Longitudinal changes in Size and Composition of Carotid Artery plaques using Ultrasound: Adaptation and validation of methods

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Thesis for the degree of Master of Science

Department of radiography

Faculty of Medicine

Supervisor: Vilmundur Guðnason

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HÁSKÓLI ÍSLANDS

Mat á breytingum í stærð og samsetningu skella í hálsæðavegg með ómun – Langsniðsrannsókn: Aðlögun og mat á aðferð

Guðlaug Björnsdóttir

Ritgerð til meistaragráðu í geislafræði

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Ágrip

Inngangur: Ómun af hálsslagæðum gerir kleift að mæla bæði stærð og samsetningu æðaskella. Tilgangur rannsóknarinnar var að innleiða aðferð og meta endurtekningarhæfni aðferðarinnar þar sem ómun er notuð til mats á stærð og samsetningu æðaskella í langsniðsrannsókn, Öldrunarrannsókn Hjartaverndar (ÖHV) og Áhættuþáttakönnun (ÁHV) Hjartaverndar.

Aðferðafræði: 219 þátttakendur úr ÖHV (76±6 ára, 36% karlar) og 10 þátttakendur úr ÁHV (60.5±5.5 ára, 70% karlar) gengust undir ómrannsókn af hálsslagæðum með u.þ.b. 5 ára millibili til skoðunar á stærð og samsetningu æðaskella. Notast var við staðlaða aðferð til að ná sem líkustum myndum milli heimsóknar1 og heimsóknar2. Myndgreiningarforritið AMS var aðlagað til að mæla breytingu í stærð og samsetningu æðaskella. Þrír rannsakendur tóku þátt í rannsókninni, mældur var bæði innan og milli rannsakenda breytileiki þar sem annarsvegar 25 þátttakendur úr ÖHV rannsókninni voru valdir og 10 þátttakendur úr ÁHV þar sem lesarar lásu tvisvar með a.m.k. viku millibili.

Niðurstöður: *Innan lesara breytileiki fyrir stærð æðaskella:* Fyrir lesara1 (bæði ÖHV og ÁHV hóparnir) breytileikastuðullinn var 12.10%, r =0.96 og 9.82%, r=0.96, enginn marktækur munur á milli úrlestra (p=0.74, p=0.23). Fyrir lesara2 (ÖHV hópurinn) breytileikastuðullinn var 18.63%, r=0.90, það var marktækur munur á milli úrlestra (p<.0001). Fyrir lesara3 (ÁHV hópurinn) breytileikastuðullinn var 16.03%, r=0.96, enginn marktækur munur milli úrlestra (p=0.72).

Innan lesara breytileiki fyrir samsetningu æðaskella: Fyrir lesara1 (báðir hóparnir ÖHV og ÁHV) breytileikastuðullinn var 7.77%, r=0.90 og 6.84%, og r=0.91, enginn marktækur munur á milli úrlestra (p=0.73, p=0.36). Fyrir lesara2 (ÖHV hópurinn) breytileikastuðullinn var 8.04%, r=0.86 enginn marktækur munur á milli úrlestra (p=0.08). Fyrir lesara3 (ÁHV hópurinn) breytileikastuðullinn var 6.97%, r=0.88 og enginn marktækur munur á milli úrlestra (p=0.20).

Breytileiki milli lesara fyrir stærð æðaskella: Fyrir lesara (1 og 2) (ÖHV hópurinn) breytileikastuðullinn var 23.29%, r=0.81, það var marktækur munur á milli lesara (p<.0001). Fyrir lesara (1 og 3) (ÁHV hópurinn) breytileikastuðullinn var 18.20%, r=0.91, það var marktækur munur á milli lesara (p=0.0023).

Breytileiki milli lesara fyrir samsetningu æðaskella: Fyrir lesara (1 og 2) (ÖHV hópurinn): var breytileikastuðullinn 8.55%, r=0.87, enginn marktækur munur á milli lesara (p=0.35). Fyrir lesara (1 og 3) (ÁHV hópurinn): var breytileikastuðullinn 7.45%, r=0.82 enginn marktækur munur á milli lesara (p=0.44).

Ályktun: Í þessari rannsókn var kynnt stöðluð aðferð bæði við ómun og úrlestur mynda til að mæla breytingu í stærð og samsetningu æðaskella. Aðferðina er hægt að nota á áreiðanlegan hátt til að mæla langtíma breytingu í stærð og samsetningu æðaskella. Lögð er áhersla á mikilvægi reynslu og þjálfunar þar sem vinnureglu eins og hér er lýst er fylgt nákvæmlega.

Abstract

Introduction: B-mode ultrasonography of the carotid arteries makes quantitative measurements of atherosclerotic plaque area and composition assessed as grey-scale median (GSM) possible. The purpose of this study was to set up a standardized ultrasound protocol to measure longitudinal changes in plaque area and composition and to determine the intra- and inter-observer variability of the measurements.

Method: A total of 219 participants from the AGES Reykjavik Study (76±6 years old, 36% males) and 10 participants from the REFINE Reykjavik Study (60.5±5.5 years old, 70% males) underwent 2D B-mode ultrasound examination of the carotid arteries approximately 5 years apart for a longitudinal assessment of plaque area and composition. Standardized protocol was used to acquire comparable images from both visits. Ultrasound was performed bilaterally on the common carotid artery, internal carotid artery and the bifurcation. A modified version of the Artery Measurement System (v1.141) was used to measure plaque area and GSM values. Three sonographers participated in this study, intra- and inter observer variability was based on re-reading ultrasound images from 25 subjects selected from the AGES Reykjavik cohort and the 10 subjects selected randomly from the REFINE Reykjavik cohort.

Results: *Intra sonographers variability for plaque area:* For sonographer1 (AGES and REFINE groups): the coefficient of variation (CoV) was 12.10%, r=0.96 and 9.82%, r = 0.96 respectively with no statistically significant difference between observations (p=0.74, p=0.23). For sonographer2 (AGES group) the CoV was 18.63%, r=0.90, statistically significant difference between observations (p<.0001). For sonographer3 (REFINE group) the CoV was 16.03%, r=0.96, no statistically significant difference between observations (p=0.72).

Intra sonographers variability for plaque GSM: For sonographer1 (AGES and REFINE groups) the CoV was 7.77%, r=0.90 and 6.84%, and r=0.91 respectively, with no with no statistically significant difference between observations (p=0.73, p=0.36). For sonographer2 (AGES group) the CoV was 8.04%, r=0.86, with no statistically significant difference between observations (p=0.08). For sonographer3 (REFINE group) the CoV was 6.97%, r=0.88, with no statistically significant difference between observations (p=0.20).

Inter sonographer's variability for plaque area: For sonographers (1 and 2) (AGES group) the CoV% was 23.29%, r=0.81, statistically significant difference between sonographers (p<.0001). For sonographers (1 and 3) (REFINE group) the CoV was 18.20%, r=0.91, statistically significant difference between sonographers (p=0.0023).

Inter sonographer's variability for plaque GSM: For sonographers (1 and 2) (AGES group) the CoV was 8.55%, r=0.87, with no statistical difference between sonographers (p=0.35). For sonographers (1 and 3) (REFINE group) the CoV was 7.45%, r=0.82, with no statistical difference between sonographers (p=0.44).

Conclusion: This study shows that ultrasound can be used consistently for assessment of changes in plaque area and GSM over time. This can be achieved by proper training of ultrasound sonographers and applying and following a strict protocol introduced in this study.

Acknowledgement

This project was made at the Icelandic Heart Association (IHA) and was funded by The Icelandic Center for Research (RANNIS) and also the National Institute of Health (NIH). The author of this thesis participated in all parts of the study, in addition four radiographers (Agnes Guðmundsdóttir, Grímheiður F. Jóhannsdóttir, Ágústa Dröfn Sigmarsdóttir and Erla Björk Hjaltadóttir) participated in the acquisition and two (Agnes and Erla Björk) in the imaging analysis with various amount of reading depending on different parts of the study. I would like to thank all of them for their contribution to this project and many members of the staff at the Icelandic Heart Association who contributed to the actual process of the research with special thanks to Agnes Guðmundsdóttir for her encouragement and support during this study. Further I would like to thank Thor Aspelund a member of staff at IHA for statistical advice. I would also like to thank the study participants for their contribution to this project this study wouldn't have happened without them.

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Table of contents

Index of Figures

Index of Tables

Abbreviations

AGES	Age Gene/Environment Susceptibility Reykjavik Study
AMS	Artery Measurement System
AS	Acoustic shadowing
BMI	Body Mass Index
CCA	Common Carotid Artery
Chol	Cholesterol
CI	Confidence Interval
CIMT	Carotid Intima-Media Thickness
cm	Centimeter
CoV	Coefficient of Variation
CT	Computer Tomography
CTA	Computed Tomography Angiography
CVD	cardiovascular disease
dB	Decibels
Diff	Difference
ECA	External Carotid Artery
FDG	Fluorodeoxyglucose
GSM	Grey Scale Median or Composition
HDL	High density lipoprotein
HTN	Hypertension
HU	Hounsfield Unit
ICA	Internal Carotid Artery
ICC	Intra Class Correlation
IHA	Icelandic Heart Association
IMT	Intima-Media Thickness
IVUS	Intra Vascular Ultrasound
L	Left
LDL	Low Density Lipoprotein
MDCTA	Multi-Detector Computed Tomography Angiography
MHz	Megahertz
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging

MSCT	Multislice computed tomography
NIA	National Institute on Aging
NIH	National Institute of Health
PET	Positron Emission Tomography
R	Right
REFINE	Risk Evaluation For Infarct Estimate Study
Rho	Correlation
SD	Standard Deviation
3D	Three dimensional
US	Ultrasound
VH	Virtual Histology

1 Introduction/Background

Carotid plaque is unequivocal evidence of atherosclerosis and reflects more advanced aspects of the atherosclerotic process than carotid intima media thickness (IMT) when measured with ultrasonography (1). It has been observed that the vessel wall thickens more slowly than the plaque itself grows, which in turn allows better detection of the progression of atherosclerosis by measuring plaque composition and size rather than IMT in longitudinal studies (2). However, qualitative plaque measurements with ultrasonography of the carotid arteries have proved to be subjective and suffer from poor reproducibility (3-5). More quantitative and objective method of plaque characterization has been made with computerized assessment of plaque echogenicity, measured as grey-scale level (6). This improved method gives plaque with high fibrous content and calcified tissue a high grey-scale value (echo-rich plaque), whereas plaque with high lipid and hemorrhage content gives a low grey-scale value (echo-lucent plaque) (6, 7). The most important outcome parameter on the grey-scale is the median value which gives information on the composition of the plaque and referred to as grey scale median (GSM). Echo-lucent (fat/lipid) plaque is more likely to rupture than echo-rich (fibrous and calcified) plaques (8-10). Most studies using this method have been limited to small sample sizes and case-control or cross-sectional study design. There is a lack of studies that have assessed the reproducibility of B-mode ultrasonography with measurements of the longitudinal changes in size and composition of carotid plaques. Furthermore, studies have used different parameters and statistical methods when assessing the reproducibility of plaque size measurements and composition making it difficult to compare results across different studies. The purpose of this study was to set up a standardized ultrasound protocol to measure longitudinal changes in plaque area and composition and to determine the intra- and inter-observer variability of the measurements. The study included participants from the Age Gene/Environment Susceptibility Reykjavik Study (AGES-Reykjavik) and also participants from the Risk Evaluation for Infarct Estimate Reykjavik Study (REFINE Reykjavik). Both studies are population-based with longitudinal measurements of carotid plaque and IMT by ultrasonography. The reproducibility was assessed by using diverse statistical methods to allow comparison with other studies.

In the following subchapters the progress of atherosclerosis and the anatomy of the common carotid artery will be address briefly. Different imaging modalities to measure plaque in the common carotid artery will be mentioned briefly but the focus will be on the ultrasound imaging of the common and the internal carotid artery and the bifurcation.

1.1 Atherosclerosis

Atherosclerosis is common and the risk of life threatening consequences of the diseases, such as myocardial infarction, stroke or gangrene in lower limbs (peripheral vascular ischemia) are common causes of mortality and morbidity (1). Atherosclerosis is a chronic disease that can affect both the young and the elderly although more common at advanced age. The disease can be progressing for years without producing any clinical symptoms (11, 12).

The main characteristic of arteriosclerosis is thickening of the arterial wall and loss of elasticity due to damage of the endothelial lining (13). The atherosclerotic progress can be divided into several stages (Figure 1). Inflammation is thought to play an important role in the progression of atherosclerotic disease and is believed to be present in the arterial wall at all stages of the disease (14). The first stage where the wall has thickened can be measured as the Intima media thickness (IMT). Intima media thickness is believed to be related to hypertension or left ventricular hypertrophy (15, 16) as well as indicate infiltration of the atherosclerotic process. As the disease progresses the formation of plaque appears. The initial plaque is minimal, depending on the progression of the formation it can go up to moderate, severe or may even cause occlusion of a blood vessel. The composition of the plaque changes over time as it goes from being comprised of more lipids to more complex lesions with more necrotic or calcified regions. The echolucency is thought to play an important role in the growth progression of the plaque. Increasing echogenicity or plaque hardening slows down the plaque growth inversely ehcolucent plaques, more composed of lipids tend to have more growth progression in plaque area (17, 18). It has been shown that the composition of plaque is more predictive than the plaque size for plaque rupture with the following formation of a thrombus (10). The plaque in symptomatic patients are more prone to rupture because the plaque is more composed of inflammatory infiltrate, smaller lipid necrotic core and it also has thin cap and increased intermediate elastin form (19-21). Smaller plaque or less obstructive ones can be more dangerous than larger plaques as they are often vulnerable to rupture because of their lipid content (22).

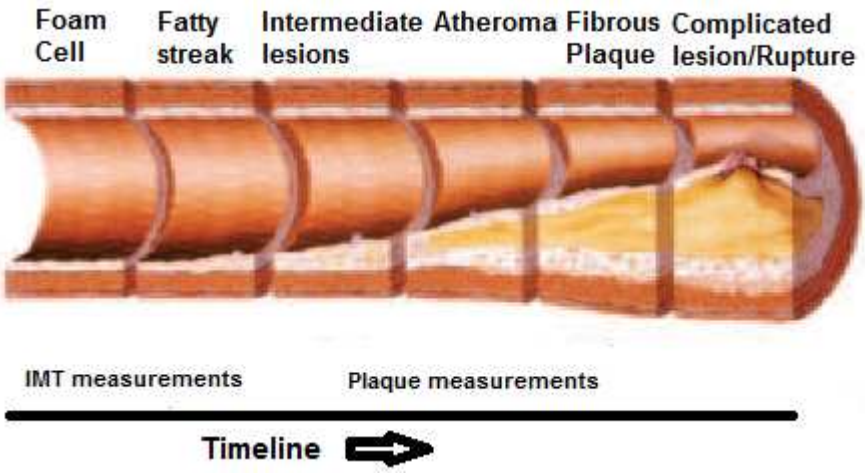


Figure 1. Schematic image showing the progression of atherosclerosis. A modified image from (14). Thickening of the arterial wall and formation of plaque starts. At the end of the plaque formation when plaque is complex the plaque can rupture.

Normal artery has three layers (Figure 2), the tunica intima which is in contact with blood through its thin monolayer of endothelial cells overlying a basement membrane. The media or the middle layer contains smooth muscle cells embedded in extracellular matrix. The outermost layers of the arteries is the adventitia which contains micro vessels, mast cells, nerve endings, fibroblast, type I collagen fibers and elastic network (12, 23).

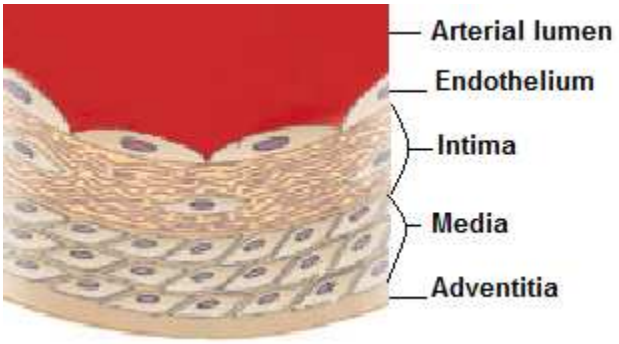


Figure 2. Schematic image of the arterial layers, modified picture from Libby (24).

During the progression of the disease macrophages are recruited into the intima layer. Macrophages take up the low density lipoprotein (LDL) in the intima layer and turn into foam cells. The smooth muscle cells from the media layer start to migrate into the intima and form a fibrous cap over the foam cells (12, 25). Atherosclerosis is a systems disease and atherosclerosis in one part of the arterial tree increases the likelihood of atherosclerosis in other parts of the arterial tree. Plaque in the common carotid artery may therefore predict the risk for atherosclerosis elsewhere in the body, such as in the coronary arterial tree or in the peripheral arteries i.e. in the lower limbs (18).

Shear stress is thought to play an important role in the progression of atherosclerosis. Low shear stress can induce changes in leukocyte so they adhere more with the activated endothelial layer (26). Formation of atherosclerosis is often in the bifurcation of an artery where the shear stress is oscillatory or where the shear stress is low like in the curvatures of coronary arteries. Increased shear stress gives a protection against the progression of atherosclerosis (26, 27).

1.2 Common carotid artery-bifurcation, Internal and external carotid artery

Left common carotid artery arises from the arch of the aorta, but the origin of the right common carotid artery is from the brachiocephalic artery see (Figure 3). Both the left and right common carotid arteries have a bifurcation located approximately at the distal part of the thyroid gland. The approximate bifurcation length varies between subjects.

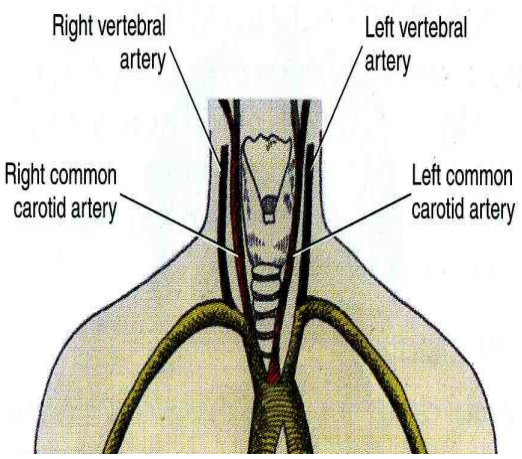


Figure 3. Arch of aorta and branches for the right common carotid artery and left common carotid artery, picture from (28).

After the bifurcation the common carotid artery (CCA) divides into the internal- and external carotid arteries (29) (Figure 4). Plaque in the common carotid artery or internal carotid artery can cause occlusion, the fibrous cap of the plaque can also rupture and the collagen gets exposed to the circulation and may cause thrombus formation. If the thrombus moves it may cause embolism and stroke (30, 31).

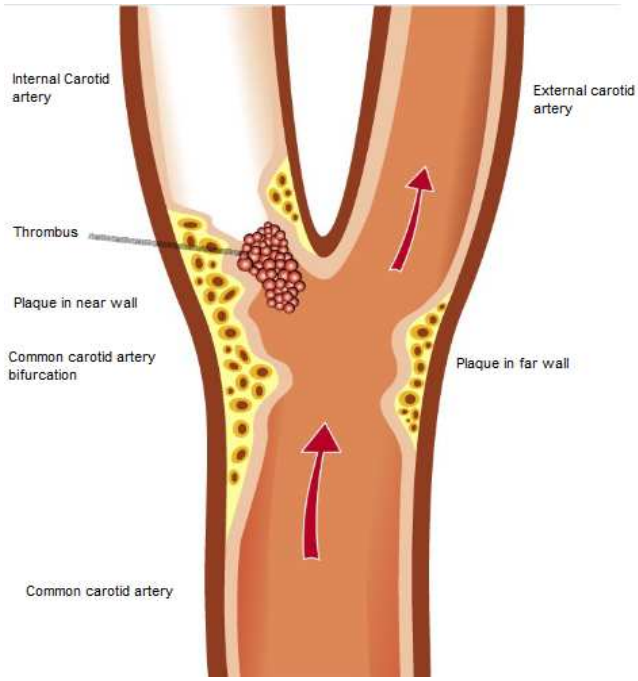


Figure 4. Schematic picture of the common-, bifurcation, internal- and external carotid artery. Plaque is visible in both near and far wall of the bifurcation and internal carotid artery. There is a thrombus in the internal carotid artery causing occlusion for the blood flow, picture from; (31).

1.3 Common carotid Intima-media-thickness (CIMT) measurement

The average intima-media-thickness (IMT) in healthy young individual is ~0.15 mm (32). To measure the IMT in the common carotid artery two parallel lines can be used to measure the distance between the arterial layers; the media-adventitia and lumen-intima on longitudinal ultrasound images, a well-known method (33). The lines can be generated using automated software with 10 mm long lines reaching proximal from the bifurcation of the CCA. The program then automatically assigns the lines to the layers. If not properly assigned it can be corrected manually (34, 35). Atherosclerosis has primarily been investigated with ultrasound by measuring the carotid intima-media thickness (CIMT) of the arterial wall and the presence of carotid artery plaques and degree of carotid artery stenosis are recognized as risk factors for cardiovascular disease (CVD), stroke and mortality (36-38). There is some evidence that the risk for atherosclerosis in coronary arteries can be effectively predicted by measuring IMT in the carotid arteries (39). Furthermore atherosclerosis in the carotid artery correlates with atherosclerosis elsewhere in the circulation including coronary arteries (7, 40-42).

Semi-automated border-detection software has been used with good results to increase the reproducibility in CIMT measurements (43). The sensitivity of the IMT has been somewhat debatable, Migrino et al. (44) showed that after a statin therapy where ultrasound was used to measure IMT and Magnetic Resonance Imaging (MRI) was used to measure borders of the adventitial/lumen, a regression was detected on the MRI images but not on the ultrasound images. The reproducibility of CIMT measurements based on inter observer variability assessment has been shown to be excellent with low mean inter-observer difference 0.0186±0.0244 mm (45). Touboul et.al. (46) measured the reproducibility of CIMT by performing repeated measurements and reported the intra class correlation coefficient of 0.97. However, CIMT measurements have not been shown to be as good risk estimator for stroke or coronary artery disease as the total plaque area in the carotid arteries (47-49).

1.4 Carotid plaque GSM (composition) and area (size)

Several studies have shown that the presence of carotid stenosis is a strong predictor for death in the general population (36, 50). Vulnerable plaques are plaques with high risk of rupture and can cause thrombosis and embolism. In the assessment of the vulnerability of atherosclerotic lesions it is common to use ultrasonographic B-mode characterization of carotid plaque morphology (7, 9). The difficulty with ultrasound acquisition and image reading is that it's very dependent on the users and it seems that variability in published measurements is often kept aside and only few statistical parameters are reported, this is summarized in Appendix II where several publications are listed with their reported statistical parameters. Ultrasound has been accepted as the commonly used imaging modality to measure plaque stenosis and mineralization or the plaque components (51). When ultrasound imaging is used the acoustic shadowing (AS) because of attenuation of ultrasound beam can affect the image quality behind the calcified structure (51, 52). In the current literature there are only few studies that have published the effect of AS (51, 53). In plaque GSM measurements, calcified regions within the plaque or in the opponent wall can cause AS and influence the GSM

results (53, 54). The GSM can indicate the instability of the plaque (echo-lucent or echogenic). The GSM scale ranges from 0 (black/blood) to ~250 which indicates highly calcified regions. The threshold for the difference between stable or unstable plaques varies somewhat between studies, the GSM cutoff can be from 32 to 50 for echo-lucent and for echogenic above ~200 (55-57).

Andersson et al. (18) reported that plaque GSM and plaque area are partly influenced by different risk factors, e.g. body mass index and high density lipoprotein (HDL) cholesterol have more influence on plaque GSM while inflammation would be more related to plaque area/size. The relationship between plaque area and age has proved to be strong. Increasing plaque area gives increased risk of myocardial infarction, stroke or other vascular death diseases than smaller plaque areas (58). In plaque area measurements the cross sectional image of the longitudinal view can be used where the plaque boundaries are traced to define area of each plaque. The total plaque burden can then be estimated as the sum of all plaque areas within the subject or arteries (1, 17, 47).

There are studies that have assessed reproducibility for both plaque area and GSM (17, 18, 59). It has been implied that measurements on near wall of carotid artery would be more difficult and less accurate than measurements in far wall possibly due to different order of the arterial layers to the incoming ultrasound beam (60, 61). Most recent studies measure both near and far wall of the common carotid artery either for the intima media thickness or plaque measurements (17, 47, 62).

Several different imaging modalities have been used to measure plaque changes over time in the carotids. In a study by Migrino et.al (44) MRI showed regression of plaque size in the CCA after 6 months statin therapy which was not detectable on CCA Ultrasound (US) images. Also, two studies measuring the effect of statin therapy on plaques with two different imaging modalities MRI and FDG-PET imaging, the difference in plaque size as an effect of statin was detectable with both modalities but FDG-PET detected the changes sooner or after 3 months but MRI after 12 months (63, 64). This suggests that FDG-PET is most sensitive in early CCA plaque detection followed closely by MRI.

A comparison between the imaging modality and histology from assessing tissue after endarterectomy has been made to measure accuracy of the imaging method in the assessment of plaque composition (3, 65-67). Examples of imaging modalities that have been used to image the carotid arteries are briefly reviewed in the following sections.

1.4.1 Magnetic resonance imaging (MRI)

Magnetic Resonance Imaging has been used from the early seventies and since then technical improvements have been extensive for all kinds of diagnostic imaging (68). This imaging modality provides highly detailed anatomy of the internal organs. One type of MRI that specially highlights the arteries or the circulatory system in the body including the carotid arteries is Magnetic Resonance Angiography (MRA) (69). The advantage with MRI is its high contrast resolution; it can reliably distinguish difference between tissues with similarities in their characteristic (70). Magnetic resonance imaging can be used to image the morphology and composition of atherosclerotic plaques (71). The spatial resolution of MR images is lower compared to CT images (70). Measurements with MRI of carotid plaques has proven to be well reproducible but has the disadvantage of being relatively time consuming, expensive and not all subjects can undergo MRI examination because of contraindications

including peacemakers, cochlear implants, nerve stimulators, ferromagnetic clips, prosthetic heart valves and claustrophobia (72). Magnetic resonance imaging can detect whether atherosclerosis is present, it has been used to measure the lipid core and fibrous cap of plaques in the CCA and compared to histological findings with good agreement (73, 74).

1.4.2 Computed tomography (CT)

Computed tomography was first used in clinical practice in early seventies and the technical development has been enormous and is constantly progressing. In the past, CT of the carotids has primarily been used to detect and estimate the degree of vessel lumen stenosis. However, recently it has been used increasingly for the assessment of plaque size and composition by using the Hounsfield Unit (HU), a measure of the density of the component (75-77). The HU scale ranges from ±1000 HU where 0 corresponds to the density of water, objects composed of more fat ingredients have negative values on the HU scale and bone or highly calcified regions have positive values up to 1000 HU (78). Spectral CT can distinguish between water, lipid, iron and calcium which are components of vulnerable plaques and there has been found a good coherence between histology and CT images of plaque surface (67). The advantage of measurements of carotid plaque volume and composition using CT is the high spatial and temporal resolution (70, 77, 79, 80). Limitations of CT in general include dependence on high doses of ionizing radiation although this depends on different types of CT studies (81).

A study by de Weert et al. (76) reported the variability between three observers with intra class correlation (ICC) for repeated volume measurements of plaques ranging from 0.53 to 0.96 (76).

1.4.3 Positron Emission Tomography (PET)

Positron Emission Tomography (PET) is a functional imaging modality that provides images of biological process to look for diseases or abnormalities by using radionuclide tracers which are connected to a carrying biologic molecule. In the early sixties the simplest form of single-PET scan was introduced and in early seventies the 2D PET scan appeared. Today the PET scan can provide three dimensional images (82, 83). This imaging modality uses various positron emission radioactive compounds or tracers. The use of ^{18}(F)-fluorodeoxyglucose (^{18}F-FDG) which was introduced in the early eighties and the (^{18}F-FDG)-PET imaging modality is commonly used in arterial plaque measurements to measure plaque progression or eruption (84-87).

The advantages of PET include that earlier stages of diseases can be visualized on molecular basis because of the use of radioactive materials or nuclear medicine (^{18}FDG) (84, 86, 88). The limitations of PET imaging include poor spatial resolution which makes accurate anatomical location of disease difficult. However, this can be compensated for by co-registering PET images with another imaging modality with higher spatial resolution, e.g. MRI or CT (87, 89). Further, the use of radionuclides is expensive due to high cost cyclotrons that are needed to produce an appropriate radionuclide with short live time which is made to reduce the ionizing radiation as possible (82). Measurements of plaque in CCA with PET/CT have shown to have a good reproducibility with correlation within and between repeated measurements ranging from 0.95 to 0.98 (90).

1.4.4 Catheter Angiography

Catheter Angiography has been used since early 20th century and the x-ray angiogram of the carotid arteries gives information about degree of stenosis or the luminal diameter implying the atherosclerotic disease. This imaging modality doesn't provide information about the composition of the plaque although it can be used to measure plaque size (87, 91). This is an invasive method that uses contrast medium to view normal and abnormal flow of the circulatory system. The injection is straight into the artery of interest through the catheter that has been placed in the artery (vessel) (28). Since angiography is an invasive method there are some contraindications and/or risk factors associated with it. Patients that are allergic to iodine contrast medium, blood clotting disorder or impaired renal functions can't undergo the angiography. Including formation of embolus or infections at the puncture side or bleeding are fairly common side effects with catheter angiography. Another factor to consider during angiography is the ionizing radiation. Angiography uses fluoroscopic equipment and to minimize the ionizing radiation it's necessary to keep the fluoroscopic time to absolute minimum. It is also important to be very precise in collimation, and if possible to use lead shields (28).

1.4.5 Intravascular ultrasound (IVUS)

The Intravascular ultrasound (IVUS) is an invasive modality that has been used when plaque can't be detected or visualized in catheter angiography. Intravascular ultrasound uses probe that is miniaturized and placed on the end of a catheter. The catheter goes into the arterial lumen and the endothelium and surroundings can be visualized (92, 93). In carotid angioplasty the utility of IVUS has been proved to increase the accuracy in the assessment of plaque or stenosis and should therefore be the modality used when evaluating carotid stenosis with catheter based intervention (94). Another IVUS imaging modality that can color map the plaque depending on the tissue type is the Virtual Histology (VH) IVUS, this method can give information about the composition of the plaque e.g. with histological components. Virtual Histology Intravascular Ultrasound color-cods the plaque into four colors that correspond to the composition (fibrous, fibro fatty, calcified and necrotic lipid core) of the plaque and the images acquired correspond to the different histological components of the plaque. A comparison between assessments of plaque in common carotid artery with VH IVUS and histological findings after endarterectomy resulted in strong correlation with high accuracy ranging from 72.4% to 99.4% (66). Intravascular ultrasound imaging studies have shown that lipid-lowering therapies using statin change plaque composition over time making it more stable (95, 96).

1.5 Vascular ultrasound

Ultrasound has been developing for several decades in diagnostic imaging (97, 98). Ultrasound is convenient for screening populations because of the real-time visualization the short acquisition time, availability, low cost and it does not use ionizing radiation (99). Ultrasound transducers transmit pulses of sound-waves on different frequencies depending on the organs or objects that are of interest. Ultrasound images are reconstructed from echoes of ultrasound waves that reflect from tissue boundaries and small irregularities within tissues. The main cause of this reflection of the sound waves is the difference of the sound speed in different elements (tissues) where the tissue density is an important factor. From these echoes a scaled map of echo-producing features is produced to display cross sectional image that corresponds to the spatial origin in the body. There are various types of transducer for different applications. The phased array transducer with narrow field of view near the probe but gives wide image deeper in the body is mainly used for cardiac imaging. The linear array transducers are commonly used and can either be linear or curvilinear (Figure 5). Linear transducers have a rectangular field of view that is wide close up to the probe and are therefore suitable for superficial organs like the common carotid artery. Curvilinear transducers share the benefit of wide view at the surface but the field of view becomes wider with depth and is therefore suitable for abdominal imaging (100).

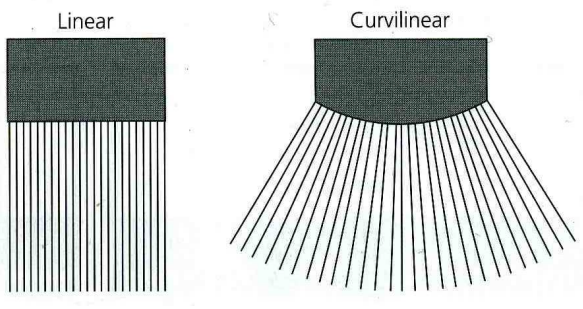


Figure 5. Linear transducers, picture from; (100)

There are three main modalities in ultrasound imaging, namely A-, B- and M-mode modalities. What modality to use depends on the object imaged (100). A-mode or the amplitude mode does not have a cross sectional display, it is a simple ultrasonic display where the ultrasound pulse is demonstrated as a horizontal line that deflects vertically with the amplitude of the echoes and gives the distance of the targets as a function of time (100).

B-mode or the brightness mode is constructed from echoes as explained earlier and is a cross sectional image of the organ boundaries. The strength or the amplitude of the echo at each point gives the image brightness (100). When B-mode imaging is applied there are two pieces of information used to display each echo in a position corresponding to that of the interface (target) that is the orientation and the position of the ultrasound beam and distance of the target from the transducer. The amplitude of the echo is demonstrated as the brightness of the image (100).

With increasing frequency and hence shorter wave length the resolution is improved but the penetration is lowered. Commonly used ultrasound frequency for superficial arteries ranges from 5 to 18 MHz (99). The bandwidth of a transducer is the range of frequencies the transducer can operate.

The image quality of each frequency is contingent on the band width of the transducers. Signal to noise ratio can be altered by changing the length and shape of the transmitted pulse (101). In ultrasound imaging there is a significant difference in the attenuation coefficient for calcified plaque versus soft plaque composed of more lipids (fibrous tissue and hemorrhagic material) attenuation can be measured in units of decibels per unit medium length (dB/cm). The attenuation coefficient for soft regions at frequencies of 2.5~7.5 MHz are 0.3~1.3 dB/cm/MHz while for calcified regions the frequencies are between 1.4~2.5 dB/cm/MHz (102).

The M-mode (motion mode) uses echoes to control the brightness of the image as B-mode does. The beam in M-mode remains fixed while each pulse corresponds to a vertical line on the image (display) and targets moving to or from the transducer show change in their vertical position while non-moving targets remain fixed (producing horizontal line) (100).

As mentioned before the ultrasound technology has been developing and the possibility of three dimensional (3D) ultrasound imaging has been of interest by the commercial companies and the researchers (103, 104). Today many manufacturers of ultrasound systems include the option of image volumes, i.e. acquire images in 3D. In B-mode imaging the acquired data is just a single image or clips but in the 3D setting there is a volume of data acquired at a time. If needed the operator can work with the volume and reformat it into a 2D image although the 2D image could include data that were not accessible when using conventional 2D B-mode imaging so the pathology can be assessed and the volume quantification is more accurate (103, 105, 106). When compared to other imaging modalities like MRI or CT the ultrasound imaging is less expensive, noninvasive does not use ionizing radiation and is generally more comfortable for the patient. Despite these advantages of the 3D ultrasound it's not yet a commonly used technique for routine examinations of the carotids due to technical limitations including the absence of software that supports accurate measurements (104, 106).

There are various sources of errors and image artifacts in ultrasound including observer (human) errors, which can be caused by inappropriate training or lack of experience to follow standard procedures. It can be difficult for the observer to make pathological diagnosis from the 2D ultrasound image of the 3D anatomy (103). A common error is when observers acquires oblique images of an organ instead of transverse or longitudinal images; this could lead to overestimation of the organ/target size (100). A protocol can be standardized and training increased to minimize the human error while careful selection of ultrasound equipment is also important. The real time use of zooming and magnification during acquisition is important since a magnified image will have more pixels per unit area. The lateral beam width can cause error; it can be reduced by optimizing the focal settings so the region of measurements has the best spatial resolution. Improving the spatial resolution can be achieved with higher ultrasound frequencies. The gain has an effect on image quality and contrast; the gain refers to the amplification applied to receive echoes. Due to rapid technical developments it is easily adjusted on the equipment (100). Low gain settings can cause reduction in received echoes, while too high gain settings can lead to hyper bright image due to noise and reduction in contrast resolution. Hence appropriate adjustments of the gain settings by the operator are essential. Proper

use of depth, focusing and magnification controls are important so the target can be optimally resolved and large within the field of view.

1.6 The Icelandic Heart Association

The Icelandic Heart Association (IHA) is a nonprofit organization initiated in 1964 and was founded to battle cardiovascular disease in Iceland. In 1967 the population based research studies by IHA started with the Reykjavik Study to identify risk factors for the development of heart- and cardiovascular diseases in the Icelandic population (107, 108). In the Reykjavik Study, individuals born in 1907 to 1935 were selected randomly of those with residence in the Reykjavik area a total of 30.795 individuals (109).

The Age/Gene Susceptibility Environmental Reykjavik Study (AGES-Reykjavik) is derived from the Reykjavik Study. It is carried out in collaboration with the IHA and the National Institute on Aging (NIA), The United States of America (USA). The AGES-Reykjavik Study is funded by the National Institute of Health (NIH), USA which is a part of the American ministry of health (107) and the Icelandic Parliament (Alþingi). The cross-sectional (baseline) part of the AGES-Reykjavik Study was carried out from 2002 to 2006 including 5764 participants of the 11.549 living individuals from the Reykjavik Study. A longitudinal follow-up of the AGES-Reykjavik Study, including almost 3600 of the same individuals was carried out from 2007 to 2011 with a mean follow-up time of 5.1 years. Harris et al. (109) and Saczynski et al. (110) have described the initial assessments of the cohort. The aim of the AGES-Reykjavik Study was to investigate environmental and genetic factors contributing to clinical and subclinical aging-associated diseases. The study includes multi-modality assessments on four main biological systems that is neurocognitive, musculoskeletal, body metabolism and the cardio-vascular system (111).

The Risk Evaluation For Infarct Estimates (REFINE) was initiated in late year 2005 and took off with full capacity in early 2006 and was carried out until spring 2011. The aim of the REFINE Study is to improve the predictability of risk factors for developing cardiovascular diseases. The cohort was composed of participants randomly selected from the greater Reykjavik area, aged between 20 to 69 years. The recruitment rate in the cross-sectional (baseline) part of the study was ~76% with a total number of participants of 6942 (112). A first longitudinal follow-up component of the REFINE Study, including almost 1331 of the same individuals was carried out from spring 2010 to early 2012 with a mean follow-up time 4.4±0.2 years. The REFINE Study includes examination on conventional cardiovascular risk factors including, blood pressure, cholesterol/lipids, blood glucose, anthropometry and questionnaire on general health (113). A subgroup of participants in the AGES-Reykjavik Study and all participants in the first follow-up component of the REFINE-Reykjavik Study underwent an US examination of the carotid arteries where an assessment of both the carotids IMT and carotid plaques was carried out.

2 The aim

The main aim of this study was to answer the question; “Can ultrasound both acquisition and image analysis be used to estimate changes in plaque area and GSM over time in a reliable manner?”

Hypothesis 1

A novel methodology based on ultrasound can be used to estimate changes in plaque size and composition over time. This was addressed by the following specific aims:

1.1. Specific aim

Ultrasound studies from 219 individuals who had measured plaque approximately ~5 years apart were used to setup and adapt both the ultrasound acquisition and the image analysis.

1.2. Specific aim for image analysis

The variability in image analysis was addressed by measuring the reproducibility in two subgroups. From the AGES-Reykjavik Study 25 subjects were selected and 10 from the REFINE-Reykjavik Study. The images were read twice by three sonographers (two sonographers for each study subgroups) with one week in between reading sessions for the assessment of intra – and inter observer variability.

1.3. Specific aim for ultrasound acquisition variability

The variability of the ultrasound acquisition was addressed by re-imaging 20 subjects from the REFINE-Reykjavik Study group, after a few minutes “stand-up” pause between the two imaging sessions. Images from the two sessions were read by the same reader and variability calculated.

3 Material and methods

3.1 Population

This study was based on two selected subgroups from two study cohorts. One from the AGES-Reykjavik Study (AGES), were ultrasound was used to set up and adapt the plaque imaging analysis methods and also to estimate reproducibility of the reading process, and another subgroup of younger population from the REFINE-Reykjavik Study (REFINE) for further evaluation of the reading reproducibility and ultrasound acquisition reproducibility.

3.1.1 The AGES-Reykjavik Study

A random sample of 219 subjects (75 men mean age=75.8, SD=4.8 and 144 women mean age=75.4, SD=5.2) with known carotid plaques from ultrasound in the baseline part of the AGES-Reykjavik Study was selected for a Visit2 (follow-up) ultrasound. The Visit1 (baseline) ultrasound data was acquired between May 2003 and September 2005. The follow-up data was acquired between February 2009 and February 2010. There were on average 5.2 years between the baseline and follow-up visits. All the subjects gave informed consent. Before the studies were initiated, approvals from the Ethics committee and National Bioethics committee were acquired (see Appendix III). Data for conventional cardio-vascular risk factors were collected for all the same individuals at baseline and follow-up to estimate interactions of plaque size and risk factors.

3.1.2 The REFINE-Reykjavik Study

A random sample of 10 subjects selected from the REFINE Study (7 men mean age=62, SD=2.9 and 3 women age=60, SD=5.7) with known carotid plaque from Visit1 (baseline) was selected for Visit2 (follow-up) ultrasound. The baseline ultrasound data was acquired in April 2006 and May 2006. The follow-up data was acquired in September 2010. There were on average 4.5 years between the baseline and follow-up visits. All the subjects gave informed consent. Before the study was initiated, approvals from the Ethics committee and National Bioethics committee were acquired (see Appendix IV). Data for conventional cardio-vascular risk factors were collected for all the same individuals at baseline and follow-up to estimate interactions of plaque size and risk factors.

3.2 Training and certification of sonographers in plaque assessment

There were three sonographers all qualified radiographers that performed the ultrasound acquisition and reading of images in this study. All of them received the same training before they initiated the reading for the reproducibility measurements. A Training and certification procedure for the plaque imaging analysis for the sonographers is described in detail in (Appendix I). In brief the training process consists of few steps; 1) the sonographer in training observers the whole reading process with a certified sonographer, 2) thereafter he/she analyses plaques of 10 to 20 subjects with a certified sonographer and 3) he/she is required to analyze plaques in 10 subjects that have been

selected as dataset for certification and meet the following statistical criteria; Correlation should be above 0.8, the coefficient of variation should not exceed 13% for intra and inter observer variability (Appendix I). Sonographer1 participated in all readings and acquisition in both the AGES and REFINE groups, sonographer2 participated in the reading reproducibility assessment in the AGES group (n=25) and sonographer3 participated in the reading reproducibility in the REFINE group (n=10). Results by sonographer1 were considered as the reference (gold standard) due to extensive experience in ultrasound of the carotid arteries (5 years and previous assessment of ~3000 number of carotid plaques).

3.3 Ultrasound image acquisition

The ultrasound acquisition was performed using an Acuson, Sequoia C256, (Siemens Medical System, Erlangen, Germany) with 8-MHz, 2D linear array transducer. To achieve this, a workstation with K-PACS V1.5.0 DICOM image software was used to view images from Visit1 (baseline) prior to and during Visit2 (follow-up) acquisition to acquire comparable Visit2 images see (Figure 6). The gain was adjusted on the Visit2 scans so the GSM would be comparable to Visit1 images.

Figure 6. The setup for the acquisition, the ACUSON sequoia and a computer with the K-PACS viewing software.

The ultrasound transducer/probe is directed at the neck, focusing on the carotid artery at a certain angulation which is seen on the Meijer's Arc (114). The Meijer's Arc is used to standardize the circumferential scan of the left and right carotid artery in order for the images to be acquired at the same angle at Visit1 and Visit2. The angles used were 180°, 150°, 120° and 90° for the right carotid artery and 180°, 210°, 240° and 270° for left carot id artery (Figure 7). All subjects were examined in the supine position with the head slightly leaned to the right when scanning the left common carotid and vice versa when scanning the right common carotid artery.

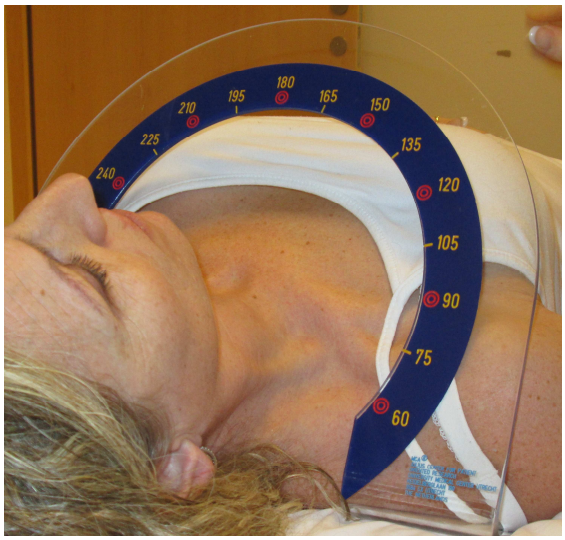


Figure 7. Meijer's Arc used to standardize the circumferential scan of the common carotid artery.

The ultrasound scan was performed on the common carotid artery (CCA), bifurcation and the internal carotid artery (ICA). Longitudinal B-mode images of the near and far wall were acquired of both sides. Lateral view image includes CCA, bifurcation and internal carotid artery (Figure 4 and Figure 8).

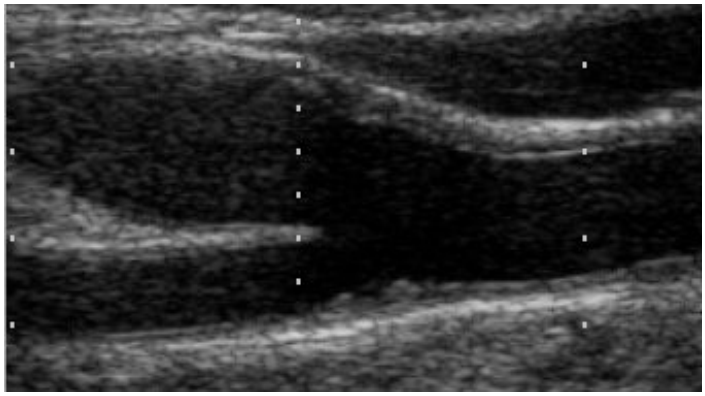


Figure 8. Optimal angle of the CCA; Internal carotid artery, external carotid artery, bifurcation and common carotid artery.

A transverse scan was also acquired to help with the interpretation of findings in general and to avoid false plaque interpretation due to tangential scan planes of the plaque in the longitudinal views (Figure 9). The definition of plaque presence was that the area with intima-media thickness (IMT) was required to be at least 50% thicker than the normal neighboring sites based on visual assessment, a commonly used definition (115). A longitudinal clip was captured of each plaque where the plaque appeared largest in area. Each clip consisted of approximately 60 to 120 frames/images.

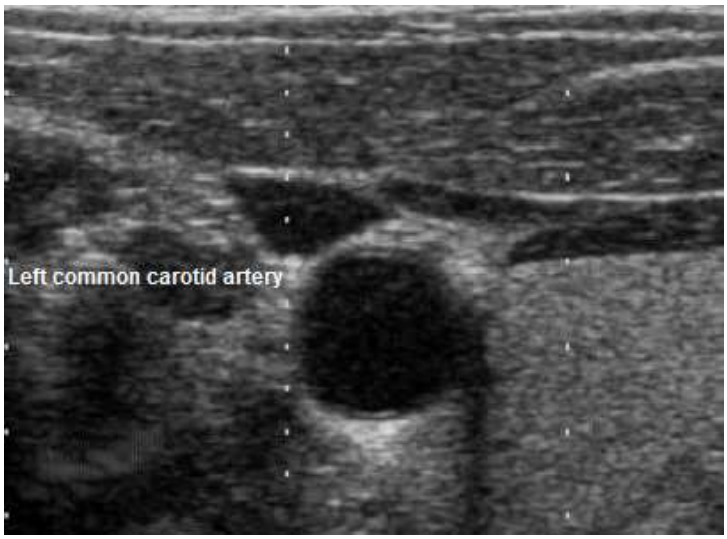


Figure 9. Transverse image of the left common carotid artery.

3.4 Image analysis

Digital images from the ultrasound acquisition were analyzed on a workstation using the Artery Measurement System (AMS) software designed by Prof. Tomas Gustavsson, (Chalmers University of Technology, Department of Signals and Systems, Göteborg) while the K-PACS V1.5.0 software was used to simultaneously compare baseline images to the follow-up images, side by side. Plaque boundaries were traced with a cursor on the screen (Figure 10).

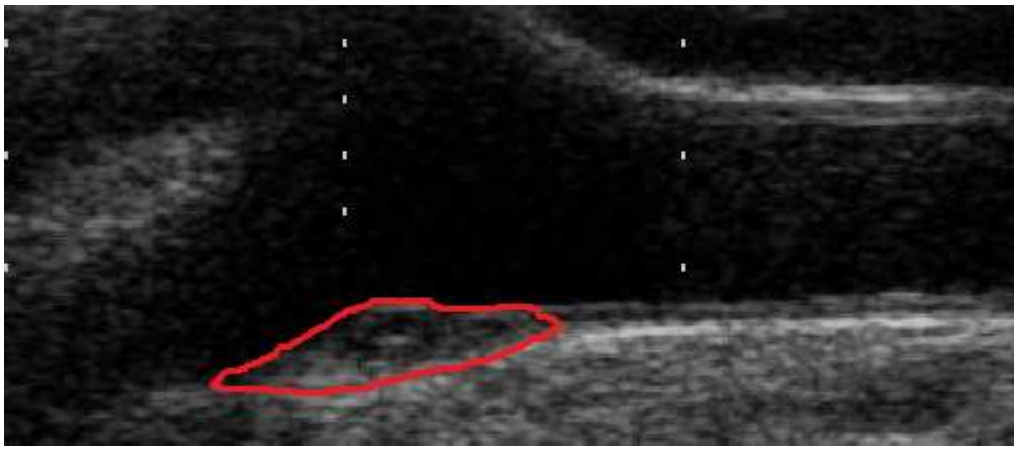


Figure 10. Internal carotid artery, external carotid artery, bifurcation and common carotid artery with traced plaque boundaries.

Plaques present on Visit1 images were traced first. From the image clip of each plaque in a longitudinal view, an image frame was selected where the plaque appeared largest in area with clear boundaries. When saving the image frame the anatomical location of the plaque was indicated in the filename. This information was then extracted from the filename into tables stored in IHA's central database. The anatomical location of the plaque was divided into the following 6 segments of the

region of interests; proximal and distal CCA far wall, proximal and distal CCA near wall and ICA both near and far walls (Figure 11). If a plaque extended over more than one anatomical segment the plaque name was assigned to the proximal parts of the plaque. This mechanism of assigning each plaque into a defined anatomical location made it possible to assess longitudinal changes in the same plaque from two time points, allowed an estimation of the influences of acoustic shadowing on far wall measurements and last but not least allow an investigation of different plaque locations have different risk profiles.

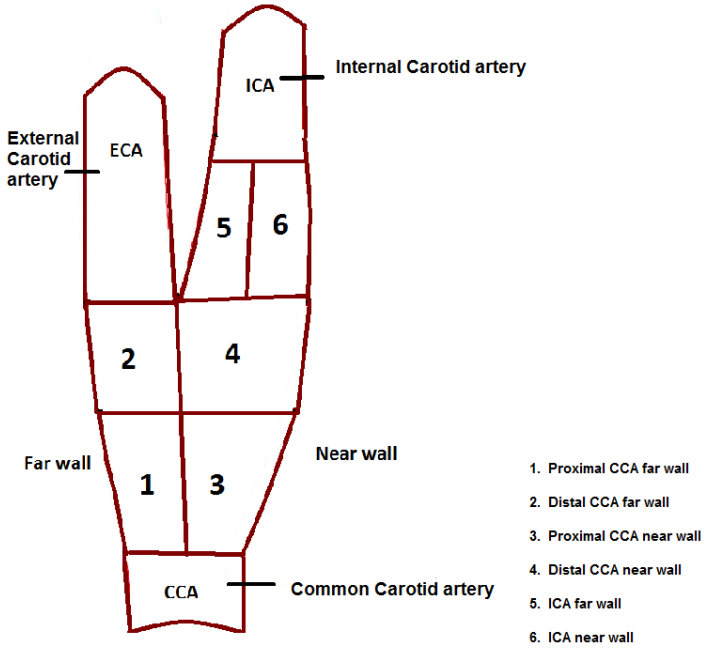


Figure 11. Simplified diagram with optimal angle of the common carotid artery (CCA) bifurcation, external carotid artery (ECA) and internal carotid artery (ICA); for the location of plaques, the region of interests in the carotid artery was divided into six different segments that plaques were assigned to.

For the plaque analysis the AMS software automatically generates values that reflect the area and composition of the defined plaques. The software carries out Gray-Weale analysis for classification of the plaque composition (116). For the Gray-Weale analysis, the grey scale of each frame was calibrated by choosing black (0) for vessel lumen (no plaque). The grey-scale gradually increases for echo-genic plaques with a maximum value of (256) indicating vessel/wall with more calcified area. Following the calibration and tracing of the plaque boundaries the program automatically assigned each plaque into four composition classes based on grey scale in pixels compromising the plaque: Class 1; composition of the plaque is fat/lipid or not calcified, class 2; calcified up to 25%, class 3; up to 75% is calcified and class 4; 75-100% is calcified. Visual classification was also made which depended on the density of the plaque (Figure 12).

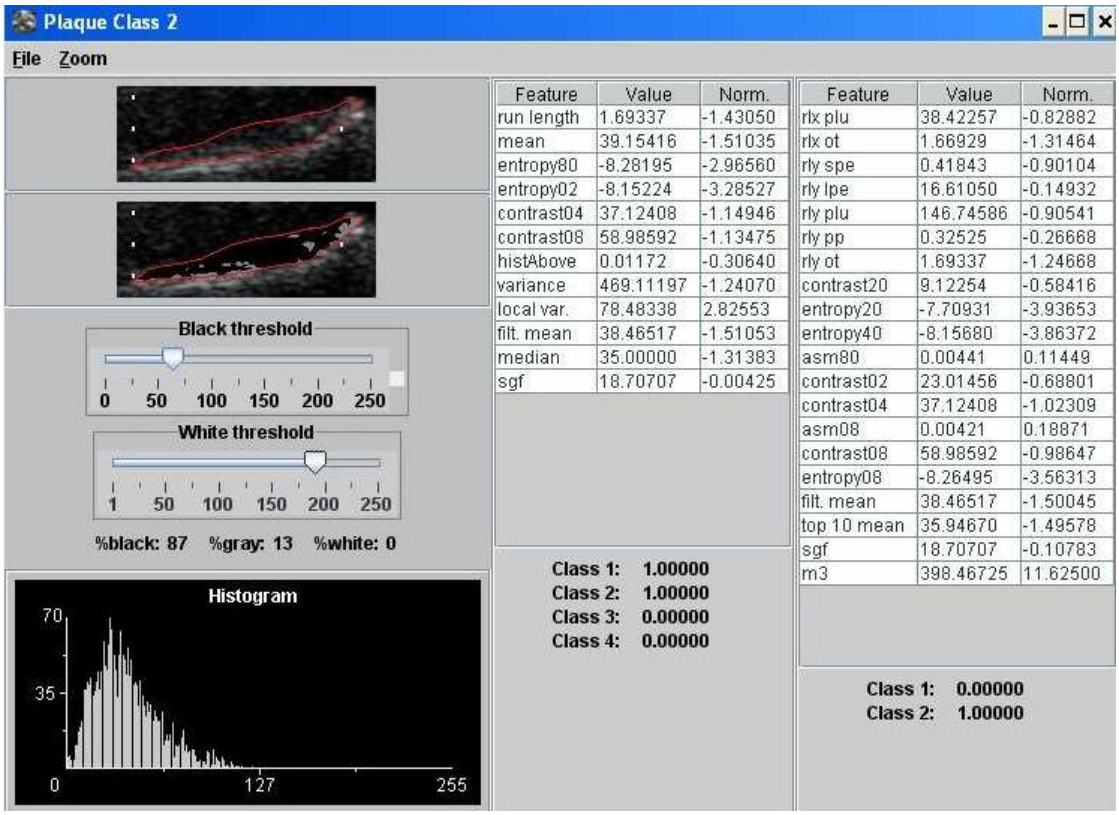


Figure 12. AMS software – Descriptive values and the classification of the plaque composition.

The plaque area (mm^2) was computed by the AMS software depending on the pixels within the traced region. Within each subject the sum of all traced plaque areas were made for Visit1 and Visit2 separately.

When tracing plaques from Visit2 the traced plaque from Visit1 was made visible on a separate computer monitor, allowing side by side comparison of the baseline and follow-up plaques. If multiple plaques were visible the same process was made for each plaque. The output from the software in addition to the plaque information in the text file was then loaded into the central database. Information on the participant identity, plaque either from Visit1 or Visit2, plaque side R (right) or L (left), frame number chosen from the clip, image angulation, plaque number depending on the anatomical location and reader initials was entered into the filename. The detailed acquisition and reading protocol for the plaque analysis at IHA is in Appendix I.

3.5 Reproducibility for plaque area and GSM measurements - AGES-Reykjavik and REFINE-Reykjavik studies

The reading variability within and between sonographers was measured using images from the two groups, first on AGES (n=25) and second, in the REFINE group (n=10). The 25 subjects from the AGES group used in the reproducibility assessment were selected from the group of 219 subjects based on total plaque burden. The total plaque burden in the group of 219 subjects was divided into five equal quintiles and then five subjects from each quintile randomly selected (Figure 13). The 25 subjects selected were read twice by two sonographers with at least one week in between readings.

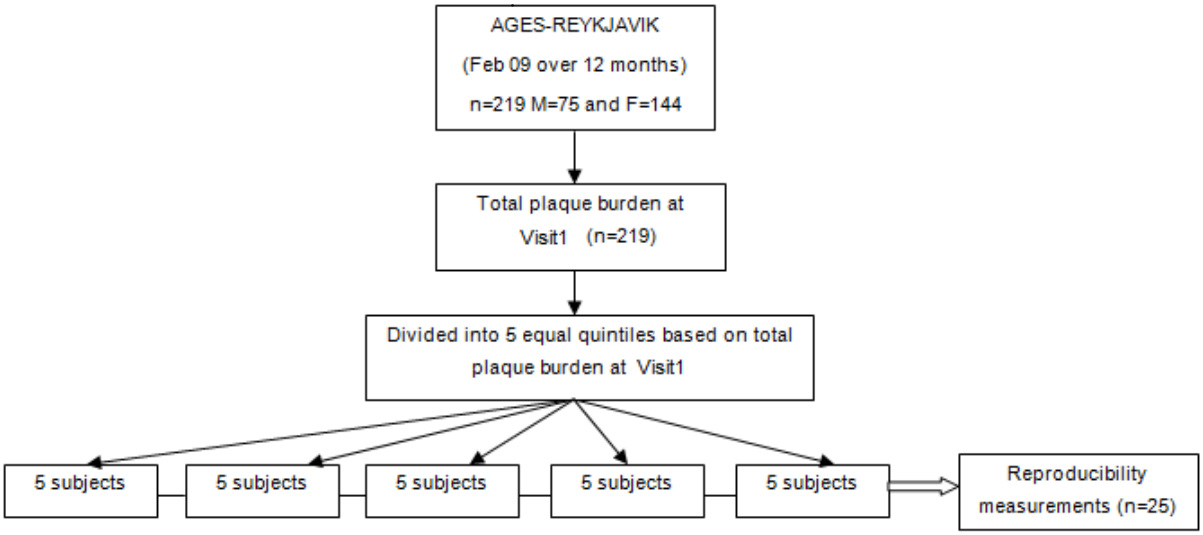


Figure 13. The cohort used for the reproducibility measurements were randomly chosen from the 219 in total depending on plaque burden at Visit1. Where M=male and F=female and n=number of participants.

From the REFINE study group, ten subjects were chosen randomly regardless of the total plaque burden although all subjects were required to have plaque present at Visit1.

3.6 Reproducibility of acquisition in the ultrasound imaging

A reproducibility assessment for the ultrasound acquisition process was made for the REFINE group where twenty individuals had two sequential imaging sessions with a pause in-between were the participants were allowed to stand up for a short while and then lie down on the examination couch again before the repeated imaging. The images were read twice by the same sonographer (sonographer1). The reading was carried out in the same manner as in the intra variability estimates described above except that the analysis was only based on Visit2 examinations.

3.7 Possible shadow effect from plaque in near wall

To test the possible effect of acoustic shadowing from plaques in near wall on the analysis of plaque in far wall a comparison was made for all subjects within the AGES study group (n=219).

When sound wave travels through highly calcified plaque, the plaque may have reduced the energy of the forward traveling sound wave so that subsequent (more distant) plaque e.g. plaque in far wall have weaker backscatter than should be expected. In this study, calcified plaques in proximal near wall could cause shadow effect on subsequent plaques in the proximal far wall. This can prevent detection of plaques in far wall, reduce estimation of plaque area and/or alter the GSM estimate. Hence, presence of shadow effect from plaques in near wall could influence the estimated total plaque burden and GSM of plaques in far wall. Prevalent shadow effect might influence estimates of plaque area and/or plaque GSM in far wall compared to clear near wall. To evaluate if the shadowing effect was influencing the results, a comparison was made on all proximal plaques in far wall with or without plaques in proximal near wall. The comparison was limited to the proximal part of the bifurcation to increase the certainty that plaque in near wall is opposite the plaque in far wall.

3.8 Statistical analysis

All statistical analysis and graphical presentations of data were made with the R-statistical software Version 2.12.1 (117).

3.8.1 Characteristics

Average age, body mass index (BMI), LDL, Cholesterol (Chol), CIMT, plaque burden and GSM of the subjects, sub-sampled from the AGES Study, for the plaque reproducibility analysis (n=25) was compared with the average of the remaining subjects (n=194) from the AGES Study using Student's t-test.

Frequencies of ever-smokers and of hypertension (HTN) within the sub-sample (n=25) and the remaining subjects (n=194) were compared with Pearson's Chi-squared test (118) to test if they were independent.

To compare differences between visits in longitudinal plaque estimation with differences between repeated readings in reproducibility estimation, the 25 subjects used in the reproducibility measurements were selected from the longitudinal study. For each individual the difference between Visit1 and Visit2 was calculated and also the difference between the two observations in the reproducibility estimates. The mean difference, standard deviation and 95% confidence intervals were calculated for each approach.

Changes in plaque area and GSM between visits by age-group were presented in a boxplot (119) where the frequency distribution is presented graphically by showing the median as a thick horizontal line between two squared boxes that represent the first and third quartiles of the frequency distribution. Further the outer most point of the likely range of variation (inner quartile range * 1.5) is shown as whiskers and suspected outliers as empty circles.

For the changes in plaque area and GSM between visits a single-classification analysis of variance (ANOVA) was used to examine if there is added variance component among age-groups. The longitudinal changes from baseline to follow-up were described as the mean difference between baseline and follow-up shown as the % of the baseline as shown in following equation:

$$\% = \frac{F - B}{B} * 100$$

Where F = Follow-up value and B = Baseline value.

3.8.2 Plaque area and GSM calculations

The total plaque area in each subject was calculated as the sum of the area of all measured plaque within the subject using the following equation:

$$A_I = \sum_{p=1}^{n} \left(A_p \right) \quad (1)$$

Where I = individual subject, A = plaque area, p = each plaque within subject. This was estimated separately within each subject for Visit1 and Visit2.

The mean plaque GSM of each subject weighted with plaque area was calculated using the following equation:

$$\overline{GSM}_I = \frac{\sum_{p=1}^{n} \left(GSM_p * A_p \right)}{\sum_{p=1}^{n} \left(A_p \right)} \quad (2)$$

Where I = Individual subject, p = each plaque within subject, GSM = greyscale median, A = plaque area. This was estimated separately within each subject for Visit1 and Visit2.

3.8.3 Variability estimates

For intra- and inter observer variability, the mean difference in total plaque area (A_I) and mean GSM_I between observations was assessed with the bland & Altman (120) method with 95% confidence intervals (95%CI). Disagreement plots show the difference between two observations as a function of the average of the observations where vertical lines show the mean, ±2 standard deviations (SD) and 95%CI of the mean difference.

For intra and inter sonographers readings the comparison between observations was shown in scatterplots with linear regression. Spearman's rank correlation coefficient (rho) was used to measure intra- and inter observer correlation with bootstrapped (121) (10 000 re-samplings) 95%CI. Percent coefficient of variation (CoV) gives the dispersion of the variables. Low CoV indicates less dispersion of variables. For two observations the CoV was calculated using the following equation:

$$CoV\% = \frac{\frac{SD}{\sqrt{2}}}{\frac{X1 + X2}{2}} * 100 \quad (3)$$

Where SD = standard deviation of the difference between observation1 and observation2, X1 = average of observation 1 and X2 = average of observation 2. A paired student t-test (117) were performed to estimate whether there was a statistical difference between observations. Statistical significance was set at $p<.05$.

3.8.4 Shadow effect

For all subjects within the AGES study group (n=219) the proximal plaques in far wall were divided into two groups; a) plaque present in near wall, b) plaque absent in near wall. Frequency distributions of measurements from each group were presented graphically in a boxplot and Students t-test was used to estimate if there was a significant difference between the averages of the two groups. Significance was set at $p<.05$.

4 Results

4.1 Characteristics of the AGES Reykjavik Study group used in the reproducibility measurements

The baseline (Visit1) characteristics for the 219 subjects (75 men, mean age=75.8, SD=4.8 and 144 women, mean age=75.4 SD=5.2) are shown in Table 1. The characteristics are shown separately for the 25 subjects selected for the setup and validation of the method and the remaining 194 subjects in the group of 219. The mean time from the baseline (Visit1) to the follow-up (Visit2) was 5.2 years (SD=0.2). The mean total plaque area and GSM for all subjects at Visit1 was 49.42 ±31.88mm^2 and 68.29±16.14 respectively. The mean total plaque area and GSM for all subjects at Visit2 was 60.39±34.72mm^2 and 65.82±13.95, the percent difference from Visit1 to Visit2 for each age-group is shown in Table 2. Total number of plaques at baseline was 657 or on average 3 plaques per subject, while at follow-up the total number of plaques was 757 or on average 3.5 plaques per subject. The group of 25 subjects used in this study did not differ statistically significantly from the group of 194 for any of the subject characteristic parameters (Table 1).

The mean difference between Visit1 and Visit2 (the longitudinal plaque changes) for the 25 subjects that were used in the reproducibility measurements for plaque area was 11.10 with 95% CI 6.32 to 15.88 and SD=11.57 and for plaque GSM -0.92 with 95% CI -5.90 to 4.10 and SD=12.06.

Table 1. Characteristics for the 219 subjects at Visit1. Categorical variables are presented in percentages and continuous variables are presented as (mean ±SD) **.

	Subjects (AGES) in plaque reproducibility analysis N=25			The remaining subjects from AGES N=194			
	Male (n=9)	**Female (n=16)**	**Total (n=25)**	**Male (n=66)**	**Female (n=128)**	**Total (n=194)**	**P - value**
Age-groups							**0.71***
-69	2	3	5	5	25	30	
70-74	2	4	6	20	33	53	
75-79	1	4	5	25	41	66	
80-84	4	5	9	14	24	38	
85+	0	0	0	2	5	7	
BMI (>30 kg/m^2) %	12.00	8.00	20.00	6.20	16.50	22.70	0.484
Hypertension * (mmHg) %**	36.00	52.00	88.00	26.80	49.50	76.30	0.285
LDL (mmol/L)	3.48 (±0.60)	3.61 (±1.05)	3.57 (±0.90)	3.3 (±0.97)	3.8 (±1.1)	3.63 (±1.1)	0.770
Chol (mmol/L)	5.31 (0.70)	5.88 (±0.99)	5.68 (±0.92)	5.20 (±1.0)	6.10 (±1.1)	5.76 (±1.2)	0.670
Eversmoker %	16.0	28.0	44.0	24.7	39.2	63.9	0.087
CIMT (mm)	1.09 (±0.11)	0.97 (±0.10)	1.01 (±0.12)	1.02 (±0.14)	0.97 (±0.14)	0.99 (±0.14)	0.303
Plaque burden (mm^2)	49.87 (±29.46)	47.74 (±38.54)	48.51 (±34.91)	50.68 (±28.88)	48.95 (±32.95)	49.54 (±31.56)	0.890
GSM (grey scale median)	64.68 (±17.03)	67.41 (±11.22)	66.43 (±13.31)	67.20 (±16.30)	69.20 (±16.60)	68.52 (±16.50)	0.480

* p-value for age-groups is based on t-test comparison on the mean age of both groups.

** mmHg =millimeters of mercury; mmol/L =millimoles per liter; mm=millimeters; mm^2=square millimeters; (SD)=Standard deviation of the mean; LDL and cholesterol measured in serum; CIMT=carotid intima media thickness. P-value <0.05 indicates a significant difference between the selected group of the 25 subjects in the reproducibility measurements and the remaining 194 subjects from the group of 219.

***Hypertension (HTN) is defined from either the use of hypertension medication or from self reported questionnaire derived from physiological measurements (systolic blood pressure, diastolic blood pressure).

4.2 Changes in plaque area and GSM between Visit1 and Visit2 within age-groups (n=219)

The changes from Visit1 to Visit2 showed the trend of increasing difference in plaque area with higher age-group. The increasing difference with higher age-group is not entirely consistent as especially in the oldest age-group where the increase in difference is far less than in younger groups. Changes in plaque GSM from Visit1 to Visit2 showed no consistent change by age-group although the oldest age-group was different from the others by far more negative difference in GSM (see Table 2). Single-classification analysis of variance (ANOVA) for changes in plaque area between visits showed that there was an added variance component with increasing age-group (p=0.00251) but not for changes in plaque GSM (p=0.7124). The mean difference between Visit1 and Visit2 within subjects both for plaque area and GSM respectively are shown in (Figure 14 and Figure 15). It should be noted that no adjustment for interventions (e.g. statin) was made.

Table 2. Longitudinal changes in plaque area and GSM between Visit1 and Visit2 within age-groups and the distribution of participants among age-groups

	-69	**70-74**	**75-79**	**80-84**	**85+**
Age-groups	Changes from Visit1 to Visit2	Changes from Visit1 to Visit2	Changes from Visit1 to Visit2	Changes from Visit1 to Visit2	Changes from Visit1 to Visit2
Participants (N)	35	59	71	47	7
Plaque area (mm^2) (%)	15.3	13.4	21.7	23.1	4.3
Plaque GSM (%)	-2.1	2.6	-0.5	-0.4	-9.5

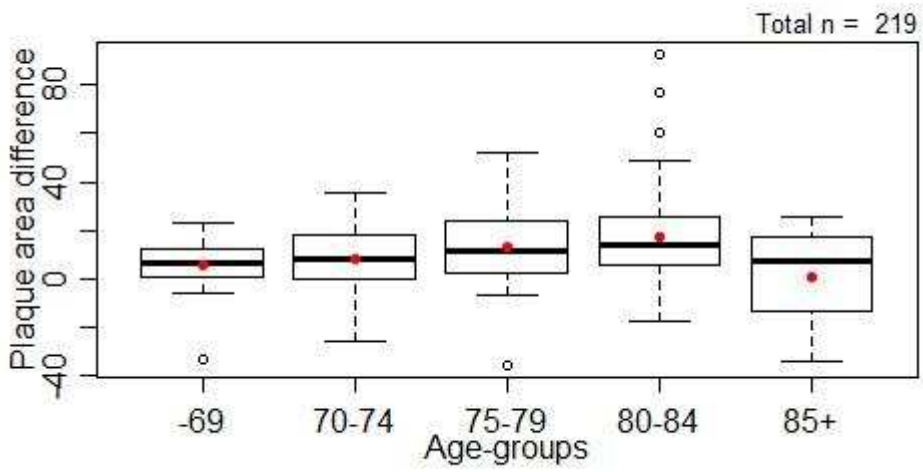


Figure 14. Boxplot showing the changes in plaque area from Visit1 to Visit2 in different age-groups, red dot indicates the mean difference.

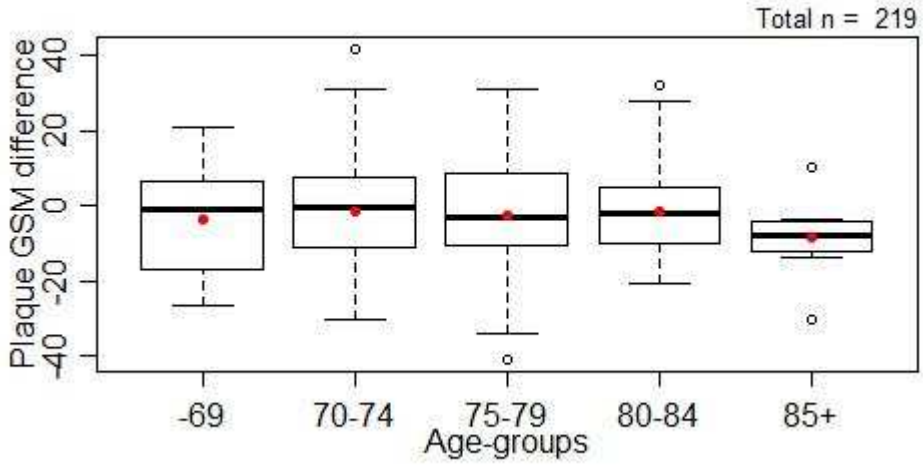


Figure 15. Boxplot showing changes in plaque GSM from Visit1 to Visit2 by age-group where red dot indicates the mean difference.

4.2.1 Numbers of identified plaques in intra- and inter- observer variability estimation from AGES study group (n = 25)

Sonographer1 identified 86 plaques in the participants from Visit1 in both readings and 105 plaques in the same participants from Visit2, also in both readings. Sonographer2 identified 89 plaques in participants from Visit1 first reading and 109 plaques in the second reading and 110 plaques identified from Visit2 first reading and 114 plaques in the second reading. The numbers of identified plaques are summarized in (Table 3).

Table 3. Number of plaques identified in intra- and inter-observer variability assessment.

	Sonographer1		*Sonographer2*	
	First reading	**Second reading**	**First reading**	**Second reading**
	Number of plaque	***Number of plaque***	***Number of plaque***	***Number of plaque***
Visit1	86	86	89	109
Visit2	105	105	110	114

4.2.2 Intra observer variability – Plaque area

Variability assessment of repeated readings of plaque area from the AGES Study (25 subjects) where each subject was analyzed twice by each sonographer, showed that sonographer1 had a mean difference between observations of -0.85mm^2 (95%CI -3.88 to 2.18) and the correlation was 0.96 (95%CI 0.91 to 0.97). The CoV was 12.10% and there was no significant difference between measurements (p=0.74). For sonographer2 the mean difference between observations was –14.75mm^2 (95%CI -19.96 to -4.53) and the correlation was 0.89 (95%CI 0.80 to 0.94) respectively. The CoV was 18.63% and there was a significant difference between measurements (p<0.0001) (Table 4 and Figure 16). The slope and intersection of the regression line in the scatterplot indicate that sonographer2 had tendency to measure the plaque area bigger in small plaques in the second reading compared to first reading (Figure 16d).

Table 4. Sonographer (1 and 2): Intra observer variability for plaque area (AGES Study n=25). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value of Visit1 and Visit2.

	Total plaque area (mm^2)			
	Mean diff (SD) **95% CI of mean**	**Rho –r** **95%CI**	**CoV %**	**P-value**
Sonographer1^{1st} vs. ***Sonographer1^{2nd}***	-0.85 (10.65) -3.88 to 2.18	0.96 (0.91 to 0.97)	12.10	0.74
Sonographer2^{1st} vs. ***Sonographer2^{2nd}***	-14.75 (18.34) -19.96 to -4.53	0.89 (0.80 to 0.94)	18.63	<0.0001

**Sonographer1^{1st}=sonographer1 first reading; Sonographer1^{2nd}=sonographer1 second reading.*
**Sonographer2^{1st}=sonographer2 first reading; Sonographer2^{2nd}=sonographer2 second reading.*

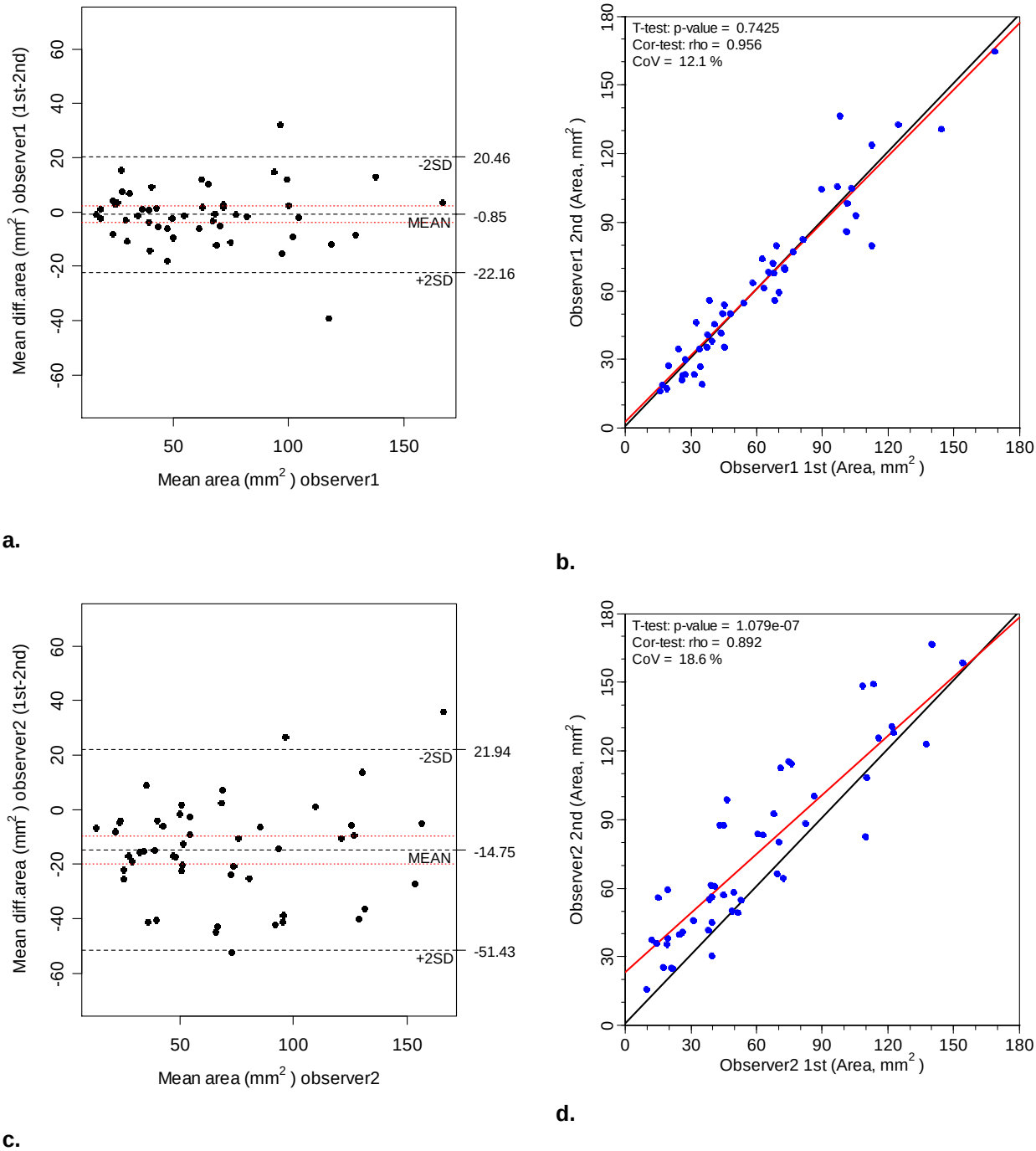


Figure 16. a and c) Intra observer variability for plaque area – Sonographer1and Sonographer2: Disagreement plot (Bland-Altman) for plaque area difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where black dotted lines show the mean, ±2 standard deviation and red dotted lines show 95% confidence limits of the mean difference. **b and d)** Intra observer variability for plaque area – Sonographer1 and Sonographer2: Scatter plot with linear regression line showing the relationship between first observation and second observation.

4.2.3 Intra observer variability – GSM

Variability assessment of repeated readings of plaque GSM from the AGES Study (n=25) where each subject was analyzed twice by two sonographers, showed that the mean difference between observations for sonographer1 was 0.33 (95%CI -1.60 to 2.25) and the correlation was 0.90 (95%CI 0.81 to 0.94). The CoV was 7.77% and there was no statistical difference between measurements (p=0.73). For sonographer2 the mean difference between observations was 1.80 (95%CI -0.25 to 3.85) and the correlation was 0.86 (95%CI 0.72 to 0.93). The CoV was 8.04% and there was no statistical difference between measurements (p=0.08) (Table 5 and Figure 17).

Table 5. Sonographer (1 and 2): Intra observer variability for plaque GSM (AGES Study n=25). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value of Visit1 and Visit2.

	Total plaque composition (GSM)			
	Mean diff (SD) 95% CI of mean	**Rho-r 95%CI**	**CoV %**	**P-value**
Sonographer1^{1st} vs. Sonographer1^{2nd}	0.33 (6.77) -1.60 to 2.25	0.90 (0.81 to 0.94)	7.77	0.73
Sonographer2^{1st} vs. Sonographer2^{2nd}	1.80 (7.20) -0.25 to 3.85	0.86 (0.72 to 0.93)	8.04	0.08

**Sonographer1^{1st}=sonographer1 first reading; Sonographer1^{2nd}=sonographer1 second reading.*

**Sonographer2^{1st}=sonographer2 first reading; Sonographer2^{2nd}=sonographer2 second reading.*

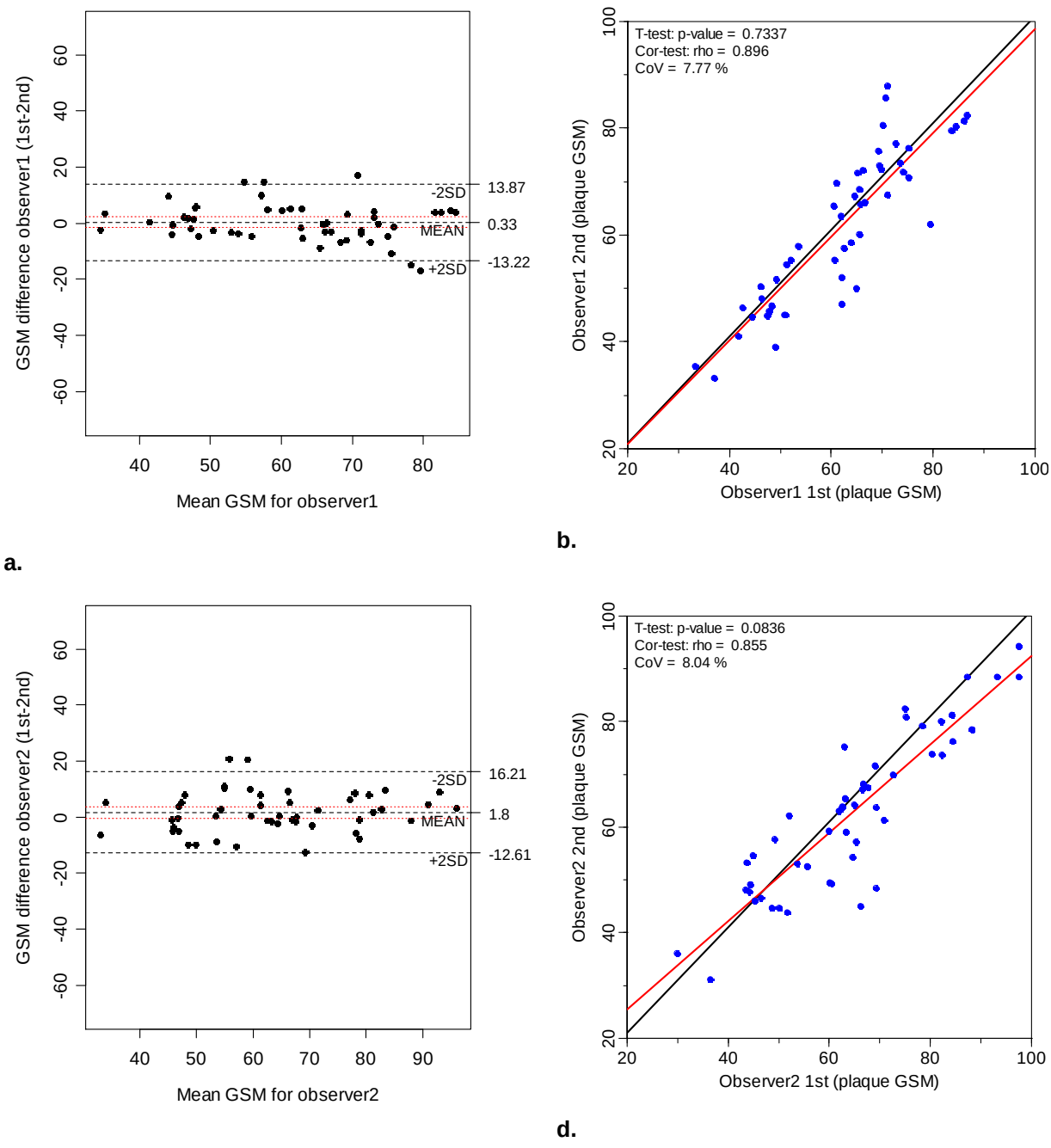


Figure 17. a and c) Intra observer variability for plaque GSM – Sonographer1 and Sonographer2: Disagreement plot (Bland-Altman) for difference in plaque GSM. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where black dotted lines show the mean, ±2 standard deviation and red dotted lines show 95% confidence limits of the mean difference. **b and d)** Intra observer variability for plaque GSM – Sonographer1 and Sonographer2: Scatter plot with linear regression line showing the relationship between first observation and second observation for GSM.

4.2.4 Inter observer variability – Plaque area

Variability assessment of repeated readings between two sonographers of plaque area from the AGES Study (25 subjects) was carried out using the second reading by each sonographer. The mean difference between sonographers 1 and 2 was -14.33 mm^2 (95%CI -20.87 to -7.80). The correlation was 0.81 (95%CI 0.67 to 0.89) and the CoV was 23.29%. The difference between sonographers was statistically significant (p<0.0001) (Table 6 and Figure 18). The regression line shows systematic difference between sonographers, where sonographer2 measures larger plaque area than sonographer1 (Figure 18).

Table 6. Inter observer variability between two sonographers for total plaque area (AGES Study n=25). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p - value Visit1 and Visit2.

	Total plaque area			
	Mean diff (SD) 95% CI of mean	**Rho –r 95%CI**	**CoV %**	**P-value**
Sonographer1^{2nd} vs. Sonographer2^{2nd}	-14.33 (22.99) -20.87 to -7.80	0.81 (0.67 to 0.89)	23.29	<0.0001

**Sonographer1^{2nd}* = sonographer1 second reading; Sonographer2^{2nd} = sonographer2 second reading.*

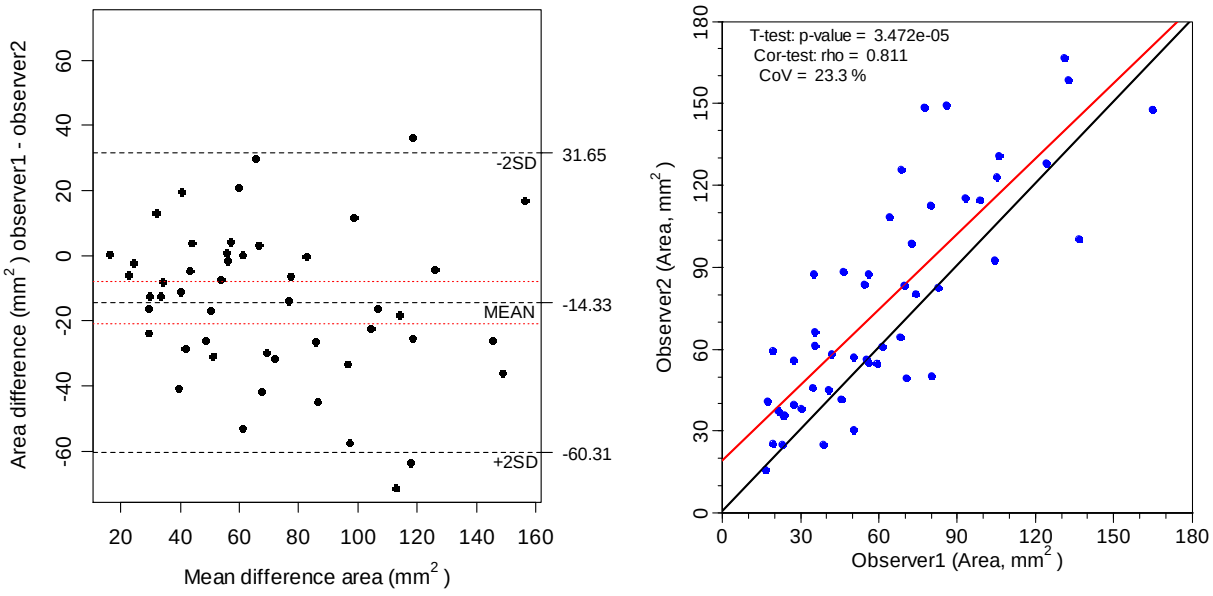


Figure 18. a) Inter observer variability plaque area – Sonographer1 vs. Sonographer2: Disagreement plot (Bland-Altman) for plaque area difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where lines show the mean, 2 ±standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Inter observer variability for plaque area – Sonographer1 vs. Sonographer2: Scatterplot with linear regression line showing the relationship between observers for total plaque area.

4.2.5 Inter observer variability – GSM

Assessment of variability of repeated readings between two sonographers for plaque GSM from the AGES Study (25 subjects) was carried out using the second reading by each sonographer. The mean difference between sonographers 1 and 2 for plaque GSM was -1.00 (-3.13 to 1.13) and the correlation was 0.87 (95%CI 0.76 to 0.93). The CoV was 8.55% and there was no significant difference between sonographers (p=0.35) (Table 7 and Figure 19).

Table 7. Inter observer variability between two sonographers for plaque GSM (AGES Study n=25). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p - value Visit1 and Visit2.

	Mean total plaque composition (GSM)			
	Mean diff (SD) 95% CI of mean	**Rho-r 95%CI**	**CoV %**	**P-value**
Sonographer1^{2nd} vs. Sonographer2^{2nd}	-1.00 (7.50) -3.13 to 1.13	0.87 (0.76 to 0.93)	8.55	0.35

**Sonographer1^{2nd} = sonographer1 second reading; Sonographer2^{2nd} = sonographer2 second reading.*

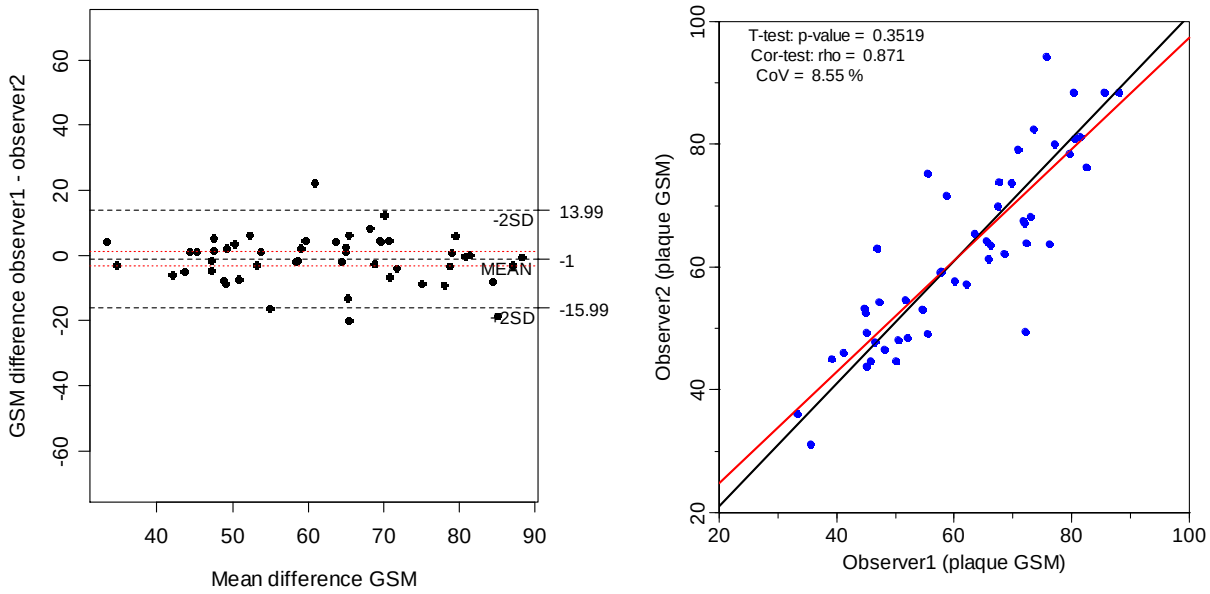


Figure 19. **a)** Inter observer variability for plaque GSM – Sonographer1 vs. Sonographer2: Disagreement plot (Bland-Altman) for GSM difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Inter observer variability for plaque GSM – Sonographer1 vs. Sonographer2: Scatterplot with linear regression line showing the relationship between sonographers for mean total GSM.

4.2.6 Anatomical plaque distribution – The AGES Study

The study group of elderly individuals (AGES) had plaques most commonly in the proximal bifurcation of the common carotid artery and the internal carotid artery both in near and far wall although more common in near wall. Plaques were also found in the internal carotid artery but more commonly in the far wall (Table 8).

Table 8. AGES n=25: Distribution of plaques at Visit1 and Visit2 stratified by location for sonographer1 first reading (n=number of plaques and % = percent of the total number of plaques).

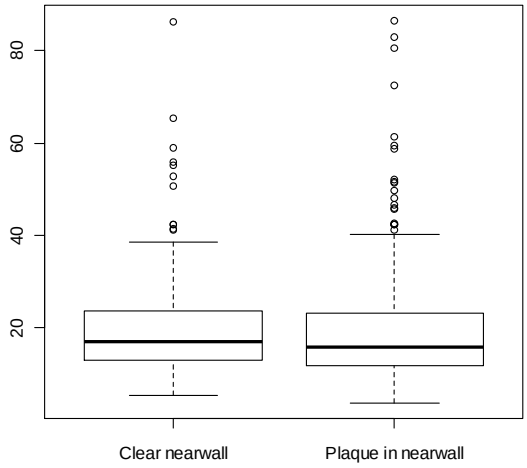
	Visit1		Visit2	
Segments	***N***	***%***	***N***	***%***
1) Proximal cca far wall	19	33.9	22	31.4
2) Distal cca far wall	1	1.8	1	1.4
3) Proximal cca near wall	23	41.1	29	41.4
4) Distal cca near wall	3	5.4	3	4.3
5) ICA far wall	6	10.7	9	12.9
6) ICA near wall	4	7.1	6	8.6
Total	56	100	70	100

*cca = Common carotid artery; N = number; % = percent of total plaques.

4.3 Possible shadow effect on plaque in far wall from plaque in near wall (AGES Study n=219)

Influence of plaque in near wall on plaque area and GSM in far wall are shown in (Figure 20). Presence or absence of plaques in the near wall had no significant influence on plaque area measurements in the far wall; there was no significant difference in plaque area in far wall irrespective of if a plaque was present in near wall or not (p=0.62). There was however a significant increase in plaque GSM in far wall if plaque was present in near wall compared to clear near wall (p= 0.0031) (Figure 20).

a) Plaque area



b) Plaque GSM

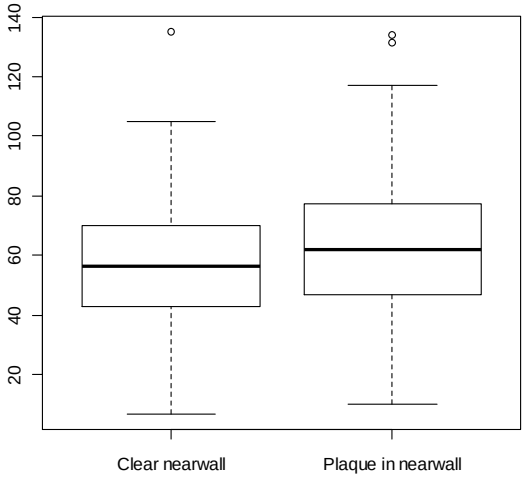


Figure 20. a) Boxplot showing the total plaque area in far wall with or without plaque present in near wall. b) Boxplot showing the GSM for plaque in far wall with or without plaque present in near wall.

4.4 Reproducibility/Validity of the method based on the REFINE study group

The characteristics from Visit1 for the 10 subjects (7 men, mean age=62.00, SD=2.94 and 3 women mean age=59.67, SD=5.69) are listed in (Table 9). The mean time from the baseline (Visit1) to the follow-up (Visit2) was 4.5 years (SD=0.05).

Sonographer1 identified 23 plaques in participants from Visit1 in both readings and 31 plaques in the same participants from Visit2, also in both readings. Sonographer3 identified 24 plaques in participants from Visit1 first reading and 23 plaques in second reading and for Visit2 he identified 31 plaques in both readings. The numbers of identified plaques are summarized in (Table 10).

Table 9. Visit1 characteristics for the 10 subjects (REFINE). Categorical variables are presented in percentages and continuous variables are presented as (mean ± SD).

	Male (n=7)	Female (n=3)	Total (n=10)
BMI (>30 kg/m^2) %	28.6	33.3	30
Hypertension (mmHg) %	0	0	0
LDL (mmol/L)	3.51(±0.90)	2.42 (±0.35)	3.18 (±0.91)
Chol (mmol/L)	5.4 (±0.92)	4.66 (±0.35)	5.18 (±0.85)
Eversmoker %	50	20	70
CIMT (mm)	0.90 (±0.14)	0.75 (±0.10)	0.86 (±0.14)
Plaque burden (mm^2)	35.6 (±21.4)	16.4 (±8.5)	29.8 (±20.17)
GSM (grey scale median)	64.2 (±11.2)	63.8 (±5.0)	64.0 (±9.4)

mmHg =millimeters of mercury; mmol/L =millimoles per liter; mm=millimeters; mm^2=square millimeters; (±SD)=Standard deviation of the mean;LDL and cholesterol measured in serum; CIMT=carotid intima media thickness.

Hypertension (HTN) is defined from either the use of hypertension medication or from self reported questionnaire derived from physiological measurements (systolic blood pressure, diastolic blood pressure).

Table 10. Number of plaques identified in intra- and inter-observer variability assessment (REFINE n=10).

	Sonographer1		***Sonographer3***	
	First reading	**Second reading**	**First reading**	**Second reading**
Visit1	23	23	24	23
Visit2	31	31	31	31

4.4.1 Intra observer variability – Plaque area

Variability assessment of repeated readings of plaque area from the REFINE Study (10 subjects) where each subject was analyzed twice by two sonographers 1 and 3, showed that sonographer1 had a mean difference between observations of 1.97mm^2 (95%CI -0.32 to 4.25) and the correlation was 0.96 (95%CI 0.85 to 0.99) and the CoV was 9.82%. There was no significant difference between observations (p=0.23). For sonographer3 the mean difference between observations was 1.03mm^2 (95%CI -2.23 to 4.28) and the correlation was 0.96 (95%CI 0.83 to 1.00) and CoV was 16.03% respectively. The difference between observations was not significant (p=0.72) (Table 11 and Figure 21).

Table 11. Sonographer (1 and 3): Intra observer variability for plaque area (REFINE Study n=10). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value of Visit1 and Visit2.

	Total plaque area			
	Mean diff (SD) 95% CI of mean	**Rho–r 95%CI**	**CoV %**	**P-value**
Sonographer1^{1st} vs. Sonographer1^{2nd}	1.97 (4.88) -0.32 to 4.25	0.96 (0.85 to 0.99)	9.82	0.23
Sonographer3^{1st} vs. Sonographer3^{2nd}	1.03 (6.95) (-2.23 to 4.28)	0.96 (0.83 to 1.00)	16.03	0.72

*Sonographer1^{1st}=sonographer1 first reading; Sonographer1^{2nd}=sonographer1 second reading.
*Sonographer3^{1st}=sonographer3 first reading; Sonographer3^{2nd}=sonographer3 second reading.

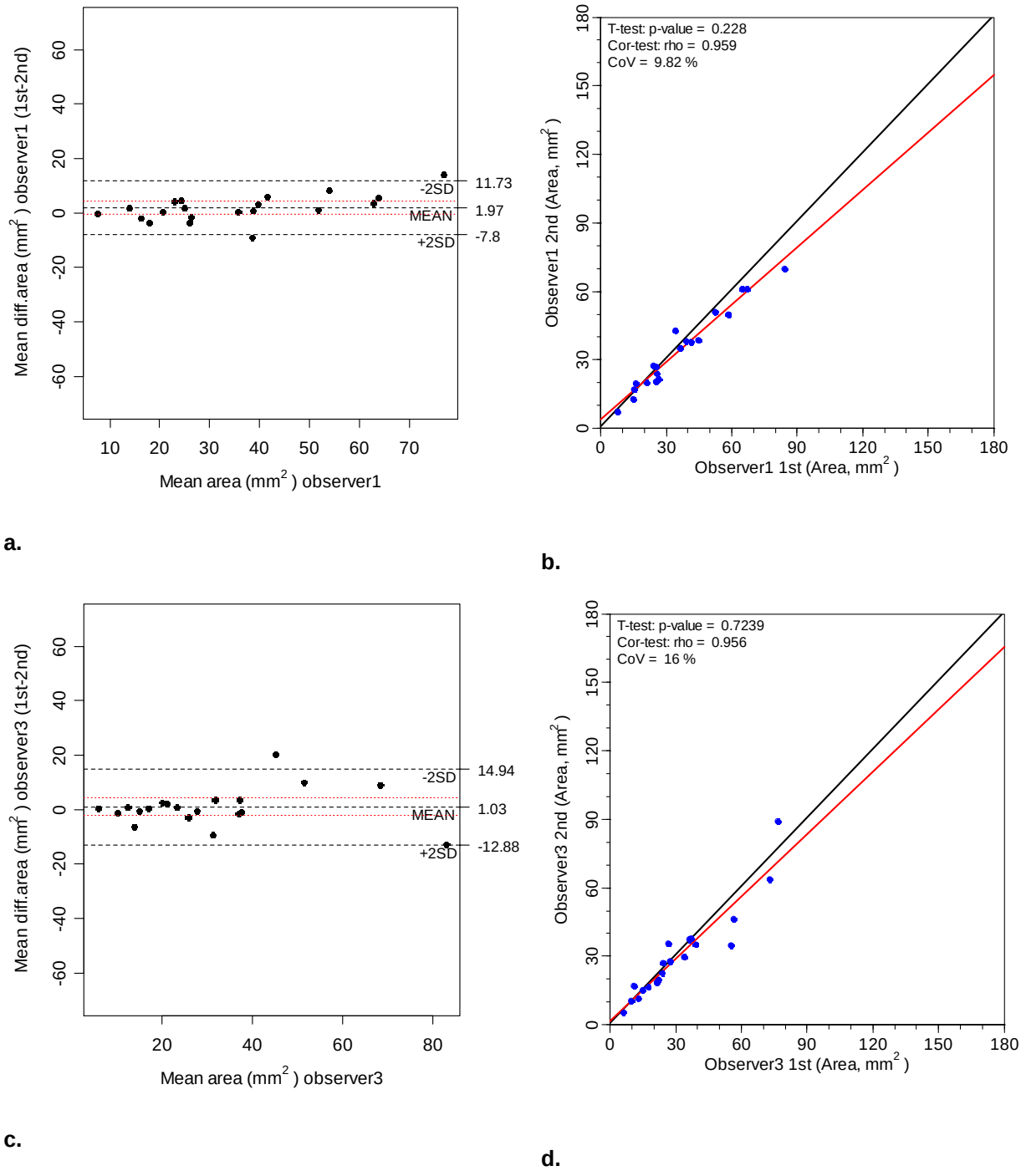


Figure 21. a and c) Intra observer variability for plaque area (REFINE n=10) – Sonographer1 and Sonographer3: Disagreement plot (Bland-Altman) for plaque area difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where black dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b and d)** Intra observer variability for plaque area (REFINE n=10) - Sonographer1 and Sonographer3: Scatter plot with linear regression line showing the relationship between 1st observation against the 2nd observation for total plaque area.

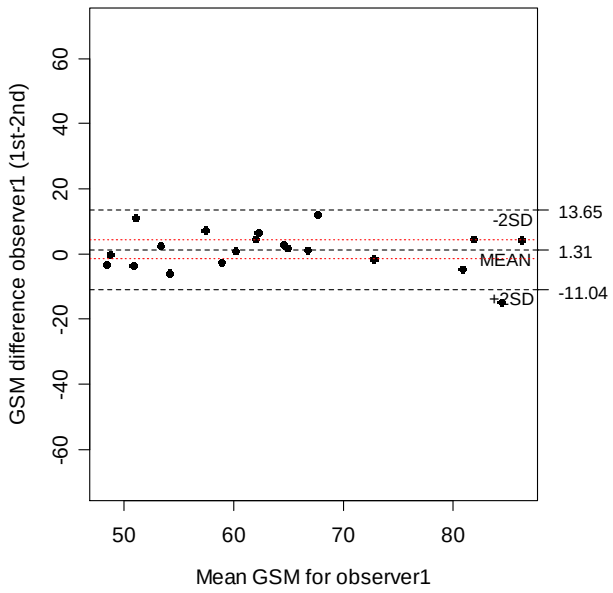
4.4.2 Intra observer variability – GSM

For the REFINE group the mean difference between observations for plaque GSM within sonographer1 was 1.31 (95%CI -1.58 to 4.20) and correlation was 0.90 (95%CI 0.76 to 0.96) and CoV was 6.84%. There was no significant difference in plaque GSM between observations (p=0.36). For sonographer3 the mean difference for plaque GSM was 1.85 (95%CI -1.05 to 4.76) and the correlation was 0.88 (95%CI 0.65 to 0.97) and the CoV was 6.97% respectively. There was no significant difference between observations for plaque GSM within sonographer3 (p=0.20) (Table 12 and Figure 22).

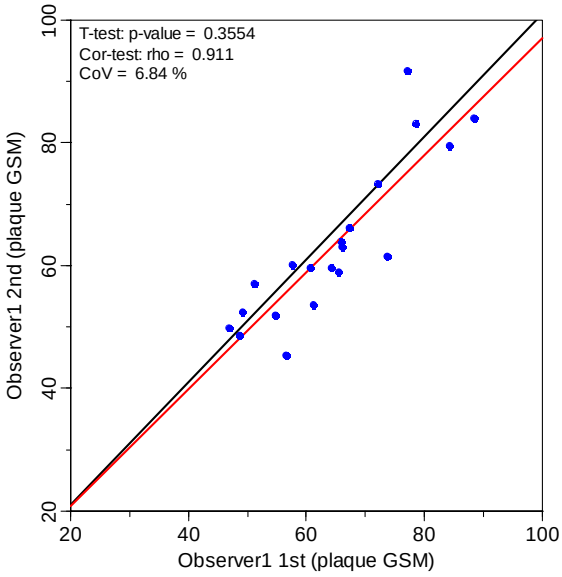
Table 12. Sonographer (1 and 3): Intra observer variability for mean plaque GSM (REFINE n=10). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value of Visit1 and Visit2.

	Total plaque composition (GSM)			
	Mean diff (SD) 95% CI of mean	**Rho-r 95%CI**	**CoV %**	**P-value**
Sonographer1^{1st} vs. Sonographer1^{2nd}	1.31 (6.2) -1.58 to 4.20	0.91 (0.76 to 0.96)	6.84	0.36
Sonographer3^{1st} vs. Sonographer3^{2nd}	1.85 (6.2) (-1.05 to 4.76)	0.88 (0.65 to 0.97)	6.97	0.20

*Sonographer1st=sonographer1 first reading; Sonographer1^{2nd}=sonographer1 second reading.
*Sonographer3^{1st}=sonographer first reading; Sonographer3^{2nd}=sonographer second reading.



a.



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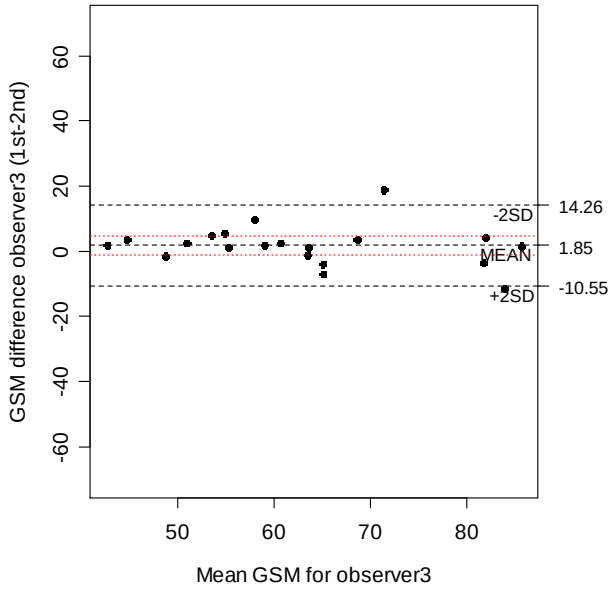

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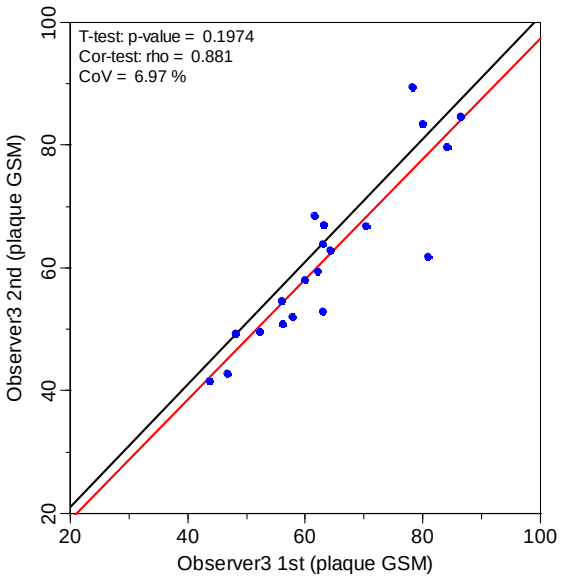

d.

Figure 22. a and c) Intra observer variability for GSM (REFINE n=10) – Sonographer1 and Sonographer3: Disagreement plot (Bland-Altman) for GSM difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b and d)** Intra observer variability for GSM (REFINE n=10) - Sonographer1 and Sonographer3: Scatterplot with linear regression line showing the relationship between 1st observation against the 2nd observation for mean total GSM.

4.4.3 Inter observer variability – Plaque area

The assessment of variability between sonographers 1 and 3 based on images from the REFINE study group showed a mean difference in plaque area of 4.04mm^2 (95%CI 0.16 to 7.91). The correlation was 0.91 (95%CI 0.72 to 0.99) and CoV 18.20%. There was a significant difference between sonographers (p=0.002) (Table 13 and Figure 23). There appears to be a systematic difference between sonographers 1 and 3, where the position of the regression line indicates that sonographer1 measures the plaque area slightly larger than sonographer3.

Table 13. Inter observer variability between two sonographers (REFINE n=10). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value for both Visit1 and Visit2 total plaque area.

	Total plaque area			
	Mean diff (SD) 95% CI of mean	**Rho –r 95%CI**	**CoV %**	**P-value**
Sonographer1^{2nd}	4.04 (8.28)	0.91	18.20	0.0023
vs.	0.16 to 7.91	(0.72 to 0.99)		
Sonographer3^{2nd}				

*Sonographer1^{2nd}=sonographer1 second reading; Sonographer3^{2nd}=sonographer3 second reading.

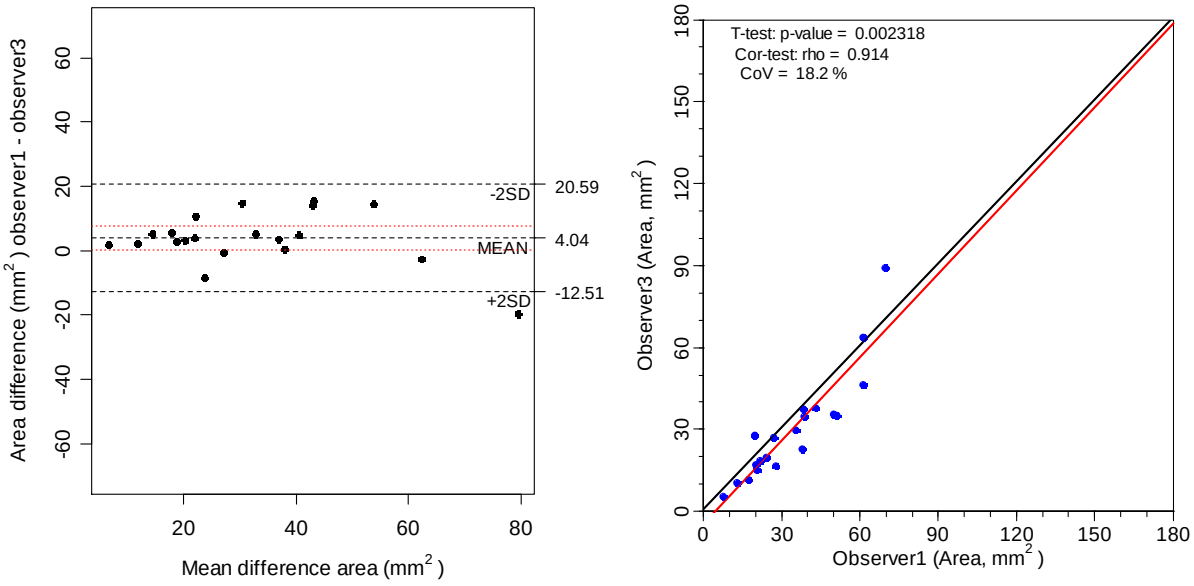

Figure 23. a) Inter observer variability (REFINE n=10) – Sonographer1 vs. Sonographer3: Disagreement plot (Bland-Altman) for plaque area difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Inter observer variability (REFINE n=10) – Sonographer1 vs. Sonographer3: Scatterplot with linear regression line showing the relationship between sonographers for total plaque area.

4.4.4 Inter observer variability – GSM

The variability in plaque GSM in the REFINE study group between sonographers 1 and 3 showed a mean difference of 1.15 (95%CI -1.93 to 4.24). The correlation was 0.82 (95%CI 0.52 to 0.97) and CoV 7.45%. The difference between sonographers was not statistically significant (p=0.44) (Table 14 and Figure 24).

Table 14. Inter observer variability between two sonographers (1 and 3) (REFINE n=10). Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value for both Visit1 and Visit2 mean plaque GSM.

	Total plaque composition (GSM)			
	Mean diff (SD) 95% CI of mean	**Rho-r 95%CI**	**CoV %**	**P-value**
Sonographer1^{2nd} vs. Sonographer3^{2nd}	1.15 (6.6) -1.93 to 4.24	0.82 (0.52 to 0.97)	7.45	0.44

**Sonographer1^{2nd} =sonographer1 second reading; Sonographer3^{2nd}=sonographer3 second reading.*

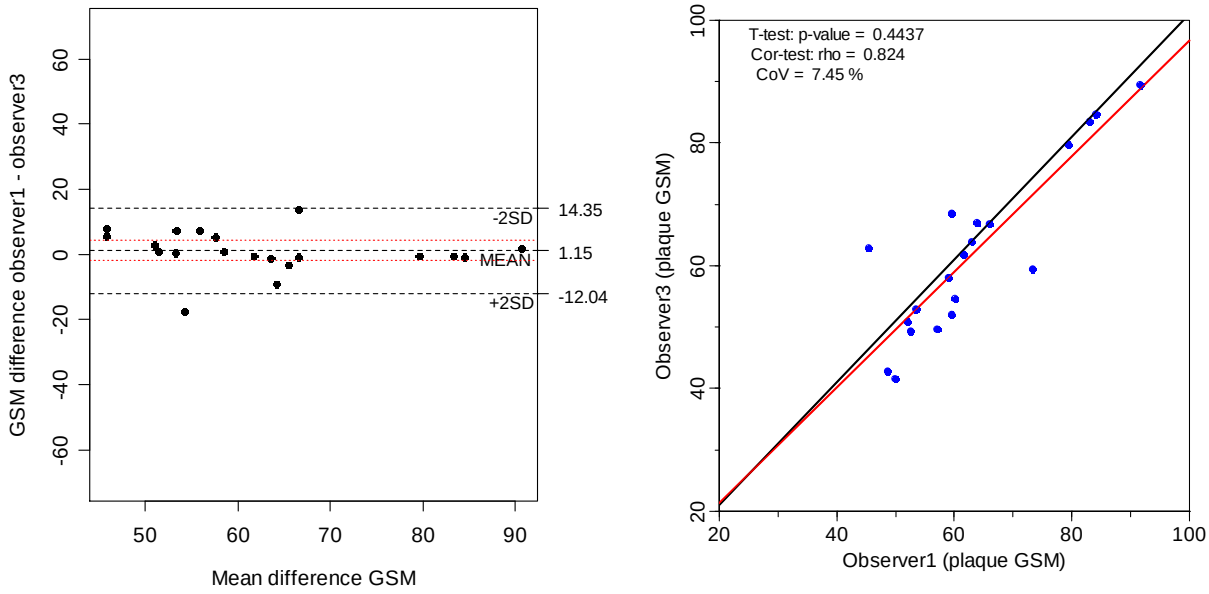

Figure 24. **a)** Inter observer variability (REFINE n=10) – Sonographer1 vs. Sonographer3: Disagreement plot (Bland-Altman) for GSM difference. Difference between the two observations (vertical axis) as a function of the average of the observations (horizontal axis) where dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Inter observer variability for GSM (REFINE n=10) – Sonographer1 vs. sonographer3; scatterplot with linear regression line showing the relationship between sonographers for plaque GSM.

4.4.5 Anatomical plaque distribution – REFINE Study

For the REFINE study group the location of plaque appearance was seen in the proximal bifurcation of the common carotid artery both near and far wall also in the far wall of internal carotid artery (Table 15).

Table 15. REFINE n=10: Distribution of plaques at Visit1 and Visit2 stratified by location (n=number of plaques and % = percent of the total number of plaques).

	Visit1		Visit2	
Segments	***n***	***%***	***N***	***%***
1) Proximal cca far wall	9	45.0	13	46.4
2) Distal cca far wall	0	0	0	0
3) Proximal cca near wall	8	40.0	12	42.9
4) Distal cca near wall	0	0	0	0
5) ICA far wall	3	15.0	3	10.7
6) ICA near wall	0	0	0	0
Total	20	100	28	100

*cca = Common carotid artery; N = number; % = percent of total plaques.

4.5 Reproducibility for the acquisition of ultrasound imaging, twenty individuals were re-imaged

The 20 subjects that participated in the repeated acquisition at baseline (Visit1) 11 men, mean age=61.5, SD=4.4 and 9 women, mean age=56.7, SD=6.1 with a mean time from baseline (Visit1) to the follow-up (Visit2) was 4.3 years (SD=0.22).

4.5.1 Acquisition variability – Plaque area

The variability in acquisition for plaque area was estimated from the REFINE study group (n=20) by comparing readings from two acquisitions. The mean difference in plaque area between acquisitions was -0.85 mm^2 (95%CI -4.11 to 2.41). The correlation was 0.95 (95%CI 0.81 to 0.99) and CoV 12.40% as shown in (Table 16 and Figure 25). There was no significant difference between acquisitions (p=0.11).

Table 16. Reproducibility for the ultrasound acquisition imaging (REFINE) 20 subjects were examined twice. Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value for both Visit1 and Visit2 total plaque area.

	Total plaque area			
	Mean diff (SD) 95% CI of mean	**Rho –r 95%CI**	**CoV %**	**P-value**
Acquistion1st vs. Acquisition2nd	-0.85 (6.96) (-4.11 to 2.41)	0.95 (0.81 to 0.99)	12.40	0.11

**Acquistion1st = First acquisition; Acquisition2nd = Second acquisition.*

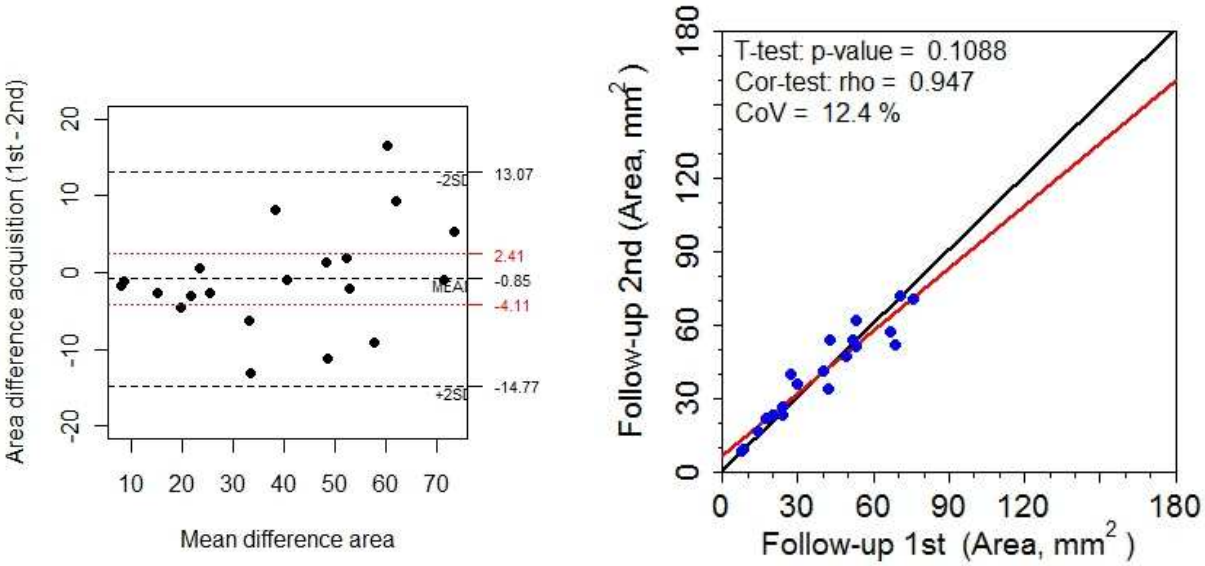

Figure 25. a) The acquisition variability for plaque area (REFINE n=20) – acquisition1 vs. acqusition2: Disagreement plot (Bland-Altman) for plaque area difference. Difference between the two acquisitions (vertical axis) as a function of the average of the acquisitions (horizontal axis) where black dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Scatterplot with linear regression line showing the relationship between first and second acquisition (n=20) for plaque area.

4.5.2 Acquisition variability – GSM

The variability in acquisition for plaque GSM was estimated from the same group of 20 subjects as outlined in the previous section by comparing the readings from two imaging sessions. Mean difference in plaque GSM between acquisition was -0.35 (95%CI -4.96 to 4.26). The correlation was 0.84 (95%CI 0.62 to 0.96) and CoV 11.17% as shown in (Table 17 and Figure 26). There was no significant difference between the two imaging sessions (p=0.88). Excluding one outlier (green point on Figure 26) resulted in a reduced CoV from 11.17% to 8.65% and a shift in the regression line (green dotted) moving closer to the expected regression (black solid) line and no significant difference between readings of images from the two imaging sessions.

Table 17. Reproducibility for the ultrasound acquisition imaging (REFINE) 20 subjects were examined twice. Mean difference, Spearman's rho, coefficient of variation (CoV%) and p-value for both Visit1 and Visit2 mean plaque GSM.

	Total plaque composition (GSM)			
	Mean diff (SD) 95% CI of mean	**Rho-r 95%CI**	**CoV %**	**P-value**
Acquistion1st vs. Acquisition2nd	-0.35 (9.86) (-4.96 to 4.26)	0.84 (0.62 to 0.96)	11.17	0.88

**Acquistion1st = First acquisition; Acquisition2nd = Second acquisition.*

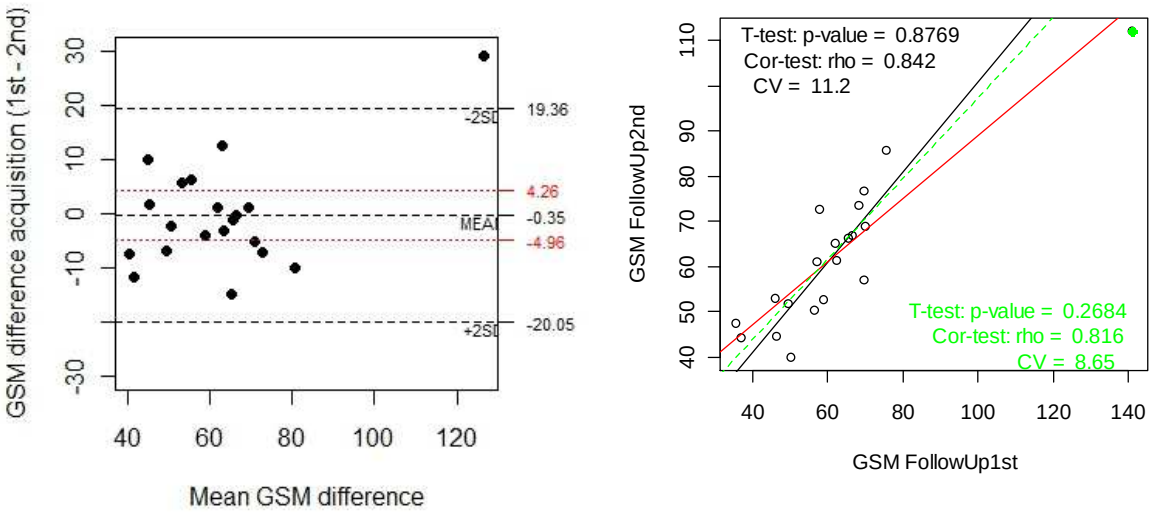

Figure 26. a) The acquisition variability for GSM (REFINE n=20) – acquisition1 vs. acqusition2: Disagreement plot (Bland-Altman) for GSM difference. Difference between the two acquisitions (vertical axis) as a function of the average of the acquisitions (horizontal axis) where black dotted lines show the mean, ±2 standard deviations and red dotted lines show 95% confidence limits of the mean difference. **b)** Scatterplot with linear regression line showing the relationship between first and second acquisition (n=20) for measurements on plaque GSM. Green dotted line shows the linear regression with one outlaw (green point) excluded.

4.5.3 Anatomical distribution from the acquisition variability - REFINE Study

For the 20 individuals that participated in the acquisition variability measurements the location of plaques appearance is more prevalent in the proximal near and far wall of the carotid artery although plaque is seen in all 6 anatomical segmentations (Table 18).

Table 18. REFINE n=20: Distribution of plaques at Visit2 stratified by location (n=number of plaques and % = percent of the total number of plaques).

	Follow-up1	
Segments	***N***	***%***
1) Proximal cca far wall	25	37.9
2) Distal cca far wall	1	1.5
3) Proximal cca near wall	27	40.9
4) Distal cca near wall	1	1.5
5) ICA far wall	8	12.1
6) ICA near wall	4	6.1
Total	66	100

*cca = Common carotid artery; N = number; % = percent of total plaques.

5 Discussion

The aim of this study was to set up a standardized protocol for reliable measurements of longitudinal changes in plaque area and plaque composition using ultrasound. Further to assess the reproducibility of the plaque measurements. Ultrasound has been found to have poor reproducibility and to be prone to observer bias compared to other imaging methods (3, 62). Achieving good reproducibility in both ultrasound acquisition and the analysis of ultrasound images is therefore a challenging task not least in a longitudinal study where images are acquired at two different time points. In this study, image analysis software was used for quantitative plaque measurements; the software was adapted to measure longitudinal changes in plaque area and composition. Baseline images were reviewed prior to and during follow-up imaging to maximize the comparability of prevalent plaques.

Most studies in current literature on the reproducibility of carotid plaque measurements using ultrasound report limited number of statistical parameters including correlation, CoV or the mean difference with limits of agreement. Further, different studies often use different statistical parameters making it difficult to compare results across studies (2, 17, 18, 122, 123). A variety of statistical parameters were used in this study to facilitate a comparison of the results with results in other studies (Appendix II).

The correlation coefficient is a commonly used statistical parameter when intra and inter observer variability is measured in plaque assessment using ultrasound images. High correlation doesn't however necessarily imply good agreement between observations or low variability. It could in fact be implying that the disagreement is consistent between the two observations and in such case the mean difference and the coefficient of variation could be high (124). Therefore in this study an estimation of several statistical parameters were made to capture if there is a divergence in the reproducibility measurements.

Several studies have estimated reproducibility using qualitative or semi-quantitative methods that rely on categorical scales, e.g., plaque occurrence, morphology and the echogenic status. The statistical methods used to calculate reproducibility in these studies have generally depended on Kappa statistics (18, 36, 125-128). A comparison of the results in this quantitative study to the results in those qualitative or semi-quantitative studies is difficult as the results in this study use continuous scales for the size and composition of plaques.

5.1 Reproducibility for both study groups - AGES and REFINE

5.1.1 Intra observer variability for plaque area

Limits for the statistical criteria for certification in this study are presented in section 3.2 and Appendix I. For within sonographer's variability measurements on plaque area the difference between two observations was plotted against the average of the observations. The limits of agreement were set to where 95% of the differences lie within ±2 standard deviation of the mean difference (124). The correlation should be above 0.8 and the CoV should not exceed 13% and there should be no significant difference between observations.

Within the subsamples of both the AGES (n=25) and REFINE (n=10) study groups sonographer1 showed low mean difference of plaque area (-0.85mm^2 and 1.97mm^2), high correlation (0.96 in both), acceptable values of CoV (12.10% and 9.82%) and there was no significant difference (p=0.74 and 0.23) between observations. Overall the intra variability for sonographer1 on plaque area measurements for both study groups is within the statistical limits set for certification.

Within the subsample of the AGES study group sonographer2 showed higher mean difference of plaque area (-14.75mm^2), slightly lower correlation (0.90), higher CoV (18.63%) when compared to results of sonographer1 and there was significant difference (p<.0001) between observations. These results do not meet the requirements for certification and may indicate that sonographer2 needs to adapt further to the protocol.

Within the subsample of the REFINE study group sonographer3 showed low mean difference (1.03mm^2), high correlation (0.96), high CoV (16.03%) and there was no significant difference (p=0.72) between observations. The results show that sonographer3 is close to acceptable repeatability only the CoV exceeds the set limit which is 13%.

The results for sonographer 1 and 3 compare reasonably well to the results in other studies. Johnsen et.al (17), reported intra observer variability for two sonographers including mean arithmetic difference of -0.2 (SD=3.1mm^2 with limits of agreement of ±8.6 mm^2) and 0.01 (3.8 mm^2 and the limits of agreement ±7.5) within subjects at age 55 to 74 years.

The correlation for the intra plaque area measurements in this study was high for all sonographers. These results compare reasonably well with other studies for example Lear et.al. (129) where intra class correlation for plaque area was 0.922 to 0.948 and Barnett et.al.(2) where 50 randomly chosen ultrasound videotapes/clips were reviewed showing intra class correlation of 0.99. Like mentioned before it can be argued that the coefficient of correlation isn't appropriate parameter to assess the agreement for these measurements (62, 124, 130). There is reasonably high correlation (0.90) but rather poor agreement within sonographer2 where the mean difference was -14.75mm^2 and the CoV 18.63%.

The CoV in this study compares well with Andersson et.al (18) where intra variability estimates for one sonographer (n=25) resulted in CoV of 11.2%.

Overall, the low intra observer variability of plaque area measurements for sonographer1 show that it is possible to generate reliable and reproducible outcome when following the protocol set up in this

study. The results for sonographers 2 and 3 are promising but further improvements or adaptations are needed.

5.1.2 Inter observer variability – plaque area

The same criteria and statistical limits apply to variability between sonographers as within sonographers for plaque area measurements.

For the subsample in the AGES group the inter observer variability for plaque area between sonographer 1 and 2 was high. The correlation was reasonable (0.81), the mean difference was rather high -14.33mm^2, CoV was high 23.29% and there was a significant difference between sonographers ($p<.0001$). According to the statistical criteria for certification, this is too high variability. The correlation was within limits but the mean difference was rather high and CoV was too high and there was a significant difference between sonographers. This high inter observer variability is not surprising in the light of differences in intra observer variability between the two sonographers where sonographer1 performed substantially better than sonographer2.

For the subsample from the REFINE study group the inter observer variability for plaque area between sonographers 1 and 3 was rather high. The mean difference was 4.04mm^2, correlation was high (0.91), CoV was high 18.20% and there was a significant difference between sonographers ($p=0.0023$). This is also too high variance between sonographers according to the certification requirements even though the correlation is within set limits. Since both sonographers had acceptable intra observer variability this indicates the need for the sonographers to adapt their readings closer to each other to lower the inter variability.

When results on inter observer variability from both study groups are compared the mean difference is lower for the REFINE study group but higher in the AGES study group and the correlation coefficients were higher in the REFINE study group. For both groups the CoV is higher than the criteria for certification and there was also a significant difference between observations. Persson et.al (131) reported high inter-observer variability between two sonographers with the mean difference for plaque area 15.9 mm^2 ±12.5 on the other hand study by Johnsen et al. (17) reported inter observer variability where the mean difference was -1.0(4.4) mm^2 and limits of agreement were ±8.6. Johnsen et.al.(17) captured a still frame of the plaque during the acquisition but in this study clips with multiple frames were collected and a still image was selected for every single plaque. Hence this study goes further in accounting for the variability caused by possibly capturing different frames.

The correlation for the inter observer plaque area measurements in this study is high for both comparisons, this is in good agreement with a study by (129).

High inter observer variability has also been published in other studies for example Joakimsen(132) where CoV was 17.9% and 22.4% although this was on plaque thickness and might not be directly comparable to the CoV in this study.

5.1.3 Intra observer variability for the GSM

As for plaque area the statistical criteria for within sonographer's variability measurements on plaque GSM were the following: the difference between two observations was plotted against the average of the observations where limits of agreement are where 95% of the differences lie within ±2 standard deviation of the mean difference. This limit of agreement is similar to other study reported by Sabetai et.al. (133) where the inter observer variability was required to have 95% of the differences within ±2SD from the mean difference. The correlation should be above 0.8 and the CoV should not exceed 13% and there should be no significant difference between observations.

Within the subsample of the AGES (n=25) and REFINE (n=10) Study groups sonographer1 showed low mean difference of GSM (0.33 and 1.31), high correlation (0.90 and 0.91), low values of CoV (7.77% and 6.84%) and there was no significant difference (p=0.73 and 0.36) between observations. These results on intra observer variability for sonographer1 for both study groups are within the statistical limits for certification and show that sonographer1 has low variability in plaque GSM measurements.

Within the subsample of the AGES study group sonographer2 showed low mean difference of GSM (1.8), high correlation (0.86), low CoV (8.04%) and there was no significant difference (p=0.08) between observations. These results also fulfill the statistical criteria for certification and indicate that sonographer2 has low variability in plaque GSM measurements.

Within the subsample of the REFINE study group sonographer3 showed low mean difference of GSM (1.85), high correlation (0.88), low CoV (6.97%) and there was no significant difference (p=0.20) between observations. The intra observer variability results for sonographer3 are also within the statistical limits and indicate that sonographer3 has low variability in plaque GSM measurements.

Overall the intra observer variability for all three sonographers on plaque GSM measurements is within statistical limits and indicates that well trained sonographers following the protocol (Appendix I) in detail has low variability.

The low mean difference in this study for intra observer plaque GSM measurements compare well to other studies commonly ranging from -0.9 to 1.7 (17, 122, 133, 134).

The CoV for intra observer plaque GSM measurements in this study is overall low and is in good agreement with other studies on GSM reproducibility including Anderson et.al. (18) that reported CoV of 8.3% (n=25), and Gronholdt et.al (122) that reported CoV of 5.5% (n=58 plaque images).

5.1.4 Inter observer variability for GSM

The same criteria and statistical limits apply to variability between sonographers as within sonographers for plaque GSM measurements.

For the subsample from the AGES group the inter observer variability for plaque GSM between sonographer 1 and 2 was low. The mean difference was -1.0, correlation was high (0.87), CoV was low (8.55%) and there was no significant difference (p=0.35) between sonographers. Hence there is good agreement between sonographers 1 and 2 in plaque GSM measurements and the results fulfill the statistical requirement in all values for certification in this study.

For the subsample from REFINE group the inter observer variability for plaque GSM between sonographers 1 and 3 was low. The mean difference was 1.15, correlation was high (0.82), CoV was low (7.45%) and there was no significant difference between sonographers (p=0.44). There is also good agreement between sonographers 1 and 3 in plaque GSM measurements and all statistical values are within the statistical limits set for the certification. Overall, the low variability on plaque GSM estimation between the three sonographers shows that following the procedure gives reliable and reproducible outcome.

This compares well to other studies e.g. Johnsen et.al. (17) where mean difference for GSM was 1.7 and limits of agreement were ±19.2 and Wijeyaratne (134) had the mean difference of 0.538. Also, the CoV in both comparisons was low as also found by Craiem et.al. (59) where CoV was 7.5%.

5.2 Reproducibility for ultrasound acquisition

The criteria and statistical limits for the variability on repeated ultrasound acquisition on both plaque area and GSM estimation were the same as for the intra- and inter observer variability for plaque area and plaque GSM: the difference between two acquisitions was plotted against the average of the acquisitions and limits of agreement are where 95% of the differences lie within ±2SD of the mean difference. The correlation should be higher than 0.8 and CoV < 13% and there should be no significant difference between acquisitions.

In the reproducibility estimate for the ultrasound acquisition, where the subjects (n=20) were scanned and measured twice by sonographer1, the mean difference of plaque area was -0.85mm^2, high correlation (0.95), acceptable CoV of 12.40% and there was no significant difference (p=0.11) between observations. The mean difference for plaque GSM was -0.35, high correlation (0.84), acceptable CoV 11.17% and there was no significant difference (p=0.88) between observations.

These results show when considering all the statistical parameters that variability in acquisition adds very little if any to the variability in the reading of plaque area and plaque GSM were images from same acquisition were read twice compared to readings of images from two different acquisitions.

The correlation in the intra-observer variability assessment in the REFINE study group of plaque area and plaque GSM for sonographer1 based on images from one and the same acquisition compared to the correlation in the intra–observer variability assessment based on images from the two acquisitions was 0.96 vs. 0.95 and 0.91 vs. 0.84 respectively, the mean difference was 1.97mm^2 vs.-–0.85 mm^2 and 1.31 vs. -0.35 respectively and the CoV was 9.82% vs. 12.4% and 6.84% vs. 11.17% respectively. The difference in mean plaque area and mean plaque GSM was not significant for either the repeated reading based on images from the same acquisition or the two different acquisitions.

The results show that the careful viewing and comparison of baseline images with the follow-up images during acquisition side by side are likely to contribute to lower variability. There is a lack of information in current literature on the effect of variability in ultrasound acquisition on quantitative plaque measurements. Therefore, to the authors of this dissertation best knowledge, this is the first

study that takes acquisition variability into account in the assessment of carotid plaque size and composition measurements using ultrasound.

5.3 General trends in plaque distribution and the possible shadow effect from plaque in near wall

In this study for both study groups the plaques were found to be more prevalent in the bifurcation of the common carotid artery than in the internal carotid artery which compares well to results from another study by Rubba et.al (53, 135). This is also in agreement with the reasoning that low shear stress in the bifurcation induces plaque formation (26, 27).

Theoretically a thick or highly calcified plaque in near wall could cause shadow effect on plaque in far wall (52). Shadow effect occurs when attenuation of the ultrasound beam is caused by intervening structures causing echoes from tissue behind to become weaker which could prevent the detection of plaques in far wall, reduce estimation of plaque area and/or alter the GSM estimate (52). The results of this study show a significant difference in far wall plaque GSM such that when near wall was clear, plaques have lower GSM values indicating less calcification while the opposite from shadow effect would be expected. Further, there was no significant difference in far wall plaque area whether or not plaque was present in near wall. Also in this approach the use of different angulations of the ultrasound probe gives the opportunity to measure plaque at the least AS affected angle. The conclusion is therefore that shadow effect is not of concern in this study.

5.4 Applicability of the method

The aim of the study was to implement a reliable and reproducible protocol to assess longitudinal changes in plaque. Fundamental requirement for reliable estimates of longitudinal changes in plaque size and composition is that the difference between repeated measurements is lower than the difference to be evaluated. To evaluate this, estimates of longitudinal plaque changes between Visit1 and Visit2 in the AGES study group were compared with the intra-observer variability for sonographer1 on 25 individuals who were participants in the AGES study. The comparison was solely based on readings by sonographer1 who fully met the requirements for plaque analysis certification.

The mean difference in plaque area for sonographer1 in the intra-observer variability estimate was -0.85mm^2 with 95%CI -3.88 to 2.18 and SD=10.65 which was considerably lower than the mean difference for longitudinal changes within the same 25 individuals who had mean difference of 11.10mm^2 with 95% CI 6.32 to 15.88 and SD=11.57 between visits.

The mean GSM difference for sonographer1 in the intra-observer variability estimate was 0.33 with 95% CI –1.60 to 2.25 and SD=6.77 compared to the mean GSM difference in longitudinal changes that was –0.92 with 95% CI -5.90 to 4.10 and SD=12.06. The SD values in the intra-observer estimates are only half the SD values in the longitudinal estimates, showing that the longitudinal measurements are meaningful.

Example of a useful approach is to look into longitudinal changes of plaque difference among age-groups. In this study the longitudinal changes without any adjustment for interventions (e.g. statin) were presented in section 4.2, Table 2. The difference between Visit1 and Visit2 for plaque area showed a trend of increasing difference with higher age-group except for the oldest group that only included 7 individuals which may explain why that group shows inconsistent results with the other age-group. For plaque GSM there was no consistent change between age-groups apart from the oldest age-group that had a higher negative value. Those results will not be discussed further since it is not the aim of this study.

Few studies have used similar methodology as presented in this study to investigate the relationship of plaque size and composition with known cardiovascular risk factors, most of them cross-sectional in design. Andersson et.al (18) studied the impact on plaque size and composition (echogenicity) from known cardiovascular risk factors, the plaque analysis was similar although they only measured the largest plaque with the exclusion of multiple plaques. Their results indicated different influences from cardiovascular risk factors on plaque growth or hardening, where smoking increased plaque calcification while systolic blood pressure and decreased HDL were more related to less calcified (echo-lucent) plaques (18).

In another study where the relationship between plaque composition and cardiovascular risk factors was examined, showed that increased plasma levels of triglyceride rich lipoprotein was associated with less calcified plaques and enhanced progression of vulnerable atherosclerotic plaques with lipid-rich cores. In this study by Gronholdt et.al (122), the acoustic shadowing part of the plaque was excluded from the plaque drawing.

At least two studies have used similar methodology as described in this study in longitudinal design to assess the relationship between cardiovascular risk factors and plaque size and composition. Johnsen et. al (17) performed a longitudinal study on plaque size and composition, although the plaque images used in the plaque estimation were captured as a still image during the acquisition and information on a maximum of six plaques per study were recorded. They reported that plaques with low growth rate between visits had increased plaque echogenicity (higher value of GSM). Further that lower HDL cholesterol increased the plaque growth (17). In a clinical study by Spence et.al (123) it was shown that plaque size is related to age with most plaque progression between 45 to 70 years while after that the progression diminishes.

The above mentioned studies show the applicability of methods similar to the one presented in this current study. The current study may have some important advances over comparable studies that result in lower variability, including the novel approach of simultaneous viewing of the ultrasound images from the baseline visit both prior to and during later ultrasound acquisitions and image analysis. Further, in this study all visible plaques in the region of interest were included, in opposition to a fixed number of plaques or a single plaque with largest diameter like reported in other studies (17, 18); hence our approach gives a more reliable estimation of total plaque burden.

In the light of a successful setup and evidence of validity of this method of measuring the longitudinal change in plaque size and composition, the Icelandic Heart Association is using this

ultrasound methodology described in current study to estimate the impact of conventional cardiovascular risk factors on the longitudinal atherosclerotic plaque changes.

5.5 Study strengths and limitations

The strengths of this study include longitudinal design in a population based setting. Highly standardized procedures were set up and applied in all stages of the image acquisition and image analysis where the baseline images were viewed during the ultrasound acquisition and the image analysis for maximizing comparability to follow-up images. The image analysis procedure included that all visible plaques within area of interest was estimated independently of size or number of total plaques. The variability was measured for both the ultrasound acquisition and image analysis but to the author's best knowledge this is the first study that estimates the contribution of both the ultrasound acquisition and image analysis in quantitative carotid plaque studies. The reproducibility was based on two study groups with different characteristics including different age span and different plaque burden. Further, the subjects in one of the study groups (AGES) were selected based on plaque size in order to reflect all levels of plaque burden in the study cohort and for the other group (REFINE) they were based on random sample from a younger cohort. The AGES subsample may have resulted in an increased observer-variability than if the sample would have been selected randomly with more homogenous plaque burden across subjects. If reproducibility results in both study groups are compared, the REFINE study group (less total plaque burden) has slightly less variance than the AGES study group (more total plaque burden). This is in contrast to findings in a study by Joakimsen et.al. (132) who reported that smaller plaque burden gave higher variability. Therefore the plaque size dependent selection of subjects in this study gives more sensitive measure of variance in the reproducibility estimation. Another, strength of current study is that multiple statistical parameters were used to detect if there was a variance between observations which might not be seen on e.g. the correlation coefficient alone. This use of multiple statistical parameters also facilitated a comparison to other studies.

Limitations include that not all sonographers fully met the criteria for certification in plaque area estimation and indicated that they were still in their learning process. Also, the imaging analysis software (AMS) that was used didn't fully support longitudinal plaque measurements and hence an adaption included entering long file names manually that were loaded into the database (described in Appendix I) which increases the likelihood of human errors in profiling the plaque identity.

5.6 Conclusion

A highly strict and standardized procedure has been implemented for both the ultrasound acquisition and imaging analysis for plaque measurements applying a novel methodology. The importance of experience and continuous practice according to such protocol is emphasized. The method can be reliably used to consistently assess changes in plaque size and composition over time at an individual level, using the protocol rigorously adhered to all stages of the procedure.

APPENDIX I

THE REFINELO Study

The Risk Evaluation For Infarct Estimates Longitudinal Component

The Carotid Ultrasound Protocol – Manual Operations

Version 2.1, 2013

Table of Contents

Index of Figures

1 INTRODUCTION

The REFINELO is a longitudinal study derived from the REFINE (Risk Evaluation for Infarct Estimate) Reykjavik Study. The REFINE Study is a cross sectional study initiated in December 2005 and has the main purpose of increasing predictability on individual basis for risk factors to develop into coronary artery disease (113). The cohort is inhabitants (aged 20-69) selected randomly from the greater Reykjavik area and approximately 6000 participants have been examined in spring 2011. The REFINELO study is composed of 1331 subjects that were randomly selected from the REFINE study and re-examined with 3 to 5 years apart.

This ultrasound protocol examination of the carotid artery has been developed to meet specific research objectives and is not suitable for routine clinical examination. All ultrasound sonographers are required to complete a standardized training program and certification to be able to perform a quantitative examination on the study participants. If the sonographer determines a severe or extensive carotid artery disease a clinical site principal investigator or a designated co-investigator is notified.

The ultrasound scanning protocol informs a standardized examination of right and left common carotid arteries, the bifurcation and the internal carotid artery. It's designed to provide images of the common carotid arteries to measure carotid intima-media thickness (CIMT), arterial stiffness together with plaque area and plaque composition by Gray-Scale-Median (GSM) values in bifurcation and internal carotid arteries. The protocol for image acquisition and measurements for CIMT and arterial stiffness are the same in the REFINE Study and the REFINELO Study except that both the baseline images and follow-up images are analyzed side by side in REFINELO by the same sonographer (repeated measure of baseline images at the same time the follow-up images are measured) for achieving maximum comparability. The protocol for CIMT and arterial stiffness is described in detail in the Manual of Operations for the REFINE Study. This Manual of Operations (MOP) is therefore focused on the longitudinal plaque imaging and the analysis of plaque area and plaque composition. For further detail of the CIMT and arterial stiffness measurements, please see the REFINE MOP.

Ultrasound sonographer has to assure that the ultrasound equipment and the back-up procedures are performing optimally prior to each scan.

1.1 ULTRASOUND TRAINING AND CERTIFICATION

1.1.1 Ultrasound Training

Training and certification for sonographers in the longitudinal REFINELO Study is similar to the REFINE Study for image acquisition and measurements of B-mode carotids Intima-Media Thickness (CIMT) and M-mode arterial stiffness (REFINE Manual Of Operations). The REFINELO Study additionally includes longitudinal measurements of plaque area and plaque composition. The training takes place on site. The new ultrasound sonographer is introduced to the proceedings and protocol, watching a few studies. He/she then starts hands on ultrasound exercises.

1.1.2 Certification

A technician/sonographer is certified when he/she shows sufficient knowledge of the execution of the ultrasound examination, provides ultrasound images from which intima-media thickness, plaques and arterial stiffness can be measured by the reader of sufficient quality. Plaque images must be of good quality and high similarity between baseline and follow-up images so the examiner can measure the changes in plaque area and composition (GSM). In acquisition the sonographer trainee is required to be able to detect and evaluate plaque on baseline images and acquire as similar (comparable) images as possible of the plaque in the follow-up examination. The ultrasound gain and angle for a prevalent plaque must be the same in the baseline imaging and the follow-up imaging.

The quality of the acquisition is evaluated by having the technician performing the entire ultrasound examinations monitored and reviewed by a qualified and experienced sonographer. The certification process usually takes around 20 – 30 scans to complete. One should allow two or three months of training time, depending on the intensity (allocated time) by which the ultrasound technician practices. Experienced technicians may need a smaller number of ultrasound examinations performed before being certified. Certification is granted by the staff of IHA and sent in written form (certificate) to the technician/sonographer. In principle a certification is granted for a 12 month period. During the study, performance is being checked at regular intervals. De-certification is being considered when performance is judged by the IHA staff as being insufficient.

2 CAROTID PLAQUE ULTRASOUND PROTOCOL

2.1 ULTRASOUND SCANNING PROTOCOL

For CIMT measurements the protocol is the same as in REFINE Study previously described (REFINE protocol V2.2, October 2008). A primary objective of the REFINELO carotid ultrasound scanning protocol is to acquire comparable follow-up standardized longitudinal clips of the bifurcation and internal carotid artery to the baseline clips. Baseline images are viewed and reviewed prior to and during follow-up examination to acquire comparable follow-up images. The gain was adjusted on the follow-up scan so the GSM would be comparable to baseline images. Plaque is measured in near and far wall in both the bifurcation and the internal carotid artery. The length of bifurcation varies between subjects.

The ultrasound acquisition was performed with two dimensional Acuson, Sequoia C256, (Siemens Medical System, Erlangen, Germany) with 8-MHz linear array transducer. The procedure was standardized to minimize the variation (3). To achieve this, a workstation with the K-PACS V1.5.0 image viewer software was used to view baseline images prior to and during follow-up acquisition to acquire comparable follow-up images (Figure 1). The manual operation on how to use the K-PACS software for viewing the baseline images is explained in later chapter on Image analysis (K-PACS 4.2.1.). For a standardized circumferential scan of the left and right carotid artery the commonly used Meijer's Arc (114) was used in order for the images to be acquired at the same angle at the baseline

and follow-up visits. Angles were either 180°, 150 °, 120° and 90° for the right and for the left 180° , 210°, 240° and 270°.

Figure 1. The setup for ultrasound acquisition, the ACUSON Sequoia and a computer with the K-PACS viewing software.

The participant lay's down in a supine position with head toward the ultrasound equipment. If requested a pillow is placed under the knees to support the lower back. The sonographer scans from the head of the table. Electrocardiogram is connected as previously described in (*REFINE chapter 2.2.5)*. A towel is placed under the neck of the participant. The Meijer's Arc marked with angle scale is positioned beneath and above the shoulders (Figure 2). The Meijer's Arc is used to standardize circumferential scan of the left and right carotid. In the beginning of the study the ultrasound equipment ACUSON is pre-set for REFINELO for the CIMT measurements.

Next, participants name is selected from a work-list and his/her date of birth confirmed. The sonographer enters his/hers ID and starts the scan. A computer is located next to the side of the ultrasound equipment for viewing baseline images prior to and during the examination. The baseline images should be set up on the computer and studied before the exam.

During follow-up always take two clips from different angle, if two clips available at baseline, then take follow-up clips from same angles. The gain on follow-up images should be set the same as on baseline clips. When there is no plaque at baseline images, a clip is acquired from two angles in follow-up. However if a new plaque(s) is present in follow-up then a clip is acquired at an angle where the plaque is largest and most clearly visualized. Clip is always acquired from two angles in the follow-up examination. Additionally to the plaque measurement, B-Mode and M-mode images are also acquired as previously described in REFINE-Study (REFINE MOP).

The angle indication should be identified on left of the screen. For the right side 4 angles marked with the capital letter R; 180°, 150°, 120° and 90° and for the left capital letter L and the angles are 180°, 210°, 240° and 270°.

For more detailed methodology of the B-mode and M-mode scan see REFINE protocol v2.2, October 2008 chapter 2.2.5.1.-2.2.5.8.

2.1.1 Equipment/Instrumentation

A two dimensional Acuson, Sequoia C256, (Siemens Medical System, Erlangen, Germany) with 8-MHz linear array transducer should normally be used for the acquisition in the REFINELO Study. There should be no upgrades of system hardware or software or any other changes made to the ultrasound equipment that can influence the consistency of the acquisition and image analysis during the course of the REFINELO Study and the REFINE Study. As in the REFINE Study, all images are routed directly following each examination to the image archive (PACS system) for storage. For reviewing the baseline images prior to the follow-up examination, a computer equipped with K-PACS V 1.6.0 Dicom image viewer is used on a computer monitor standing next to the ultrasound equipment.

2.1.2 Back-up procedure for the acquisition

Since the REFINELO Study images will be directly stored in a digital format, the possibility exists that images are lost due to failure of the digitalization process. When that happens all recently acquired images are lost. Therefore a back-up procedure is installed, issuing that all carotid ultrasound examinations are stored on videotape. There must be back-up tapes for all days of the week (Monday to Friday) and appropriately labeled" (The back-up procedure see chapter from REFINE protocol 2.2.3. back up procedure). When the US acquisition is finished, click on Review to view the whole study and make sure all images and clips is stored correctly before sending the study into the PACS system.

2.1.3 Personnel

For the ultrasound scanning duties, at least three sonographers are required to be able to perform the procedures and they should equally share scanning time during the course of the study to maintain a high skill level and consistency in examinations.

2.1.4 Plaque acquisition

The K-PACS V1.6.0 Dicom viewer next to the ultrasound system is used to view baseline images (Figure 2). The baseline clip of the bifurcation and internal carotid artery on both sides is viewed thoroughly for inspection of possible plaques. Another clip is acquired at the same angle in the follow-up study. The gain is set the same as at baseline and the focus is set in middle of the common carotid artery (CCA) or if the quality of either near or far wall is bad put the focus positioning on the low quality wall.

The clip images are labeled in the left corner off the image frame with side and angle. Two clips are acquired from both sides (even in cases when only one was acquired at baseline) but only one at each angle.

Figure 2. Follow up acquisition. Baseline images are viewed with a Dicom viewer for maximizing comparability of baseline and follow-up images.

3 Ultrasound Image Analysis (Plaque – Reading)

3.1. Equipment – Software

Digital images from the ultrasound acquisition were analyzed on a workstation using the Artery Measurement System (AMS) II V1.141 © Peter Holdfeldt software designed by Professor Tomas Gustavsson, (Chalmers University of Technology, Department of Signals and Systems, Göteborg) while the K-PACS V1.5.0 software was used simultaneously to compare with the images of the other acquisition (baseline or follow-up).

3.2 Training of readers/sonographers

The training process for plaque imaging analysis consists of the following few steps;

a) Sonographer in training observers the whole path of the reading process with a certified sonographer (approximately 20 full studies).
b) Sonographer in training reads approximately 10 to 20 cases with a certified sonographer watching over her/his shoulders until they seem to have full agreement on the plaque tracings.
c) Sonographer in training reads through 10 cases that have been selected to be re-read for quantitative quality assessment of the reading to measure both inter and intra variability.
d) For within and between sonographers variability measurements on plaque area and GSM the correlation should be above 0.8 and the CoV should not exceed 13%.
e) Within and between sonographers variability measurements should have no significant difference between observations ($p>0.05$).
f) If the sonographer in training meets these statistical tolerances listed in d) and e) he or she qualifies to read on his/her own and every six months this dataset should be re-read.

All sonographers were experienced in imaging the common carotid artery using ultrasound equipment.

4 Image analyses

Digital images from the ultrasound acquisition are analyzed on a workstation using the Artery Measurement System (AMS) software designed by Professor Tomas Gustavsson, (Chalmers University of Technology, Department of Signals and Systems, Göteborg). The K-PACS V1.5.0 Dicom viewer is used simultaneously with the AMS image for comparison of baseline- and follow-up images. For plaque analysis the software automatically generates descriptive values that reflect the size and composition (GSM) of plaques. Among these are size (area mm^2, length, and height), grey-level median/mean and grey-level variance. The Software also carries out Gray-Weale analysis for classification of the plaque composition (116). Gray scale value for Echo-lucent plaque is ~0 (vessel) and gradually increasing for echo-rich plaque at ~256 (vessel wall/more calcified area). The definition of plaque presence is the area with intima-media thickness (IMT) 50% thicker than the normal neighboring sites by visual assessment, a commonly used definition (115). From the image series of each plaque in a longitudinal view, an image frame should be selected where the plaque appears largest in area while still having clear boundaries. The grey scale of each frame should be calibrated by selecting black (0) and white (256) for the darkest and brightest parts of the image.

Plaque boundaries should be traced with a cursor on the screen for both baseline and follow-up images. The software will be used to automatically assign each plaque into four classes:

Class 1; composition of the plaque is fat/lipid or not calcified

Class 2; calcified up to 25%

Class 3; up to 75% is calcified

Class 4; 75-100% is calcified.

The output from the software will be extracted from a text file and loaded into a central database.

4.1 User name and Password

Turn on the ultrasound computer (UL_03) password to login written on piece of paper at the table. **Another password needs be written on the E://drive** ultra5544 or raforn

Two other ultrasound computers (UL_01 and UL_02) are used for plaque reading and the password to login for both computers is written on a piece of paper at the table.

4.2 Software for Image analysis

4.2.1 K-PACS software;

Double click on K-PACS icon on desktop - Accept the comment for "*please note*"

Make sure the *network* tab is marked (not the database tab)

In ***patient-id*** box – write the participants id-number

- The folders that appear will include every imaging study that the participant has undergone
  - Highlight and double click the US older date for baseline (baseline images should appear)

- Screen thoroughly through all the examination and decide whether plaque present or not, close baseline (if plaque present go to the next subchapter AMS) ***and***;
- Double click on the "Hreinsa conquest " a desktop icon (before opening the follow-up on K-PACS)
- In the K-PACS:

-Highlight and double click on the newer-date and follow-up images should appear

After viewing the images in the K-PACS software, remember to keep it open on the right screen.

4.2.2 Preparation for AMS software;

- In DICOM-view Preloaded Images on the **//E: drive**;
  - Take a copy of the images and sort it for the AMS software.
    - choose the id-folders name press F2 and ctrl.C for copying
- Go into Command Prompt to sort the images for the AMS;
  - double click the icon "rada" on the desktop,

-window with the c:/Documents and Settings/car2> appears

-write rada spacebar ID (paste_id) ENTER ENTER

-when the c:/word> in the command prompt window:

- Double Click on the **AMS** software icon on the desktop

- Keep the AMS software on the left screen

4.2.3 AMS software;

- Click in the **Reading** box:

  -Assign your initials (XX) in the ***reader*** box (Figure 6).
- Go into the blue box called **IMAGE**
  - Both studies appear under this folder, baseline either under the "001" folder both image and clips or straight under "image" and "clips". The follow-up folders are usually under "002" image and clip separately
- Choose View clip and carefully choose frame where the plaque is clear and biggest. While working in the AMS software it's necessary to view the follow-up clip on the K-PACS and choose frame where there is most similarity between baseline and follow-up images (for arterial lining and also the plaque position and length). If there is no match between baseline and follow-up clips it's possible to take a still image (IMT) just make sure the arterial lining (angle) positioning is the same. If the still image is saved the frame name will be F0 (zero).

- Still in **View** window choose the frame that represents the plaque best:
  - Go into File:
    - press on the icon "**save frame as**" (Figure 4).

- The Path should indicate:
    - o For baseline; C:/work/DICOM/IMAGES/clips
    - o For follow-up; C:/work/DICOM/IMAGES/002/clips
- Check the "**specify filename**" write filename in the column: **ID-B (F)-R (L)-F (XXX)-P (XX)-IN**

ID: Participants number

B: for Baseline or

F: for Follow up

R - for right side: 90°, 120°, 150° and 180° angle

L - for left side: 270°, 240°, 210° and 180° angle

F (number 1 to ~130): stands for frame number from the clip

P (number): stands for the plaque position (Figure 3). Plaque number should always indicate the initial (proximal) part of the plaque even though it goes over another anatomical position and if it starts in the CCA it will take either P1 or P2

IN: write the examiners (reader) initials

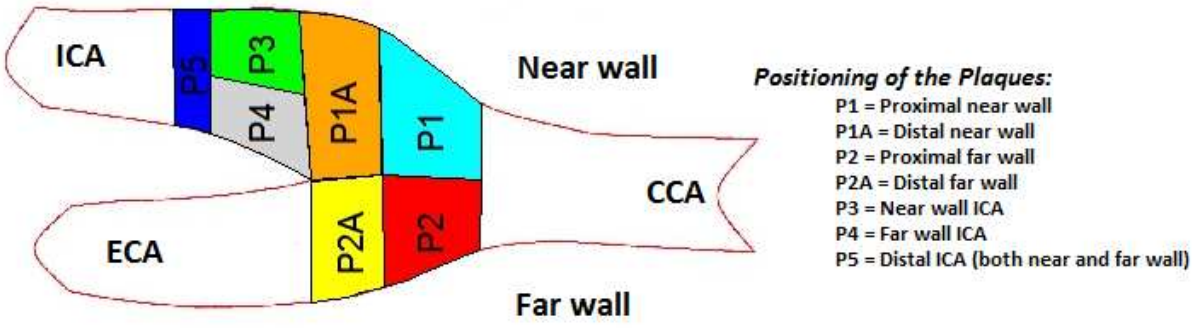

Figure 3. Simplified diagram with optimal angle of the common carotid artery (CCA) bifurcation, external carotid artery (ECA) and internal carotid artery (ICA); for the location of plaques, the region of interests in the carotid artery was divided into seven different segments that plaques were assigned to.

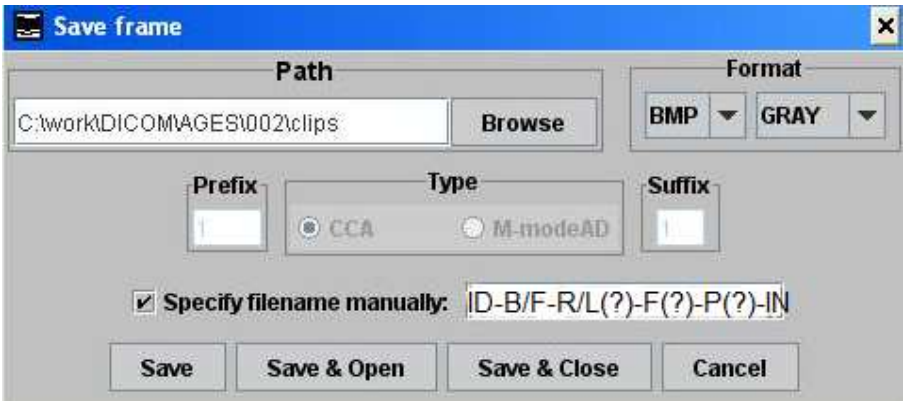

Figure 4. Save image – After viewing the plaque and deciding the frame where the plaque is clearest.

• Take a copy of the specify filename and press **Save&Open** **(**Figure 4).

o Close the View window

•Saved frame should include:

• CCA space and file name at the top of a picture

• In the empty boxes beside the filename (Figure 5).

-write the **ID** number

-write **year-month-date**

-**initials** of the sonographer

-make sure these numbers are correct **13.832022** (Figure 5).

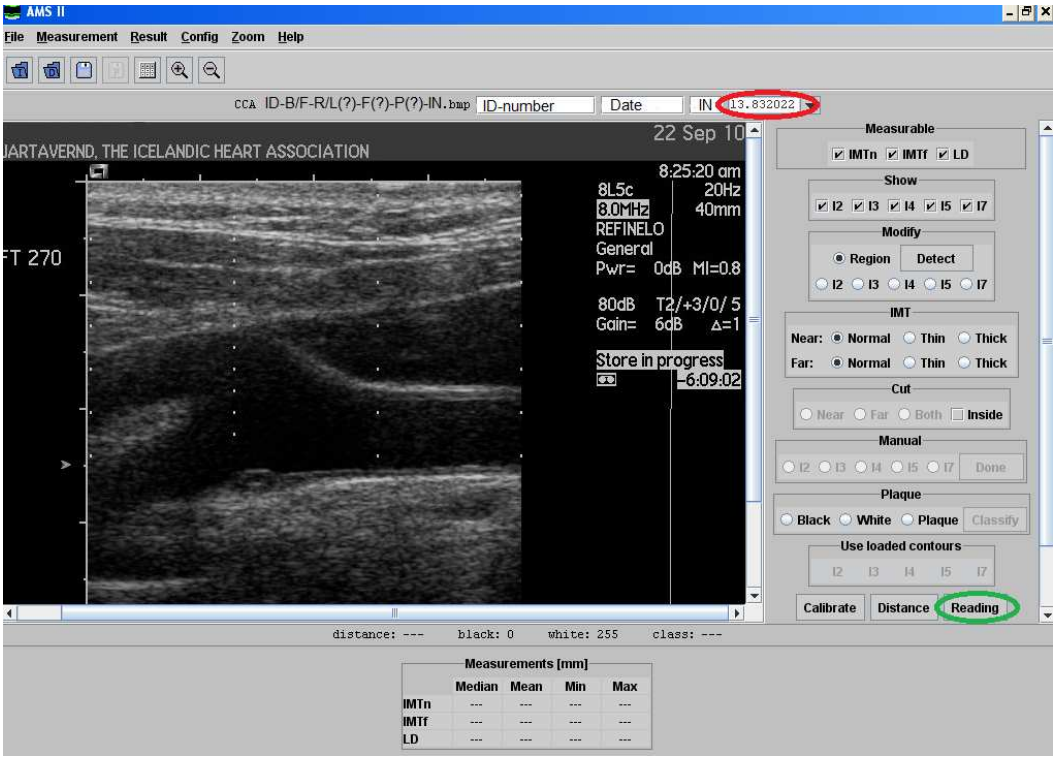

Figure 5. Saved frame – go into the „Reading“

• The saved image after filling above tabs click on the “**Reading**” column, few things to consider (Figure 6):

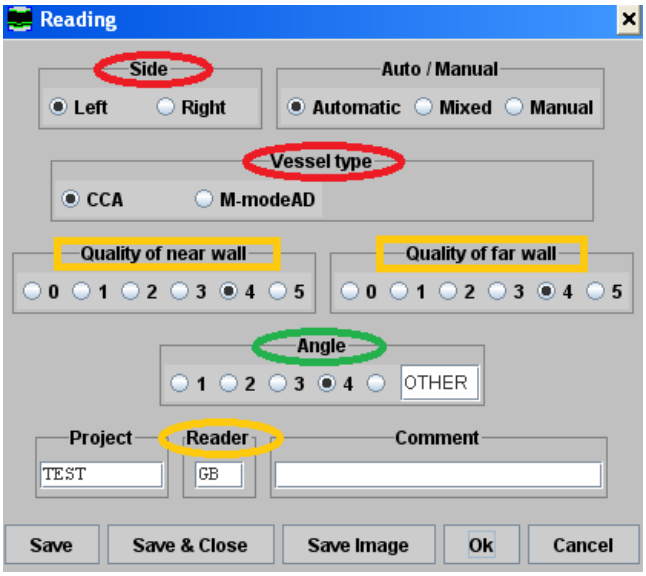

Figure 6. Reading - Make sure the information in the boxes are correct before the plaque boundaries are drawn and the classified.

1. **Side**;
    a. Left or Right (mark reference side)
2. **Vessel type**;
    a. Mark – CCA
3. Give the plaque boundaries **quality** either in near or far wall from 0 to 4
        i. 4= well cleared boundaries
        ii. 3= good quality of the boundaries
        iii. 2= boundaries moderately clear
        iv. 1= boundaries hardly visible
        v. 0= not traceable boundaries
    b. plaque in near wall mark – quality of near wall
    c. plaque in far wall mark – quality of far wall
        i. zero (0) for the opponent wall
4. **Angle**
    a. Angle of the clip where the image was saved from:
        i. Right side
            1. (90°); 2. (120°); 3. (150°); 4. (180°)
        ii. Left side
            1. (180°); 2. (210°); 3. (240°); 4. (270°)
5. **Reader**
    a. Initials of the reader

⇨ Press **OK** and continue working on the saved frame (image).

###############

1. Calibrate the image under plaque column:
        i. **Black**; mark black and draw a box over the darkest part of the image-value should be close to 0
        ii. **White**; mark white and draw a box over the brightest part of the image although be careful don't take the scaling into the white calibration-value should be close to ~220
        iii. Plaque
            1. Draw the plaque boundaries – try to find clear lines of the plaque beginning and ending (to increase the reproducibility)
2. Plaque drawing
    o Decide where you want to start; LEFT click the mouse again and again until the plaque boundary has all been drawn (should have red dots around it). If you want to change the drawing while drawing then -RIGHT click mouse until you want to start over again. If you want to begin from start you press **plaque** button again (drawing will disappear).

3. After drawing the plaque boundaries
    1. Press "**classify**"
        a. The software gives out a box (Figure 7).
    2. Press OK

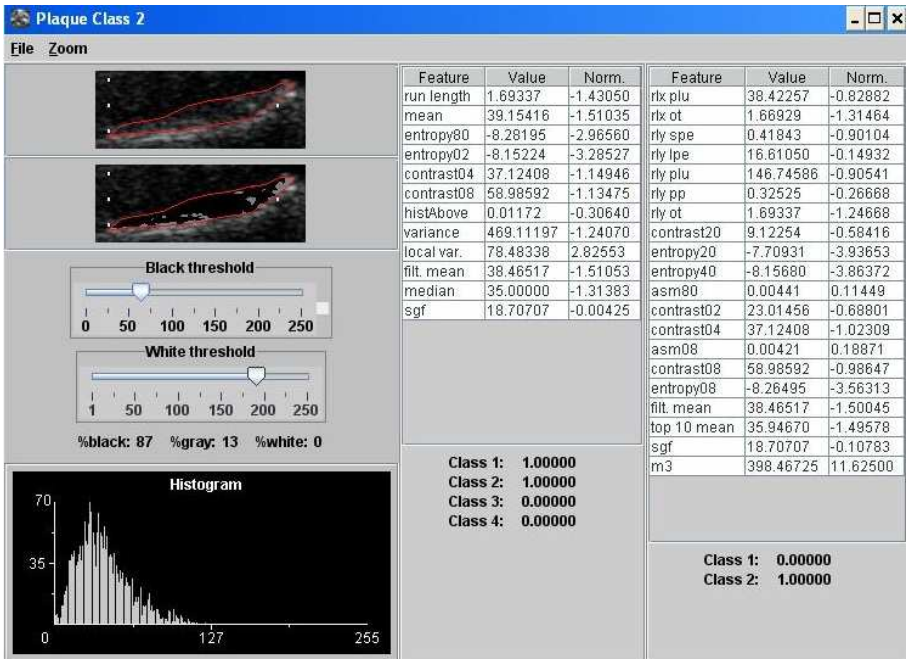

Figure 7. Automated classified plaque: Carefully view the histogram. The grey scale is on x-axis, 0 for black and 255 for white and the y-axis has the percentage. Decide where the plaque should be classified. If the distribution is normal from ~0 to ~127 it should be class2 and if the distribution is between ~127 to ~255 it should be class 3 and if all the plaque is close to 255 Class 4.

3. **Press the icon** P for classifying the plaque into the four groups (Figure 8):
    i. Class 1; Composition of the plaque is fat/lipid or not calcified
    ii. Class 2; Calcified up to 25%
    iii. Class 3; Up to 75% is calcified
    iv. Class 4; 75 – 100% is calcified

Figure 8. Visual classification from the drawn plaque

4. Choose appropriate Class (based on the histogram information)
5. Press save

6. Go to "**Reading**" column and:
    a. Save image
        i. Choose .jpg instead of .bmp
            1. Choose the folder for "E:\MYNDIR\REFINELO_PLAQUE_DRAWING".
            2. Save image - ENTER

- To make sure the plaque was measured look into the review box on the AMS software "C:/AMS/cca.txt" and the plaque measurements should appear.

- If there are multiple plaques present the same process is made for every single plaque until every plaque has been drawn.

- When drawing the follow-up plaques you can call for the saved image of traced plaque by choosing the folder: "E:\MYNDIR\REFINELO_PLAQUE_DRAWING" and double click the image filename to view the baseline drawing while drawing the follow-up plaque.

5 Registering all reading information in "Innranet"

5.1 Information on the IMT and plaque readings:

-Fill in all tabs in HVIS "innranet" path **http://newhvis.hjarta.is/hvis/**

- ***CU-B (carotid ultrasound baseline 1162):***
  -Fill in all the columns and use the K-PACS software to view the baseline study
- ***CU-F (carotid ultrasound follow-up 1163):***
  -make sure the information that is filled in is correct
- ***CU-PB (plaque baseline 1164):***
  -Fill in all traced baseline plaques and say no to other tabs (columns)
- ***CU-PF (plaque follow-up 1165):***
  -Fill in all traced follow-up plaques and say no to other tabs (columns)

- Make sure that the **ULBP** blood pressure has been filled out and the **TON** for tonometry is filled out before closing the case.

- Reading finished:
    - In the K-PACS
        - Go into the database and highlight the correct id (both baseline and follow-up) and right click the mouse and delete folders.
    - AMS – close the program
    - Delete the folder on the DICOM-view Preloaded Images on the **//E: drive**;
        - Highlight the id folder and click on delete button – press Enter
        - Highlight the id folder and right click the mouse (or press the "delete" key), remember to make sure the dotted lines are around the file when deleting XXXXXXXXXX

5.1.1 A few bugs in the AMS software

The AMS software has some "pitfalls" if you need to delete the drawn plaque/image there are few steps to go through;

1. C:\AMS\cca.txt
    a. -delete the line with correct filename (remember to get the dotted lines around the folders name)
        i. After deletion save the folder.
2. C:\AMS\plaque.txt
    a. - delete the line with correct filename (remember to get the dotted lines around the folders name)
        i. After deletion save the folder.
3. C:\AMS\plaque
    a. – delete the line with correct filename (remember to get the dotted lines around the folders name)
4. C:\AMS\contour
    a. – delete line with correct filename (remember to get the dotted lines around the folders name)
        i. Remember the dotted lines around the filename should appear when you are deleting the file
5. E:\MYNDIR\REFINELO_PLAQUE_DRAWING
    a. –highlight the plaque name and press delete
        i. Remember the dotted lines around the filename should appear when you are deleting the file.

Appendix II

Reproducibility/variability measurements on plaque area and/or GSM - Published studies with various statistical parameters: sample sizes, mean difference, correlation, coefficient of variation and p - value.

	Plaque area					Plaque composition (GSM)			
	Sample Size	**Mean diff. (SD) 95% CI of mean**	**Rho –r 95%CI**	**CoV %**	**P-value**	**Mean diff (SD) 95% CI of mean**	**Rho-r 95%CI**	**CoV %**	**P-value**
Andersson,2009 (18)									
Intra observer	25	-	-	11.2	-	-	-	8.3	-
Barnett,1997 (2)									
Intra observer	25	-	0.94	-	-	-	-	-	-
Inter observer	50	-	0.99	-	-	-	-	-	-
Craiem,2009 (59)									
Inter observer	55 (plaques)	0.087 mm^2 log	0.97	6.6	<.001		0.99	7.5	<.001
For different 2mm thick layers									
Inter observer	55 (plaques)	-	-	-	-		0.81 to 0.99	7.0 to 12.0	<.001
Denzel,2003 (136)									
Inter observer							0.881		<0.01
Gronholdt,1998 (122)									
Intra observer	58 (plaques)	-	-	-	-	-0.4 (-1.5 to 0.1)	-	5.3	-
Intra observer	10 (plaques)	-	-	-	-	0.6% (-1.5% to 2.6%)	-	3.5	-
Intra observer	10 (plaques)	-	-	-	-	1.1% (-0.8% to 3.1%)	-	2.2	-
Joakimsen,1997 (132)									
Intra observer									
Inter observer									
Johnsen,2005 (17)									
Intra observer 1	-	0.2 (3.1)mm^2 Limit of agreement ±6.1	-	-	-	-0.1 (6.1) Limit of agreement ±12.0	-	-	-
Intra observer 2	-	0.01 (3.8)mm^2 Limit of agreement ±7.5	-	-	-	-0.9 (8.7) Limit of agreement ±17.1	-	-	-
Inter observer	-	-1.0 (4.4)mm^2 Limit of agreement ±8.6	-	-	-	-1.7 (9.8) Limit of agreement ±19.2	-	-	-

	Plaque area					Plaque composition (GSM)			
	Sample Size	**Mean diff. (SD) 95% CI of mean**	**Rho –r 95%CI**	**CoV %**	**P-value**	**Mean diff (SD) 95% CI of mean**	**Rho-r 95%CI**	**CoV %**	**P-value**
Lear,2007 (129)									
Intra observer	-	-0.21 mm^2	0.922 to 0.948	-	-	-	-	-	-
Inter observer	-	3.61 mm^2	0.850 to 0.901	-	-	-	-	-	-
Persson,1992 (131)									
Intra observer (for IMT area)	13	17.3±13.1	0.92	11.9 to 17.3	<.001	-	-	-	-
Inter observer (for IMT area)	20	15.9±12.5	0.92	11.9 to 17.3	<.001	-	-	-	-
Sabetai,2000 (133)									
Inter observer	-	-	-	-	-	-0.05 (95% CI -1.7 to 1.6)	-	-	-
Spence,2002 (137)									
Senior technician	25	-	0.94	-	-	-	-	-	-
Junior technician	25	-	0.85	-	-	-	-	-	-
Wijeyaratne,2003 (134)									
Intra observer	50 ((plaques))	-	-	-	-	0.469 (95%CI -0.93 to +1.8)	-	-	-
Inter observer	50 (images)	-	-	-	-	0.538 (95%CI -1.2 to +2.3)	-	-	-

APPENDIX III

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Efni: Varðar: 07-062-V6 Öldrunarrannsókn Hjartaverndar: AGES II. áfangi.
AGES Gene/Environment Suceptibility (AGES) Reykjavík study second phase.

Á fundi sínum 24.03.2009 fjallaði Vísindasiðanefnd um umsókn þína dags. 05.03.2009, vegna viðbótar nr. 6 við ofangreinda rannsóknaráætlun.

Í bréfinu kemur m.a. fram að óskað er eftir leyfi til að bæta við ómun og segulómun af hálsslagæðum í 2. áfanga Öldrunarrannsóknarinnar. Fyrirhugað er að gera þessa rannsóknir á 230 þátttakendum frá apríl 2009 til desember 2009. Um er að ræða rannsókn þar sem annars vegnar verður notuð ómun ásamt nýjum myndúrvinnslubúnaði sem þróaður hefur verið af prófessor Tomas Gustafsson.

Vísindasiðanefnd hefur farið yfir bréf þitt og innsend gögn og gerir ekki athugasemdir við tilgreindar breytingar. Rannsakendum er bent á að á fylgiskjal nr. 5, upplýsingar vegna þátttöku, vantar logo Hjartaverndar.

Viðbót nr. 6 ásamt fylgigögnum við ofangreinda rannsókn, er endanlega samþykkt af Vísindasiðanefnd.

Með kveðju,
f.h. Vísindasiðanefndar,

dr. med., Björn Rúnar Lúðvíksson, læknir, formaður

MÓTTEKIÐ
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Hjartavernd
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Forstöðulæknir Rannsóknarstöðvar Hjartaverndar
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Reykjavík, 14. maí 2002
Tilvísun: 2002050228 MS/--

Persónuvernd hefur borist bréf yðar, dags. 30. apríl 2002, ásamt umsókn um heimild til vinnslu persónuupplýsinga vegna Öldrunarrannsóknar sem er næsti áfangi í Hóprannsókn Hjartaverndar sem staðið hefur síðan 1967.

Í umsókn yðar kemur fram að einungis verður leitað til þeirra einstaklinga sem eftir lifa af Hóprannsókn Hjartaverndar og að *upplýsts samþykkis* þeirra verði aflað. Þegar af þeirri ástæðu gerir Persónuvernd ekki athugasemdir við framangreint.

Virðingarfyllst

Margrét Steinarsdóttir
lögfræðingur

APPENDIX IV

Móttekið 04/11/05
BÞ.

VÍSINDASIÐANEFND
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Rannsóknarstöð Hjartaverndar
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201 Kópavogi

Reykjavík 1. nóvember 2005
Tilvísun: VSNb2005090009/03-13 Umsóknir frá Hjartavernd/BH/--

Varðar: 05-112-S1 Áhættuþáttakönnun Hjartaverndar 2005-6.

Vísindasiðanefnd þakkar svarbréf þitt, dags. 14.10.2005 vegna áðursendra athugasemda við ofangreinda rannsóknaráætlun sbr. bréf nefndarinnar dags. 06.10.2005. Í bréfinu koma fram svör og skýringar til samræmis við athugasemdir Vísindasiðanefndar. Með bréfinu fylgdu ný og endurbætt upplýsinga- og samþykkisbréf ásamt afriti af persónuleikaprófi.

Fjallað var um svarbréf þitt og önnur innsend gögn á fundi Vísindasiðanefndar 01.11.2005 og voru þau talin fullnægjandi.

Rannsóknaráætlunin er endanlega samþykkt af Vísindasiðanefnd, en nefndin áréttar þó, vegna svars rannsakenda við athugasemd Vísindasiðanefndar nr. 3, að nefndinni þurfa að berast til umfjöllunar upplýsingar um hverja sendingu lífsýna til erlendra aðila þegar þar að kemur, þar sem m.a. koma fram upplýsingar um fyrirhugaðan flutning lífsýna úr landi, ábyrgðarmann með vörslu þeirra og eyðingu, eða endursendingu þeirra til vörsluaðila ef við á.

Vísindasiðanefnd bendir rannsakendum vinsamlegast á að birta VSN tilvísunarnúmer rannsóknarinnar þar sem vitnað er í leyfi nefndarinnar í birtum greinum um rannsóknina. Jafnframt fer Vísindasiðanefnd fram á að fá send afrit af birtum greinum um rannsóknina. Rannsakendur eru minntir á að tilkynna rannsóknarlok til nefndarinnar.

Með kveðju,
f.h. Vísindasiðanefndar,

Ólöf Yrr Atladóttir, framkvæmdastjóri

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Rannsóknarstöð Hjartaverndar
Dr. Vilmundur Guðnason, forstöðulæknir
Holtasmára 1
201 Kópavogur

Reykjavík 15. desember 2009
Tilv.: VSNb2005090009/03.13

Efni: Varðar: 05-112-V5 Áhættuþáttakönnun Hjartaverndar 2005-6

Á fundi sínum 15.12.2009 fjallaði Vísindasiðanefnd um umsókn þína dags. 09.12.2009, vegna viðbótar nr. 5 við ofangreinda rannsóknaráætlun.

Í bréfinu kemur m.a. fram að sótt er um leyfi til að bjóða þátttakendum í Áhættuþáttakönnun Hjartaverndar í viðbótarrannsókn; Langsniðsáfanga Áhættuþáttakönnunarinnar, sem felur í sér að mestu leiti endurtekningu á mælingum Áhættuþáttakönnunarinnar sem hófst árið 2006 og stendur enn yfir.

Vísindasiðanefnd hefur farið yfir bréf þitt og innsend gögn og gerir ekki athugasemdir við tilgreindar breytingar. Viðbót nr. 5 ásamt fylgigögnum við ofangreinda rannsókn, er endanlega samþykkt af Vísindasiðanefnd.

Með kveðju,
f.h. Vísindasiðanefndar,

dr. med., Björn Rúnar Lúðvíksson, læknir, formaður

Rannsóknarstöð Hjartaverndar
Vilmundur Guðnason, forstöðulæknir
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Reykjavík, 1. júlí 2005
Tilvísun: 2005060329 BG/--

Persónuvernd hefur borist erindi yðar, dags. 1. júní 2005, þar sem tilkynntar eru breytingar á rannsóknaráætlun í tenglum við rannsóknina „MONICA 2001 áhættuþáttaskráning Rannsóknarstöðvar Hjartaverndar í þéttbýli og dreifbýli," sem Persónuvernd veitti leyfi fyrir þann 13. ágúst 2001 (mál nr. 2001/629).

Óskið þér eftir samþykki Persónuverndar á þeim breytingum sem tilgreindar eru í erindinu, sbr. eftirfarandi:

1. Nafni rannsóknarinnar verði breytt í: Áhættuþáttakönnun Hjartaverndar 2005-6.
2. Áhættuþáttakönnun sú sem lýst er í umsókn Hjartaverndar til vísindasiðanefndar „MONICA 2001 áhættuþáttaskráning Rannsóknarstöðvar Hjartaverndar í þéttbýli og dreifbýli" nái einnig til fólks á aldrinum 20 til 65 ára.
3. Auk rannsókna sem getið er um í áætluninni verði gerð ómskoðun af hálsslagæðum af þátttakendum á aldrinum 40-65 ára.
4. Lagður er fyrir spurningalisti til að greina persónuleika þátttakenda.
5. Spurningalista um heilsufar og lyf verði breytt til samræmis við spurningalista í Öldrunarrannsókn Hjartaverndar.
6. Samþykkisyfirlýsingu verði breytt til samræmis við Öldrunarrannsókn Hjartaverndar.
7. Upplýsingablaði breytt með viðbótarupplýsingum um hálsæðaómskoðun og persónuleikapróf.
8. Niðurstöður verði nýttar til að bæta áhættureiknivél Hjartaverndar.

Persónuvernd hefur kynnt sér fyrirhugaðar breytingar og telur þær rúmast innan skilmála þegar útgefins leyfis fyrir rannsókninni, dags. 13. ágúst 2001 (mál nr. 2001/629). Er þá horft til þess að samþykkisyfirlýsingin hefur verið endurskoðuð með tilliti til umræddra breytinga á rannsóknaráætluninni. Varðandi fjölgun þátttakenda með breytingar á úrtaki rannsóknarinnar minnir Persónuvernd hins vegar á samþykkisreglur nr. 170/2001.

Virðingarfyllst

Björn Geirsson

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