present study a novel quantification method for assessment of IPN was evaluated. This method is different from the previously described quantification method by Xiong et al. which was based on time intensity curves and peak intensity values. Carotid CEUS is a dynamic examination in which contrast microbubbles flow in and out of the atherosclerotic plaque during the ultrasound clip. Using the peak intensity value, information on contrast-enhancement before and after the peak intensity is neglected. Therefore, an IPN quantification method that is solely based on time intensity curves and peak intensity values may have some limitations. For the quantification of IPN in this study, perfusion features were calculated using information of a maximum intensity projection image and a time intensity curve. Maximum intensity projections represent the maximum brightness per pixel in the ROI over a predefined time frame, whereas time intensity curves represent the mean brightness in the selected ROI per individual ultrasound frame. Both analyses include information of consecutive ultrasound frames within the selected time frame. This reduces the chance that information on plaque per-

fusion is missed. Moreover, the use of all data from the time frame instead of one peak intensity value may reduce the variability of the IPN quantification. The exclusion of saturation artifacts from the plaque ROI may further improve quantification of IPN. The motion compensation tools were developed to minimize the chance that the assessment of IPN would be influenced by contrast in the carotid lumen or saturation artifacts.

This study has some limitations. First, the study population was relatively small. Second, in this study an arbitrary cut-off value of 50% calcification was used to define plaques with severe calcification. Third, the ultrasound derived IPN will be depicted larger than the true size of IPN because of the point spread function of the ultrasound signal. In addition, CEUS to assess IPN using bolus injections of microbubble contrast agents is a dynamic diagnostic modality. This leads to variability in the amount of contrast-enhancement between clips. An attempt to avoid underestimation of contrast-enhancement in the atherosclerotic plaque was made by using the clip with visually the highest amount of contrast-enhancement. Overestimation of contrast-enhancement was avoided by the exclusion of artifacts from the regions of interest. Fourth, logarithmic compressed data was used for analysis of IPN features. Using linear data would allow better curve fitting and calculating accurate results for slope of the fitted curve. However, the perfusion of atherosclerotic plaques is intermittent and is not equal to perfusion characteristics of large organs (e.g. myocardium or liver). Therefore, using linear data for the calculation of the IPN features would probably not significantly alter the results. Nevertheless, the sensitivity of the IPN features to discriminate between stable plaques and vulnerable plaques should be investigated using prospective follow-up data. Fifth, the amount of IPN may vary in different regions within the atherosclerotic plaque. Therefore, using two dimensional analysis (and thus single plane views) the different IPN features may underestimate or overestimate the condition of the atherosclerotic plaque. The development of 3 dimensional matrix probes would provide a solution for this problem. Last, plaques in the far wall or below contrast-pools were excluded from analysis because of pseudo-enhancement [20,21]. Development of new pulse-sequences that overcome this artifact will probably provide a solution for this limitation of CEUS [32].

CONCLUSIONS

Semi-automated motion compensated quantification of carotid IPN on CEUS is feasible, reproducible and provides multiple features on carotid IPN. Semi-automated neovessel count, IPN area, and IPN area ratio correlate well with visual scoring of IPN. Accurate quantitative assessment of IPN may be important to recognize and to monitor changes during therapy in vulnerable atherosclerotic plaques.

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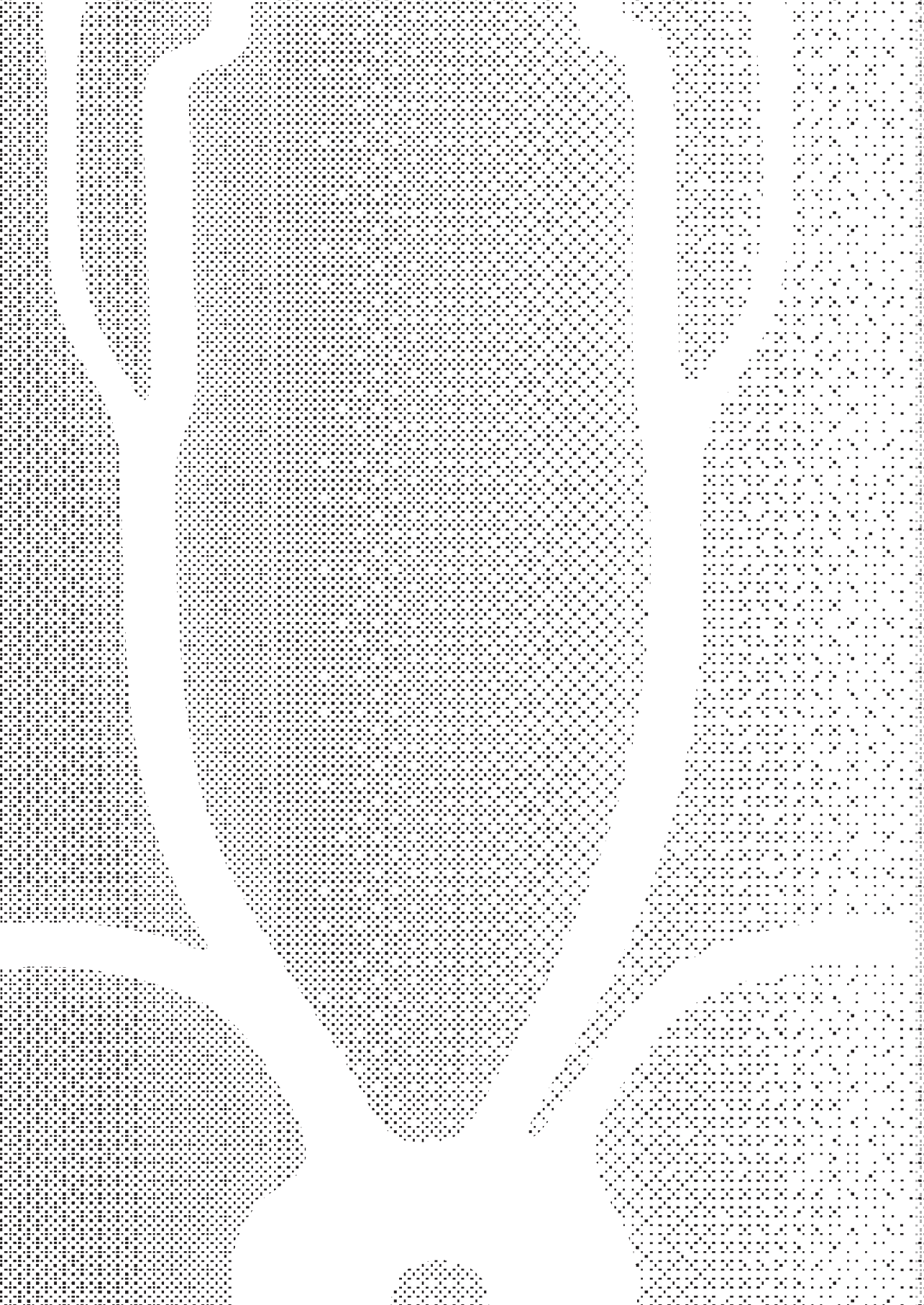

CHAPTER 7

INTRAPLAQUE NEOVASCULARIZATION ON CAROTID CONTRAST-ENHANCED ULTRASOUND: A HISTOLOGICAL STUDY AND ITS PITFALLS

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In preparation

INTRODUCTION

Annually, 15 million people worldwide suffer a stroke [1]. Approximately 25% of the ischemic strokes are caused by atherothrombotic events [2]. One of the underlying causes of atherothrombotic events is atherosclerotic plaque rupture leading to loose particles of debris in the bloodstream. These thrombo-embolisms get stuck in the smaller intracranial arteries. Adequate tissue perfusion distal from this thrombo-embolic obstruction may be threatened and thus may lead to ischemia and infarction of the brain tissue.

The risk of rupture of atherosclerotic plaques depends on several atherosclerotic plaque characteristics. The plaque with increased risk to rupture, i.e. the vulnerable plaque, is characterized by a lipid-rich atheromatous core that is covered by a thin fibrous cap with macrophage and lymphocyte infiltration and decreased smooth muscle cell content [3]. More recent studies indicate that intraplaque neovascularization, i.e. intraplaque vasa vasorum, and intraplaque hemorrhage are signs of plaque vulnerability. Both intraplaque hemorrhage and intraplaque neovascularization (IPN) were found to be independent predictors of future cardiovascular events [4-7].

Vasa vasorum are present in the tunica adventitia of most muscular arteries. A major role of these vasa vasorum is to provide nutrients to the vessel wall. However, a number of stimuli may trigger the vasa vasorum to proliferate through the tunica media to the atherosclerotic plaque [8-10]. These immature neovessels promote the formation of this atherosclerotic plaque. The relation of intraplaque neovascularization with early signs of atherosclerosis dates back more than a century ago. Köster et al. suggested in 1876 that the intimal proliferation and medial hypertrophy was related to the presence of vasa vasorum in the media and intima [11]. Over the last decade, the role of intraplaque neovascularization in the progression of atherosclerosis and its role in destabilization of atherosclerotic plaques has become more clear [12,13].

These potential crucial roles encourage the ongoing investigation on direct imaging of IPN. These investigations include contrast-enhanced ultrasound (CEUS) of the carotid arteries [14]. Using CEUS intraplaque neovessels can be identified with the advantages of high spatial and temporal resolution. The aim of this study was to correlate IPN parameters derived using CEUS with histological parameters for IPN.

METHODS

Study protocol

The study protocol was approved by the local ethical committee and all study participants provided informed consent. Thirty-three patients were included in this prospective study. All patients had a > 70% stenosis of the internal carotid artery and were scheduled for elective carotid endarterectomy (CEA). The indication for CEA was made in consensus by the treating neurologist and vascular surgeon. Clinical characteristics of the study population were recorded. All patients underwent a carotid CEUS examination of the on the day prior to CEA.

Ultrasound protocol

Both standard carotid ultrasound and CEUS of the carotid arteries was performed using a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, USA), equipped with a L9-3 transducer. Standard carotid ultrasound was performed prior to CEUS of the carotid arteries. Standard carotid ultrasound and CEUS were performed according to a standardized scanning protocol according to the American Society of Echocardiography consensus statement [15]. In short, first the left common carotid artery (CCA), carotid bulb, internal carotid artery (ICA) and external carotid artery (ECA) were evaluated using B-Mode ultrasound, Color Doppler imaging and Pulsed Wave Doppler imaging. The right carotid artery was evaluated in the same way. CEUS of the carotid arteries was performed after standard carotid ultrasound. CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). The contrast mode of the ultrasound system, using amplitude modulation and a mechanical index of 0.06-0.08, was used to optimize the CEUS examination. First, the left CCA, carotid bulb, ICA and ECA were evaluated, with special emphasis for the present atherosclerotic plaques. Again, the right carotid artery was evaluated in the same way. Bolus injections of the contrast agent were repeated when necessary up to a maximum total dose of 9.6 ml. Both carotid arteries were examined, focusing on the presence of atherosclerotic plaques. If plaques were present, the plaque was visualized in the longitudinal axis of the carotid artery. Still frames and cineclips were digitally stored.

Ultrasound analysis

All standard carotid ultrasound clips and CEUS clips were reviewed offline by two observers using Xcelera PACS (V3.1, Philips Medical Systems Nederland B.V., Best, the Netherlands). Both observers were blinded from patient specific data. The analysis of the clips was performed in a predefined sequence. Using the CEUS clips, the atherosclerotic plaques were scored for the presence of IPN. Per carotid artery with ≥1 atherosclerotic plaque on the near wall of the carotid artery, the clip with visually the highest amount of

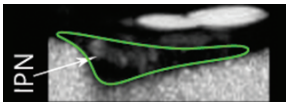
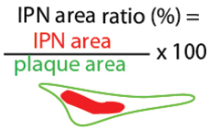
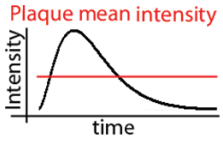
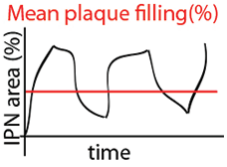
contrast enhancement was selected for analysis, to avoid underestimation of contrast-enhancement. Contrast enhancement was scored in the frames starting from the high mechanical index flash. Atherosclerotic plaques that could have been affected by pseudo enhancement, an artifact that hinders the evaluation of IPN in plaques on the far wall or behind contrast-pools, were excluded from analysis. Dynamic contrast-enhancement in the atherosclerotic plaque was considered to represent IPN. Fixed echogenic signals were considered to be tissue acoustic reflectors (i.e. saturation artifacts). These artifacts were neglected to avoid overestimation of contrast-enhancement. For each carotid artery the presence of IPN was graded as follows: Grade (0) no IPN, was used to indicate no appearance of microbubble contrast agent in the carotid lesion; Grade (1) limited or moderate IPN, was used to indicate limited to moderate visible appearance of moving microbubbles in the carotid lesion; or Grade (2) severe IPN, was used to indicate extensive vascularization of the atherosclerotic plaque with clear visible appearance of microbubbles.

Semi-automated quantification of IPN was performed using dedicated software. DICOM files of side-by-side B-Mode and contrast mode ultrasound clips were imported in the software to assess different plaque perfusion parameters after selection of the time-frame. The software calculated several parameters based on region-of-interest (ROI) analysis after motion compensation of the carotid artery. These calculations were based on maximum intensity projections and time intensity curves which resulted in the following parameters: 1. IPN surface area in mm2 (IPN-SA), 2. IPN surface area ratio in % (IPN-SA ratio), 3. mean percentage of the plaque filled with contrast over time (MPCP), 4. plaque mean intensity (PMI) and 5. plaque area in mm2. Table 1 provides a short description of how the parameters were derived. The reproducibility of the parameters of this software was good to excellent. Intra-observer intra-class correlation ranged from 0.835 to 0.984. Inter-observer intra-class correlation ranged from 0.682-0.968. Bland-Altman plots showed a low variability of repeated measurements. The reproducibility of the measurements was reported by Akkus et al [16].

Immunohistological processing protocol

Histological specimens were obtained during carotid surgery to assess the presence of IPN. The histological specimens were fixed in buffered formaldehyde (3,5-4%) for 24 to 48 hours. After fixation with formaldehyde the histological specimens were processed to obtain 1 mm thick blocks, decalcified and embedded in paraffin. Serial cross-sections of 5-µm thickness were obtained and stained with Hematoxylin–eosin as a routine stain. To confirm the presence of IPN, immunohistochemistry for endothelial cells was performed using mouse anti CD31 antibody (clone JC70, Cell Marque, Rocklin, California). Secondary antibodies were labeled with horseradish peroxidase and visualized with diaminobenzidin. Negative controls were obtained by omitting the primary antibodies.

Table 1

	Example of a maximum intensity projection (MIP) of a region of interest (ROI) in the atherosclerotic plaque. This projection is used for calculation of the intraplaque-neovascularization (IPN) parameters
	Graphical description of IPN surface area (IPN-SA). IPN-SA is calculated as the absolute enhancement of contrast in the atherosclerotic plaque ROI.
	Graphical description of IPN surface area ratio (IPN-SA ratio). IPN-SA ratio is calculated as the absolute IPN area relative to the total atherosclerotic plaque ROI area.
	Graphical description of plaque mean intensity (PMI). PMI is calculated as the mean intensity value of the atherosclerotic plaque ROI over time.
	Graphical description of mean percentage of the plaque filled with contrast over time (MPCP). MPCP is calculated as the mean IPN area ratio over time.

Immunohistological analysis

Immunohistological analysis was performed by two independent observers on an analogue microscope. For each cross-sectional 1 mm block, a slice stained with Hematoxylin-eosin was analyzed to estimate the atherosclertotic plaque burden. The block which provided the 5 micrometer section with the greatest atherosclerotic plaque burden was annotated as "analysis block 1". The blocks 1 millimeter distal and 1 mm proximal of "analysis block 1" were annotated as "analysis block 2" and " analysis block 3". The blocks 2 millimeter distal and 2 mm proximal of " analysis block 1" were annotated as "analysis block 4" and "analysis block 5" (Figure 1). In addition to the blocks selected based on the plaque burden, two blocks were selected in the proximal and distal plaque shoulder. The block selected for the distal atherosclerotic plaque shoulder was annotated as "analysis block 6". The block selected for the proximal atherosclerotic plaque shoulder was annotated as "analysis block 7".

Absolute quantification of intra-plaque neovascularization was performed on the selected CD-31 stained 5 micrometer sections in ascending order. For each 5 micrometer section 3 hotspots with intra-plaque neovascularization were selected. Hotspots were

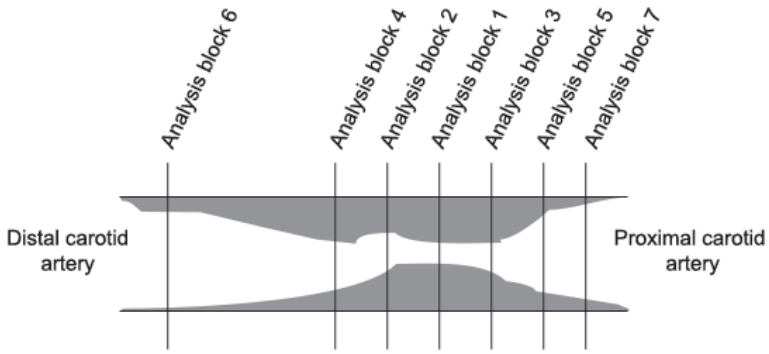


Figure 1
Schematic overview of the method used to select the histological blocks which were used for to calculate histological IPN parameters.

defined as regions with visually the highest density of intra-plaque neovessels in a 40x maginification field of view. Neovessels were defined as structures that 1. had a lumen, 2. lumen border was enhanced with CD-31 stain for at least 2/3 of the circumferential border and 3. at least one endothelial cell was detected in the luminal border. All neovessels that met the definition criteria were manually counted. If less than 3 hotspots were detected in a 5 micrometer section, the remaining hotspot(s) were assigned a value of 0 neovessels.

A total of 4 histological parameters were calculated for further analysis: 1. total number of neovessels in the hotspots of blocks 1-5 (i.e. the greatest plaque burden), 2. total number of neovessels in the hotspots off blocks 6-7 (i.e. the plaque shoulders), 3. the mean number of neovessels per hotspot of blocks 1-5, 4. the mean number of neovessels per hotspot of blocks 6-7. The total number of neovessels was calculated as the sum of the number of neovessels of the selected hotspots. The mean number of neovessels was calculated by dividing the total number of neovessels of the selected hotspots by the number of hotspots after exclusion of hotspots with 0 microvessels.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 21, IBM Corp, Armonk, New York, USA). Continuous variables are presented as mean (SD) or median [Inter-quartile range]. Categorical variables and ordinal variables are expressed as number (percentage). Intra-observer variability and inter-observer variability for the histological analysis was assessed using intra-class correlation coefficient and Bland-Altman plots. Intra-observer variability was calculated for 20 randomly selected 5 micrometer sections. Inter-observer variability was assessed for the 5 micrometer sections of 14 carotid arteries. Correlations between histological parameters and CEUS

parameters were calculated using Spearman's Rho. A p-value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Table 2 provides an overview of the patients characteristics (n=33, 12% females). Mean age of the patients was 65 ± 8 years. No complications occurred during the standard carotid ultrasound examination and the CEUS examination of the carotid arteries. In 5 patients the carotid specimen was not suitable for further processing. One patient had uninterpretable ultrasound images. These 6 patients were excluded from further analysis.

Table 2 Patient characteristics

Characteristic	Data (n = 33 patients)
Age (years)	65 ± 8
Female gender	4 (12%)
Height (cm)	177 ± 9
Weight (kg)	89 ± 17
Body mass index (kg/m^2)	28 ± 6
Cardiovascular risk factors	
Diabetes	6 (18%)
Dyslipidemia	8 (24%)
Hypertension	19 (58%)
Smoker (current or former)	22 (67%)
Medication	
Platelet aggregation inhibitor	33 (100%)
Beta-blocker	16 (49%)
Statin	29 (88%)
Oral antidiabetics	4 (12%)
Insulin	3 (9%)
Operation indication	
Transient Ischemic Attack	17 (52%)
Stroke	11 (33%)
Ischemic ocular event	2 (6%)
Asymptomatic high grade stenosis	3 (9%)

Carotid ultrasound analysis

A total of 27 carotid arteries were included in the analysis. Results of the semi-automated assessment of IPN and other plaque characteristics are summarized in table 3. Visual assessment of IPN showed mild to moderate IPN in 15 carotid arteries (56%) and severe IPN in 12 carotid arteries (44%).

Table 3 Ultrasound findings

Ultrasound characteristic	Data (n = 27 arteries)
IPN surface area (mm^2)	2.8 (1.5-6.9)
IPN surface area ratio (%)	38 (10-60)
MPCP (%)	15 (9-30)
Plaque mean intensity	1.53 (0.86-3.55)
Plaque calcification (%)	11 (0-42)
Plaque area (mm^2)	12 (9-16)

MPCP = Mean percentage of the plaque filled with contrast over time.

Immunohistological analysis

Results of the immunohistological analysis are summarized in table 4. Reproducibility of the measurements was excellent. Median difference between measurements performed by the same reader was 9.3 % and the Bland-Altman plot showed no systematic error between the the repeated measurments(Figure 2A). Intra-observer intraclass correlation coefficient was excellent (0.979, $p<0.001$) (Figure 2B). Median difference between measurements performed by two independent readers was 10.4 % and the Bland-Altman plot showed no systematic error between the two readers (Figure 2C). Inter-observer intraclass correlation coefficient was excellent (0.945, $p<0.001$) (Figrue 2D).

Table 4 Histological findings

Immunohistological parameter	Data (n = 27 arteries)
Greates plaque burden: total number of neovessels	87 ± 35
Greates plaque burden: mean number of neovessels	11 ± 4
Plaque shoulders: total number of neovessels	37 ± 26
Plaque shoulders: mean number of neovessels	8 ± 4

Correlations between CEUS and histology

Table 5 provides an overview of the correlations between the IPN parameters derived using carotid contrast enhanced ultrasound and the histological parameters. No statistically significant correlations were detected between any of the IPN parameters and histological parameters (table 5).

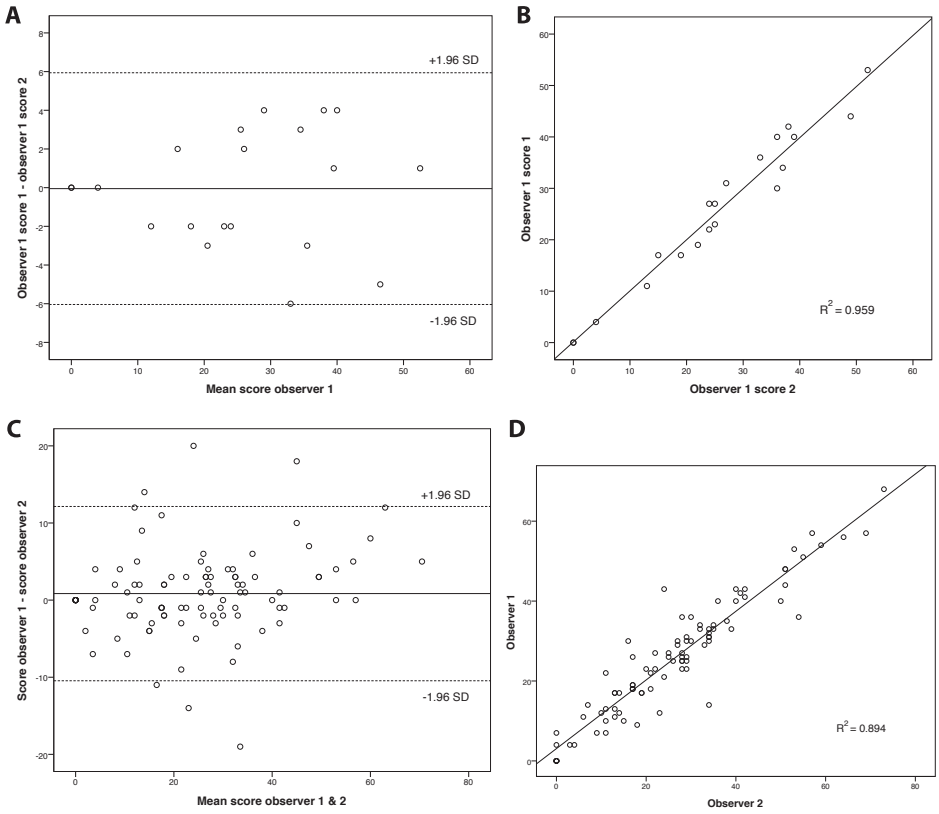


Figure 2

Intra-observer and inter-observer variability and agreement plots for histological scoring method. Panel a:Intra-observer correlation plot. Panel b: Bland-Altman plot of intra-observer variability of histological score. Panel c: Inter-observer correlation plot. Panel d: Bland-Altman plot of inter-observer variability of histological score.

DISCUSSION

The results in this study carry out an important message: the way carotid IPN is assessed in CEUS clips in this study does not correlate with the way neovessels were counted in histological samples in this study. No statistically significant correlations between CEUS parameters and histological parameters were detected and no trend was seen in the results toward any direction. Although the methods to quantify IPN on CEUS and the methods to quantify neovessels in histological samples seemed delicate when this study was performed, there may be some methodological considerations which limit the value to extrapolate the current data from this study.

The first limitation is the selection bias due to strict inclusion criteria for participating patients. In this study only those patients with 1. advanced atherosclerotic plaques and 2. an indication for carotid surgery were included. Over 90% of the patients had

Table 5 Correlation between ultrasound findings and histological findings

	Total number of neovessels around greatest plaque burden	Mean number of neovessels around greatest plaque burden	Total number of neovessels in plaque shoulders	Mean number of neovessels in plaque shoulders
IPN-surface area				
Correlation coefficient	0.110	0.026	-0.036	-0.099
p value	0.584	0.899	0.862	0.624
IPN-surface area ratio				
Correlation coefficient	0.022	-0.046	-0.088	-0.188
p value	0.913	0.818	0.668	0.347
Plaque mean intensity				
Correlation coefficient	0.010	0.019	-0.288	-0.335
p value	0.959	0.924	0.154	0.088
MPCP				
Correlation coefficient	-0.039	-0.005	-0.203	-0.319
p value	0.848	0.979	0.320	0.104

MPCP = mean percentage of the plaque filled with contrast over time.

symptomatic carotid atherosclerotic plaques and only 3 patients had asymptomatic high grade carotid stenosis. Therefore it may be possible that in this study the majority of atherosclerotic plaques had vulnerable characteristics such as IPN. This is supported by the results from visual IPN assessment: a total of 0 patients had no IPN on CEUS clips. Inclusion of patients with low-grade carotid stenosis or subclinical atherosclerotic plaques may have led to other results. However, those patients do not have an operation indication and therefore it is not possible to obtain histological samples in that group.

The second limitation is the sampling error in the analysis of CEUS clips and in the analysis of the histological samples (figure 3). In CEUS, only plaques that are not hampered by pseudo-enhancement are eligible for analysis [17]. Pseudo-enhancement appears behind contrast pools and therefore only near-wall atherosclerotic plaques can be used in the analysis with the restriction that these plaques are not located behind other contrast pools such as jugular vein. This results in significant loss of information. In addition, the 2D-CEUS clips are recorded in a longitudinal direction covering a slice thickness of approximately 2mm. On the other side, the histological specimens are processed in a cross-sectional direction, which is perpendicular to the imaging direction. As a result, a relatively small area is covered by both CEUS clips and the histological samples (figure 3). The sampling error is further increased by using hotspot analysis for quantification of neovessels on histological samples. Using hotspot analysis, a chance was introduced that area's that were included in the hotspots were not included in the 2D-CEUS clips resulting in a mismatch between histological samples and CEUS clips. In addition, using hotspot analysis the histological parmeters may have the same value in atherosclerotic

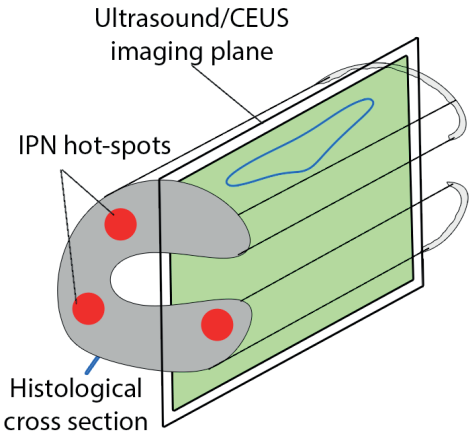


Figure 3
Schematic overview of sampling error between analysis of the CEUS clips and the analysis of the histological samples. Grey area represents the cross-sectional orientation for histological analysis, the red dots represent the hotspots which are selected for counting the intraplaque neovessels. The green plane represents the imaging plane which is recorded using CEUS. Within this plane, the blue line represents the region-of-interest (ROI) in which the amount of intraplaque neovascularization is quantified.

plaques with unequal numbers of neovessels and unequal densitity of neovessels due to the inhomogeneous distribution of neovessels in atherosclerotic plaques.

The third limitation is caused by another mismatch between the data derived from CEUS clips and the number of neovessels in histological samples. Using CEUS it is possible to visualize microbubbles in the arterial wall and in the atherosclerotic plaque (i.e. IPN) [14,18,19]. Since microbubbles are pure intravascular tracers, the presence of microbubbles within atherosclerotic plaques indicates the presence of vasculature [20,21]. In addition, enhancement of microbubbles is evidence of actual perfusion of the vasculature. For multiple reasons, the absence of plaque enhancement is no evidence for the absence of intraplaque vasculature: 1. due to low flow and low absolute concentration of microbubbles in the blood, the chance of a microbubble passing through the intraplaque microvasculature is small and 2. during the CEUS examination, the perfusion status of the intraplaque neovessels is not known and therefore it may be possible that there is no continuous perfusion of intraplaque neovessels. The data derived from histological samples contain quantitative information on the amount of neovessels in hotspots, but no data is available on the perfusion status of the intraplaque neovessels.

Fourth, using CEUS it is not possible to distinguish adventitial vasa vasorum from IPN. Analysis of enhancement in carotid CEUS clips is based maximum intensity projections and time intensity curves of regions of interest. Although it may be obvious that central ROI enhancement is indicative for IPN, enhancement at the edges of the ROI may be caused by microbubbles flowing through adventitial vasa vasorum. During carotid

endarterectomy, only the intima and parts of the media are removed and the advetitial layer remains in situ. Therefore, the histological samples do not contain information on the presence and density of adventitial vasa vasorum. This leads to another mismatch between what is detected on carotid CEUS clips and what is quantified in histological samples.

Another limitation is the quality of the histological samples. During carotid surgery, the intima and parts of the media layer are removed from the carotid artery. In ideal situations, the atherosclerotic plaque can be removed in-toto without opening the lumen (i.e. en-bloc resection). The main advantage of this technique is the preservation of plaque anatomy. Unfortunately, perioperative conditions may limit the surgeon in performing this technique and often the surgeon decides to cut open the atherosclerotic plaque into the lumen for varying reasons. In these cases, the plaque anatomy is lost and the plaque may be resected in several smaller pieces. As a result, reconstruction of plaque anatomy and exact location (e.g. proximal/distal, anterior/posterior, medial/lateral) within the carotid artery is more complicated.

Although some limitations are inevitable, there may be several methodological options to reduce the impact of the current limitations. These methological options may be able to reduce the impact of the sampling error by adjusting the carotid ultrasound analysis protocol and immunohistological analysis protocol or may be able to compare the current data according to another protocol.

The current analyses protocols could be improved by further reduction of the impact of the sampling error. First, in the current carotid ultrasound analysis protocol only one clip with visually the highest enhancement of contrast in the atherosclerotic plaque was analysed to avoid underestimation of IPN. To reduce the sampling error, multiple clips from different angles of insonation could be analysed. This may provide a more veracious impression on the distribution of IPN through the atherosclerotic plaque. However, the imaging window for carotid ultrasound insonation is limited to approximately 90 degrees and pseudo-enhancement caused by jugular vein may further limit the imaging window.

Second, the current immunohistological analysis protocol could be improved by abandoning hotspot analysis and introduce complete plaque analysis which would allow to calculate a more robust plaque neovessel density. In addition, histological analysis could be limited to the field which was most likely in the field of carotid ultrasound insonation. Although based on several assumptions, the quarter of a cross-sectional histological block which contains the surgical cut through the atherosclerotic plaque is most likely the quarter which was located closest to the skin. Therefore, it is also the quarter which was most likely to be insonated using carotid ultrasound and analysed for the presence and amount of IPN. In-vivo MRI and ex-vivo MRI examinations of the atherosclerotic plaque may assist in reconstruction of the actual in-vivo anatomy of the

atherosclerotic plaque. Therefore these examinations may be helpful in defining which quarter of the cross-sectional histological blocks was most likely imaged using carotid ultrasound. Unfortunately, these additional examinations are not available for all carotid plaques in the current dataset.

In addition, other protocols to analyse the current data could provide valuable information. Prior studies investigated the differences in plaque composition between proximal and distal atherosclerotic plaque [22]. That study showed that the proximal atherosclerotic plaque had more vulnerable plaque characteristics than the distal atherosclerotic plaque. In the current study, it may be possible to divide the carotid ultrasound analysis in a proximal and a distal atherosclerotic plaque part and to correlate this to the amount of vessels in the proximal and distal atherosclerotic plaque in the histological samples. Unfortunately, this option in itself does not reduce the sampling error.

SUMMARY

The data in this study show that the way carotid IPN is assessed in CEUS clips in this study does not correlate with the way neovessels were counted in histological samples. Several methodological considerations limit the value to extrapolate the current data. Nevertheless, so far this study showed that histological studies with carotid CEUS have several potential pitfalls. Future studies should avoid the pitfalls presented in the current study.

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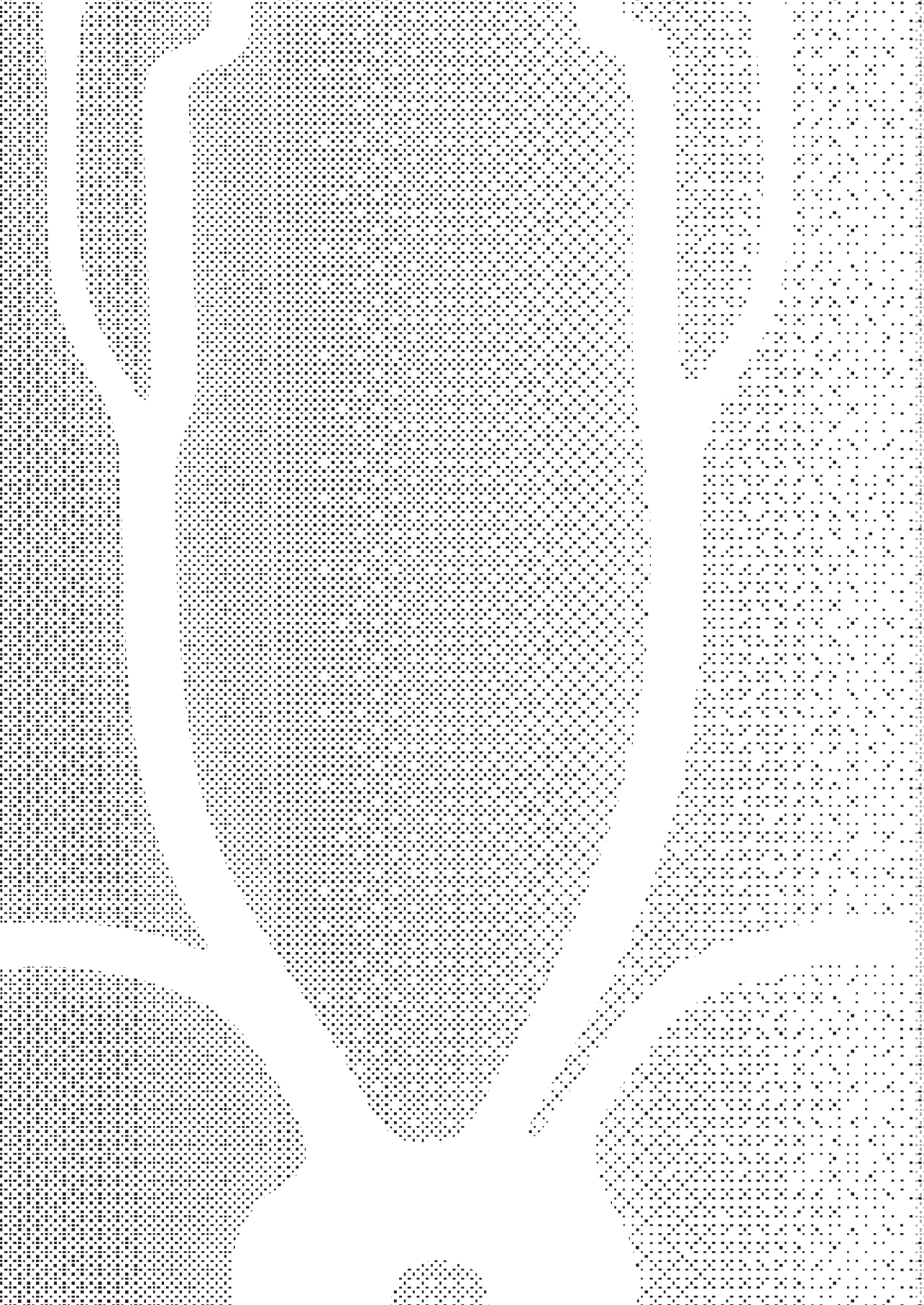
PART IV



CHAPTER 8

ASSESSMENT OF SUBCLINICAL ATHEROSCLEROSIS AND INTRAPLAQUE NEOVASCULARIZATION USING QUANTITATIVE CONTRAST-ENHANCED ULTRASOUND IN PATIENTS WITH FAMILIAL HYPERCHOLESTEROLEMIA

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ABSTRACT

Objective: Patients with heterozygous familial hypercholesterolemia (FH) are at severely increased risk of developing atherosclerosis at relatively young age. The aim of this study was to assess the prevalence of subclinical atherosclerosis and intraplaque neovascularization (IPN) in patients with FH, using contrast-enhanced ultrasound (CEUS) of the carotid arteries.

Methods: The study population consisted of 69 consecutive asymptomatic patients with FH (48% women, mean age 55 ± 8 years). All patients underwent carotid ultrasound to evaluate the presence and severity of carotid atherosclerosis, and CEUS to assess IPN. IPN was assessed in near wall plaques using a semi-quantitative grading scale and semi-automated quantification software.

Results: Carotid plaque was present in 62 patients (90%). A total of 49 patients had plaques that were eligible for the assessment of IPN: 7 patients (14%) had no IPN, 39 (80%) had mild to moderate IPN and 3 (6%) had severe IPN. Semi-automated quantification software showed no statistical significant difference in the amount of IPN between patients > 50 years and patients ≤ 50 years and between patients with a defective low-density lipoprotein receptor (LDLR) mutation and patients with a negative LDLR mutation. Plaques with irregular or ulcerated surface had significantly more IPN than plaques with a smooth surface ($p < 0.05$).

Conclusion: Carotid ultrasound demonstrated atherosclerotic plaque in 90% of asymptomatic patients with FH without known atherosclerosis. IPN assessed with CEUS, was present in 86% of these patients. Irregular and ulcerated plaques exhibited significantly more IPN than plaques with a smooth surface.

INTRODUCTION

Heterozygous familial hypercholesterolemia (FH) is the most prevalent autosomal dominant inherited disorder of the lipoprotein metabolism, resulting in increased LDL cholesterol levels. Patients with FH are at severely increased risk of developing atherosclerosis at relatively young age. The introduction of statin treatment has partly reduced the risk of myocardial infarction and stroke in patients with FH [1-4]. Still, patients with FH receiving long term statin treatment may have a substantial amount of subclinical atherosclerosis [5]. There is a large variation in the extent and severity of atherosclerotic disease, and therefore presymptomatic evaluation of atherosclerosis may be especially useful in patients with FH.

Carotid ultrasound is a widely available, low-cost and free from ionizing radiation imaging modality for the evaluation of (subclinical) atherosclerosis. Previous studies have demonstrated that ultrasound assessment of carotid intima-media thickness and plaque provides valuable information for monitoring the response to treatment and for risk-stratification in patients with FH [6,7]. Contrast-enhanced ultrasound (CEUS) is an advanced form of ultrasound imaging using a microbubble contrast agent to provide improved detection of plaques and can be used to visualize intraplaque neovascularization (IPN) [8-10]. Recent data indicate that IPN is a marker of plaque instability. A recent pathology study of unstable carotid lesions demonstrated that IPN and intraplaque hemorrhage are predictors of rupture of atherosclerotic plaques [11]. This confirms the suggestion that these histological characteristics are considered to be components of the vulnerable atherosclerotic plaque [12]. Information on IPN in patients with FH is at present not available, but may be relevant for a better understanding of the pathophysiology of atherosclerosis, development of new treatment approaches, and risk stratification. The aim of this study was therefore to assess the prevalence of subclinical atherosclerosis and IPN in a consecutive group of patients with FH. Carotid ultrasound was used to evaluate the presence and severity of carotid atherosclerosis, and CEUS was used to assess IPN.

METHODS

Patient population and study protocol

The study population consisted of 69 asymptomatic patients with heterozygous FH. Inclusion criteria were: heterozygous FH, and age ≥ 18 years. Exclusion criteria were: known atherosclerosis, contra-indications for the use of ultrasound contrast agent, such as unstable angina, acute cardiac failure, acute endocarditis, known right-to-left shunts and known allergy to microbubble contrast agents. FH was diagnosed according to

the criteria of van Aalst-Cohen [13], which can be summarized as either the presence of a documented LDL-receptor mutation, or an LDL-cholesterol level above the 95th percentile for gender and age in combination with the presence of typical tendon xanthomas in the patient or in a first degree relative, or an LDL-cholesterol level above the 95th percentile for gender and age in a first degree relative or proven coronary artery disease in the patient or in a first degree relative under the age of 60. Genetic screening for the presence of mutations in the low-density lipoprotein receptor (LDLR) gene, apolipoprotein B (APOB) gene and proprotein convertase subtilisin/kexin gene was routinely performed for all patients. Carotid ultrasound and CEUS were performed to investigate the prevalence of subclinical atherosclerosis and the density of IPN. The study protocol was approved by the local ethical committee and all patients provided informed consent. A single carotid ultrasound and CEUS examination takes approximately 30 min.

Carotid ultrasound acquisition

Carotid ultrasound including color Doppler, and CEUS were performed with a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, USA), equipped with a L9-3 transducer. For standard carotid ultrasound a frequency of 7 MHz was used. Image acquisition was performed using a scanning protocol according to the American Society of Echocardiography consensus [14]. In short, both left and right carotid arteries were examined with the patient in a supine position with the head turned at a 45° angle to the contralateral side. The left and the right common carotid artery (CCA), carotid bifurcation (BIF), internal carotid artery (ICA), external carotid artery (ECA), and vertebral arteries were imaged with B-mode ultrasound, color Doppler and pulse-wave Doppler. All anatomical sites were examined from different angles of view. Gain and imaging depth were adjusted per patient to obtain optimal ultrasound images. Each side was extensively evaluated for the presence of plaques.

CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). For CEUS a frequency of 3.5 MHz was used. The contrast mode of the ultrasound system, using amplitude modulation, a minimum frame-rate of 13/second and a mechanical index of 0.06, was used to optimize the CEUS examination. CEUS clips were recorded with a dual display mode for simultaneous B-mode ultrasound and CEUS view. The ultrasound contrast agent was injected in boluses of 0.5 ml. Each contrast agent bolus was followed by a saline flush using 2.0 ml NaCl 0.9% solution. Bolus injections were repeated when necessary to a maximum dose of 9.6 ml, depending on the number of CEUS cineclips that were required for complete imaging of the carotid arteries. Both carotid arteries were examined, focusing on the presence of plaques. If plaques were present, the plaque was visualized in the longitudinal axis of the carotid artery. The longitudinal image plane with visually the largest plaque area was used to record cineclips. Still frames and cineclips with a maximum of 20 s were digitally stored.

Carotid ultrasound analysis

Carotid ultrasound studies were reviewed offline. Carotid plaque screening was performed using the standard carotid ultrasound images, color Doppler, and CEUS. Atherosclerotic plaque was defined as a focal structure encroaching into the lumen by at least 0.5 mm or 50% of the surrounding carotid intima-media thickness, or demonstrates a thickness >1.5 mm as measured from the media-adventitia interface to the intima–lumen interface [15]. The presence of plaque and maximum plaque thickness was recorded for each carotid segment. All analyses were performed at the near wall of the carotid artery. For intima-media thickness measurements this will give less reproducible results [16] but since the plaque thickness as defined above is at minimum the double of the resolution of the ultrasound this will not be the case for the current plaque measurements. Maximum plaque thickness was measured perpendicular to the longitudinal axis of the carotid artery. The carotid segments were defined as CCA, BIF, ICA and ECA. Additionally for each segment with signs of atherosclerosis the presence of calcification, wall irregularities and/or ulceration were recorded. Plaque ulceration was defined as a disruption in the plaque-lumen border ≥1 × 1 mm.

IPN was assessed using CEUS clips. To minimize the contribution of pseudo-enhancement to the assessment of IPN, only near wall plaques were used. Clips without atherosclerotic plaques or with plaques that could have been affected by pseudo-enhancement were not eligible for the assessment of IPN. Pseudo-enhancement is an artifact that is mainly seen in the far-wall of the carotid artery and mimicks contrast-enhancement which may lead to overestimation of IPN [17,18]. In general that means that plaques below contrast pools (e.g. the carotid lumen or jugular lumen) were excluded from assessment of IPN. The amount of IPN was assessed using a semi-quantitative grading scale and semi-automated quantification software. For each carotid artery with ≥1 atherosclerotic plaque on the near wall of the carotid artery, the clip with visually the highest amount of contrast enhancement was selected for analysis (Fig. 1). In patients with bilateral plaques, the thicker plaque was selected for patient based analysis. Visual assessment of IPN was performed using a 3 point grading scale as 0 = no IPN, 1 = mild to moderate IPN and 2 = severe IPN. Semi-automated quantification of IPN was performed using dedicated software (Fig. 2). The software was based on a simple and user-friendly user interface created using MevisLab (MeVis Medical Solutions AG and Fraunhofer MEVIS, Bremen, Germany). DICOM files of side-by-side B-Mode and contrast mode ultrasound clips were imported in the software to assess different plaque perfusion features after selection of the time-frame. Several plaque perfusion features were calculated after motion compensation of regions of interest. The calculations were based on maximum intensity projection images and time intensity curves. Features that were calculated included IPN surface area in mm2 (IPN-SA), IPN surface area ratio in % (IPN-SA ratio), mean percentage

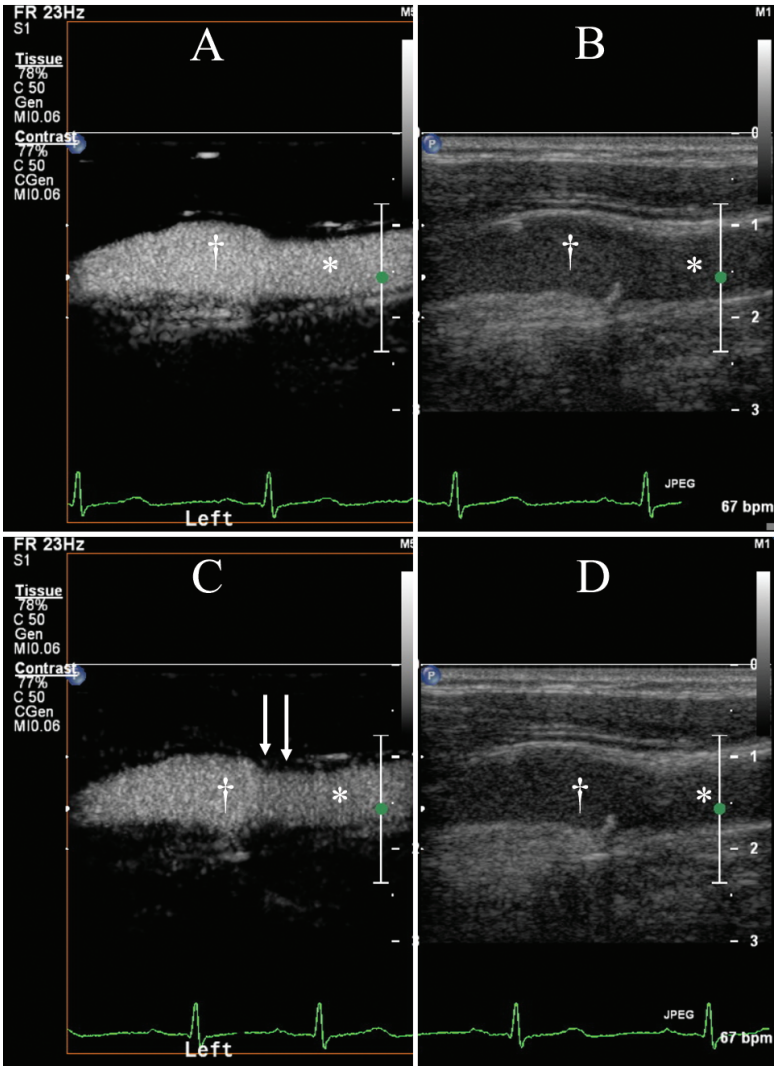

Figure 1
Example of side-by-side contrast enhanced ultrasound (CEUS) examination. * = common carotid artery, † = carotid bulb. Panel A: CEUS clip of the left carotid artery shortly after contrast injection. Panel B: corresponding B-mode clip of the left carotid artery recorded simultaneously with the CEUS clip. Panel C: A CEUS clip of the same carotid artery recorded a few seconds following contrast administration. The arrows indicate contrast-enhancement in a small atherosclerotic plaque in the carotid bulb. Panel D: corresponding B-mode clip of the left carotid artery recorded simultaneously with the CEUS clip.

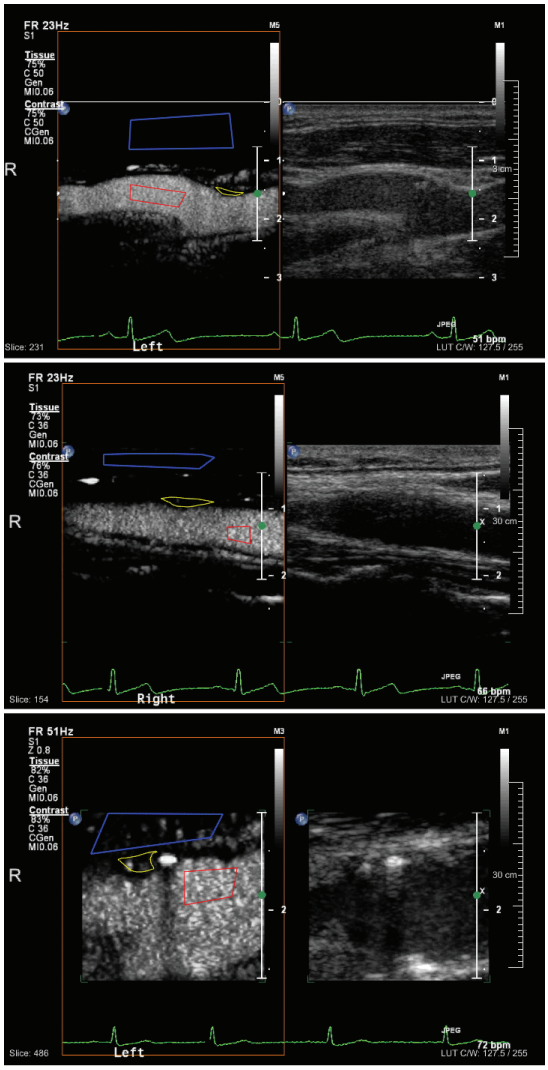

Figure 2

Semi-automated quantification of IPN in patients with FH. The examples shown are from 3 different patients with FH. Side-by-side contrast-enhanced ultrasound clips were used to quantify the amount of contrast enhancement in the atherosclerotic plaques. Yellow regions of interest: atherosclerotic plaque. Red regions of interest: lumen, used to adjust for lumen intensity. Blue regions of interest: background, used to adjust for background intensity.

of the plaque filled with contrast over time (MPCP), the plaque mean intensity (PMI), and plaque area. Details of the quantification features have been described previously [19].

Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS, Chicago, USA). Continuous variables are presented as mean (SD) or median (Inter-quartile range). Categorical variables are expressed as number (percentage). Mann–Whitney U test was performed to compare the output of the semi-automated software in selected patient groups. Spearman's rank correlation coefficients were calculated to test for correlations between IPN features and continuous variables. A p value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Patient characteristics

The clinical characteristics of the study population are summarized in Table 1. Sixty-six patients (96%) received statin treatment for a mean duration of 11 ± 8 years. Due to side effects of the statin medication, 28 patients (41%) did not receive a maximum dose or

Table 1 Patient characteristics (n=69)

Male gender	36 (52%)
Age, years	55 ± 8
Current or former smoker	30 (43%)
Hypertension	11 (16%)
Diabetes mellitus	2 (3%)
Body mass index (kg/m2)	26 ± 4
Identified genetic mutation causing FH	46 (67%)
Tendon xanthomas	18 (26%)
Arcus cornealis	19 (28%)
Highest total cholesterol without therapy, mmol/L	8.6± 2.1
Current total cholesterol, mmol/L	5.6 ± 1.9
LDL, mmol/L	3.7 ± 1.7
HDL, mmol/L	1.5 ± 0.4
Triglycerides, mmol/L	1.3 ± 0.7
Statin use	66 (96%)
Ezetimibe use	38 (57%)
Age of first statin use, years	44 ± 10
Duration of statin use, years	11 ± 8

Data are presented as number of patients (percentage) or as mean ± standard deviation.

no statin at all. In 63 patients (91%) results of the genetic screening for the presence of mutations in the LDLR gene were available. A total of 27 patients (39%) had LDLR-negative mutations. A total of 19 patients (28%) had LDLR-defective or APOB mutations which in general lead to a milder phenotype (Table 2). The remaining 17 patients (25%) had unidentified mutations.

Table 2 Quantitative assessment of intraplaque neovascularization (n=68 carotid arteries)

IPN features			
	Median	IQR	Range
IPN -SA (mm^2)	1.9	2.1	11.1
IPN -SA ratio (%)	48	55	98
MPCP (%)	26	40	97
PMI	3.0	4.9	20.0
Plaque area (mm^2)	4.0	3.9	29.1

IPN-SA = Intraplaque neovascularization surface area, IQR = Inter quartile range, MPCP = Percentage of the plaque filled with contrast over time, PMI = Plaque mean intensity.

Carotid ultrasound results

Using the standard ultrasound clips, carotid plaque was detected in 157 of 552 carotid segments (28%). The mean plaque thickness was 2.4 ± 0.8 mm. A total of 96 carotid segments (61%) with plaque exhibited signs of calcification. An irregular plaque surface was present in 46 carotid segments with plaque (29%). Fourteen of the carotid segments with atherosclerotic plaques (9%) had an ulcerated plaque surface. In 89 carotid arteries (64%) ≥1 atherosclerotic plaque was detected.

CEUS results

A total of 68 carotid arteries (49%) were eligible for the assessment of IPN. The remaining 70 carotid arteries (51%) had plaques that were affected by the pseudo-enhancement artifact (n = 19, 14%) or had no atherosclerotic plaque (n = 51, 37%). Visual assessment showed no IPN in 7 carotid arteries (10%), mild to moderate IPN in 58 carotid arteries (85%) and severe IPN in 3 carotid arteries (4%). The output of the semi-automated IPN quantification software is summarized in Table 2. The median number of frames used for IPN quantification was 255 (IQR = 148). The selected time-frames had a median duration of 11 s (IQR = 7). Plaques with an irregular or ulcerated surface (n = 18, 27%) had a significantly higher visual IPN score ($p < 0.05$) and had significantly more IPN as assessed by semi-automated quantitative software ($p < 0.05$) (Fig. 3).

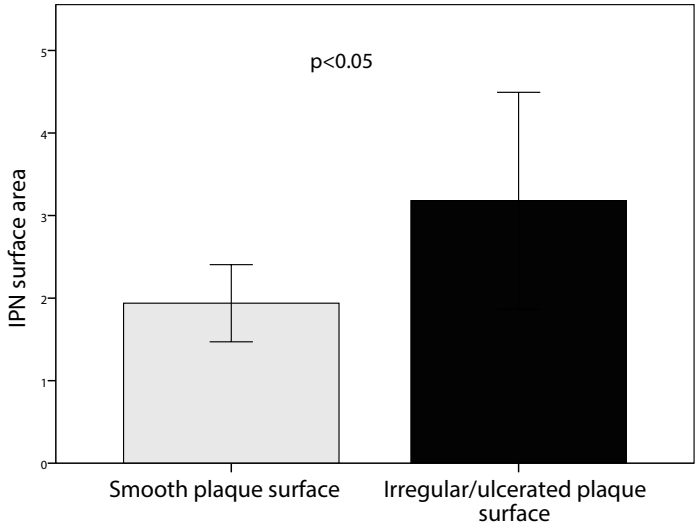

Figure 3
IPN in plaques with a smooth surface compared to plaques with an irregular/ulcerated surface. Semi-automated quantification software calculated the surface area enhanced during CEUS examinations. Plaques with an irregular or ulcerated surface had significantly more IPN than plaques with a smooth surface ($p<0.05$).

Patient-based carotid ultrasound and CEUS results

A total of 62 patients (90%) exhibited carotid atherosclerotic plaque. In these patients the median number of carotid segments involved was 2 (IQR: 1–4). In 49 patients (71%) the plaques were eligible for the assessment of IPN. Of these, a total of 19 patients (39%) had bilateral plaques, 30 patients (61%) had unilateral plaques. Visual assessment showed no IPN in 7 patients (14%), mild to moderate IPN in 39 patients (80%) and severe IPN in 3 patients (6%). The quantitative evaluation of IPN is summarized in Table 3. No statistically significant differences in the amount of IPN were detected between patients aged ≤50 years and patients aged >50 years ($p = 0.072$). In addition, correlations between IPN

Table 3 Quantitative assessment of intraplaque neovascularization (n=49 patients)

IPN features			
	Median	IQR	Range
IPN -SA (mm^2)	2.0	2.3	11.1
IPN -SA ratio (%)	45	57	98
MPCP (%)	22	41	97
PMI	2.6	5.0	20.0
Plaque area (mm^2)	4.3	4.1	29.2

IPN-SA = Intraplaque neovascularization surface area, IQR = Inter quartile range, MPCP = Percentage of the plaque filled with contrast over time, PMI = Plaque mean intensity.

features and patients age were not statistically significant ($p > 0.14$). Comparison of the amount of IPN between patients with and without LDLR rest function (i.e. the residual capacity of the genetically mutated LDLR to bind LDL) showed no statistically significant difference between the groups. The current total cholesterol levels and the highest total cholesterol level without medical therapy showed no statistically significant correlations with the IPN features ($p > 0.5$).

DISCUSSION

In the present study the prevalence of subclinical atherosclerosis and IPN was assessed using standard ultrasound and CEUS in a consecutive group of patients with FH. All patients were asymptomatic and had no known atherosclerosis. Carotid plaque was present in 90% of these patients despite the entire study population receiving long-term statin treatment. The majority of the patients (86%) had ≥1 plaques with IPN. Semi-automated quantification software was used to precisely assess the amount of contrast-enhancement in the plaques. Atherosclerotic plaques with an irregular or ulcerated surface had significantly more IPN than plaques with a smooth surface ($p < 0.05$).

Limited data is available on the prevalence of carotid atherosclerosis in asymptomatic patients with FH and information on IPN in these patients is currently lacking. It has become clear that IPN is a marker of the instable or vulnerable atherosclerotic plaque [11-20]. The intraplaque neovessels are probably formed to meet the increased metabolic demands of the developing atherosclerotic plaque. However, due to their poor structural integrity the intraplaque neovessels may serve a pathologic role [21]. These microvessels are immature thin-walled vessels that may leak lipids, glucose and red blood cells which attracts macrophages and may lead to progression into a more advanced plaque type [22]. Plaques with a high IPN density are consequently at an increased risk of intraplaque hemorrhage and plaque rupture which may lead to cerebrovascular events [23]. Information on IPN may be particularly relevant in patients with FH, for a better understanding of the pathophysiology and progression of atherosclerosis, development of new treatment approaches, and risk stratification.

Previous studies have reported on the prevalence of plaque and IPN in patients without FH. Staub et al. [24] used CEUS to study the prevalence of IPN in 159 patients (61% men, age 64 ± 11 years) who underwent carotid ultrasound for a clinical indication. Established cardiovascular disease was documented in 61% of the patients. Standard ultrasound and CEUS demonstrated ≥1 plaques in 111 (76%) patients; the maximum plaque thickness was 2.8 ± 0.8 mm. CEUS demonstrated IPN in 54% of the patients. Patients with IPN more frequently had a history of cardiovascular disease as compared to patients without IPN

[24]. Xiong et al. [25] studied IPN with CEUS in 104 patients (80% men, age 64 ± 9 years) who underwent carotid ultrasound for a clinical indication and had ≥1 plaque thicker than 2.0 mm. A relatively large proportion (34%) of the patients were symptomatic (i.e. had a previous transient ischemic attack and/or stroke). Per inclusion criterion, all patients had a carotid plaque. IPN was present in 80% of the symptomatic patients, and in 30% of the asymptomatic patients. The authors concluded that symptomatic patients had more intense contrast enhancement in the plaque than asymptomatic patients suggesting that CEUS may be used for plaque risk stratification [25].

In comparison to these 2 previous studies, the present study included asymptomatic patients with heterozygous FH, without known atherosclerosis. The patients had no clinical indication for carotid ultrasound, and were asked to participate in this research project. The current study included a higher proportion of women (48%) than in the previous studies 24-25. Additionally, the mean age of our patients was substantially lower (55 ± 8 years), and nearly all patients received statin therapy (96%). Despite this, the mean plaque thickness was comparable with the previously reported findings. The patients with FH more frequently exhibited IPN as compared with the 2 previous reports. This finding suggests that patients with FH exhibit more vascularized atherosclerotic plaques, which may be of the vulnerable plaque type. This is supported by the fact that irregular and ulcerated plaques exhibited more IPN. In this study dedicated quantification software was used to accurately assess the amount of IPN. Previously, Xiong et al. [25] used a semi-automated quantitative scoring system for IPN, but that method lacked a motion compensation tool to adjust for pulsatility of the carotid artery and motion due to breathing of the patient [25]. The semi-automated quantification software used in this study was equipped with motion compensation tools and provided multiple features on contrast-enhancement of the atherosclerotic plaques. This allows a more accurate evaluation of IPN, and may be used to monitor changes in IPN density over time or following treatment.

The current findings are in accordance with clinical observations that patients with FH are at severely increased risk of developing symptomatic atherosclerosis at relatively young age. Statin treatment significantly reduces morbidity and mortality in these patients. The beneficial effect of statin therapy is perhaps not only caused by the inhibition of cholesterol synthesis in the liver, but may also have a stabilizing effect on atherosclerotic plaques. In animal models of atherosclerosis, statin treatment has been shown to inhibit IPN [26]: 14 pigs were randomized to a normal diet (n = 5), a high cholesterol (HC) diet (n = 5) or a HC diet with simvastatin (n = 4). After 12 weeks, the animals were sacrificed and micro-CT demonstrated an increased density of vasa vasorum in coronary specimens of animals on a HC diet whereas animals on a HC diet and statin therapy had a lower density of vasa-vasorum. A recent study in 28 patients (82% male, mean age 55 ± 6 years) with coronary and/or carotid atherosclerosis has reported a similar

effect of statin treatment [27]. Dynamic contrast-enhanced magnetic resonance imaging of the carotid arteries demonstrated a significant reduction of adventitial vasa vasorum signal after one year of statin treatment. Efforts to develop novel treatment strategies to reduce progression of atherosclerosis and inhibit IPN may have substantial clinical benefits.

This study has limitations. First, the study population was relatively small. Further studies are needed to confirm the findings in larger populations. Second, no age and gender matched control subjects were available to compare the amount of IPN between patients with FH and patients without FH. Future studies should aim to investigate whether there are difference in the amount of IPN in patients with FH and patients without FH. Third, in the comparison between age groups, the cut-off value of 50 year was chosen arbitrary without scientific arguments. A different cut-off value to divide the patients in age groups could possibly lead to different results. Fourth, prospective follow-up studies are needed to evaluate the prognostic implications of carotid plaque and carotid IPN in patients with FH. Finally, this study did not investigate the prevalence and density of adventitial microvascularization. Future studies should aim to investigate this topic in patients with FH.

CONCLUSION

Carotid ultrasound demonstrated atherosclerotic plaque in 90% of asymptomatic patients with FH without known atherosclerosis. IPN assessed with CEUS, was present in 86% of these patients. Irregular and ulcerated plaques exhibited significantly more IPN than plaques with a smooth surface.

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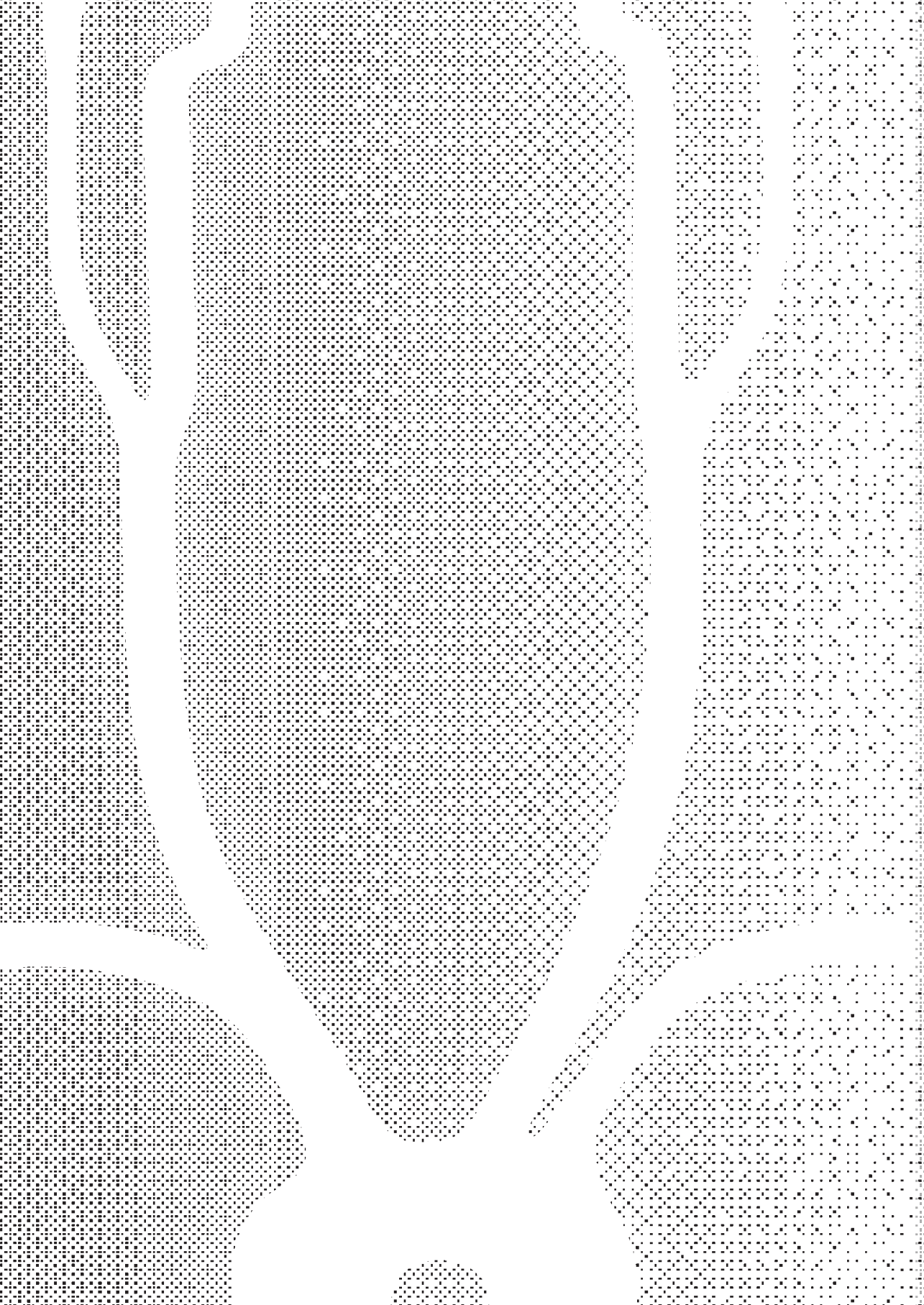

CHAPTER 9

ASSESSMENT OF CAROTID ATHEROSCLEROSIS, INTRAPLAQUE NEOVASCULARIZATION, AND PLAQUE ULCERATION USING QUANTITATIVE CONTRAST-ENHANCED ULTRASOUND IN ASYMPTOMATIC PATIENTS WITH DIABETES MELLITUS

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ABSTRACT

Aims: Patients with diabetes mellitus (DM) are at severely increased risk of developing atherosclerosis. Intraplaque neovascularization (IPN) and plaque ulceration are markers of the vulnerable plaque, which is at increased risk of rupture and may lead to cardiovascular events. The aim of this study was to assess the prevalence of subclinical carotid atherosclerosis, intraplaque neovascularization (IPN), and plaque ulceration in asymptomatic patients with DM.

Methods and results: A total of 51 asymptomatic patients with DM underwent standard carotid ultrasound in conjunction with contrast-enhanced ultrasound (CEUS) to assess the prevalence of subclinical atherosclerosis, IPN, and plaque ulceration. Subclinical atherosclerosis was defined as the presence of atherosclerotic plaque, according to the Mannheim consensus. Semi-automated quantification software was used to assess IPN in suitable plaques. Plaque ulceration was defined as a disruption of the plaque-lumen border ≥1x1 mm. A total of 408 carotid segments in 102 carotid arteries were investigated. Forty-six (90%) patients had subclinical atherosclerotic plaques, with a median plaque thickness of 2.4 mm (Inter quartile range 1.9-3.0). CEUS revealed IPN in 88% of the patients. In 10 carotid segments (8%) the plaque had an ulcerated surface. The presence of IPN could not be predicted by the presence of clinical characteristics including complications of DM ($p>0.05$).

Conclusion: Patients with DM have a high prevalence (90%) of subclinical carotid atherosclerosis. Severe IPN and plaque ulceration, which are markers of the vulnerable plaque type, were detected in respectively 13% and 9% of these patients.

INTRODUCTION

Diabetes mellitus (DM) is an important cause of microvasculopathy and macrovasculopathy, which may lead to complications in several organ systems [1]. Vascular complications of DM are characterized by inflammation and the formation of atherosclerotic plaques [2]. Several histological components, including intraplaque neovessels (IPN) and plaque ulceration, have been related to atherosclerotic plaque progression in diabetic patients [3]. The presence of IPN is a hallmark of the vulnerable atherosclerotic plaque [4–6]. Detection of those vulnerable plaques may improve risk stratification of patients with DM.

Ultrasound is a non-invasive and relatively low-cost method to assess subclinical atherosclerotic disease in the carotid arteries. Using contrast-enhanced ultrasound (CEUS), the presence of IPN and plaque ulceration can be assessed [7–9]. So far, several studies have shown that the amount of IPN and plaque ulceration are associated with future cardiovascular events [4,5,10]. Nevertheless, no prospective studies in asymptomatic patients have been performed yet, and no studies provide specific reports of the prevalence of subclinical atherosclerosis, IPN or plaque ulceration in patients with DM. Therefore, the purpose of this study was to assess the prevalence of subclinical atherosclerosis, IPN and plaque ulceration in asymptomatic patients with diabetes mellitus. Carotid ultrasound and CEUS were used to assess subclinical atherosclerosis and vulnerable plaque characteristics.

METHODS

Patient population and study protocol

A Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, USA), equipped with a L9-3 transducer was used for acquisition of carotid ultrasound clips. Image acquisition was performed using a scanning protocol according to the American Society of Echocardiography consensus [12]. In short, both left and right carotid arteries were examined with the patient in a supine position with the head turned at a 45° angle to the contralateral side. The left and the right common carotid artery (CCA), carotid bifurcation (BIF), internal carotid artery (ICA), external carotid artery (ECA), and vertebral arteries were imaged using standard carotid ultrasound. Standard carotid ultrasound included B-mode ultrasound, color Doppler and pulse-wave Doppler. All anatomical sites were examined from different angles of view. Gain and imaging depth were adjusted per patient to obtain optimal ultrasound images. Each side was extensively evaluated for the presence of plaques.

For CEUS the contrast mode of the ultrasound system was used to optimize the contrast enhanced ultrasound clips. The contrast mode of the ultrasound system was based on amplitude modulation, a mechanical index of 0.06 and a dual display mode for simultaneous B-mode ultrasound and CEUS view. CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). The ultrasound contrast agent was injected in boluses of 0.5 ml. Each contrast agent bolus was followed by a saline flush using 2.0 ml NaCl 0.9% solution. Bolus injections were repeated when necessary to a maximum dose of 9.6 ml. Both carotid arteries were examined, focusing on the presence of atherosclerotic plaques. If plaques were present, the plaque was visualized in the longitudinal axis of the carotid artery. Still frames and cineclips with a maximum of 20 seconds were digitally stored.

Carotid ultrasound acquisition

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Carotid ultrasound analysis

The carotid ultrasound studies were reviewed offline. First, carotid plaque screening was performed in the near and far-wall of the carotid arteries using all ultrasound clips of the standard carotid ultrasound examination and the CEUS examination. Atherosclerotic plaque was defined according to the consensus statement as a focal structure encroaching into the lumen of at least 0.5 mm or 50% of the surrounding carotid intima-media thickness, or demonstrates a thickness >1.5 mm as measured from the media-adventitia interface to the intima-lumen interface [13]. The presence of plaque and maximum plaque thickness was recorded for each carotid segment (i.e. CCA, BIF, ICA and ECA). The maximum atherosclerotic plaque thickness was measured manually perpendicular to the carotid longitudinal axis. In addition, for each segment with signs of atherosclerosis the presence of calcification, wall irregularities and/or ulceration were recorded. Plaque ulceration was defined as a disruption in the plaque-lumen border ≥1×1mm [14].

IPN was assessed using the CEUS clips in near wall plaques. Clips without atherosclerotic plaques or with plaques that could have been affected by pseudo-enhancement were not suitable for the assessment of IPN. Pseudo-enhancement is an artifact that is mainly seen in the far-wall of the carotid artery and mimics contrast-enhancement which may lead to overestimation of IPN [15,16]. The artifact also appears behind other contrast pools (e.g. jugular vein). For each carotid artery with ≥1 atherosclerotic plaque on the near wall of the carotid artery, the clip with visually the highest amount of contrast enhancement was selected for analysis (Figure 1). In patients with bilateral plaques, the thickest plaque was selected for patient based analysis. IPN was assessed using a semi-quantitative visual grading scale and using semi-automated quantification software. The visual grading scale was based on a 3 point grading scale as 0 = no IPN, 1 = mild to moderate IPN and 2 = severe IPN. Semi-automated quantification of IPN was performed using dedicated software (Figure 1) [17]. In short, several plaque perfusion features were calculated after motion compensation of regions of interest (i.e. atherosclerotic plaque) and selection of the time-frame. The calculations were based on maximum intensity projection images and time intensity curves. Features that were calculated included IPN surface area in mm2 (IPN-SA), IPN surface area ratio in % (IPN-SA ratio), mean percentage of the plaque filled with contrast over time (MPCP), the plaque mean intensity (PMI) and plaque area. The reproducibility of the parameters of this software was good to excellent. Intra-observer intra-class correlation ranged from 0.835 to 0.984. Inter-observer intra-class correlation ranged from 0.682-0.968. Bland-Altman plots showed a low variability of repeated measurements [17].

Statistical analysis

Statistical analysis of the data was performed using SPSS 20.0 for Windows (SPSS, Chicago, USA). Continuous variables are presented as mean (standard deviation) or median [Inter-quartile range]. Categorical variables are expressed as number (percentage). The

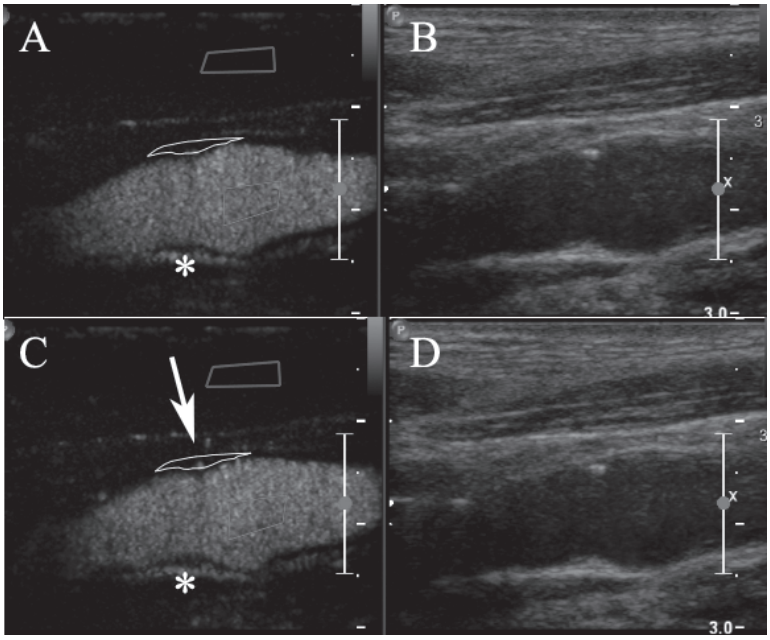

Figure 1
Example of semi-automated quantification of intraplaque neovessels (IPN) in carotid atherosclerosis. Panel A: contrast-enhanced ultrasound still frame with a yellow plaque region of interest (ROI), a red lumen ROI and a blue background ROI, recorded seconds after infusion of microbubble contrast agents. Panel B: corresponding B-mode still frame. Panel C: contrast-enhanced ultrasound still frame with a yellow plaque region of interest (ROI), a red lumen ROI and a blue background ROI (left), recorded seconds after the frames in the panels A&B. Contrast enhancement is observed in the plaque ROI (arrow). Panel D: corresponding B-mode still frame. Note: pseudo-enhancement appears in the far wall of the carotid artery (see *)

carotid atherosclerotic plaque with the highest thickness was selected for analysis with or between patients. Mann-Whitney U test was performed to compare the output of the semi-automated software in selected patient groups. A p value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Patient characteristics

The clinical characteristics of the study population are reported in Table 1. The study population had a mean age of 61 ± 6 years and contained 31 males (61%). On average, both males and females were overweight (BMI 29 ± 6). Males had a higher waist-to-hip ratio than females (0.99 ± 0.07 vs. 0.89 ± 0.08). A total of 45 patients (88%) had T2DM, the remaining six patients (12%) had T1DM (n=5, 10%) or ODM (n=1, 2%). Forty-nine

Table 1 Study population characteristics

Characteristic	Data (n=51)
Age (y)	61 ± 6
Female gender	20 (39%)
Height (cm)	174 ± 11
Weight (kg)	88 ± 20
BMI (kg/m^2)	29 ± 6
Waist-hip ratio	0.95 ± 0.09
Glucose (mmol/L)	9.1 ± 3.8
Glycated hemoglobin	
HbA1c (%)	7.7 (7.3-8.5)
HbA1c (mmol/mol)	58 (55-67)
HbA1c <7.5% or HbA1C <59mmol/mol	23 (45%)
Creatinin (µmol/L)	78 ± 26
Estimated glomerular filtration rate (ml/min)	81 (77-90)
Total cholesterol (mg/dL)	181 ± 46
Low density lipoprotein cholesterol (mg/dL)	104 ± 46
High density lipoprotein cholesterol (mg/dL)	54 ± 23
Triglycerides (mg/dL)	168 ± 115
Urine Albumin (mg/L)	7 (2-30)
Urine Albumin/Creatinin ratio (g/mol)	0.9 (0.4-3.2)
Hypertension	35 (69%)
Dyslipidemia	30 (59%)
Smoking (current/former)	18 (35%) / 6 (12%)
Familial history of cardiovascular disease	12 (24%)
Oral anti-diabetics	31 (61%)
Insulin	38 (75%)
Platelet aggregation inhibitor	8 (16%)
Diuretics	18 (35%)
Beta-blocker	11 (22%)
ACE-inhibitor	22 (43%)
Calcium antagonist	9 (18%)
Statin	41 (80%)
Ezetimibe	5 (10%)
Diabetic retinopathy	12 (24%)
Diabetic nephropathy	17 (33%)
Diabetic neuropathy	19 (37%)
Diabetic macrovasculopathy	9 (18%)
Coronary artery disease	7 (14%)
Peripheral artery disease	4 (8%)

BMI = Body mass index. Continuous variables are presented as mean ± standard deviation or as median (inter-quartile range). Categorical variables are presented as number of patients (percentage).

patients (96%) used oral anti-diabetics or insulin or a combination of both. Secondary complications due to DM were reported in 36 patients (71%). Nine patients (18%) were known to have vascular complications due to DM.

Carotid ultrasound findings

Using standard carotid ultrasound, subclinical atherosclerotic plaques were detected in 74 carotid arteries (73%). In these carotid arteries, 128 of 408 carotid segments (31%) had atherosclerotic plaque. The carotid segment which was most involved was the carotid bulb (n=68, 53%). In a total of 10 carotid segments (8%) the atherosclerotic plaque had an ulcerated plaque surface and plaques in 43 segments (34%) exhibited signs of calcification.

Assessment of IPN was performed using carotid CEUS. In a total of 69 carotid arteries (68%) atherosclerotic plaques were detected using CEUS. After exclusion of 34 carotid arteries (49%), which had only plaques that either were affected or could have been affected by pseudo-enhancement, the atherosclerotic plaques in 35 carotid arteries (51%) were suitable for assessment of IPN. Visual assessment of IPN showed no IPN in 4 of these arteries (12%), mild to moderate IPN in 26 carotid arteries (74%) and severe IPN in 5 carotid arteries (14%). Table 2 provides an overview of automated quantification and visual assessment of IPN for the individual carotid arteries and for the total patient group.

Table 2 Automated quantification and visual assessment of IPN in carotid atherosclerotic plaques

	All carotid arteries (n=35)	All patients (n=24)
IPN-SA (mm^2)	2.11 (1.24-2.88)	2.37 (1.29-3.08)
IPN-SA ratio (%)	38.4 (14.3-57.2)	36.1 (10.9-53.4)
MPCP (%)	14.0 (7.6-31.5)	11.0 (7.0-27.6)
PMI	1.34 (0.80-3.61)	1.34 (0.86-2.44)
Plaque area (mm^2)	6.17 (3.58-10.17)	6.67 (4.85-10.5)
Visual IPN grade		
No IPN	4 (12%)	3 (13%)
Mild to moderate IPN	26 (74%)	18 (75%)
Severe IPN	5 (14%)	3 (13%)

IPN-SA = intraplaque neovascularization surface area, IPN-SA ratio = intraplaque neovascularization surface area ratio, MPCP = mean percentage of the plaque filled with contrast over time, PMI = plaque mean intensity. Continuous variables are presented as median (inter-quartile range). Categorical variables are presented as number of patients (percentage)

Table 3 Ultrasound findings in the study population

Ultrasound characteristics	Standard carotid ultrasound (n=51)	Standard carotid ultrasound + Contrast-enhanced carotid ultrasound (n=51)
Presence of atherosclerotic plaques	43 (84%)	46 (90%)
Presence of atherosclerotic plaques in the right carotid artery	37 (73%)	41 (80%)
Presence of atherosclerotic plaques in the left carotid artery	37 (73%)	38 (75%)
Presence of bilateral atherosclerotic plaques	31 (61%)	35 (69%)
Median number of carotid segments with atherosclerotic plaque per patient	2 (1-4)	3 (2-4)
Median mean atherosclerotic plaque thickness (mm)	2.1 (1.7-2.6)	2.4 (1.9-3.0)
Median maximum atherosclerotic plaque thickness (mm)	2.3 (1.9-3.0)	1.9 (1.7-2.3)
Presence of plaque ulceration	3 (6%)	4 (8%)

Continuous variables are presented as median (inter-quartile range). Categorical variables are presented as number of patients (percentage).

Patient based analysis

Table 3 provides an overview of the carotid ultrasound findings per patient. Using standard carotid ultrasound and CEUS, subclinical atherosclerosis was detected in 46 patients (90%). In 4 of these patients (9%), the plaque surface was ulcerated. A total of 24 patients (47%) had atherosclerotic plaques which were suitable for assessment of IPN. Analysis of differences in the amount of IPN was performed between patient groups with and without secondary complications due to DM. There was no statistically significant difference in the amount of IPN in patients with any secondary complications compared to patients without any secondary complications (all $p>0.05$). There was no statistically significant difference in the amount of IPN between 1. patients with or without diabetic retinopathy, 2. patients with or without diabetic nephropathy and 3. patients with or without diabetic neuropathy (all $p>0.05$). Patients without diabetic macrovasculopathy had higher values for mean percentage of the plaque filled with contrast over time (MCPCP) ($p<0.05$). Other automated quantification features showed no statistically significant difference for patients with or without diabetic macrovasculopathy ($p>0.05$).

DISCUSSION

The present study investigated the prevalence of subclinical atherosclerosis, IPN and plaque ulceration in patients with DM. Using standard carotid ultrasound and CEUS of the carotid arteries, subclinical atherosclerosis was detected in 90% of the asymptomatic patients, despite regular statin use (80%) and values of HbA1c within the normal-range

in 45% of the patients. A total of 8% of the atherosclerotic plaques were found to have ulcerations. In the patients with atherosclerotic plaques that were suitable for the assessment of IPN, 88% of the carotid arteries showed signs of IPN, of which 5 (14%) had severe IPN. Semi-automated quantification software was used to accurately assess the amount of IPN in these arteries. The prevalence and amount of IPN in the asymptomatic patients with DM were relatively high. The amount of IPN could not be reliably predicted by clinical characteristics including the presence of secondary complications due to DM (all $p>0.05$).

This study demonstrates that subclinical carotid atherosclerosis is highly prevalent in patients with DM. Carotid CEUS revealed that a substantial proportion of the carotid plaques in patients with DM contain IPN, and thus may be of the vulnerable plaque type. In fact, the presence of plaque ulceration demonstrates that in a minority of the patients, the plaque surface was disrupted. A high density of IPN and carotid plaque ulcerations have been related to the occurrence of future cardiovascular events. The results demonstrate the necessity for the development of therapeutic strategies that focus on stabilization of vulnerable atherosclerotic plaques in patients with DM. Eventually, IPN as detected using carotid CEUS may develop into a target to monitor therapeutic strategies to stabilize atherosclerotic plaques.

Recent studies indicated the value of IPN in predicting future cardiovascular events in patients who recently suffered a cardiovascular event [4,10]. The presence of IPN in atherosclerotic plaques may contribute to destabilization of the atherosclerotic plaques, leading to an increased risk of rupture [18]. Another parameter that is linked to plaque vulnerability is the presence of plaque ulceration. The presence of ulcerations in atherosclerotic plaques was associated with an increased risk of stroke [5,6]. Unfortunately, limited data is available on IPN and plaque ulceration in asymptomatic patients. This study included 51 asymptomatic patients with DM and demonstrates that these patients have a high prevalence of subclinical atherosclerosis (90%). IPN and plaque ulceration, which are markers of the vulnerable plaque type, were detected in respectively 88% and 9% of these patients. This probably indicates that these patients are at an increased risk for future cardiovascular events.

Previous studies have reported on the prevalence of IPN in other patient groups. Staub et al. performed standard carotid ultrasound and CEUS in 147 patients (mean age 64 ± 11 years) with a clinical indication for a carotid duplex ultrasound examination [19]. The majority of the population had an increased cardiovascular risk profile and 31% of the patients had DM. Atherosclerotic plaques were detected in 76% of the patients (maximum plaque thickness 2.8 ± 0.8 mm). IPN was noted in 60 patients (54%). Xiong et al. investigated the correlation of carotid plaque vascularization with clinical symptoms [20]. They assessed the amount of IPN using semi-automated contrast-enhancement analysis in 35 symptomatic and 69 asymptomatic patients (mean age 63 ± 9 years) with at least

one carotid atherosclerotic plaque >2mm thickness. A total of 35 of these patients (34%) had DM. The amount of IPN was statistically significant lower in the asymptomatic patients, but no data was reported according to individual cardiovascular risk factors such as DM. Although previous studies. reported on IPN in their populations, a direct comparison to the present study seems not appropriate. First, in the previious studies a different grading scale was used for the evaluation of IPN, whereas this study used quantitative assessment of IPN. Second, the previous studies included patients with a clinical indication for carotid ultrasound whereas this study included asymptomatic patients who were recruited for CEUS as part of the research protocol. Third in one of those studies a considerable number of patients had known coronary artery disease (n=77, 52%) [19], whereas the present study included only 14% patients with known coronary artery disease.

Carotid CEUS may have a role in the risk stratification of patients with DM. Patients with DM are at severely increased risk of developing atherosclerosis. Previous studies have demonstrated that a substantial proportion of patients with DM have plaques with IPN [3,21]. The presence of IPN and plaque ulceration in patients with DM is an adverse sign, because these are indicators of plaque instability [21]. Unstable or vulnerable plaques are at increased risk of rupture, which may lead to acute clinical events. The present results demonstrate that CEUS allows in-vivo assessment of vulnerable plaque characteristics (IPN and plaque ulceration) in patients with DM. The detection of IPN and plaque ulceration using CEUS could be implemented in clinical practice to identify plaques that are at high-risk of causing clinical events. Additional prospective follow-up studies are needed to develop accurate risk stratification models, including clinical parameters and information provided by CEUS, for these patients. The results also demonstrate the necessity for the development of therapeutic strategies that focus on stabilization of vulnerable atherosclerotic plaques. There are indications that carotid CEUS may provide valuabel information on cronary risk status. A recent study by Deyama et al. [22] suggest that a higher grade of carotid IPN assessed using CEUS was associated with more complex and extensive coronary lesions and a higher prevalence of multivessel coronary artery disease as assessed by coronary angiography. However, the results in the study by Deyama et al. may have been influenced by pseudoenhancement. In the current study, the amount of IPN could not be reliably predicted by clinical characteristics including the presence of diabetic retinopathy, nefropathy and neuropathy. The lack of association between IPN and secondary complications of DM may be related to the vascularization paradox in patients with DM, which is based on observations that angiogenesis within the plaque is induced and arteriogenesis is impaired. Further studies are needed to fully elucidate this issue.

This study has several limitations. First, the study was limited by the relatively small number of patients (n=51) and carotid arteries (n=102). Second, 34 carotid arteries were

excluded from the analysis because of apparent or possible pseudo-enhancement. New pulse-sequences are currently being developed to overcome the hindering artifact. This new method is based on counter-propagation interation between a low-frequency and a high-frequency ultrasound wave [23]. The use of artifact-free contrast detection method may improve the accuracy of carotid CEUS, especially in far-wall plaques. In addition, other technical improvements such as the development of a 3D ultrasound probe could further improve visualization of IPN using carotid CEUS. Third, follow-up studies are needed to evaluate the prognostic value of carotid CEUS for the prediction of clinical outcome in patients with DM.

In conclusion, carotid ultrasound demonstrated subclinical atherosclerosis in 90% of asymptomatic patients with DM. Severe IPN and plaque ulceration, which are considered to be markers of plaque vulnerability, were present in respectively 13% and 9% of these patients. No associations were found between the amount of IPN and the presence of complications of DM.

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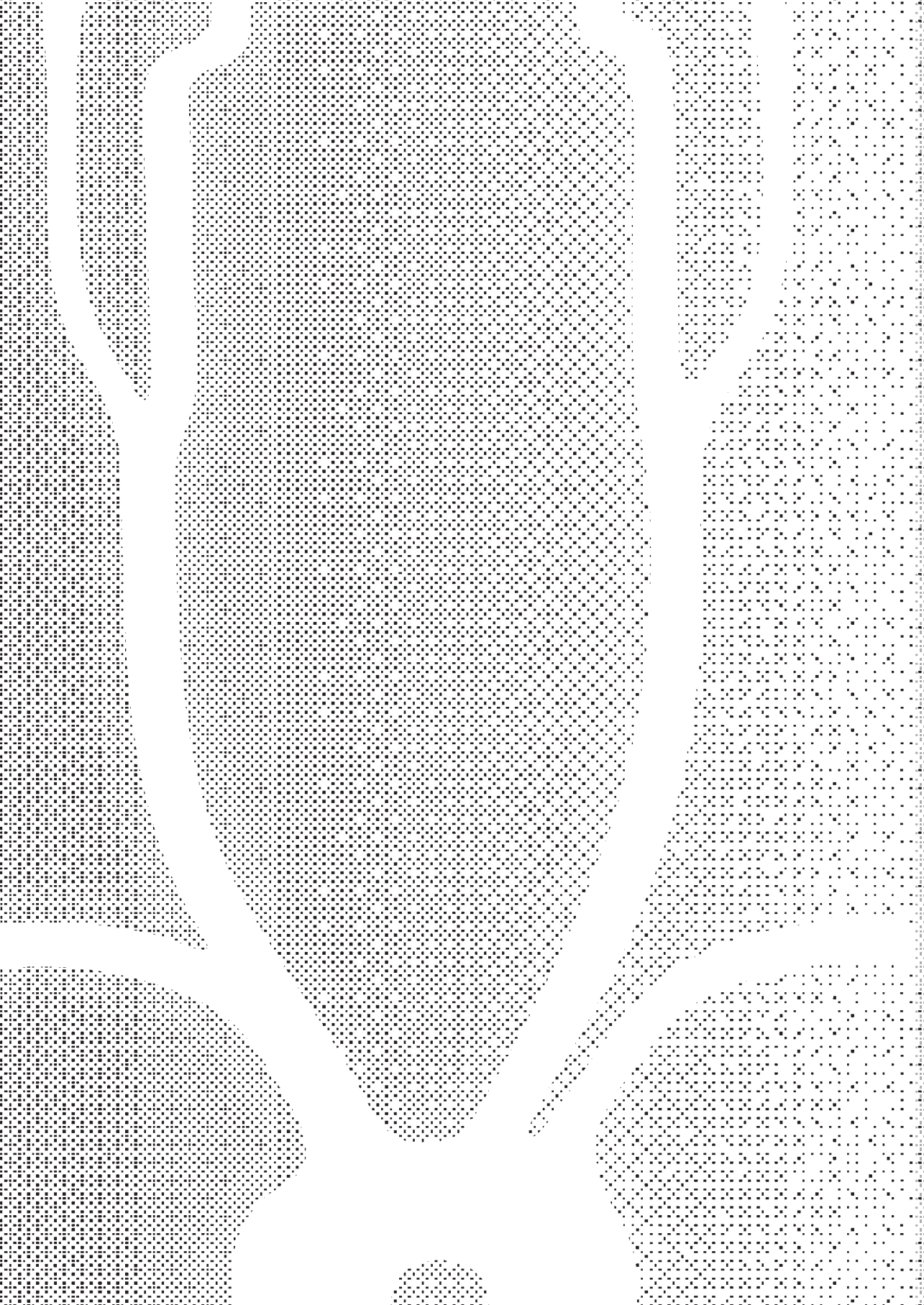

CHAPTER 10

IMPACT OF GENDER ON THE DENSITY OF INTRAPLAQUE NEOVASCULARIZATION: A QUANTITATIVE CONTRAST-ENHANCED ULTRASOUND STUDY

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ABSTRACT

Objective: Atherosclerosis is the main underlying cause of the majority of cardiovascular events. Although cardiovascular diseases (CVD) are a major challenge in both males and females, gender specific differences in the prevalence of CVD have been observed. This may indicate that there are differences in the development of atherosclerosis between males and females. The presence of intraplaque neovessels (IPN) is an imaging marker for plaque vulnerability. The aim of this study was to investigate the impact of gender on IPN.

Methods: A total of 159 patients with ≥1 cardiovascular risk factor were included in this prospective study (mean age 56.9 ± 8.7 years, 47% females). Patients had no symptoms of carotid atherosclerotic disease. All patients underwent a standard carotid ultrasound examination in conjunction with contrast-enhanced ultrasound (CEUS). The presence of atherosclerotic plaques was assessed according to the Mannheim consensus. IPN was assessed using a visual grading scale and semi-automated quantification software.

Results: Subclinical atherosclerosis was detected using standard carotid ultrasound and CEUS in 64 females (86%) and in 79 males (93%) ($p = 0.177$). The mean atherosclerotic plaque sizes were not significantly different ($p = 0.068$). Semi-automated quantification of IPN demonstrated that females had significant more IPN compared to males ($p < 0.05$). After adjustment for clinical variables this association remained significant ($p < 0.05$).

Conclusion: In this population at increased risk for CVD, females had significantly more IPN compared to males. This suggests that the females had a more vulnerable atherosclerotic plaque type.

INTRODUCTION

Each year, more than 10 million people die due to stroke or coronary heart disease and atherosclerosis is the main underlying cause of these cardiovascular events (CVE) [1,2]. Although cardiovascular diseases (CVD) are a major challenge in both males and females, gender specific differences in the prevalence of CVD have been observed [3]. This may indicate that there are differences in the development of atherosclerosis between males and females. Several studies have reported on gender specific differences in atherosclerotic plaque composition. Females tend to have a more stable atherosclerotic plaque type than males [4-6]. Vulnerable atherosclerotic plaques have an increased risk of rupture, and may cause cardiovascular events. These vulnerable atherosclerotic plaques are characterized by a lipid rich necrotic core and a thin fibrous cap, intraplaque hemorrhage and the presence of intraplaque neovessels (IPN) [7-9]. Contrast enhanced ultrasound (CEUS) is an advanced form of ultrasound that allows dynamic assessment of IPN in carotid atherosclerotic plaques [10,11]. So far, no studies have investigated whether there are gender-specific differences in the density of IPN. Therefore the aim of this study was to investigate the impact of gender on IPN assessed using carotid CEUS in asymptomatic patients at increased risk of atherosclerosis.

METHODS

Patient population and study protocol

This study was set-up as a prospective observational study. Between April 2010 and May 2013 patients from the outpatient clinics of Cardiology and Vascular Medicine in the Erasmus Medical Center were asked to participate in this study. Inclusion criteria were age ≥18 years, and ≥1 cardiovascular risk factor (i.e. hypertension, diabetes mellitus, dyslipidemia, smoking, and/or a family history of cardiovascular disease). Dyslipidemia was defined as total serum cholesterol ≥200 mg/dl. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg or treatment with antihypertensive medication. Diabetes mellitus was defined as the use of anti-diabetic medication or a diagnosis of diabetes mellitus according to current guidelines [12]. None of the patients had symptoms of carotid atherosclerotic disease. Exclusion criteria were contra-indications for the use of ultrasound contrast agent, such as unstable angina, acute cardiac failure, acute endocarditis, known right-to-left shunts and known allergy for microbubble contrast agents. Clinical characteristics were documented and a physical examination was performed prior to the ultrasound examination, including weight, height, waist-to-hip ratio (WHR) and blood pressure measurements. The study protocol was approved by the local ethical committee and all patients provided written informed

consent. All patients underwent a standard ultrasound examination in conjunction with CEUS of the carotid arteries.

Carotid ultrasound acquisition

Standard carotid ultrasound and CEUS were performed with a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, USA), equipped with an L9-3 transducer. Standard carotid ultrasound included acquisition of B-mode clips and color Doppler clips. Image acquisition was performed using a standard scanning protocol according to the American Society of Echocardiography consensus statement [13]. In short, both left and right carotid arteries were examined with the patient in a supine position with the head supported at a 45° angle turned to the contralateral side. The left and the right common carotid artery (CCA), carotid bifurcation (BIF), internal carotid artery (ICA), external carotid artery (ECA), and vertebral arteries were imaged by B-mode ultrasound, color Doppler and pulse-wave Doppler. All anatomical sites were examined from different angles of view and a short axis sweep was executed to detect atherosclerotic plaques. Each side was extensively evaluated for the presence of carotid plaques. Gain and imaging depth were adjusted per patient to obtain optimal ultrasound images.

After standard carotid ultrasound, CEUS was performed using intravenous administration of SonoVue™ contrast agent (Bracco S.p.A., Milan, Italy). The contrast mode of the ultrasound system was selected using amplitude modulation to enhance the ultrasound contrast and suppress the tissue. A mechanical index of 0.06–0.08 was used to optimize the CEUS examination. CEUS clips were acquired according to a standard acquisition protocol. CEUS clips were recorded with a side-by-side display mode for simultaneous standard B-mode ultrasound and CEUS view. The ultrasound contrast agent was injected in boluses of 0.5 ml followed by a flush with 2.0 ml NaCl 0.9% solution. After administration of contrast agent, high-quality contrast images could be obtained for approximately 1 min. Bolus injections of the contrast agent were repeated when necessary up to a maximum total dose of 9.6 ml. Both carotid arteries were examined, focusing on the presence of atherosclerotic plaques. If plaques were present, the plaque was visualized in the longitudinal axis of the carotid artery. Still frames and cineclips were digitally stored.

Carotid ultrasound analysis

Standard carotid ultrasound clips and CEUS clips were reviewed offline. First, carotid plaque screening was performed using standard carotid ultrasound clips including color Doppler and CEUS clips. Atherosclerotic plaque was defined as a focal structure encroaching into the lumen of at least 0.5 mm or 50% of the surrounding CIMT, or demonstrating a thickness >1.5 mm as measured from the media–adventitia interface to the intima–lumen interface [14]. The presence of atherosclerotic plaques was recorded for each side and

all individual carotid segments (i.e. CCA, BIF, ICA, ECA). Maximum atherosclerotic plaque thickness was measured perpendicular to the carotid longitudinal axis. In addition, for each segment that exhibited atherosclerotic plaques, the presence of wall irregularities and/or ulcerations was recorded. Atherosclerotic plaque ulceration was defined as a disruption in the plaque-lumen border ≥1 × 1 mm. If plaques were present, carotid luminal diameter was measured proximal from the atherosclerotic plaque.

Assessment of IPN was performed using the CEUS clips. Due to pseudo-enhancement artifact only near-wall plaques were eligible for assessment of IPN [15,16]. Clips without atherosclerotic plaques or with atherosclerotic plaques that could have been affected by pseudo-enhancement were not eligible and as a consequence excluded from further analysis. Therefore, all far wall plaques and all near-wall plaques that were behind a contrast-pool (e.g. jugular vein) were excluded from assessment of IPN. Per carotid artery with ≥1 atherosclerotic plaque on the near wall of the carotid artery, the clip with visually the highest amount of contrast enhancement was selected for analysis (Fig. 1). In patients with bilateral plaques, the thickest plaque was selected for patient based analysis. The amount of intraplaque neovascularization (IPN) was visually assessed according using a previously published 3 point ordinal scale as 0 = absent IPN, 1 = mild to moderate IPN and 2 = extensive IPN [17]. Semi-automated quantification of IPN was performed using dedicated software. DICOM files of side-by-side B-Mode and contrast mode ultrasound clips were imported in the software to assess different plaque perfusion parameters after selection of the time-frame. The software calculated several parameters based on region-of-interest (ROI) analysis after motion compensation of the

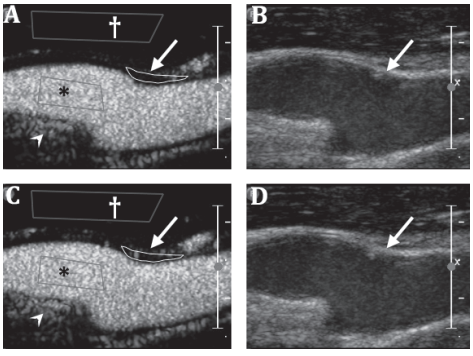


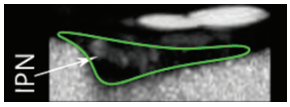
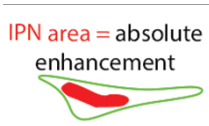
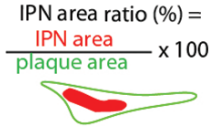
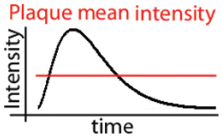
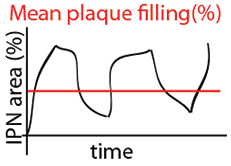
Figure 1
Side-by-side images of CEUS and corresponding B-mode carotid ultrasound. An atherosclerotic plaque was detected in the carotid bulb (arrows). CEUS image A and B-mode image B were recorded simultaneous just after injection of contrast. Minimal contrast-enhancement is observed within the atherosclerotic plaque (image A, arrow). CEUS image C and B-mode image D were recorded approximately 3 seconds after images A and B. Contrast-enhancement in the atherosclerotic plaque is observed (image C, arrow). Regions of interest in the lumen (*) and in the background (†) were used for standardization of the contrast enhancement. Quantification was performed using semi-automated quantification software. The arrowheads in CEUS images A&C are pointing at pseudo-enhancement in the far wall of the carotid artery due to non-linear propagation artifacts.

carotid artery. These calculations were based on maximum intensity projections and time intensity curves which resulted in the following parameters: 1. IPN surface area in mm2 (IPN-SA), 2. IPN surface area ratio in % (IPN-SA ratio), 3. mean percentage of the plaque filled with contrast over time (MPCP), 4. plaque mean intensity (PMI) and 5. plaque area in mm2 [18]. Table 1 provides a short description of how the parameters were derived. The reproducibility of the parameters of this software was good to excellent. Intra-observer intra-class correlation ranged from 0.835 to 0.984. Inter-observer intra-class correlation ranged from 0.682 to 0.968. Bland–Altman plots showed a low variability of repeated measurements. The reproducibility of the measurements was reported by Akkus et al. [18].

Statistical analysis

Statistical analysis of the data was performed using SPSS PASW software for Windows (Version 20.0.0, SPSS Inc., Chicago, IL, USA). Continuous and ordinal data were expressed as mean ± standard deviation or median and inter-quartile range. Categorical variables were expressed as counts and percentages and/or median value. Differences in patient characteristics were analyzed. Depending on their distribution, continuous variables

Table 1 Definitions of different plaque perfusion parameters

	Example of a maximum intensity projection (MIP) of a region of interest (ROI) in the atherosclerotic plaque. This projection is used for calculation of the intraplaque-neovascularization (IPN) parameters
	Graphical description of IPN surface area (IPN-SA). IPN-SA is calculated as the absolute enhancement of contrast in the atherosclerotic plaque ROI.
	Graphical description of IPN surface area ratio (IPN-SA ratio). IPN-SA ratio is calculated as the absolute IPN area relative to the total atherosclerotic plaque ROI area.
	Graphical description of plaque mean intensity (PMI). PMI is calculated as the mean intensity value of the atherosclerotic plaque ROI over time.
	Graphical description of mean percentage of the plaque filled with contrast over time (MPCP). MPCP is calculated as the mean IPN area ratio over time.

were compared using the student's T test or the Mann-Whitney U test. Differences between proportions were compared using the Chi-squared test. Correlations between serum cholesterol levels, insulin use waist-hip ratio and IPN parameters were tested using Spearman's Rho. Differences in the amount of IPN between males and females were assessed using the non-parametric Mann-Whitney U test. Linear regression was used for univariate and multivariate analysis of the association between the IPN parameters and gender. In the multivariate analysis the association between IPN parameters and gender was adjusted for age, BMI, hypertension, diabetes mellitus, hypercholesterolemia and smoking status. A p value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

The clinical characteristics of the study population (n = 159) are summarized in Table 2. A total of 74 females (47%) and 85 males (53%) of similar age were included in this study (mean age 56.9 ± 8.7 years). Both females and males were overweight (mean BMI 27.7 ± 4.7 versus mean BMI 27.1 ± 4.8, respectively, p = 0.430). The serum total cholesterol and serum HDL were significantly higher in females, whereas the serum triglycerides were significantly higher in males. There was no difference in statin use between females and males. All patients underwent bilateral standard carotid ultrasound and CEUS of the carotid arteries. No complications occurred during the standard carotid ultrasound and the CEUS examination.

Carotid ultrasound and CEUS results

Findings from the standard carotid ultrasound examination and CEUS are summarized in Table 3. Subclinical atherosclerotic plaques were detected using standard carotid ultrasound in 211 carotid arteries (66%) in 126 patients (79%). A total of 365 carotid segments (29%) showed subclinical atherosclerosis. The CCA was involved in 76 carotid arteries (36%), the BIF was involved in 196 arteries (92%), the ICA was involved in 80 carotid arteries (38%) and the ECA was involved in 12 carotid arteries (6%). Bilateral subclinical atherosclerosis was detected in 85 patients (54%). No significant differences in standard carotid ultrasound findings between females and males were detected, especially no differences in carotid luminal diameter (Table 3).

Using the combination of standard carotid ultrasound and CEUS subclinical atherosclerotic plaques were detected in 249 carotid arteries (78%) in 143 patients (90%). A total of 472 carotid segments (37%) showed subclinical atherosclerosis. The CCA was involved in 128 carotid arteries (51%), the BIF was involved in 237 carotid arteries (95%), the ICA was involved in 94 carotid arteries (38%) and the ECA was involved in 12 carotid

Table 2 Patient characteristics

	Total (n=159)	Females (n=74)	Males (n=85)	p-value
Age (years)	56.9 ± 8.7	56.7 ± 8.7	57.0 ± 8.7	0.858
Length (cm)	174 ± 11	166 ± 7	181 ± 8	<0.001
Weight (kg)	83 ± 17	74 ± 14	90 ± 16	<0.001
BMI (kg/m^2)	27.4 ± 4.7	27.1 ± 4.8	27.7 ± 4.6	0.430
Systolic blood pressure (mmHg)	135 ± 18	138 ± 18	133 ± 17	0.087
Diastolic blood pressure (mmHg)	77 ± 10	78 ± 11	76 ± 9	0.332
Waist-hip ratio	0.92 ± 0.09	0.86 ± 0.07	0.96 ± 0.07	<0.001
Diabetes mellitus	51 (32%)	20 (27%)	31 (36%)	0.325
Hypertension	60 (38%)	28 (38%)	32 (38%)	0.980
Dyslipidemia	112 (70%)	50 (68%)	62 (73%)	0.459
Familial history of CVD	68 (43%)	31 (42%)	37 (44%)	0.835
Current or former smoker	74 (47%)	30 (41%)	44 (52%)	0.275
Platelet aggregation inhibitor use	35 (22%)	19 (26%)	16 (19%)	0.277
Beta-blocker therapy	28 (18%)	11 (15%)	17 (20%)	0.418
ACE inhibitor therapy	31 (20%)	12 (16%)	19 (22%)	0.351
Calcium channel blocker therapy	17 (11%)	9 (12%)	8 (9%)	0.555
Diuretic therapy	28 (18%)	14 (19%)	14 (17%)	0.657
Statin therapy	126 (80%)	56 (77%)	70 (82%)	0.379
Ezetimibe therapy	40 (26%)	15 (21%)	25 (30%)	0.205
Oral anti-diabetic therapy	32 (20%)	15 (21%)	17 (20%)	0.932
Insulin therapy	38 (24%)	13 (18%)	25 (29%)	0.089
Glucose (mg/dL)	119 ± 50	113 ± 49	124 ± 54	0.208
Creatinin (µmol/L)	79 ± 19	69 ± 15	87 ± 19	<0.001
Total cholesterol (mmol/L)	5.2 ± 1.5	5.4 ± 1.8	4.9 ± 1.2	0.037
LDL cholesterol(mmol/L)	3.2 ± 1.5	3.4 ± 1.7	3.1 ± 1.2	0.229
HDL cholesterol(mmol/L)	1.4 ± 0.4	1.6 ± 0.5	1.3 ± 0.4	<0.001
Triglycerides (mmol/L)	1.5 ± 1.0	1.3 ± 0.8	1.6 ± 1.1	0.034

Data are presented as number of patients (percentage) or as mean ± standard deviation. Chi-squared test was used to compare proportions and students' T test was used to compare continuous variables. ACE = angiotensin converting enzyme, BMI = body mass index, CVD = cardiovascular disease, LDL = low density lipoprotein, HDL = high density lipoprotein.

arteries (5%). Bilateral subclinical atherosclerosis was detected in 102 patients (64%). No significant differences in CEUS findings between females and males were detected (Table 3).

The ultrasound clips of 101 patients (64%) were eligible for the assessment of IPN. Results of the assessment of IPN are summarized in Table 3. No significant correlations were found between serum cholesterol levels, insulin use and waist–hip ratio and IPN parameters ($p > 0.05$).

Gender based analysis of intraplaque neovascularization

Visual assessment of IPN showed no differences between females and males (p = 0.269). Semi-automated quantification of IPN showed that IPN-SA ratio was significantly higher

Table 3 Ultrasound findings

	Total (n=159)	Females (n=74)	Males (n=85)	p-value
Standard carotid ultrasound				
Subclinical atherosclerotic plaques (n)	126 (79%)	55 (74%)	71 (84%)	0.153
Carotid segments with atherosclerotic plaques (n)	2 (3)	2 (3)	2 (3)	0.154
Median atherosclerotic plaque thickness (mm)	2.1 (0.9)	2.0 (0.8)	2.2 (0.9)	0.308
Maximum atherosclerotic plaque thickness (mm)	2.4 (1.1)	2.3 (1.2)	2.5 (1.3)	0.557
Plaque surface				0.336
Regular	90 (71%)	43 (78%)	47 (66%)	
Irregular	27 (22%)	9 (16%)	18 (25%)	
Ulcerated	9 (7%))	3 (6%)	6 (9%)	
Standard carotid ultrasound + CEUS				
Subclinical atherosclerotic plaques	143 (90%)	64 (86%)	79 (93%)	0.177
Carotid segments with atherosclerotic plaques	3 (2)	3 (2)	3 (2)	0.150
Median atherosclerotic plaque thickness (mm)	1.8 (0.7)	1.8 (0.7)	1.9 (0.7)	0.316
Maximum atherosclerotic plaque thickness (mm)	2.4 (1.2)	2.2 (1.3)	2.4 (1.3)	0.419
Carotid luminal diameter	7.3 (2.0)	7.2 (1.7)	7.3 (2.1)	0.286
Plaque surface				0.618
Regular	97 (68%)	46 (72%)	51 (65%)	
Irregular	32 (22%)	13 (20%)	19 (24%)	
Ulcerated	14 (10%)	5 (8%)	9 (11%)	
Assessment of IPN				
Patients eligible for assessment of IPN (n)	101 (64%)	44 (59%)	57 (67%)	0.321
Visual IPN grade	1 (1)	2 (1)	1 (1)	0.269
IPN surface area (mm2)	2.1 (2.2)	2.3 (2.7)	2.0 (2.3)	0.229
IPN surface ratio (%)	40 (51)	49 (48)	29 (46)	0.030
MPCP (%)	20 (29)	26 (29)	12 (30)	0.064
Plaque mean intensity	1.82 (3.35)	2.56 (3.90)	1.41 (2.75)	0.037
Lumen mean intensity	115.01 (47.70)	118.49(48.28)	112.63 (46.8)	0.585
Atherosclerotic plaque area (mm2)	5.2 (4.5)	4.8 (3.2)	6.2 (6)	0.068

Categorical variables are presented as number of patients (percentage). Continuous and ordinal variables are presented as median (inter-quartile range). Chi-squared test was used to compare proportions and Mann-Whitney U test was used to compare continuous and ordinal variables. CEUS = contrast-enhanced ultrasound, IPN = intraplaque neovasuclarization, MPCP = mean percentage of the plaque filled with contrast over time.

in females when compared to males (females: 49%, males: 29%; p = 0.030). PMI was significantly higher in females when compared to males (females: 2.56, males: 1.41; p = 0.037). No statistically significant differences in plaque area measurement were present between females and males (p = 0.068). The IPN-SA (p = 0.229) and MPCP (p = 0.064) showed no statistically significant differences between females and males. Findings from the linear regression analysis are summarized in Table 4. Univariate analysis showed a statistically significant association between gender and the IPN-SA ratio (β = −12.269, p = 0.046). After adjustment for possible covariates this association remained significant (β = −13.557, p = 0.031). In univariate and multivariate analysis, no statistically significant associations were found between gender and IPN-SA, PMI and MPCP.

Table 4 Univariate and multivariate analysis of the impact of gender on IPN

	Univariate analysis [a]		Multivariate analysis [b]	
	Beta	p-value	Beta	p-value
IPN surface area	-0.377	0.383	-0.232	0.593
IPN surface ratio	-12.269	0.046	-13.557	0.031
MPCP	-5.339	0.290	-5.749	0.261
PMI	-0.573	0.464	-0.722	0.370

Univariate and multivariate analysis of the impact of gender on the amount of IPN in carotid atherosclerotic plaques assessed using CEUS. [a] = Beta for univariate association of gender with IPN feature. [b] = Beta for association of gender with IPN feature after adjustment for age, BMI, hypertension, diabetes mellitus, dyslipidemia and smoking status. BMI= body mass index, CEUS = contrast-enhanced ultrasound, IPN = intraplaque neovascularzation, MPCP = mean percentage of the plaque filled with contrast over time, PMI = plaque mean intensity.

DISCUSSION

This is the first study to investigate the impact of gender on IPN detected using CEUS. In a population with ≥1 cardiovascular risk factor, IPN was detected and quantified using CEUS of the carotid arteries. Subclinical atherosclerotic plaques were detected in 86% of the females and in 93% of the males, and the plaque size was comparable between females and males. Quantification of the amount of IPN showed that females had significantly more IPN compared to males. Multivariate analysis demonstrated that after adjustment for baseline variables this association was still significant. The presence of more IPN in females in this study may indicate that these females have a more vulnerable atherosclerotic plaque phenotype than males.

Prior studies investigated whether histological plaque characteristics are related to gender [4]. The study by Hellings et al. included 450 patients whom where scheduled for carotid surgery (mean age 67.3 ± 8.8 years, 315 males, symptomatic carotid atherosclerosis was present in 76% of the patients). Differences in atherosclerotic plaque composition were assessed in males and females. Males had significantly more atheromatous and fibroatheromatous atherosclerotic plaques than females. In addition, the atherosclerotic plaques of males exhibited more infiltration of macrophages whereas the plaques of females had more infiltration of smooth muscle cells. Based on these results, the authors concluded that females undergoing carotid surgery have more stable plaques than males. Sangiorgi investigated whether there were gender related differences in inflammatory plaque components in 457 patients (mean age 69.7 ± 7.1 years, 325 males, symptomatic carotid atherosclerosis was present in 50% of the patients). Using CD68 and CD3 monoclonal antibodies they characterized the inflammatory cells in the plaques. The numbers of CD68 and CD3 positive cells were counted in high magnification fields. These analyses demonstrated significantly more inflammation in atherosclerotic plaques of males. Multivariate analysis demonstrated that the difference in amount of inflammatory cells in atherosclerotic plaques between males and females was related to other variables such as stenosis severity and symptom status [6].

The findings of the current study do not confirm the hypothesis that females have more stable atherosclerotic plaques than males. In the present study, females had significantly more IPN than males. This discrepancy between the results of this study and previous studies [4,6] may be explained by several factors. First, the study populations were different: the present study included asymptomatic patients with subclinical atherosclerotic plaque, whereas previous studies included patients with a high-grade carotid stenosis who were referred for carotid endarterectomy. Second, plaque features in the present study were assessed in-vivo with CEUS whereas plaque characteristics in previous studies were assessed ex-vivo using histology. Third, plaque vulnerability in the present study was defined by IPN, whereas plaque vulnerability in previous studies

was defined by macrophages, smooth muscle cells, collagen, calcifications and luminal thrombus.

A number of studies have shown that IPN plays an important role in the progression and destabilization of atherosclerotic plaques [19-21]. The process of intraplaque neovascularization is triggered by multiple pro-angiogenic stimuli. One of the most important factors is medial hypoxia caused by progression of atherosclerosis [22]. Due to the hypoxic state, the physiologic vasa vasorum are triggered by VEGF and other pro-angiogenic factors to sprout through the adventitia to the atherosclerotic plaque. The intraplaque microvasculature contributes to the delivery of metabolic substrates and, more important, to the delivery of inflammatory cells and mediators. In addition, the intraplaque microvessels are immature and thin walled. This results in a fragile network which is at risk to rupture, causing intraplaque hemorrhage [23]. A recent follow-up study demonstrated that a high density of IPN in atherosclerotic plaques has been related to future CVE [8]. Therefore, the presence of more IPN in females in this study may indicate that these females have a more vulnerable atherosclerotic plaque phenotype than males and are at increased risk of future CVE.

This study has limitations. First of all, 29% of the patients with subclinical atherosclerosis were excluded from analysis of IPN due to pseudo-enhancement in the far wall of the carotid artery. Nevertheless, there was no statistically significant difference in the amount of exclusions between males and females. In addition, per carotid artery ≥1 atherosclerotic plaque on the near-wall of the carotid artery only one clip was selected for analysis. This could have led to selection bias. Second, this study diagnosed atherosclerotic plaque according to current guidelines for carotid ultrasound [14]. Therefore, other processes that cause vessel wall thickening such as media hypertrophy or vasculitis could have been falsely diagnosed as atherosclerotic plaque. This may have altered the results. Third, there was no data on the hormonal status of the females in this study. Therefore we cannot assess whether differences in IPN may also be related to sex-hormones. Fourth, no follow-up data was available. Therefore, the clinical value of the detection of IPN using CEUS in asymptomatic patients is still unknown and needs to be tested prospectively in future research.

CONCLUSION

In this asymptomatic population with ≥1 cardiovascular risk factor, subclinical atherosclerosis was detected in 90% of the patients. Assessment of IPN using CEUS showed that females had statistically significantly more IPN than males. This could indicate that these females had more vulnerable atherosclerotic plaques than males.

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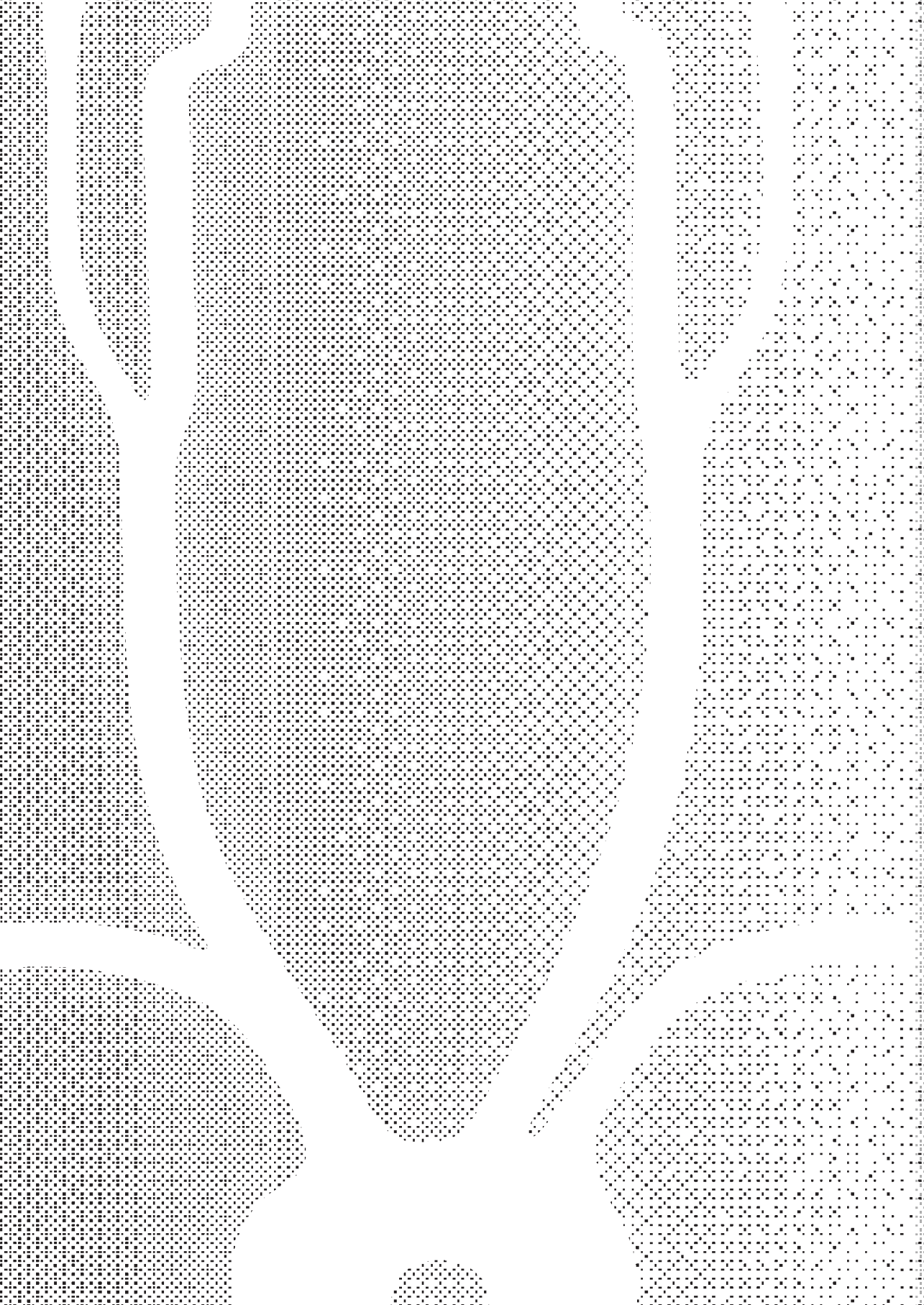

CHAPTER 11

UTILITY OF CONTRAST-ENHANCED ULTRASOUND FOR THE ASSESSMENT OF THE CAROTID ARTERY WALL IN PATIENTS WITH TAKAYASU OR GIANT CELL ARTERITIS

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ABSTRACT

Aims: Carotid contrast-enhanced ultrasound (CEUS) was recently proposed for the evaluation of large-vessel vasculitides (LVV), particularly to assess vascularization within the vessel wall. The aim of this pilot study was to evaluate the potential of carotid colour Doppler ultrasound (CDUS) and CEUS in patients with LVV.

Methods and results: This prospective study included seven patients (mean age 48 ± 14 years, all females) with established LVV (Takayasu arteritis or giant cell arteritis). All patients underwent CDUS and CEUS (14 carotid arteries). Intima-media thickness, lumen diameter, Doppler velocities, vessel wall thickening, and lesion thickness were assessed. CEUS was used to improve visualization of the lumen-to-vessel wall border, and to visualize carotid wall vascularization. Four (57%) patients [7 (50%) carotid arteries] exhibited lesions, and the average lesion thickness was 2.0 ± 0.5 mm. According to the Doppler peak systolic velocity, 5 (35%) carotid arteries had a <50% stenosis, 1 (7%) had a 50-70% stenosis, and 1 (7%) had a ≥70% stenosis. The contrast agent improved the image quality and the definition of the lumen-to-vascular wall border. Carotid wall vascularization was observed in 5 (71%) patients [9 (64%) carotid arteries]. Five (36%) carotid arteries had mild-to-moderate vascularization, and 4 (29%) had severe wall vascularization.

Conclusion: Carotid CDUS allows the assessment of anatomical features of LVV, including vessel wall thickening and degree of stenosis. Carotid CEUS improves the visualization of the lumen border, and allows dynamic assessment of carotid wall vascularization, which is a potential marker of disease activity in patients with LVV.

INTRODUCTION

Takayasu arteritis and giant cell arteritis are the most prevalent large vessel vasculitides (LVV) [1]. LVV is defined by a predominant but not exclusive involvement of large arteries. Takayasu arteritis and giant cell arteritis occur predominantly in women, and histopathological findings are similar [2]. Vascular imaging may play an important role in the diagnosis and follow-up of patients with LVV [3]. For the evaluation of disease activity and monitoring of treatment response, a reproducible, robust marker of disease activity is desired [4].

Contrast-enhanced ultrasound (CEUS) is increasingly being used for vascular imaging indications [5]. Multiple studies have demonstrated that CEUS improves the visualization of the vessel lumen, leads to improved detection of atherosclerotic plaques, and allows visualization of intraplaque vascularization [6,7]. Carotid CEUS was recently proposed as a novel imaging modality for the evaluation of LVV, particularly to assess vascularization (vasa vasorum) within the vessel wall [8,9]. The aim of this pilot study was to evaluate the potential of carotid CEUS in patients with LVV. The hypothesis of this study was that carotid CEUS may be used in patients with LVV to improve visualization of the lumen, and assess vascularization within the carotid wall.

METHODS

Patient population and study protocol

The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands. All patients provided informed consent. Consecutive patients with an established diagnosis of LVV (Takayasu arteritis or giant cell arteritis) were asked to participate in this prospective pilot study. Diagnoses were based on suggestive clinical features and radiological arterial imaging. All patients underwent a carotid CDUS examination in conjunction with CEUS. Exclusion criteria were contraindications for the use of ultrasound contrast agent, such as unstable angina, acute cardiac failure, acute endocarditis, known right-to-left shunts, and known allergy for microbubble contrast agents.

Carotid ultrasound acquisition

The carotid CDUS and CEUS examination were performed using a Philips iU-22 ultrasound system (Philips Medical Systems, Bothell, USA), equipped with a L9-3 transducer. The patient was positioned in supine position with the head at a 45° angle turned to the contra-lateral side. For CDUS, a standardized image acquisition protocol based on the American Society of Echocardiography consensus statement was used [10]. In short,

first the left common carotid artery (CCA), carotid bulb, internal carotid artery (ICA) and external carotid artery (ECA) were evaluated using B-mode ultrasound, color Doppler imaging and pulsed wave Doppler imaging. The right carotid artery was evaluated in the same way.

For the CEUS examination, the ultrasound system was switched to its contrast mode. The contrast mode was using amplitude modulation techniques and a mechanical index of 0.06-0.08 to optimize the contrast enhanced ultrasound images. Side-by-side display mode with a simultaneous B-mode and CEUS image was used for optimal coordination of the ultrasound examination. Other presets were as follows: gain 30%, compression 60, imaging depth 3.0 cm. These presets were adjusted per patient to obtain optimal quality of the ultrasound clips. CEUS was performed using intravenous administration of SonoVue™ ultrasound contrast agent (Bracco S.p.A., Milan, Italy). The ultrasound contrast-agent was injected in boluses of 0.5 ml, the bolus administration was repeated when necessary. First, the left CCA, carotid bulb, ICA and ECA were evaluated, with special emphasis for the present carotid lesions and atherosclerotic lesions. The right carotid artery was evaluated in the same way. For both standard carotid ultrasound and CEUS of the carotid arteries, cineclips were digitally stored and reviewed offline.

Carotid ultrasound analysis

Carotid ultrasound studies were reviewed offline by 2 independent observers unaware of the clinical data. Discrepancies in their evaluation were resolved by consensus. The image quality of CDUS and CEUS clips was independently scored based on a grading scale as (1) good, (2) moderate, (3) poor or (4) uninterpretable. In accordance with previously published studies, the carotid intima media-thickness (CIMT) was measured in the far wall of the distal 1 centimeter of the CCA [11]. Semi-automated CIMT measurement was performed using Qlab quantification software (Philips Healthcare, Best, the Netherlands). For each side, the CIMT measurement was performed 3 times on selected still frames on different R-peaks of the ECG signal. The mean value of 3 measurements from the left and right carotid artery was used in further statistical analysis. Using the CDUS clips the CCA, carotid bulb, ICA and ECA were extensively evaluated for the presence of lesions. The maximum lesion thickness was measured perpendicular to the luminal flow. The presence of lesions was recorded for each side. Stenosis severity was assessed using the criteria of the Society of Radiologists in Ultrasound [12]. In short, abnormal CIMT or lesion and peak systolic velocity (PSV) <125 cm/sec was considered indicative of <50% diameter stenosis; lesion and PSV of 125-230 cm/sec as 50%-69% stenosis; lesion and PSV >230 cm/sec as ≥70% stenosis; no detectable patent lumen, and no flow at spectral, power, and color Doppler was considered total occlusion.

Using the CEUS clips, the carotid arteries were scored for the presence of wall vascularization. Carotid walls that could have been affected by pseudo enhancement, an

artifact that hinders the evaluation of vascularization in the far wall or vascular wall below contrast-pools, were excluded from analysis [13,14]. The presence and extent of wall vascularization was visually assessed using a previously published grading method [15]. Dynamic contrast-enhancement in the carotid wall was considered to represent carotid wall vascularization. The presence of blood flow activity was identified on the basis of the movement of the echogenic reflectors (microbubble contrast agent) observed in the microvessels in the carotid lesions. Fixed echogenic signals were considered to be tissue acoustic reflectors. For each carotid artery the presence of wall vascularization was graded as follows: Grade (0) no vascularization, was used to indicate no appearance of microbubble contrast agent in the carotid lesion; Grade (1) limited or moderate vascularization, was used to indicate limited to moderate visible appearance of moving microbubbles in the carotid lesion; or Grade (2) severe vascularization, was used to indicate extensive wall vascularization with clear visible appearance of microbubbles. If there was a discrepancy in the scores of the independent readers, a consensus was reached.

Statistical analyses

Statistical analyses were performed using SPSS for Windows (version 17.0, SPSS, Chicago, USA) and Excel (Excel 2003, Microsoft, Redmont, USA). Continuous variables are reported as mean ± standard deviation. Categorical variables are expressed as number (%). The Chi-square test was used to evaluate differences between proportions. A p value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Patient characteristics

The patient characteristics (mean age 48 ± 14 years, all female) are summarized in Table 1. There were 5 patients with established Takayasu arteritis, and 2 patients with Giant cell arteritis. Three (43%) patients had a history of previous vascular stenting, and 3 (43%) had previous vascular surgery. However, none of the patients had previous vascular stenting or surgery of the carotid artery. Five (71%) patients used some form of immunosuppressive therapy. All CDUS and CEUS studies were performed without adverse reactions.

Image quality

The image quality of the CDUS studies was good in 6 (43%), moderate in 8 (57%) and poor in 0 carotid arteries. CEUS resulted in significantly improved image quality ($P<0.005$). All 14 CEUS studies were of good image quality, and none of moderate or

Table 1 Clinical Characteristics.

Case No.	Age, y	Sex	Disease	Vascular stenting	Vascular surgery	Aspirin	Clopido-grel	Oral antico-agulation	Prednison	Metotrexate	Azatioprine	Blood pressure		Carotid bruit	
												right	left	right	left
1	31	F	Takayasu	+	+	+	+	+	0	0	0	150/90	150/90	0	0
2	64	F	Takayasu	+	+	+	0	0	0	0	0	110/80	NM	+	+
3	33	F	Takayasu	0	0	+	0	0	0	+	0	130/70	116/74	0	0
4	53	F	Giant cell	+	0	+	0	0	+	0	0	160/80	-	0	0
5	34	F	Takayasu	0	+	0	0	0	+	0	+	110/70	120/70	0	0
6	57	F	Takayasu	0	0	+	0	0	+	0	+	NM	NM	0	0
7	67	F	Giant cell	0	0	+	0	0	+	0	0	120/64	110/64	0	0
Summary	48±14	100% (7/7)	71% (5/7)	43% (3/7)	43% (3/7)	86% (6/7)	14% (1/7)	14% (1/7)	57% (4/7)	14% (1/7)	29% (2/7)	130±19/ 76±9	124±15/ 75±10	14% (1/7)	14% (1/7)

+ = present, 0 = absent, - = not measured, F = female, NM = not measurable.

Table 2 Carotid Ultrasound Findings.

Case No.	IMT (mm)		Lumen diameter at systole (mm)		Lumen diameter at diastole (mm)		Doppler peak systolic velocity (cm/s)		Lesion		Lesion thickness (mm)		Carotid wall vascularization	
	right	left	right	left	right	left	right	left	right	left	right	left	right	left
1	0.54	0.58	5.3	6.0	4.8	5.2	66	69	0	0	-	-	+	+
2	1.10	1.32	3.1	3.6	2.8	3.4	283	126	+	+	3.1	2.4	+	+
3	0.51	0.48	5.9	5.7	5.3	5.2	98	106	0	0	-	-	0	+
4	0.60	0.59	6.0	5.9	5.2	5.4	74	73	+	0	1.7	-	0	0
5	0.48	0.51	4.8	5.5	4.1	4.6	136	137	0	0	-	-	0	0
6	0.84	0.89	6.4	5.8	5.8	5.2	74	92	+	+	1.4	1.7	++	++
7	0.63	0.70	6.9	6.4	5.9	5.9	40	57	+	+	2.1	1.8	++	++
Summary	0.67±0.20	0.72± 0.26	5.5± 1.2	5.6± 0.8	4.8±1.0	5.0±0.7	110±76	94±28	57% (4/7)	43% (3/7)	2.0±0.6	2.0±0.3	57% (4/7)	71% (5/7)

+ = present, ++ = strongly present, 0 = absent, - = not applicable, IMT = intima-media thickness.

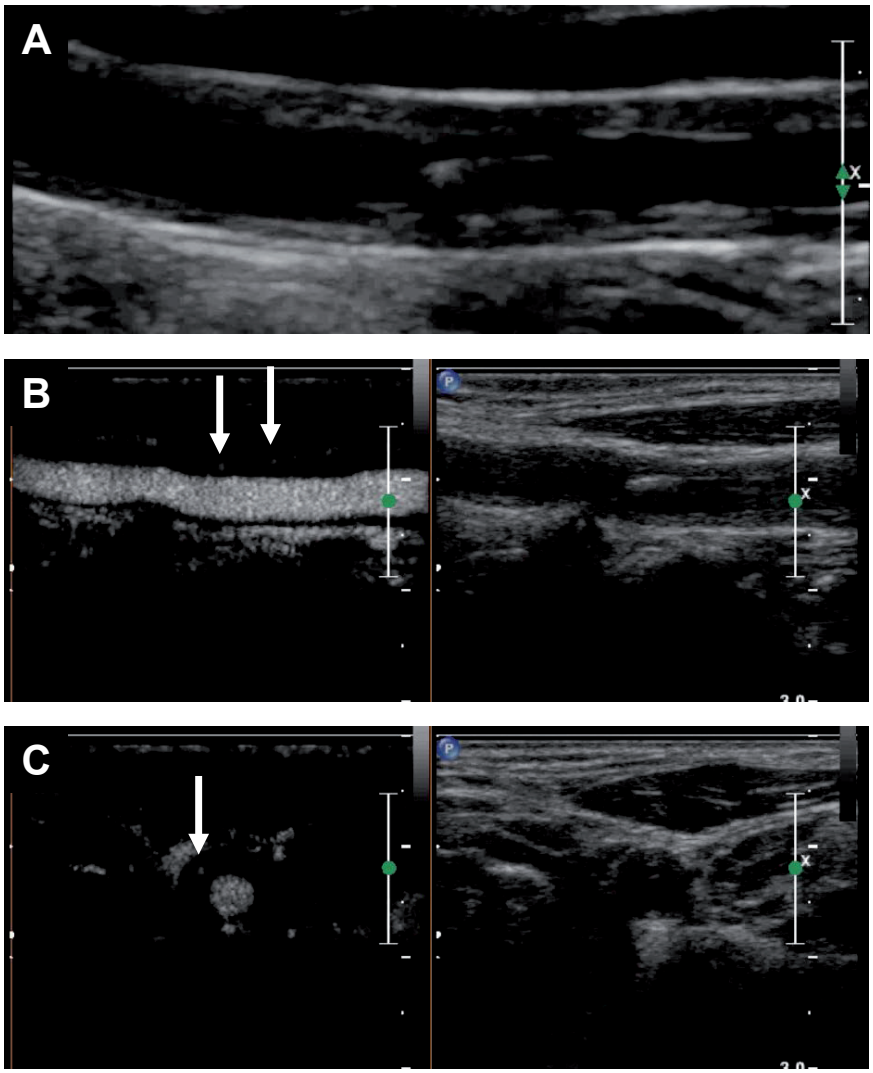

Figure 1

Carotid B-mode ultrasound and contrast enhanced ultrasound (CEUS) in a 64-year old woman with Takayasu arteritis (Case no. 2 from Table 1). A: Longitudinal B-mode ultrasound demonstrating marked carotid wall thickening. B: Longitudinal side-by-side carotid CEUS (left panel) and B-mode ultrasound (right panel). The microbubble contrast agent improved the borders of the vascular lesion, the arrows indicate microbubble contrast agent within the vascular wall lesions, indicating wall vascularization (vasa vasorum). C: Transversal side-by-side carotid CEUS (left panel) and B-mode ultrasound (right panel). The contrast agent improved delineation of the carotid lumen; there is marked circumferential thickening of the vascular wall. The arrows indicate microbubble contrast agent within the vascular wall lesions, indicating carotid wall vascularization.

poor quality. The use of contrast agent specifically led to an improved delineation of the carotid lumen and a better definition of the borders of carotid lesions. None of the ultrasound studies was judged to be uninterpretable; hence 14 carotid arteries were available for the analysis.

Carotid ultrasound findings

The carotid ultrasound findings are summarized in Table 2. The CIMT was on average 0.70 ± 0.24 mm (range 0.48 mm to 1.32 mm). The lumen diameter at systole was 5.5 ± 1.0 mm (range 3.1 mm to 6.9 mm), and the lumen diameter at diastole was 4.9-0.9 mm (range 2.8 mm to 5.9 mm). Seven (50%) carotid arteries demonstrated lesions, with an average lesion thickness of 2.0 ± 0.5 mm. According to the Doppler peak systolic velocity, 5 carotid arteries had a <50% diameter stenosis, 1 had a 50-70% diameter stenosis, and 1 carotid artery had a ≥70% diameter stenosis. Carotid CEUS demonstrated vascularization within the carotid wall in 9 (64%) carotid arteries. Five (36%) carotid arteries exhibited mild to moderate vascularization, and 4 (29%) had severe vascularization of the carotid wall. Figure 1 demonstrates the CDUS and CEUS findings in a patient

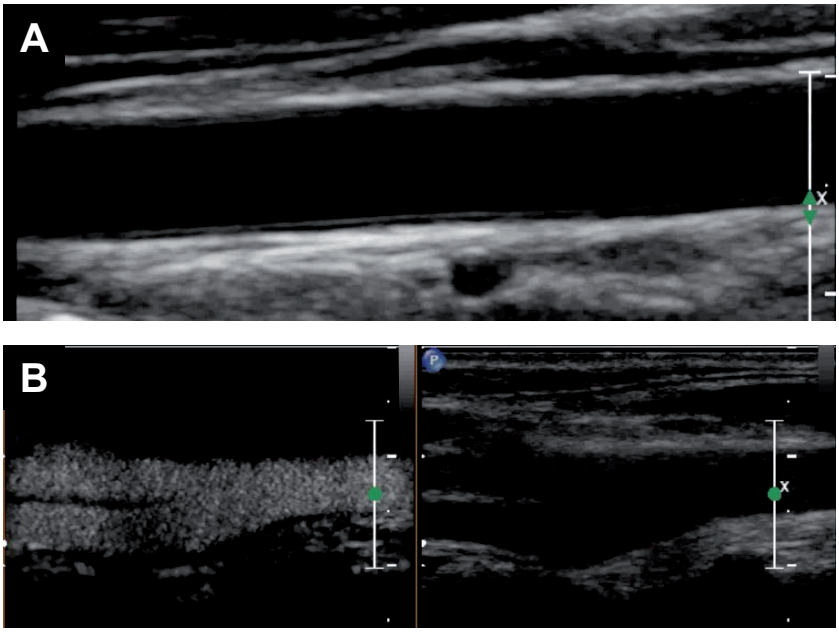

Figure 2
Carotid B-mode ultrasound and contrast enhanced ultrasound (CEUS) in a 34-year old woman with Takayasu arteritis (Case no. 5 from Table 1). A: Longitudinal B-mode ultrasound demonstrating a normal carotid wall thickness in the common carotid artery. B: Longitudinal side-by-side carotid CEUS (left panel) and B-mode ultrasound (right panel) of the carotid bifurcation. There were no vascular lesions, and no contrast enhancement at the vascular wall, suggesting absence of pathological wall vascularization

with Takayasu arteritis and vascular lesions with signs of wall vascularization. Figure 2 demonstrates the CDUS and CEUS images in a patient with Takayasu arteritis without involvement of the carotid artery.

DISCUSSION

The main findings of the present study are that carotid CEUS improves the visualization of the lumen border, and allows assessment of carotid wall vascularization, which is a potential marker of disease activity in patients with LVV. Takayasu and giant cell arteritis are relatively rare diseases and the diagnosis and evaluation of disease activity may be challenging. Traditionally, invasive angiography has been used as the main imaging method in the diagnosis and management of LVV. Invasive angiography allows an accurate visualization of the vascular lumen, however changes in lumen diameter occur relatively late in the disease. Additionally the use of iodinated contrast agent and ionizing radiation are limitations of this technique. Recently, computed tomography angiography has replaced invasive angiography, but this method has similar limitations as invasive angiography [3]. FDG-positron emission tomography (PET) and magnetic resonance angiography (MRA) have overcome some limitations, and provide information on vessel wall thickness and disease activity. However, FDG-PET and MRA involve respectively ionizing radiation and gadolinium contrast agent. Repeated use of these imaging modalities in often young female patients with known or suspected LVV is not desired.

Recently, carotid CEUS has been proposed, in 2 case-reports, as a potentially useful imaging modality in the assessment of disease activity in patients with Takayasu arteritis. In the report by Giordana et al. [8] carotid CEUS was used in a 35-year old woman to diagnose Takayasu disease and to monitor the response to treatment. The authors initially observed circumferential wall thickening of the right common carotid artery with multiple vasa vasorum before initiation of treatment. At 3 and 6 months after initiation of treatment, carotid CEUS was repeated, and a progressive decrease of inflammatory activity within the carotid artery wall under steroid treatment was observed. Next, Magnoni et al. [9] described a case of a 35-year old woman with Takayasu arteritis. Using carotid CEUS, an improved image quality with a greater enhancement of vessel wall lumen and higher definition of the borders of the vascular lesion was observed. CEUS demonstrated the presence of a large amount of contrast signal within the carotid lesions, as visualized by moving bright spots and linear flow of microbubbles within the vascular lesions. This phenomenon was principally seen on the adventitial side of the vessels and was considered to represent the contrast agent's bubble signal coming from neovessels. This pilot study confirms and extends the results from these 2 case-

reports. Carotid CDUS and CEUS were used to evaluate 7 patients (14 carotid arteries) with established LVV. Carotid CDUS allowed assessment of anatomical features of LVV, including vessel wall thickening and degree of stenosis. CEUS was relatively easily incorporated into the standard carotid CDUS imaging protocol. Carotid CEUS improved the visualization of the lumen border, leading to a better definition of vascular lesions. CEUS allowed assessment of carotid wall vascularization, by visualization of contrast microbubbles through the vascular wall and vascular lesions. The presence and intensity of the contrast-enhancement within the vascular wall are potential markers of disease activity in patients with LVV. Additionally, if the described carotid lesions are detected in patients without known LVV, this has to be considered in the differential diagnosis.

In the present study, no histology was obtained to validate the carotid CEUS findings. Previous studies in patients with symptomatic carotid stenosis undergoing carotid endarterectomy have compared CEUS findings with histology. A recent overview [7] of the literature demonstrated that 6 studies [16-21] including a total of 95 patients have compared carotid CEUS findings with histopathological analysis of the plaque after surgery. It was shown that plaque with a higher amount of contrast enhancement had a significantly increased density of small diameter (20-30 µm) microvessels in the corresponding region on histology [18,19]. Histological staining for specific vascular (CD31, CD34, hemosiderin, and von Willebrand factor) and angiogenic markers (vascular endothelial growth factor) showed a correlation between intraplaque contrast enhancement and the amount of staining [17,19,21]. Thus, contrast enhancement was shown to correlate with the presence and degree of intraplaque vascularization.

This study has several limitations. The number of included patients and carotid arteries was relatively small. After this pilot study, larger studies are needed to confirm the present findings. This study excluded the far wall of the carotid artery from the analysis of wall vascularization. Evaluation of the far wall vascularization may be influenced by the pseudo-enhancement artifact, leading to over-estimation of the contrast activity at the far wall [13,14]. Currently new ultrasound pulse sequences are being developed to overcome this limitation of CEUS.

In conclusion, carotid CDUS allows assessment of anatomical features of LVV, including vessel wall thickening and degree of stenosis. Carotid CEUS improves the visualization of the lumen border, and allows dynamic assessment of carotid wall vascularization, which is a potential marker of disease activity in patients with LVV.

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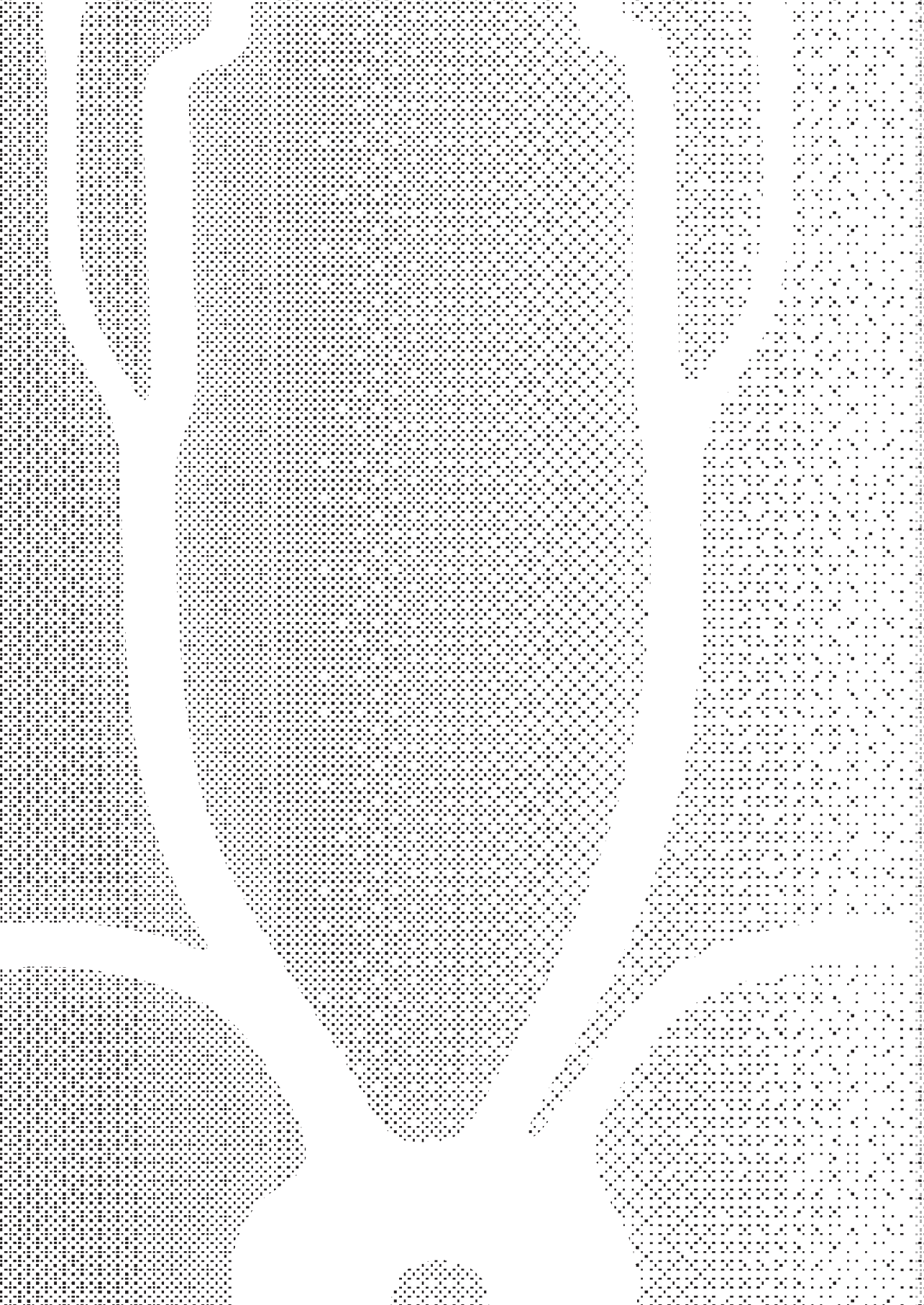
PART V



CHAPTER 12

SUMMARY & DISCUSSION

SUMMARY AND DISCUSSION

The challenges of cardiovascular risk prediction

During the last 28 years, the chances to survive a cardiovascular event have increased dramatically due to ongoing improvements in diagnostic and treatment strategies. Nevertheless, atherosclerosis, as the main cause of cardiovascular diseases and cardiovascular events, is a growing problem in the modern western world [1]. It is important to detect those individuals who are at increased risk of cardiovascular events [1-3]. A considerable amount of cardiovascular events can potentially be prevented by life-style modifications and if necessary medical therapy [4,5].

To detect individuals at increased risk of suffering a cardiovascular event, several cardiovascular risk prediction models have been developed [6-14]. Those models are based on conventional cardiovascular risk factors such as gender, age, lipid profiles, blood pressure, smoking status and the presence of diabetes mellitus [15]. These models have limited accuracy due to general assumptions that combinations of risk factors work additively not synergistically [11,16]. Cardiovascular risk prediction based on patient characteristics lacks specificity to accurately predict cardiovascular events in individuals. Therefore, clinicians are not able to predict who will suffer from a cardiovascular event. This inability of clinicians to anticipate on these cardiovascular events has led to an enormous amount of studies with the same research question: how can we improve cardiovascular risk prediction [17-20]?

A method to improve cardiovascular risk prediction is the addition of surrogate markers for atherosclerosis to existing cardiovascular risk prediction models. Examples of such surrogate markers can be found in the fields of genetics, chemical biomarkers and imaging biomarkers. It has been suggested that the addition of imaging biomarkers for (subclinical) atherosclerosis may provide a better risk estimation [16,21]. After all, the actual condition of the vessel wall provides information about the exposure to known and unknown cardiovascular risk factors and risk modifiers. Imaging biomarkers aim to detect atherosclerosis and characterize the atherosclerotic plaques. Characterization of atherosclerotic plaques would ideally distinguish stable atherosclerosis (i.e. an atherosclerotic plaque with low risk to cause cardiovascular events) from vulnerable atherosclerosis (i.e. an atherosclerotic plaque with an increased risk to rupture and cause cardiovascular events) [22-24].

The research in this thesis focused on the value of imaging biomarkers, more precisely on imaging biomarkers acquired using carotid ultrasound and carotid contrast-enhanced ultrasound (CEUS). Although the research described in this thesis did not aim to provide the best imaging biomarker for cardiovascular risk assessment, it did close

some doors for established imaging biomarkers and it opened doors for other new imaging biomarkers.

The quest for new methods to improve cardiovascular risk prediction

Carotid intima-media thickness (CIMT), as assessed by B-mode ultrasound, is a well-established imaging biomarker for the presence of subclinical atherosclerosis [25]. Large epidemiological studies have shown the relation of the vessel wall thickness with the occurrence of both stroke and myocardial infarction [26-30]. In chapter 2 of this thesis we presented a systematic review and meta-analysis on the relation of CIMT with future cardiovascular events and the additional value of CIMT to existing cardiovascular risk prediction models. Using published and unpublished data from cohort studies worldwide, with this study we were able to provide not only the overall relation of CIMT with future stroke, future myocardial infarction and a combined endpoint, as well as summary data on the improvement of cardiovascular risk prediction models when CIMT was implemented [31]. The results showed that despite a clear relationship between CIMT and future cardiovascular events, the additional value of CIMT to existing cardiovascular risk prediction models (equal to models such as Framingham Risk score and SCORE) was small and not significant. Although this study focused on the situation in which CIMT is only measured once and changes of CIMT over time were not taken into consideration, this paper in addition to existing literature on CIMT progression may have closed the door for CIMT as an additive marker to traditional cardiovascular risk prediction models [32]. CIMT did not succeed to provide additional information over clinical variables for the prediction of future cardiovascular events.

The reason why CIMT failed maybe multi-factorial. First of all, the normal values of the CIMT are debatable by means of widespread variations among general populations and the variations between CIMT acquisition protocols [33,34]. Second, thickening of the intimal-medial complex does not necessarily represent subclinical atherosclerosis. Last, but certainly not least, is the reason that if thickening of the vessel wall is caused by atherosclerosis, it is still unclear whether this process progresses into stable atherosclerotic plaques or vulnerable atherosclerotic plaques. Although thickening of the CIMT may represent the first atherosclerotic changes, the specificity of CIMT thickening for prediction of cardiovascular events is too low to be of incremental value for accurate cardiovascular risk assessment [31].

Other parameters that can be assessed using standard carotid ultrasound include the presence or absence of atherosclerotic plaques. Atherosclerotic plaques are a marker of atherosclerosis in an advanced stage and thus might be more specific than CIMT. Inaba et al. performed a meta-analysis to investigate whether plaque detection can predict future myocardial infarction more accurately than the CIMT. Although plaque detection

was found to have a significant higher accuracy than CIMT, the absolute incremental value of plaque detection in cardiovascular risk prediction models was limited [19]. Further improvement of cardiovascular risk assessment could potentially be found in the characterization of atherosclerotic plaques [35].

Several plaque characteristics are associated with an increased risk of plaque rupture and thus may cause cardiovascular events such as myocardial infarction and stroke [23,24,36]. These characteristics include a lipid rich necrotic core, a thin fibrous cap, the presence of intraplaque hemorrhage and the density of intraplaque neovessels (IPN). Identification of patients with vulnerable atherosclerotic plaques would allow starting early medical treatment to prevent plaque rupture [37]. To achieve that goal, many studies focus on detecting and quantifying multiple characteristics or a single characteristic of the vulnerable atherosclerotic plaques [38].

The assessment of IPN can be performed using multiple imaging modalities such as dynamic contrast-enhanced magnetic resonance imaging and contrast-enhanced ultrasound (CEUS) [39,40]. In chapter 3 of this thesis we reviewed the knowledge about CEUS for carotid atherosclerosis imaging [41]. The non-invasive and non-ionizing characteristics in addition to the relative low-costs of CEUS, make this technique attractive.

As shown in chapter 3, several studies have investigated the presence of IPN in patients. Histological validation studies showed good correlation between the presence of IPN on CEUS and the presence of IPN in surgical samples of the atherosclerotic plaques [42-46]. In addition, studies showed that the amount of IPN in symptomatic carotid atherosclerosis was higher than in asymptomatic atherosclerosis and that the amount of IPN is associated with the cardiovascular history of patients [47,48]. However, there is no consensus between investigators how to score the amount of IPN. Most investigators have used a subjective visual scoring method based on a two-point or a three-point grading scale and only a few studies made an attempt quantify the enhancement of contrast in atherosclerotic plaques. The lack of a standardized method to score the presence of IPN limits the applicability of carotid CEUS. Development of automated quantification software would possibly provide an answer to the subjectivity of visual scoring methods and the reproducibility of IPN scoring methods.

Carotid contrast-enhanced ultrasound: the opportunities

Detection of atherosclerosis

There are more advances of CEUS in addition to the ability of CEUS to demonstrate IPN in carotid atherosclerosis. In-vitro and in-vivo studies have showed that CEUS may have additional value for the detection of wall irregularities [49,50]. These studies compared standard B-mode and color Doppler with CEUS measurements for assessment of stenosis grade and wall irregularities in animal models or patients with established ath-

erosclerosis. The data showed that CEUS may have incremental value for the evaluation of atherosclerosis [49,50]. However, no data was reported on the additional value of CEUS for the detection of subclinical atherosclerosis. In chapter 4 of this thesis, we reported a study on the incremental value of CEUS on top of standard carotid ultrasound for the detection of subclinical atherosclerotic plaques [51]. An extensive screening was performed to detect subclinical atherosclerosis in 100 consecutive patients. For this screening all patients underwent standard carotid ultrasound in conjunction with CEUS. The results showed that 21% of the patients had an increased CIMT. In 77% of the patients, subclinical atherosclerotic plaques were detected using standard carotid ultrasound. Using the combination of standard carotid ultrasound and CEUS, an additional 11 patients were found to have subclinical atherosclerotic plaques. These results led to the conclusion that CEUS has additional value for the detection of subclinical atherosclerosis in asymptomatic patients. The prevalence of subclinical atherosclerosis reported in this study was relatively high. Large epidemiological studies in general population cohorts have reported a prevalence of carotid atherosclerotic plaque up to 42%-55% [52,53]. This discrepancy may be explained by the clinical characteristics of the study population used in chapter 4. Although all patients were asymptomatic for carotid atherosclerotic disease, the inclusion criteria of this study included the presence of ≥ 1 cardiovascular risk factor. Therefore, all patients had an increased risk for the development of atherosclerosis [51]. Extrapolation of the data on the prevalence of atherosclerosis may therefore be limited. Nevertheless, the data on the additional value of CEUS for the detection subclinical atherosclerosis may have clinical implications. The data showed that a subset of 11 patients (13%) with signs of subclinical atherosclerosis were missed without the use of CEUS. The use of standard carotid ultrasound only to investigate the presence of subclinical atherosclerosis will therefore be limited with a suboptimal sensitivity, independent from which population is selected.

According to the current guidelines, patients with atherosclerosis (either symptomatic or asymptomatic) are at high risk for future cardiovascular events [2]. As we have shown in chapter 4, a substantial amount of patients with subclinical atherosclerotic plaques is missed with standard carotid ultrasound only. Addition of CEUS to the standard ultrasound protocol would decrease the number of patients that would be missed and thus CEUS would decrease the number of patients that are falsely not stratified as being at high risk [51]. In chapter 5 we tested this hypothesis [54]. In 100 consecutive patients the PROCAM risk score was calculated to divide the patients into low, intermediate or high cardiovascular risk. Consequently, the presence of subclinical atherosclerosis was assessed using standard carotid ultrasound in conjunction with CEUS. The results showed that subclinical atherosclerosis was detected in 89 patients, whereas only 11 patients were considered to be at high cardiovascular risk according to the PROCAM risk score. The addition of standard carotid ultrasound with CEUS lead to reclassification

of 79 patients to high cardiovascular risk. Although the study claims that 79 patients were reclassified to the high cardiovascular risk category, it is not clear whether all these patients are correctly reclassified. It would require long-term follow up data to determine whether the reclassification of these patients to high cardiovascular risk could be justified. So far, no prospective follow up data is available to determine the clinical value of subclinical atherosclerotic plaques in asymptomatic patients detected using carotid CEUS. Therefore, it is not known whether all patients were correctly reclassified into the high cardiovascular risk category.

A number of studies have shown a relation between the presence of subclinical atherosclerotic plaques and future cardiovascular events [52,53]. These general population based cohort studies reported hazard ratios up to 1.77 for future cardiovascular events. The event rates for patients with plaques was up to 2.5 times increased as compared to the patients without plaque [52]. However, the fraction of patients with atherosclerotic plaque whom suffered a cardiovascular event after 8 years follow up ranged from 11% up to 18%. The highest incidence of cardiovascular events was observed in patients with carotid stenosis >25% (event rate 18%) [52,55]. Therefore, the presence of atherosclerotic plaque lacks specificity to predict future cardiovascular events. Characterizing vulnerable plaque components (such as the presence of ulcerations or the amount of IPN) in patients with subclinical atherosclerosis may increase the specificity and accuracy of the prediction of cardiovascular events.

Quantification of carotid intraplaque neovascularization

As stated in chapter 3, the lack of standardized ways to score the amount of IPN in atherosclerosis limits the applicability of carotid CEUS. Automated quantification tools could provide a solution for this problem. Chapter 6 of this thesis presents a custom-developed semi-automated software tool for the quantification of IPN in carotid atherosclerosis. This study aimed to develop software that was able to calculate several carotid IPN features, taking into consideration the limitations of previously published semi-automated quantification tools [48,56].

The previously published papers which used semi-automated quantification tools, investigated the value of carotid CEUS in patients with established atherosclerosis [48,56]. The limitations of the methods used for quantification of IPN in those papers have some similarities. Assessment of IPN is performed using analysis of regions of interest (i.e. the atherosclerotic plaque). However, both papers did not use a tool to compensate for the motion of the carotid artery (and thus the motion of the atherosclerotic plaque). Therefore, movements of the atherosclerotic plaque, the lumen and the vessel wall may have influenced the results of these studies [48,56]. Another shared major limitation of the previously published quantification methods is the calculation of atherosclerotic plaque enhancement. Both papers used data from time-intensity curve analysis with special

interest for peak enhancement [48,56]. Peak-enhancement from time-intensity curves is widely used in the evaluation of perfusion of large organs using contrast-enhanced ultrasound [57]. However, the perfusion characteristics of large organs varies from the perfusion characteristics of atherosclerotic plaques. Looking at carotid CEUS clips, atherosclerotic plaque perfusion has a more intermittent perfusion pattern. An intermittent perfusion pattern leads to multiple peaks on a time intensity curve [58]. Distillation of only one peak value from a time intensity curve with multiple peaks leads to loss of potentially valuable perfusion data. Therefore, the methods that we have developed and which were presented in chapter 6 of this thesis had to overcome those two major limitations. To correct for the motion of the carotid artery, a motion compensation tool was implemented in the software [59]. This tool was able to minimize the influence of false positive plaque enhancement due to carotid pulsatility and breathing motions [59]. Furthermore, the development of new tools for the quantification of contrast-enhancement in regions of interest included features based on data from maximum intensity projections, time-intensity curves and a vascular structure analysis method previously described by Hoogi et al. [60].

The development process resulted in 5 potentially relevant features for the quantification of carotid IPN. In chapter 6, we performed the validation of these features and the reproducibility of the semi-automated quantification software was tested. In a subset of 25 patients with established carotid atherosclerosis, the amount of IPN was assessed visually and using the semi-automated quantification software. The new IPN features were associated with visual scoring grades for IPN. The tests for reproducibility of the results demonstrated good to excellent intra-and inter-observer reproducibility and low intra- and inter-observer variation.

With this results, the software was able to provide several objective and reproducible features for IPN in carotid atherosclerosis on CEUS. The implication of this software would lead to a more homogenous method to report results in studies investigating the value of CEUS for detection of IPN. It would allow studies to compare results and simplify the interpretation of the results of other studies. In addition, clinical trials which would aim to investigate changes in density of IPN over time would benefit this software.

However, the software tool has limitations that are inherent to the use of carotid CEUS. Due to far-wall pseudo-enhancement, plaques that are only visible at the far-wall of the carotid artery may demonstrate false positive contrast enhancement [61,62]. Therefore, plaques in the far-wall of the carotid artery had to be excluded. Consequently, the software should not be used for quantification of IPN in far-wall atherosclerotic plaques. Another limitation of the software is the 2-dimensional approach to quantify a plaque component that is characterized by a 3-dimensional architecture [63]. Although there are no commercial systems available which provide reliable 3-dimensional CEUS clips, the development of 3-dimensional CEUS is ongoing. Nevertheless, the principles of the new

quantification tools for IPN on 2-dimensional CEUS may serve as foundation for the development of tools for quantification of IPN on 3-dimensional CEUS.

In chapter 7 of this thesis, we presented a series of cases in which we explored histological validation of automated quantification of IPN in carotid CEUS. Unfortunately, there were no significant correlations between the amount of IPN in carotid CEUS clips and the amount of IPN in histological samples. Due to limitations of both 2D carotid CEUS and the histological processing protocol, the lack of a correlation between IPN features on CEUS clips and the amount of IPN in histological samples does not necessarily lead to the conclusion that carotid CEUS is an invalid method to assess carotid IPN. Therefore, extrapolation of the data from these cases is at this moment not reliable. As stated in the discussion of this study, improvement of both protocols may lead to a reduction of the mismatch between the protocols. The improvements of the protocols are in progress, and future results from this project may provide an answer on the original question without being hampered by a number of major limitations.

Carotid contrast-enhanced ultrasound: the current applications

Several studies investigated the use of CEUS for the assessment of IPN in the carotid arteries [41]. Previously published studies reported increased amounts of IPN in symptomatic patients compared to asymptomatic patients. Ideally, the symptomatic patients would have been identified prior to their event. However, no studies investigated the characteristics and amount of IPN in patients without prior cardiovascular events or patients without established carotid atherosclerotic disease. Assessment of IPN in asymptomatic patients may allow early identification of patients at increased risk for plaque rupture and cardiovascular events. In chapter 8 we evaluated the role of CEUS in patients with familial hypercholesterolemia (FH). The amount of subclinical atherosclerosis and IPN in 69 asymptomatic statin-treated patients with FH was assessed using CEUS of the carotid arteries [64]. Patients with FH are at severely increased risk for developing premature atherosclerosis [65]. Statin use reduces the risk of cardiovascular events in patients with FH to a minimum [66]. Still, a substantial amount of patients develop subclinical atherosclerosis. Recently published studies reported a prevalence of almost 25% of obstructive coronary artery disease in treated asymptomatic patients with FH [67,68]. Information on subclinical atherosclerosis in the carotid arteries, including the amount of IPN could further improve risk stratification of patients with FH. Using CEUS, subclinical atherosclerotic plaques were detected in 90% of asymptomatic FH patients. In these patients, the amount of IPN was quantified using custom developed software. No significant relations were found between clinical characteristics of the patients with FH and the amount of IPN. Plaques with an irregular or ulcerated surface exhibited increased amounts of IPN when

compared to plaques with a smooth surface. This supports the hypothesis that IPN is a marker for the vulnerable atherosclerotic plaque.

The absence of associations between clinical characteristics of patients with FH and the amount of IPN may have several reasons. First of all, 96% of the patients used statins. In addition to the cholesterol lowering effect of statins, statins may also reduce the amount of IPN [63]. This may have had influence on plaque morphology of the individual patients. Second, 20% of the patients with subclinical atherosclerosis were not eligible for the quantification of IPN due to pseudo-enhancement. This resulted in a limited number of patients that were included in the statistical analysis. Follow up data from these patients with FH will provide an answer to the question whether the amount of IPN has prognostic value.

Patients with diabetes mellitus (DM) are also at increased risk for developing premature atherosclerosis. Therefore, DM is considered to be an important determinant of cardiovascular risk [8,69]. Characterization of atherosclerotic plaques may further improve cardiovascular risk stratification of these patients. Chapter 9 of this thesis we presented a study investigating the value of CEUS for the detection and characterization of atherosclerotic plaques in patients with DM. In a total of 51 patients with DM the prevalence of subclinical carotid atherosclerosis was assessed using CEUS. The presence of plaque ulceration and the amount of IPN were assessed to characterize subclinical atherosclerotic plaques. Subclinical atherosclerotic plaques were detected in 90% of these patients. IPN was detected in 88% of the atherosclerotic plaques whereas ulcerations were detected in 8% of the atherosclerotic plaques (Oord DM). This supports the idea that the angiogenic tendency of DM plays an important role in the pathogenesis of the complications of diabetes mellitus [70,71].

CEUS may have clinical implications in cardiovascular risk stratification of patients with DM. It could be used to identify those patients with vulnerable atherosclerotic plaques. Prospective follow-up data will determine the prognostic value of CEUS for assessment of the vulnerable atherosclerotic plaque in patients with DM. The lack of associations between vulnerable plaque characteristics and the clinical characteristics of patients with DM (such as complications of DM) may partly be explained by the limitations of CEUS. Due to far-wall pseudo-enhancement a considerable amount of carotid arteries had to be excluded (i.e. 49%). This limitation in addition to the small number of patients included in this study may have had influence on the results. In addition, the prevalence of subclinical atherosclerosis and IPN in this study was relatively high. Subclinical atherosclerotic plaques were detected in 90% of the patients and in only 12% of those patients there was no sign of IPN. This high prevalence of subclinical atherosclerosis and IPN may be caused by the selection of cases in this study: only patients whom were referred to the hospital to control the DM were included in this study. Therefore, this study included only patients with advanced DM.

Studies investigating the prevalence of CVD among males and females showed that there are gender specific differences in both the prevalence of atherosclerosis and the composition of atherosclerotic plaques [72-74]. Histological examination of advanced atherosclerotic plaques showed that atherosclerotic plaques of males had significantly more inflammation and lipid necrotic core when compared to advanced atherosclerotic plaques of females [73,74]. In chapter 10 we reported a study in which we investigated whether there are gender specific differences in the amount of IPN in patients at increased risk of atherosclerosis but without known carotid atherosclerosis [75]. A total of 159 patients (74 females and 85 males) were included and underwent bilateral carotid CEUS for the assessment of subclinical atherosclerosis and to quantify IPN. The ultrasound clips of 101 patients (64%) were eligible for the assessment of IPN. The amount of IPN was significantly higher in females, and therefore females had more vulnerable atherosclerotic plaques than males ($p<0.05$). After adjustment for covariates the difference in the amount of IPN in females and males remained statistically significant ($p<0.05$).

The reported results are conflicting with prior studies investigating this topic [73,74]. This discrepancy may have several reasons. First, the design of prior studies may have led to a selection bias. These studies included only patients with advanced atherosclerotic plaques and an indication for carotid endarterectomy, whereas the study in reported in chapter 10 included only asymptomatic patients with an increased cardiovascular risk and without a history of carotid atherosclerotic disease. Second, those studies investigated other characteristics of the vulnerable atherosclerotic plaque (i.e. the amount of inflammation and lipids within atherosclerotic plaques) and did not investigate the amount of IPN in the histological specimens. Our study had also some limitations. In addition to the limitations that are inherent to the use of carotid CEUS (e.g. pseudo-enhancement and its consequences on eligibility of ultrasound clips for assessment of IPN), we did also not assess data on the hormonal status of females in this study. Therefore, it was not possible to evaluate the relationship between IPN and sex-hormones.

In addition to the use of carotid CEUS for characterization of atherosclerotic plaques, it may also play a role in monitoring other vascular pathologies such as giant cell arteritis or Takayasu arteritis (i.e. the large vessel vasculitides (LVV)). These diseases are characterized by inflammation of the arterial wall which may lead to intimal thickening and formation of intimal neovascularization [76-78]. The intimal neovasculature serve a pro-inflammatory and anti-ischemic role in vasculitides [78]. Therefore, monitoring the amount of intimal neovasculature may provide information on disease activity and may guide medical therapy. In chapter 11 of this thesis we presented the results of a pilot study, in which carotid arterial wall characteristics were assessed in patients with Takayasu arteritis (n=5) or giant cell arteritis (n=2) [79]. Carotid CEUS improved carotid wall visualization when compared to standard carotid ultrasound. In a total of 5 patients (71%) vessel wall vascularization was detected.

The results reported in this study show that it is possible to assess several imaging features of LVV using the combination of standard carotid ultrasound and CEUS. However, it is not clear if these features provide prognostic information about disease progression and therapeutic success. Future prospective studies including a larger sample of patients with suspected LVV with carotid involvement could provide an answer which imaging features are important prognostic markers. In addition, repeated assessment of the imaging features before and after initiation of medical therapy could help to determine if carotid ultrasound and CEUS provide helpful non-invasive information about success of therapies.

Carotid contrast-enhanced ultrasound: the remaining challenges

Although the work described in this thesis focusses on the clinical application of carotid CEUS, there is still work to do before this technique is ready for broad clinical implementation. Technical improvements may facilitate solutions for the current limitations of carotid CEUS. In addition, further research on microbubble contrast agents may lead to evolution of CEUS from diagnostic imaging modality to a combined diagnostic and therapeutic imaging modality.

Prognostic value of findings on carotid CEUS

Chapters 8 to 11 of this thesis described the implementation of carotid CEUS in asymptomatic high-risk patients without known carotid disease. The studies in these chapters focused on detecting and characterizing subclinical atherosclerotic plaques. Using CEUS it was possible to detect and quantify characteristics of the vulnerable atherosclerotic plaque such as the amount of IPN and the presence of plaque ulcerations. Although previous studies showed that these characteristics are related to past cardiovascular events and cerebrovascular symptoms, the prognostic value in asymptomatic patients is still unclear [47,48]. Future follow-up data from the studies we described in chapters 8 to 11 will provide more information on the prognostic value of IPN and plaque ulcerations detected using carotid CEUS.

Improvement of carotid CEUS

Assessment of IPN using carotid CEUS is limited to plaques that are not affected by pseudo-enhancement [61,62]. Pseudo-enhancement is an artifact caused by the non-linear scatter characteristics of microbubble contrast agents. This artifact affects all atherosclerotic plaques which are located behind pools of microbubble contrast agents such as the carotid lumen and the lumen of the jugular vein. Due to this artifact, not all carotid CEUS examinations are eligible for the assessment of IPN. In the studies presented in this thesis, pseudo-enhancement lead to exclusion of 29% to 48% of the patients with sub-

clinical atherosclerotic plaques. Development of new pulse sequences which are free of the hindering artifact would improve carotid CEUS. Pulse sequences based on counter-propagating wave interaction or based on subharmonic or ultraharmonic imaging do not produce the artifact [80-82]. However, at this moment none of these pulse sequences are commercially available for contrast-enhanced ultrasound imaging.

Another limitation of the CEUS technique is the absence of options for real-time 3 dimensional (3D) CEUS imaging. Current studies on 3D CEUS imaging use post-processing techniques for reconstruction of a 3D CEUS image [83,84]. Post-processing techniques may be of limited value when examining a dynamic atherosclerotic plaque characteristic such as IPN. Future studies should focus on developing a technique which allows real-time 3D CEUS imaging of the atherosclerotic plaque perfusion. This would provide valuable information on the presence of IPN and perfusion characteristics of IPN.

Improvement of microbubbles

The microbubble contrast-agents used for carotid CEUS consist of gas filled phospholipid shells. These shells can be targeted to disease specific processes such as angiogenesis or inflammation, allowing microbubbles to specifically adhere to diseased organs [85,86]. For assessment of the vulnerable plaque it would be interesting to target bubbles for example to processes such as angiogenesis and inflammation. In addition to targeting microbubbles, it is possible to replace the inert gas within the microbubbles with high dosages of drugs or genes. These bubbles can be destroyed using ultrasound beams [85]. The combination of targeting microbubbles and loading them with drugs or genes allows a non-invasive site specific delivery of drugs or genes (i.e. therapeutic microbubbles). Future studies should investigate the possibilities to deliver high dosages of drugs using targeted microbubbles to stabilize vulnerable atherosclerotic plaques.

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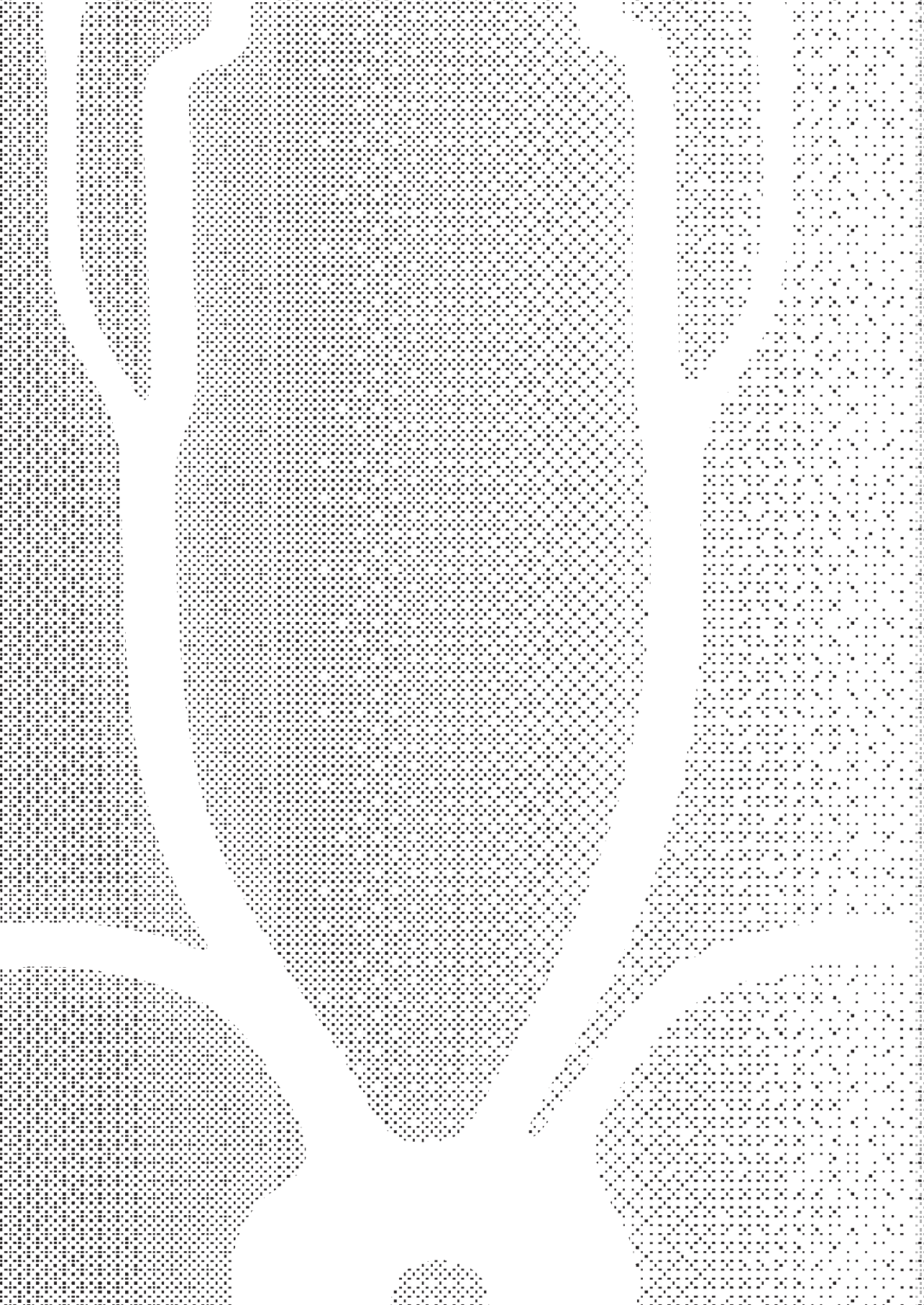

CHAPTER 13

NEDERLANDSE SAMENVATTING

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Dankzij veel onderzoek naar betere diagnostiek en therapie is de kans om een cardiovasculair event, zoals een hart- of herseninfarct, te overleven de laatste decennia fors toegenomen. Helaas blijven hart- en vaatziekten in veel Westerse landen de belangrijkste doodsoorzaak. De grootste veroorzaker van hart- en herseninfarcten is aderverkalking ofwel atherosclerose. Atherosclerose is een vaatwand ziekte van de slagaderen die zorgt voor de ontwikkeling van atherosclerotische plaques. Deze plaques kunnen scheuren, met het risico op stolsel vorming in het lumen van de aangedane slagader. Deze stolsels kunnen het volledige bloedvat afsluiten met als gevolg bijvoorbeeld een hart- of herseninfarct.

Risicofactoren voor het ontwikkelen van hart- en vaatziekten zijn onder andere roken, een hoge bloeddruk, suikerziekte, een hoog cholesterol, overgewicht en hart- en vaatziekten binnen de directe familie. Door modificatie van deze risicofactoren kan het risico op het ontwikkelen van hart en vaatziekten verlaagt worden. Zodoende kan een gedeelte van de cardiovasculaire events voorkomen worden door aanpassing van de leefstijl en zo nodig medicamenteuze therapie. Het is dan ook belangrijk om onderzoek te doen naar welke personen een verhoogd risico hebben op het krijgen van een hart- of herseninfarct, zodat er tijdig advies ten aanzien van de leefstijl gegeven kan worden of medicatie gestart kan worden.

Met behulp van grote bevolkingsonderzoeken zijn er risico stratificatie modellen ontwikkeld. Deze modellen voorspellen voor een individu het 5 of 10 jaar cumulatieve risico op het ontwikkelen van hart- en vaatziekten, gebaseerd op de aan- of afwezigheid van verschillende risicofactoren. Het voorspellen van het risico gebaseerd op alleen standaard karakteristieken van patiënten (zoals bijvoorbeeld man/vrouw, leeftijd, roken, hoge bloeddruk) heeft zijn beperkingen. Door meerdere redenen is de nauwkeurigheid van deze modellen niet optimaal. Zodoende is het lastig voor dokter om zeer specifiek die groep patiënten te detecteren die een hoge kans hebben op het ontwikkelen van een hart- of herseninfarct. Dit heeft er toe geleid dat er veel onderzoek wordt gedaan naar het verbeteren van de cardiovasculaire risico stratificatie modellen.

Het verbeteren van de modellen kan bereikt worden door het toevoegen van surrogaat markers die duiden op het bestaan van atherosclerose of wijzen op een sterk verhoogd risico op het bestaan van atherosclerose. Voorbeelden hiervan zijn onder andere genetische markers, biochemische markers maar ook afbeeldingsmarkers. Met behulp van verschillende afbeeldingstechnieken kan aderverkalking in diverse stadia worden vastgesteld. Het detecteren van aderverkalking biedt een uitgelezen kans om de toestand van de vaatwand te beoordelen. De aanwezigheid van aderverkalking geeft namelijk inzicht in de totale blootstelling aan bekende en onbekende risicofactoren en factoren die het risico verlagen.

In **hoofdstuk 2** van deze thesis hebben we onderzocht of het toevoegen van CIMT, gemeten met standaard echografie van de halsslagaderen, een toegevoegde waarde heeft aan bestaande cardiovasculaire risico stratificatie modellen. Hiervoor hebben we literatuuronderzoek gedaan naar de uitkomsten van grote bevolkingsonderzoeken. De resultaten van dit onderzoek toonde aan dat, ondanks een significante relatie tussen intima-media dikte en het optreden van hart- of herseninfarcten, er geen toegevoegde waarde is voor deze afbeeldingsmarker in het voorspellen van het risico op hart- en vaatziekten. Het toevoegen van CIMT aan bestaande modellen leidde niet tot een significant betere risicostratificatie ten op zichten van de bestaande modellen.

Het detecteren van verschillende karakteristieken van atherosclerotische plaques zou mogelijk een beter onderscheid kunnen maken tussen patiënten met een hoog- en patiënten met een laag risico. Het is namelijk bekend dat niet iedere atherosclerotische plaque hetzelfde risico heeft op scheuren. Er bestaan stabiele atherosclerotische plaques (plaques met een laag risico op scheuren) en kwetsbare atherosclerotische plaque (plaques met een hoog risico op scheuren). De kwetsbare atherosclerotische plaques worden gekarakteriseerd door een lipiden rijke kern welke wordt afgedekt door een dunne fibreuze kap, de aanwezigheid van bloedingen in de kern van de plaque en de aanwezigheid van microvasculatuur in de atherosclerotische plaque. De aanwezigheid van microvasculatuur in een atherosclerotische plaque kan in beeld worden gebracht met behulp van contrast-echografie van de halsslagaderen. In **hoofdstuk 3** van dit proefschrift hebben we bestaande literatuur over contrast-echografie van de halsslagaderen samen gevat. Hieruit blijkt dat verschillende groepen, verschillende methodes gebruiken voor het beoordelen van de aanwezigheid van microvasculatuur in een atherosclerotische plaque, en dat er vaak gebruik wordt gemaakt van subjectieve visuele beoordelingsmethodes. Deze subjectiviteit zou mogelijk afnemen wanneer er gebruik gemaakt zou worden van automatische kwantificatie software.

In **hoofdstuk 4** en **hoofdstuk 5** van het proefschrift gaan we in op de toegevoegde waarde van contrast-echografie van de halsslagaderen voor het detecteren van subklinische atherosclerotische plaques. Wanneer er alleen gebruik wordt gemaakt van standaard echografie van de halsslagaderen worden er kleine hypo-echogene atherosclerotische plaques gemist. Door gebruik te maken van contrast echografie van de halsslagaderen wordt er een scherpe contour van het lumen zichtbaar waarbij ook de kleine donkere plaques die uitpuilen in het lumen zichtbaar worden. Uit de analyses blijkt dat er bij significant meer patiënten atherosclerose wordt gedetecteerd wanneer er gebruik wordt gemaakt van de combinatie van standaard echografie en contrast-echografie van de halsslagaderen. Daarbij lijkt het cardiovasculaire risico in hoog risico patiënten onderschat te worden wanneer er gebruik wordt gemaakt van risico modellen zonder beeldvorming. Recente richtlijnen voor cardiovasculaire risico stratificatie wijzen erop dat de aanwezigheid van subklinische atherosclerose automatisch lijdt tot een hoog

cardiovasculair risico. Uit de resultaten gepresenteerd in **hoofdstuk 5** blijkt dat het grootste gedeelte van patiënten met een vasculair risicoprofiel worden in een andere risicogroep terecht komen wanneer er beeldvorming middels standaard echografie en contrast-echografie van de halsslagaderen toegevoegd wordt aan een standaard cardiovasculair risico model.

Zoals eerder gesteld wordt er in de bestaande literatuur over het detecteren van microvasculatuur in atherosclerotische plaques middels contrast-echografie van de halsslagaderen gebruik gemaakt van subjectieve visuele beoordelingsmethodes. In **hoofdstuk 6** van dit proefschrift presenteren wij een geautomatiseerde kwantificatie methode voor het beoordelen van microvasculatuur in plaques in de halsslagaderen. In het ontwikkelingsproces van deze methode is er rekening gehouden met diverse factoren welke de beoordeling van contrast-echografie van de halsslagaderen zouden kunnen beïnvloeden. Voorbeelden hiervan zij het bewegingspatroon van de halsslagader, de aanwezigheid van storingen in de beeldvorming en het patroon van contrast in atherosclerotische plaques. Uiteindelijk heeft dit proces geleid tot een software module die met minimale handmatige input een beoordeling geeft over de hoeveelheid microvasculatuur in atherosclerotische plaques. Hierbij corrigeert de module voor het bewegingspatroon van de halsslagader en berekend de module verschillende scores voor microvasculatuur gebaseerd op tijds-intensiteit curves en maximum intensiteitsanalyses. Met behulp van contrast-echografie beelden van 25 patiënten met ernstige atherosclerose in de halsslagaderen werd er onderzocht of de automatische kwantificatie scores correleren met een visuele consensus score voor de hoeveelheid microvasculatuur in de atherosclerotische plaque. Hieruit kwam naar voren dat er een associatie zit tussen beide methodes en dat de automatische kwantificatie methode in hoge mate reproduceerbare resultaten leverde zowel voor twee verschillende metingen van eenzelfde beoordelaar als voor twee metingen van verschillende beoordelaars. Deze software module maakt het mogelijk om op een gestandaardiseerde wijze te rapporteren over bevingen ten aanzien van contrast-echografie van de halsslagaderen, ook tussen verschillende studiegroepen.

In **hoofdstuk 7** presenteren wij de voorlopige resultaten van een onderzoek naar de overeenkomst van de scores die de geautomatiseerde software module produceert met de hoeveelheid microvasculatuur in een atherosclerotische plaque onder de microscoop (histologisch onderzoek). In deze studie werd gebruik gemaakt van de echobeelden van atherosclerotische plaques van patiënten die geopereerd werden aan de halsslagader. Door deze operatie zijn we in staat om (delen van) de atherosclerotische plaque onder de microscoop te bekijken. Door onder de microscoop de hoeveelheid microvaatjes te tellen en dit af te zetten tegen de bevindingen op de echobeelden krijgen we een idee over de correlatie tussen beiden. Uit onze voorlopige resultaten blijkt dat er geen enkele relatie zit tussen de hoeveelheid contrast die aan kleurt op echobeelden en de

hoeveelheid microvaatjes geteld onder de microscoop. Door limitaties van zowel de contrast-echo techniek en de methode om microvaatjes te tellen onder een microscoop is extrapolatie van deze data op dit moment onbetrouwbaar. Wel is duidelijk dat de protocollen voor zowel contrast-echografie van de halsslagaderen en voor histologisch onderzoek beter op elkaar moeten worden afgestemd om een wanverhouding tussen beide methode met de mogelijkheid tot verkeerde interpretatie te voorkomen.

Met behulp van boven omschreven software hebben we onderzoek gedaan naar het voorkomen van subklinische atherosclerotische plaques, de hoeveelheid intra plaque microvasculatuur en de aanwezigheid van plaque ulceraties in asymptomatische patienten met een verhoogd cardiovasculair risico profiel. In **hoofdstuk 8** presenteren we de resultaten van dit onderzoek in patiënten met familiaire hypercholesterolemie (FH). FH is een genetische afwijking die er toe lijdt dat patiënten een hoog serum cholesterol hebben. Onbehandeld hebben deze patiënten een ernstig verhoogd risico op prematuur vaatlijden. Onze resultaten tonen dat er bij de meerderheid van de patiënten, ondanks strikte behandeling middels statines, sprake is van subklinische atherosclerose. Er lijkt geen verband te zitten tussen de hoeveelheid intra plaque microvasculatuur en verschillende karakteristieken van patiënten met FH. Wel is er in atherosclerotische plaques met een ulceratie meer intra plaque microvasculatuur dan in plaques met een glad oppervlakte.

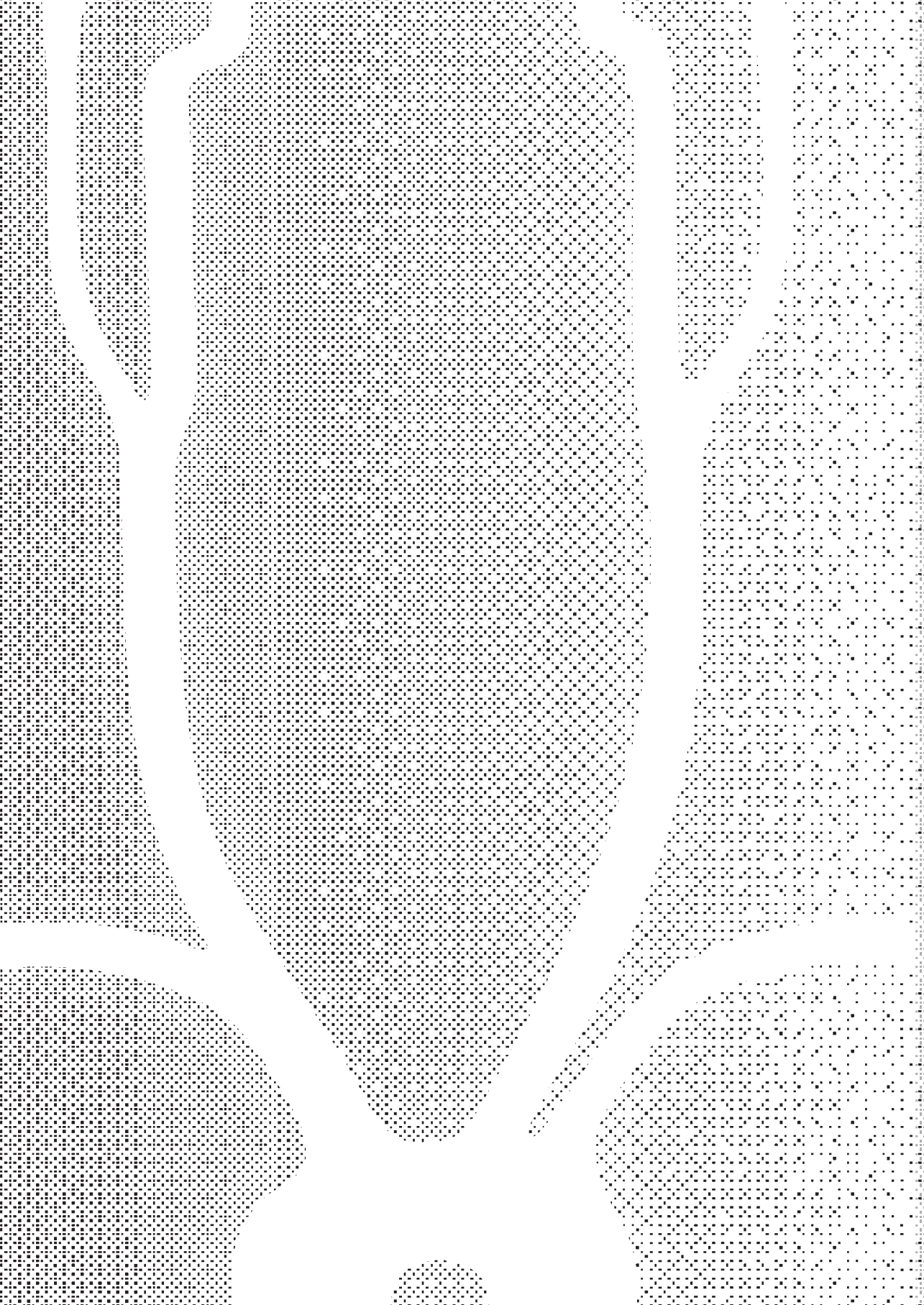
In **hoofdstuk 9** gaan we in op de onderzoeksresultaten binnen een groep patiënten met diabetes mellitus (DM). Naast schade door DM aan de vaatwand, kan DM ook leiden tot schade aan de nieren, schade aan de ogen en schade aan de zenuwen. Binnen de groep diabeten hebben we onderzocht of er een relatie bestaat tussen het optreden van deze complicaties en de hoeveelheid vaatschade. Hieruit kwam naar voren dat er geen relatie lijkt te zijn tussen de verschillende complicaties DM en het aanwezig zijn van subklinische atherosclerose of de hoeveelheid intra plaque microvascularisatie of de aanwezigheid van plaque ulceraties. Wel werd duidelijk dat in 90% van de diabeten tekenen zijn van subklinische atherosclerose.

Al langer is duidelijk dat er een verschil bestaat in de prevalentie van hart- en vaatziekten tussen mannen en vrouwen. In **hoofdstuk 10** van dit proefschrift hebben we onderzocht of er ook een verschil zit in de hoeveelheid intra plaque microvascularisatie tussen mannen en vrouwen. Hiervoor hebben we bij 159 patiënten, 74 vrouwen en 85 mannen, een contrast-echo gemaakt waarin we specifiek hebben gekeken naar de aan- of afwezigheid van atherosclerose en de hoeveelheid intra plaque microvasculatuur. Uit deze studie komt naar voren dat, na correctie voor de verschillen in de aanwezigheid van bekende cardiovasculaire risicofactoren, er bij vrouwen meer intra plaque microvasculatuur aanwezig is. Dit zou betekenen dat de vrouwen in de populatie die we hebben onderzocht meer kwetsbare atherosclerotische plaques hebben dan de mannen. Wel dient er de kanttekening gemaakt te worden dat nog onduidelijk is wat de prognos-

tische waarde is van de hoeveelheid intra plaque microvasculatuur. Daarbij is ook nog onduidelijk wat precies de onderliggende oorzaak is voor het optreden van meer intra plaque microvasculatuur in vrouwen dan in mannen.

Hoofdstuk 11 van dit proefschrift focust zich op een ander probleem dan atherosclerose: vaatwand ontsteking als gevolg van Takayasu arteritis of groot-cel arteritis. Bij deze ziektebeelden kan de vaatwand van de halsslagaderen ontstoken raken waarbij er verdikking van de vaatwand optreedt. Door ontsteking van de vaatwand treedt er mogelijk hyperplasie op van het bestaande fysiologische netwerk van vasa-vasorum, de vaten die de vaatwand van bloed voorzien. Middels contrast-echografie van de halsslagaderen zouden we betrokkenheid van de halsslagaderen en ziekte activiteit in de halsslagaderen kunnen bepalen. Om dit te evalueren werd er bij 7 patiënten met vaatwand ontsteking een contrast-echo van de halsslagaderen gemaakt. De resultaten van deze studie toonden dat contrast echografie een betere visualisatie gaf van het lumen van de halsslagaderen en dat er bij 5 van de 7 patiënten in verschillende gradatie sprake was van microvasculatuur in de vaatwand.

Tot slot hebben we de bevindingen van alle hoofdstukken samengevat in **hoofdstuk 12**. In dat hoofdstuk worden de belangrijkste bevindingen van het proefschrift afgezet tegen wat al bekend was, worden de resultaten in context geplaatst met de gebruikte methodologie en wordt er besproken op welke gebieden toekomstig onderzoek naar contrast-echografie van de halsslagaderen zich zou kunnen richten.



LIST OF PUBLICATIONS & PHD PORTFOLIO

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PHD PORTFOLIO

Name PhD student:	Stijn Cornelis Hendirkus van den Oord
Erasmus MC department:	Cardiology
Research school:	Cardiovascular Research School (COEUR), Erasmus MC
Title thesis:	Clinical evaluation of contrast-enhanced carotid ultrasound imaging
Promotors:	Prof.dr. A.F.W. van der Steen Prof.dr. E.J.G. Sijbrands
PhD period:	2010-2014

PhD training

Courses

	12 ECTS	Year
1.	Animal Imaging Workshop	2011
2.	Vascular Medicine	2011
3.	Biomedical English Writing and Communication	2011
4.	Neurovascular and Peripheral Vascular Diseases	2012
5.	Intensive Care Research	2012
6.	Introduction to Data-Analysis	2012
7.	Principles of Research in Medicine and Epidemiology	2012
8.	Cardiovascular Imaging and Diagnostics	2013

Teaching

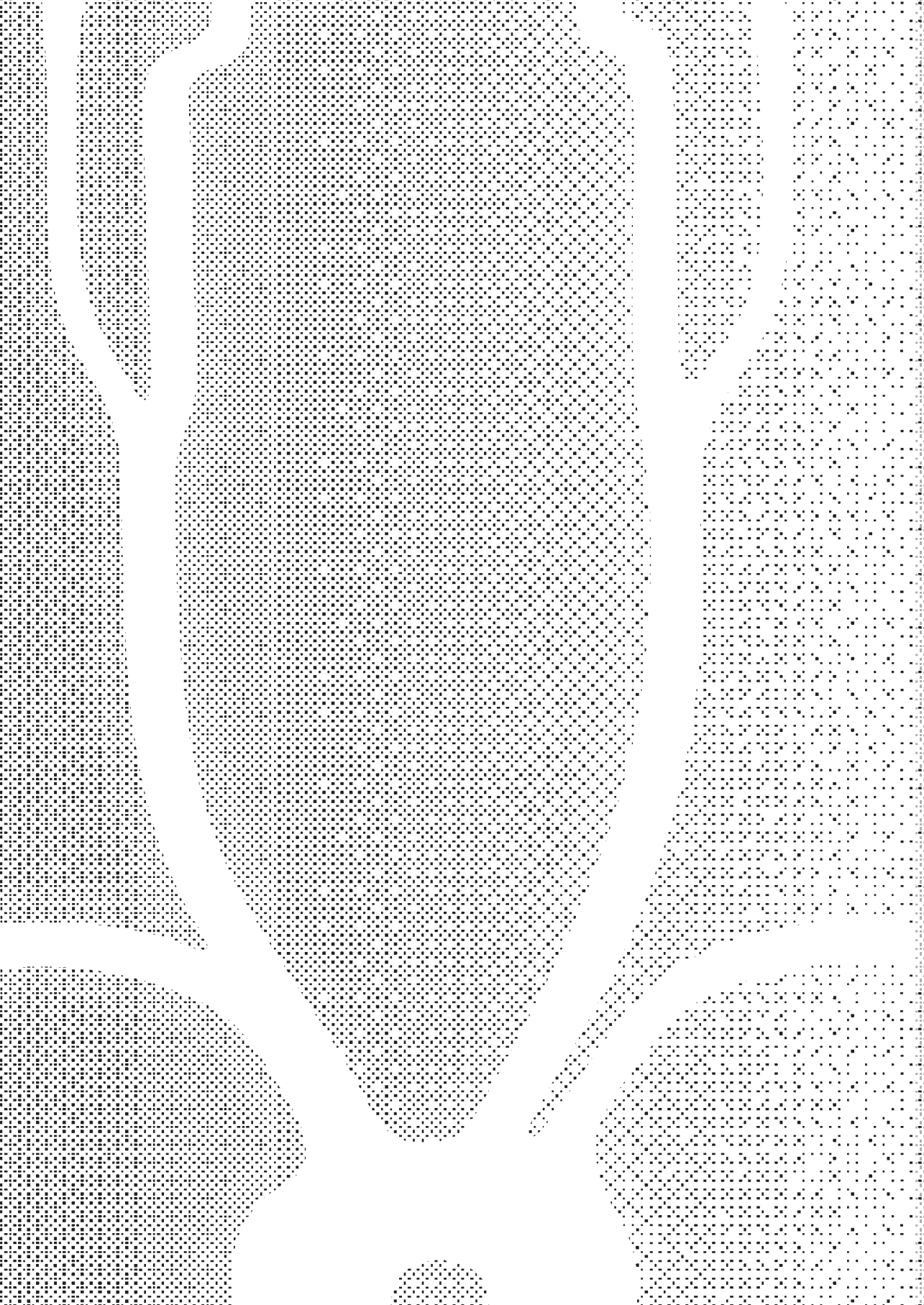
	5 ECTS	Year
1.	Supervising minor students "Medical Delta"	2011
2.	Junior Medschool	2011
3.	Staflunch	2011
4.	Supervising 2nd year medical students writing systematic review	2012
5.	Lecturing minor students "Keuzevak Cardiologie"	2012
6.	Staflunch	2012
7.	Supervising 2nd year medical students writing systematic review	2013
8.	Supervising 4th year medical student writing bachelor thesis	2013
9.	Staflunch	2013
10.	Staflunch	2014

COEUR Research Seminars

	4 ECTS	**Year**
1.	Coarctation Aorta	2010
2.	Imaging Atherosclerosis	2010
3.	Identification of Novel Genetic Regulators of Vessel Formation	2011
4.	Detection of Early Atherosclerosis	2011
5.	Diagnosis and Risk of Ischemic Heart Disease (oral presentation)	2011
6.	Pulmonal Hypertension	2013
7.	Carotid Atherosclerosis	2013
8.	Gender Differences in Cardiovascular Disease	2013

Symposia & Conferences

	17 ECTS	**Year**
Oral presentations		
1.	NVVC najaarscongres, Arnhem	2012
2.	EUROECHO & other Imaging Modalities, Athens	2012
3.	Ultrasound Contrast Imaging Symposium, Rotterdam	2014
Poster presentations		
1.	CTMM Annual Meeting, Utrecht	2010
2.	CTMM Annual Meeting, Utrecht	2011
3.	EUROECHO, Budapest	2011
4.	Ultrasound Contrast Imaging Symposium, Rotterdam	2012
5.	EUROECHO & other Imaging Modalities, Athens	2012
6.	Ultrasound Contrast Imaging Symposium, Rotterdam	2013
7.	American Heart Association, Scientific Sessions, Dallas	2013
8.	EUROECHO & other Imaging Modalities, Istanbul	2013
Attendance		
1.	AHA Telereview, Rotterdam	2010
2.	Wetenschapsdagen Inwendige Geneeskunde, Antwerpen	2011
3.	Ultrasound Contrast Imaging Symposium, Rotterdam	2011
4.	ACC Telereview, Rotterdam	2011
5.	Cardiology and Vascular Medicine; an ESC update, Rotterdam	2011
6.	Refereeravonden, Rotterdam	2011
7.	ESC Webinar Imaging Carotid Atherosclerosis	2011
8.	Symposium "Vasculair spreekuur", Rotterdam	2011
9.	Wetenschapsdagen Inwendige Geneeskunde, Antwerpen	2012
10.	Cardiology and Vascular Medicine; an ESC update, Rotterdam	2012
11.	Refereeravonden, Rotterdam	2012
12.	International Contrast Ultrasound Society Bubble Conference, Chicago	2013
13.	Refereeravonden, Rotterdam	2013



CURRICULUM VITAE

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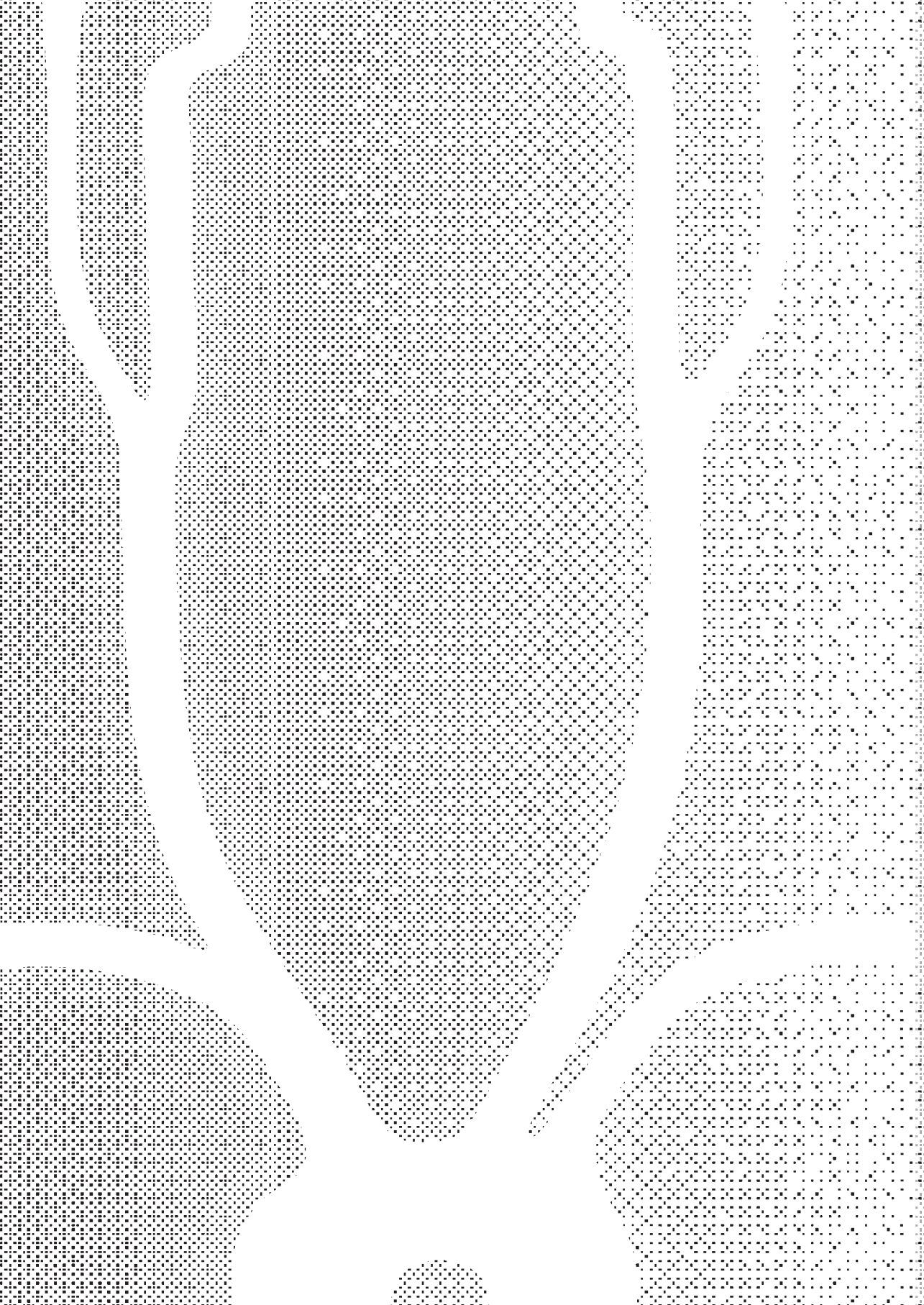
Stijn Cornelis Hendrikus van den Oord werd geboren op 22 april 1986 te Prinsenbeek. In 2004 behaalde hij zijn zijn VWO diploma (Newman College, Breda). In datzelfde jaar begon hij aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In 2008 startte hij zijn co-schappen waarna hij in 2010 zijn artsexamen behaalde. Aansluitend werkte hij als arts-onderzoeker op de afdelingen Cardiologie en Biomedical Engineering in het Erasmus Medisch Centrum te Rotterdam alwaar hij promotie onderzoek deed naar contrast-echografie van de halsslagaderen (promotoren: Prof.dr. A.F.W. van der Steen en Prof.dr. E.J.G. Sijbrands, copromotor: Dr. A.F.L. Schinkel). Het resultaat van dit promotie onderzoek is beschreven in dit proefschrift. In Januari 2014 startte Stijn als ANIOS op de afdeling Cardiologie van het Erasmus Medisch Centrum. Per Oktober 2014 zal hij starten aan de opleiding tot cardioloog (opleiders: Prof. dr. J.J. Deckers en Dr. T.W. Galema).



DANKWOORD

DANKWOORD

Het is bijna klaar. Het boekje is bijna af. Het enige dat nog rest is het dankwoord. Tevens het enige gedeelte in het proefschrift dat ik volledig zelfstandig geschreven heb. Onderzoek is namelijk teamwork en behoudens het dankwoord is alles in dit proefschrift tot stand gekomen door fijne samenwerkingen, goede supervisie en niet aflatende steun van verschillende personen. Ik wil iedereen die heeft bijgedragen aan het tot stand komen van dit werk hartelijk danken.

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Mijn copromotor dr. A.F.L. Schinkel is de drijvende kracht geweest achter al het onderzoek gepresenteerd in dit proefschrift. Beste Arend, jij hebt mij geïntroduceerd in de wereld van het wetenschappelijke onderzoek. Met de VASA studie hebben we een unieke database op kunnen bouwen. Mede door jou begeleiding heb ik geleerd om resultaten van onderzoek uit deze database in de vorm van papers te publiceren. Ik waardeer jou altijd

positieve instelling wanneer er in mijn ogen onmogelijke verzoeken kwamen vanuit reviewers of wanneer er een paper werd afgewezen voor publicatie. Daarnaast hebben we samen een aantal imaging congressen bezocht om onze resultaten te presenteren. Dit werd altijd gecombineerd met etentjes in goede restaurants. Ik ben erg blij dat jij mij overhaalde om te gaan promoveren. Dit heeft voor mij vele deuren geopend. Ik heb veel van je geleerd en ik hoop in de komende jaren nog veel van je te kunnen leren.

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Prof. dr. A van der Lugt. Beste Aad, je hebt een unieke gave om op vrijwel alle problemen die we tegenkwamen een oplossing paraat te hebben. Je to-the-point e-mail antwoorden op mijn vaak iets langere e-mails, toverde vaak een glimlach op mijn gezicht. Het is een eer voor me dat ik mijn proefschrift tegenover je mag verdedigen.

Prof. dr. M.J.A.P. Daemen. Beste Mat, ik waardeer je altijd kritische blik ten aanzien van contrast-echografie van de halsslagaderen. Dit heeft mij scherp gehouden. Dank voor je begeleiding gedurende de analyses in het histologie project.

Prof. dr. J.W. Jukema, Prof. M.A. Averkiou en dr. F.J. ten Cate wil ik bedanken voor het plaats nemen in de grote commissie.

Dr. F.J. ten Cate. Beste Folkert, mede door jou expertise op het gebied van contrast-echografie is de VASA studie van de grond gekomen. Ik ben blij dat ik je af en toe kon helpen als je computer weer niet deed wat je wilde. Dank voor de fijne samenwerking!

Dr. G.L. ten Kate. Beste Luit, jij was ruim een jaar bezig aan de VASA studie toen ik begon. Je hebt al het voorwerk verricht, zodoende kon ik een vliegende start maken. Ik ben je daar dankbaar voor. Je hebt in februari dit jaar je proefschrift met succes verdedigd, ik hoop dat ik op 8 oktober eenzelfde prestatie neer kan zetten.

Zeynettin Akkus. Dear Zeynettin, the past years we have worked together on the same topic. This led to a close collaboration that resulted in a series of clinical and tehnological papers. It was always a pleasure to step by on the 23rd floor, have a coffee together and think of new ideas and improvements that could be made. The 8th of october we will both defend our thesis, I'm looking forward to it!

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I want to thank all my colleagues on the Department of Biomedical Engineering. Special thanks to Verya and Alex, both of you were always available for a coffee when I needed some distraction from writing papers. Mijn dank gaat ook uit naar Rita, Gracia en Mieke die een hoop administratief werk van mij afgenomen hebben de afgelopen 4 jaar.

Edith Padberg. Beste Edith, bedankt dat je de vele afspraken met Eric altijd op korte termijn geregeld kreeg.

Dr. H.M.M. van Beusekom, Kim en Rory. Beste Heleen, Kim en Rory, we hebben lang samengewerkt aan het histologie project en vele uurtjes achter de microscoop gezeten. Op het punt dat ik vermoedde dat we tijd te kort zouden komen hebben we samen naar logistieke oplossingen gezocht. Ik realiseer me dat ik in die tijd veel druk op jullie heb gezet om alles op tijd klaar te krijgen. Ik wil jullie danken dat jullie zo hard gewerkt hebben, dat alles precies op tijd af was.

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