THE ROLES OF HORMONES AND MEDICATION IN ATHEROSCLEROSIS

A POPULATION IMAGING APPROACH

BLERIM MUJAJ

The roles of Hormones and Medication in Atherosclerosis a population imaging approach

BLERIM MUJAJ

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Medicatie in Atherosclerose
een populatieonderzoek

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to obtain the degree of Doctor from the

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Chapter 1

General Introduction

1. General Introduction

1.1 The role of atherosclerosis in stroke

Cardiovascular diseases (CVD) are the main causes of morbidity and mortality worldwide (1, 2). Of all CVD deaths, stroke is the second leading cause after myocardial infarction (3, 4). Approximately 87% of strokes are of ischemic origin, and the remaining 13% are comprised of intracerebral or subarachnoid hemorrhages (5).

An ischemic stroke is defined as a syndrome of sudden, focal or global neurological deficit that lasts longer than 24 hours. Although ischemic strokes can be caused by cardiac thrombo-emboli, vasculitis or dissection, the atherosclerotic disease is the most common cause of ischemic stroke. Especially, when located in the carotid arteries, atherosclerosis is thought to contribute to at least 20-25% of all ischemic strokes (4, 6).

Atherosclerosis is characterized by thickening of the arterial wall due to the accumulation of lipids, calcium, and fibrous material (atherosclerotic plaques) under the influence of risk factors (e.g. smoking, obesity) (7).

An important location in the trajectory of the carotid artery that is commonly affected by atherosclerosis is the bifurcation (i.e. the point at which the common carotid artery splits into the external and internal carotid artery). Atherosclerotic

plaque formation at this location is an important source for the formation of thrombus and or subsequent embolization that may lead to ischemic stroke (8). (9, 10). Over the years, specific characteristics of the atherosclerotic plaque have been identified to predispose for abovementioned formation of thrombus or emboli. These include the plaque size, but more importantly the presence of certain components in the plaque, such as intraplaque hemorrhage, lipid core, or calcification. Imaging techniques are essential to visualize and quantify these specific characteristics of the plaque and may aid in determining the progression of atherosclerosis.

Hence, the general aim of this thesis is to contribute to our understanding of the etiology and pathophysiology of carotid atherosclerosis by means of in-vivo, state-of-the-art imaging of atherosclerosis in a sample of community-dwelling middle-aged and elderly people.

1.2 Magnetic Resonance Imaging of carotid atherosclerosis

Current guidelines for the prevention of the strokes in patients with carotid atherosclerosis are based on the assessment of the carotid degree of luminal stenosis (11, 12) according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) and European Carotid Surgery Trial (ECST) criteria, and is used as a parameter for stratification of severity of atherosclerosis in clinical decision making.

Over the last decades, advanced non-invasive visualization of carotid artery atherosclerosis using medical imaging has been hugely improved. Given the topography and relative superficial localization of the carotid artery in the neck, various non-invasive imaging modalities, including ultrasound, computed tomography an magnetic resonance, have been optimized to visualize atherosclerotic disease (13). Especially, magnetic resonance imaging (MRI) techniques have enabled feasible non-invasive characterization of atherosclerosis, including quantification of carotid stenosis and advanced plaque imaging, (determination of intraplaque hemorrhage, lipid core, and calcification). MRI accuracy has been validated against a histological background with high reproducibility (14). (15). Several MRI-based prospective studies found that non-calcified components of the plaque, such as lipid necrotic core or intraplaque hemorrhage, correlate strongly with the occurrence of cerebrovascular events (6, 16, 17).

1.3 The role of hormones and cardiovascular medication in carotid atherosclerosis

Various risk factors have been found to influence the initiation of the atherosclerotic process in the carotid artery, led by atherogenic lipoproteins and inflammatory pathophysiological mechanisms. Additional risk factors including hypertension, diabetes mellitus, and smoking, contribute to the development and

further progression of the plaque size and volume, thereby increasing the risk of plaque rupture and consequently ischemic stroke (18). Interestingly, lipid metabolism (lipoproteins) is strongly influenced by circulating hormones, such as insulin and sex hormones (19, 20), and circulating hormones have been linked with risk of cardiovascular disease (21, 22). Whether hormones are directly implicated in development or progression of the atherosclerotic plaque remains unknown, but would provide important information on the risk prediction of cardiovascular disease (21, 22).

To prevent cardiovascular events different treatment options are available, including drug therapy. Initially, at early stages of the disease a lifestyle modification, diet change, smoking cessation, and more physical activity are recommended (23). In more advanced stages medication treatment is provided. At this stage, drug treatment is initiated to prevent hard endpoints, such as stroke, but whether such treatment directly influences the underlying atherosclerotic disease remains unknown. Current guidelines for the management of patients having one or more cardiovascular risk factors recommend the prescription of antihypertensive medication, lipid-lowering medication, and antithrombotic medication (24). Although, these medical treatments have established their beneficial effect on preventing CVD events and lower the overall cardiovascular risk, their effect on existing carotid atherosclerotic plaque remains unknown.

Beyond that, although the drug treatment provides beneficial effects, understanding the mechanisms on how they interplay with atherosclerotic plaque characteristics and constituent components, may provide extensive understanding on pathophysiology of these processes. Whether medical treatment trigger molecular transitions into the carotid plaque and this way plaque modifications remains unknown. Understanding such pathways may further help to improve medical treatment strategies which would later be translated to reduce the CVD event rates and would be an important element to improve the prevention of ischemic events.

1.4 Outline of this thesis

In ***Chapter 2,*** the capacity of magnetic resonance imaging (MRI) to quantify carotid artery calcification is assessed and compared to the golden standard for calcification assessment, i.e. computed tomography.

Circulating hormones play an important role in cardiometabolic health and are strongly linked to cardiovascular disease (21, 25, 26). The third chapter focuses on the impact of circulating hormones on carotid plaque composition. ***Chapter 3.1*** demonstrates the role of the serum insulin and glucose on carotid plaque composition, whereas ***Chapter 3.2*** highlights the role of endogenous estradiol

and testosterone levels in carotid plaque composition and the risk of stroke in subjects with carotid atherosclerosis.

The fourth chapter is focused on the role of medical treatment used for prevention of cardiovascular events. The goal of ***Chapter 4.1*** was to elucidate associations of lipid-lowering medication (statins) with composition of the plaque. ***Chapter 4.2*** assessed the effect of antithrombotic treatment on carotid plaque composition.

Finally, in ***Chapter 5,*** the main findings of the studies included in this thesis are summarized and put into clinical context. Finally, directions for future research are given.

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Chapter 2

Comparison of CT and MRI detection and quantification of carotid artery calcification

Chapter 2

Comparison of CT and MRI for the detection and quantification of carotid artery calcification and their comparative association with the history of stroke

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Abstract

Background

Carotid artery atherosclerosis is an important risk factor for stroke. As such, quantitative imaging of carotid artery calcification, as a proxy of atherosclerosis, has become a cornerstone of current stroke research. Yet, population-based data comparing the main imaging modalities (computed tomography and magnetic resonance imaging) for the detection and quantification of calcification remain scarce.

Methods

A total of 684 participants from the population-based Rotterdam Study underwent both a CT-examination and an MRI-examination of the carotid artery bifurcation to quantify the amount of carotid artery calcification (mean interscan interval: 4.9 ± 1.2 years). We investigated the correlation between the amount of calcification measured on CT and an MRI using Spearman's correlation coefficient, Bland-Altman plots, and linear regression. In addition, using logistic regression modeling, we assessed the association of CT- and MRI-based calcification volumes with a history of stroke.

Results

We found a strong correlation between CT- and MRI-based calcification volumes (Spearman's correlation coefficient:0.86, p-value ≤0.01). Bland-Altman analyses

showed a good agreement, though CT-based calcification volumes were systematically larger. Finally, calcification volume assessed with either imaging modality was associated with a history of stroke with similar effect estimates (odds ratio (OR) per 1-SD increase in calcification volume: 1.52 (95%CI:1.00;2.30) for CT, and 1.47 (95%CI:1.01;2.14) for MRI.

Conclusion

CT-based and MRI-based volumes of carotid artery calcification are highly correlated, but MRI-based calcification is systematically smaller than those obtained with CT. Despite this difference, both provide comparable information with regard to a history of stroke.

BACKGROUND

Atherosclerosis located at the bifurcation of the carotid artery is an important risk factor for stroke (1-5). As such, quantification of the severity of carotid atherosclerosis has become an increasingly important topic in stroke research. Multiple non-invasive imaging techniques, including ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI), are currently available to obtain measures of the extent of atherosclerosis (6). An important advantage of CT and MRI is that both modalities offer possibilities for detailed characterization and quantification of the atherosclerotic plaque (7). The mostly studied characteristic of the atherosclerotic plaque is calcification, given that it is one of the most prominent plaque characteristics and represents a reliable marker of the underlying plaque burden (8). For the visualization of calcification, non-contrast CT is acknowledged to be superior to any other imaging modality (9). Yet, thanks to rapid technological advances, non-contrast MRI now also allows for the detection and quantification of calcification in the atherosclerotic plaque (10) and has the major advantage over CT that it does not involve radiation exposure. Moreover, with MRI it is possible to visualize additional plaque characteristics such as intraplaque hemorrhage or lipid-rich necrotic core which provide unique additional information on the disease. Despite these potential advantages of MRI, it remains unclear whether calcification volumes obtained with MRI are comparable to those measured with CT. Against this

background, we set out to quantify and compare CT-based and MRI-based carotid artery calcification in terms of absolute volumes and with respect to the history of stroke as a relevant clinical outcome, in participants from the population-based Rotterdam Study.

MATERIAL AND METHODS

Setting

This study was carried out within the framework of the Rotterdam Study, a prospective population-based study among middle-aged and elderly persons (11). Between 2003 and 2006, all participants that visited the research center were invited to undergo multi-detector computed tomography (MDCT) to quantify vascular calcification in multiple vessels, including the carotid artery bifurcation (12). In total 2,524 participants were scanned.

From October 2007 onwards, carotid MRI was incorporated in the Rotterdam Study. Between 2007 and 2012, we invited 2,666 participants to undergo an MRI examination of the carotid arteries to study atherosclerotic disease. These participants were selected on the basis of the presence of atherosclerosis in at least one carotid artery on ultrasound examination (defined as intima-media thickness >2.0 mm in one or both carotid arteries), which is regularly performed in all Rotterdam Study participants. In total 1,982 participants underwent carotid MRI. From these 1,982, 808 participants had also undergone a CT-examination. Due to

image artifacts or low image quality (n=31, or errors in the MRI registration process needed for analysis (n=93) 124 participants were excluded, leaving 684 participants with usable CT and MRI data for the current study. The mean time interval between CT scan and MRI scan was 4.9 years (standard deviation 1.2 years).

Assessment of CT-based calcification

We performed a non-enhanced CT-examination (16-or 64-slice MDCT Somatom Sensation, Siemens, Forchheim, Germany) that reached from the aortic arch to the intracranial vasculature, to visualize calcification in the extracranial carotid arteries. The detailed information regarding the scan protocol is described elsewhere (12). In short, the following scan parameters were used: 16 x 0.75 mm collimation, 120 kVp, 100 effective mAs, and 0.5 s rotation time, with a normalized pitch of 1. Images were reconstructed with an effective slice width of 1 mm, a reconstruction interval of 0.5 mm, and a medium sharp convolution kernel (12). Calcification in the extra-cranial carotid artery was measured bilaterally within three centimeters proximal and distal of the bifurcation and was automatically quantified with dedicated commercially available software (syngo calcium scoring, Siemens, Germany) (12). Calcification volumes in both carotid arteries were expressed in cubic millimeters (mm^3) (13) (Figure 1).

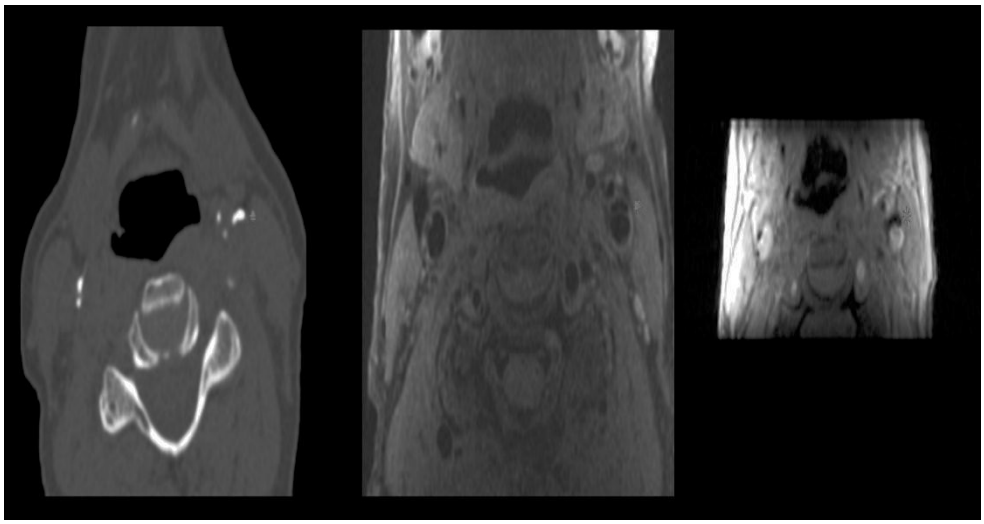

Figure 1 Example of calcification in the left carotid artery bifurcation (indicated by the red star) on CT (left image) and on MRI (middle image; PDw-FSE-BB sequence, and right image; magnitude image of the 3D-phase contrast sequence).

Assessment of MRI-based calcification

MRI imaging of the carotid arteries was performed on a single 1.5-T scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phased-array surface coil (Machnet, Eelde, The Netherlands). The high-resolution images were obtained using a standardized protocol (14). First, both carotids were identified by means of two-dimensional (2D) time-of-flight MR angiography. Second, high-resolution MRI sequences were planned to image the carotid bifurcations on both sides. These sequences consisted of four 2D sequences in the axial plane, namely a proton density weighted (PDw)-fast spin echo (FSE)-black blood (BB) sequence (in-plane resolution 130/160 x 130/128 = 0.8 x 1 cm); a PDw-FSE-BB with an increased in-plane resolution

(in-plane resolution 130/224 x 130/160 = 0.5 x 0.8 cm); a PDw-echo planar image (EPI) sequence (in-plane resolution 130/160 x 70/160 = 0.8 x 0.4 cm); and a T2w-EPI sequence (in-plane resolution 130/160 x 70/160 = 0.8 x 0.4 cm). Additionally, we performed two 3D sequences, namely a 3D-T1w-gradient echo (GRE) sequence (in-plane resolution 180/192 x 180/180 = 0.9 x 1 cm), and a 3D phased-contrast MR angiography (in-plane resolution 180/256 x 180/128 = 0.7 x 1.4 cm) (supplementary table 3). The total scanning time was approximately 30 min (14). Calcification was evaluated bilaterally within three centimeters proximal and distal of the bifurcation (12). All calcification measurements on MRI were performed by one trained physician under the supervision of an experienced neuroradiologist. We performed an intra- and inter-observer reproducibility analysis on a random set of 30 MRI examinations. The intra- and inter-agreement was very good [Cohens' Kappa: 0.91 (95% CI 0.82-0.99) and 0.94 (95% CI 0.86-0.99)], respectively. We defined calcification as a hypointense region in the plaque on all sequences. We manually annotated and segmented calcification in all plaques using a standardized approach. First, we pre-processed all images using a method that has been described extensively before (15). This starts with a bias correction to reduce the intensity inhomogeneity characteristic in MRI (15). Subsequently, the carotid artery in all images was rigidly registered to the black-blood image space using the Elastix tool (15). For the registration of the sequences, a Region of Interest (ROI) around the artery in black blood was used. This

ROI was obtained semi-automatically by uniformly growing an extracted carotid artery centerline, which requires three marked seed points at the common, internal and external parts of the artery (15). Then calcification was manually delineated in every consecutive slice using an annotation tool developed in Mevislab (MeVisLab, MeVis Medical Solutions AG). Fourth, the total volume of calcification was calculated by counting the number of voxels within the annotated areas and multiplying this by the voxel volume (Figure 1). This provided volumes of calcification in cubic millimeters.

Assessment of history of stroke

At study entry, all participants were interviewed, and a history of stroke was assessed. Moreover, after enrollment, all participants are continuously followed for the occurrence of stroke (16). All potential stroke events were reviewed by research physicians and verified by an experienced stroke neurologist (17). At the time of CT scan, 38 participants had suffered a prior stroke (16).

Statistical analysis

Due to skewed distributions of the calcification data, we used natural log (Ln) transformed values after we added 1.0 mm^3 to the non-transformed data in order to deal with calcification scores of zero (Ln (calcification volume +1.0 mm^3)) (16). Our analysis strategy consisted of four steps. First, we investigated the correlation of CT-based calcification volumes with MRI-based calcification volumes using Spearman's

correlation coefficient. Second, we used linear regression to assess the relation between CT-based and MRI-based calcification volumes while adjusting for the time interval between the scans. Given the substantial time interval between the CT and MRI examinations, we furthermore performed a sensitivity analysis in which we analyzed the correlation between CT-based and MRI-based calcification volumes only for those persons with an interval equal or less than 3 years (n = 128). We performed post-hoc sensitivity analysis while adjusting for CT-scanner type also.

Third, we assessed the agreement between CT-based and MRI-based calcification volumes using a Bland-Altman analysis. Fourth, as a proof-of-principle, we investigated the association of CT-based and MRI-based calcification volumes (per 1-SD increase) related with a history of stroke using logistic regression while adjusting for age, sex and the time interval between CT and MRI, and studied whether the results were comparable for both modalities All analyses were carried out using IBM SPSS Statistics version 21 (International Business Machines Corporation, Armonk, New York).

RESULTS

Table 1 shows the baseline characteristics of the study population. The mean age of participants at the time of CT examination was 68.1 years (SD: 6.1 years). There were 41.5% female participants. We found no calcification in 60 participants (8.8%). There were no instances in which calcification was found on either CT or MRI and not on

the other modality. The mean Ln-transformed calcification volume for CT was 3.98 mm^3 (SD: 1.86 mm^3), and 2.70 mm^3 (SD: 1.36 mm^3) for an MRI.

Table 1 Baseline characteristics of study participants

Sample size	684
Woman	41.5%
Age, years at CT scan	68.8±6.1
Age, years at MRI scan	74.2±6.1
CT calcification volumes, mm^{3*}	3.98±1.87*
MRI calcification volumes, mm^{3*}	2.70±1.37*
Smoking (current)	40.2%
Systolic blood pressure (mm/Hg)	146.81±19.46
Diastolic blood pressure (mm/Hg)	79.84±10.85
Diabetes Mellitus	13.3%
Serum total cholesterol (mmol/L)	5.6±0.9
HDL cholesterol (mmol/L)	1.4±0.3
Antihypertensive medication use	37.7%
Statin medication use	31.1%
Stroke events	5.6%

Values are means with standard deviations for continuous variables and percentages for dichotomous or categorical variables.
* Ln-transformed volumes (Ln (calcification volume+1mm^3)).
Abbreviation: CT = computed tomography, HDL = high-density lipoprotein, MRI = magnetic resonance imaging.

We found a strong correlation between CT and MRI calcification volumes (Spearman's correlation coefficient:0.86) (Figure 2, supplementary table 1, and supplementary table 2). This correlation was similar when we investigated the left

and right side separately (supplementary table 1). After performing linear regression with adjustment for the time interval between the CT and MRI scan, the prominent relation between CT-based and MRI-based calcification volumes remained present [beta per 1-SD increase in CT-based calcification volume: 0.65 (95% confidence interval (CI): 0.63–0.68)]. After performing the analyses in those persons with a time interval between the scans of less or equal to 3 years, the association between CT-based and MRI-based calcification volumes was similar [beta per 1-SD increase in CT-based calcification volume: 0.65 (95% CI: 0.58–0.72)]. Adjustment for CT-scanner type did not influence the results (data not shown).

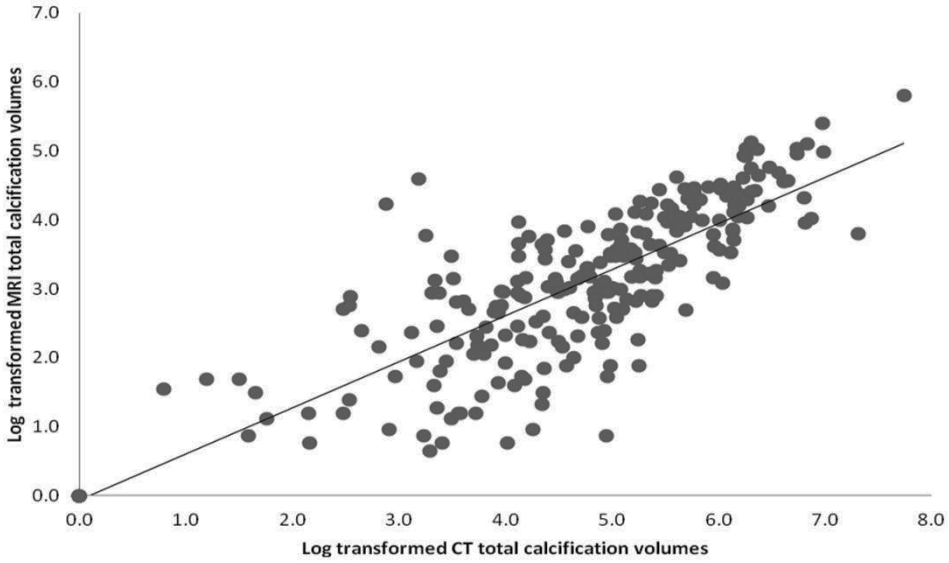

Figure 2 Scatter plot of Ln-transformed CT-based and MRI-based calcification volumes, indicating a positive correlation between both detected and quantified calcification volumes.

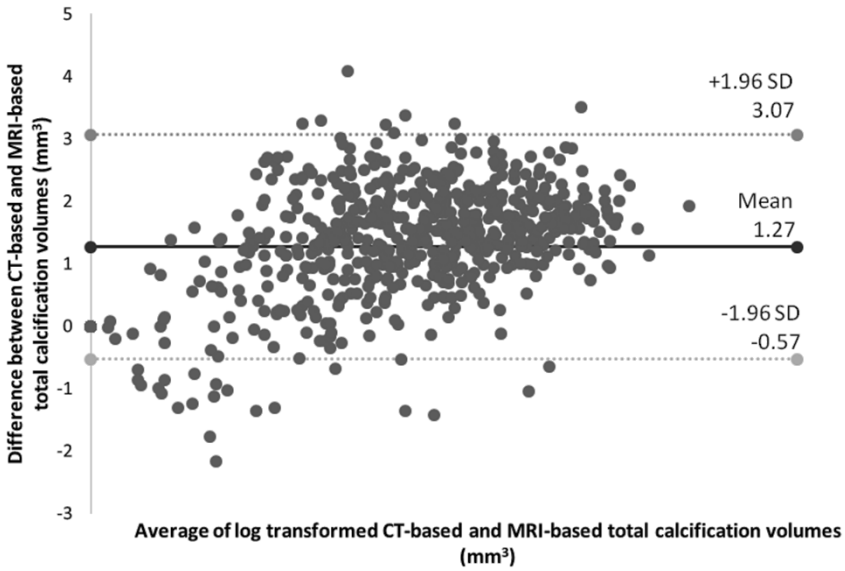

Figure 3 Bland-Altman plot of the difference of CT-based and MRI-based Ln-transformed total calcification volumes, with a mean absolute difference (bold continues line) and 95% confidence interval of mean differences (dashed lines).

Figure 3 shows the Bland-Altman plot for the relation between the absolute differences in Ln-transformed calcification volumes and the mean of the two measurements of 1.27 mm^3 (standard deviation: 0.92). We found that the CT-based calcification volumes were consistently larger than those obtained from MRI.

When investigating the relationship between calcification and a history of stroke, we found that both CT-based and MRI-based calcification volumes were associated with a history of stroke [CT - odds ratio per 1-SD increase: 1.52 (95% CI: 1.00–2.30), MRI – odds ratio per 1-SD increase: 1.47 (95% CI: 1.01–2.14)] (Table 2).

Table 2 Association of calcification volumes with stroke

	Odds ratio (95%CI)	p-value
Model 1		
CT calcification volumes	1.63 (1.09-2.46)	0.01
MRI calcification volumes	1.55 (1.07-2.24)	0.01
Model 2		
CT calcification volumes	1.52 (1.00-2.30)	0.04
MRI calcification volumes	1.47 (1.01-2.14)	0.04

Model 1 - scan time difference. Model 2 –adjusted for age, sex and scan time difference. Values represent odd ratios with 95% CI per 1 standard deviation increase in calcification volumes.
Abbreviation: CT = computed tomography, MRI = magnetic resonance imaging.

DISCUSSION

In this large population-based sample of persons with subclinical atherosclerosis, we found that CT-based and MRI-based volumes of carotid artery calcification are highly correlated, but MRI-based calcification is systematically smaller than those obtained with CT. Despite this difference, both provide comparable information with regard to a history of stroke.

We found that CT-based and MRI-based calcification volumes were highly correlated. Yet, we also found that the volumes measured with MRI were systematically smaller than those measured on CT. This was especially interesting in light of the fact that the MRI was performed on average 4 years later than the CT.

Given that our scanning protocol on CT was specifically designed for the visualization of vascular calcification combined with that CT is currently the gold standard for the assessment calcification, it is likely that with MRI the amount of calcification is systematically underestimated (6). The reason for this could the differences between CT-based and MRI-based calcification volume may be explained by differences in image analysis to a certain extent. Additionally, differences in spatial resolution between CT and MRI might be a potential explanation for this difference. In this light, it is important to note that CT images were analyzed automatically using dedicated commercially available software, whilst MRI images were analyzed manually for the presence and amount of calcification. To our knowledge, there are no studies that have compared CT and MRI on the detection and quantification of carotid artery using a non-invasive population-based approach. Previous research performed on the comparison between CT and MRI in 50 patients with recent TIA or minor stroke, demonstrated a correlation between CT-based and MRI-based calcification volumes of the only *p*: 0.55 (18). We demonstrate that with the use of dedicated MRI-multi-sequences for the detection of calcification the correlation between CT-based and MRI-based calcification volume is substantially improved. Finally, another important topic to consider with regard to the difference between CT and MRI is the blooming effect of calcifications which is known to occur on CT (19). Especially for calcifications with very high Hounsfield units, a gradient over multiple adjacent pixels is necessary

to reach a low Hounsfield unit. This effect may lead to slight overestimation of the calcification area. On the other hand, MRI is known to underestimate the amount of calcification, because a certain amount of calcification is required before the MR-signal disappears. In this context, it is important to acknowledge that possible micro-calcifications in the atherosclerotic plaque may be missed (20).

As a proof of principle, we investigated the association of CT-based and MRI-based calcification with a history of stroke and found that both related to this outcome with comparable effect estimates. We chose history of stroke because the relationship between carotid artery calcification and stroke has been well-established (16, 21) (22). Importantly, despite the fact that MRI systematically underestimates the amount of calcification compared to CT, we found comparable risk estimates for CT-based and MRI-based calcification volumes with respect to a history of stroke. This suggests that when assessing clinical outcomes, the value of MRI-based calcification is similar to that of CT.

Our findings have implications that should be considered in the choice for MRI or CT for the assessment of vascular calcification. First, while assessing atherosclerosis with MRI it is directly possible to visualize other plaque characteristics in addition to calcification, including intra-plaque hemorrhage and lipid-rich necrotic core which provide unique additional information on the disease. Second, MRI has the major advantage over CT that it does not involve harmful radiation exposure. Third, the

systematic underestimation of calcification on MRI may pose a problem, specifically in situations where one is particularly interested in the exact amount of calcification. Fourth, drawbacks of MRI, in general, are its absolute contraindications (i.e. metal objects in the body), and the fact that MRI is more time-consuming, more expensive and less widely available than CT. Taken together, the pros and cons of both imaging modalities should be carefully considered for all research and clinical applications involving the assessment of vascular calcification.

The strengths of our study include the relatively large sample size of community-dwelling individuals, all with varying degrees of carotid atherosclerosis, and the standardized assessment of calcification volumes on both modalities. Yet, some limitations should also be taken into account of which the first is the time interval between the CT scan and the MRI scan, with a mean interval of 4.9 years. We acknowledge that the interscan interval represents a potential limitation of the current study and that during this interval there may have been slight changes in plaque composition. Yet, we would like to emphasize that in all instances the CT-scan was made before the MRI-scan and that calcification is a plaque component that generally remains present and shows only very slow progression over time (23, 24). Therefore, it seems unlikely that the amount of calcification at the time of MRI would differ substantially from that at the time of the CT. This is further supported by the fact that adjustment for the time interval did not change the results; and

secondly by our finding that MRI volumes were consistently estimated somewhat smaller than CT volumes, whereas a large influence of the time interval would induce an opposite difference. Another potential limitation is that we used two types of MDCT scanners (16-slice and 64-slice) to assess calcification. Yet, adjustment for scanner-type did not change the association.

CONCLUSION

In summary, CT-based and MRI-based volumes of carotid artery calcification are highly correlated, but MRI-based calcification is systematically smaller than those obtained with CT. Despite this difference, both provide comparable information with regard to a history of stroke.

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Supplementary material

Supplementary Table 1 Relation between calcification volume on CT and MRI

	MRI left carotid	MRI right carotid	MRI total volumes
CT left carotid	0.77		
CT right carotid		0.78	
CT total volumes			0.86

Correlation is significant at the 0.01 level (2-tailed).
Abbreviation: CT = computed tomography, MRI = magnetic resonance imaging.

Supplementary Table 2 Relation between calcification volume on CT and MRI, between the subjects with <3 years and >3 years difference on CT and MRI scans.

	MRI <3 years n=128	MRI >3 n=556	MRI total volumes n=684
CT <3 years	0.79		
CT >3 years		0.87	
CT total volumes			0.86

Correlation is significant at the 0.01 level (2-tailed).
Abbreviation: CT = computed tomography, MRI = magnetic resonance imaging.

Supplementary table 3 Parameters of the MRI Protocol

	2-D				3-D*	
	FSE-BB		EPI		PC-MRA	GRE
	PDw		PDw	T2w		T1w
	Thin slice	High Resolution				
TE, (ms)	9.8	12.7	24.3	60	4.3	1.8
TR, (ms)	4800	2000	12000	12000	13	15.7
ETL	6	4	-	-	-	-
Field of View, (cm)	13x13	13x13	13x7	13x7	18x18	18x18
Matrix	160x128	224x160	160x160	160x160	256x128	192x180
Slice thickness, (mm)	0·9	1·2	1·2	1·2	1·0/0·5†	1·0/0·5†
No. of slices	51	19	41	41	26/52	124/248
NEX	2	3	20	25	1	1
Scan time, (min.sec)	3·36	4·04	4·00	5·00	6·13	6·02

FSE-BB indicates Fast Spin Echo Black Blood; EPI, Echo Planar Imaging; PC-MRA, Phased-Contrast Magnetic Resonance Angiography; GRE, Gradient Recalled Echo; PD, proton density; TR, repetition time; TE, echo time; ETL, echo train length; NEX, No. of excitations; 2-D, two-dimensional; 3-D, three-dimensional.

* = Axial images are reconstructed from the 3-D volume

† = Images are interpolated from 1·0 mm to 0·5 mm

Chapter 3

Role of the hormones on carotid plaque composition

Chapter 3.1

Serum insulin levels are associated with vulnerable plaque components in the carotid artery

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Submitted/Revision

ABSTRACT AND KEYWORDS

Background

To investigate the association between fasting serum insulin and glucose levels with atherosclerotic plaque composition in the carotid artery. Impaired insulin and glucose levels are implicated in the etiology of cardiovascular disease, however, their influence on the formation and composition of atherosclerotic plaque remains unclear.

Methods

In 1740 participants (mean age 72.9 years, 46% women, 14.4 % diabetes mellitus) from the population-based Rotterdam Study, we performed carotid MRI to evaluate the presence of calcification, lipid core, and intraplaque hemorrhage in carotid atherosclerosis. All participants also underwent blood sampling to obtain information on serum insulin and glucose levels. Using logistic regression models, we assessed the association of serum insulin and glucose levels (per standard deviation (SD) and in tertiles) with the different plaque components, while adjusting for sex, age, intima-media thickness, and cardiovascular risk factors. **Results**

High serum insulin levels were associated with the presence of intraplaque hemorrhage [adjusted odds ratio (OR): 1.32 (95% confidence interval (CI) 1.01–1.75)] and with a lower frequency of lipid core [adjusted OR: 0.69 (95%CI: 0.54–0.88)]. We found no association with the presence of calcification. Sensitivity analyses restricted

to individuals without diabetes mellitus yielded similar results. No associations were found between serum glucose levels and any of the plaque components.

Conclusions

High serum insulin levels are associated with the presence of intraplaque hemorrhage, and with a lower frequency of lipid core in carotid atherosclerosis. These findings suggest a complex role for serum insulin in the pathophysiology of carotid atherosclerosis and in plaque vulnerability.

INTRODUCTION

Dysregulations in insulin and glucose metabolism, the pathophysiological underpinnings of diabetes mellitus, are associated with an increased risk of cardiovascular disease due to the accelerated accumulation of atherosclerosis (1, 2). Despite abundant evidence for a role of diabetes in the pathophysiology of atherosclerosis and clinical cardiovascular events, insights into the contribution of early disruptions in serum levels of insulin and glucose on the development of atherosclerosis remain scarce. Moreover, levels of serum insulin and their atherogenic properties are even conflicting (3-5).

Another important topic of interest within the field of atherosclerosis, for which the role of serum levels of insulin and glucose are even more elusive, pertains to plaque composition. Plaque composition is directly related to the chances of a plaque to rupture and potentially result in clinical cardiovascular events (6-9). The vulnerability of a plaque to rupture is assessed by evaluation of the presence of vulnerable, non-calcified plaque components such as lipid core or intraplaque hemorrhage (9), and the presence of calcification, which is regarded as a more plaque-stabilizing component (3-5). In-vivo visualization of the atherosclerotic plaque and its components can be non-invasively accomplished by magnetic resonance imaging (MRI) (10).

Against this background, we investigated the association between insulin and glucose levels with atherosclerotic plaque composition in the carotid artery in a large population-based cohort of subjects with subclinical atherosclerosis.

METHODS

Study population

The Rotterdam Study is a prospective population-based cohort (11). Between 2007 and 2012 participants with carotid atherosclerosis were invited to undergo an MRI scan of the carotid arteries. Participants were selected for MRI based on the results of carotid artery ultrasound examination (intima-media thickness ≥2.5 mm in one or both carotid arteries) performed in all participants of the Rotterdam Study. From the 2666 invited participants, 272 refused to participate, and another 363 did not undergo MRI scan due to claustrophobia (n=57), physical limitations (n=191), and MRI contraindication (n=115). From the remaining 1982 participants that underwent MRI scan, 242 were excluded due to bad image quality (n=95), the absence of plaque (n=41), or incomplete examinations due to claustrophobia during scanning (n=106). Hence, 1740 participants were included in the analyses. The Rotterdam Study complies with the Helsinki Declaration and has been approved by the Medical Ethics Committee of the Erasmus MC and by the Dutch Ministry of Health, Welfare and Sports, implementing the "Wet Bevolkings Onderzoek: ERGO (Population Screening

Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Carotid scanning and analysis of plaque components

A magnetic resonance 1.5 Tesla scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phased-array surface coil (Machnet, Eelde, the Netherlands) was used to perform bilateral multisequence imaging of the carotid arteries, with a standardized scanning protocol, that required an approximate total scanning time of 30 minutes. Details of the scanning protocol, reading procedure, and reproducibility is described in detail elsewhere (12, 13). Two independent readers, with three years of experience visually evaluated the carotid artery images for the presence of three plaque components, namely intraplaque hemorrhage (IPH), lipid core, and calcification. IPH was defined as the presence of a hyperintense region in the atherosclerotic plaque on 3D-T1w-GRE. Lipid core presence was defined as a hypointense region, not classified as IPH or calcification, in the plaque on PDw-FSE or PDw-EPI and T2w-EPI images or a region of relative signal intensity drop in the T2w-EPI images compared with the PDw-EPI images. Calcification was defined as the presence of a hypointense region in the plaque on all sequences (14). Subjects were recorded as positive for the presence of any plaque component if the component was identified in one or both carotid arteries. To assess the intra-scan variability, 40 participants underwent a second MRI scan (average time between scans 15 ± 9 days).

For interobserver reproducibility analyses, random MRI examinations were selected (*n* = 50) and read by a second observer. Intra-scan and interobserver agreement were calculated by using Cohens' Kappa statistics. The intra-scan agreement was good for all measurements. The Kappa value for the presence of IPH was 0.95 (95% CI 0.88–0.99); for lipid core 0.85 (95% CI 0.74–0.96) and for calcification 0.91 (95% CI 0.82–0.99). The interobserver agreement was good for all measurements. The Kappa value for IPH was 0.86 (95% CI 0.72–0.99); for lipid core 0.86 (95% CI 0.72–0.99) and for calcification 0.94 (95% CI 0.86–0.99) (13).

Assessment of fasting insulin and glucose levels

The venous blood samples were taken, after overnight fasting from all participants at the research center and stored at −80°C in a number of 5-mL aliquots. Serum fasting glucose levels were determined by using the glucose hexokinase method within 1 week after sampling (15). Serum fasting insulin level was determined in samples that had been kept frozen and were measured on a Roche Modular Analytics E170 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) by electrochemiluminescence immunoassay technology. This assay does not cross-react with proinsulin or C-peptide. The intraassay repeatability showed a coefficient of variation of 1.0%. The day-to-day variation of the assay (i.e., intermediate precision) yielded a coefficient of variation of 3.6%. These numbers indicate the excellent

reliability of the insulin assay in our study (16). The blood measurements were made for all participants at study entry and the mean time interval between blood measurements and carotid MRI scan was 7.9 years (standard deviation of 4.0 years).

Other risk factors in the Rotterdam Study

The information about other cardiovascular risk factors as relevant covariables was obtained by interview, physical examination, and blood sampling between the years 1998 and 2008 (11). Diabetes mellitus was defined as fasting blood glucose >6.9 mmol/L, nonfasting glucose >11.0 mmol/L, or use of glucose-lowering medication. Systolic and diastolic blood pressure was measured using a random-zero sphygmomanometer on the right arm and two measurements were averaged for the analysis. Smoking status was assessed by interview and categorized into never, past, and current smoking. Body mass index (BMI) was calculated based on the weight in kilograms divided by height in meters squared. Total cholesterol and high-density lipoproteins (HDL) levels were measured using standard laboratory techniques. The information on the use of antihypertensive medication and lipid-lowering medication was obtained from pharmacy records (11). History of stroke or coronary heart disease (CHD) was self-reported at study entry and verified by clinical data from the medical records or the occurrence during study follow-up but before the time of carotid MRI scanning.

Statistical analysis

The distribution of continuous and categorical variables was described using means (standard deviations [SD]), medians (interquartile ranges [IQRs]), or percentages. We performed a natural logarithmic transformation to normalize the distributions of serum insulin and glucose. To investigate the association between fasting insulin and glucose levels with intraplaque hemorrhage (IPH), lipid core, and calcification, a three-step statistical analysis approach was used. First, we investigated the association between fasting insulin and glucose levels with the presence of each component in one or both carotid arteries using logistic regression models. In model 1, adjusted for sex, age, intima-media thickness and the time difference between insulin and glucose measurements and MRI scan. In model 2, additionally adjusted for smoking, serum high-density lipoprotein, serum total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication, and insulin or glucose levels, dependent on the determinant under investigation. In model 3, additionally adjusted for the use of lipid-lowering medication (13), vitamin K antagonists and antiplatelet agents (17). Second, we categorized serum insulin and glucose levels into tertiles and investigated the association of tertiles of insulin and glucose (lowest tertile was used as the reference category) with carotid plaque components using regression model 1. Third, we performed the following three sensitivity analyses. In the first analysis, we investigated all above-mentioned

associations only in participants that had their MRI-scan and blood measurements within one year in order to assess the potential effect of the time delay between the measurements. In the second analysis, we reassessed all associations in participants that were free of diabetes mellitus at the time of the MRI. In the third analysis, we stratified all analyses for sex to investigate whether associations are different between males and females. Additionally, we investigated the association between serum insulin and glucose and intima-media thickness using regression models. All analyses were carried out using IBM SPSS Statistical package version 21 (Chicago, IL, USA).

RESULTS

Table 1 shows the population characteristics at the MRI scan. The mean age of the population was 72.9 years (9.1 years) and 46.0 percent were women. A total of 251 (14.4%) participants were diagnosed with diabetes mellitus at baseline. The median (IQR) fasting insulin level was 74 (50–98) pmol/L and the median (IQR) fasting glucose level was 5.6 (5.2–6.0) mmol/L.

Associations between fasting insulin and glucose levels with the different plaque components are summarized in Table 2. We found that higher fasting insulin levels were associated with the presence of intraplaque hemorrhage (fully adjusted odds ratio (OR) per 1-SD increase in insulin level: 1.39 [95% confidence interval (CI): 1.09–1.76]) (Table 2, model 2). Further adjustment for antithrombotic treatment increased

the estimate and empowered the association (OR per 1-SD increase: 1.43 [95% confidence interval (CI): 1.14–1.81]) (Table 2, model 3). Higher fasting insulin levels also related to a lower frequency of lipid core (fully adjusted OR per 1-SD increase in insulin level: 0.88 [95% CI: 0.72–1.09]) (Table 2, model 3). We found no association between fasting glucose levels with any of the plaque components.

When investigating tertiles of insulin and glucose levels, we found that the high insulin level tertile was associated with a higher frequency of intraplaque hemorrhage (adjusted OR of highest versus lowest tertile: 1.32 [95% CI: 1.01–1.75]) and a lower frequency of lipid core (adjusted OR of highest versus lowest tertile:

Table 1 Baseline characteristics of the study population (n=1740)

Characteristics	*Insulin ≤57*	*Insulin 58–89*	*Insulin >90*	*P-value*	*All*
Number in category	587	615	538		1740
Age, years (SD)	74.0±8.8	73.4±9.1	71.1±9.4	<0.001	72.9±9.1
Women, %	48.9	47.2	41.4	0.03	46.0
Smoking, current %	43.3	41.1	42.9	0.41	42.4
Diabetes mellitus, %	8.2	11.4	24.7	<0.001	14.4
Fasting glucose, mmol/L (SD)	5.3 (5.0–5.7)	5.6 (5.2–5.9)	5.9 (5.4–6.5)	<0.001	5.6 (5.2–6.0)
Fasting insulin, pmol/L	44 (35–51)	75 (66–85)	121(100–165)	<0.001	74(50–98)
Systolic blood pressure, mm/Hg (SD)	144±20	147±20	144±20	0.02	145±20
Diastolic blood pressure, mm/Hg (SD)	79±10	81±10	81±11	0.001	80±10
BMI, kg/m^2 (SD)	25.7±3.0	27.1±3.1	29.2±3.7	<0.001	27±3.5
Total cholesterol, mmol/L (SD)	5.6±1.0	5.7±1.0	5.5±1.0	0.004	5.6±1.0
HDL cholesterol, mmol/L (SD)	1.5±0.3	1.4±0.3	1.2±0.3	<0.001	1.4±0.3
Antihypertensive medication, %	32.0	36.6	50.4	<0.001	39.3
Statin use, %	25.6	28.6	33.3	0.02	29.0
Vitamin K antagonists, %	5.8	5.7	5.2	0.90	5.6
Antiplatelet agents; %	27.6	26.5	28.4	0.76	27.5
Intima-media thickness, mm	3.2±0.6	3.2±0.6	3.2±0.7	0.77	3.2±0.6
Degree of stenosis, (%)	12.3 (0.0–25.9)	14.5 (0.0–26.4)	15.2 (0.0–28.0)	0.16	14.5 (0.0–26.8)
History of stroke, %	4.6	7.2	6.5	0.02	6.3
History of coronary heart disease, %	12.8	10.1	11.5	0.01	11.4
Presence of calcification, %	85.7	80.2	81.0	0.02	82.3
Presence of lipid core, %	47.2	45.5	38.8	0.01	44.0
Presence of intraplaque hemorrhage, %	32.5	35.9	35.5	0.41	34.7

Values are means with standard deviations and median (interquartile ranges) for continuous variables and percentages for dichotomous or categorical variables. P-values were derived by Fisher's exact test or analysis of variance (ANOVA).

Table 2 Association serum insulin and glucose levels with carotid artery plaque composition (n=1740)

Insulin	***IPH OR (95%CI)***	***Lipid core OR (95%CI)***	***Calcification OR (95%CI)***
Model 1	1.27 (1.05–1.55)	0.76 (0.63–0.90)	0.93 (0.74–1.16)
Model 2*	1.39 (1.09–1.76)	0.88 (0.72–1.09)	1.05 (0.80–1.38)
Model 3	1.43 (1.14–1.81)	0.88 (0.72–1.09)	1.05 (0.80–1.38)
Glucose			
Model 1	1.04 (0.55–1.96)	0.51 (0.29–0.91)	1.19 (0.58–2.47)
Model 2†	0.49 (0.20–1.21)	1.08 (0.49–2.37)	1.22 (0.44–3.34)
Model 3	0.48 (0.19–1.19)	1.10 (0.50–2.41)	1.18 (0.43–3.24)

Odds ratio (OR), given with a 95% confidence interval (CI), express the relationship between serum insulin and glucose (per SD increment) with intraplaque hemorrhage (IPH), lipid core and calcification. Model 1 = adjusted for sex, age, intima-media thickness and the time difference between insulin and glucose measurements and MRI scan. Model 2 = model 1 + smoking, high-density lipoprotein, total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication and *glucose or †insulin levels. Model 3 = model 2 + use of lipid-lowering medication, vitamin K antagonists and antiplatelet agents.

0.69 [95% CI: 0.54–0.88]) compared to the low tertile (Figure 1). Again, for glucose, we did not find any association with the various plaque components (Figure 2).

When restricting analyses only to participants who had both measurements (insulin and glucose measurements and MRI scan) within one year (n=212), the results were in similar trend (Table S1). Similarly, the results did not change when we excluded participants with a diagnosis of diabetes mellitus at the time of MRI (n=1489) (Table S2). Furthermore, after stratifying the analyses by sex similar effect estimates, between males and females, for the association of serum insulin levels with IPH and

lipid core. Whereas no association was observed for calcification in both sexes. Also, when assessing the relationship between glucose and plaque components no association was observed in either sex (Table S3). Moreover, when assessing the relationship between serum insulin or glucose and carotid intima-media thickness no association was found (Table S4).

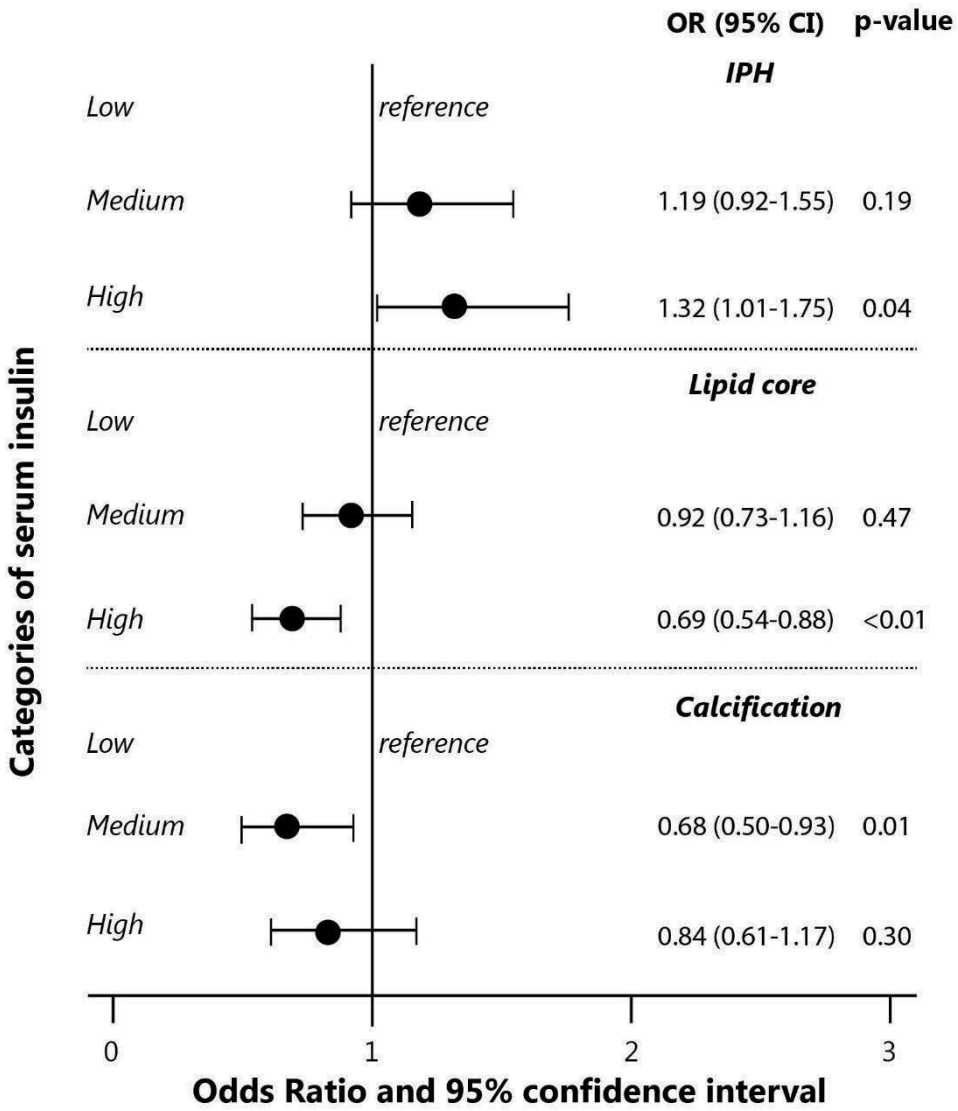

Figure 1 Association of serum insulin levels (tertiles) with plaque composition in the carotid artery. Values on the x-axis represent the odds ratios and 95% confidence interval. The values are adjusted for sex, age, and intima-media thickness. Statistics was performed using logistic regression using total sample of subjects n=1740. *P-trend* over categories of insulin for intraplaque hemorrhage was 0.04, for lipid core was 0.003 and for calcification was 0.32.

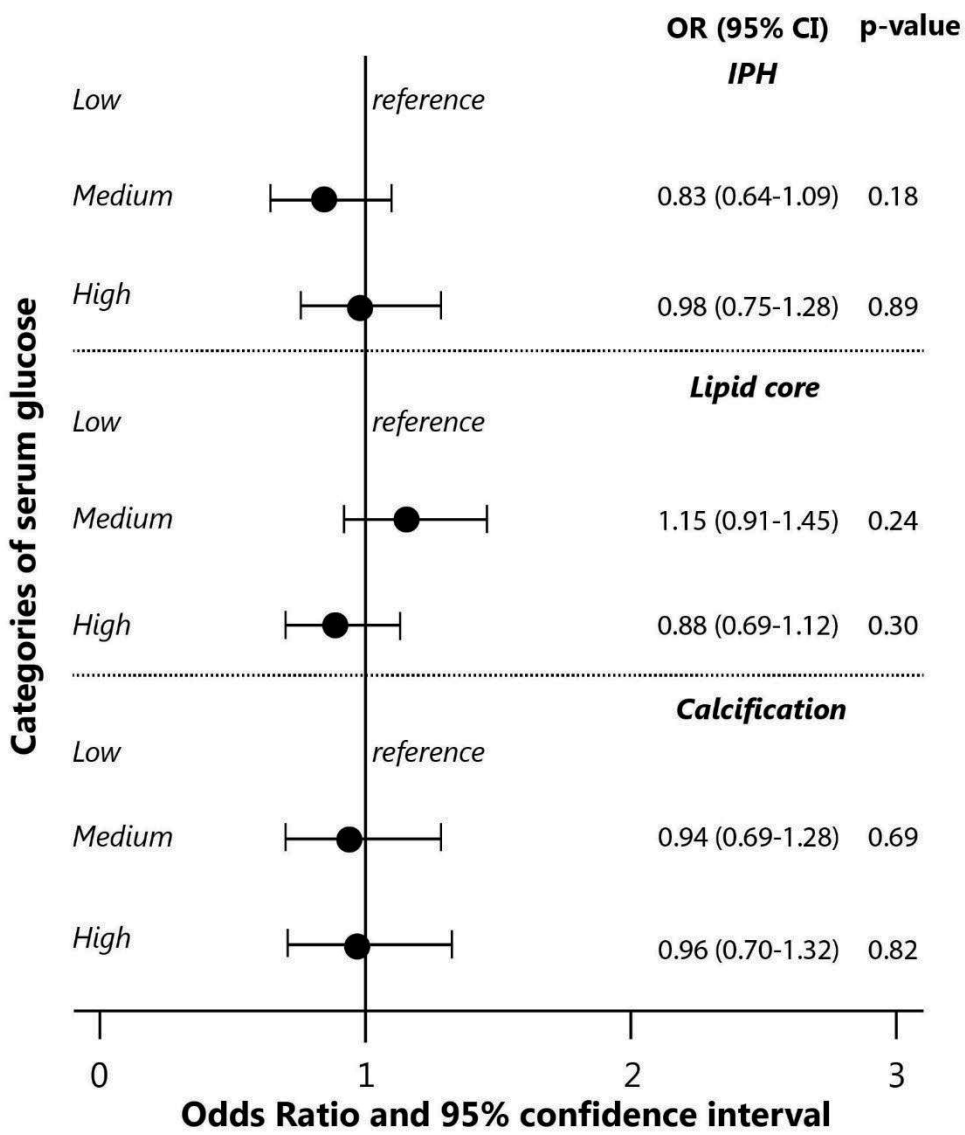

Figure 2 Association of serum glucose levels (tertiles) with plaque composition in the carotid artery. Values on the x-axis represent the odds ratios and 95% confidence interval. The values are adjusted for sex, age, and intima-media thickness. Statistics was performed using logistic regression using total sample of subjects n=1740. *P-trend* over categories of glucose for intraplaque hemorrhage was 0.87, for lipid core was 0.33 and for calcification was 0.81.

DISCUSSION

In this large population-based study of subjects with subclinical carotid atherosclerosis, we observed that higher fasting serum insulin levels were associated with the presence of intraplaque hemorrhage and a lower frequency of lipid core within the carotid atherosclerotic plaque. We did not find an association between fasting glucose levels and any of the carotid plaque components.

Until now, most of the evidence linking insulin and glucose to atherosclerosis comes from studies in which atherosclerotic cardiovascular clinical endpoints, such as ischemic heart disease or ischemic stroke, were investigated (18-20). Hyperinsulinemia was found to increase the risk of ischemic heart disease among 4637 middle-aged men from the Quebec Cardiovascular Study (18), and the risk of acute coronary and cerebrovascular events in 1521 men enrolled in Kuopio Ischemic Heart Disease Risk Factor Study (19). Our results extend on these findings by showing that preclinical changes in serum insulin levels relate to a more vulnerable composition of the carotid atherosclerotic plaque. More specifically, we demonstrated that high serum insulin levels, especially relate to the presence of intraplaque hemorrhage, the plaque component which is regarded as the most vulnerable (21). Similarly, insulin was found to increase intraretinal hemorrhage and extraretinal neovascularization in rats (22). Previous animal studies described insulin to play a pleiotropic effect on the vascular system, through vascular endothelial

growth factor (VEGF), which plays a pivotal role in angiogenesis (22, 23). High levels of insulin increase the levels of VEGF, which in turn induce abnormal neovascularization that is prone to leakage and hemorrhage (22). In addition, interestingly, we also found that high serum insulin levels related to a lower frequency of lipid core, which is generally also considered an indicator of unstable plaque. This finding may potentially be explained by the insulin lowering effect on plasma oxidized LDL/LDL cholesterol ratio (24).

In contrast to high or low serum insulin levels, it may be speculated that physiological concentration levels (median levels 66–85 pmol/L) potentially behave protectively against atherosclerosis. Observations in our study showed that medium levels of serum insulin were not associated with any vulnerable plaque component, but were associated with a lower presence of calcification, which may support the hypothesis that medium levels of serum insulin have the protective effect (Figure 1). In the same line, a recent animal study examined the role of insulin in atherosclerotic plaque reported the protective effect of insulin on atherosclerosis (21). In this study, the insulin effect was tested on atherosclerosis in a mouse model, and insulin was found to decrease the plaque burden and increased plaque stability via nitric oxide synthase (NOS) mechanisms (21). Furthermore, it was found that insulin reduced macrophage accumulation, plaque necrosis, and increased collagen and smooth muscle cell accumulation (21). However, it seems that only disrupted levels of serum insulin, low

and high insulin levels, link serum insulin with atherosclerosis. Previously, animal studies demonstrated also that impaired insulin signaling by genetic modification accelerated atherosclerosis (25-27).

Surprisingly, we found no effects on serum glucose levels in carotid plaque composition. In the context of glucose, our findings do not support previous reports that link higher glucose levels to an increased risk of vascular diseases (28). However, a recent meta-analysis of 102 prospective studies that investigated the relationship between fasting glucose levels and risk of vascular diseases concluded that glucose concentrations were non-linear and modestly associated with the risk of vascular diseases among individuals without diabetes (20), meaning that glucose levels below and higher than 7.0 mmol/L were associated with increased risk for coronary heart disease and ischemic stroke (20).

In terms of clinical practice, our findings may have clinical implications given that these suggest that fasting serum insulin conveys information on the atherosclerotic plaque composition that may ultimately be used for risk stratification of patients in daily practice.

The major strength of our study includes the largest population-based sample of individuals with subclinical carotid atherosclerosis and the MRI-based assessment carotid plaque composition. Given the accurate diabetes assessments within the Rotterdam Study, we were able to address for the first time the association between

subclinical variations of insulin and glucose levels with atherosclerotic disease. Nevertheless, our study should be interpreted in the context of some limitations. First, the cross-sectional study design limits us to draw causal inferences between fasting insulin and atherosclerotic plaque components. Second, in a substantial part of our study population, the time interval between insulin and glucose measurements and MRI scanning was more than 2 years. However, limiting our analyses to the subgroup of participants with available measurements of MRI and serum insulin levels in the same year did show different associations.

CONCLUSION

In conclusion, high serum insulin levels are associated with the presence of intraplaque hemorrhage, and with a lower frequency of lipid core in carotid atherosclerosis, suggesting that serum insulin may play a role in the vulnerability of carotid atherosclerotic plaque. Further studies are required to confirm our findings in a longitudinal design.

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Supplemental material

Table S1 Association serum insulin and glucose levels with carotid artery plaque composition in the ≤ 1-year difference between MRI and insulin measurements (n=212)

Insulin	***IPH OR (95%CI)***	***Lipid core OR (95%CI)***	***Calcification OR (95%CI)***
Model 1	1.50 (0.85–2.64)	0.41 (0.23–0.74)	0.98 (0.55–1.75)
Model 2*	2.02 (0.97–4.19)	0.41 (0.20–0.84)	0.96 (0.45–2.06)
Model 3	2.41 (1.11–5.21)	0.40 (0.20–0.83)	0.97 (0.45–2.11)
Glucose			
Model 1	1.03 (0.20–5.44)	0.20 (0.36–1.08)	1.38 (0.25–7.58)
Model 2†	0.22 (0.20–2.84)	0.49 (0.05–4.88)	0.22 (0.02–2.64)
Model 3	0.16 (0.01–2.43)	0.52 (0.05–5.20)	0.21 (0.02–2.66)

Odds ratio (OR), given with a 95% confidence interval (CI), express the relationship between serum insulin and glucose (per SD increment) with intraplaque hemorrhage (IPH), lipid core and calcification. Model 1 = adjusted for sex, age, intima-media thickness and the time difference between insulin and glucose measurements and MRI scan. Model 2 = model 1 + smoking, high-density lipoprotein, total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication and *glucose or †insulin levels. Model 3 = model 2 + use of lipid-lowering medication, vitamin K antagonists and antiplatelet agents.

Table S2 Association serum insulin and glucose levels with carotid artery plaque composition in individuals free of diabetes mellitus (n=1489)

Insulin	***IPH OR (95%CI)***	***Lipid core OR (95%CI)***	***Calcification OR (95%CI)***
Model 1	1.32 (1.05–1.66)	0.78 (0.64–0.96)	0.98 (0.75–1.29)
Model 2*	1.46 (1.12–1.90)	0.86 (0.68–1.09)	1.13 (0.82–1.56)
Model 3	1.49 (1.14–1.95)	0.87 (0.69–1.11)	1.12 (0.81–1.54)
Glucose			
Model 1	0.71 (0.20–2.57)	0.90 (0.30–2.67)	1.31 (0.32–5.38)
Model 2†	0.40 (0.10–1.54)	1.85 (0.56–6.13)	0.92 (0.20–4.28)
Model 3	0.41 (0.11–1.59)	1.86 (0.56–6.19)	0.94 (0.20–4.41)

Odds ratio (OR), given with a 95% confidence interval (CI), express the relationship between serum insulin and glucose (per SD increment) with intraplaque hemorrhage (IPH), lipid core and calcification. Model 1 = adjusted for sex, age, intima-media thickness and the time difference between insulin and glucose measurements MRI scan. Model 2 = model 1 + smoking, high-density lipoprotein, total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication and *glucose or †insulin levels. Model 3 = model 2 + use of lipid-lowering medication, vitamin K antagonists and antiplatelet agents.

Table S3 Association serum insulin and glucose levels with carotid artery plaque composition stratified by sex

Females (n=800)		***IPH OR (95%CI)***	***Lipid core OR (95%CI)***	***Calcification OR (95%CI)***
Insulin	Model 1	1.16 (0.87–1.57)	0.72 (0.55–0.94)	0.95 (0.68–1.32)
	Model 2	1.25 (0.87–1.79)	0.82 (0.60–1.13)	1.04 (0.70–1.56)
	Model 3*	1.26 (0.88–1.81)	0.81 (0.58–1.12)	1.02 (0.67–1.54)
Glucose	Model 1	1.80 (0.61–5.32)	0.64 (0.24–1.72)	1.15 (0.33–4.08)
	Model 2	1.13 (0.24–5.30)	3.84 (0.92–16.13)	2.27 (0.38–13.69)
	Model 3†	1.11 (0.23–5.28)	4.19 (0.98–17.88)	2.42 (0.39–15.17)
Males (n=940)		***IPH OR (95%CI)***	***Lipid core OR (95%CI)***	***Calcification OR (95%CI)***
Insulin	Model 1	1.35 (1.04–1.74)	0.90 (0.68–1.17)	0.92 (0.67–1.24)
	Model 2	1.52 (1.11–2.06)	0.93 (0.70–1.22)	1.08 (0.75–1.58)
	Model 3*	1.57 (1.15–2.14)	0.93 (0.71–1.24)	1.07 (0.74–1.56)
Glucose	Model 1	0.74 (0.33–1.67)	0.46 (0.22–0.95)	1.18 (0.48–2.85)
	Model 2	0.31 (0.10–0.98)	0.64 (0.24–1.71)	0.85 (0.25–2.88)
	Model 3†	0.30 (0.09–0.96)	0.65 (0.24–1.72)	0.84 (0.25–3.14)

Odds ratio (OR), given with a 95% confidence interval (CI), express the relationship between serum insulin and glucose (per SD increment) with intraplaque hemorrhage (IPH), lipid core and calcification. Model 1 = adjusted for age, intima-media thickness and the time difference between insulin and glucose measurements MRI scan. Model 2 = model 1 + smoking, high-density lipoprotein, total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication and *glucose or †insulin levels. Model 3 = model 2 + use of lipid-lowering medication, vitamin K antagonists and antiplatelet agents.

Table S4 Association serum insulin and glucose levels with intima-media thickness (n=1740)

Insulin	***Beta***	***95% CI***	***p-value***
Model 1	0.009	-0.048–0.066	0.75
Model 2	-0.004	-0.071–0.063	0.91
Model 3*	0.006	-0.061–0.072	0.87
Glucose			
Model 1	0.034	-0.154–0.222	0.72
Model 2	-0.012	-0.266–0.242	0.93
Model 3†	-0.014	-0.267–0.238	0.91

Effect size, given with a 95% confidence interval (CI), express the relationship between serum insulin and glucose (per SD increment) intima-media thickness. Model 1 = adjusted for sex, age and the time difference between insulin and glucose measurements and MRI scan. Model 2 = model 1 + smoking, high-density lipoprotein, total cholesterol, systolic and diastolic blood pressure, body mass index, use of antihypertensive medication and *glucose or †insulin levels. Model 3 = model 2 + use of lipid-lowering medication, vitamin K antagonists and antiplatelet agents.

Chapter 3.2

Associations of endogenous estradiol and testosterone levels with plaque composition and risk of stroke in subjects with carotid atherosclerosis

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ABSTRACT

Rationale: Sex steroids may play a role in plaque composition and in stroke incidence.

Objectives: To study the associations of endogenous estradiol and testosterone with carotid plaque composition in elderly men and postmenopausal women with carotid atherosclerosis, as well as with risk of stroke in this population.

Methods and Results: Data of 1023 postmenopausal women and 1124 men (≥45 years) with carotid atherosclerosis, from prospective population-based Rotterdam Study, were available. At baseline, total estradiol (TE) and total testosterone (TT) were measured. Carotid atherosclerosis was assessed by ultrasound, whereas plaque composition (presence of calcification, lipid core and intraplaque hemorrhage) was assessed by MRI. TE and TT were not associated with calcified carotid plaques in either sex. TE was associated with presence of lipid core in both sexes (in women odds ratio (OR), 95% CI: 1.48 [1.02, 2.15], in men OR, 95% CI: 1.23 (1.03, 1.46]), whereas no association was found between TT and lipid core in either sex. Higher TE (OR, 95% CI: 1.58 [1.03, 2.40]) and lower TT (OR, 95% CI: 0.82 [0.68, 0.98]) were associated with intraplaque hemorrhage in women but not in men. In women, TE was associated with increased risk of stroke (hazard ratio (HR), 95% CI: 1.98 [1.01, 3.88], whereas no association was found in men. TT was not associated with risk of stroke in either sex.

Conclusions: TE was associated with presence of vulnerable carotid plaque as well as increased risk of stroke in women, whereas no consistent associations were found for TT in either sex.

ABBREVIATIONS

BMI= body mass index

DHEA= dehydroepiandrosterone

FAI= free androgen index

HT= hormone therapy

SHBG= sex hormone-binding globulin

TE= total estradiol (17-β Estradiol)

TSH= thyroid stimulating hormone

TT= total testosterone

INTRODUCTION

Ischemic stroke, a major cause of death and long-term disability among men and women, inflicts a considerable economic burden to society (1). Within the etiology of ischemic stroke, atherosclerotic disease of the carotid artery, and particularly plaque composition are important risk factors (2). Carotid atherosclerotic plaque can be composed of various components, such as a lipid pool (with/without necrosis), calcification, and intraplaque hemorrhage (3). Plaques that contain lipid deposits can lead to development of so-called vulnerable plaques in which hemorrhage can develop. This in turn can lead to plaque instability, further progress to rupture, ultimately precipitating embolism (4, 5) and subsequently increase risk of stroke (2). Sex differences have been observed in plaque composition as well as in stroke incidence (6). Stroke incidence is about 30% higher in men than in women (98). In line with this, women have higher rates of stable fibro-calcific atherosclerotic plaques, while plaques found in men tend to be more complex with higher rates of unstable lesions- intraplaque hemorrhage and presence of necrotic lipid core (7). Nevertheless, in women the risk for stroke roughly doubles during the 10 years after menopause (8). These sex- and menopause-differences in plaque composition and risk of stroke might be driven by endogenous sex hormones (9, 10). Experimental evidence suggests a direct action of estradiol on the vascular system, affecting many mechanisms that impact

plaque composition and occurrence of atherothrombotic ischemic stroke, including lipid metabolism, inflammation, oxidative stress, fibrinolysis, and thrombosis (11). Testosterone may slow down atherosclerosis through inhibiting carotid intima-media thickening , atheroma formation (12) and immunomodulation of plaque development and stability (13). To date, no study has investigated the association between circulating estradiol, testosterone and plaque composition in human populations. Also, limited evidence exists on endogenous estradiol, testosterone and risk of stroke, and particularly in high risk populations such as subjects with presence of carotid atherosclerosis who are at increased risk of developing stroke (14).

The aim of our study was to investigate the associations of endogenous estradiol and testosterone with carotid plaque composition in middle-aged and elderly men and postmenopausal women with carotid atherosclerosis, as well as with risk of stroke in this population.

Methods and materials

Study Population

The Rotterdam Study (RS) is a prospective, population-based cohort study among individuals aged ≥ 45 in Ommoord municipality of Rotterdam, The Netherlands. The rationale and design of RS is described in detail elsewhere (15). In brief, all inhabitants of the Ommoord district aged 55 years or older were invited to

participate (n =10,215). At baseline (1990-1993), 7,983 participants were included (RS-I). In 2000, all persons living in the study district who had become 55 years of age (n=3011) were additionally enrolled (RS-II). A second extension of the cohort was initiated in 2006, in which 3,932 participants aged 45 years or older were included (RS-III). Follow-up visits were held every 3-5 years. The Rotterdam Study has been approved by the Medical Ethics Committee according to the Wet Bevolkingsonderzoek: ERGO (Population Study Act: Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of The Netherlands. All participants gave informed consent to participate in the study and to obtain information from treating physicians and pharmacies, separately. The present study used data from the third visit of the first cohort (RSI-3, year 1997-1999) and the baseline examinations of the second (RSII-1, year 2000-2001) and third cohort (RSIII-1, year 2006-2008).

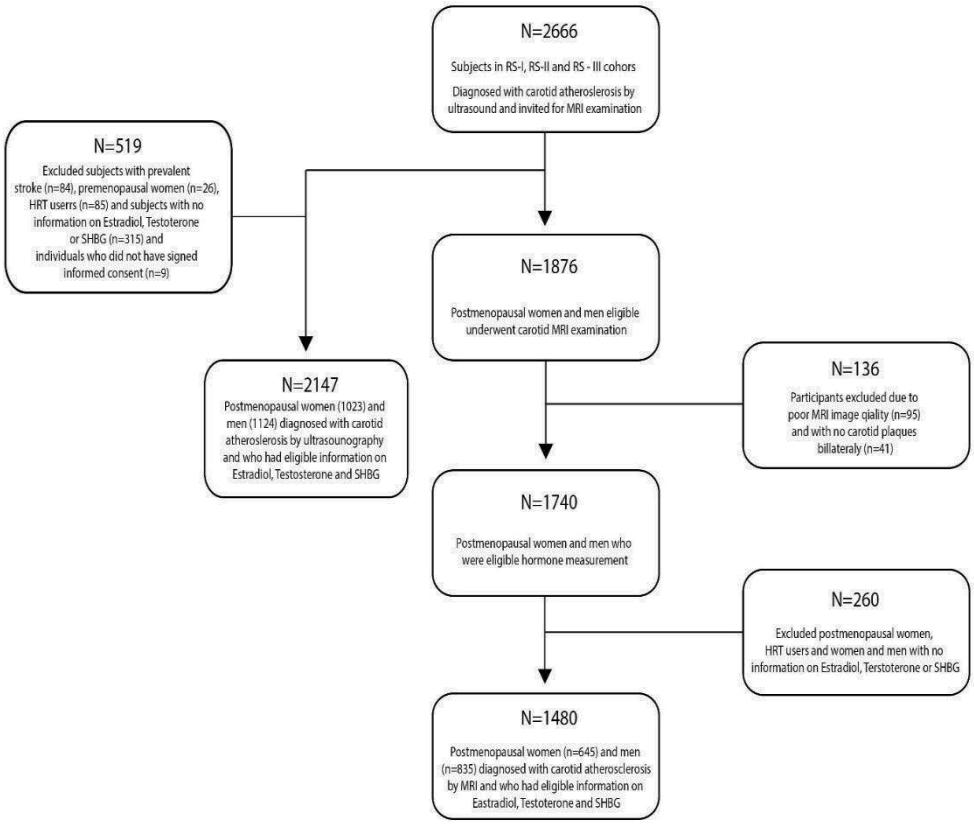
Population for Analyses

Sex steroids and Plaque Composition

All subjects (n=2,666) diagnosed with carotid atherosclerosis by carotid artery ultrasound (intima-media thickness > 2.0 mm in one or both carotid arteries) were invited to magnetic resonance imaging (MRI) of carotid arteries. Subjects were not examined due to various reasons (n=790): MRI contraindications (n=115), physical limitations (n=191), claustrophobia (n=163), refusal to participate (n=272), and loss

to follow-up (n=49). Of the remaining 1876 participants, 95 were excluded due to poor image quality, 41 because of absence of carotid plaque bilaterally, 215 subjects did not have information on TE, TT levels and 45 women previously used HT or did not have information on HT use. Therefore, 645 postmenopausal women and 835 men were included in final analysis for the association of sex steroids with plaque composition and subsequent carotid features (Figure 1).

Figure 1. Flowchart of study participants



Sex Steroids and Stroke Incidence

From 2,666 participants diagnosed with carotid atherosclerosis, 315 subjects were excluded due to missing information on TE and TT, and 84 subjects with prevalent stroke, 85 women who used HT/did not have information on HT use and 26 women who were not postmenopausal, leaving 2,147 subjects, 1,124 men and 1,023 women, for final analysis for the association of sex steroids and stroke incidence (Figure 1).

Information on sex steroid measurement, carotid scanning and analysis of plaque components, stroke assessment and assessment of covariates can be found in Appendix 1.

Statistical analyses

Main Analysis

Continuous variables are reported as mean ± standard deviation (SD) unless stated otherwise and categorical variables were presented as percentages. To achieve normal distribution, skewed variables (TT, SHBG, FAI, triglycerides, glucose, insulin, CRP, and TSH) were natural log transformed. All analyses were stratified by sex. Logistic regression models were used to evaluate whether sex steroids were associated with plaque composition. All sex hormones variables were assessed continuously (per SD increase) in separate models. As the minimum detection limit for TE was 18.35 pmol/liter, and 32.7 percent of women had values of TE lower than

18.35, we analyzed TE as dichotomized variable (18.35 pmol/liter as reference category) in women. Therefore, in our analyses we compared women having undetectable TE levels (18.35 pmol/liter) being the reference group, versus women having detectable TE levels (>18.35 pmol/liter). Undetectable estradiol was not an issue in men since only 4 (0.48%) men participants had value of TE lower than the detection limit, and therefore we analyzed TE as continues variable (presented per 1SD of estradiol increase). For men participants with estradiol levels lower than the detection limit, the value of 18,35 was assigned. In the sensitivity analysis, exclusion of men individuals with undetectable TE did not affect any of the investigated associations (data not shown) (16). In the basic model (Model 1), we adjusted for age at baseline, time from hormone measurement at baseline and MRI scan (men: mean= 7.80 ±4.20 years and women: mean=7.43 ± SD=4.13 years), BMI, and sex steroids for each other. The main role of SHBG is the transport of sex steroids within the blood stream to extravascular target tissues. By binding testosterone and estradiol, SHBG regulates the balance between bioavailable testosterone and estrogens, and thus, might act as a confounder in the associations of TT and TE with plaque composition and risk of stroke. Also, upstream hormones, but not downstream hormones (which might act as mediators) in the cascade of sex hormone synthesis may act as confounders (Online figure I). Therefore, for TT we adjusted for SHBG, for TE we adjusted for SHBG and TT, for FAI we adjusted for E

and for SHBG we adjusted for TT and E). Model 2 was additionally adjusted for serum total cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (presence versus absence), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent cardiovascular disease at time of magnetic resonance imaging (yes vs no), smoking status (yes vs. no) and alcohol consumption (continuous). Cox proportional hazard modelling was used to evaluate whether TE and TT were associated with risk of stroke in subjects who were diagnosed with carotid atherosclerosis based on ultrasonography. Hazard ratios (HR) and 95% confidence intervals (95% CIs) were reported. We used same models as in the analysis for plaque composition as outcome. However, for these analyses, we corrected for age and prevalent cardiovascular disease (excluding stroke) at the time when sex steroid was measured.

There were missing values on one or more covariates. Because the missing values were likely to be missing at random and for avoidance of loss in efficiency, missing values were imputed using a multiple imputation technique (5 imputation sets). Rubin's method was used for the pooled coefficients (odds ratio (OR) or HR) and 95% CIs (17). A P-value lower than 0.05 was considered as statistically significant, but to account for multiple testing, we adjusted the p-value from 0.05 to 0.025 by applying the Bonferroni correction for the number of exposures studied (N=2). All

analyses were done using SPSS statistical software (SPSS, version 21.0; SPSS Inc, Chicago, Illinois).

Sensitivity Analysis

We performed a series of sensitivity analyses using imputed data. First, we repeated the analysis on sex hormones and plaque composition by using tertiles of endogenous sex hormone, and, to study the relations across increasing tertiles, trend tests were computed by entering the categorical variables as continuous variables in the logistic regression models. To account for the specific effects of lipid particles on carotid plaque composition and risk of stroke we substituted total cholesterol with HDL-C, TG, and LDL-C. We created additional models adjusting further for TSH (continuous), glucose and insulin (continuous), CRP (continuous), DHEA (continuous) and maximal carotid plaque thickness (continuous). Number of pregnancies, age of menarche and type of menopause (non-natural vs. natural) are associated with sex hormone levels, therefore, we built another model adjusting further for these factors in women. In both men and women, effect modifications of sex hormones by BMI, age and years since menopause (in women) were tested by adding an interaction term in the final multivariable model in addition to performing stratified analysis. Since there was a time difference between time of sex steroids measurements and MRI assessment, we stratified analysis by median time difference between the two assessments (men: median=9.91years, women:

9.00 years). Also, to account for effects of statin use on plaque composition, we further corrected for statin use frequency and duration, and in stratified analysis we excluded statin users. Also, we performed analysis excluding individuals with prevalent CVD at time of MRI scan. Additionally, we investigated whether SHBG and FAI were associated with plaque composition and risk of stroke. Also, we restricted the analysis on sex steroids and stroke to the participants who had also information on plaque composition (n=1498). Among these subjects we further investigated the effect of carotid plaque characteristics and risk of stroke. We created additional models adjusting for carotid plaque thickness and for plaque composition (presence of calcification, lipid core and intraplaque hemorrhage in individual models, as well as the three variables in the model). We also restricted our analysis on sex hormones and risk of stroke to subtypes of stroke (ischemic only, and ischemic and unspecified types of stroke). Finally, we performed the analysis on sex hormones and risk of stroke in women and men without atherosclerosis. Atherosclerosis has shown to lead to changes in expression of sex hormones receptors and thus, might modify the effect of sex hormones on risk of stroke (18). Further, the "timing hypothesis" indicates that estradiol might have adverse effects mainly in women with underlying atherosclerosis (18, 19).

Results

Table 1 summarizes the baseline characteristics of the participants included in the main analysis (the association between sex steroids and carotid plaque composition). A total study population 1480, included 645 (43.58%) postmenopausal women and 835 (56.42%) men. The mean age of women was 65.54 years (SD 7.22), and men 63.79 (SD 6.72). Women were on average 17.29 years (SD 9.11) into menopause, and majority of the women (66.4%) experienced natural menopause. Calcified carotid plaques were present in 1220 (82.4%) participants, 532 women (82.5%) and 688 (82.4 %) men. Lipid core was detected in 651 (43.9%) participants, 238 (36.9%) women, and 413 (49.46%) men. Intraplaque hemorrhage was observed in 521 (35.2 %) individuals, 187(29.0%) women and 334(40.0 %) men. Selected characteristics of study participants for the analysis on sex steroids and risk of stroke were similar to the study population for plaque composition as outcome (Online Table I). As expected, considering that 36.9% of women and 25.7 % of men who did not perform MRI had contraindication or physical limitations, we found differences in age (women: mean age 68 vs. 65.5; men: 65.2 vs. 63.7) and incidence rate of stroke (women: 8.1/1000 person-years vs. 4.8/1000 person years; men: 9.3/1000 person-years vs. 4/1000 person-years) among participants who did not attend and who attended MRI visit (Online table II).

Table 1. Characteristics of the Study Population

	Women (n=645)	**Men** (n=835)
Age at baseline, mean (SD), y	65.54 (±7.22)	63.79 ±6.72
Age at time of MRI scan, mean (SD), y	73.44 (±9.07)	71.60±9.10
Carotid plaques		
Calcium plaques, yes	532 (82.5 %)	688 (82.4 %)
Lipid plaques, yes	238 (36.9 %)	413 (49.5 %)
Plaque hemorrhage, yes	187 (29.0 %)	334 (40.0 %)
BMI, kg/m^2	26.85(±4.10)	26.96 (±3.13)
Smoking		
yes	153 (23.7%)	199 (23.8 %)
no	487 (75.5%)	633 (75.8%)
Alcohol intake g/day	8.40 (±13.39)	14.86 (±17.48)
Health indicators		
Systolic BP, mmHg	139.13 (±20.47)	141.90 (±19.84)
Antihypertensive therapy with indication, yes	231 (35.8%)	303 (32.2%)
Total cholesterol, mmol/l	6.0 (5.35-6.70)	5.63 (4.9-6.30)
LDL, mmol/l	3.975 (±0.97)	7.70 (±1.55)
HDL, mmol/l	1.51 (±0.39)	1.23 (±0.32)
Triglycerides, mmol/l	1.36(1.02-1.81)	1.45 (1.08-2.00)
Insulin, pmol/l	67.0 (48.0-98.0)	73.0 (51.0-107.0)
Glucose, mmol/l	5.40 (5.1-5.9)	5.60 (5.20-6.20)
CRP mg/l	1.70 (0.6-3.5)	1.40 (0.56-2.90)
Serum lipid lowering medication, yes	148 (22.9%)	181 (21.7%)
Prevalent diabetes mellitus	57 (8.8%)	117 (14.01%)
Prevalent CVD at baseline	36 (5.6%)	89 (10.7%)
Prevalent CVD at MRI	73 (11.3%)	166 (19.9%)
Hormones		
Estradiol, pmol/l	32.06 (18.35-56.85)	100.10 (76.38-129.3)
Testosterone, nmol/l	0.80 (0.60-1.08)	16.66 (13.09-20.88)
SHBG, nmol/l	59.09 (42.14-79.99)	43.21 (33.87-55.31)
DHEA, nmol/L	9.64 (6.26-14.13)	9.51 (6.31-13.99)
FAI	1.32 (0.92-2.03)	38.72 (31.84-46.61)
TSH mU/l	2.00 (1.28-3.17)	1.82 (1.27-2.60)
Women-specific variables		
Age at menopause, years[1]	48.16 ±5.85	NA
Years since menopause	17.29 (±9.11)	NA
Menopause type, natural menopause[1]	428 (66.4%)	NA
Age at menarche, years	13.56 (±1.69)	NA
Number of pregnancies	2 (1-3)	NA

Values are reported as number (percentage) for categorical variables and means ±SD or median (25th–75th quartile) for continuous variables. Age at menopause, age at menarche, type of menopause and years since menopause were not available for all women, the present values are based on 631, 637, 638, and 631 women respectively. BMI indicates body mass index; BP, blood pressure; CRP, c-reactive protein; CVD, cardiovascular disease; FAI, free androgen index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MRI, magnetic resonance imaging; NA, not applicable; SHBG, sex hormone binding globulin; and TSH, thyroid-stimulating hormone.

Sex steroids and Plaque Composition

No associations were found between sex steroids and calcified carotid plaques in either sex (Table 2). After correcting for potential confounding factors, detectable TE levels in women (TE levels > 18.35 comparing to ≤18.35 pmol/l , odds ratio (OR) and 95% CI 1.48 [1.02, 2.15]) and higher TE levels in men per 1SD TE increase, OR and 95% CI 1.23 [1.03,1.46]) were associated with higher prevalence of lipid core in carotid plaques (Table 2). No associations were found between TT and presence of lipid core in either sex (Table 2). Detectable TE levels (TE levels > 18.35 comparing to ≤18.35 pmol/l, OR and 95% CI 1.58 [1.03, 2.40]) and lower levels of TT (per 1SD TT increase OR and 95% CI 0.82 [0.68, 0.99] respectively) were also associated with higher prevalence of intraplaque hemorrhage in postmenopausal women irrespective of potential confounding factors. No association was found between TE, TT and intraplaque hemorrhage in men (per 1SD TE increase, OR and 95% CI 0.94 [0.81, 1.09] and per 1SD TT increase OR and 95% CI 0.84 [0.69, 1.01]) (Table 2).

Table 2. Association between sex hormones and carotid plaque composition

WOMEN (n=645)	**Calcified plaques OR (95% CI)**	**Lipid Core, OR (95% CI)**	**IPH OR (95% CI)**
Estradiol†			
Model 1	0.97 (0.77; 1.23)	1.40 (0.98; 2.00)	1.49 (1.01; 2.23)
Model 2	1.06 (0.66; 1.71)	1.48 (1.02; 2.15)	1.58 (1.03; 2.40)
Testosterone‡			
Model 1	1.08 (0.96; 1.22)	0.99 (0.84; 1.17)	0.87 (0.73; 1.05)
Model 2	1.03 (0.81; 1.31)	0.99 (0.84; 1.18)	0.82 (0.68; 0.99)
MEN (n=835)	**Calcified plaques OR (95% CI)**	**Lipid Core, OR (95% CI)**	**IPH OR (95% CI)**
Estradiol†			
Model 1	0.94 (0.79;1.09)	1.22(1.06;1.39)*	0.95 (0.83;1.10)
Model 2	0.94 (0.77;1.14)	1.23(1.03;1.46)*	0.94 (0.81;1.09)
Testosterone‡			
Model 1	1.04 (0.93;1.17)	1.00 (0.84;1.19)	0.86 (0.71;1.02)
Model 2	1.06 (0.84;1.34)	1.00 (0.84;1.19)	0.84 (0.69;1.01)

Model 1: Age, time difference between hormone measurement and MRI scan, body mass index (BMI), sex hormones for each other (for Estradiol we adjusted for SHBG and TT, for TT we adjusted for SHBG). Model2: Model 1+ total serum cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (yes vs. no), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent CVD at time of MRI, smoking status (yes vs. no) and alcohol consumption (continuous).† In women detectable TE levels (> 18.35 pmol/l) were compared to undetectable TE levels (≤18.35 pmol/l), in men results are presented per 1SD of estradiol increase. ‡Per 1SD increase of serum testosterone; *Results are presented as Odds ratio (OR) and 95% Confidence interval (CI 95%); *Statistically significant results are bold (at level p<0.05); *Association remains significant at a Bonferroni corrected $P < .025$ for 2 tests.

Association between sex steroids and risk of stroke

During a median follow-up of 10.0 years, we identified 57 incident cases of stroke in women and 56 new cases of stroke in men. In the multivariable model (Model 2), women with detectable TE (TE levels > 18.35) as compared to women with undetectable TE ≤18.35 pmol/l had an increased risk of stroke in women (HR and 95% CI 1.98 [1.01, 3.88]), whereas no association was found between TE and risk of stroke in men (Table 3). Also, no associations were found between TT and risk of stroke in either sexes (Table 3).

Table 3. Association between sex steroids and risk of stroke

	WOMEN (n=1023) HR (95% CI) (57 stroke cases)	**P value**	**MEN (n=1124) HR (95 % CI)** (56 stroke cases)	**P value**
[a]Estradiol				
Model 1	1.99 (1.42-2.79)	0.04	0.96 (0.83-1.11)	0.77
Model 2	1.98 (1.01-3.88)	0.04	0.98 (0.75-1.29)	0.91
[b]Testosterone				
Model 1	1.06 (0.93-1.21)	0.67	1.13 (0.78-1.66)	0.52
Model 2	1.04 (0.79-1.37)	0.77	1.10 (0.75 -1.62)	0.61

Model 1: Age, body mass index (BMI), sex hormones for each other (for TT we adjusted for SHBG, for E we adjusted for SHBG and TT); Model 2: Model 1+ total serum cholesterol (continuous), statin use (yes vs. no), prevalent diabetes mellitus (yes vs. no), systolic blood pressure (continuous), antihypertensive medication (yes vs. no), prevalent CVD before the date of hormone measurement, smoking status (yes vs. no) and alcohol consumption (continuous); [a] in women detectable TE levels (> 18.35 pmol/l) were compared to undetectable TE levels (≤18.35 pmol/l), in men results are presented per 1SD of estradiol increase; [b] per 1SD testosterone increase; Results are presented as hazard ratio (HR) and 95% confidence interval (CI 95%); Statistically significant results are bolded; Analysis done in subjects diagnosed with carotid atherosclerosis using ultrasonography.

Sensitivity analysis

Only the association between TE and lipid core presence in men remained significant after we applied the Bonferroni correction ($p<0.025$). In sensitivity analyses, using tertiles of sex hormones showed same results as the main analysis (Online figure Ia, Ib, Ic). Also, substituting total cholesterol for other blood lipids, adjusting further for number of pregnancies, age of menarche and type of menopause, statin frequency use, as well as for glucose, insulin, TSH, CRP, DHEA, maximal carotid plaque thickness and exclusion of individuals with prevalent CVD at the time of MRI scan did not materially affect any of the associations (Online table IIIa, IIIb and 4). Also, in the stratification analysis, no significant interactions were found for sex steroids with BMI, age, years since menopause or with carotid intima media thickness (Online table IIIa, IIIb and 4). Furthermore, the significant results we observed in the main analysis on sex steroids and plaque composition did not materially change when the analysis were restricted to participants with lower than the median (9 years) of the time difference in between sex steroids and MRI measurements. Also, the analyses stratified for time interval between sex hormones and MRI showed in general no differences in the results in either sex. Only for calcification and intraplaque hemorrhage, the effect estimate in women between estradiol and presence of calcification was larger in the stratum with short time interval compared with long time interval (Calcified plaques: OR, 1.70 (0.92-

3.17) versus 0.36 (0.14-0.91), IPH (2.27 (1.15-4.48) versus 1.24 (0.69-2.20)) (Online table IIIa, IIIb). The results on sex steroids and plaque composition did not materially change when we further corrected for statin use frequency and duration (Online table IIIa, IIIb). After statin users were excluded from the analysis, the association between TE and lipid core presence and intraplaque hemorrhage was not anymore significant in women, but the magnitude of the effect did not materially change (Online table IIIa, IIIb). Also, no association was observed between SHBG and FAI with plaque composition and risk of stroke in either sex (Online table V, VI). The results on sex steroids and risk of stroke did not materially change when restricting the analysis to subjects with available data on plaque composition (Online table IV). In these subjects' carotid plaque thickness and characteristics of carotid plaques did not affect the direction and magnitude of the association between TE and TT and risk of stroke (Online table VII). Exclusion of hemorrhagic stroke cases, and further of non-specified types, did not materially affect the associations of sex hormones with risk of stroke. (Online table IV). Restriction of the analysis on sex hormones and risk of stroke to participants without atherosclerosis diminished the association between TE and risk of stroke among women (Online table VIII).

Discussion

In this population-based study among postmenopausal women and elderly men, we show sex differences in the association between estradiol, carotid plaque composition and risk of stroke. While TE was associated with higher prevalence of lipid core in both men and women, TE was associated with higher odds of having intraplaque hemorrhage and risk of stroke in women but not in men. No consistent association was observed between testosterone and plaque composition and risk of stroke in either sex (Illustration 1).

Estradiol, plaque composition and risk of stroke

Sex differences in the physiological actions of estradiol may underlie the sex differences we observed in our study, but another factor contributing can be the differences in sex hormone levels between men and women. In our sample, the distribution of estradiol and testosterone were different among men and women, with men having higher levels of both estradiol (median 100.10 pmol/L as compared to 32.06 pmol/L in women) and testosterone: median 16.66 nmol/l as compared to 0.80 in women nmol/l). Future studies with larger sample and wide distribution (range) of estradiol and testosterone levels in men and women are needed to understand further the role of sex hormones in atherosclerosis and risk of stroke. Our results on vulnerable plaque composition and increased risk of stroke with increasing levels of estradiol in women far from menopause (on average 17 years into menopause) are in line with the "timing hypothesis", which

theorizes that estradiol has harmful vascular effects in elderly women in contrast to neutral or beneficial effects in younger women (20, 21). Also, in line with the "timing hypothesis", the lack of association between total estradiol and risk of stroke in postmenopausal women without carotid atherosclerosis indicated that endogenous estradiol might have deleterious effects only in women with underlying atherosclerosis. Thus, the carotid atherosclerosis might act as an effect modifier on the association between TE and risk of stroke which needs to be addressed by future studies.

Findings from animal studies based on monkey models in pre-menopause show estradiol to prevent fatty streak deposition and progression of atherosclerotic plaque (22). In contrast, in monkey models of female subjects 2 years into menopause (comparable to six postmenopausal years for women), no beneficial effect of estradiol was observed on the progression of coronary artery plaque (23). Similarly, studies among premenopausal women or in women in menopausal transition show that women using hormone therapy (HT) have a lower incidence of carotid atherosclerotic lesions (24), and slower progression of CIMT (25), while studies in postmenopausal women report no or deleterious effects (26, 27). Also, TE has beneficial effects in the vascular system in premenopausal women, whereas in postmenopausal women, large clinical trials have reported use of exogenous estradiol, which increases circulating estrogen levels, to increase risk of stroke (28,

29). Women's Health Initiative (WHI) clinical trials reported harmful effect of HT on ischemic stroke risk in women older than 50 years of age (30, 31). Observational data from the Nurses' Health Study confirmed those findings (32).

Although, emerging evidence supports the "timing hypothesis", the pathways still remain unclear. It is early to say if switch from protective to harmful estradiol effect is due to changes in ER signaling (11) or it is a consequence of age-related hyper-inflammatory state (33). Some experimental studies suggest molecular mechanisms that may contribute to hyper-inflammatory state and possibly promote pro-inflammatory effect of estrogens in the aging vasculature (34). Also, the direct anti-\atherogenic effect of estrogen are present, absent, or reversed, depending on the state of the arterial endothelium. Coronary artery fatty streaks and small plaques are common in women at the time of perimenopausal transition, while advanced atherosclerotic plaques are common in aging women and in women 5–15 years after menopause. Endothelium changes related to atherosclerosis progression in elderly women might be another explanation why hormone replacement therapy initiated at the complicated plaque stage (beyond about 60 years of age) can have either no beneficial effects or deleterious effects (23).

In men, TE was associated with presence of lipid core, however, no association was observed with calcified carotid plaque, intraplaque hemorrhage or risk of stroke. Muller et al (35), demonstrated that higher E was associated with progression of

CIMT of the common carotid artery. Study done in men with DM II showed inverse association between E and carotid atherosclerosis (36), while another study did not find any correlation between E and atherosclerosis in men (37). Evidences on association between E and risk of stroke in men are limited. In the Honolulu-Asia Aging Study, elevated serum E was cross-sectionally associated with frequency of lacunar infarcts found on MRI (38). Study done in elderly men demonstrated positive correlation between E and stroke, however, they did not adjust for other sex hormones (39). In line with our findings, two case-control studies did not find any correlation between E levels and stroke (40, 41).

Total testosterone and plaque composition and risk of stroke

In the present study, no association was observed between TT and FAI, plaque composition and risk of stroke in either sex.

Animal models of atherosclerosis report contradictory results in both men and women. Few studies show that T has no effect (42) or beneficial effect on atherosclerosis in male animals (43), while in females exogenous androgen treatment may be atherogenic (44). Similarly, while cross-sectional studies in men and women have demonstrated inverse relation between T, CIMT and carotid plaque (45), longitudinal studies and clinical trials show no impact of T to CIMT progression, coronary artery calcification and plaque area (46). Limited and conflicting evidence exist also on T and stroke incidence. In a prospective

observational study of 3443 elderly men with a median follow-up of 3.5 years, men with low-normal T levels had increased risk of incident stroke and TIA combined (47). In contrast, T therapy use in cohort of veterans with significant medical comorbidities was associated with increased risk ischemic stroke (48).

Strengths and limitations

To the best of our knowledge, this is the first and most comprehensive study to examine the associations of estradiol and testosterone and carotid plaque composition in sample of postmenopausal women and men. Our sample was drawn from middle-aged and elderly subjects from the general population, which is one of the major strengths of our study. Total testosterone was measured using chromatography-tandem mass spectrometry, at the moment considered to be the gold standard method. High-resolution protocol-based MRI sequences are used to evaluate carotid plaque composition. Nevertheless, limitations of our study need to be discussed. First, a cross-sectional study design does not allow us to address the temporality of the observed associations, hence, we cannot draw any conclusions with regard to the causality of the observations. Second, in the RS there are no measures of bioavailable estradiol, which could have strengthened our results. Also, TE was measured using an immunoassay with a detection limit of 18.35 pmol/L, which is considered suboptimal particularly in postmenopausal women. Therefore, the analyses were done by categorizing the values into 0 if estradiol levels were

≤18.35 pmol/l and 1 if the values were >18.35 pmol/l. Categorization of a continuous variable introduces loss of information and power. Third, we found difference between participants who attend MRI visit and who did not attend. However, majority of subjects who did not perform MRI had contraindication or physical limitations (38.73 %), therefore they might have been sicker comparing to the participants who were eligible to attend MRI. Also, it has been shown that using a selected source population for a cohort study usually leads to bias towards the null (49). Fourth, as our sample is population-based we chose not to administer gadolinium contrast to the participants, although lipid core is more easily detected with a contrast-enhanced MRI (50). However, in validation studies non-contrast-enhanced MRI sequences have shown a good accuracy and reproducibility (51). Last, MRI assessment was measured only once and that measurement was taken after the exposure was measured, and therefore time difference between sex steroid measurements and MRI assessment exists. However, we did not find any difference in the results when main analysis was stratified by the time difference between the two assessments. Also, this study was carried out in middle-aged and elderly patients, and in older age hormone levels are more stable over time (52). Furthermore, the results on sex steroids and risk of stroke were on same direction as the results expected from sex steroids and plaque composition. Finally, the number of significant results on the association of TE, with plaque composition and

risk of stroke before correcting for multiple testing, but not after, as well as the presence of several methodological issues, suggests that our results are hypothesis-generating and should stimulate further studies on the possible influence of sex hormones on atherosclerosis and stroke risk.

In summary, our findings suggest that endogenous estradiol may play a role in the development of vulnerable carotid plaque composition and increases risk of stroke in middle-aged elderly women. We hypothesize that endogenous estradiol levels in middle-aged elderly women can lead to plaque instability by increasing lipid content and intra-plaque hemorrhage which on the other hand can increase the risk of stroke. The mechanisms by which estradiol levels can lead to adverse changes in plaque composition and subsequent risk for stroke in postmenopausal women remain largely unknown. Since the characteristics of carotid plaque did not alter the association between estradiol and risk of stroke, factors other than plaque composition parameters we evaluated can contribute to increased risk of stroke associated with high levels of estradiol in middle-aged and elderly women. Thus, further studies are needed to replicate our findings and to explore mechanisms of action of estradiol in carotid artery atherosclerosis, but also in other blood vessels. Additionally, our results encourage future research investigating whether estradiol might help to predict ischemic stroke in women with subclinical atherosclerosis, and thus, whether estradiol levels might be used in stroke risk prediction models.

Considering HT alters endogenous estradiol levels, our results raise a concern on whether the exogenous estradiol could have similar effect. Therefore, until future studies replicate or refute our findings, we believe HT should be taken with caution among women with similar characteristics to our study; postmenopausal women who already have diagnosis of carotid atherosclerosis and are further from menopause.

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Chapter 4

Cardiovascular therapy on carotid plaque composition

Chapter 4.1

Statin use is associated with carotid plaque composition: The Rotterdam Study

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Abstract

Background

Statins represent a key treatment for cardiovascular disease. Nevertheless, the direct effects of statin treatment on the composition of atherosclerotic plaques remain elusive.

Objectives

We aimed to investigate the association of statin treatment with the presence of different plaque components located in the carotid arteries within a population-based setting.

Methods

From the population-based Rotterdam Study, 1740 participants with carotid atherosclerosis (mean age 72.9 years, 46% women) underwent MRI of the carotid arteries to determine the presence of calcification, lipid core, and intraplaque hemorrhage. Information for the duration and dosage of statin use was obtained from pharmacy records for all participants. We used logistic regression models to study the association of statin use with the presence of plaque components.

Results

Statin treatment was associated with a higher presence of calcification (OR: 1.73 [95%CI:1.22-2.44]). Longer duration of use strengthened this association (OR: 1.82 [95%CI:1.00-3.33] for 10 to 48 months, and OR 1.74 [95%CI: 1.09-2.77] for > 48

months, compared to OR: 1.65 [95%CI:0.94-2.89] for ≤ 10 months). Current statin treatment was also associated with a lower presence of lipid core (OR: 0.66 [95%CI:0.42-1.04]), but only when using statins for 10 months or less. Any dosage of statins was associated with a higher presence of calcification, whilst only high dosages (DDD > 1.33) were associated with a lower presence of lipid core.

Conclusions

Active, high-dosage statin use seems to beneficially influence the composition of carotid atherosclerosis by shifting the composition from vulnerable plaque with a lipid core to more stable calcified plaque.

INTRODUCTION

Atherosclerosis in the carotid artery is the most important cause of stroke (1-3). Within the complex etiological framework of atherosclerosis, a key role is played by serum low-density lipoprotein (LDL) cholesterol, specifically in the initiation and progression of the disease (2). Following this, lowering the concentration of LDL cholesterol using statins has become a cornerstone for primary prevention of stroke and cardiovascular events overall (4, 5).

Several trials have demonstrated a direct effect of statins on the formation of coronary artery disease and a lower risk of coronary events (6-8). This direct effect of statins is thought to be due to the beneficial influence of statins on plaque stability by increasing the amount of calcium at the cost of vulnerable plaque components such as lipid core (9).

In contrast to the extensive research in the field of the coronary arteries, studies on the effects of statin treatment on atherosclerosis in the carotid arteries are far more limited, especially from a general-population perspective. Yet, especially in light of the increased risk of stroke that carotid atherosclerosis harbors (10), it is paramount to also disentangle the effect of statin treatment on carotid artery atherosclerosis. Moreover, it is important to highlight that findings regarding the physiopathology of coronary artery disease may not be directly generalizable to the carotid arteries,

given that correlation for atherosclerosis across vessel beds is only moderate (11, 12). Magnetic resonance imaging (MRI) allows detailed characterization of different plaque components, including lipid core, intraplaque hemorrhage (IPH), and calcification (13, 14).

Against this background, we investigated the association of statin use with specific components of the carotid plaque in a large population-based sample of persons with subclinical atherosclerosis.

METHODS

Setting

The current study is embedded within The Rotterdam Study, a prospective population-based cohort study, in participants of ≥ 45 years living in Ommoord, a district of Rotterdam (15). The Rotterdam Study has been approved by the medical ethics committee, according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants provided written informed consent.

Study population

Participants were selected on the basis of a carotid artery ultrasound examination (intima-media thickness > 2.5 mm in one or both carotid arteries) which is performed in all participants of the Rotterdam Study. Between the year 2007 and

2012, 2666 participants were invited to undergo an MRI examination of the carotid arteries. From the invited participants, 684 participants did not undergo MRI scanning due to claustrophobia (n=57), physical limitations (n=191), MRI contraindications (n=115), refusal to participate (n=272) and no show or lost follow-up (n=49), leaving 1982 participants. From these, we excluded another 242 participants due to poor image quality (n=95), scan interruption due to claustrophobia (n=106) and absence of plaque bilaterally (n=41), leaving 1740 participants in the present analyses.

2.3 Carotid scanning and analysis of plaque components

MRI imaging was performed using a 1.5 Tesla scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phase-array surface coil (Machnet, Eelde, the Netherlands). A standardized scanning protocol was used with a total scanning time of approximately 30 minutes. The protocol included 4 sequences in axial plane: a proton density weighted (PDw)-fast spin echo (FSE)-black blood (BB) sequence (in-plane resolution 130/160 x 130/128 = 0.8 x 1 cm); a PDw-FSE-BB with an increased in-plane resolution (in-plane resolution 130/224 x 130/160 = 0.5 x 0.8 cm); a PDw-echo planar imaging (EPI) sequence (in-plane resolution 130/ 160 x 70/160 = 0.8 x 0.4 cm); a T2 weighted-EPI sequence (in-plane resolution 130/160 x 70/160 = 0.8 x 0.4 cm) and two three-dimensional (3D) sequences: a 3D-T1

weighted (T1w)-gradient echo sequence (in-plane resolution 180/192 x 180/180 = 0.9 x 1 cm), and a 3D phased-contrast magnetic resonance angiography (3D-PC-MRA) (in-plane resolution 180/256 x 180/128 = 0.7 x 1.4 cm). Details of the scan protocol, scan reading procedure, and reproducibility are described in detail elsewhere (16). We assessed plaque characteristics in all plaques with a maximum thickness of ≥2.5 mm on MRI. On the proton density weighted fast spin echo images, maximum carotid wall thickness was measured, and degree of luminal stenosis was calculated using the North American Symptomatic Carotid Endarterectomy Trial criteria (17). The carotid images were evaluated for the presence of three different plaque components, calcification, lipid core, and IPH. Calcification was defined as the presence of a hypointense region in the plaque on all sequences (18-20). IPH was defined as the presence of a hyperintense region in the atherosclerotic plaque on 3D-T1w-GRE (21, 22). Lipid core presence was defined as a hypointense region, not classified as IPH or calcification, in the plaque on PDw-FSE, PDw-EPI and T2w-EPI sequence, and with relative signal intensity drop on the T2w-EPI sequence (18, 19, 23). All sequences were used in parallel to record the presence of plaque components. Subjects were recorded as positive for the presence of any plaque component if the component was identified in one or both carotid arteries. To test the intra-subject variability, 40 participants underwent a second MRI scan (average time between scans 15±9 days) (16). For an inter-

observer reproducibility analysis, MRI examinations were selected randomly (n=50) and read by a second independent observer with three years of experience. Inter-observer agreement and intra-scan agreement were calculated using Cohens' Kappa statistics. The intra-subjects agreement was good for all measurements. The Kappa values were 0·95 (95% CI 0·88-0·99) for the presence of intraplaque hemorrhage; 0·85 (95% CI 0·74-0·96) for lipid core and 0·91 (95% CI 0·82-0·99) for calcification (16). Moreover, interobserver agreement was good for all measurements. The Kappa values were 0·86 (95% CI 0·72-0·99) for intraplaque hemorrhage; 0·86 (95% CI 0·72-0·99) for lipid core presence and 0·94 (95% CI 0·86-0·99) for calcification (16)

Assessment of statin treatment

Information on statin treatment dispensing was obtained from fully computerized linked pharmacies in the study area. All prescriptions for statin therapy filed from January 1, 1991, until October 26, 2012, were available and included the product name of the drug, the anatomical therapeutic chemical code (ATC code), the amount dispensed, the prescribed dose regimen, and the date of dispensing. For every dispensing of statins, the duration of use (prescription episode) was calculated by dividing the number of dispensed tablets by the prescribed daily number. On the date of carotid MRI scanning, every participant was classified into

one of the following mutually exclusive categories: 'current use' if the measurement occurred within a prescription episode; 'past use' if the participant had previously stopped using statins; or 'never use' if the participant had not used statins during the study period. Next, we created tertiles of the duration of cumulative exposure to statins among statin users. This resulted in the following categories: current use ≤10 months; current use 10 - 48 months, current use >48 months and past use ≤10 months; past use 10 – 48 months, past use >48 months since the end of the last prescription episode. To facilitate direct dose comparisons between drugs from the same therapeutic drug group, the daily defined dose (DDD) of statin therapy was expressed (24). Finally, we created tertiles of discontinuation of statin use among past users as follows: ≤3 months, 3-16 months and >16 months.

Other measurements in the Rotterdam Study

Information on other relevant measurements was obtained by interview, physical examination, and blood sampling (15). Smoking status was categorized into never, the past, and current smoking. Diabetes mellitus was defined as fasting blood glucose >6.9 mmol/L, nonfasting glucose >11.0 mmol/L, or use of glucose-lowering medication. Systolic and diastolic blood pressure was measured using a random-zero sphygmomanometer on the right arm. Two measurements were performed and the average of the two was used in the analyses. Body mass index (BMI) was calculated based on weight in kilograms divided by height in meters

squared. Serum total cholesterol and high-density lipoprotein (HDL) levels were measured using standard laboratory techniques. The use of antihypertensive medication and vitamin K antagonists (VKA) was obtained from pharmacy records (15).

Statistical analysis

We used a three-step statistical analysis approach to investigate the association between statin use and the presence of different plaque components. First, we used two logistic regression models to assess the association of statin use (never, former, current) with the presence of calcification, lipid core, and IPH in any of the two carotid arteries. In the first model, we adjusted these analyses for age and sex. In the second model, we additionally adjusted for smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, BMI, total cholesterol, HDL, use of antihypertensive medication, and use of vitamin K antagonists. Factors were selected based on previous literature and univariate analyses. Vitamin K antagonists were handled as a potential confounder given that these accelerate the deposition of calcification in the arterial wall through the competitive lowering of vitamin K receptor binding. Second, we investigated whether the duration of statin use was associated with any of the three plaque components, using the same regression models. For the duration of statin use, we compared the six categories as defined above (based on tertiles of use), vs. never use. Third, we investigated whether the

DDD of statin treatment was associated with any of the three plaque components. Fourth, we created tertiles of DDD of statin treatment and compared the three tertile categories with never use. Finally, we investigated the association of discontinuation of statin use with plaque composition (based on tertiles of discontinuation).

Additionally, we performed sensitivity analyses to address confounding by indication, we re-analyzed all associations in participants without prevalent cardiovascular diseases (participants with a confirmed history of stroke, myocardial infarction, and coronary heart disease) (Table 1) (4, 5). Finally, we performed stratified analyses for age below and above 70 years of age and sex, to investigate whether associations differed by these factors. All analyses were carried out using IBM SPSS Statistical package version 21 (Chicago, IL, USA).

RESULTS

Table 1 presents the baseline characteristics of the study population. Among study subjects a total of 30.2% on statin treatment at the time of MRI. In statin users, the median duration of exposure to statins was 48 months (interquartile range, 10 – 110 months) with varying DDD regimes. Whereas in the statin past users the median statin discontinuation was 16 months (interquartile range, 3 – 48 months).

Table 1 Baseline characteristics of the study population (n=1740)

	Never user N=1014	**Current user N=526**	**Past user N = 200**
Age (years)	72.05±9.9	73.60±8.0	75.44±6.8
Women (%)	48.8	38.2	52.0
Current smoking (%)	30.8	33.5	30.0
Diabetes mellitus (%)	8.2	24.0	21.0
Systolic blood pressure (mm/Hg)	144.6±20	146.1±21	148.4±20
Diastolic blood pressure (mm/Hg)	80.7±10	79.2±10	80.1±12
BMI (kg/m^2)	27.0±3.6	27.6±3.2	27.9±3.7
Total cholesterol (mmol/L)	5.83±0.8	5.22±1.1	5.65±1.1
HDL cholesterol (mmol/L)	1.46±0.3	1.29±0.3	1.37±0.3
Antihypertensive medication use (%)	27.4	59.1	47.5
Vitamin K antagonists use (%)	4.2	8.4	5.0
History of stroke (%)	2.5	12.0	10.5
History of myocardial infarction (%)	1.9	21.5	20.0
History of CHD (%)	1.4	26.8	22.0
Degree of stenosis (%) mean±SD	16.1±17.5	22.3±23.3	20.4±21.4
Wall thickness, mm	3.1±0.6	3.3±0.7	3.3±0.8
Presence of calcification	78.7	88.4	84.5
Presence of lipid core	45.0	42.4	43.5
Presence of IPH	30.7	39.5	42.0

Values are means with standard deviations for continuous variables and percentages for dichotomous or categorical variables. Abbreviations: BMI = body mass index, CHD = coronary heart disease, HDL = High-density lipoprotein

Statin use and plaque composition

Current statin use was associated with the presence of calcification and lipid core (age, sex and carotid wall thickness adjusted odds ratios [OR]: 1.77, and 0.78, respectively). After additional adjustment for cardiovascular risk factors, only the association between current statin use and the presence of calcification remained statistically significant (OR: 1.73 [95%CI: 1.22-2.44]) (Table 2). We found no association between current statin use and the presence of IPH. Moreover, we found no association of past statin use with any of the plaque characteristics.

Table 2 Association between statin treatment and carotid artery plaque composition

Statin use	**Calcification OR (95%CI)**	**Lipid core OR (95% CI)**	**IPH OR (95% CI)**
Never use	Ref	Ref	Ref
Current use	1.73 (1.22-2.44)	0.91 (0.72-1.16)	1.07 (0.82-1.40)
Past use	1.13 (0.73-1.75)	0.99 (0.71-1.37)	1.35 (0.94-1.92)
Global P-value over statin use categories	<0.05	>0.05	<0.05

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use. Abbreviations: CI = confidence interval, IPH = intra-plaque hemorrhage, OR = odds ratio.

Duration of statin use and plaque composition

Longer duration of statin use was associated with a higher presence of calcification (for statin use of 10 months OR: 1.65 [95%CI:0.94-2.89]), (for statin use of 10 to 48

months OR: 1.82 [95% CI: 1.00-3.33]), and (for statin use of more than 48 months OR: 1.74 [95% CI: 1.09-2.77]) (p-trend 0.01) (Table 3). We also found an association between the current statin use of ≤10 months with a lower presence of lipid core (OR: 0.54 [95% CI: 0.35-0.83]). After additional adjustment this association was not significant (OR: 0.66 [95% CI: 0.42-1.04]). We found no associations for any length of past use with any of the plaque components (Table 3).

Table 3 Association between duration of statin treatment and plaque composition

Statin use	**Calcification OR (95%CI)**	**Lipid core OR (95% CI)**	**IPH OR (95% CI)**
Never use	Ref	Ref	Ref
Current ≤ 10 months	1.65 (0.94-2.89)	0.66 (0.42-1.04)	1.25 (0.76-2.04)
Current 10–48 months	1.82 (1.00-3.33)	0.95 (0.64-1.40)	0.81 (0.52-1.27)
Current > 48 months	1.74 (1.09-2.77)	1.00 (0.74-1.34)	1.14 (0.83-1.58)
Past ≤ 10 months	0.82 (0.44-1.52)	1.04 (0.63-1.71)	1.89 (1.11-3.22)
Past 10 – 48 months	1.50 (0.65-3.45)	1.28 (0.72-2.25)	1.79 (0.97-3.29)
Past > 48 months	1.37 (0.65-2.90)	0.76 (0.45-1.29)	0.71 (0.39-1.30)
Global P-value over statin use categories	< 0.05	> 0.05	< 0.05

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use. Abbreviations: CI = confidence interval, IPH = intra-plaque hemorrhage, OR = odds ratio.

Dosage of statin use and plaque composition

We found a dose-response relation between the DDD of statin use and a higher presence of calcification and a lower presence of lipid core (Figure 1). The high statin dosage did not associate with a presence of IPH, even after adjustment for cardiovascular risk factors (Figure 1).

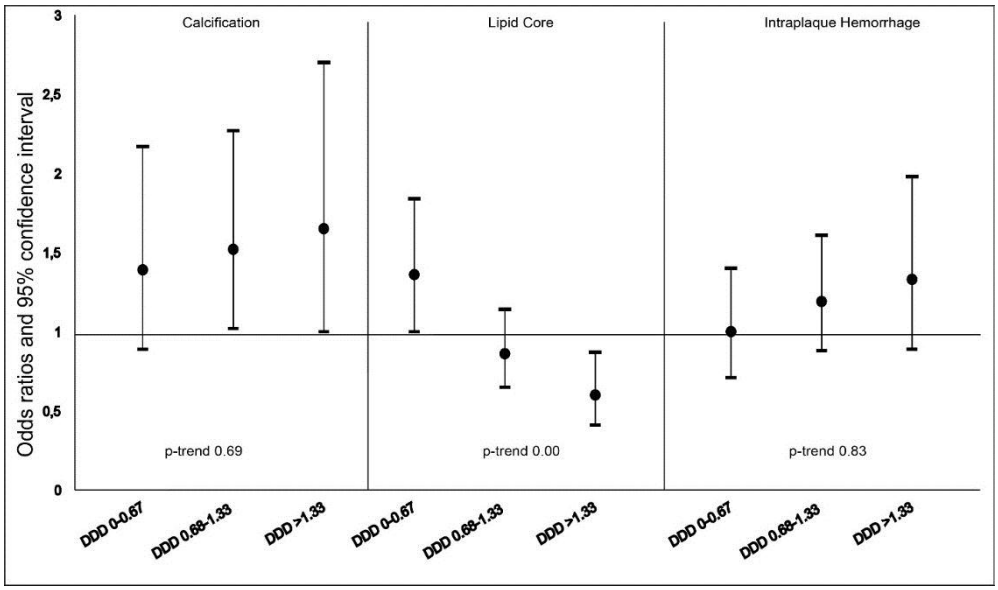

Figure 1. Association of statin treatment, according to defined daily dosage (DDD) on carotid composition. In all three sections are represented the association of the calcification, lipid core, and intraplaque hemorrhage. Values on the y-axis represent the odds ratios per DDD tertiles. The values are adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, and vitamin K antagonists use.

Discontinuation of statin use and plaque composition

When we examined associations between the discontinuation of statin use in past users, we found a positive trend of ≤3 months of statin discontinuation with a higher presence of calcification (OR: 1.48 [95% CI: 0.60-3.61]) and the positive trend

of statin discontinuation of more than >16 months with a lipid core. Interestingly, we also found that discontinuation of longer than 3 months (i.e. 3-16 months) was associated with a higher presence of IPH (OR: 1.66 [95% CI: 1.05-2.64]) (Supplementary Table 1).

Sensitivity and stratified analyses

After excluding participants with a history of CVD, the results did not substantially change (Supplementary Table 2). When we compared the association of statin use with the presence of calcification among persons younger than ≤70 vs. those older than >70, we found that current use and past use was associated most prominently with the presence of calcification and IPH in persons younger than ≤70 (Supplementary Table 3). We found no differences between men and women (Supplementary Table 4).

DISCUSSION

In this large sample of middle-aged and elderly community-dwelling individuals, we observed that current use of a statin is related to a higher presence of calcification and a lower presence of lipid core in the carotid atherosclerotic plaque. A longer duration of statin use was primarily associated with the presence of calcification. Moreover, higher dosages of statins were related to a higher presence

of calcification and a lower presence of lipid core. We found no influence of past statin use on carotid plaque components.

The current findings suggest that statins may play an important role in the remodeling of the atherosclerotic plaque composition and potentially change the natural course of atherosclerotic disease in the carotid artery. Thus far, evidence on the role of statins comes from several clinical studies investigating the effect of statins on cardiovascular disease (6-8), mainly targeting coronary arteries and the risk of coronary heart disease (25-27). Primarily, these studies consistently demonstrated that plaques tend to regress (i.e. Diminish in size) under the influence of statins and statins plays a role in risk reduction of atherosclerotic cardiovascular events (28-32). However, evidence from trials with patients, targeting carotid artery, demonstrated that statin treatment markedly reduced intima-media thickness, plaque volume, lipid core and increased calcification (28, 33-35). Collectively, these studies examined the effect of statins and attributed the beneficial role of statins based on their ability to reduce cardiovascular events but did not elucidate the direct effect of statins on plaque composition. Our study differed from these prior investigations, particularly as we investigated in detail the plaque components of atherosclerotic plaques. In this context, our findings indicate that statins inhibit progression of vulnerable components (i.e. lipid core in the atherosclerotic plaque), promotes calcification of the plaque and here with the

transition from vulnerable to the stable plaque. Similar findings have been reported by a Multicenter CONFIRM study, which investigated the effects of statins on coronary plaque composition (36). In support of our findings a similar trend with calcification, lipid core, and intraplaque hemorrhage has been reported by the study, within Rotterdam study, when assessed the effect of statins on the incidence or the persistence of calcification, lipid core and IPH after 4 years of follow-up in longitudinal design, but owing to a small study sample limited the capacity to detect associations (37). Although our study design was cross-sectional, we may hypothesize that statins remodel the atherosclerotic plaque composition, thereby, accelerate calcification of the plaque under statin treatment whilst at the same time, decrease lipid core. However, pathological observations suggest the central role of the vascular smooth muscle cells and macrophage apoptosis as driving mechanisms of calcification (38). Aside from lipid regression within a plaque statin treatment may promote calcification in the plaque, herein plaque stabilization (25). Similarly, recent PET studies related effects of statins with reduced vascular inflammation and vascular calcification (39, 40). Therefore, it is likely that the beneficial role of statins may be attributed to the capacity of statins to remodel the composition of the atherosclerotic plaque towards more calcification and less non-calcified components. Importantly, the amount of calcification as a measure of

plaque-stability and a potential indicator of lower risk of subsequent clinical events requires further research (41).

Statin treatment produces observable changes in the plaque after short periods of use, nevertheless, it remains unclear to what extent of the duration of use statins can alter plaque morphology into a stable plaque phenotype. In the current study, we were able to consider the three different cutoffs for the duration of use and dosage. These findings suggest that statins are capable of promoting calcification in the short term of use, in low and high dosages, whereas the regression of lipid core can be achieved only through high dosages (42). Alternatively, weighing the p-trends we may hypothesize that duration of use significantly relates to calcification component and dosage of use relates to lipid component. At this point, an important clinical finding has been reported from a middle-aged patient who presented with moderate ischemia and coronary computed tomography (CTA) revealed a large amount of atherosclerotic plaque in the proximal left circumflex coronary artery. Among the others, the patient has been put in statin high dosage treatment (43). After 4 years the same patient returned back, the coronary CTA was performed, and it showed a marked reduction of plaque amount (43). Similarly, our findings may suggest that a longer duration and high dosages of statin treatment should be considered to lower the risk of atherosclerotic CVD on primary prevention (44-46). Furthermore, we highlight that duration and daily dosage of

statin treatment are key factors to determine the direction of plaque remodeling process and plaque morphology.

In contrast to our findings with current statin use, we did not find evidence for beneficial effects of past statin use suggesting a narrower window of exposure to treatment in order to accrue these effects. This is underlined by our finding that discontinuation of 3 months or longer was associated with a higher presence of vulnerable components, namely lipid core, and intraplaque hemorrhage. This may thus indicate that cessation of statin treatment will eventually lead to the continuation of plaque development, which could be comparable to the development prior to statin use. These findings are in line with a recent study in which the discontinuation of statin use led to an increased risk of myocardial infarction and cardiovascular mortality (47). Following this, future longitudinal studies that take into account the effect of statin use on clinical outcomes such as cardiovascular events and mortality, are warranted.

The major strength is that our study is the first population-based study, with a long duration of exposure to statins, to investigate the association of statin use with carotid plaque components in a relatively large study sample. All data were collected irrespective of the current study. Furthermore, we were able to characterize in detail each specific component of the carotid atherosclerotic plaque

using MRI. Additionally, we were able to take into consideration the duration and dosing of statin therapy. Yet, some considerations with regard to our study should also be addressed. First, although we conducted a sensitivity analysis confounding by indication in participants with a history of CVD should be considered. Second, due to the observational nature of the study, our findings should be regarded as hypothesis-generating and non-conclusive. Third, we were not able to consider the quantitative measures of each component within the atherosclerotic plaque. The quantitative assessments may provide additional, unique information on plaque composition and that future endeavors will also involve quantitative plaque assessments. Fourth, the potential of the MRI or CT versus PET/CT on detection of microcalcification is weak (48), therefore the interpretation of our results on calcification should be in the context of the macrocalcification.

Conclusions

In summary, we found that current statin treatment and high dosages of statins seem to beneficially influence the composition of the atherosclerotic plaque in the carotids by lowering the presence of the vulnerable lipid core component and contributing to a higher presence of stable calcified plaques.

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Supplementary Material

Supplementary Table 1 Association between discontinuation of statin treatment and carotid artery plaque composition

Statin use	Calcification OR (95%CI)	Lipid core OR (95% CI)	IPH OR (95% CI)
Never use	Ref	Ref	Ref
D ≤3 months	1.48 (0.60-3.61)	0.93 (0.51-1.69)	0.72 (0.35-1.47)
D 3-16 months	0.88 (0.50-1.56)	0.84 (0.54-1.31)	1.66 (1.05-2.64)
D > 16 months	0.77 (0.35-1.71)	1.56 (0.86-2.81)	1.37 (0.72-2.59)

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use. Abbreviations: CI = confidence interval, D = Statin discontinuation, IPH = intra-plaque hemorrhage, OR = odds ratio.

Supplementary Table 2 Association of statin treatment on carotid plaque composition in individuals free of stroke, MI, and coronary heart disease

Statin use	Calcification OR (95%CI)	Lipid core OR (95% CI)	IPH OR (95% CI)
Never use	Ref	Ref	Ref
Current use	2.12 (1.37-3.28)	0.92 (0.69-1.24)	1.26 (0.91-1.75)
Past use	1.09 (0.65-1.83)	0.90 (0.60-1.35)	1.41 (0.91-2.18)

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use. Abbreviations: CI = confidence interval, D = Statin discontinuation, IPH = intra-plaque hemorrhage, OR = odds ratio.

Supplementary Table 3 Association of statin treatment with carotid plaque components, stratified by age

Statin use	Calcification OR (95%CI)	Lipid core OR (95% CI)	IPH OR (95% CI)
≤70 years			
Never use	Ref	Ref	Ref
Current use	2.57 (1.51-4.36)	1.14 (0.75-1.75)	1.46 (0.89-2.41)
Past use	3.77 (1.26-11.31)	1.03 (0.49-2.15)	2.73 (1.25-5.96)
>70 years			
Never use	Ref	Ref	Ref
Current use	1.29 (0.81-2.04)	0.81 (0.60-1.09)	0.91 (0.66-1.26)
Past use	0.78 (0.47-1.28)	0.97 (0.67-1.42)	1.10 (0.73-1.64)

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use.
Abbreviations: CI = confidence interval, D = Statin discontinuation, IPH = intra-plaque hemorrhage, OR = odds ratio.

Supplementary Table 4 Association of statin treatment with carotid plaque components, stratified by sex

Statin use	Calcification OR (95%CI)	Lipid core OR (95% CI)	IPH OR (95% CI)
Male			
Never use	Ref	Ref	Ref
Current use	1.66 (1.06-2.61)	0.91 (0.66-1.24)	1.02 (0.72-1.44)
Past use	1.11 (0.59-2.08)	1.14 (0.71-1.82)	1.53 (0.92-2.53)
Female			
Never use	Ref	Ref	Ref
Current use	1.88 (1.09-3.26)	0.95 (0.64-1.40)	1.21 (0.79-1.86)
Past use	1.18 (0.64-2.20)	0.91 (0.57-1.45)	1.20 (0.72-2.00)

All estimates were adjusted for age, sex, carotid wall thickness, smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, blood pressure-lowering medication use and vitamin K antagonists use.
Abbreviations: CI = confidence interval, D = Statin discontinuation, IPH = intra-plaque hemorrhage, OR = odds ratio.

Chapter 4.2

Antithrombotic treatment is associated with intraplaque hemorrhage in the atherosclerotic carotid artery: a cross-sectional analysis of The Rotterdam Study

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ABSTRACT AND KEYWORDS

Aim

Antithrombotic treatment plays a key role in stroke prevention, but their direct effects on the composition of carotid artery atherosclerotic plaques are unknown. To investigate the association of antithrombotic treatment with carotid artery plaque composition, with a specific focus on an intraplaque hemorrhage.

Methods

From the population-based Rotterdam Study, 1740 participants with carotid atherosclerosis on ultrasound (mean age 72.9 years, 46.0 women) underwent MRI of the carotid arteries to assess plaque composition. Information on the use of oral anticoagulants (vitamin K antagonists) and antiplatelet agents (salicylates), including duration of use and dosage, was obtained from pharmacy records for all participants. We used logistic regression models to assess the association between the use of anticoagulants and antiplatelet agents, and the different plaque components adjusting for confounders.

Results

Current and past use of vitamin K antagonists (adjusted odds ratio (OR): 1.88 [95% confidence interval (CI): 0.74-4.75], and OR 1.89 [95%CI: 0.91-3.93]) and antiplatelet agents (OR: 1.22 [95%CI: 0.91-1.62]), and OR: 1.23 [95%CI: 0.86-1.75]) showed positive trend with a higher presence of intraplaque hemorrhage. Also, a longer

duration of use was associated with a higher frequency of intraplaque hemorrhage (OR: 3.15 [95%CI: 1.23-8.05]) for the use of vitamin K antagonists, and longer duration of the use for antiplatelet agents showed a positive trend (OR: 1.21 [95%CI: 0.88-1.67]). We also found that higher levels of INR above 2.97 for vitamin K antagonists (OR: 1.48 [95%CI: 1.03-2.15]) and higher daily defined dosage than 1.0 for antiplatelet agents (OR: 1.50 [95%CI: 1.21-1.87]) were related to a higher frequency of intraplaque hemorrhage. We found no association with lipid core or calcification.

Conclusions

The use of antithrombotic treatment relates to a higher frequency of intraplaque hemorrhage in carotid atherosclerotic plaques.

ABBREVIATIONS:

DDD = daily defined dosage,

INR = international normalized ratio,

IPH = intraplaque hemorrhage,

MRI = magnetic resonance imaging,

PDw-FSE = proton density fast spin echo image,

PDw-EPI = proton density echo planar image,

T2w-EPI = T2 weighted echo planar image,

3D-T1w-GRE = 3D T1 weighted gradient echo sequence.

INTRODUCTION

Atherosclerotic disease in the carotid artery is considered a key risk factor for ischemic stroke. In recent years considerable efforts have been put in the development of strategies to prevent the occurrence of both new and recurrent ischemic strokes (1-5). An important cornerstone of these strategies is the prescription of oral anticoagulants and antiplatelet agents, given their beneficial effect on lowering the risk of cardiovascular events, including strokes (6). Despite this benefit of oral anticoagulants and antiplatelet agents (7), their effects on the development or changes in already existing atherosclerotic carotid plaque are unknown (8). Emerging technological development in magnetic resonance imaging (MRI) enables a detailed characterization of carotid plaque components like intraplaque hemorrhage (IPH), lipid core and calcification (8-10), which play an important role in future thromboembolic events (11-14). Recently, in a relatively small sample of symptomatic patients, it was highlighted that antithrombotic treatment may exert a potentially harmful effect on the composition of carotid atherosclerotic plaques (8). Specifically, the use of antiplatelet agents was related to a higher presence of intraplaque hemorrhage, which is a plaque component that is known to be more prevalent in plaques that are prone to rupture (8). However, several important questions on this topic remain as previous studies were in symptomatic patients with advanced atherosclerosis. First, the influence of using

vitamin K antagonists (VKA), which is also frequently prescribed for prevention of cardiovascular events of cardiac origin like as atrial fibrillation, on the composition of carotid atherosclerosis remains unclear. Second, the duration and dosage of use of vitamin K antagonists or antiplatelet agents may substantially affect the carotid plaque composition but has not been studied before. Therefore, we investigated in a large sample of subjects with subclinical atherosclerosis from the Rotterdam Study, the associations between oral antithrombotic treatment and carotid plaque composition, with a special focus on intraplaque hemorrhage.

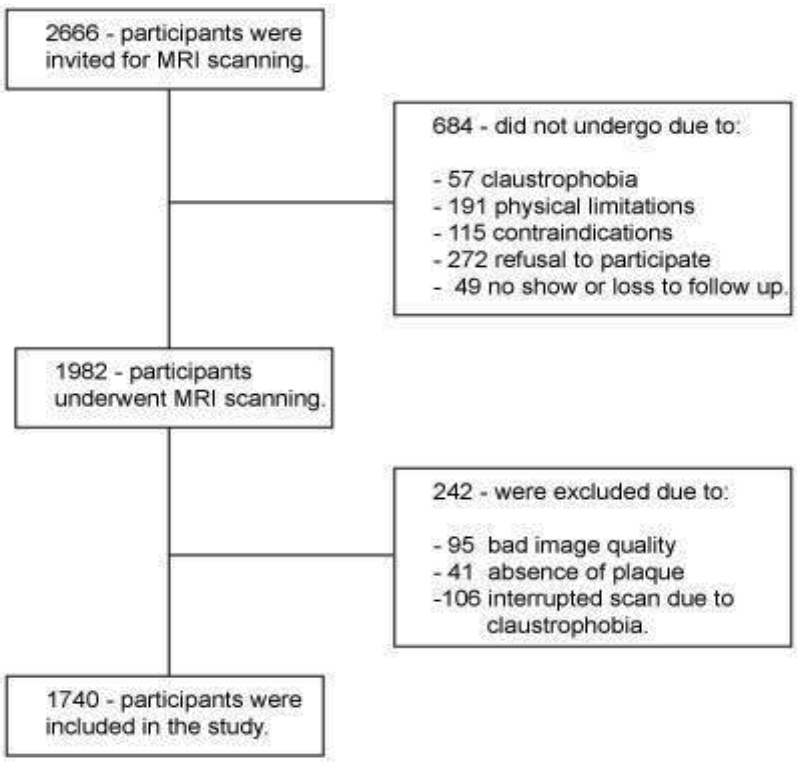
METHODS

Study population

This study is embedded within a prospective population-based cohort, The Rotterdam Study (15). Between the year 2007 and 2012 participants with carotid atherosclerosis were invited to undergo an MRI scan of the carotid arteries. Previously, participants were selected on the basis of a carotid artery ultrasound examination (intima-media thickness ≥ 2.5 mm in one or both carotid arteries) performed in all participants of the Rotterdam Study. From the 2666 invited participants, 684 did not undergo MRI scan, and 1982 did (74%). From them, 242 participants were excluded and 1740 were included in the present study (Figure 1). The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Dutch Ministry of Health, Welfare and Sports, implementing

the "Wet Bevolkings Onderzoek: ERGO (Population Screening Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Figure 1 Flowchart of the study population



Carotid scanning and analysis of plaque components

A 1.5 Tesla scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phase-array surface coil (Machnet, Eelde, the Netherlands) was used to perform bilateral imaging of the carotid artery, with a standardized scanning protocol, which required an approximate total scanning time of 30 minutes. The protocol included four sequences in axial plane: a proton density weighted (PDw)-fast spin echo (FSE)-black blood (BB) sequence (in-plane resolution 130/160 x 130/128 = 0.8 x 1 cm); a PDw-FSE-BB with an increased in-plane resolution (in-plane resolution 130/224 x 130/160 = 0.5 x 0.8 cm); a PDw-echo planar imaging (EPI) sequence (in-plane resolution 130/ 160 x 70/160 = 0.8 x 0.4 cm); a T2 weighted-EPI sequence (in-plane resolution 130/160 x 70/160 = 0.8 x 0.4 cm) and two three-dimensional (3D) sequences: a 3D-T1 weighted (T1w)-gradient echo sequence (in-plane resolution 180/192 x 180/180 = 0.9 x 1 cm), and a 3D phased-contrast magnetic resonance angiography (3D-PC-MRA) (in-plane resolution 180/256 x 180/128 = 0.7 x 1.4 cm) (16). More details of the scanning protocol, reading procedure, and reproducibility are described in detail elsewhere (17). The images of the carotid were evaluated for the presence of three different plaque components: Intraplaque hemorrhage, lipid core, and calcification. Calcification was defined as the presence of a hypointense region in the plaque on all sequences (16, 18, 19). Intraplaque hemorrhage was defined as the presence of a hyperintense region in the atherosclerotic plaque on

3D-T1w-GRE (20, 21). Lipid core presence was defined as a hypointense region, not classified as intraplaque hemorrhage or calcification, in the plaque on PDw-FSE or PDw-EPI and T2w-EPI images or a region of relative signal intensity drop in the T2w-EPI images compared with the PDw-EPI images (18, 19, 22). Two independent MRI readers, with 3 years of experience, not being aware of any of the clinical characteristics of the participants, including the medication recorded subjects as positive for the presence of any plaque component if the component was identified in one or both carotid arteries. Testing the intra-subject variability, 40 participants underwent a second MRI scan (average time between scans 15±9 days) (17). For an interobserver reproducibility analysis, MRI examinations were selected randomly (n=50) and read by a second independent observer with three years of experience. Further, using Cohens' Kappa statistics interobserver and an intra-scan agreement was calculated. The intrasubject agreement was good for all measurements. The Kappa value for the presence of intraplaque hemorrhage was 0.95 (95% CI 0.88 – 0.99); for lipid core was 0.85 (95% CI 0.74 – 0.96) and for calcification was 0.91 (95% CI 0.82 – 0.99) (17). The interobserver agreement was good for all measurements. The Kappa value for intraplaque hemorrhage was 0.86 (95% CI 0·72 – 0.99); for lipid core presence 0.86 (95% CI 0.72 – 0.99) and for calcification 0.94 (95% CI 0.86 – 0.99) (17).

Assessment of antithrombotic treatment

Dispensing information for the vitamin K antagonists and antiplatelet agents (acetylsalicylic acid [ATC code B01AC06] and carbasalate calcium [ATC code B01AC08]) was obtained using a computerized platform linking the study database and the pharmacies in the study area. All prescriptions for vitamin K antagonists and antiplatelet agents from January 1, 1991, until October 26, 2012, included the product name of the drug, the anatomical therapeutic chemical code (ATC code), the amount dispensed, the prescribed dose regimen, and the date of dispensing (23, 24). The average measured international normalized ratios (INR) (25) were obtained for vitamin K antagonists use and daily defined dosage (DDD) was obtained for antiplatelet agents use (26). Among the antithrombotic users, we distinguished the main therapeutic indications such as atrial fibrillation, recent coronary or cerebrovascular events.

At the time of the MRI, all subjects were classified into one of the following mutually exclusive categories: 'current user' if the subject was an active user at time of MRI; 'past user' if the subject discontinued the use before the MRI scan date, or 'never user' if subject never used any of these drugs. We used tertiles for the duration of use to classify the use of vitamin K antagonists and antiplatelet agents. This resulted in the vitamin K antagonists duration of use ≤ 3 months; duration of use 3 – 11 months, duration of use > 11 months, and for the antiplatelet agents' duration of use ≤ 30 months; duration of use 30 – 72 months, duration of use > 72 months since

the end of the last prescription episode. To facilitate direct dose-dependent relation between drugs from the same therapeutic drug group, we used the international normalized ratios (25) for the vitamin K antagonists use and a daily dose of antiplatelet agents expressed in daily defined dosage (DDD) (26). We created three categories of measured INR and DDD and compared them separately with never use. The international normalized ratios categories were set based on tertiles and antiplatelet daily defined dosage categories were set based on the median. Only five subjects, previously treated with vitamin K antagonists due to atrial fibrillation and with the recent coronary syndrome, were in concomitant use of dual therapy at the time of MRI and none in past use category. Considering the prolonged effect of anticoagulation medication, we have not reallocated any subject from past use into current use categories among vitamin K antagonists users as a minimum discontinuation period was 22 days, but we reclassified 3 subjects in past use category, among antiplatelet agent users, to current use category since the minimum discontinuation period was 3 – 5 days (25, 27).

Other risk factors in the Rotterdam Study

Information on the other cardiovascular risk factors as relevant measurements was obtained by interview, physical examination, and blood sampling (15). Among the other measures smoking status was categorized into never, the past, and current smoking, diabetes mellitus was defined as fasting blood glucose > 6.9 mmol/L,

nonfasting glucose > 11.0 mmol/L, or use of glucose-lowering medication, systolic and diastolic blood pressure was measured using a random-zero sphygmomanometer on the right arm and two measurements were performed and the average of the two was used in the analyses. BMI was calculated based on weight in kilograms divided by height in meters squared. Serum total cholesterol and HDL levels were measured using standard laboratory techniques. We were able to consider the use of antihypertensive medication and statin use, which were obtained from pharmacy records (15).

Statistical analysis

We used the means (standard deviations [SD]), medians (interquartile ranges [IQRs]) and percentages to describe the distribution of continuous and categorical variables, respectively. To investigate the association between antithrombotic treatment and plaque components, intraplaque hemorrhage, lipid core, and calcification, a three-step statistical analysis approach was used. Initially, we prepared three models, using logistic regression, to assess the association of antithrombotic treatment (never, current, past) with the presence of each component in one or both carotid arteries. In the first model, we adjusted these analyses for age and sex and in second model we adjusted for other risk factors. Additionally, in model three for vitamin K antagonists users, we adjusted for the international normalized ratios levels. Further, as a second step, we examined whether the duration of antithrombotic treatment

was associated with any of the plaque components. Third, we assessed whether the international normalized ratios levels for vitamin K antagonists and daily defined dosages for antiplatelet agents were associated with each plaque component. Finally, we performed sensitivity analyses and stratified analyses. First, to address confounding by indication, we restricted analyses among subjects without known cardiovascular diseases (CVD), only by excluding the participants with known CVD history and performed the propensity-score matching for untreated and treated participants. Cardiovascular diseases were defined as known and verified the history of stroke, myocardial infarction or coronary heart disease. Second, we performed stratified analyses for sex and age below and above 70 years of age, to investigate whether associations are different between sex and age groups. Antithrombotic treatment is usually prescribed to subjects at risk for or with a history of cardiovascular diseases.

All analyses were carried out using IBM SPSS Statistical package version 21 (Chicago, IL, USA).

RESULTS

The study population characteristics are provided in Table 1. The mean age of the population was 72.9 years (SD: 9.1 years) and 46.0% were women. At the time of MRI, a total of 6.8% of the participants was using vitamin K antagonists treatment and 29.9% used antiplatelet (salicylates) treatment. The median vitamin K antagonists use

was 11 months (interquartile range, 3 – 43 months), and median antiplatelet agents use was 72 months (interquartile range, 30 – 123 months). The intraplaque hemorrhage was more frequently found in the users of antithrombotic treatment compared to never-users (Table 2).

Table 1 Baseline characteristics of the study population (n=1740)

Age, years (SD)	72.9 ± 9.1
Women, %	46.0
Smoking, current %	31.5
Diabetes mellitus, %	14.4
Systolic blood pressure, mm/Hg (SD)	145 ± 21
Diastolic blood pressure, mm/Hg (SD)	80 ± 11
BMI, kg/m^2 (SD)	27.3 ± 3.5
Total cholesterol, mmol/L (SD)	5.6 ± 1.0
HDL cholesterol, mmol/L (SD)	1.4 ± 0.3
Use of antihypertensive medication, %	39.3
Use of statins, %	29.0
Vitamin K antagonists	
Current, %	6.8
Past, %	9.0
Antiplatelet agents use	
Current, %	29.9
Past, %	11.9
Wall thickness, mm	3.2 ± 0.6
Degree of stenosis (%)	14.4 (0.0 - 26.8)
History of stroke, %	6.3
History of coronary heart disease, %	11.4

Abbreviations: BMI = body mass index, CHD = coronary heart disease, HDL = High-density lipoprotein, IPH = intraplaque hemorrhage. Values are means with standard deviations or median (interquartile ranges) for continuous variables and percentages for dichotomous or categorical variables.

Table 2 Presence of the components in the carotid artery plaque according to antithrombotic treatment

	IPH	Lipid core	Calcification
Vitamin K antagonists			
Never Use	31.9	43.6	82.0
Current use	50.4	47.9	87.4
Past use	48.1	44.9	81.4
Antiplatelet agents			
Never Use	28.6	43.8	78.7
Current use	42.5	44.4	87.9
Past use	44.4	44.0	86.0

Abbreviations: IPH = intra-plaque hemorrhage. The values represent percentages.

Antithrombotic therapy and carotid plaque composition

Table 3 summarizes the associations of oral vitamin K antagonists and antiplatelet agents use with the three plaque components. Although not statistically significant, we found a trend that current and past use of vitamin K antagonists (adjusted odds ratio (OR): 1.88 [95% confidence interval (CI): 0.74-4.75], and OR 1.89 [95%CI: 0.91-3.93], respectively) (Table 3) and current and past use of antiplatelet agents (OR: 1.22 [95%CI: 0.91-1.62]), and OR: 1.23 [95%CI: 0.86-1.75], respectively) related to a higher presence of intraplaque hemorrhage (Table 3). We found no associations between antithrombotic treatment with a lipid core or calcification.

Table 3 Association between antithrombotic treatment and carotid artery plaque composition

	IPH OR (95%CI)	**Lipid core OR (95%CI)**	**Calcification OR (95%CI)**
Vitamin K Antagonists		Model 1	
Never Use	Ref	Ref	Ref
Current use	1.48 (1.00-2.19)	1.01 (0.69-1.49)	0.96 (0.54-1.71)
Past use	1.55 (1.10-2.19)	0.97 (0.69-1.36)	0.66 (0.42-1.03)
		Model 2	
Never Use	Ref	Ref	Ref
Current use	1.34 (0.87-2.06)	1.06 (0.70-1.59)	0.88 (0.48-1.61)
Past use	1.46 (1.01-2.12)	0.99 (0.70-1.41)	0.59 (0.37-0.94)
		Model 3	
Never Use	Ref	Ref	Ref
Current use	1.88 (0.74-4.75)	0.87 (0.36-2.11)	1.09 (0.33-3.53)
Past use	1.89 (0.91-3.93)	0.85 (0.42-1.73)	0.70 (0.28-1.77)
Antiplatelet agents use		Model 1	
Never Use	Ref	Ref	Ref
Current use	1.46 (1.16-1.84)	0.92 (0.74-1.15)	1.51 (1.10-2.07)
Past use	1.57 (1.14-2.17)	0.94 (0.69-1.29)	1.19 (0.77-1.84)
		Model 2	
Never Use	Ref	Ref	Ref
Current use	1.22 (0.91-1.62)	1.00 (0.77-1.30)	1.07 (0.74-1.54)
Past use	1.23 (0.86-1.75)	0.91 (0.66-1.27)	1.03 (0.65-1.61)

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios.
Model 1 – adjusted for age, sex. Model 2 – model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Duration of use and dosage of antithrombotic therapy

The use of vitamin K antagonists for 3 months or less was not associated with the presence of intraplaque hemorrhage whereas the use of vitamin K antagonists for more than 3 months was significantly related to the presence of intraplaque hemorrhage. This relation became more prominent when we additionally adjusted for international normalized ratios levels (adjusted OR: 3.15 [95% CI: 1.23-8.05]) (Table 4). The use of antiplatelet agents for less than or more than 30 months was not related to the presence of intraplaque hemorrhage (adjusted OR: 1.21 [95% CI: 0.88 – 1.67]) (Table 4).

Table 4 Association between duration of use of antithrombotic treatment and IPH in the carotid artery

	IPH		
Vitamin K antagonists	**Model 1 OR (95%CI)**	**Model 2 OR (95%CI)**	**Model 3 OR (95%CI)**
Never Use	Ref	Ref	Ref
Duration ≤ 3 months	0.81 (0.48-1.37)	0.76 (0.43-1.35)	1.21 (0.51-2.86)
Duration 3 – 11 months	1.63 (0.99-2.69)	1.62 (0.96-2.76)	2.54 (1.11-5.82)
Duration > 11 months	1.95 (1.35-2.82)	1.74 (1.16-2.60)	3.15 (1.23-8.05)
Antiplatelet agents use			
Never Use	Ref	Ref	
Duration ≤30 months	1.30 (0.92-1.83)	1.14 (0.78-1.66)	
Duration 30–72 months	1.54 (1.10-2.16)	1.32 (0.91-1.92)	
Duration >72 months	1.57 (1.21-2.04)	1.21 (0.88-1.67)	

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios.
Model 1 – adjusted for age, sex. Model 2 – model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Furthermore, when considering the effect of international normalized ratios and daily defined dosages, the dose-response relation was found in both groups. Among oral vitamin K antagonist users, international normalized ratios levels higher than 2.97 were significantly associated with the presence of intraplaque hemorrhage, whereas among the antiplatelet users daily defined dosages levels higher than 1.0 were significantly associated with the presence of intraplaque hemorrhage (Table 5 and Figure 2).

Table 5 Association of antithrombotic treatment with IPH, according to international normalized ratios (INR) for vitamin K antagonists and daily defined dosage (DDD) for antiplatelet agents

	IPH
Vitamin K antagonists	**OR (95%CI)**
INR ≤ 2.68	1.56 (0.94-2.56)
INR 2.69-2.97	1.54 (0.93-2.54)
INR > 2.97	1.48 (1.03-2.15)
Antiplatelet agents	
DDD ≤ 0.99	0.71 (0.29-1.76)
DDD 1.00 – 1.99	1.50 (1.21-1.87)
DDD > 2.00	2.24 (1.03-4.87)

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios. The values are adjusted for age and sex.

When restricting our analyses only to subjects without a history of cardiovascular diseases, we found a prominent positive trend with intraplaque hemorrhage, among current and past users of vitamin K antagonists, and a positive trend with intraplaque hemorrhage among current and past users of antiplatelet agents (Supplementary

Table 1). Further, propensity-score matched analyses yielded similar results (Supplementary Table 2).

Additionally, investigating for age differences, we found a more prominent association of vitamin K antagonists current use with intraplaque hemorrhage in the younger age group ≤70 for both drug groups (Supplementary Table 3). Moreover, when investigating the sex differences, we found a prominent trend between vitamin K antagonists use and intraplaque hemorrhage in females compared to males and similar trend between antiplatelet agents use and intraplaque hemorrhage for both sexes (Supplementary Table 4).

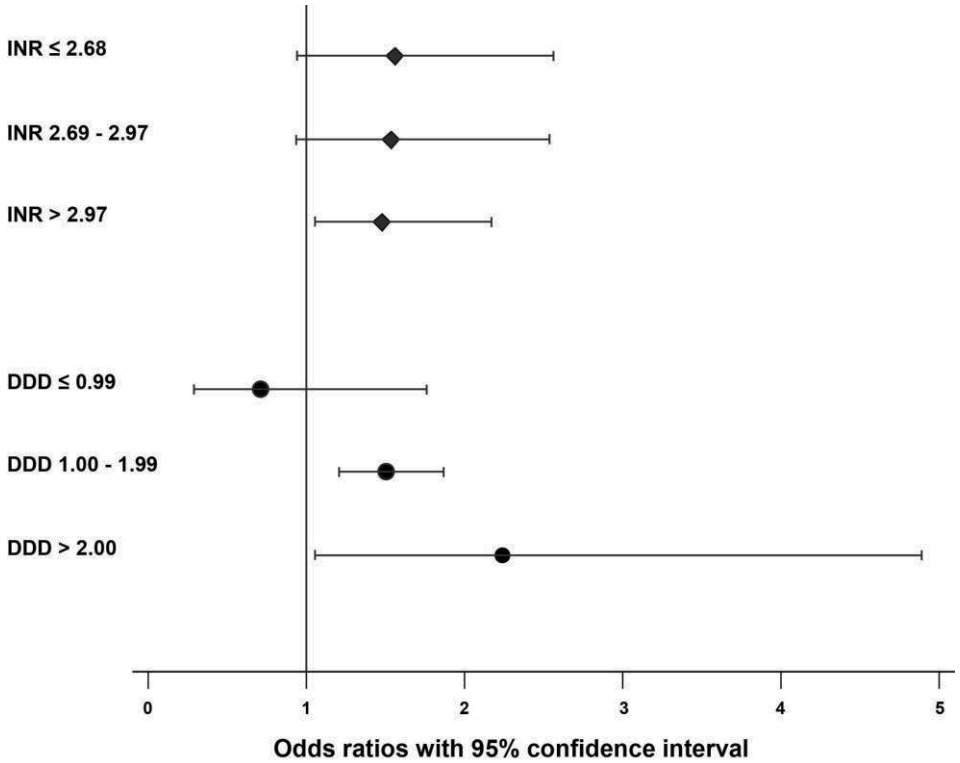


Figure 2 Association of antithrombotic treatment with IPH, according to international normalized ratios (INR) for vitamin K antagonists and daily defined dosage (DDD) for antiplatelet agents. Values on the y-axis represent the odds ratios and 95% confidence interval. The values are adjusted for age and sex. The p-trend in both groups < 0.001.

DISCUSSION

In this large population-based sample of individuals with subclinical carotid atherosclerosis, we observed that current and past use of antithrombotic treatment is associated with intraplaque hemorrhage in the carotid artery plaques. Moreover, we found that longer duration of use and higher dosages of antithrombotic treatment were related to a higher frequency of intraplaque hemorrhage.

The association of antithrombotic treatment with intraplaque hemorrhage in atherosclerotic plaques has been studied before, but only in high-risk, symptomatic patients. In these studies, it was found that the use of antithrombotic treatment related to the presence of intraplaque hemorrhage (8, 28, 29). Moreover, a histopathological study on carotid endarterectomy specimens demonstrated an effect of antithrombotic treatment on the presence of intraplaque hemorrhage, in particular of vitamin K antagonists (28). Apart from this, the earlier study also found that before surgical intervention the users of antiplatelet agents, suffered a higher presence of multiple hemorrhages in plaques (68% compared to 17%) compared to patients who did not use (29). Finally, a recent report from the PARISK-study highlighted an association of antiplatelet agents with intraplaque hemorrhage (8). Our results further elucidate the effect of antithrombotic treatment on intraplaque hemorrhage in carotid atherosclerosis on multiple levels and corroborate findings of studies with symptomatic high-risk patients. First, we demonstrated that already in the subclinical phase of carotid atherosclerosis there is a prominent association of antithrombotic treatment. Second, duration of use is important with regard to the presence of intraplaque hemorrhage. Third, independently of other risk factors higher levels of international normalized ratios and daily defined dosages influence on the presence of intraplaque hemorrhage.

One of the main mechanisms underlying the relation of antithrombotic treatment with a higher presence of intraplaque hemorrhage may be the leakage of neovessels (neovascularization) in the plaque under influence of antithrombotic treatment (8). Histological observations suggest that intraplaque hemorrhage arises from the adventitia (30), as inward sprouting neovessels towards the plaque and neovessels are considered immature and highly susceptible to leakage (31). In this context, we hypothesize that antithrombotic treatment may predispose persons to extended leakage from neovessels, due to their antithrombotic effects. Additionally, our findings may be explained through prior histopathological evidence, which suggests that oral vitamin K antagonists relate to hemorrhage from the neovessels within the plaque whereas antiplatelet agents increase densities of these neovessels (32, 33). Similarly, a capillary bleeding in humans has been found in patients using coumarin-type anticoagulation (34). On the other hand, animal studies exhibited that capillary dilation and permeability, or capillary bleeding, increase when using coumarin-type anticoagulants (35-37). Although vitamin K antagonists inhibit the vitamin K conversion cycle and salicylates inhibit the platelet aggregation and induce vasodilation, both drugs may contribute similarly with their effects in regard to intraplaque hemorrhage formation (25, 27).

These findings seem paradoxical to current knowledge as the goal of antithrombotic treatment is to prevent cardiovascular events. Of note is that the beneficial effect of

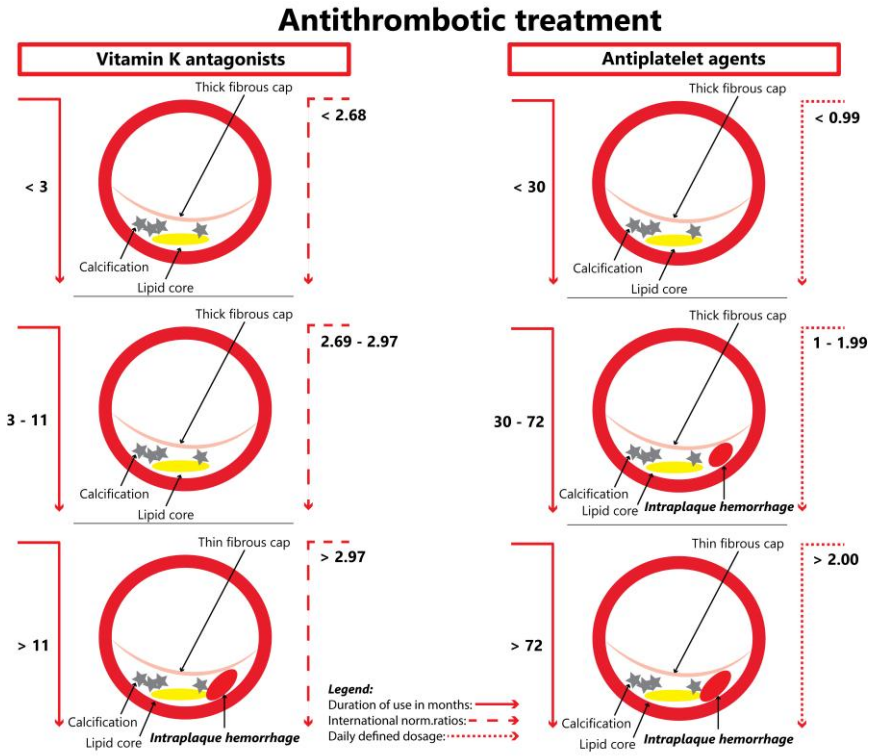
antithrombotic treatment (7) is well documented under medical conditions such as ischemic cardiovascular or cerebrovascular diseases (6, 38, 39). Yet, our findings emphasize that antithrombotic treatment should be used with care and in low dosage (7) and not prescribed unless the clinical benefits outweigh the risks. With regard to the clinical effects of antithrombotic treatment, a meta-analysis of two large trials (International stroke trial and Chinese acute stroke trial), revealed that antithrombotic treatment reduced the risk of ischemic stroke only in the 6-12 weeks directly after the stroke, but established no beneficial effect after this period (40). Due to the observational nature of the study, the findings should be regarded as hypothesis generating and further confirmation is required.

The major strengths of the current study include the long follow-up time, the duration and dosage of exposure to antithrombotic treatment for all study participants, as well as the standardized MRI-based assessment of carotid atherosclerotic plaques. This is the first population-based study to investigate the association of antithrombotic treatment with plaque components in the carotid artery within a relatively large study sample. Moreover, the MRI enabled to characterize in great detail each specific component of the carotid plaque. Nonetheless, some limitations of our study should also be taken into account. First, we did not have data on the use of new oral anticoagulants (NOAC's). Second, as in many observational studies investigating the effect of specific medication, we should

acknowledge the issue of confounding-by-indication given that antithrombotic treatment is more often prescribed to persons with an increased risk of cardiovascular diseases. However, sensitivity analysis did not show prominent differences in the results. Third, although salicylates are prescribed by doctors, such drugs are available 'over the counter', and used for other therapeutic indications for e.g. like analgesics. Fourth, the cross-sectional design limits our ability to draw causal inferences between antithrombotic treatment and intraplaque hemorrhage.

Conclusion

The use of antithrombotic treatment relates to a higher frequency of intraplaque hemorrhage in carotid atherosclerotic plaques. Further studies are warranted to replicate this finding in a longitudinal design.



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Supplementary material

Supplementary Table 1 Association between antithrombotic treatment and IPH in individuals free of stroke, myocardial infarction, and coronary heart disease (n= 1360)

	IPH		
Vitamin K antagonists	**Model 1 OR (95%CI)**	**Model 2 OR (95%CI)**	**Model 3 OR (95%CI)**
Never Use	Ref	Ref	Ref
Current use	1.44 (0.86-2.41)	1.40 (0.80-2.43)	3.12 (0.92-10.60)
Past use	1.34 (0.89-2.00)	1.31 (0.85-2.03)	2.34 (0.95-5.74)
Antiplatelet agents			
Never Use	Ref	Ref	
Current use	1.54 (1.15-2.05)	1.15 (0.81-1.61)	
Past use	1.33 (0.90-1.96)	1.16 (0.77-1.77)	

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios. Model 1 - adjusted for age, sex. Model 2 - model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Supplementary Table 2 Association between antithrombotic treatment and IPH in propensity-score matched individuals

	IPH		
Vitamin K antagonists	**Model 1 OR (95%CI)**	**Model 2 OR (95%CI)**	**Model 3 OR (95%CI)**
Never Use (N=247)	Ref	Ref	Ref
Vitamin K antagonists use (N=247)	1.39 (0.96 – 2.00)	1.49 (1.00-2.26)	2.23 (0.97 – 5.18)
Antiplatelet agents			
Never Use (N=378)	Ref	Ref	
Antiplatelet use (N=378)	1.16 (0.86-1.57)	1.16 (0.84-1.60)	

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios. Model 1 - adjusted for age, sex. Model 2 - model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Supplementary Table 3 Association between antithrombotic treatment and IPH in the carotid artery, stratified by age

	IPH		
Age ≤ 70 years (n=604) Vitamin K antagonists	**Model 1 OR (95%CI)**	**Model 2 OR (95%CI)**	**Model 3 OR (95%CI)**
Never Use	Ref	Ref	Ref
Current use	6.29 (2.05-19.30)	5.17 (1.55-17.20)	7.72 (0.99-60.35)
Past use	1.26 (0.54-2.95)	1.10 (0.45-2.67)	1.70 (0.24-12.06)
Antiplatelet agents			
Never Use	Ref	Ref	
Current use	1.76 (1.13-2.74)	1.70 (0.97-2.99)	
Past use	2.19 (1.05-4.60)	1.83 (0.83-4.01)	
Age > 70 years (n=1136) Vitamin K antagonists			
Never Use	Ref	Ref	Ref
Current use	1.39 (0.92-2.09)	1.34 (0.84-2.13)	1.48 (0.50-4.35)
Past use	1.79 (1.23-2.62)	1.70 (1.12-2.58)	1.84 (0.81-4.15)
Antiplatelet agents			
Never Use	Ref	Ref	
Current use	1.51 (1.16-1.98)	1.12 (0.81-1.57)	
Past use	1.62 (1.14-2.31)	1.20 (0.80-1.78)	

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios.

Model 1 - adjusted for age, sex. Model 2 - model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Supplementary Table 4 Association between antithrombotic treatment and IPH in the carotid artery, stratified by sex

	IPH		
Female (n=800) Vitamin K antagonists	**Model 1 OR (95%CI)**	**Model 2 OR (95%CI)**	**Model 3 OR (95%CI)**
Never Use	Ref	Ref	Ref
Current use	1.36 (0.69-2.65)	1.19 (0.58-2.42)	4.11 (0.86-19.51)
Past use	1.39 (0.82-2.36)	1.42 (0.81-2.51)	3.44 (1.11-10.63)
Antiplatelet agents			
Never Use	Ref	Ref	
Current use	1.46 (1.01-2.12)	1.20 (0.77-1.86)	
Past use	1.35 (0.85-2.17)	1.15 (0.70-1.93)	
Male (n=940) Vitamin K antagonists			
Never Use	Ref	Ref	Ref
Current use	1.57 (0.96-2.55)	1.43 (0.83-2.46)	1.02 (0.30-3.41)
Past use	1.67 (1.06-2.64)	1.47 (0.89-2.40)	1.11 (0.41-3.03)
Antiplatelet agents			
Never Use	Ref	Ref	
Current use	1.45 (1.08-1.95)	1.21 (0.83-1.75)	
Past use	1.84 (1.17-2.89)	1.32 (0.80-2.18)	

Abbreviations: CI = confidence interval, INR = international normalized ratio, IPH = intra-plaque hemorrhage, OR = odds ratios.

Model 1 - adjusted for age, sex. Model 2 - model 1 + smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, body mass index, total cholesterol, high-density lipoprotein, antihypertensive medication use, statin use and carotid wall thickness. Model 3 – model 2+ average INR.

Supplementary Table 5 Comparison of the baseline characteristics between untreated and treated subjects in unmatched sample

Vitamin K Antagonists	Untreated (N=1465)	Treated (N=275)	p-value	Standardized differences
Age, years (SD)	72.0 ± 9.2	77.6 ± 7.4	0.00	0.670
Women, %	47.1	40.0	0.00	–0.143
Smoking, current %	32.1	28.4	0.05	–0.080
Diabetes mellitus, %	13.6	18.9	0.00	–0.144
Systolic blood pressure, mm/Hg (SD)	145 ± 20	147 ± 21	0.04	0.097
Diastolic blood pressure, mm/Hg (SD)	80 ± 10	79 ± 11	0.06	–0.095
BMI, kg/m^2 (SD)	27.2 ± 3.5	27.9 ± 3.8	0.01	0.191
Total cholesterol, mmol/L (SD)	5.6 ± 1.0	5.3 ± 1.0	0.00	–0.3
HDL cholesterol, mmol/L (SD)	1.4 ± 0.3	1.3 ± 0.3	0.02	–0.333
Use of antihypertensive medication, %	37.2	50.5	0.00	0.270
Use of statins, %	27.0	39.6	0.00	0.269
Wall thickness, mm	3.2 ± 0.6	3.4 ± 0.8	0.00	0.281

Continues variables are reported as means ± standard deviation. Dichotomous variables are reported as percentages.

Supplementary Table 6 Comparison of the baseline characteristics between untreated and treated subjects in matched sample

Vitamin K Antagonists	Untreated (N=247)	Treated (N=247)	p-value	Standardized differences
Age, years (SD)	75.3 ± 8.0	77.5 ± 7.6	0.00	0.281
Women, %	44.9	39.3	0.01	–0.113
Smoking, current %	30.4	30.4	0.56	0
Diabetes mellitus, %	16.2	20.2	0.02	0.103
Systolic blood pressure, mm/Hg (SD)	149 ± 21	147 ± 21	0.13	–0.095
Diastolic blood pressure, mm/Hg (SD)	79 ± 11	79 ± 11	0.72	0
BMI, kg/m^2 (SD)	27.3 ± 3.1	28.0 ± 3.7	0.01	0.205
Total cholesterol, mmol/L (SD)	5.4 ± 1.0	5.4 ± 1.0	0.55	0
HDL cholesterol, mmol/L (SD)	1.3 ± 0.3	1.3 ± 0.3	0.44	0
Use of antihypertensive medication, %	48.6	49.0	0.33	0.008
Use of statins, %	42.1	36.8	0.02	–0.108
Wall thickness, mm	3.3 ± 0.8	3.4 ± 0.8	0.24	0.125

Continues variables are reported as means ± standard deviation. Dichotomous variables are reported as percentages.

Supplementary Table 7 Comparison of the baseline characteristics between untreated and treated subjects in unmatched sample

Antiplatelet agents	Untreated (N=1013)	Treated (N=727)	p-value	Standardized difference
Age, years (SD)	70.9 ± 9.3	75.6 ± 8.1	0.00	0.538
Women, %	47.8	43.5	0.00	–0.086
Smoking, current %	32.4	30.3	0.18	–0.045
Diabetes mellitus, %	13.2	16.1	0.00	0.082
Systolic blood pressure, mm/Hg (SD)	143 ± 20	148 ± 20	0.00	0.25
Diastolic blood pressure, mm/Hg (SD)	81 ± 10	79 ± 11	0.00	–0.190
BMI, kg/m^2 (SD)	27.3 ± 3.6	27.3 ± 3.5	0.86	0
Total cholesterol, mmol/L (SD)	5.8 ± 0.9	5.3 ± 1.0	0.00	–0.525
HDL cholesterol, mmol/L (SD)	1.4 ± 0.3	1.3 ± 0.3	0.00	–0.333
Use of antihypertensive medication, %	30.1	52.1	0.00	0.458
Use of statins, %	13.3	50.9	0.00	0.879
Wall thickness, mm	3.1 ± 0.6	3.3 ± 0.7	0.00	0.306

Continues variables are reported as means ± standard deviation. Dichotomous variables are reported as percentages.

Supplementary Table 8 Comparison of the baseline characteristics between untreated and treated subjects in matched sample

Antiplatelet agents	Untreated (N=378)	Treated (N=378)	p-value	Standardized difference
Age, years (SD)	74.9 ± 8.4	74.5 ± 8.4	0.48	–0.047
Women, %	45.5	48.1	0.17	0.052
Smoking, current %	30.7	33.1	0.15	0.051
Diabetes mellitus, %	16.4	16.7	0.84	0.008
Systolic blood pressure, mm/Hg (SD)	147 ± 19	147 ± 20	0.77	0
Diastolic blood pressure, mm/Hg (SD)	80 ± 9.9	80 ± 10	0.74	0
BMI, kg/m^2 (SD)	27.3 ± 3.5	27.4 ± 3.6	0.77	0.028
Total cholesterol, mmol/L (SD)	5.6 ± 0.9	5.6 ± 1.0	0.28	0
HDL cholesterol, mmol/L (SD)	1.3 ± 0.4	1.3 ± 0.3	0.84	0
Use of antihypertensive medication, %	42.1	43.1	0.57	0.020
Use of statins, %	28.6	30.4	0.26	0.039
Wall thickness, mm	3.2 ± 0.6	3.2 ± 0.7	0.78	0

Continues variables are reported as means ± standard deviation. Dichotomous variables are reported as percentages.

Chapter 5

General Discussion

In this chapter, the main findings of the thesis are briefly summarized, methodological considerations related to the studies are discussed, and the clinical implications of the findings along with directions for future research will be outlined.

MAIN FINDINGS

QUANTIFICATION OF CALCIFICATION USING MRI

Carotid artery atherosclerosis is an established risk factor for stroke (1, 2). A key aim of the prevention of cardiovascular events, including stroke (3), is the early identification and treatment of subjects at high risk (4). Calcification is considered as a surrogate imaging marker of underlying atherosclerosis (5). Computed tomography (CT) and magnetic resonance imaging (MRI) enable detection and visualization of the carotid plaque components, although non-contrasted CT is superior to any other imaging modality (6) in the detection of calcification, yet, it cannot reliably distinguish between intraplaque hemorrhage (IPH) and lipid core (7, 8). While non-contrast enhanced MRI is useful to discriminate plaque components such as IPH and lipid core, recent technological developments now also allows for detection and quantification of calcification (9). However, whether these measurements are as accurate as those obtained with CT remains unclear. To address this gap, in chapter 2.1 we compared the absolute volumes of calcification, within atherosclerotic plaque in the carotid artery, measured by CT

and MRI, among individuals with subclinical atherosclerosis in a population-based study (The Rotterdam Study). Our results indicated that MRI-based calcifications volumes are comparable and highly correlated (correlation coefficient: 0.86) with CT-based volume. Aiming to provide better evidence for the correlation, we investigated whether calcification volumes measured with both modalities provide similar risk estimates in relation to the history of stroke, in another way confirming the correlation of the calcification volumes between MRI and CT.

Further, considering that our study was performed in subjects with subclinical atherosclerosis often asymptomatic and with a low stenosis degree of less than 28%, MRI was capable to detect the calcification even in small carotid plaques. Additional arguments in support to our findings come from the results of a study in symptomatic subjects and stenosis degree less or higher than 70% in the carotid arteries, suggesting a strong correlation of up to 0.73, between CT and MRI in the measurements of calcifications (10).

In summary, magnetic resonance imaging (MRI) is a reliable tool for assessing of volumetric quantities of carotid plaque components, including calcification and soft tissue components, and may be considered a relevant tool for clinical examination, research, and clinical decision-making.

ROLE OF CIRCULATING HORMONES IN CAROTID ATHEROSCLEROSIS.

Specific hormonal levels have been associated with the composition of the atherosclerotic plaque and its progression hence increasing the risk for cardiovascular events (13, 14). Hormones as risk factors (studied in chapter 3.1 and 3.2) play an important role in the presence of vulnerable components of atherosclerotic plaque. As plaque vulnerability is directly related to the changes of a plaque-to-rupture and potential risk of clinical events (15), serum insulin levels might provide relevant information for early risk assessment of patients.

Insulin

Diabetes mellitus is a well-established risk for cardiovascular diseases, including ischemic stroke (16, 17). Dysregulation of the insulin and glucose levels are the main features of the diabetes mellitus pathophysiology. Diabetes mellitus is associated with an increased risk of cardiovascular diseases due to accelerated atherosclerosis (18-21). The leading paradigm on the role of diabetes mellitus (22), is attributed to the impaired and high glucose levels (23, 24), namely hyperglycemia (25), to be the main cause of vascular complications, which later translates consequently to cardiovascular events (26). However, a recent meta-analysis of 102 prospective studies reported that diabetes mellitus confers excess risk for vascular complications and in subjects without diabetes, glucose levels were non-linearly associated with risk of vascular diseases, namely the levels

below and above of 7.0 mmol/L were associated with increased risk for coronary heart disease and ischemic stroke (27), which suggests that the deleterious effect of diabetes mellitus is not limited to glucose levels. Our results in this thesis, elucidate further the role of insulin in atherosclerotic plaque and provide further evidence regarding the pathway linking insulin levels with cardiovascular diseases, perhaps through its association with specific characteristics of the atherosclerotic plaque.

Estradiol

Sex-related differences in risk factor profiles of the atherosclerotic process remain understudied. In women, the risk for stroke roughly doubles during the 10-years after the menopausal transition (28). These gender differences might be driven by endogenous gender hormones and after menopause may influence the plaque composition to increase the risk of stroke thereafter. Previously, experimental evidence suggested a direct effect of estradiol on the vascular system, affecting plaque composition and occurrence of ischemic stroke. In contrast, testosterone may slow down atherosclerosis through inhibiting carotid intima-media thickening, atheroma formation, hence plaque stability (29). Further, impaired levels of estradiol in the postmenopausal woman may explain the gender differences in terms of cardiovascular risk. Our study furthermore provides a body of evidence by showing an increased risk of stroke in women with established

carotid atherosclerosis. This way unraveling a cascade of mechanisms of ischemic etiology between the role of hormones on atherosclerotic plaque and stroke.

ROLE OF CARDIOVASCULAR TREATMENT IN THE COMPOSITION OF THE ATHEROSCLEROTIC PLAQUE IN THE CAROTID ARTERY

Statins

Statin therapy is a crucial treatment for cardiovascular diseases (30, 31), specifically of ischemic origin. In this context, low-density lipoprotein (LDL) cholesterol plays a key role (30). Statins proved to be potent drugs capable of lowering the concentrations of LDL cholesterol (32) and reducing cardiovascular events (33). The beneficial effect (34) of statins expand beyond lipid-lowering effects and might also occur by a direct effect on plaque composition (i.e. increasing the calcification and reducing the presence of vulnerable components such as lipid core) (35). In contrast to extensive research in coronary arteries (36) on the effect of the of statin therapy and mainly on calcification using computed tomography, the evidence on the effect of statin on atherosclerosis in the carotid arteries and other atherosclerotic plaque components (lipid core and intraplaque hemorrhage), is scarce. Moreover, although there was sufficient proof of statins to reduce atherosclerosis burden and increase the plaque stability, less is known about how statins influence the atherosclerotic plaque composition in the carotid arteries. In this chapter, I focused to elaborate further the effect of statins,

duration of use and dosage of use. My findings suggest that statins play implies changes to a specific composition phenotype of the atherosclerotic plaque. Statins were related to the high presence of calcification and lower presence of lipid core. Furthermore, our findings on the duration and dosage of the use of statins provide novel insights into the time-dependent effects of statins in the plaque. Thus, longer duration of use increases the presence of calcification whereas only higher dosages of statins are associated with higher levels of calcification and lower levels of lipid core. This way I highlight that duration and dosage of use could be determining factors of the plaque remodeling process and plaque morphology. In addition, in chapter 4.1, we proposed three different cutoffs for the duration of use and dosage that may serve as an initial reference for different treatment regimes in primary and secondary prevention (37).

Antithrombotics

Oral anticoagulants and antiplatelet agents provide a beneficial effect in lowering the risk for cardiovascular events, including stroke (38). As such, they play a key role in stroke prevention (39, 40). But despite the benefits of oral anticoagulants (38), vitamin K antagonists and antiplatelet agents; their effects on the development or changes in already existing atherosclerotic plaques were unknown. Considering that bleeding is a recognized side effect of antithrombotic treatment, in this chapter, I elaborated the evidence gaps in terms of duration

and dosage of use, considering that antithrombotic treatment requires long-term use. Previous studies have potentially suggested the link between the use of anticoagulant and antiplatelet with the occurrence of intraplaque hemorrhage (41, 42). Antithrombotic treatment is the key in the prevention of the cardiovascular events and such treatment is prescribed to patients at high-risk of due to therapeutic indications such as atrial fibrillation, deep venous thrombosis, coronary heart disease, but not due to atherosclerosis. However, given that our data provide novel insights on plausible mechanisms on how these drugs potentially influence on the levels of vulnerable plaque components such as intraplaque hemorrhage, which may lead to plaque instability, their use in primary prevention warrants reconsideration and in secondary prevention could require careful monitoring of the atherosclerotic plaques. However, the clinical significance of these results should be confirmed with the long-term evaluation of cardiovascular outcomes (43).

METHODOLOGICAL CONSIDERATIONS

The studies described in this thesis were conducted within The Rotterdam Study, a prospective population-based cohort study in middle-aged and older adults (44). Observational population-based studies provide a unique opportunity to study the incidence and etiology of disease, also ability to study disease in subclinical phase, and findings may be generalized to a large portion of the

population (45). The strengths of our studies include a large number of study participants, well-characterized components of the atherosclerotic plaque within the carotid artery, and extensive information in all study participants on exposure, outcome, and potential confounders. Although several advantages, the following drawbacks should be considered. Observational studies may be subject to specific types of bias, including selection bias. In our studies, we used selection criteria for MRI scan of having a thickening of 2.5 mm of the carotid intima-media thickness on ultrasound, widely accepted as optimal criteria for the assessment of subclinical atherosclerosis in a population-studies (46, 47), for an additional MRI scan on selected participants of the Rotterdam Study. When the cutoff 2.5 mm was used a selection bias might have been introduced. However, when we elaborated the MRI scans the absence of plaque bilaterally was found in several participants, that were excluded from the analyses. This information may confirm that the use of low cutoff of 2.5 mm along with the standardized protocol with ultrasound, during the screening and selection process, and MRI scanning protocol has minimized the potential selection bias. Further, potential confounders may have had some measurement error or misclassification and could have been affected by measurement error. Then, in chapter 4 information bias on drug use in context of subjects' drug adherence to treatment or the use of medication such as aspirin for other therapeutic indications e.g. analgesic,

might be an issue as potentially introduced unmeasured bias, and due to number of subjects with a known history of CVD concerns of confounding by indication may be valid. However, to address these types of bias and potential confounding, we have performed multiple level testing and extensive sensitivity analyses to control for the misleading effects of bias and confounders.

In this thesis, several studies are characterized by cross-sectional design analyses. The known limitation of such analyses is the lack of ability to establish a temporal effect, i.e. namely whether determinant precedes the outcome. Specifically, this applies to chapters 3.1, 4.1 and 4.2. Nonetheless, using pharmacy records we were able to accurately investigate the retrospective duration of use of these treatments, chapters 4.1 and 4.2, that might provide to some extent evidence for the temporal relationship between exposure and outcome. Yet, here it can be only speculated but does not reflect a causal explanation of these processes. However, prospective analyses in chapter 3.2 with long term follow-up of participants provided evidence for the temporal relation between the exposure and outcome.

Another important aspect of the methodological consideration is related to MRI qualitative and quantitative measurements of the carotid plaque components. The validity of the imaging measurements has been addressed with special attention through inter-and-intraobserver variability. The results of the inter-and

-intraobserver agreement have been extensively described in chapter 2 and chapter 4 of this thesis.

CLINICAL IMPLICATIONS AND FUTURE RESEARCH

In the final part of this thesis, I focus on the following aspects which may be important in future research.

Magnetic Resonance Imaging (MRI) as a reliable imaging tool for carotid plaque assessment

MRI as an imaging technique to assess the imaging biomarkers of atherosclerosis has advantages, including being non-invasive and free of radiation exposure as with CT. In chapter 2.1 of this thesis, we used a standardized multisequence MRI imaging protocol, which provides important evidence of the capacity of the MRI to characterize the atherosclerotic plaque components, including calcification. Computed Tomography (CT) is an established imaging tool which is used in clinical and research settings but with some important shortcomings. It seems that CT is unable to characterize intraplaque hemorrhage (48), a representative feature of vulnerable plaques. Also, given the need for radiation exposure, CT seems less suitable for prospective serial imaging in asymptomatic patient populations. However, MRI does have the potential for serial imaging in the asymptomatic patient population for screening strategies without radiation

exposure. Furthermore, it may optimize clinical diagnosis and decision-making strategies in symptomatic patients with carotid atherosclerosis when it is used in clinical settings. Yet, further improvement of reproducible characterization of atherosclerotic plaque using MRI imaging is needed, including plaque component volumes. Despite the presence or absence of component, the volumetric presence of specific component might be the main determinant of the plaque rupture or vice-versa. Semi-automated or automated analysis of MRI images, including volume quantification, would reduce the extensive laborious work of imaging processing and reading and additional would provide possibilities to research coexistence of different plaque components and their relation with clinical outcomes, such as stroke. In the future involving serial MRI studies are probably more promising to understanding subclinical plaque transformation into rupture-prone plaque in asymptomatic patients, giving the prominent role of MRI use for preventive strategies of ischemic stroke. Further efforts should be put in investigating the potential role of carotid plaque component on the risk of ischemic stroke, especially their volumetric presence within the carotid artery.

Hormone levels as risk factors for atherosclerosis

The studies included in this thesis showed that circulating hormones (insulin and sex hormones) are associated with specific aspects of atherosclerosis (Chapters 3.1 and 3.2), independent of cardiovascular risk factors. The observed association between the participants with high levels of insulin were similar to those who were free of diabetes mellitus, indicating that serum insulin might be directly involved in the atherosclerotic plaque development, already before the DM is clinically overt. Although our study is a novel concept in the field, further research is warranted to understand the mechanisms of actions and the potential link between dysregulated serum insulin levels and atherosclerotic plaque development. The previous study addressed the role of the serum insulin on cardiovascular diseases and found out that hyperinsulinemia is an independent risk factor of ischemic heart disease (13). Whether the role of hyperinsulinemia on CVD, is indirectly through worsening of the atherosclerotic plaque, through increased presence of vulnerable components e.g. intraplaque hemorrhage and lipid core, requires further longitudinal confirmation. Alternatively, longitudinal studies with throughout characterization of atherosclerosis burden in subjects free of diabetes mellitus may evaluate the direct influence of serum insulin on the development of vulnerable components and possibly relate their influence on the risk of rupture and consequently to CVD event.

On the other hand, results on circulating estradiol suggest that impaired levels, after menopause, are implicated with cardiovascular events. This further may explain the cardiovascular risk profile difference between men and women in middle-age when men are at higher risk for cardiovascular diseases than women. After menopause, hormone imbalance is breached, and women are exposed to a comparable or even higher risk for cardiovascular events than men. Importantly, our results report an association between estradiol and risk of stroke in women and regardless of whether an association is causal or not, yet it still can be very useful for prediction. Indirectly, observations between estradiol and atherosclerotic plaque components may further link the involvement of estradiol effect on atherosclerosis in overall and carotid atherosclerotic plaque in particular. Although the high levels of estradiol may act as an effect modifier, by influencing vulnerable components development indirectly, they may increase the risk of stroke directly.

In the future, from the clinical point of view, incorporating the level of circulating hormones in risk estimation scores might provide additional information and contribute to better risk assessment for cardiovascular diseases.

Cardiovascular treatment and the carotid atherosclerotic plaque

Although atherosclerosis has been considered as a progressive and chronic disease, statin treatment has shown to regress atherosclerotic plaque with high-

dose lipid-lowering therapy. In line with previous studies (49, 50) our results add to this in showing that statin treatment could transform the composition of the atherosclerotic plaque into a more stable phenotype when given in high dosages and long duration of use. A molecular mechanism by which statin statins reduce the lipid component and enhance plaque calcification, in other words, transform plaque into the more stable phenotype, remains to be elucidated. Statins provide more observable changes into plaque only when given with high-dose and long-term use, indicating that both dose and duration of use might determine the characteristics and composition of the plaque. In the future, this might be relevant clinical information when assigning patient in statin treatment for primary prevention of ischemic events due to risk of atherosclerotic thrombo-embolic events. Whether the effect of statin on the atherosclerotic plaque is direct, or whether it occurs due to their capacity to reduce the systemic lipid levels and/or reduce anti-inflammatory processes remains unclear.

Besides statins, antithrombotic treatment is often used in cardiovascular disease prevention. Bleeding is a well-known side effect of antithrombotic treatment, but thrombotic occlusion or thrombus embolization plays a major role in the pathogenesis of ischemic stroke and myocardial infarction. High-risk patients or patients with co-morbidity routinely are treated with antithrombotic treatment to prevent cardiovascular events. Moreover, intraplaque hemorrhages within

atherosclerotic plaques represent a higher risk for future vascular events and in chapter 4.2 we have provided evidence to document the possible effect of antithrombotic treatment on the presence of intraplaque hemorrhage. In addition, our results add further evidence on how high dosages and longer durations influence the presence of intraplaque hemorrhage. In the future, the next step is to investigate the potentially harmful effect of antithrombotic treatment on atherosclerotic plaque composition and cardiovascular events with further confirmation and clinical follow-up.

CONCLUSION

MRI is a promising imaging modality for the early detection and serial imaging of carotid atherosclerosis with excellent soft-tissue characterization and comparable abilities to CT in the detection of calcification. This thesis extends the current knowledge on the atherosclerotic plaque in the carotid artery with specific aspects of the role of hormones and cardiovascular treatment. Looking at a broader perspective, in this thesis we presented novel insights of the association of the circulating hormones with specific features of the plaque composition and as well as the potential influences of cardiovascular treatment in the progression or regression of vulnerable characteristics within the carotid plaque. Moreover, future research is needed to replicate our results in other longitudinal designs and populations in different geographical regions and to explore underlying

mechanisms, and potentially establish of causality between exposure and outcome.

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Chapter 6

Summary / Samenvatting

SUMMARY

In **chapter 1**, the general introduction, I provide the general background of carotid atherosclerosis, rational and the aim of the thesis. Utilizing the multi-sequenced MRI images from the approximately 1,800 participants of the Rotterdam Study, with underlying atherosclerotic burden, I examined the roles of hormones and the cardiovascular treatment on the carotid plaque composition.

In chapter 2, I focused on the imaging capability of the MRI, in detection and quantification of the calcification, as a reliable biomarker of underlying atherosclerotic burden. **Chapter 2** reviewed the CT-based and MRI-based detection of the calcification, considering the CT as a golden standard for detection and quantification of calcification. Although, MRI is powerful in the detection and quantification of the soft tissue components, such as lipid core and intraplaque hemorrhage, the detection of calcification was seen as the main limitation. Quantitative measurements of the calcification have been regarded as optimal parameters to compare and validate the MRI-based capacity in the detection and quantification of the calcification against the CT-based imaging. Moreover, volumetric measurements of calcification obtained by MRI-based scan in relation to the history of ischemic stroke as an important clinical outcome provided comparable risk estimates as calcification volumetric measurements obtained by CT-based scan,

demonstrating that the utilization of MRI for the assessment of vascular calcification is similar to that of CT.

Chapter 3 is devoted to the role of the circulating hormones on the carotid plaque composition. In **chapter 3.1** I focused on investigating the association of the serum insulin levels with atherosclerotic plaque components. Diabetes mellitus is an established risk factor for vascular diseases. Two distinct characteristics of diabetes mellitus such as impaired levels of insulin and glucose have been implicated with cardiovascular complications. Still, their impact on the atherosclerotic plaque has not been previously investigated. Our results, presented in this chapter, associated only with impaired serum insulin levels, high and low, with the vulnerable plaque components, but not with impaired levels of the serum glucose. These indicate that impaired serum insulin levels are implicated with the presence of vulnerable components and act as an important part in the pathophysiology of atherosclerotic changes within carotid artery. **Chapter 3.2** describes the function of sex hormones in the composition of the carotid plaque and their potential implications with the risk of stroke. Gender differences in risk profiles between men and women for cardiovascular diseases might be explained by endogenous hormones. Women after the menopause are imposed on higher cardiovascular risk, and sex steroids may take on a role to influence on development of more vulnerable carotid plaque and thereby increase the stroke incidence when taking into account the levels of total

estradiol. Still, total testosterone was not found to impose a higher risk in either gender.

Chapter 4 I aimed to investigate the effect of cardiovascular treatment on the carotid plaque composition. Statins, in **chapter 4.1**, through their pleiotropic effect proved to influence on the composition of atherosclerotic plaque, by shifting the composition towards more stable phenotype, and increasing the presence of calcification and reducing the presence of lipid core. Although statins are able to change the composition, their duration and dosage of use yielded to be determining factor of their effect on the remodeling of atherosclerotic plaque and plaque stabilization process. Recounting to the vulnerable plaque concept, plaque component such as intraplaque hemorrhage is thought to predispose the plaque to rupture. In this context, **chapter 4.2**, I investigated the association of antithrombotic treatment with the presence of intraplaque hemorrhage within the atherosclerotic plaque. Antithrombotic treatment may impose a harmful role in the composition of carotid plaque by increasing the risk of neovascularization and through extended leakage to higher presence of intraplaque hemorrhage, which at a later stage may translate to a higher risk for plaque rupture. Yet, antithrombotic treatment is the main frontier of cardiovascular events prevention and present findings require further confirmation.

In **chapter 5**, the main findings, as well as methodological considerations, are discussed along with potential clinical implications and future research.

SAMENVATTING

In **hoofdstuk 1**, de algemene inleiding, schets ik de algemene achtergrond van carotis-atherosclerose en geef ik de rationale en het doel van het proefschrift weer. Gebruikmakend van multi-sequence MRI-beelden van de ongeveer 1.800 deelnemers aan de Rotterdam Study met onderliggende atherosclerotische belasting, onderzocht ik het effect van hormonen en de cardiovasculaire medicatie op de samenstelling van de carotis-plaque.

In **hoofdstuk 2** richtte ik me op de mogelijkheden van MRI om calcificaties, een betrouwbare biomarker van atherosclerotische belasting, te detecteren en kwantificeren. CT wordt beschouwd als de gouden standaard hiervoor. Hoewel MRI erg geschikt is voor de detectie en kwantificering van zacht weefsel-componenten, zoals de lipidenkern of een intra-plaque bloeding, werd de detectie van calcificaties gezien als de belangrijkste beperking van MRI. Volumetrische metingen van calcificaties op basis van MRI leidden tot vergelijkbare risicoschattingen als CT met betrekking tot voorgeschiedenis van ischemische beroerte. Dit toont aan dat MRI evenwaardig is aan CT voor de beoordeling van vasculaire calcificaties.

Hoofdstuk 3 is gewijd aan de rol van de circulerende hormonen in de samenstelling van carotis-atherosclerose. In **hoofdstuk 3.1** heb ik me gericht op het de associatie van serum-insulinespiegels met componenten van atherosclerotische plaque.

Diabetes mellitus is een gekende risicofactor voor hart- en vaatziekten. Gestoorde insuline- en glucose-spiegels, twee kenmerken van diabetes mellitus, zijn betrokken bij cardiovasculaire complicaties. Toch werd hun impact op de atherosclerotische plaque niet eerder onderzocht. Op basis van de resultaten gepresenteerd in dit hoofdstuk, zijn de kwetsbare plaque-componenten geassocieerd aan gestoorde serum-insulinespiegels (zowel te hoge als te lage spiegels), maar niet aan gestoorde glucose-spiegels. Dit geeft aan dat gestoorde serum-insulinespiegels een belangrijke rol spelen in de pathofysiologie van atherosclerotische veranderingen van de arteria carotis. **Hoofdstuk 3.2** beschrijft het effect van geslachtshormonen op de samenstelling van de carotis-plaque en hun mogelijke implicaties voor het risico op een beroerte. Verschillen tussen mannen en vrouwen in het risico op hart- en vaatziekten kunnen worden verklaard door endogene hormonen. Postmenopauzale vrouwen hebben een hoger cardiovasculair risico. Geslachtssteroïden kunnen een rol spelen bij de ontwikkeling van carotis-atherosclerose en hierdoor de incidentie van beroerte verhogen rekening houdend met de totale estradiolspiegels. Anderzijds bleek totaal testosteron niet gepaard te gaan met een hoger risico, in geen van beide geslachten.

In **Hoofdstuk 4** wilde ik het effect onderzoeken van cardiovasculaire medicatie op de samenstelling van carotis-atherosclerose. Statines bleken in **hoofdstuk 4.1** door hun pleiotrope effect de samenstelling van atherosclerotische plaque te beïnvloeden:

ze leidden tot een stabieler fenotype door de hoeveelheid calcificatie te vergroten en de lipidenkern te verkleinen. De dosis en duur van blootstelling waren bepalende factoren in hun effect op de hermodellering van atherosclerotische plaques. Het "kwetsbare plaque-concept" stelt dat componenten van de plaque, zoals intra-plaque bloedingen, de plaque vatbaar maken voor scheuren. In dit verband onderzocht ik in **hoofdstuk 4.2** de associatie van antitrombotische medicatie met intra-plaque bloeding. Antitrombotische medicatie kan een schadelijke invloed hebben op de samenstelling van de carotis-plaque, door het risico op neovascularisatie te vergroten en door een toename in intra-plaque bloedingen, hetgeen zich zou kunnen vertalen in een hoger risico op plaque-scheuren. Antitrombotische medicatie is echter een zeer belangrijke tool in de preventie van cardiovasculaire events en de huidige bevindingen dienen nog onafhankelijk bevestigd te worden.

In **hoofdstuk 5**, tenslotte, worden de belangrijkste bevindingen, methodologische overwegingen, mogelijke klinische implicaties en toekomstig onderzoek besproken.

Chapter 7

Appendices

WORDS OF GRATITUDE

The Ph.D. trajectory has been a challenging and enjoyable journey of my life. The way you encounter difficulties and joy, struggle with ups and downs, learn new things and need support, but also provides opportunities to meet new people, make new friends, colleagues, and collaborators. It also offers privileges to work as a team with excellent scientists, who directly or indirectly contributed to coming to the end of this track and therefore they deserve a special appreciation.

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An important moment during the period of my Ph.D. was when I left Erasmus MC and joined the KU Leuven, to work in the group headed by Emeritus Professor Jan A. Staessen. Dear Jan, I want to thank you for everything, and I must admit that working with you means to learn a lot, generate brilliant ideas, assess science from different perspectives and gain huge experience. I was very fortunate to become part of your team and work in a smooth, effective and productive environment. Thank you.

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From the beginning, I have dedicated this thesis is to my parents Ibush Mujaj (late father) and Hasibe Mujaj (mother), who has been my true inspiration and motivation to pursue this academic track and aim this goal. I was a very fortunate child to be born and raised by brilliant parents. I'm here today to say them thank you, and all my achievements are credited and dedicated to them.

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Sincerely yours, 3 December 2019

Blerim Mujaj Rotterdam, The Netherlands

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Yu CG, Wei FF, Yang WY, Zhang ZY, **Mujaj B**, Thijs L, Feng YM, Boggia J, Nawrot TS, Struijker-Boudier HAJ, Staessen JA. Central hemodynamics in relation to blood lead in young men prior to chronic occupational exposure. Blood Press. 2019 May 10:1-12. doi: 10.1080/08037051.2019.1610654.

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Yatabe MS, Yatabe J, Asayama K, Staessen JA, **Mujaj B**, Thijs L, Ito K, Sonoo T, Morimoto S, Ichihara A. The rationale and design of reduction of uncontrolled hypertension by Remote Monitoring and Telemedicine (REMOTE) study. Blood Press. 2018 Apr;27(2):99-105. doi: 10.1080/08037051.2017.1406306.

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MANUSCRIPTS IN PREPARATION:

Mujaj B, Bos D, Kavousi M, Lugt AV, Staessen JA, Franco OH, Vernooij MW. Serum insulin levels are associated with vulnerable plaque components in the carotid artery. *Submitted/Revision*.

Mujaj B, Zhang ZY, Yang WY, Thijs L, Wei FF, Verhamme P, Delles C, Butler J, Sever P, Cleland J, Zannad F, Staessen JA, on behalf of the Heart Omics in Ageing Investigators. Aspirin Use Increases the Risk for Incident Heart Failure: A Patient-Level Meta-Analysis. *Submitted*.

Mujaj B, Zhang ZY, Thijs L, Clark A, Mischak H, Nkuipou-Kenfack E, Cleland J, Zannad F, Staessen JA, on behalf of the Heart 'Omics' in AGEing (HOMAGE) investigators. Novel urinary peptidomics classifiers predict fatal heart failure incidents in heart failure patients: The Heart 'Omics' in AGEing (HOMAGE).
Manuscript in preparation.

PHD PORTFOLIO

PhD student	**Blerim Mujaj**
Erasmus MC Department	Epidemiology, Radiology
Promotors	Prof. dr. O.H. Franco
	Prof. dr. M.W. Vernooij
Co-promotor	Dr. D. Bos

Training	**Year**	**ECTS**
Master of Science in Clinical Epidemiology, NIHES Erasmus Medical Center, Rotterdam, the Netherlands		
Research period Master of Science	2014-2015	38.1
General courses		
Study Design	2014	4.3
Biostatistical Methods I: Basic Principles	2014	5.7
Clinical Epidemiology	2014	5.7
Methodologic Topics in Epidemiological Research	2014	1.4
Biostatistical Methods II: Classical Regression Models	2014	4.3
Principles of Research in Medicine	2014	0.7
Methods of Clinical Research	2014	0.7
Methods of Public Health Research	2014	0.7
Clinical Trials	2014	0.7
Health Economics	2014	0.7
Case-control Studies	2014	0.7
Introduction to Global Public Health	2014	0.7
Primary and Secondary Prevention Research	2014	0.7
Causal Inferences	2014	0.7
Markers and Prediction Research	2014	0.7

The Practice of Epidemiological Analysis	2014	0.7
Fundaments of Medical Decision Making	2014	0.7
Advanced courses		
Bayesian Statistics	2015	1.4
Woman's Health	2015	0.9
Principles of Epidemiological Data-analysis	2015	0.7
Planning and Evaluation of Screening	2015	1.4
Skills Courses		
English Language	2014	1.4
Introduction to Medical Writing	2015	1.1
Courses for the Quantitative Researcher	2015	1.4

Conferences – Oral presentations

European Atherosclerosis Society Congress, Prague, Czech *CT and MRI on carotid artery calcification*	2017	0.7
Kosovo Society of Cardiology Congress, Prishtina, Kosovo *CT and MRI on carotid artery calcification*	2017	0.7
European Society of Cardiology Congress, Barcelona, Spain *Antithrombotic treatment and intraplaque hemorrhage*	2017	0.7
European Society of Cardiology Congress, Munich, Germany *Serum insulin levels and carotid artery plaque composition*	2018	0.7
European Atherosclerosis Society Congress, Maastricht, NL *Serum insulin levels and carotid artery plaque composition*	2019	0.7

Invited speaker

Kosovo Society of Cardiology Congress, Prishtina, Kosovo	2019	0.7
Serum insulin levels and carotid artery plaque composition		

Conferences – Poster and moderated poster presentations

European Society of Cardiology Congress, Barcelona, Spain	2017	0.7
Statins and carotid atherosclerosis		
Scandinavian Society of Atherosclerosis Annual Meeting	2018	0.7
Statins and carotid atherosclerosis		
CVOT Summit, Munich Germany	2018	0.7
Serum insulin levels and carotid artery plaque composition		
ESC Heart and Stroke Conference*, Berlin Germany*	*2019*	*0.7*
Serum insulin levels and carotid artery plaque composition		
European Endocrinology Society Congress, Lyon, France	2019	0.7
Serum insulin levels and carotid artery plaque composition		
European Heart Failure Society Congress, Athens, Greece	2019	0.7
Novel urinary peptidomics classifiers predict mortality in HF patients		

Seminars and meetings

Cardiovascular Group Meetings	2015-2017	1.0
Seminars at Department of Epidemiology	2015-2017	1.0
2020 Epidemiology Meetings	2015-2017	1.0
ESC, Cardiovascular pharmacotherapy, All about clinical trials	2016	1.0

European Stroke Organization, Summer school	2016	1.0
EAS, Advanced Course in Atherothrombosis	2017	1.0
EAS, Advanced Course Hot Topics in Clinical Lipidology	2018	1.0
ESC, Aorta and peripheral circulation WG, Head to toes	2018	1.0

Peer Review for Scientific Journals

AHA Journal of Hypertension	0.5
AHA Atherosclerosis, Thrombosis and Vascular Biology Journal	0.5
BMC Cardiovascular Disorders Journal	0.5

Scholarship and grants

ERAWEB Scholarship	2014
European Stroke Organization, Summer school grant	2016
European Atherosclerosis Society, Travel grant	2017
European Society of Cardiology, Travel grant	2017
4th CVOT Summit, Travel grant	2018
European Endocrinology Society, Basic science grant	2019
European Atherosclerosis Society, Travel grant	2019
European Heart Failure Society, Travel grant	2019
5th CVOT Summit, Travel grant	2019

Awards

European Society of Endocrinology, ***Young Investigator Award*** 2019
presented at 21st Congress of European Society of Endocrinology,
held on 18-21 May 2019, in Lyon – France.

Supervision

Kaltrina Kolgeci, Exchange Student

ABOUT THE AUTHOR

Blerim Mujaj was born and raised-up in Prishtina, today the capital of the Republic of Kosovo. In the year 1998, he graduated cum laude from secondary school ¨Dr. Ali Sokoli¨ in Prishtina. In the academic year 1998/99, he registered in the Medical Faculty at the University of Prishtina but in the same year, the education process was stopped due to the war situation in Kosovo. After the war, in the year 2000/2001, the education system was re-established, and the University of Prishtina continued its educational process. Blerim graduated as a medical doctor (Doktor i Mjekësisë) from Medical Faculty of the University of Prishtina in the year 2008. After graduation, he continued for 6-months working as a physician (not-in-training) at the University Clinical Center of Kosovo, to obtain a physician's license. After licensing, Blerim has worked as a physician at the Main Family Medicine Center (Health House) in the small town of Hani Elezit. Later he joined the Emergency Medicine Center in Prishtina. During his time at the Emergency Medicine Center, he received a European License in Advanced Life Support, issued by the European Resuscitation Council. In the meantime, Blerim's scientific interest grew and he spent 3-months as a research assistant at European Homograft Bank in Brussels and was trained in cardiovascular tissue recovery and banking. The next year 2014, he received ERAWEB scholarship and was enrolled in the doctorate program at the Department of Epidemiology and Radiology of Erasmus MC, Erasmus University, in Rotterdam, the Netherlands. In September 2015 he graduated from a NIHES Master's program in Clinical Epidemiology. From 2015 onwards, Blerim continued his Ph.D. project as described in this thesis under the supervision of Prof. dr. O.H. Franco. In year 2016 he completed the clinical training in occupational medicine. Later, from the middle of 2017, he joined the KU Leuven – Department of Cardiovascular Sciences, Unit Hypertension and Cardiovascular Epidemiology, Leuven, Belgium, led by Emeritus Professor Jan A. Staessen, to work in the multi-center research project Heart Omics in Aging (HOMAGE). In 2019, he won the Young Investigator Award presented by European Society of Endocrinology, during the annual congress of the society in Lyon, France.