Imaging of arterial wall inflammation

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Imaging of Arterial Wall Inflammation

Aart Christiaan Strang
Imaging of Arterial Wall Inflammation

ACADEMISCH PROEFSCHRIFT

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aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
Prof. dr. D.C. van den Boom
ten overstaan van een door het college van promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 14 april 2015, te 14:00 uur

door

Aart Christiaan Strang

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1

GENERAL INTRODUCTION

Aart C. Strang
GENERAL INTRODUCTION

Background

Cardiovascular diseases are the most prevalent cause of death world-wide, killing more than 17.5 million people in 2012. With ‘developing’ countries adopting the western lifestyle, these figures are expected to rise [1].

Cardiovascular diseases (CVD) typically commence with endothelial dysfunction caused by traditional CVD risk-factors such as smoking, hypertension, dyslipidemia and systemic inflammation. The leaky endothelium allows cholesterol carrying lipoproteins to enter the sub-endothelial space, causing an inflammatory cascade which attracts monocytes into the vessel wall. These monocytes transform into macrophages and become foam cells after phagocytosis of the lipid depositions. The degree of inflammation in the vessel wall is directly related to plasma lipoprotein levels, especially the plasma level of low density lipoprotein (LDL) [2]. Plasma LDL cholesterol therefore is regarded as a principal determinant of CVD risk, which can be eliminated by decreasing LDL cholesterol levels using HMG-CoA reductase inhibitors (statins) [3,4]. Next to plasma LDL cholesterol levels, an individual’s CVD risk is routinely estimated by evaluating traditional risk factors for CVD, such as age, gender, blood pressure, smoking and diabetes; all these parameters are fed into one of the existing risk engines such as the Framingham risk algorithm [5]. Since the contribution of each risk factor may vary, important risk factors are not included in these engines (e.g. body mass index) and even more risk factors have not been identified yet, alternative strategies have been developed to improve CVD risk detection.

MRI imaging

Because imaging strategies allow detection of phenotypical presence of CVD, they have emerged as the most promising surrogate markers that allows both display presence of latent CV-disease as well as providing a tool to estimate future CVD risk. After intima media thickness (IMT) measured using B-mode ultrasonography, magnetic resonance imaging (MRI) has proven to have a superior reproducibility in measuring vessel wall dimension [6]. Among advantages of MRI are the higher imaging resolution, measurement of the entire vessel wall, endothelial sheer stress measurements and even identification of atherosclerotic plaque measurement, especially since the 3 Tesla scanner has become more widely available [7]. Besides its use as a readout of treatment efficacy in lipid lowering drugs, MRI is increasingly being used to demonstrate effects of chronic inflammatory diseases and diabetes mellitus on CVD risk.

Arterial wall atherosclerosis as a product of inflammation becomes clinically relevant after intimal hyperplasia causes stenosis and thereby prevents blood flow through the arterial lumen or if the atherosclerotic plaque ruptures and causes a downstream arterial thrombotic event. MRI allows detection of patients at risk for a cardiovascular event via identification of stenosis, and detection of vulnerable plaque prone to rupture.
by identification of plaque components, especially necrosis, which is associated with plaque vulnerability [8]. Both stenosis and plaque rupture are mediated and caused by inflammatory activity in the arterial vessel wall [2,9]. Unfortunately, MRI is less well equipped to simultaneously address functional plaque properties, comprising the inflammatory activity in the plaque.

**FDG-PET imaging**

$^{18}$F-fluorodeoxyglucose positron emission tomography with computed tomography (FDG-PET/CT) imaging allows measurement of arterial wall metabolic activity, which at the level of the atherosclerotic vessel wall has been shown to closely correlate with inflammation since tracer uptake is predominantly found in macrophage rich areas [10] and has been associated with both macrophage content [11] and inflammatory gene expression [12]. In this respect, it has been proposed to use PET/CT, apart from its predominantly oncological applications, to assess inflammatory activity in atherosclerotic plaques. Especially in chronic inflammatory diseases like rheumatoid arthritis (RA), effects of anti-inflammatory regimens on the arterial wall inflammation may provide insight in the pathophysiology of the increased CVD risk in chronic inflammatory diseases. In addition, it allows to detect the effect of these anti-inflammatory regimens on the inflammatory activity within the atherosclerotic vessel wall, which is likely to translate into alterations of the CVD risk. By directly measuring effects on arterial wall inflammation, the complex interactions of the chronic inflammatory disease, traditional risk factors and even undesired effects of novel anti-inflammatory drugs, mostly biologicals, will be bypassed. In this scenario, FDG-PET/CT may provide an answer to the question if the pro-atherogenic changes in the lipid profile as observed in anti-TNF therapy [13], and even stronger in anti-IL-6 antibody therapy [14], result in increased or decreased risk of CVD if anti-inflammatory effects are weight against undesired effects on the lipid profile.

**HDL cholesterol**

In an attempt to decrease the arterial wall inflammatory activity in presence or absence of chronic inflammatory diseases, among more subtle methods than biologicals, high density lipoprotein (HDL)-targeted therapies appear to be promising. In contrast to the pro-atherogenic lipoprotein LDL, the cholesterol contained within HDL is inversely associated with risk of coronary heart disease and is a key component of predicting CVD risk [15,16]. Next to its principal role in reverse cholesterol transport, the transport of cholesterol from peripheral cells to the liver, HDL’s main component apolipoprotein-A1 (ApoA-I) improves endothelial function [17] and it modulates platelet function and coagulation [18]. Many HDL-targeted therapies aim to systemically increase plasma HDL levels. Nicotinic acid, fibrates, cholesteryl ester transfer protein (CETP)-inhibitors and infusion of HDL-derived proteins, ApoA-I mimetic peptides and direct HDL-
infusions have proven to increase these plasma HDL levels [19], but until recently, only fibrates are associated with cardiovascular benefit in clinical endpoint studies [20]. First clinical endpoint trial results of CETP-inhibitors are still awaited [21,22]. Nevertheless, the convincing evidence of anti-atherosclerotic effects of HDL derived from in vitro and animal data are firm and deserve a more sophisticated application, aimed to increase HDL or ApoA-I near or in the arterial wall to decrease or fade out arterial wall inflammation, thereby tame and as a consequence decrease the CVD risk.

OUTLINE OF THE THESIS

This thesis consists of three parts. The first part focusses on reproducibility and applicability of MRI in imaging of the carotid artery as a surrogate marker for CVD risk. In chapter 2 we investigated if increased in-plane imaging resolution improves reproducibility of 3T MRI carotid artery wall dimension measurements in 31 patient with advanced atherosclerotic disease. In addition, we investigated reproducibility of carotid artery atherosclerotic plaque component identification in the same group of patients, as disclosed in chapter 3. In chapter 4, carotid artery wall dimensions using MRI are compared in 59 subjects with a history of CVD accompanied with or without type 2 diabetes mellitus at baseline and after 2 years of follow-up while using stringent guideline CVD preventing medication. Using multivariate linear regression, we set out to identify subjects that respond best and worst to guideline treatment.

In the second part, FDG-PET/CT is subjected to measure arterial vessel wall inflammation. Before use in clinical research, in chapter 5 we attempt to provide guidance for interpretation of arterial wall FDG-PET/CT by defining reference ranges for the degree of arterial wall inflammation, as well as interscan variability, interobserver and intraobserver agreement. Twenty-five healthy subjects, 23 subjects with an intermediate risk for coronary heart disease (CHD) and 35 subjects with overt CVD were scanned. These data allowed application of FDG-PET/CT to detect the effect of biological and non-biological treatment on in patients with RA in remission for at least 6 months, as described in chapter 6. Using FDG-PET/CT, next to MRI measured carotid artery wall dimensions which enables plaque detection, we are able to illustrate if arterial wall inflammation in RA in remission is extinguished as well and if degree if inflammation approximates levels in healthy subjects or CVD subjects instead. Chapter 7 illustrates the complexity of RA treatment, especially if standard treatment fails. In this chapter we identified the pathway via which tocilizumab, an interleukin-6 receptor blocker, generates undesired pro-atherogenic lipid changes, while it strongly decreases systemic inflammation.

In the third part, HDL-targeted therapies are explored in animal models, after reviewing clinical effects of genetic defects and gain-of-function mutations resulting in changed function of proteins involved in HDL’s metabolism and thereby revealing its role and importance in CVD risk reduction (chapter 8). In an attempt to profit from
HDL’s properties, two strategies were engineered to increase HDL concentrations near the inflamed arterial wall. In chapter 9, pro-endothelial and antithrombotic effects of a newly developed ApoA-I antibody coating on a metal surface were tested in vitro. This coating was additionally applied to coronary artery stents, implanted in the iliac arteries of 13 rabbits, in a side-to-side comparison with bare metal stents (BMS) in vivo. In chapter 10 we tested the benefits of hydrophilic polymer coated compared to hydrophobic polymer coated prosthetic arterial grafts in 8 goats. We tested if HDL, specifically attracted to the hydrophilic coating, is involved in guarding patency after 28 days and if adhesion of endothelial cells leucocytes and thrombocytes differ between the two coatings because HDL is bound.

REFERENCES

MRI TO QUANTIFY ARTERIAL WALL CHARACTERISTICS
INCREASING SPATIAL RESOLUTION OF 3T MRI SCANNING IMPROVES REPRODUCIBILITY OF CAROTID ARTERIAL WALL DIMENSION MEASUREMENTS

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Rob J. van der Geest, John J.P. Kastelein, Eric de Groot, Erik S.G. Stroes,
Aart J. Nederveen

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ABSTRACT

Object: To improve carotid 3T MRI dimension measurements in patients with overt atherosclerotic carotid artery disease.

Materials and Methods: In 31 patients with advanced atherosclerotic carotid artery disease two high resolution (0.25 x 0.25 mm²; HR) and two routinely used low resolution (0.50 x 0.50 mm²; LR) carotid 3T MRI scans were performed within one month. After manual delineation of carotid wall contours in a dedicated image analyses program in 8 slices covering the atherosclerotic plaque, image reproducibility, as well as the within- and between-reader variability were determined.

Results: We found significantly higher intraclass correlation coefficients for total wall volume, mean wall area as well as mean wall thickness for the HR measurements (all p < 0.05). We found a significant lower signal-to-noise (SNR) and contrast-to-noise ratio for the HR compared to the LR measurements. The carotid arterial wall dimension measurements of all parameters were significantly lower for the HR compared to the LR measurements. No significant differences were observed between the within-reader and between-reader reproducibility for HR versus LR measurements.

Conclusion: Increasing the in-plane resolution improves the reproducibility of 3T MRI carotid arterial wall dimension measurements. The use of HR imaging will contribute to a reduced sample size needed in intervention trials using MRI scanning of the carotid artery as surrogate marker for atherosclerosis progression.
INTRODUCTION

Primary and secondary prevention of cardiovascular disease is a major public health priority. The long-term, slowly progressive character of atherosclerosis offers an opportunity for early detection and treatment. Although statin therapy has proved highly effective in reducing the risk of cardiovascular events [1], residual risk remains high, and early accurate risk assessment combined with the ongoing development of novel pharmacological agents is needed to further improve primary and secondary prevention strategies. MRI has evolved as an excellent non-invasive method to obtain arterial wall dimension measurements. Improvement of MRI wall dimension measurements is thought to contribute to earlier detection of disease, improvement of risk stratification algorithms and more reliable, individual monitoring. In addition, clinical trials using MRI wall dimension measurements as a surrogate endpoint can assess the efficacy of newly developed pharmacotherapies in a relative early stage of the developmental process, guiding further possible large multi-center endpoint trials.

Previously, we have reported good correlation between 3T MRI and B-mode ultrasound carotid intima-media thickness measurements [2] in the common carotid artery, the latter being a validated surrogate endpoint in cardiovascular intervention trials [3-5]. In agreement, several other studies have demonstrated a high reproducibility [6-9] and feasibility to utilize carotid MRI as a surrogate endpoint in clinical trials [10-12]. Currently, an in-plane resolution of approximately 0.50 x 0.50 mm² is routinely used in clinical trials at 1.5 Tesla and 3 Tesla [10,11]. These resolution parameters are commonly used in carotid MRI given the trade-off between resolution, signal-to-noise ratio (SNR), contrast-to-noise (CNR) and acquisition time. These parameters are highly interdependent: higher resolution allows observation of smaller details, but typically reduces SNR, and/or increases imaging time. Therefore, increasing the imaging resolution could potentially reduce measurement reproducibility due to the lower SNR. 3T MRI has shown to yield superior SNR compared to 1.5 Tesla MRI scanners [13,7,9,14], and Balu et al. recently demonstrated that an 8 instead of a 4 channel coil configuration led to an additional improvement of the SNR [15] for carotid plaque scanning.

In an effort to further improve carotid MRI dimension measurements, we hypothesized that increasing the in-plane spatial resolution using a 3T MRI scanner equipped with a dedicated 8 channel carotid coil would improve carotid measurement reproducibility, despite the expected lower SNR. Previous studies have shown that with an increase in resolution, vessel wall MRI dimension measurements decrease and thereby more closely approach the histological or B-mode ultrasound validation measurements [16,17]. We therefore aim to show that increasing the in-plane spatial resolution will result in smaller size carotid MRI dimension measurements. To this end, we performed repeated 3T MRI scans with a dedicated 8 channel carotid coil at 0.50 x 0.50 mm² and 0.25 x 0.25 mm² in-plane resolution in patients with overt carotid atherosclerosis.
MATERIALS AND METHODS

Participants

Using previously described echo duplex measurements [2,5], consecutive individuals with one or more atherosclerotic events were screened for the presence of significant atherosclerotic disease in one of the carotid arteries. Individuals with 30 to 70% stenosis of the carotid artery were eligible for inclusion. All participants were scheduled for a scan and a rescan. Scans were performed between September 2009 and April 2011. The study complied with the Declaration of Helsinki. Written informed consent was obtained from all participants and the institutional review board of the Academic Medical Center in Amsterdam (the Netherlands) approved the protocol.

3T MRI and image analysis

MRI scans were obtained on a 3T whole-body scanner (Intera, Philips Medical Systems, Best, The Netherlands), using an 8 channel dedicated bilateral carotid artery coil (Shanghai Chenguang Medical Technologies, Shanghai, China). LR and HR measurements were compared. The non-interpolated pixel size 0.5 x 0.5 mm$^2$ is referred to as the low resolution (LR) and the non-interpolated pixel size 0.25 x 0.25 mm$^2$ is referred to as the high resolution (HR) setting. Positioning of the image stack was performed using axial magnetic resonance angiography images acquired with a time of flight (TOF) sequence covering the carotid arteries at both sides (field of view (FOV) 10 x 10 cm, 40 slices of 2 mm thickness). These images together with the ultrasound duplex data were used for planning the T1w, PDw and TOF sequences using a FOV of 6 x 6 cm, at the centre of the carotid plaque in the carotid artery with the most profound plaque burden. This could be either side of the neck. Subsequently, T1w, PDw and TOF images were acquired using ECG-gated unilateral axial turbo spin echo sequences for both the HR and LR images during each scan session. Full scan parameters are displayed in Table 1. Overview images showing the image stacks superimposed over the carotid artery were saved for planning of the repeat scan. Images were saved according to the DICOM protocol. Standardized equipment and protocols were used for image storage and data management. Quantitative image analyses were performed using dedicated measurement software (VesselMass, Leiden University Medical Center, Leiden, The Netherlands) [6]. Before any quantitative analysis was performed one reader (RD) corrected all scan and rescan images for possible Z-axis displacement using T1w, PDw and TOF images. After localisation of the carotid bifurcation both proximal and caudal scan and rescan images were compared.

Readers were fully blinded for patient characteristics and scan session. After overall inspection of T1w, PDw and TOF images, all delineation procedures of the lumen wall boundaries and the outer wall boundaries were performed manually with the aid of the VesselMass software on T1w images. Total wall volume (TWV, mm$^3$), mean wall area (MWA, mm$^2$), normalized wall index (NWI) and the mean wall thickness...
Table 1. Scan parameters for the HR and LR carotid wall measurements

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HR Black-blood T1w</th>
<th>HR Black-blood PDW</th>
<th>HR Bright blood TOF</th>
<th>LR Black-blood T1w</th>
<th>LR Black-blood PDW</th>
<th>LR Bright blood TOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>TSE</td>
<td>TSE</td>
<td>FFE</td>
<td>TSE</td>
<td>TSE</td>
<td>FFE</td>
</tr>
<tr>
<td>ECG gating</td>
<td>Yes, end diastole</td>
<td>Yes, end diastole</td>
<td>Yes, gate delay 200 ms</td>
<td>Yes, end diastole</td>
<td>Yes, end diastole</td>
<td>Yes, gate delay 200 ms</td>
</tr>
<tr>
<td>Image mode</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
</tr>
<tr>
<td>Scan plane</td>
<td>Axial</td>
<td>Axial</td>
<td>Axial</td>
<td>Axial</td>
<td>Axial</td>
<td>Axial</td>
</tr>
<tr>
<td>TR (ms)</td>
<td>1 heart beat</td>
<td>2 heart beats</td>
<td>35</td>
<td>1 heart beat</td>
<td>2 heart beats</td>
<td>19</td>
</tr>
<tr>
<td>TE (ms)</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>ETL</td>
<td>8</td>
<td>12</td>
<td>-</td>
<td>8</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>FOV (mm)</td>
<td>60 x 60</td>
<td>60 x 60</td>
<td>60 x 60</td>
<td>60 x 60</td>
<td>60 x 60</td>
<td>60 x 60</td>
</tr>
<tr>
<td>Matrix size</td>
<td>240 x 240</td>
<td>240 x 240</td>
<td>240 x 240</td>
<td>120 x 120</td>
<td>120 x 120</td>
<td>120 x 120</td>
</tr>
<tr>
<td>Resolution (mm)</td>
<td>0.25 x 0.25</td>
<td>0.25 x 0.25</td>
<td>0.25 x 0.25</td>
<td>0.5 x 0.5</td>
<td>0.5 x 0.5</td>
<td>0.5 x 0.5</td>
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<tr>
<td>Slice thickness (mm)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Flip angle</td>
<td>90</td>
<td>90</td>
<td>20</td>
<td>90</td>
<td>90</td>
<td>20</td>
</tr>
<tr>
<td>Number of slices</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Blood suppression</td>
<td>DIR</td>
<td>DIR</td>
<td>Inflow suppression (veins)</td>
<td>DIR</td>
<td>DIR</td>
<td>Inflow suppression (veins)</td>
</tr>
<tr>
<td>Fat suppression</td>
<td>SPAIR</td>
<td>SPAIR</td>
<td>-</td>
<td>SPAIR</td>
<td>SPAIR</td>
<td>-</td>
</tr>
<tr>
<td>NEX</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Scan time (minutes)*</td>
<td>4.4</td>
<td>6.6</td>
<td>2</td>
<td>2.2</td>
<td>3.3</td>
<td>1</td>
</tr>
</tbody>
</table>

TSE = turbo spin-echo, FFE = fast field echo, FOV = field of view, DIR = double inversion-recovery, NEX = number of excitations. * Scan times at heart rate of 60 min⁻¹

(MWT, mm) were assessed. Normalized wall index was calculated by dividing the wall area by the outer wall boundary area. To assess the between-reader reproducibility, two readers (DFvW and ACS) independently analysed all (both HR and LR scans and rescans) fully blinded MRI image stacks. To assess the within-reader reproducibility, one reader (DFvW), blinded to previous outcome data, analysed all (both HR and LR scans and rescans) fully blinded image stacks in a second session, 2 months after the first analysis.
Signal-to-noise ratios (SNR) were calculated as SNR = \( S/\delta \), where \( S \) is the true signal intensity corrected for the noise contribution and \( \delta \) is the true SD of the noise. \( \delta \) was calculated from the measured SD of the noise (SD\(_n\)) and the numbers of receivers: SD\(_n\) = 0.7\( \delta \) [15]. Corrected signal intensity \( S \) was obtained from the measured magnitude signal (\( S_m \)) and the measured magnitude of the background noise (\( S_n \)): \( S = (S_m^2 - S_n^2)^{1/2} \). [18] The magnitude (\( S_m \)) and the SD (SD\(_n\)) of the background noise were measured in a region of interest free of signal and free of artefacts in the corner of the image. Contrast-to-noise ratios (CNR) between wall and lumen were calculated as CNR = SNR\(_{wall}\)-SNR\(_{lumen}\).

**Statistical Analysis**

Continuous variables are expressed as mean ± SD. The SD of the paired differences (SDpd) and the coefficients of variability (CV) between the scan and the rescan were calculated for TWV, MWA, NWI, and MWT. CV was calculated by dividing the SDpd by the population mean for each parameter. Bland-Altman plots were used to illustrate systematic bias between scan and rescan images [19] for the HR and LR imaging measures (TWV, MWA, NWI, and MWT). An additional Bland-Altman plot with centralised values was used. Centralisation was performed by subtracting the mean of each imaging measure (TWV, MWA, NWI, and MWT) from the individual values contributing to the mean. The agreement between successive MRI scans, as well as the between reader and within reader agreement was assessed using intra-class correlation coefficients (ICC). An ICC of <0.40 indicated poor, one between 0.40 and 0.75 indicated fair to good, and one of >0.75 indicated excellent reproducibility[20]. Differences between the ICC of different parameters were tested with a Levene’s test. Z-values were calculated by subtracting the mean of each imaging measure (TWV, MWA, NWI, and MWT) from the individual values contributing to the mean divided by the standard deviation of each imaging measure (TWV, MWA, NWI, and MWT). Correlations between scan and rescan of calculated Z-values of all HR and LR measurements were determined. All statistical analyses were performed using PASW statistics 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

**RESULTS**

Fifty-one individuals with one or more atherosclerotic events were screened for the presence of significant atherosclerotic disease in one of the carotid arteries. Thirty-one individuals with a 30 to 70% stenosis of the carotid artery were included. Full baseline characteristics of the patient population are listed in supplementary table 1. All participants were scheduled for a scan and a rescan session. Two patients cancelled the second appointment and withdraw consent. A HR repeat scan was successfully performed in 27 patients and a LR scan was successfully performed in 25 patients. One LR scan could not be completed due to a panic attack, whereas two other subjects
didn’t complete their LR scan due to discomfort. The average interval between the scan and rescan sessions was 26 days. No significant clinical events were reported during the study.

Figure 1 depicts an example of the scan and rescan T1w images used for the HR and LR carotid MRI dimension measurements. Prior to the analyses, 2 HR and 1 LR data sets were excluded based on a poor image quality. After exclusion, 27 full repetitive sets of the HR scans, and 25 repetitive sets of the LR scans were available for the final analyses. This corresponds to 832 initial available images of which 808 images (97%) were of sufficient quality to be analysed. From these images twenty-one image stacks of the available 101 needed a Z-axis correction. Fourteen image stacks were shifted by one slice, 3 image stacks by two slices, 3 image stacks by three slices and 1 image stack needed a Z-axis correction of 4 slices. Supplementary figure 1 shows the alignment of a scan (A) and rescan (B) image stack were Z-axis displacement was corrected.

**HR scanning improves the reproducibility of carotid MRI dimension measurements**

Table 2 depicts the measurement variability and the reproducibility of the HR and LR scans.
Table 2. HR and LR carotid wall measurements with variability and reproducibility

<table>
<thead>
<tr>
<th></th>
<th>Paired-differences</th>
<th>CV (%)</th>
<th>Intraclass correlation coefficient</th>
<th>p*</th>
<th>Scan</th>
<th>Rescan</th>
<th>p†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>LR</td>
<td>HR</td>
<td>LR</td>
<td>HR</td>
<td>LR</td>
<td></td>
</tr>
<tr>
<td>TWV (mm³)</td>
<td>2.9 ± 63.5</td>
<td>11.2 ± 114.2</td>
<td>8.6 14.1</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MWA (mm²)</td>
<td>0.2 ± 4.4</td>
<td>1.4 ± 7.6</td>
<td>8.3 13.1</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NWI</td>
<td>0.004 ± 0.030</td>
<td>0.002 ± 0.039</td>
<td>4.6 5.8</td>
<td>0.98</td>
<td>0.96</td>
<td>ns</td>
<td>0.65</td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>0.02 ± 0.14</td>
<td>0.03 ± 0.20</td>
<td>6.8 9.1</td>
<td>0.99</td>
<td>0.98</td>
<td>&lt;0.05</td>
<td>2.06</td>
</tr>
</tbody>
</table>

Data are presented as mean with ± SD or as mean with (95% CI). CV = coefficient of variation, p* = p-value for Levene’s test between HR and LR intraclass correlation coefficient, p† = p-value for paired T-test between HR and LR measurements. Other abbreviations as in text.
The ICC of both the HR and LR carotid MRI dimension measurements were all greater than 0.75 (excellent). However, we found a significant smaller measurement variability for the TWV (p<0.05), the MWA (p<0.05) and the MWT (p<0.05) for the HR carotid MRI dimension measurements. Supplementary figure 2 depicts the Bland-Altman plots for all parameters of the carotid MRI dimension measurements. The significant lower measurement variability is illustrated by the smaller 95% limits of agreement for the HR scans. Figure 2 shows the correlation between Z-values of the scan and rescan measurements of both the HR and LR carotid MRI dimension measurements.

HR scanning reduces carotid MRI dimension measurements
Results for the scan and rescan TWV, MWA, NWI and MWT of both the HR and LR scans are provided in Table 2. We found significant lower dimensions for all HR carotid dimensions measurements compared to the LR carotid dimensions measurements. This is also illustrated by the significant bias (p<0.01) in the Bland-Altman plot of the centralized HR and LR carotid MRI dimension measurements (figure 3).

HR scanning decreases the SNR ratio of carotid MRI dimension measurements
We found a significantly lower mean SNR of the arterial wall for the HR compared to the LR carotid dimension measurements (17.8 ± 8.1 vs. 52.2 ± 13.4; p<0.001). The mean contrast-to-noise ratio (CNR) between the arterial wall and arterial lumen was 15.7 ± 7.2 for the HR and 48.0 ± 13.1 for the LR carotid dimension measurements (P<0.001). Mean SNR and mean CNR of the LR measurements were lower to previously published values [2].

Figure 2. Correlation between HR and LR measurements.
Correlation between Z-values of the scan and rescan HR and LR measurements.
Within-reader and between-reader reproducibility

The ICC values for the within-reader reproducibility and the between-reader analysis of all MRI parameters were all greater than 0.75 (range 0.86 – 0.99; excellent). No significant differences were found between the HR and LR within-reader and between-reader reproducibility of the carotid MRI dimension measurements (supplementary table 2).

DISCUSSION

This study showed that increasing the spatial resolution above the commonly used 0.50 x 0.50 mm$^2$ in-plane resolution improves the reproducibility of carotid wall measurements at 3T MRI. We found consistently higher measurement reproducibility for the TWV, MWA, and MWT for the HR compared to the LR measurements. Vessel wall dimension measurements were smaller for HR measurements compared to LR measurements suggesting more accurate HR carotid wall measurements due to the reduced partial volume effects of the higher resolutions scans. As expected, we found a lower SNR and CNR for the HR compared to the LR measurements and did not find any differences between the within-reader and between-reader reproducibility between the HR and the LR carotid wall measurements. Overall, these data suggest that a 0.25 x 0.25 mm$^2$ in-plane resolution has a higher accuracy and reproducibility compared to the commonly used 0.50 x 0.50 mm$^2$ in-plane resolution when performed at 3T MRI. We demonstrated that the lower SNR and CNR do not counteract the advantages of HR carotid wall measurements, nor affect the between and within-reader reproducibility, resulting in an overall improvement of measurement reproducibility.

Overall, our LR interscan reproducibility results are in line with previous studies reporting a high overall interscan reproducibility with ICCs above 0.90 for carotid...
dimension measurements [9,8,6,21,7,22,23]. However, comparing our reproducibility results directly with previous investigations is hindered by the heterogeneous setup and limited reported measures of the various studies performed in this field. TWV is the most commonly reported measure in reproducibility studies [6,8,9,21] and is directly dependent of the length of the arterial segment used for the reproducibility results. Therefore it provides little information about arterial plaque burden of the carotid arteries. Other measures such as MWT, NWI and MWA are more suitable to quantify and compare atherosclerotic plaque burden between studies. For example, in a study of Saam et al. carotid MRI measurements reproducibility was determined using four repetitive scan using 16 cross-sectional images resulting in a higher TWV, whereas the intima-media thickness is higher (0.90mm vs 1.10mm) in our population [21]. Vidal et al. compared 1.5T MRI carotid measurements with 3T MRI measurements reporting a similar TWV to our results while including a larger arterial segment covering both the internal and external carotid arteries [9]. Studies reporting other measures of arterial wall quantification report lower MWT, MWA [22,7,6] and NWI [7]. The recent reproducibility study of Li et al. performed a study in a population with a MWT of 1.08 mm and a NWI of 0.41 compared to our results of 2.06mm and 0.65 respectively, confirming the high atherosclerotic burden in our population [7]. In addition to the difference in baseline atherosclerotic plaque burden, different field strengths, single or multi-contrast scanning protocols, different carotid MRI coils and patients with different baseline characteristics hinder a direct comparison. The ICCs presented by Kang et al., ranging between 0.90 and 0.95 for both the lumen and vessel wall, were obtained at 1.5T in a relatively small group of patients (n=8) [8], without presenting plaque volume or size measurements. Saam et al. report higher ICCs (>0.95) and lower CV (5.8%) for TWV for carotid MRI dimension measurements at 1.5T in a patient population with four repetitive scans. However, all scans were analysed in an open simultaneous setting, making a comparison with fully blinded readers difficult [21]. Studies performed at 3.0 Tesla by Dehnavi et al showed high reproducibility (ICCs >0.95) for TWV, but was performed in healthy volunteers [6]. Li et al. recently showed excellent reproducibility (ICCs >0.95) and lower CV for TWV, MWA, NWI and MWT compared to our results in a less diseased population using scans performed at 0.5 x 0.5 mm$^2$ in-plane resolution, comparable to our LR scans [7].

To determine the effect of increasing the in-plane resolution we have performed an internal comparison with a LR and HR scanning protocol without any changes in coils, scan sequence parameters, patient population and other factors potentially affecting reproducibility. Therefore, this carefully standardized study allows us to conclude that increasing the spatial in-plane resolution improves measurement reproducibility and reduces variability for carotid MRI dimension measurements. Apparently, the improved SNR and CNR of the increased field strength and the use of the 8 channel coil is sufficient to improve the carotid arterial wall dimensions reproducibility while retaining feasible scan times. As expected, the between-reader and within-reader
reproducibility of our data were high, due to the use of dedicated software. These findings are consistent with previously published data [6]. The exclusion rates of our study were lower [9] or similar [7] compared to other studies.

Another important observation of our study was the smaller size of carotid dimension measurements for the HR compared to the LR measurements. Keenan et al. recently described that a vessel wall measurements with a HR MRI scan (0.195 × 0.190 mm²) approaches the vessel wall size determined by histology 7% more closely compared to a LR (0.43 × 0.43 mm²) MRI scan [16]. Similar findings of overestimated dimension measurements were also demonstrated by comparing vessel wall MRI imaging (0.78 x 0.49 mm²) with intravascular ultrasonography [17]. Together, these findings imply that smaller carotid wall measurements of higher resolution MRI scans approach the true vessel wall size more accurately. Our finding of smaller size carotid dimension measurements for the HR scans, therefore suggests a higher measurement accuracy and is probably due to the reduced partial volume effects of the HR measurements.

Since the objective of our study was to determine the reproducibility of the different in-plane resolution scans in patient with advanced atherosclerosis only the most diseased atherosclerotic carotid artery was included. One should realize that scanning both arteries will either greatly increase the FOV resulting in a lower in plane resolution if performed in a similar time-span. Increasing the scanning time could resolve this issue by enlarging the FOV and maintaining the in-plane resolution.

**Limitations**

One of the limitations of our study is the fact that the LR measurements were compared with HR measurement with a similar FOV, but a longer scanning time (table 1). The additional time might have affected the comparison between the LR and HR scans by introducing other confounding factors such as increased motion artefacts. Although we did not investigate the effect of possible additional motion artefacts on reproducibility specifically, the better overall HR reproducibility did suggest that possible motion artefacts did not outweigh the benefits of increasing the spatial resolution. Likewise, the difference in SNR between the HR and LR measurements did not negatively influence the overall better HR reproducibility. Clinical trials using imaging parameters as surrogate endpoint use the standard deviations of the paired differences for the power calculations of future trials. These trials are often performed in patients with overt atherosclerosis making it possible to detect contraction of the atherosclerotic plaque. Our data show that a sample size of 45 subjects would be needed to detect a 4% difference in MWT with the HR scanning protocol compared to 90 subjects with the LR scanning protocol or 469 subjects when using ultrasound IMT. These numbers indicate the potential reduction in sample size and subsequent costs of clinical cardiovascular intervention trials when HR MRI measurements are used as a surrogate endpoint.
CONCLUSIONS

In a population with overt atherosclerosis, increasing the in-plane resolution robustly improves the reproducibility of 3T MRI carotid arterial wall dimension measurements. Considering the relatively small changes in atherosclerotic disease progression, precise and accurate measurements are of key importance for clinical monitoring, cardiovascular treatment trials and risk stratification of individual patients. Increasing the spatial in-plane resolution at 3T MRI can improve carotid vessel wall dimension measurements in clinical care and reduce the number of subjects needed for clinical cardiovascular intervention trials.

ACKNOWLEDGEMENTS

We thank Sandra van den Berg and Raschel Snoeks for their assistance in data acquisition.

REFERENCES


### Supplementary Table 1. Baseline characteristics of the study population

#### Patient characteristics

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<tr>
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<td>68.8 ± 7.5</td>
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<tr>
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<tr>
<td>Body mass index, kg/m2</td>
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<tr>
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</tr>
<tr>
<td>Hypertension, n (%)</td>
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<tr>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
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<td>HDL-cholesterol, mmol/l</td>
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<td>Triglycerides, mmol/l</td>
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<td>HbA1c, %</td>
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</tr>
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<td>CRP, mg/L</td>
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#### Medication use

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<td>AT II-inhibitors, n (%)</td>
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<td>Anticoagulants, n (%)</td>
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</tr>
<tr>
<td>Biguanides, n (%)</td>
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</tr>
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<td>β-blockers, n (%)</td>
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<td>Calcium channel blockers, n (%)</td>
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<td>Insulin, n (%)</td>
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</tr>
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<td>Statins, n (%)</td>
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#### Carotid ultrasound characteristics

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<tr>
<td>IMT&lt;sub&gt;BULB&lt;/sub&gt;, mm</td>
<td>1.73 (0.72)</td>
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<tr>
<td>IMT&lt;sub&gt;ICA&lt;/sub&gt;, mm</td>
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</tr>
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</table>

Data are presented as mean ± (SD) or numbers with the corresponding percentage.
Triglyceride and CRP concentrations are presented as median with the [25<sup>th</sup> to 75<sup>th</sup> percentile].
LDL = low-density lipoprotein; HDL = high-density lipoprotein; HbA1c = glycosylated haemoglobin; CRP = C-reactive protein. ACE = Angiotensin converter enzyme; AT II = Angiotensin receptor; IMT<sub>CC</sub> = common carotid intima-media thickness; IMT<sub>BULB</sub> = carotid bulb intima-media thickness; IMT<sub>ICA</sub> = internal carotid artery intima-media thickness.
**Supplementary Table 2.** Within-reader and between-reader reproducibility for the HR and LR carotid wall measurements

<table>
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<td>0.96 (0.93 – 0.98)</td>
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<td>0.95 (0.90 – 0.99)</td>
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<tr>
<td>NWI</td>
<td>0.92 (0.87 – 0.96)</td>
<td>0.97 (0.94 – 0.98)</td>
<td>ns</td>
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<tr>
<td>MWT (mm)</td>
<td>0.86 (0.76 – 0.92)</td>
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<tr>
<td><strong>Within-observer variability</strong></td>
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<td>TWV (mm$^3$)</td>
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<tr>
<td>MWA (mm$^2$)</td>
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<tr>
<td>NWI</td>
<td>0.98 (0.97 – 0.99)</td>
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<tr>
<td>MWT (mm)</td>
<td>0.99 (0.95 – 0.99)</td>
<td>0.97 (0.93 – 0.99)</td>
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</table>

Data presented are intraclass correlation coefficients with (95% CI). Abbreviations as in text. $p = p$-value for Levene’s test between HR and LR measurements.
Supplementary Figure 1. Z-axis displacement correction of scan and rescan images
Representative set of cross-sectional PD weighted images used for reproducibility analyses of the scan (A) and rescan (B). Only the images corrected for Z-axis displacement (within dotted box) are used for the final analysis.
Supplementary Figure 2. Reproducibility of scan-rescan for the HR and LR measurements. Bland-Altman plots of the HR and LR carotid arterial wall dimensions measurements TWV, MWA, NWI and MWT. No significant measurement bias was found. The dashed lines indicate 95% limits of agreement.
INCREASING THE SPATIAL RESOLUTION OF 3T CAROTID MRI HAS NO BENEFICIAL EFFECT FOR PLAQUE COMPONENT MEASUREMENT REPRODUCIBILITY
ABSTRACT

Purpose: Different in-plane resolutions have been used for carotid 3T MRI. We compared the reproducibility, as well as the within- and between reader variability of high and routinely used spatial resolution in scans of patients with overt atherosclerotic carotid artery disease.

Materials and Methods: In 31 patients with advanced carotid atherosclerosis a high (0.25 × 0.25 mm²; HR) and routinely used (0.50 × 0.50 mm²; LR) spatial resolution carotid MRI scan were performed within one month. A fully blinded closed and a simultaneously open segmentation were used to quantify the lipid rich necrotic core (LRNC), calcified and loose matrix (LM) plaque area.

Results: We found a significant lower signal-to-noise ratio for the HR compared to the LR measurements. No significant differences were observed between measurement reproducibility for HR versus LR measurements, nor did we find any significant difference between the within-reader and between-reader reproducibility. The same applies for differences between the open and closed reads. All intraclass correlation coefficients between scans and rescans for the LRNC, calcified and LM plaque area with the open segmentation method were excellent (all above 0.75).

Conclusions: Carotid 3T MRI provides compositional information of carotid atherosclerotic plaques. Increasing the spatial resolution does not improve carotid plaque component reproducibility in patients with advanced atherosclerotic carotid disease and awaits further developments in MRI hardware and fieldstrength.
INTRODUCTION

Primary and secondary prevention of cardiovascular disease is a major public health priority. The long-term, slowly progressive character of atherosclerosis offers an opportunity for early detection and treatment. Although statin therapy has proved highly effective in reducing the risk of cardiovascular events [1], residual risk remains high, and early accurate risk assessment combined with the ongoing development of novel pharmacological agents is needed to further improve primary and secondary prevention strategies.

MRI has evolved as an excellent non-invasive method to visualize the carotid artery. Improvement of MRI carotid artery measurements is thought to contribute to earlier detection of disease, improvement of risk stratification algorithms and more reliable, individual monitoring of disease progression. In addition, clinical trials using MRI carotid artery measurements as a surrogate endpoint can assess the efficacy of newly developed pharmacotherapies in a relative early stage of the developmental process, guiding further possible large multi-center endpoint trials. High-resolution MRI enables accurate and highly reproducible measurement of increased carotid artery wall dimension measurements [2,3]. However, the occurrence of stroke in patients with mild to moderate (<70%) carotid stenosis suggests that lumen narrowing is not the strongest classifier of atherosclerotic disease severity [4]. In line, high-risk features of atherosclerosis, such as intraplaque hemorrhage and a lipid rich necrotic core (LRNC), have been reported across all stenotic categories (0%-99%)[5,6]. In fact, complex lesions develop in substantial numbers in the absence of high-grade stenosis [5].

In addition to carotid artery wall dimensions measurements, multi-contrast MRI protocols can accurately determine plaque components [2,7], although no consensus exists regarding the most accurate segmentation method and reading methodology. Saam et al. previously determined the between-scan reproducibility of carotid plaque components measurements at 1.5 T in a severely diseased population using an open-simultaneously manual segmentation method between the repeated measurements [8]. More recently, Li et al. investigated the between-scan reproducibility in a broader population at 3 Tesla, only analysing components present in at least one-time point [9]. Both studies were performed with an in-plane resolution of approximately 0.50 × 0.50 mm [3]. These resolution parameters are commonly used in carotid MRI as a tradeoff between resolution, signal-to-noise ratio (SNR), and acquisition time. These three imaging parameters are highly interdependent: higher resolution allows observation of smaller details, but typically reduces SNR, and increases imaging time. Therefore, increasing the resolution could potentially reduce measurement reproducibility due to the lower SNR. While maintaining the same resolution, it was shown that 3T MRI yields superior SNR compared to 1.5T MRI scanners [10–12]. In addition, Balu et al. demonstrated that an 8 instead of a 4 channel coil configuration led to an additional improvement of the SNR for carotid plaque scanning [13].
In an effort to further improve MRI carotid plaque component measurements, we hypothesized that increasing the in-plane spatial resolution using a 3T MRI scanner equipped with a dedicated 8 channel carotid coil would improve carotid plaque component measurements reproducibility, despite the expected lower SNR. Secondly, since no consensus exists for the optimal segmentation method we compared two different manual segmentation methods for carotid plaque component image analysis. For this aim, we performed prospective repeated 3T MRI scans with a dedicated 8 channel carotid coil at 0.50 × 0.50 mm² and 0.25 × 0.25 mm² in-plane resolution in patients with overt carotid atherosclerosis.

MATERIALS AND METHODS

Ethics statement
The review board of the Academic Medical Center in Amsterdam, the Netherlands approved the study protocol. Written informed consent was obtained from all participants. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Participants
Individuals with one or more atherosclerotic events were screened for the presence of significant atherosclerotic disease in one of the carotid arteries using echo duplex measurements. Individuals with 30 to 70% stenosis of the carotid artery were included. All participants were scheduled for a scan and a rescan within one month. Scans were performed between September 2009 and April 2011.

3T MRI
MRI scans were obtained on a 3T whole-body scanner (Intera, Philips Medical Systems, Best, The Netherlands), with the use of an 8 channel dedicated bilateral carotid artery coil (Shanghai Chenguang Medical Technologies, Shanghai, China). Positioning of the image stack was performed using axial magnetic resonance angiography images acquired with a time of flight (TOF) sequence covering the carotid arteries at both sides (field of view (FOV) 10 × 10 cm, 40 slices of 2 mm thickness). These images together with the ultrasound duplex data were used for planning the T1w, T2w, PDw and TOF sequences using a FOV of 6 × 6 cm, at the center of the carotid plaque in the carotid artery with the most profound plaque burden. This could be either side of the neck. Subsequently, T1w, T2w, PDw and TOF images were acquired using ECG-gated unilateral axial turbo spin echo sequences for both the HR and LR images during each scan session. Overview images showing the image stacks superimposed over the carotid artery were used to to plan the repeat scan. Images were saved according to the DICOM protocol. Standardized equipment and protocols were used for image storage.
and data management. Before any quantitative analysis was performed one reader (RD) corrected all scan and rescan images for possible Z-axis displacement using T1w, PDw and TOF images. After localisation of the carotid bifurcation both proximal and caudal scan and rescan images were compared. For this study low resolution (LR) and high resolution measurements (HR) were compared. Therefore PDw, T2w, T1w and TOF sequences were repeated for the two different resolutions at each scan session. The non-interpolated pixel size of 0.5 × 0.5 mm$^2$ is referred to as the low resolution (LR) and the non-interpolated pixel size of 0.25 × 0.25 mm$^2$ is referred to as the high resolution (HR) setting. Table 1 displays the full scan parameters.

### 3T MRI Image Analysis

A fully blinded segmentation method was compared to a segmentation method with side-by-side workstations enabling a direct comparison between the scan and rescan images. With the fully blinded segmentation method (referred to as closed segmentation), the scans and rescans were analysed one-by-one, in a fully random order, without knowledge of patient characteristics and scan session. With the side-by-side workstation segmentation method (referred to as open segmentation) scan and rescan were analysed simultaneously, without knowledge of patient characteristics and scan session. To prevent recall bias, the open segmentation method was performed two months after the last blinded analysis.

Delineation of LRNC, calcification and loose matrix (LM) boundaries were performed manually with the aid of a dedicated vessel wall analysis package VesselMass (VesselMass, Leiden University Medical Center, Leiden, The Netherlands) [14]. All four weightings were used to identify the different plaque components according to previously published literature [7]. Briefly, isointense to hyperintense areas on T1w and PDw images with varying intensities on T2w and TOF images was considered to correspond with the LRNC. Calcification was defined by a hypointense signal on all 4 weightings. Loose matrix was delineated when plaques had hyperintense areas on T2w and PDw, isointense to hypointense areas on T1w and isointense areas on TOF images. If scan and rescan images during the open segmentation analysis could not be interpreted similarly according to these criteria, they were discussed in an expert meeting for a definite decision. If no agreement could be reached in accordance with the image delineation strategy outlined above the scan and rescan images were analysed unchanged independent of a possible mismatch between scan and rescan images.

To assess the between-reader reproducibility of the closed segmentation method, two readers independently analysed all (both HR and LR scans and rescans) fully blinded MRI image stacks. To assess the within-reader reproducibility, one reader blinded to previous outcome data, analysed all (both HR and LR scans and rescans) fully blinded image stacks in a second session, 2 months after the first analysis.
<table>
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</table>

HR = high resolution; LR = low resolution; TSE = turbo spin-echo, FFE = fast field echo, FOV = field of view, DIR = double inversion-recovery, NEX = number of excitations. * Scan times at heart rate of 60 min⁻¹
Signal-to-noise ratios (SNR) were calculated as $SNR = S/\sigma$, where $S$ is the true signal intensity corrected for the noise contribution and $\sigma$ is the true SD of the noise. $\sigma$ was calculated from the measured SD of the noise ($SD_n$) and the numbers of receivers: $SD_n = 0.76 \times \sigma$. Corrected signal intensity $S$ was obtained from the measured magnitude signal ($S_m$) and the measured magnitude of the background noise ($S_n$): $S = (S_m^2 - S_n^2)^{1/2}$. The magnitude ($S_n$) and the SD ($SD_n$) of the background noise were measured in the corner of the image in a region free of signal and free of artefacts. Contrast-to-noise ratios (CNR) between wall and lumen were calculated as $CNR = SNR_{\text{wall}} - SNR_{\text{lumen}}$.

**Statistical Analysis**

Continuous variables are expressed as mean ± SD. The SD of the paired differences (SDpd) between the initial and the rescan were calculated for the LRNC, calcification and LM plaque area. Bland-Altman plots were used to illustrate systematic bias between scan and rescan images [15]. The agreement between successive MRI scans, as well as the between observer and within observer agreement was assessed using intra-class correlation coefficients (ICC). An ICC of <0.40 indicated poor, one between 0.40 and 0.75 indicated fair to good, and one of >0.75 indicated excellent reproducibility [16]. Differences between the ICCs for the LRNC, calcified plaque and LM plaque area between the HR and LR scans and between the open and closed segmentation method were tested with a Levenes test. All statistical analyses were performed using PASW statistics 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

**RESULTS**

Fifty-one individuals with one or more atherosclerotic events were screened for the presence of significant atherosclerotic disease in one of the carotid arteries. Thirty-one individuals with a 30 to 70% stenosis of the carotid artery were included. Full baseline characteristics of the patient population are listed in table 2. All participants were scheduled for a scan and a rescan session. Two patients cancelled the second appointment and withdrew consent. One LR scan could not be completed due to a panic attack, whereas two other subjects did not complete their LR scan due to discomfort. The average interval between the scan and rescan sessions was 26 days. No significant clinical events were reported during the study. Prior to the analyses, 2 HR and 1 LR data sets were excluded based on a poor image quality. After exclusion, 27 full repetitive sets of the HR scans, and 25 repetitive sets of the LR scans were available for the final analyses. This corresponds to 832 initial available images of which 808 images (97%) were of sufficient quality to be analysed. From these images twenty-one image stacks of the available 101 needed a Z-axis correction. Fourteen image stacks were shifted by one slice, 3 image stacks by two slices, 3 image stacks by three slices and 1 image stack needed a Z-axis correction of 4 slices. Figure 1 depicts an example of corrected Z-axis
### Table 2. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>N = 31</td>
</tr>
<tr>
<td>Age, years</td>
<td>68.8 ± 7.5</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>15 (48)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.4 ± 2.9</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>15 (48)</td>
</tr>
<tr>
<td>Myocardial infarction, n (%)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Stroke, n (%)</td>
<td>14 (45)</td>
</tr>
<tr>
<td>Peripheral artery disease, n (%)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>144 ± 20</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.86 ± 1.10</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>2.85 ± 1.16</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.45 ± 0.45</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.20 [0.88 – 1.63]</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.28 ± 0.96</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.6 [1.0 – 3.8]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medication use</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE-inhibitors, n (%)</td>
<td>4 (13)</td>
</tr>
<tr>
<td>AT II-inhibitors, n (%)</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Anticoagulants, n (%)</td>
<td>26 (84)</td>
</tr>
<tr>
<td>Biguanides, n (%)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>β-blockers, n (%)</td>
<td>10 (32)</td>
</tr>
<tr>
<td>Calcium channel blockers, n (%)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Cardiac glycosides, n (%)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>4 (13)</td>
</tr>
<tr>
<td>Insulin, n (%)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>23 (74)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ultrasound dimension measurements</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IMT&lt;sub&gt;CC&lt;/sub&gt;, mm</td>
<td>1.10 (0.56)</td>
</tr>
<tr>
<td>IMT&lt;sub&gt;BULB&lt;/sub&gt;, mm</td>
<td>1.73 (0.72)</td>
</tr>
<tr>
<td>IMT&lt;sub&gt;ICA&lt;/sub&gt;, mm</td>
<td>1.02 (0.56)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MRI dimension measurements</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total wall volume, mm³</td>
<td>735.0 (431.8)</td>
</tr>
<tr>
<td>Mean wall area, mm²</td>
<td>53.0 (29.6)</td>
</tr>
</tbody>
</table>
displacement. Table 3 displays the number of components identified on the scan and rescan images according to the resolution and stratified by segmentation method.

**HR scanning decreases the SNR of carotid MRI dimension measurements**
A significantly lower mean SNR of the arterial wall was detected for the HR compared to the LR carotid measurements (17.8 ± 8.1 vs. 52.2 ± 13.4; p <0.001). We found a mean contrast-to-noise ratio (CNR) between the arterial wall and arterial lumen of 15.7 ± 7.2 for the HR and of 48.0 ± 13.1 for the LR carotid measurements (p <0.001).

**Plaque component measurements**
Table 4, 5 and 6 comprises the LRNC, the calcified and the loose matrix plaque area for the HR and LR scans. We found no difference between the LRNC area and calcified plaque area between the HR and LR scans. Plaque loose matrix areas are significant smaller for the HR measurements compared to the LR measurements for the open (p<0.05) and closed (p<0.05) segmentation method. Figure 2 shows an example of a HR scan and rescan containing a LRNC, whereas figure 3 shows a calcified plaque area on a HR and LR scan.

**Reproducibility and variability of plaque component measurements**
Figure 4 shows the Bland-Altman plots for the LRNC, calcified plaque area and loose matrix area measurements between the scan and rescan for the open and closed segmentation method of the HR and LR scans. The ICC of the HR LRNC area with the closed segmentation was 0.58 (0.0 – 0.81), indicating a fair to good reproducibility. All other ICCs of the HR and LR scans and rescans for the open and closed segmentation method were greater than 0.75 (table 4-6). No significant differences were found in the measurement variability for the LRNC area, the calcified plaque area and the loose matrix plaque areas between the HR and LR scans. Likewise, no significant differences were found in the measurement variability between the open and closed read.

**Table 2. Baseline characteristics of the study population (Continued)**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized wall index</td>
<td>0.659 (0.099)</td>
</tr>
<tr>
<td>Mean wall thickness, mm</td>
<td>2.07 (0.63)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± (SD) or numbers with the corresponding percentage. Triglyceride and CRP concentrations are presented as median with the [25th to 75th percentile]. LDL = low-density lipoprotein; HDL = high-density lipoprotein; HbA1c = glycosylated haemoglobin; CRP = C-reactive protein. ACE = Angiotensin converter enzyme; AT II = Angiotensin receptor; IMTcc = common carotid intima-media thickness; IMTbulb = carotid bulb intima-media thickness; IMTica = internal carotid artery intima-media thickness.
Table 3. Number of identified carotid plaque components with corresponding scan-rescan mismatch

<table>
<thead>
<tr>
<th></th>
<th>High Resolution</th>
<th>Low Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scan-rescan</td>
<td>Mismatch</td>
</tr>
<tr>
<td>Total scans available</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>LRnC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Calcified plaque</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Loose matrix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>14</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are presented as number.

Table 4. Open and closed segmented LRnC surface area measurements for HR and LR carotid arterial wall measurements

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>LR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>6.2 (5.8)</td>
<td>4.6 (5.2)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>5.7 (4.4)</td>
<td>6.2 (5.7)</td>
<td>ns*</td>
</tr>
<tr>
<td>Rescan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>5.6 (5.2)</td>
<td>5.7 (4.9)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>6.8 (4.5)</td>
<td>5.1 (5.5)</td>
<td></td>
</tr>
<tr>
<td>Variability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD paired differences (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.6 (6.2)</td>
<td>1.1 (4.1)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>1.1 (2.7)</td>
<td>1.1 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Reproducibility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.58 (0.0 – 0.81)</td>
<td>0.80 (0.43 – 0.93)</td>
<td>ns‡</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>0.89 (0.57 – 0.98)</td>
<td>0.98 (0.90 – 0.99)</td>
<td>ns‡</td>
</tr>
</tbody>
</table>

Data are presented as mean with ± SD. ICCs are given with the corresponding 95% confidence interval. * = p-value for paired t-test between scan and rescan HR and LR measurements. ‡ = p-value for Levene’s test between HR and LR measurements. HR = high resolution; LR = low resolution; ICC = intraclass correlation coefficient; SD = standard deviation.
Figure 1. Representative set of cross-sectional PDw images used for reproducibility analyses of the scan (A) and rescan (B). Only the images corrected for Z-axis displacement (within dotted box) are used for the final analysis.

Figure 2. Representative sample of scan and rescan HR images containing a LRNC. Panel A and B show the difference in intensity between a T1w (panel A, panel D) and a T2w (panel B, panel E) image for the LRNC of a scan and rescan respectively. Panel C (scan) and panel F (rescan) show the manual delineation of the LRNC with the closed segmentation method.
Table 5. Open and closed segmented calcified surface area measurements for HR and LR carotid arterial wall measurements

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>LR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scan measurements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>3.8 (5.5)</td>
<td>5.0 (7.7)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>4.2 (6.0)</td>
<td>4.7 (5.9)</td>
<td>ns*</td>
</tr>
<tr>
<td>Rescan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>3.7 (5.0)</td>
<td>4.5 (6.5)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>4.4 (6.0)</td>
<td>4.8 (6.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Variability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD paired differences (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.1 (2.7)</td>
<td>0.5 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>0.2 (1.3)</td>
<td>0.1 (0.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.93 (0.85– 0.97)</td>
<td>0.94 (0.86– 0.97)</td>
<td>ns‡</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>0.99 (0.98 – 0.99)</td>
<td>0.99 (0.99 – 1.00)</td>
<td>ns‡</td>
</tr>
</tbody>
</table>

Data are presented as number with percentage or mean with SD. ICCs are given with the corresponding 95% confidence interval. * = p-value for paired t-test between scan and rescan HR and LR measurements. ‡ = p-value for Levene’s test between HR and LR measurements. HR = high resolution; LR = low resolution; ICC = intraclass correlation coefficient; CV = coefficient of variation; SD = standard deviation.

Table 6. Open and closed segmented loose matrix surface area measurements for HR and LR carotid arterial wall measurements

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>LR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scan measurements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>4.6 (4.8)</td>
<td>8.5 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>5.7 (4.8)</td>
<td>9.5 (5.9)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rescan measurements (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>4.3 (5.2)</td>
<td>7.2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>6.9 (4.8)</td>
<td>8.9 (5.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Variability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD paired differences (mm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.4 (4.5)</td>
<td>1.3 (6.1)</td>
<td></td>
</tr>
<tr>
<td>Open segmentation</td>
<td>1.1 (1.6)</td>
<td>0.7 (2.4)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Open and closed segmented loose matrix surface area measurements for HR and LR carotid arterial wall measurements (continued)

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>HR</th>
<th>LR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed segmentation</td>
<td>0.76 (0.40 – 0.91)</td>
<td>0.76 (0.40 – 0.91)</td>
<td>ns‡</td>
</tr>
<tr>
<td>Open segmentation</td>
<td>0.97 (0.91 – 0.99)</td>
<td>0.96 (0.88 – 0.99)</td>
<td>ns‡</td>
</tr>
</tbody>
</table>

Data are presented as mean with ± SD. ICCs are given with the corresponding 95% confidence interval. * = p-value for paired t-test between scan and rescan HR and LR measurements. ‡ = p-value for Levene’s test between HR and LR measurements. HR = high resolution; LR = low resolution; ICC = intraclass correlation coefficient; CV = coefficient of variation; SD = standard deviation.

**Within-reader and between-reader reproducibility**

The ICC values for the within-reader reproducibility and the between-reader reproducibility of all MRI parameters are given in table 7. No significant differences were found between the HR and LR within-reader and between-reader reproducibility of the carotid MRI dimension measurements.

**DISCUSSION**

This study shows that increasing the spatial resolution of carotid MRI above the commonly used 0.50 × 0.50 mm² in-plane resolution lowers the SNR and CNR, but does not improve measurement reproducibility of atherosclerotic plaque components. These findings are in contrast to a recent paper demonstrating an improved reproducibility for carotid plaque dimension measurements using 3T carotid MRI [17]. Furthermore, we could not demonstrate any difference in reproducibility between the closed and open segmentation method. No differences between the within-reader and between-reader reproducibility between the HR and the LR carotid plaque component measurements were found. Measurements of calcified and loose matrix plaque area show an excellent between-reader and within-reader reproducibility. The exclusion rates of our study are lower [10] or similar [9] compared to other studies.

Comparing our reproducibility data with previous studies is hindered by the heterogeneous setup of the various studies previously performed. Saam et al. investigated the reproducibility of interscan plaque composition analysing the scans simultaneously with an in-plane resolution of 0.50 × 0.50 mm² in 20 patients at 1.5 T [8]. Baseline carotid dimension measurements (normalized wall index 0.6 ± 0.1), were similar to our population characteristics (normalized wall index 0.7 ± 0.1), making a comparison possible with the open LR reproducibility data. ICCs for calcification measurements (ICC 0.95, CI0.9-1.0) and the LRNC measurements (ICC 0.99, CI 0.98-1.00) were within the same range as our LR open segmentation ICCs for calcified
Figure 4. Bland-Altman plots of the high resolution and low resolution carotid plaque composition measurements for all parameters and for the open and closed segmentation method. No significant measurement bias was found. The dashed lines indicate 95% limits of agreement.
baseline characteristics (normalized wall index 0.33± 0.1; mean wall thickness 0.86 ± 0.19) indicate this study was performed in less diseased patients, which is further corroborated by the wider selecting criteria, including patients with a carotid stenosis > 15%, hindering a direct comparison.

The lack of improved plaque component reproducibility at higher resolution in our data indicate that the decrease in CNR and SNR, especially noticeable on the T2w images, outweighed the advantages of the higher spatial resolution. Future developments in MR coil design, fast imaging and reconstruction methods or the use of 7T MRI may compensate for the loss in SNR at higher resolutions [18], enabling the use of higher resolution for carotid MRI.

No significant improvement between the ICCs of the open segmentation method compared to the ICCs of the closed method could be demonstrated. However, lower confidence intervals for the ICCs of the open segmentation method indicate that a beneficial effect may be present in a larger population suggesting that the open segmentation method is the preferable choice for carotid MRI component analyses.

The larger CIs in the closed read might be explained, at least partially, by the scan-rescan plaque component identification mismatch (component identified in the scan or rescan, but not in the rescan or scan). Although mismatches occurred less frequent with the open segmentation method, repeated images with mismatches were included in the final analyses, in contrast to previously published studies [3,8]. Taken together, clinical intervention trials using carotid plaque imaging as surrogate endpoint can reduce the impact of these issues by reviewing scans with an open segmentation method fully blinded for intervention and time-point.

Literature on accuracy for carotid artery plaque composition using different resolutions with carotid MRI is lacking or scarce. Keenan et al. recently described a 7% larger area in low resolution (0.43 × 0.43 mm²) measurements compared to
high resolution measurements (0.195 × 0.190 mm$^2$) in an ex vivo study validating vessel wall size with histology [19]. Similar findings of overestimated dimension measurements were also demonstrated by comparing vessel wall MR imaging (0.78 × 0.49 mm$^2$) with high resolution intravascular ultrasonography [20]. Using a low and high resolution scanning protocol, without any changes in coils, scan sequence parameters, patient population and other factors potentially affecting scan comparisons we find that 88.9% of all mean HR scans (scan, rescan and second observer) had a lower plaque component surface area compared to the mean LR scans. More specifically, the significant lower surface area for plaque loose matrix composition with the HR scanning protocol suggests that increasing the spatial in-plane resolution improves plaque loose matrix composition measurement accuracy, which is probably due to a reduced partial volume effect at the increased resolution.

**CONCLUSION**

Our data indicate that the reproducibility of carotid artery plaque composition with an open segmentation method is excellent. Increasing the spatial resolution above the routinely used 0.50 × 0.50 mm$^2$ does however not improve measurement reproducibility for carotid plaque component measurements contrary to earlier findings for plaque dimension measurements [17]. LR measurements on a 3T MRI scanner equipped with a dedicated carotid coil combined with an open segmentation method are the preferred choice for clinical intervention trials using plaque component areas as surrogate endpoint. Plaque composition analyses can be added in clinical trials quantifying functional and structural properties proving information beyond the dimensions of the atherosclerotic plaque.

**ACKNOWLEDGEMENTS**

We gratefully acknowledge Sandra van den Berg, Raschel van Luijk-Snoeks and Paul Groot of the Department of Radiology of the Academic Medical Center, Amsterdam, The Netherlands.

**REFERENCES**


GUIDELINE TREATMENT RESULTS IN REGRESSION OF ATHEROSCLEROSIS IN TYPE 2 DIABETES MELLITUS

Aart C. Strang, Diederik F. van Wijk, Henri J.M.M. Mutsaerts, Erik S.G. Stroes, Aart J. Nederveen, Joris I. Rotmans, Ton.J. Rabelink, Frieke M.A. Box, Ph.D.

Published in Diab Vasc Dis Res. 2015 Jan; epub
ABSTRACT

Background: Efficacy of guideline cardiovascular disease (CVD) prevention regimens may differ between patients with or without type II diabetes mellitus (DM2). We therefore compared change in carotid artery (CA) wall dimensions in DM2 and non-DM2 patients with a history of a major CVD event, using magnetic resonance imaging (MRI).

Methods: Thirty DM2 patients and 29 age and sex matched non-DM patients with a history of stroke or myocardial infarction and a CA stenosis (15%-70%) were included. In all patients, treatment was according to CV-risk management guidelines. At baseline and follow-up, CA wall dimensions were measured using 1.5T MRI.

Results: After 2 years of follow-up, total wall volume (TWV) of the CA in DM2 patients decreased by 9.6% (P = 0.016). In contrast, stabilization rather than regression of CA wall dimensions was observed in non-DM patients over a 2-year period. Body mass index (BMI) was identified as a predictor of TWV decrease.

Conclusions: Guideline treatment arrests atherogenesis in non-DM patients and even decreases vessel wall dimensions in DM2 patients. Baseline BMI predicts CVD prevention efficacy expressed as decrease in TWV. These data emphasize the importance of optimal CV-prevention, particularly in diabetes patients with a high BMI.
INTRODUCTION

With a prevalence of 10%, diabetes mellitus (DM) is responsible for 1.3 million deaths per year worldwide [1]. In patients with type II diabetes mellitus (DM2), cardiovascular disease (CVD) accounts for up to 60% of mortality. Accelerated atherosclerosis in patients with DM2 has been partly attributed to direct effects of hyperglycemia [2,3].

Except hyperglycemia, DM2 patients and non-DM2 patients share the same risk factors for CVD including central obesity, hypertension and combined dyslipidemia [4–7]. Despite these similarities, the effects of several diabetes-related metabolic disturbances may lead to different atherosclerotic structure and architecture. For example, the high prevalence of hypertriglyceridemia and low levels of HDL-cholesterol in diabetes patients is accompanied by abnormalities in lipoprotein particle structure. Indeed, the LDL-cholesterol particles in diabetes patients are predominantly present in small dense, oxidized and glycated forms that possess the most atherogenic properties [8]. Next to the specific dyslipidemia, endothelial dysfunction contributes to chronic vessel wall inflammation and accelerated atherogenesis in diabetes. These high levels of inflammation are held responsible for other structure and architecture of the atherosclerotic vessel wall, making it more prone to plaque rupture [9,10].

This supposed difference in atherosclerotic structure and architecture may result in different efficacy of CVD prevention regimens on atherosclerosis in DM2 patients compared with non-DM patients. The efficacy of CVD-risk reducing regimens in patients with and without DM2 has been investigated extensively. Despite the established role of elevated HbA1c levels in cardiovascular (CV) morbidity and mortality, a direct effect of interventions to decrease HbA1c on endpoints or even surrogate endpoints like carotid intima media thickness (CIMT) decrease still has not been established [11]. The use of platelet inhibitors and antihypertensive medication, however, has been associated with a significant reduction of atherogenesis in DM2 patients [12], as assessed as change in CIMT.

In the present study, we compared progression of carotid atherogenesis over a 2-year period in patients with overt atherosclerotic disease of the carotid arteries (CA) and a history of a major cardiovascular event, in absence and presence of DM2. During the 2-year period, all patients received routine CVD-preventive regimens. To assess the changes in atherosclerotic burden in vivo, we measured CA wall dimensions using MRI, expressed as total wall volume (TWV) in both DM2 and non-DM patients. In addition we identified predictors for the observed change in TWV.

MATERIALS AND METHODS

Patient selection

The protocol was approved by the institutional review board at the University Medical Center of Leiden, the Netherlands and the study was conducted in conformance with the Declaration of Helsinki. All patients provided written informed consent. Patients
were recruited from the outpatient clinics of the department of vascular medicine, internal medicine, surgery, ophthalmology, endocrinology and geriatrics of the University Center between 2007 and 2009. Patients with a history of stroke (transient ischemic attack included) or myocardial infarction were screened by ultrasonography for CA stenosis. Thirty patients met the inclusion criteria of DM2, a CA stenosis between 15% and 70% and an HbA1c of above 7.0% (53 mmol/mol). Twenty-nine non-diabetic patients with a history of CVD and a CA stenosis between 15% and 70% were selected from a database, matched for sex and age. All participants were invited for blood withdrawal and a carotid MRI-scan. Two years after the baseline visit, participants were re-invited for a two-year follow-up visit, during which blood withdrawal and MRI-scan were repeated.

**CV-prevention protocol during 2-year follow-up**

At the initiation of the study, all participants continued receiving CV-preventive treatment according to the Guideline Cardiovascular Risk Management from the Dutch Society of the Internal Medicine [13]. The therapeutic regimen included four times per year monitoring and control visits for lifestyle advice, a target LDL-cholesterol < 2.5 mmol/L (97 mg/dL), arterial blood pressure below 140/90 mmHg in both patient groups and glycated hemoglobin (HbA1c) levels of below 7% (53 mmol/mol) in DM2 patients.

**Risk factor measurements**

At baseline and 2-years follow-up visit, Ethylenediaminetetraacetic acid (EDTA) anti-coagulated peripheral blood samples were drawn from antecubital veins. Hematologic, inflammatory nephrologic and lipidologic analyses were performed using routine laboratory analyzers. At both visits, patient information was collected concerning medical history, use of medication and information on dietary- and smoking habits. Blood pressure was measured using oscillometric method (Omron Electronics, The Netherlands).

**Image acquisition**

MRI images were obtained on a 1.5T MRI scanner (Philips Interna, Philips Healthcare, Best, the Netherlands). In all subjects, left common carotid artery, carotid bifurcation and internal carotid artery were scanned. Localization of these structures was performed by acquiring axial time-of-flight images. Nine transversal images were acquired with the flow-divider positioned in the 5th (center) image, with a slice thickness of 3mm. Five MRI sequences were acquired in the corresponding region: T1-weighted turbo field echo (TFE), T2-weighted turbo spin echo (TSE), time of flight angiography (TOF), proton density weighted (PDW) TSE. Scan sequence parameters are listed in table 1 supplementary materials.
**Image review procedure**

Image analysis was performed using dedicated software (Vessel-Mass v3, Leiden University Medical Center, Leiden, the Netherlands). Before quantitative analysis, image region at baseline and follow-up scans of each patient were compared. Images outside overlapping area proximal or distal to the carotid bifurcation (Z-axis) between baseline and follow-up were excluded from analysis. Also, all images from all sequences were scored on a quality scoring system. Main quality criterion was that outer and inner carotid artery border was recognizable.

**Quantitative image analysis**

A predefined delineation protocol was used to optimize manual vessel wall delineation. Quantitative image analysis was performed using dedicated software (Vessel-Mass v3, Leiden University Medical Center, Leiden, the Netherlands). The reader was blinded for subject characteristics and date of scan. Baseline and follow-up scans were analyzed randomly. Outer vessel wall border and inner vessel wall border were delineated from caudal to cranial. The surface area within the outer border is called total vessel area (TVA) and the surface area within the inner border is called lumen area (LA; Figure 1). At the first image superior to the carotid bifurcation, the internal carotid artery was used for vessel wall analysis.

Vessel wall calculations were performed using text export function from Vessel-Mass into Microsoft Excel 2003 (Microsoft, Redmond, Washington, US) and calculations were performed using an automatic macro. Total wall volume (TWV) was calculated by taking vessel wall area (VWA = TVA - LA), multiply by slice thickness (3mm) and summing volumes from the whole stack (mostly counting 9 slices).

**Statistical analysis**

All are expressed as mean ± standard deviation (SD). Differences in baseline and follow-up values or levels are tested using paired nonparametric test: Wilcoxon signed ranks test. Differences between non-DM subjects and diabetic subjects are tested using independent samples t-tests. Based on a difference of 15% in TWV between non-DM subjects and diabetic subjects [14], and an assumed SD of 20%, power of 0.8 and Type I error probability of 0.05, a minimum of 25 patients must be included in each group.

Change in TWV was calculated by deducing individual follow-up TWV from individual baseline TWV and expressed as mean differences. Predictors of change in TWV were assessed using multivariate linear regression. Potential predictors or confounders were used as explanatory variable and excluded using backward elimination. All measures were adjusted for body mass index (BMI), LDL-cholesterol, HDL-cholesterol, pulse pressure and HbA1c. All statistical tests were performed using SPSS Statistics (version 19, IBM, USA).
Baseline characteristics are listed in table 1. The majority of patients were male, with a mean age of 65 (± 8.0) years for patients with DM2 and 63 (± 8.3) years for non-DM subjects. The DM2 patients had higher levels of blood glucose, HbA1c, BMI, pulse pressure and triglycerides, whereas levels of total cholesterol, LDL-cholesterol and HDL-cholesterol were lower. A significantly larger proportion of DM2 patients used statins and antihypertensive drugs compared to non-DM patients (p=0.020 and 0.029 respectively). Next to more frequent usage of statins, DM2 patients used more potent statins compared to non-DM2 subjects (p=0.025).

At baseline, no significant differences between DM2 patients and non-DM patients were observed in LA, TVA, TWV (Figure 2).

**RESULTS**

Baseline characteristics are listed in table 1. The majority of patients were male, with a mean age of 65 (± 8.0) years for patients with DM2 and 63 (± 8.3) years for non-DM subjects. The DM2 patients had higher levels of blood glucose, HbA1c, BMI, pulse pressure and triglycerides, whereas levels of total cholesterol, LDL-cholesterol and HDL-cholesterol were lower. A significantly larger proportion of DM2 patients used statins and antihypertensive drugs compared to non-DM patients (p=0.020 and 0.029 respectively). Next to more frequent usage of statins, DM2 patients used more potent statins compared to non-DM2 subjects (p=0.025).

At baseline, no significant differences between DM2 patients and non-DM patients were observed in LA, TVA, TWV (Figure 2).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-DM (n=29)</th>
<th>DM2 (n=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male; %)</td>
<td>20 (69%)</td>
<td>17 (57%)</td>
<td>0.389</td>
</tr>
<tr>
<td>Age in years</td>
<td>63.0 ± 8.3</td>
<td>65.5 ± 8.0</td>
<td>0.196</td>
</tr>
<tr>
<td>BMI in kg/m²</td>
<td>25.9 ± 3.3</td>
<td>28.7 ± 3.4</td>
<td>0.006</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>22 (79%)</td>
<td>18 (60%)</td>
<td>0.147</td>
</tr>
<tr>
<td>Hip to waist ratio</td>
<td>0.926 ± 0.083</td>
<td>0.989 ± 0.121</td>
<td>0.076</td>
</tr>
<tr>
<td>Follow-up time days</td>
<td>760 ± 109</td>
<td>792 ± 148</td>
<td>0.416</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin users</td>
<td>22</td>
<td>29</td>
<td>0.020</td>
</tr>
<tr>
<td>Potency 1 (simvastatin 5 mg eq.)</td>
<td>1</td>
<td>1</td>
<td>0.981</td>
</tr>
<tr>
<td>Potency 2 (simvastatin 10 mg eq.)</td>
<td>1</td>
<td>1</td>
<td>0.981</td>
</tr>
<tr>
<td>Potency 3 (simvastatin 20 mg eq.)</td>
<td>5</td>
<td>14</td>
<td>0.025</td>
</tr>
<tr>
<td>Potency 4 (simvastatin 40 mg eq.)</td>
<td>13</td>
<td>10</td>
<td>0.430</td>
</tr>
<tr>
<td>Potency 5 (simvastatin 80 mg eq.)</td>
<td>2</td>
<td>2</td>
<td>0.981</td>
</tr>
<tr>
<td>Potency 6 (simvastatin &gt;80 mg eq.)</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>15</td>
<td>24</td>
<td>0.029</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>6</td>
<td>11</td>
<td>0.252</td>
</tr>
<tr>
<td>Diuretics</td>
<td>3</td>
<td>13</td>
<td>0.007</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>3</td>
<td>7</td>
<td>0.299</td>
</tr>
<tr>
<td>Angiotensin-inhibitors</td>
<td>3</td>
<td>9</td>
<td>0.104</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>3</td>
<td>5</td>
<td>0.707</td>
</tr>
<tr>
<td>Anidiabetic treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin class</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Biguanide class</td>
<td>-</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Sulfonylurea class</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Others</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP systolic</td>
<td>139.3 ± 20.6</td>
<td>146.7 ± 20.3</td>
<td>0.188</td>
</tr>
<tr>
<td>BP diastolic</td>
<td>82.2 ± 8.0</td>
<td>77.1 ± 10.0</td>
<td>0.050</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>57.2 ± 17.4</td>
<td>69.6 ± 16.3</td>
<td>0.010</td>
</tr>
<tr>
<td>Renal function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>79.0 ± 11.5</td>
<td>84.7 ± 24.0</td>
<td>0.268</td>
</tr>
<tr>
<td>MDRD (ml/min/1.73m²)</td>
<td>85.5 ± 12.5</td>
<td>80.7 ± 20.1</td>
<td>0.282</td>
</tr>
<tr>
<td>Urine albumin (mg/L)</td>
<td>8.4 ± 11.4</td>
<td>73.7 ± 167.5</td>
<td>0.063</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.5 ± 1.2</td>
<td>10.6 ± 4.2</td>
<td>0.002</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.52 ± 0.40</td>
<td>7.33 ± 1.34</td>
<td>0.000</td>
</tr>
</tbody>
</table>
At follow-up, a significant decrease was observed in the vessel wall dimension in patients with DM2 compared to baseline. TWV decreased 9.6% ($p=0.016$; Figure 2). In non-DM patients, the TWV decreased 2.2% only ($p=0.259$).

BMI was the single significant predictor for change in TWV in both groups ($p=0.046$ ;Table 2). The predictive role of BMI remained significant after adjustment for the contribution of LDL-cholesterol, HDL-cholesterol, pulse pressure and HbA1c at baseline.

No predictors for within-group variation in TWV ($\Delta$TWV) in DM2 patients could be identified in using multivariate linear regression analysis. In non-DM patients, HbA1C was identified as predictor for within-group variation despite insignificant difference in TWV in this group at follow-up compared with baseline, even after adjustment for contribution of baseline BMI, LDL-cholesterol and pulse pressure (Table 2).

**Table 1.** Baseline characteristics of non-DM (SM-) and DM2 patients (DM+) (continued)

<table>
<thead>
<tr>
<th>Lipid metabolism</th>
<th>Non-DM (n=29)</th>
<th>DM2 (n=30)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.07 ± 1.09</td>
<td>4.13 ± 0.73</td>
<td>0.000</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.14 ± 0.95</td>
<td>2.46 ± 0.65</td>
<td>0.004</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.43 ± 0.61</td>
<td>2.06 ± 1.28</td>
<td>0.026</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.59 ± 0.32</td>
<td>1.09 ± 0.27</td>
<td>0.000</td>
</tr>
<tr>
<td>Non-HDL-cholesterol (mmol/L)</td>
<td>3.48 ± 1.08</td>
<td>3.04 ± 0.68</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation.

**Table 2.** Significant predictors for change total wall volume (TWV).

<table>
<thead>
<tr>
<th>Significant predictor</th>
<th>B Coefficient</th>
<th>Model $R^2$</th>
<th>Model $p$ value</th>
<th>Predictor $p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>0.476</td>
<td>0.036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>1.179</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.026</td>
<td></td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>(adjusted for LDL-cholesterol, HDL-cholesterol, pulse pressure and HbA1c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DM2 patients

No significant predictors when adjusted for LDL-cholesterol, HDL-cholesterol, pulse pressure and HbA1c

| Non-DM patients       | 0.639         | 0.028       |                 |                     |
| (Constant)            | 1.179         |             |                 |                     |
| HDL cholesterol       | 0.268         |             | 0.056           |                     |
| HbA1c                 | -0.315        |             | 0.014           |                     |
| (adjusted for BMI, LDL-cholesterol and pulse pressure) |
Figure 2. Carotid artery wall dimensions at baseline and at follow-up in non-DM (DM-) and DM2 patients (DM+). Mean and standard deviation (SD) dimensions are shown. *= P<0.05.

No significant changes in mean LDL-cholesterol, glucose and HbA1c were observed between baseline and follow-up in both DM2 and non-DM patient groups (Figure 1 supplemental data).

DISCUSSION

In the present study, we show a decrease in the total wall volume (TWV) and mean wall area of the carotid artery in DM2 patients with CA stenosis after two years of guideline-based cardiovascular prevention therapy. In contrast, a stabilization of the vessel wall parameters was observed in non-DM patients with CA stenosis receiving
similar CV-prevention. BMI was identified as predictor for a decrease in TWV after 2 years of follow-up.

**Decrease in vessel wall dimensions in DM2 patients after two years**

The decrease in carotid artery wall dimensions expressed as TWV at follow-up in DM2 patients illustrates that particularly in these patients guideline-treatment is efficacious at CVD risk reduction. Such a decrease in TWV may not only lead to a decrease in major cardiovascular events, but might also reduce mortality, especially if treatment is continued for a life-time period.

Identification of predictive parameters for TWV decrease in DM2 patients was performed by multivariate linear regression. In this way, plasma glucose, HbA1c levels and the diagnosis diabetes mellitus fully participated in analysis and were ruled out as bias. By doing so, BMI was identified as the only predictor for TWV decrease.

To be specific, having a high BMI is associated with stronger decrease of TWV, even if adjusted for alternative traditional cardiovascular risk factors. The latter suggest that DM2 patients with a high BMI benefit the most from guideline cardiovascular prevention regimens.

Indeed, high BMI has been widely associated with increased cardiovascular mortality and morbidity in both DM2- and non-DM patients [15]. BMI was in addition identified as predictor for 6-year progression of carotid intima-media thickness (IMT) in a population of young adults, independent from other components of metabolic syndrome. High BMI in patients with established cardiovascular morbidity has been associated with superior medication treatment response compared with patients with normal BMI [16]. In addition, a stronger decrease in BMI in patients with coronary artery disease (CAD) results in a higher decrease in coronary atherosclerosis, especially in patients with metabolic syndrome [17]. These findings support the hypothesis that patients with obesity have atherosclerotic lesions with higher inflammatory activity that allows stronger inhibitory effect of statins, resulting in a stronger reduction in vessel wall dimension [18]. The presence of diabetes may contribute to even higher inflammatory lesions. Opposite effect of BMI was reported by Tani et al, who showed that high BMI attenuates the anti-atherosclerotic effects of pravastatin, supposed to be caused by the presence of more pro-atherosclerotic stimuli in obese patients, inhibiting the effects of pravastatin [19].

No predictors were identified for the within-group variation in TWV in DM2 patients. This could partly be attributed to lack of variation and in part due to adjusting for several risk-factors.

**Vessel wall dimensions in non-DM patients after two years**

In non-DM patients, CA wall dimensions were unchanged after two years follow-up, indicating that atherogenesis was arrested during the two-year follow-up. Though,
HbA1c was identified as significant predictor, even after adjustment for traditional risk factors. High levels of HbA1c were associated with a stronger decrease in TWV than low levels of HbA1c. We thus can conclude that long-term exposure to these high levels of glycated hemoglobin is associated with increased atherosclerosis, resulting in higher cardiovascular disease and death from any cause, which is in line with recent literature concerning patients without the diagnosis of diabetes [20].

It remains unclear why higher HbA1c robustly predicts a decrease in vessel wall dimensions, despite adjustment for BMI, LDL-cholesterol and pulse pressure. High HbA1c in these patients may be accompanied with elevated levels of LDL-cholesterol. Although not identified as a predictor, the association of elevated LDL-cholesterol levels and accelerated atherogenesis has been widely acknowledged [21–24].

**Study limitation**

MRI-imaging was performed using a 1.5T MRI. Recently published studies that use MRI to determine changes in the carotid artery wall often use field strengths of 3T or above. However, the inferior field strength in a head-to-head comparison has proven to be equally utilizable for morphometric measurements, despite lower signal-to-noise ratio’s [25,26].

Our method for patient selection using ultrasonography to detect common carotid artery stenosis may have yielded a heterogeneous group of patients. Since carotid ultrasound measurement of plaque only includes the interior quadrant of the carotid artery, this technique may overestimate the actual stenosis rate as measured by MRI [27]. The smaller wall dimensions of the included patients as observed by MRI may have reduced the measured effect size.

**CONCLUSION**

Our study demonstrates that especially patients with DM2 with high levels of BMI benefit from strict adherence to treatment guidelines. Although all patients were on treatment during study initiation, DM2 patients demonstrated a decrease in vessel wall dimensions, which may be translated into cardiovascular risk modulation. Our results therefore urge clinicians to progressively eliminate effects of risk factors by tenaciously supporting improvement in prescribing drug therapy according to up-to-date cardiovascular guidelines.

**ACKNOWLEDGEMENTS**

We would like to gratefully and sincerely thank dr. R.J. van der Geest for supplying quantification software (Vessel-Mass) and for his excellent technical engineering and technical advice. We also would like to thank Anne Cappon for her assistance with image analysis.
REFERENCES


Supplementary Figure 1. Mean plasma levels in DM2 patients (DM+) and non-DM patients (DM-) at baseline and follow-up. No significant differences were observed between baseline and follow-up parameters.

Supplementary Figure 2. Example of decrease in carotid artery (CA) wall dimensions in diabetics (top row) and unchanged CA wall dimensions in non-diabetics at follow-up compared with baseline. ICA=internal carotid artery, ECA=external carotid artery.
<table>
<thead>
<tr>
<th></th>
<th>T1 TFE</th>
<th>T2 TSE</th>
<th>TOF</th>
<th>PDW-TSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR/TE (ms)</td>
<td>10/3.9 ms</td>
<td>2 heartbeats/50 ms</td>
<td>31/3.0</td>
<td>2 heartbeats/20 ms</td>
</tr>
<tr>
<td>Flip angle (deg)</td>
<td>15</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Field of view (mm)</td>
<td>140x140</td>
<td>140x140</td>
<td>140x140</td>
<td>140x140</td>
</tr>
<tr>
<td>Matrix size</td>
<td>256x256</td>
<td>256x256</td>
<td>256x256</td>
<td>256x256</td>
</tr>
<tr>
<td>Reconstructed width</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>In-plane resolution</td>
<td>0.39x0.39 mm</td>
<td>0.39x0.39 mm</td>
<td>0.39x0.39 mm</td>
<td>0.39x0.39 mm</td>
</tr>
<tr>
<td>Imaging duration</td>
<td>3:35 min</td>
<td>1:53 min</td>
<td>4:52 min</td>
<td>2:58 min</td>
</tr>
</tbody>
</table>

Parameters of MR pulse sequences at a heartbeat of 70 per minute
FDG-PET/CT TO QUANTIFY ARTERIAL WALL INFLAMMATION
DIAGNOSTIC REFERENCE RANGES FOR CAROTID AND AORTA ATHEROSCLEROTIC PLAQUE INFLAMMATION AS ASSESSED BY FDG-PET/CT
ABSTRACT

Background: ¹⁸F-fluorodeoxyglucose positron emission tomography with computed tomography (FDG-PET/CT) is increasingly applied in atherosclerosis imaging. The degree of carotid and aorta arterial wall inflammation as assessed with FDG-PET/CT is expressed in a target-to-background-ratio (TBR). We aimed to provide reference values for TBR in cardiovascular disease (CVD) risk groups.

Methods: We performed FDG-PET/CT scanning of the left and right carotid artery and aorta in 25 healthy control subjects, 23 patients with an increased coronary heart disease (CHD) risk, and 35 patients with a documented history of atherosclerotic CVD. We quantified FDG uptake using the TBR and translated the 95% confidence interval per group as reference values for the corresponding group. In addition, we assessed interscan variability and interobserver and intraobserver agreement.

Results: The upper limit of normal of TBR max for the carotid in healthy controls was 2.16, which was higher in 30% of patients at increased risk and in 46% in CVD patients. For the ascending aorta, TBR max’s upper limit was 2.87 in healthy controls, which was exceeded by 35% of patients with increased risk for CHD and by 43% of patients with CVD. Interscan variability over 3 weeks was low, whereas intra- and interobserver agreement was high across all arterial segments imaged and in all three groups separately.

Conclusions: The study data provide guidance for the interpretation of FDG-PET/CT parameters of arterial wall inflammation in the spectrum of healthy to diseased human subjects.
INTRODUCTION

Atherosclerosis is an inflammatory disease affecting the arterial wall. Typically, atherosclerotic plaques develop in a slumbering fashion over many decades prior to the acute onset of cardiovascular events, primarily myocardial infarction and stroke. In an effort to discriminate those who will suffer from an event from those destined to remain event-free, several noninvasive imaging modalities are being evaluated for their ability to predict cardiovascular disease (CVD) risk.

Mirroring inflammation’s crucial role in atherogenesis, 18F-fluordeoxyglucose positron emission tomography with computed tomography (FDG-PET/CT) is increasingly applied to increase our insight in atherosclerotic plaque biology [1–10], identify patients at risk for CVD [11,12] and monitor therapeutic efficacy of anti-atherosclerotic or anti-inflammatory strategies [13–17]. In the context of atherosclerosis, FDG uptake is associated with arterial wall inflammation since tracer uptake is predominantly found in macrophage rich areas [10] and has been associated with both macrophage content and inflammatory gene expression [3].

Whereas the number of FDG-PET/CT atherosclerosis imaging studies is rapidly expanding, reference ranges the target to background ratio (TBR) are lacking. In the present paper, we set out to provide a reference range for arterial wall inflammation as assessed by FDG-PET/CT in healthy subjects, patients at risk for coronary heart disease (CHD) and patients with a documented history of CVD. In addition, we evaluated interscan variance and intra- and interobserver agreement in our study participants.

METHODS

Subject population

We selected subjects for three study groups; (i) healthy control subjects, (ii) patients with an increased cardiovascular risk as attested by a 10 years coronary heart disease (CHD) Framingham risk score of >10%, and (iii) patients with a documented history of atherosclerotic cardiovascular disease; i.e. myocardial infarction, transient ischemic attack (TIA), stroke and/or significant carotid stenosis. Patients were on stable medication for at least 6 weeks prior to study participation. Exclusion criteria for all subjects were age <40 years, diabetes mellitus, ongoing inflammatory diseases, use of anti-inflammatory drugs, serum creatinine >2.0 mg/dL, or alanine transaminase and or aspartate transaminase >2 times the upper limit of normal. In all subjects FDG-PET/CT was performed between January 2012 and May 2013 at the Academic Medical Center, Amsterdam, The Netherlands. Ten subjects underwent an initial scan and a repeat scan after 3 weeks to assess interscan reproducibility. All subjects provided written informed consent. The study was approved by the local institutional review board and conducted according to the principles of the International Conference on Harmonisation–Good Clinical Practice guidelines.
Biometric and Biochemical Measurements

Presence of cardiovascular risk factors, and use of medication were assessed by a questionnaire. Brachial artery blood pressures were measured using an oscillometric blood pressure device. Presence of hypertension was defined as a systolic blood pressure (SBP) >140 mmHg, a diastolic blood pressure (DBP) >90 mmHg or use of antihypertensive medication. Weight and length were measured to calculate body mass index (BMI). EDTA plasma obtained through venous blood samples were obtained after overnight fasting and stored using standardized protocols. Plasma total cholesterol (TChol), high-density lipoprotein cholesterol (HDL-c) and triglyceride (TG) levels were analyzed using commercially available enzymatic methods. Low-density lipoprotein cholesterol (LDL-c) levels were calculated using the Friedewald equation.

FDG-PET/CT imaging and analysis

FDG-PET/CT scans of the carotid arteries and aorta were performed on a dedicated PET/CT scanner (Philips, Best, the Netherlands). Subjects fasted for at least 6 hours prior to infusion of 200 MBq of FDG (5.5 mCi). After 90 min of FDG circulation, PET/CT imaging was initiated with a low-dose non-contrast enhanced CT with a slice thickness of 3 mm for attenuation correction and anatomic co-registration.

PET/CT images were analyzed with dedicated analysis software (OsiriX, Geneva, Switzerland; http://www.osirix-viewer.com/). Two readers analyzed all images to assess inter-observer variability. One reader analyzed all images twice to assess intra-observer variability. The images were blinded for patient data.

In the left and right carotid arteries FDG uptake was assessed (Figure 1) starting from 1 slice caudal of the carotid bifurcation downwards. In each carotid artery at least 5 regions of interest (ROI) were drawn, delineating the outer arterial wall. In the aorta, FDG uptake was assessed in at least 5 ROIs per subsequent slice, starting from 1 slice cranial of the pulmonary arteries upwards. Of each ROI, the mean and maximum standardized uptake values (SUVs) were obtained. The SUV represents the FDG activity in the ROI (in kBq/ml), adjusted for the administered FDG dose corrected for decay (in MBq) and divided by body weight (in kg). Out of the ROIs, mean and maximum SUVs were averaged for each arterial segment (SUV_{mean} and SUV_{max}). Background activity was assessed within the venous blood pool (for the carotid arteries in corresponding slices in the jugular veins and for the aorta in corresponding slices in the vena cava superior) as an average of 5 ROIs. The target-to-background-ratio (TBR) of was calculated as: TBR_{mean or max} = SUV_{target (mean or max)} / SUV_{Background Venous Activity}.

In addition, most diseased arterial segment TBR (TBR_{mds}) was calculated as a mean of three contiguous slices with the highest SUV_{max}, thus: TBR_{mds} = SUV_{target (3 slices highest max)} / SUV_{Background Venous Activity}.
**Figure 1.** Analysis of FDG uptake in the arterial wall.
Representative CT, FDG-PET and FDG-PET/CT images from the region of interest of the carotid arteries. The schematic overview (second column) shows the ratio between the target (arterial wall) and background (blood pool) of the whole vessel wall and the most diseased segment.

<table>
<thead>
<tr>
<th>Representative images</th>
<th>Image analysis</th>
<th>Image parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CT</strong></td>
<td>Per Arterial Segment</td>
<td>$TBR_{mean} = \frac{\text{mean SUV mean of Arterial Segment}}{\text{mean SUV mean of Bloodpool}}$</td>
</tr>
<tr>
<td><strong>FDG-PET</strong></td>
<td>Per Most Diseased Segment</td>
<td>$TBR_{max} = \frac{\text{mean SUV max of Arterial Segment}}{\text{mean SUV mean of Bloodpool}}$</td>
</tr>
<tr>
<td><strong>FDG-PET/CT</strong></td>
<td></td>
<td>$TBR_{mds} = \frac{\text{mean SUV max of Most Diseased Segment}}{\text{mean SUV mean of Bloodpool}}$</td>
</tr>
</tbody>
</table>
Statistical Analysis
Continuous variables are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR), unless otherwise specified. Differences in demographic, biometrical and biochemical parameters between healthy control subjects, patients at increased CHD risk and patients with CVD were assessed using one way analysis of variance (ANOVA). Differences in FDG-PET/CT imaging parameters between the different groups were assessed with a multivariate model to account for age, gender, hypertension (SBP >140 mmHg, DBP >90 mmHg or use of antihypertensive medication), TChol, HDL-c, BMI, and smoking. We estimated the reference ranges for the FDG-PET/CT parameters based on log-normal distribution. We defined the upper limit of the 95% prediction interval of the healthy controls as the upper limit of normal (ULN). The agreement between successive FDG-PET/CT scans and analyses were assessed using intraclass correlation coefficients (ICC, r) and Bland-Altman plots. The SD of the paired differences (SDpd) and the coefficient of variation (COV) between the initial en repeat scans were calculated. COV was calculated by dividing the SDpd by the mean value of the population for each parameter. Alpha <0.05 was defined as statistically significant. All data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois).

RESULTS
Clinical characteristics
Overall, we included 83 study participants (aged 61 ± 8 years), consisting of 25 healthy control subjects, 23 patients at intermediate risk for CHD (median Framingham risk score 14% [IQR 4]), and 35 patients with a history of atherosclerotic CVD documented as significant carotid artery stenosis (n=13), TIA (n=9), stroke (n=9), and/or myocardial infarction (n=25). Clinical characteristics are outlined in Table 1. Fourteen percent of patients with CVD smoked, while none of the healthy subjects and patients with increased CHD risk were active smokers. Tchol, LDLc and TG were higher in patients with increased CHD risk and patients with CVD than in healthy subjects. Other parameters were comparable between groups.

Imaging parameters
The $TBR_{max}$ values of the carotid arteries and ascending aorta are provided in Table 2, whereas $TBR_{mean}$ and $TBR_{mds}$ are shown in supplementary Table 1. Carotid index TBR displayed a significant stepwise increase from healthy control to patients at increased CHD risk and patients with CVD (p<0.001 for both comparisons; Figure 2). Corresponding differences between groups were observed for aortic TBR (p<0.001 for both comparisons; Figure 2). The reference ranges of carotid and aorta FDG-PET parameters are shown in Table 3. The upper limit of normal $TBR_{max}$ was 2.16 for the carotids 2.87 for the ascending aorta, which exceeded in 46% and 43% of the CVD patients, respectively.
### Table 1. Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy controls (n=25)</th>
<th>Patients with CHD risk (n=23)</th>
<th>Patients with CVD (n=35)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60 ± 11</td>
<td>59 ± 6</td>
<td>63 ± 7</td>
<td>ns</td>
</tr>
<tr>
<td>Gender, %male (n)</td>
<td>60 (15)</td>
<td>74 (17)</td>
<td>77 (27)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg m⁻²)</td>
<td>25 ± 3</td>
<td>26 ± 3</td>
<td>27 ± 4</td>
<td>ns</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>134 ± 16</td>
<td>135 ± 9</td>
<td>133 ± 8</td>
<td>ns</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81 ± 10</td>
<td>82 ± 8</td>
<td>81 ± 7</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking, %active (n)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (5)</td>
<td>0.026</td>
</tr>
<tr>
<td>TChol, mmol/L</td>
<td>5.32 ± 0.96</td>
<td>7.33 ± 2.81</td>
<td>5.99 ± 3.16</td>
<td>0.040</td>
</tr>
<tr>
<td>LDLc, mmol/L</td>
<td>3.24 ± 0.97</td>
<td>5.42 ± 2.63</td>
<td>4.18 ± 3.11</td>
<td>0.011</td>
</tr>
<tr>
<td>HDLc, mmol/L</td>
<td>1.65 ± 0.37</td>
<td>1.21 ± 0.25</td>
<td>1.24 ± 0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG</td>
<td>0.98 ± 0.53</td>
<td>1.63 ± 0.54</td>
<td>1.49 ± 0.70</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD; BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, ns = not significant

### Table 2. Imaging parameters (TBR_max)

<table>
<thead>
<tr>
<th>TBR_max per arterial segment</th>
<th>Controls (n=25)</th>
<th>Patients with CHD risk (n=23)</th>
<th>Patients with CVD (n=35)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left carotid artery</td>
<td>1.51 ± 0.20</td>
<td>1.89 ± 0.20</td>
<td>2.00 ± 0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Right carotid artery</td>
<td>1.58 ± 0.26</td>
<td>1.99 ± 0.34</td>
<td>2.09 ± 0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carotid index vessel</td>
<td>1.63 ± 0.25</td>
<td>2.04 ± 0.32</td>
<td>2.16 ± 0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>2.36 ± 0.25</td>
<td>2.84 ± 0.43</td>
<td>2.93 ± 0.58</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* multivariate analysis adjusted for age, gender, hypertension, smoking, BMI, total cholesterol, HDL-c, LDL-c and triglycerides.

### Table 3. TBR reference ranges

<table>
<thead>
<tr>
<th>Arterial segment</th>
<th>Controls (n=25)</th>
<th>Patients with CHD risk (n=23)</th>
<th>Patients with CVD (n=35)</th>
<th>ULN</th>
<th>Range</th>
<th>&gt;ULN</th>
<th>Range</th>
<th>&gt;ULN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid index</td>
<td>TBR_mean 1.76</td>
<td>1.26–2.01 22%</td>
<td>1.32–2.41 40%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TBR_max 2.16</td>
<td>1.52–2.67 30%</td>
<td>1.55–2.95 46%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRB_mid 2.31</td>
<td>1.55–2.93 26%</td>
<td>1.75–3.05 46%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>TBR_mean 2.41</td>
<td>1.58–2.73 22%</td>
<td>1.57–2.98 26%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TBR_max 2.87</td>
<td>2.09–3.77 35%</td>
<td>1.99–4.17 43%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRB_mid 2.99</td>
<td>2.16–3.90 30%</td>
<td>2.02–4.35 43%</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Reproducibility

We also assessed scan and observer agreement in healthy controls and patients with increasing disease severity and interscan agreement in patients. Table 4 shows the interscan variability within 3 weeks for 10 patients, and the intra- and inter-observer variability data for all subjects at all three arterial segments, indicating that all ICC values are >0.95 with narrow 95% confidence intervals. Bland-Altman plots for observer variability display no fixed or proportional bias (Figure 3). Corresponding agreements were found when analyzing the variability data for TBRmean and TBRmds, and for all three groups separately (Supplementary Table 2).

DISCUSSION

In the present study, we demonstrate the distinctiveness of FDG-PET/CT and provide a reference range for carotid and aortic TBR’s. Using standard operating and reader procedures, we confirm reproducibility of scan and observer agreement for PET imaging in cardiovascular patients, and demonstrate similar agreement data in healthy control subjects across all three arterial segments imaged.

The present imaging data are in line with previous FDG-PET/CT studies in cardiovascular patients (Figure 4). For the carotids, we show a mean TBRmax of 2.16 ± 0.36 ranging between 1.55 and 2.95 in CVD patients. In line, others reported carotid TBRmax’s of 1.90 ± 0.20 (n=20) [18], 1.83 ± 0.20 (n=35) [19], 2.00 ± 0.30 (n=82) [20] and 1.90 ± 0.27 (n=10) [1] in CVD patients. With respect to the aorta, mean TBRmax was 2.93

Figure 2. Carotid index and ascending aorta TBRmax.
Scatterplot showing the significant stepwise increase in TBRmax of the carotid index and ascending aorta from healthy controls to patients at CHD risk and patients with actual CVD. The provided p-values were determined after adjusting for traditional cardiovascular risk factors on multivariate analysis. ULN represent the upper limit of the 95% prediction interval.
± 0.58 ranging between 1.99 and 4.17 in our CVD patients, which corresponded to previous studies providing TBR\textsubscript{max} of 2.60 ± 0.60 \((n=66)\) \[16\] and 2.84 ± 0.69 \((n=10)\) \[1\].

Evaluation of previous FDG-PET/CT studies in cardiovascular patients revealed that imaging acquisition protocols are quite uniform across various studies following two landmark papers \[18,21\], whereas there still is substantial variation in image analysis and presentation of outcome parameters throughout literature. Lack of consistency in methods limits comparisons between these studies due to two main issues.

First, which arterial segments are imaged and subsequently used for analysis? Whereas the TBR values are similar for the left and right carotid artery, the TBR of the ascending aorta is in general significantly higher than in the carotid arteries. This has important implications for the index vessel, i.e. the arterial segment with the highest TBR at baseline. Due to the fact that the aortic TBR is most often the highest, the aorta would have been selected as index vessel in more than 80% of the subjects in our study. This percentage corresponds to a previous study showing a similar distribution \[16\].

Such an approach might potentially mask changes over time in the carotid arteries and

### Table 4. Scan and observer reproducibility for TBR\textsubscript{max}

<table>
<thead>
<tr>
<th></th>
<th>Left carotid artery</th>
<th>Right carotid artery</th>
<th>Ascending aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interscan variability in 10 patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scan 1</td>
<td>2.00 ± 0.33</td>
<td>2.12 ± 0.42</td>
<td>3.04 ± 0.61</td>
</tr>
<tr>
<td>Scan 2</td>
<td>1.98 ± 0.35</td>
<td>2.10 ± 0.39</td>
<td>3.01 ± 0.69</td>
</tr>
<tr>
<td>SDpd</td>
<td>0.07</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>COV</td>
<td>3.5%</td>
<td>2.4%</td>
<td>2.3%</td>
</tr>
<tr>
<td>wsCV</td>
<td>6.2%</td>
<td>2.2%</td>
<td>6.2%</td>
</tr>
<tr>
<td>Interscan ICC [CI]</td>
<td>0.97 [0.88 – 0.99]</td>
<td>0.98 [0.92-1.00]</td>
<td>0.98 [0.93-1.00]</td>
</tr>
<tr>
<td>Intraobserver variability in 83 subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Read 1A</td>
<td>1.83 ± 0.35</td>
<td>1.91 ± 0.39</td>
<td>2.73 ± 0.52</td>
</tr>
<tr>
<td>Read 1B</td>
<td>1.82 ± 0.36</td>
<td>1.93 ± 0.41</td>
<td>2.71 ± 0.50</td>
</tr>
<tr>
<td>Paired difference</td>
<td>0.01 ± 0.05</td>
<td>0.03 ± 0.09</td>
<td>0.02 ± 0.05</td>
</tr>
<tr>
<td>COV</td>
<td>2.7%</td>
<td>4.7%</td>
<td>1.8%</td>
</tr>
<tr>
<td>wsCV</td>
<td>5.7%</td>
<td>2.8%</td>
<td>2.1%</td>
</tr>
<tr>
<td>Intra ICC [CI]</td>
<td>0.99 [0.98 – 1.00]</td>
<td>0.99 [0.97 – 1.00]</td>
<td>0.99 [0.99 – 1.00]</td>
</tr>
<tr>
<td>Interobserver variability in 83 subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Read 2*</td>
<td>1.83 ± 0.35</td>
<td>1.93 ± 0.38</td>
<td>2.69 ± 0.47</td>
</tr>
<tr>
<td>Paired difference</td>
<td>0.03 ± 0.11</td>
<td>0.05 ± 0.14</td>
<td>0.07 ± 0.14</td>
</tr>
<tr>
<td>COV</td>
<td>6.0%</td>
<td>7.3%</td>
<td>5.2%</td>
</tr>
<tr>
<td>wsCV</td>
<td>4.1%</td>
<td>3.0%</td>
<td>2.1%</td>
</tr>
<tr>
<td>Inter ICC [CI]</td>
<td>0.96 [0.93 – 0.97]</td>
<td>0.96 [0.95 – 0.98]</td>
<td>0.97 [0.96 – 0.99]</td>
</tr>
</tbody>
</table>

* for interobserver variability analysis, read 2 was compared with read 1A
Figure 3. Bland-Altman plots for observer agreement.
Bland-Altman plots display no fixed or proportional bias for TBR_{\text{max}} in interscan, intra- and inter-observer agreement as shown for the carotid index (A-c) and ascending aorta (D-F).
ignores the variation in plaques at different arterial segments. Thus, our data reinforce to describe the (changes in) carotid and aortic TBR's separately.

Secondly, which part of the arterial segment is analyzed? The TBR can be retrieved from the whole vessel, a most diseased segment, active segments or via a plaque-based analysis [14]. We show clear differences between the TBR derived from the whole vessel, such as TBR_{mean} and TBR_{max}, and the TBR of a most diseased segment. This mds is retrieved from three contiguous slices with the highest SUV_{max}. Thus, when comparing the TBR_{mds} over time or after a therapeutic intervention it must we ensured that in the follow-up scans the mds is matched to baseline and not based on potentially three other contiguous slices with the highest SUV_{max}. Whereas in the case of whole vessel TBR's this might not be an issue, TBR_{mean} and TBR_{max} might ignore more moderate changes within the arterial wall. Hence, FDG-PET/CT atherosclerosis imaging studies should carefully consider their surrogate endpoints based on the expected change (i.e. regression of plaque or anti-inflammatory) and the included subjects (i.e. patients with or without significant plaques).

The present data underscore the reproducibility of scan and observer agreement for PET imaging in humans. Whereas these findings strengthen the robustness of FDG-PET/CT imaging in atherosclerosis, several pending issues need further attention. First, we confirm a low TBR variation of less than 3.5% over 3 weeks time, whereas most intervention studies comprise 3 months. Yet, previous placebo-controlled interventions studies show relative small variations over 3 to 6 months [14,16]. Second, the specificity of FDG uptake in atherosclerosis is still under debate. Whereas FDG is a highly sensitive marker, it lacks intrinsic specificity for target tissue. The majority of data poses that FDG uptake mainly indicates (hypoxia-induced) inflammation, yet these studies are merely performed in an in vitro setting or via ex vivo analysis [1,3,7,10,22]. Reconstruction of PET with magnetic resonance imaging (MRI), instead of the current used low-dose CT, would allow for an improved interpretation of plaque FDG uptake in vivo.

Finally, using the mean and standard deviation of TBR_{max} of the carotid index and ascending aorta, we assessed the number of CVD patients needed to include in drug trials using FDG-PET/CT as surrogate endpoint (Figure 5). We reaffirm that PET
Figure 5. Sample size calculation for intervention studies using FDG-PET/CT as primary outcome measure. This graph shows the number of CVD patients needed to include in an intervention study based on the estimated reduction in TBR shown separately for the carotid index and aorta. Test significance level of 0.05, 2-sided test and power of 80%.

imaging requires few CVD patients to show significant differences between groups [21], nonetheless, we would like to emphasize the increased number of CVD patients necessary once the aortic TBR is used as read-out parameter.

CONCLUSION

We provide a reference value for the degree of arterial wall inflammation in humans as assessed with FDG-PET/CT and corroborate the reproducibility of this imaging approach. With the number of FDG-PET/CT atherosclerosis imaging studies increasingly rapidly, the present data can serve as a guide in the interpretation of these studies.

REFERENCES


### Supplementary Information

**Supplementary Table 1.** Additional imaging parameters (TBR\text{mean} and TBR\text{mds})

<table>
<thead>
<tr>
<th>Arterial segment</th>
<th>Controls (n=25)</th>
<th>Patients with CHD risk (n=23)</th>
<th>Patients with CVD (n=35)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBR\text{mean}</td>
<td>TBR\text{mds}</td>
<td>TBR\text{mean}</td>
<td>TBR\text{mds}</td>
</tr>
<tr>
<td>Left carotid artery</td>
<td>1.42 ± 0.17</td>
<td>1.62 ± 0.23</td>
<td>1.52 ± 0.19</td>
<td>1.97 ± 0.32</td>
</tr>
<tr>
<td>Right carotid artery</td>
<td>1.31 ± 0.18</td>
<td>1.64 ± 0.27</td>
<td>1.58 ± 0.20</td>
<td>2.10 ± 0.40</td>
</tr>
<tr>
<td>Carotid index vessel</td>
<td>1.43 ± 0.16</td>
<td>1.72 ± 0.27</td>
<td>1.60 ± 0.20</td>
<td>2.16 ± 0.38</td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>1.82 ± 0.28</td>
<td>2.44 ± 0.28</td>
<td>2.10 ± 0.30</td>
<td>2.94 ± 0.45</td>
</tr>
</tbody>
</table>
Supplementary Table 2. Observer reproducibility in separate groups

<table>
<thead>
<tr>
<th>ICC [CI]</th>
<th>All subjects (n=83)</th>
<th>Healthy controls (n=25)</th>
<th>Patients at CHD risk (n=23)</th>
<th>Patients with CVD (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Left carotid artery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBR&lt;sub&gt;mean&lt;/sub&gt;</td>
<td>Intraobserver</td>
<td>0.97 [0.96 – 0.98]</td>
<td>0.79 [0.51 – 0.91]</td>
<td>0.98 [0.96 – 0.99]</td>
</tr>
<tr>
<td></td>
<td>Interobserver</td>
<td>0.98 [0.97 – 0.99]</td>
<td>0.98 [0.95 – 0.99]</td>
<td>0.95 [0.88 – 0.98]</td>
</tr>
<tr>
<td>TBR&lt;sub&gt;mds&lt;/sub&gt;</td>
<td>Intraobserver</td>
<td>0.99 [0.99 – 1.00]</td>
<td>0.97 [0.93 – 0.99]</td>
<td>0.99 [0.97 – 1.00]</td>
</tr>
<tr>
<td></td>
<td>Interobserver</td>
<td>0.96 [0.93 – 0.97]</td>
<td>0.79 [0.48 – 0.91]</td>
<td>0.98 [0.94 – 0.99]</td>
</tr>
<tr>
<td><strong>Right carotid artery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBR&lt;sub&gt;mean&lt;/sub&gt;</td>
<td>Intraobserver</td>
<td>0.98 [0.97 – 0.99]</td>
<td>0.92 [0.82 – 0.97]</td>
<td>0.99 [0.97 – 1.00]</td>
</tr>
<tr>
<td></td>
<td>Interobserver</td>
<td>0.93 [0.90 – 0.96]</td>
<td>0.99 [0.95 – 0.99]</td>
<td>0.90 [0.75 – 0.96]</td>
</tr>
<tr>
<td>TBR&lt;sub&gt;mds&lt;/sub&gt;</td>
<td>Intraobserver</td>
<td>0.99 [0.99 – 1.00]</td>
<td>0.99 [0.98 – 1.00]</td>
<td>1.00 [0.99 – 1.00]</td>
</tr>
<tr>
<td></td>
<td>Interobserver</td>
<td>0.98 [0.96 – 0.99]</td>
<td>0.99 [0.96 – 0.99]</td>
<td>0.96 [0.89 – 0.98]</td>
</tr>
<tr>
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<tr>
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<td>Intraobserver</td>
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<td>1.00 [0.99 – 1.00]</td>
<td>1.00 [0.97 – 1.00]</td>
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<tr>
<td></td>
<td>Interobserver</td>
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<td>0.98 [0.95 – 0.99]</td>
<td>0.98 [0.94 – 0.99]</td>
</tr>
<tr>
<td>TBR&lt;sub&gt;mds&lt;/sub&gt;</td>
<td>Intraobserver</td>
<td>1.00 [0.99 – 1.00]</td>
<td>1.00 [0.99 – 1.00]</td>
<td>1.00 [0.98 – 1.00]</td>
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<tr>
<td></td>
<td>Interobserver</td>
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<td>0.97 [0.88 – 0.99]</td>
<td>0.98 [0.95 – 0.99]</td>
</tr>
</tbody>
</table>
ARTERY WALL INFLAMMATORY ACTIVITY REMAINS ELEVATED IN PATIENTS WITH RHEUMATOID ARTHRITIS IN REMISSION DURING ANTI-TNF THERAPY


Manuscript in preparation
ABSTRACT

Background: Patients with rheumatoid arthritis (RA) have increased cardiovascular disease (CVD) risk which has been attributed to the chronic systemic inflammation. Anti-inflammatory regimens have proven to effectively decrease RA disease activity, but the effect on inflammatory activity in the (atherosclerotic) arterial wall remains elusive. Using MRI and FDG-PET/CT to measure artery wall dimensions and inflammation, we evaluated the atherogenic process in RA patients in remission during either MTX or MTX/anti-TNF combination therapy.

Methods: We included 24 RA patients in remission after biological therapy, of whom 15 used methotrexate only (MTX) and 9 required continued MTX and anti-TNF combination therapy to remain in remission. In addition, 19 age- and sex-matched controls as well as 14 patients with cardiovascular disease were included. We assessed atherosclerotic burden and arterial wall inflammation by carotid arterial wall dimensions with MRI assessed as normalized wall index (NWI) and PET/CT assessed as target-to-background ratio (TBR).

Results: NWI in RA patients was higher compared to controls (0.40 ± 0.04 vs 0.32 ± 0.03; p < 0.001), albeit it was smaller than that observed in CVD patients (0.51 ± 0.05; p < 0.001 vs all other). FDG uptake on PET/CT was significantly higher in RA patients using biologicals (n=9) compared to those using MTX only (n=7) (TBR MTX 1.52 ± 0.16 vs anti-TNF 1.93 ± 0.37; p = 0.046), despite comparable RA disease activity scores (DAS MTX 1.39 [1.31-1.54] vs anti-TNF 1.59 [1.46-2.72]; ns).

Conclusions: Remissive RA patients in need of continued biological therapy are characterized by an increased inflammatory activity in the arterial wall, compared to the absence of inflammatory activity in remissive RA patients requiring MTX only. This local pro-inflammatory activity of the atherosclerotic arteries in remissive RA patients requiring biological implies a persistent pro-atherogenic state, reinforcing the need for optimal CV-prevention notwithstanding the clinical remission achieved in these patients.
INTRODUCTION

Patients with rheumatoid arthritis (RA) have an increased cardiovascular disease (CVD) risk, clinically translated in a CV-age 10 years higher than age-matched subjects from the general population [1]. Apart from the traditional risk factors for CVD [2], chronic and systemic inflammation has been suggested to play a causal role in this increased risk [3], since a direct role for arterial wall inflammation has been demonstrated at least during active stages of the disease [4]. Improvements in therapeutic interventions following the introduction of immune modulating biological therapies have led to lower disease activity and disability in RA [5,6]. The decrease in RA related systemic inflammation has however not translated into a significant benefit in cardiovascular comorbidity yet [7–9]. Conversely, short-term treatment with anti-tumor necrosis factor-α (anti-TNFα) therapy has been shown to reduce the inflammatory activity in the vessel wall in RA patients [10]. However, it remains to be elucidated whether long-term disease remission abolishes the increased inflammatory activity in the arterial wall and the concomitant excess cardiovascular risk in patients with RA.

Since clinical endpoint studies require long follow-up and cardiovascular risk algorithms are only applicable in large cohorts, CVD risk has been estimated in vivo based on the use of surrogate markers for CVD. Presence of vessel wall atherosclerosis as product of inflammation in the vessel wall by measuring by measuring IMT using ultrasonography has been substituted by magnetic resonance imaging (MRI). The use of MRI allows high resolution imaging and measurement of arterial wall dimensions of an entire artery of interest, mostly the carotid artery [11,12]. More recently, 18F-fluorodeoxyglucose positron emission tomography (18FDG-PET) combined with x-ray computed tomography (CT) has increasingly been used to assess arterial wall inflammatory activity in the carotid artery and aortic arch as well [13]. In addition to the MRI-derived vessel wall dimensions, FDG-PET/CT has further improved CVD risk prediction [14].

In the current study we aim to assess the structural and functional artery wall properties in subjects with RA in remission for at least 6 months following effective therapy. To this end, we measured vessel wall dimensions using MRI and vessel wall inflammation using FDG-PET/CT. The combination of these modalities allows superior assessment of CVD risk in RA compared to healthy control subjects and patients with a history of CVD. In addition, we dissect the effects on arterial wall dimensions and inflammation in subjects with RA in remission dependent and independent of anti-TNFα therapy to maintain RA-remission.

METHODS

Study population

In the present study we compared atherosclerotic burden between subjects with remissive RA, matched healthy subjects and subjects with overt cardiovascular disease, all over 18 years of age. Subjects with RA in remission were referred from
the outpatient clinic of the department of clinical immunology and rheumatology at the Academic Medical Center in Amsterdam, The Netherlands. Inclusion criteria were: Disease activity score (DAS28; disease activity score in 28 joints [15]) below 2.7 for more than 6 months and presence of RA for more than 2 years. Patients not treated with immune-modulating biological agents (anti-TNF α therapy, interleukin blockers and T/B-cell inhibitors) were selected as being ‘remissive’ due to biological agent treatment in the past, ceased for at least 6 months or more. Exclusion criteria were medical history of cardiovascular diseases and the use of lipid-modifying medication and a body mass index of 30 and above.

Control subjects and subjects with overt cardiovascular disease were selected from a historical cohort at the department of Vascular Medicine at the Academic Medical Center in Amsterdam, The Netherlands. Healthy volunteers were matched for age and gender. Subjects with overt cardiovascular disease had a history of myocardial infarction and were on standard guideline prevention treatment, including lipid-modifying therapy.

Written informed consent was obtained from all subjects after explanation of the study. The study was approved by the institutional review board of the Academic Medical Center of the University of Amsterdam, The Netherlands and carried out according to the Declaration of Helsinki.

**Patient baseline data collection and parameters of Rheumatoid Arthritis**

All subjects visited the hospital for baseline assessment of their RA and cardiovascular risk profile. After an overnight fast, blood was collected for baseline laboratory investigations. Basal fasting glucose, HbA1c, total cholesterol, HDL and LDL cholesterol, triglycerides and a haematology panel were assessed in fasting plasma using standard laboratory procedures. Highly sensitive C-reactive protein (CRP), was determined using a commercialized immunoturbidimetric analysis. Physical examination including blood pressure was performed and medical- and family history was recorded.

Disease severity using DAS28 (disease activity score in 28 joints) [15] was recorded. Most recent ultrasound (US) data was collected from the AMC stop study, assessing joint damage.

**MR Imaging**

MRI scans were obtained on a 3.0 Tesla whole-body scanner (Intera, Philips Medical Systems, Best, The Netherlands), using an 8 channel dedicated bilateral carotid artery coil (Shanghai Chenguang Medical Technologies, Shanghai, China). Positioning of the image stack was performed using axial magnetic resonance angiography images acquired with a time of flight (TOF) sequence covering the carotid arteries at both
sides (field of view (FOV) 10 x 10 cm, 40 slices of 2 mm thickness). A stack of ten 3mm thick T1 weighed slices then was scanned with the top slice 6-8mm below the carotid bifurcation. Region of interest for vessel wall dimension measurement was performed at a standardized section of the common carotid artery. Images were saved according to the DICOM protocol. Standardized equipment and protocols were used for image storage and data management.

Quantitative image analyses were performed using dedicated measurement software (VesselMass, Leiden University Medical Center, Leiden, The Netherlands). Readers were blinded for patient characteristics and scan session. Normalized wall index (NWI = mean wall area/outer wall area), lumen area (LA, mm²), outer wall area (OWA, mm²), mean wall area (MWA, mm²), mean wall thickness (MWT, mm) and were measured or calculated as described previously [12].

**18F-FDG PET-CT imaging**

18F-FDG PET/CT imaging was performed in all RA patients and healthy controls. Scans were made using a Gemini time-of-flight multi-detector helical PET/CT scanner (Philips, Best, the Netherlands) as previously described [16]. Briefly, after fasting at least 6 hours, 250 MBq of FDG (6 mCi) was injected intravenously and allowed to circulate for 90 minutes, allowing uptake into metabolically active tissues. After unenhanced CT scans for attenuation correction and anatomic co-registration, PET data were acquired in 3-dimensional mode, covering the left and right common carotid arteries with the use of VUE Point FX time-of-flight reconstruction. The estimated dose of radiation per patient was 9.8 mSv per scan.

PET/CT images stripped of metadata were analyzed at the core laboratory by blinded experienced readers using OsiriX (Geneva, Switzerland; http://www.osirix-viewer.com). FDG uptake was assessed in the arterial wall of the left and right carotid artery. In each artery 5 ROIs were drawn, delineating the arterial wall. Maximum standardized uptake values (SUV) were averaged for each artery. The target-to-background-ratio (TBR) was calculated from the ratio of maximal arterial SUV and mean venous background activity (within the superior caval vein (correction for aorta) and the jugular vein (correction for carotids)), in line with previously described methods [16].

**Statistical analysis**

All data analysis was performed using SPSS Statistics (version 21, Armonk, USA) Continuous variables were tested for normality of distribution using a Shapiro-Wilk test and are presented as mean ± SD or medians with inter quartile range [IQR] unless stated otherwise. Independent samples t-tests were used for the comparison of parameters between groups, with the exception of skewed data, where a Mann-
Whitney U test were used. A two-sided P-value below 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of all patients

29 Patients with remissive RA were screened for participation. 4 Patients were excluded due to the presence of other cardiovascular risk factors, 1 patient was excluded for receiving anti-TNFα therapy without having active arthritis. The 24 included subjects were compared to 19 healthy control subjects matched for age, gender and BMI. For the MRI study, 14 matched subjects with overt CVD were selected from an existing cohort.

No differences were found in age, gender distribution and BMI. Baseline laboratory investigation (including, kidney function) were comparable between all groups. LDL cholesterol was increased in RA subjects, as has been previously reported [17]. Systolic and diastolic blood pressures were also higher in both RA and CVD subjects, as expected from previous findings (Table 1A) [18].

Baseline disease characteristics of RA patients

Of the 24 included patients, 15 had successfully discontinued their anti-TNF α therapy for 6 months or longer without disease relapse (average disease activity score 1.67±0.55). The groups where not different in age, gender, BMI and lipid levels. In concordance with previous literature [19], the group still requiring anti-TNF α therapy had significantly longer disease duration, as well as active disease years (See Table 1B).

MRI measured coronary artery wall dimensions

Average common carotid artery wall dimensions as measured by normalized wall index (NWI), mean wall thickness (MWT), and mean wall area (MWA) were significantly larger in patients with RA in remission, compared to control subjects, but smaller compared to subjects with overt CVD: NWI: Control 0.32 ± 0.03, RA (all) 0.40 ± 0.04 (p < 0.001 vs control), CVD 0.51 ± 0.05 (p < 0.001 vs all other groups)) (figure 1). MWA: Control 0.15 ± 0.05 , RA 0.21 ± 0.06 (p = 0.004 vs control), CVD 0.38 ± 0.13 (p < 0.001 vs all other groups), MWT: Control 0.68 ± 0.15, RA 0.93 ± 0.17 (p < 0.001 vs control), CVD 1.4±0.31 (p<0.001 vs all other groups). (Supplementary figure 1) No differences were observed between the subjects on MTX only and those requiring continued anti-TNFα therapy (NWI: 0.43 ± 0.03 versus 0.40 ± 0.04 p = 0.067) (figure 1).

FDG-PET/CT measures

In both subgroups of RA patients, as well as the control subjects, arterial wall inflammation was assessed with FDG-PET CT. No differences in TBRmax were found between RA patients and control subjects (data not shown). In contrast, when comparing
RA subgroups, the mean of TBRmax in the aorta (AA) as well as the common carotid arteries (CC) was significantly higher in RA patients on anti-TNF therapy compared to patients with MTX only (AA: 2.55 ± 0.49 versus 1.96 ± 0.21, p = 0.020; CC: 1.93 ±

**Table 1A.** Baseline characteristics of the study cohort

<table>
<thead>
<tr>
<th></th>
<th>A: Control subjects (n=16)</th>
<th>B: RA remission All (n=24)</th>
<th>C: CVD patients (n=14)</th>
<th>P-value A vs B</th>
<th>P-value B vs C</th>
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</thead>
<tbody>
<tr>
<td>Age at visit (years)</td>
<td>57 ± 4</td>
<td>57 ± 9</td>
<td>61 ± 8</td>
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<td>ns</td>
</tr>
<tr>
<td>Gender male (in %)</td>
<td>13%</td>
<td>17.2%</td>
<td>14%</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.0 ± 2.3</td>
<td>26.4 ± 5.0</td>
<td>26.6 ± 4.8</td>
<td>0.049</td>
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<tr>
<td>Currently smoking</td>
<td>10.5%</td>
<td>17.2%</td>
<td>35.7%</td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>6.2%</td>
<td>34.5%</td>
<td>85%</td>
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<td></td>
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<tr>
<td>Diabetes</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td></td>
<td></td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>128.6 ± 15.6</td>
<td>149.8 ± 20</td>
<td>136.5 ± 19.3</td>
<td>&lt;0.001</td>
<td>0.039</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77.1 ± 9.6</td>
<td>90.1 ± 11.28</td>
<td>83.0 ± 7.4</td>
<td>&lt;0.001</td>
<td>0.046</td>
</tr>
<tr>
<td>CRP (mmol/L)</td>
<td>2.30 ± 4.47</td>
<td>1.8 ± 1.8</td>
<td>1.32 ± 1.92</td>
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<td>ns</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.55 ± 0.85</td>
<td>5.8 ± 0.9</td>
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<td>ns</td>
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<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.49 ± 0.70</td>
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<td>HDL-cholesterol (mmol/L)</td>
<td>1.72 ± 0.39</td>
<td>1.5 ± 0.5</td>
<td>1.49 ± 0.57</td>
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<td>ns</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.95 ± 0.35</td>
<td>1.4 ± 0.5</td>
<td>1.33 ± 0.56</td>
<td>0.001</td>
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</tbody>
</table>

Data are presented as mean ± SD. ns = not statistically significant, BMI = body mass index, BP = blood pressure.

**Table 1B.** Baseline characteristics of the patients with Rheumatoid Arthritis

<table>
<thead>
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<th></th>
<th>MTX (15)</th>
<th>Anti-TNF α (9)</th>
<th>P-value</th>
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<tr>
<td>Age (years)</td>
<td>57±11</td>
<td>58±8</td>
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<td>Gender male (%)</td>
<td>13 (N=2)</td>
<td>22 (N=2)</td>
<td>ns</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.8 ± 4.4</td>
<td>28.2 ± 5.7</td>
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</tr>
<tr>
<td>Systolic BP</td>
<td>144 ± 20</td>
<td>156 ± 22</td>
<td>ns</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.9 ± 0.9</td>
<td>4.0 ± 0.92</td>
<td>ns</td>
</tr>
<tr>
<td>DAS 28</td>
<td>1.39 [1.31-1.54]</td>
<td>1.59 [1.46-2.72]</td>
<td>ns</td>
</tr>
<tr>
<td>CRP (mmol/L)</td>
<td>1.7 ± 2.0</td>
<td>1.4 ± 1.0</td>
<td>ns</td>
</tr>
<tr>
<td>RA disease duration (months)</td>
<td>94 [48-109]</td>
<td>150 [102-199]</td>
<td>0.083</td>
</tr>
<tr>
<td>Active RA duration (months)</td>
<td>46 [21-71]</td>
<td>109 [71-173]</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or median [95% confidence interval] ns = not statistically significant, BMI = body mass index, BP = blood pressure, DAS 28 = disease activity score in 28 joints, RA = rheumatoid arthritis
**Figure 1.** Vessel wall dimensions expressed as normalized wall index (NWI).

In the left panel, RA patients are divided into therapy of methotrexate (MTX) with or without the use of anti-TNF α and compared to control subjects (control) and patients with overt cardiovascular diseases (CVD). Data are presented as mean ± SD (whiskers). In the right panel, RA patients are displayed together (RA (all)).

**Figure 2.** Artery wall inflammation.

Arterial wall inflammation is expressed as mean maximum target-to-background ratio (TBR(max)) and as TBR of the most diseased section of the artery (TBR(MDS)). Data of the carotid arteries are displayed in top panels, data of aorta below. RA patients are divided into therapy of methotrexate (MTX) with or without the use of anti-TNF α (anti-TNF) and compared to control subjects (control). Data are presented as mean ± SD (whiskers).
0.37 versus 1.52 ± 0.16, p = 0.046). (Figure 2, left panels). The mean TBRmax in the most diseased segment (MDS) showed the same pattern for all segments (AA: 2.60 ± 0.52 versus 2.01 ± 0.24, p = 0.029; CC: 2.04 ± 0.38 versus 1.58 ± 0.18, p = 0.037). (Figure 2, right panels)

**DISCUSSION**

In the present study we show that despite a persistent remission of RA, structural changes to the vessel wall are present in RA patients compared to healthy controls. In remissive RA patients requiring persistent biological therapy, the arterial wall inflammation measured by FDG-PET/CT was significantly increased compared to age and sex-matched controls. In contrast, arterial wall inflammation in remissive RA patients using only MTX was comparable to that in control subjects. These data imply that the continued need for biological therapy to maintain remission in RA points towards a persistent inflammatory drive, with a concomitant increased inflammatory state in atherosclerotic arterial walls.

**MRI measured carotid artery wall dimensions**

The increased arterial wall dimensions in RA compared to control subjects are in line with published data, indicating that cumulative inflammatory burden contributes to the development of carotid atherosclerosis through a synergistic interaction with conventional CVD risk factors in patients with RA [20]. The early and stringent use of potent anti-inflammatory drugs such as the anti-TNF α therapy was thought to prevent the onset of structural arterial wall abnormalities altogether [21]. In fact, anti-TNF therapy has even been reported to regress arterial wall dimensions in RA [22,23], most likely reflecting a decrease of the inflammatory burden within atherosclerotic lesions. However, notwithstanding the fact that artery wall dimension was smaller than those observed in CVD-patients, there was still a significant elevation compared to healthy, age-matched control subjects. Based on the MRI image only, it is not feasible to dissect whether this reflects damage which occurred during active disease only, or whether RA is characterized by a long-lasting, low-grade pro-inflammatory reaction, which persists even during clinical RA-remission. In support of the former, the vessel wall dimension correlated to the years of active RA-disease, supporting the concept that the dimension increase may represent predominantly damage due to ‘old’ atherogenic stimuli.

**FDG-PET/CT measured carotid artery wall inflammation**

To dissect ‘historical’ versus ongoing inflammatory activity, we also performed FDG-PET/CT in those patients who consented. In the overall RA group, there was no significant difference in TBR signal compared to controls. However, TBR was significantly increased in RA patients requiring persistent biological treatment to maintain the remissive state. In contrast, TBRs in remissive RA patients using MTX only
were comparable to those observed in controls. These data indicate that RA patients requiring biological maintenance therapy still have active ongoing inflammation within their artery walls, which implies an increased cardiovascular risk in these patients [14]. The increased inflammatory activity of the artery wall in remissive patients using potent anti-inflammatory treatment may, at first glance, seem counter-intuitive. Thus, treatment with anti-TNF therapy has been shown to potently attenuate the inflammatory disease activity in joints affected by RA [24] as well as reduce the pro-inflamatory activity in the arterial wall of RA patients [25]. Our present observation that remissive RA patients on MTX only have lower TBR compared to those requiring persistent biological treatment imply a persistent pro-inflammatory drive in these patients, which leads to inflammatory activity in the atherosclerotic arterial wall.

From a pathophysiologic standpoint, studies have emerged to support the persistence of a systemic inflammatory drive in subgroups of RA patients. Thus, a certain level of joint destruction has been suggested to elicit a prolonged, inflammatory state. Van Vollenhoven et al speculated that this observation may be explained by differences in synovial tissue mass. Thus, if joint damage progresses beyond a ‘certain’ stage, epigenetic changes in the damaged synoviocytes may result in autonomous disease progression [26]. Underlining this concept is the observation that immunological markers, such as CD4+ T cells, are predictive of relapse, suggesting that chronic inflammation changes the phenotype of inflammation-related cells (IRCs) [27]. In view of recent data, it is likely that these ‘local’ stimuli contribute to a systemic pro-inflammatory phenotype of circulating mononuclear cells, which have a greater likelihood of transmigrating towards other affected areas in the body, such as atherosclerotic plaques. The ensuing higher level of inflammation in the vessel walls of these patients may thus reflect accumulation of the mononuclear cells in the arterial wall, which has previously been shown to correlate to arterial wall inflammation measured with PET/CT [28]. In this scenario, it seems imperative to start aggressive immune-therapy early in order to prevent joint destruction at an early stage [19,29–31].

Study limitations
The group size of RA patients was limited. At present, the group size is being expanded to further substantiate the intriguing difference in PET/CT activity in RA patients with and without anti-TNF therapy. In the ongoing experiments, phenotyping of circulating monocytes occurs to further unravel the contribution of monocyte hyper-responsiveness as a causal mechanism for arterial wall inflammation.

CONCLUSION
In the present study we substantiate a significantly increased artery wall dimension in patients with RA. Notwithstanding clinical remission in all RA patients studied, we show persistent arterial wall inflammation in remissive RA patients requiring persistent
biological therapy. The increased inflammatory activity in the arterial wall implies a persistent pro-inflammatory drive in these patients, the mechanism of which needs further evaluation. Since inflammatory activity in the atherosclerotic wall has been associated with an increased CV-risk, our data suggest that remissive RA-patients requiring continued biological therapy also deserve optimal CV-preventive treatment.

REFERENCES


## SUPPLEMENTARY DATA

### Supplementary table 1. Scan parameters for 3T imaging of the carotid artery.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>Black-blood PDW</th>
<th>Bright blood TOF</th>
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<td>Inflow suppression (veins)</td>
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<td>Scan time (minutes)*</td>
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</tbody>
</table>

TSE = turbo spin-echo, FFE = fast field echo, FOV = field of view, DIR = double inversion recovery, NEX = number of excitations. * Scan times at heart rate of 60 min⁻¹
Supplementary figure 1. Vessel wall dimensions expressed as mean wall area (MWA) and mean wall thickness (MWT). RA patients are divided into therapy of methotrexate (MTX) with or without the use of anti-TNF α (anti-TNF) and compared to control subjects (control) and patients with overt cardiovascular diseases (CVD). Data are presented as mean ± SD (whiskers).
7

PRO-ATHEROGENIC LIPID CHANGES AND DECREASED HEPATIC LDL RECEPTOR EXPRESSION BY TOCILIZUMAB IN RHEUMATOID ARTHRITIS


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ABSTRACT

Objectives: Blocking the interleukin-6 pathway by tocilizumab (TCZ) has been associated with changes in the lipoprotein profile, which could adversely impact cardiovascular (CV) risk in patients with rheumatoid arthritis (RA). In the present study, we addressed the effect of TCZ on lipoproteins in both fasting and non-fasting state in RA patients and tested effect of TCZ on LDL receptor (LDLr) expression \textit{in vitro}.

Methods: Twenty patients with active RA and an inadequate response to TNF blockers received monthly TCZ intravenously. On week 0, 1 and 6 blood was drawn before and after an oral fat-load and the lipid profiles were measured. Effects of TCZ on LDLr expression in transfected HepG2 cells were subjected.

Results: After 6 weeks of TCZ, total cholesterol increased by 22% (4.8±0.9 to 5.9±1.3 mmol/L; \(<p<0.001\)), LDLc by 22% (3.0±0.6 to 3.6±0.8 mmol/L; \(<p<0.001\)) and HDLc by 17% (1.4±0.4 to 1.7±0.7 mmol/L; \(<p<0.016\)). Fasting triglycerides (TG) increased by 48% (1.0±0.4 to 1.4±0.8 mmol/L; \(p=0.011\)), whereas postprandial incremental area-under-the-curve TG increased by 62% (\(p=0.002\)). Lipid changes were unrelated to the change in disease activity or inflammatory markers. \textit{In vitro}, LDLr expression was found to be significantly decreased following TCZ incubation (\(<p<0.001\)) in cultured liver cells.

Conclusions: TCZ adversely impacts on both LDLc as well as fasting and postprandial TG in patients with RA. The changes in hepatic LDLr expression following TCZ imply that adverse lipid changes may be a direct hepatic effect of TCZ. The net effect of TCZ on CV-morbidity has to be confirmed in future clinical trials.
INTRODUCTION

Rheumatoid arthritis (RA) has been widely acknowledged to increase cardiovascular morbidity and mortality, irrespective of established classical risk factors [1,2]. Apart from the chronic inflammatory state as a driver of the excess cardiovascular risk [3], additional pro-atherosclerotic pathways including changes in the coagulation and fibrinolytic system and impaired endothelial regenerative capacity have been implicated [4]. Aggressive management of inflammatory activity in RA patients has been projected to reduce this augmented cardiovascular risk [5].

Tocilizumab (TCZ), a humanized monoclonal antibody blocking interleukin-6 (IL-6) signaling by binding both soluble and membrane bound IL-6 receptors (CD126), has been shown to effectively decrease RA disease activity [6,7]. Upon treatment, TCZ has however been reported to increase total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), high-density cholesterol (HDLc) and triglycerides [8–15]. It has been put forward that these modifications largely reflect a physiological adaptation following reversal of the chronic inflammatory state, in spite of a lack of studies addressing the underlying mechanisms of the lipid changes following TCZ administration [16]. These changes in lipid profile occur within a week after treatment initiation, which coincides with inhibition of inflammation [17]. The magnitude of the lipid changes associated with TCZ however generally exceeds those following treatment with other potent anti-inflammatory regimens such as tumor necrosis factor (TNF) inhibition [18].

Together with the observation that IL-6 may impact on LDL removal by the liver [19], we hypothesized that TCZ induced an adverse lipid profile by direct effects on hepatic clearance pathways. Therefore, we investigated the effect of 6 weeks TCZ treatment in RA patients on the fasting and non-fasting lipid profile. Changes in cholesterol synthesis, intestinal absorption, lipolysis and clearance were measured. To validate hepatic changes, the direct effect of TCZ on LDLr expression was tested in HepG2 cells.

METHODS

Patient selection

Twenty-one patients with RA were recruited from the outpatient clinic of the Academic Medical Center/University of Amsterdam (AMC) and enrolled in the study. The study protocol was approved by the institutional review board at the AMC. Written informed consent was obtained from all participants.

The first four recruited patients received TCZ as part of the ACT-RAY study: a phase IIIb study, designed to evaluate the efficacy and safety of TCZ in patients with RA who do not respond to methotrexate (MTX) [20]. The current study comprises an investigator-driven, open-label study. Patients who were assigned to TCZ treatment in combination with MTX (oral or parenteral; at least 10 mg/week) or another conventional disease
modifying anti-rheumatic drug (DMARD) (eg. leflunomide, hydroxychloroquine, or sulfasalazine) for at least twelve weeks and at a stable dose for at least six weeks prior to treatment were included. Patients were allowed to use oral corticosteroids at a dose of ≤10 mg/day prednisone (or equivalent) but only if a stable dose was used for at least 25 out of 28 days prior to treatment. The RA-patients had no history of overt cardiovascular disease. Amongst the major exclusion criteria were concomitant use of lipid lowering drugs including statins, fibrates, nicotinic acid, fish oils, and antioxidants and a history of excessive alcohol consumption, type I/II diabetes, obstructive biliary disorders, and history of genetic hyperlipoproteinaemia. A control group of 38 healthy subjects was recruited via advertisements and was used to compare mean baseline plasma levels. These healthy controls did not actively participate in the study protocol and were matched for sex, age and BMI.

**Patient data and blood collection**

Patients visited the research unit 1 day before start of TCZ, 1 week after start of TCZ, and 6 weeks after start of TCZ. Treatment with TCZ at a dose of 8mg/kg intravenously every 4 weeks was initiated after the first study visit. Before TCZ treatment and 3 months after treatment initiation, disease activity score evaluated in 28 joints (DAS28) was assessed to monitor disease activity [21]. Use of concomitant medication was also registered at the first visit. At each visit, fasting blood was drawn, followed by an oral fat load test, as described previously [22]. Briefly, patients ingested cream (35 g fat per 100 mL), at a dose of 30 g of fat per square meter of body surface area, after which blood samples were taken at time points 2, 3, 4, and 6 hours.

**Biochemical analyses**

Plasma was isolated by centrifugation at 2000 rpm for 15 minutes at 4°C, within 30 minutes after collection and stored at -80°C. Plasma levels of TC, LDLc, HDLc, TG, apolipoprotein A1 (ApoA-I) and apolipoprotein B (ApoB) were determined using commercially available assays on a chemistry analyzer (Cobas Mira Plus, Roche Diagnostics, Basel, Switzerland) using commercialized kits from Rendox Laboratories-US (Kearneysville, West Virginia) for TC, TG, ApoA-I, and ApoB and from Wako Chemicals (Neuss, Germany) for measurement of LDLc and HDLc.

Additionally, individual patient samples were fractioned using fast performance liquid chromatography (FPLC) analysis to determine lipoprotein cholesterol and triglyceride content [23]. In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol and TG (4-Amino-antipiperine-peroxidase) PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 mL/min. Ethylenediaminetetraacetic acid (EDTA) plasma was diluted 1:1 with Tris buffered saline and 60 μL sample/buffer
mixture was loaded on a Superose 6 HR 10/30 column (GE Health care, Life sciences division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 mL/min.

As reflection of the change in intestinal TG uptake and transport via chylomicrons, we measured post-prandial Apolipoprotein B 48 (ApoB48) in fasting blood samples and two and four hours after fatload using an ELISA kit (Shibayagi ltd, Shibukawa, Japan).

Serum levels of campesterol, lathosterol, and sitosterol were measured using standard Gas Chromatography – Mass Spectrometry (GC-MS) analysis to determine changes in whole body cholesterol synthesis [24], and intestinal cholesterol absorption [25]. Campesterol, lathosterol and sitosterol levels were adjusted for simultaneously measured total cholesterol levels. Inflammatory activity, highly sensitive C-reactive protein (CRP), and serum amyloid A (SAA) were determined using immunoturbidimetric analysis, and Enzyme-linked immunosorbent assay (ELISA) (Anogen, Ontario, Canada) respectively. Glucose levels were measured using a standard hexokinase/G6PDH biosensor (Roche Modular P800, Roche Diagnostics, Mannheim, Germany).

**Calculation of atherogenic indexes**

Atherogenic indexes were calculated using the fasting lipid levels from the visits before and six weeks after TCZ treatment, measured commercially available assays on a chemistry analyzer (Cobas Mira Plus, Roche Diagnostics, Basel, Switzerland). Non-HDL cholesterol was calculated by subtracting HDLc level from TC level. TC:HDLc ratio was calculated by dividing TC by HDLc. ApoB:ApoA-I ratio was calculated by dividing the ApoB level by the ApoA-I level.

**LDL-receptor promoter expression using luciferase reporter**

In vitro effects of TCZ on hepatic LDL receptor expression was performed using HepG2 cells. These cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin at 5% CO₂ and 37°C. For transient transfection using lipolfection agent Roti®-Fect (Carl Roth GmbH), cells were grown in 24-well plates to 80% confluency. A promoter-reporter gene construct carrying the firefly luciferase under control of the human LDL receptor promoter (further on referred as pGL2-LDLR) was kindly provided by J. Kotzka (from German Diabetes-Center, Düsseldorf, Germany). 0.5 μg of plasmid DNA was applied per well. To correct for transfection efficiency, 12.5 ng/well phRL-TK (Promega) were co-transfected. Cells were subsequently incubated with transfection medium (Opti-MEM®, 10% FCS, Roti®-Fect) for 24h followed by a 12h-incubation in serum-reduced full medium (1% FCS). 24 h after stimulation with 10 ng/ml IL-6 as well as 100 μg/ml tocilizumab or 100 ng/ml adalimumab (both antibodies 1h prior to IL-6 stimulation), cells were lysed by applying Passive Lysis Buffer (Promega), and luciferase activity was detected in a luminometer (Berthold Mithras LB 940) using the dual luciferase reporter assay (Promega).
**Statistical analysis**

A sample size of 17 patients was calculated based on the previously reported effect of TCZ on LDLc. Based on a difference of 20% and an assumed standard deviation (SD) of 20%, power of 0.8 and Type I error probability of 0.05, 20 patients were included.

Differences in blood levels of lipoproteins, apolipoproteins, sterols, inflammatory markers, and atherogenic indexes were non-parametrically tested using Wilcoxon signed ranks test.

Differences between patients and healthy control were statistically tested using Mann Whitney tests. Incremental area under curve (IAUC) of TG levels after fatload was calculated for each individual patient. Differences in IAUCs between visits were tested using Wilcoxon signed ranks test. Luciferase reported LDLr expression differences between groups were tested using a Student’s t-test and expressed as mean with SD.

To test if the change in mean plasma levels of markers of inflammation (ESR, CRP, SAA) and DAS28 after TCZ was related to the change in mean plasma lipid and sterol levels, a Spearman’s rho correlation analysis was used. All results are expressed as means with SD.

**RESULTS**

Of the 21 patients included in the study, one patient dropped out due to abdominal discomfort following oral fat ingestion at the first visit. No serious adverse events were observed during follow-up. After six weeks, TCZ treatment resulted in a substantial decrease in mean CRP from 13.23 (20.66) to 0.17 (0.42) (P<0.010) (table 1). Mean DAS28 after 12 weeks decreased from 4.65 (0.73) to 2.83 (1.30) P<0.001).

**Table 1. Baseline characteristics**

<table>
<thead>
<tr>
<th>Patient characteristics (mean)</th>
<th>Patients (n=20)</th>
<th>Controls (n= 38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (♀ in %)</td>
<td>75%</td>
<td>74%</td>
<td>0.923</td>
</tr>
<tr>
<td>Age (SD) (years)</td>
<td>53.4 (12)</td>
<td>53.2 (7)</td>
<td>0.413</td>
</tr>
<tr>
<td>BMI (SD) (kg/m²)</td>
<td>24.3 (4)</td>
<td>24.4 (5)</td>
<td>0.757</td>
</tr>
<tr>
<td>DAS28 (SD) (before treatment)</td>
<td>4.7 (0.7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Co-medication for RA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate (10-25mg; n)</td>
<td>17</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Prednisolone (5-10mg; n)</td>
<td>7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Non-steroid anti-inflammatory drugs (n)</td>
<td>8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hydroxychloroquine (n)</td>
<td>1</td>
<td>-</td>
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Mean baseline characteristics of all patients with rheumatoid arthritis treated with tocilizumab compared to matched healthy controls. (BMI = body mass index; DAS28 = disease activity score evaluated in 28 joints; SD = standard deviation)
Plasma lipid profiles are depicted in figure 1. Following TCZ, TC increased by 22% (4.8 (0.9) to 5.9 (1.3) mmol/L; P<0.001), LDLc by 22% (3.0 (0.6) to 3.6 (0.8) mmol/L; P<0.001) and TG by 48% (1.0 (0.4) to 1.4 (0.8) mmol/L; P=0.002). This increase in fasting TG largely represented VLDL particles, which displayed a 36% increase in TG-

Figure 1. Mean TC, LDLc, TG, HDLc, ApoA-I and ApoB levels in 20 patients before, one week and six weeks after start of TCZ treatment, compared to matched healthy controls. After TCZ, TC increased by 22%, LDLc by 22%, TG by 48% and HDLc by 17%. (n.s.=not significant; *= P < 0.05; **= P <0.01; ***= P<0.001 using Wilcoxon signed ranks test, bars indicate mean level, error bars indicate SD)
content (P=0.027) with concomitantly larger diameter of the VLDL particles (VLDL TG peak shifted towards larger particles (P=0.008; figure 2)). HDLc also increased by 17% (1.4 (0.4) to 1.7 (0.7) mmol/L; P=0.004) after six weeks compared to baseline (figure 1). Following TCZ, TC and LDLc levels were higher than levels in matched controls (P<0.001 for both), whereas HDLc and TG were comparable between groups. In line, ApoB and ApoA-I increased by 13% (111 (23) to 125 (26) mg/dL) and 10% (157 (25) to 172 (37) mg/dL) respectively (P=0.005 and P=0.002; figure 1). The atherogenic indices also increased after TCZ treatment. Non-HDL increased from 3.28 (0.77) to 4.19 (0.99) (P<0.001), the TC: HDL ratio increased from 3.49 (0.84) to 3.98 (1.49) (P=0.044), but the ApoA-I: ApoB ratio was unchanged (table 2). Markers for cholesterol synthesis

Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Week 0 Mean ± SD</th>
<th>Week 6 Mean ± SD</th>
<th>Change</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td><strong>Lipoprotein cholesterol peak</strong></td>
<td></td>
<td></td>
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<tr>
<td>VLDL (min)</td>
<td>26.38 ± 0.36</td>
<td>26.30 ± 0.42</td>
<td>-0.30%</td>
<td>0.008</td>
</tr>
<tr>
<td>LDL (min)</td>
<td>32.14 ± 0.93</td>
<td>32.12 ± 0.70</td>
<td>-0.06%</td>
<td>0.619</td>
</tr>
<tr>
<td>HDL (min)</td>
<td>48.02 ± 1.47</td>
<td>47.86 ± 0.94</td>
<td>-0.33%</td>
<td>0.711</td>
</tr>
<tr>
<td><strong>Apolipoproteins</strong></td>
<td></td>
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<tr>
<td>Apolipoprotein A-I (mg/dL)</td>
<td>157.0 ± 25.5</td>
<td>172.2 ± 38.1</td>
<td>+10%</td>
<td>0.003</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>110.6 ± 23.4</td>
<td>124.6 ± 25.9</td>
<td>+13%</td>
<td>0.005</td>
</tr>
<tr>
<td>Fasting ApoB48 (µg/mL)</td>
<td>6.14 ± 5.21</td>
<td>10.82 ± 9.41</td>
<td>+76%</td>
<td>0.010</td>
</tr>
<tr>
<td>Post-prandial ApoB48 (µg/mL)</td>
<td>6.26 ± 5.21</td>
<td>13.68 ± 12.75</td>
<td>+119%</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Atherogenic indices</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Non-HDL cholesterol (mmol/L)</td>
<td>3.28 ± 0.77</td>
<td>4.19 ± 0.99</td>
<td>+28%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC:HDL ratio</td>
<td>3.55 ±0.84</td>
<td>3.98 ± 1.49</td>
<td>+17%</td>
<td>0.044</td>
</tr>
<tr>
<td>ApoB:ApoA-I ratio</td>
<td>0.70 ± 0.15</td>
<td>0.74 ± 0.18</td>
<td>+6%</td>
<td>0.227</td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
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<tr>
<td>CRP (mg/L)</td>
<td>13.23 ± 20.66</td>
<td>0.17 ± 0.42</td>
<td>-99%</td>
<td>0.010</td>
</tr>
<tr>
<td>SAA (ng/ml)</td>
<td>13.62 ± 10.56</td>
<td>2.78 ± 3.04</td>
<td>-80%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>21.73 ± 15.52</td>
<td>4.37 ± 4.80</td>
<td>-80%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.21 ± 0.49</td>
<td>5.05 ± 0.47</td>
<td>-3%</td>
<td>0.076</td>
</tr>
</tbody>
</table>

Mean levels of lipoprotein cholesterol content, triglyceride content and the mean cholesterol time-to-peak, inflammatory markers and apolipoprotein levels. SD = standard deviation; P values were calculated using Wilcoxon signed ranks test. All levels are measured at week 6, except from rheumatoid arthritis disease activity markers Disease Activity Score in 28 joints (DAS 28), tender joint count and swollen joint count.
Figure 2. Fast Performance Liquid Chromatography (FPLC) curve of lipoproteins in 20 patients before and 6 weeks after TCZ. Curves represent mean lipoprotein triglyceride (TG) content (panel A) and mean cholesterol content (panel B) of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL and free glycerol (in panel A only) divided based on retention times in minutes. Panel A contains a detail inlay of the TG content in VLDL, where a peak shift towards larger particles is demonstrated. Peak area represents measured content, derived from electrical current in microvolt (mV) x min. Black solid curve represents content at week 0, with black dashed line indicating standard deviation (SD); red solid line represents content at week 6, with red dashed line indicating SD.

(lathosterol/cholesterol ratio) and cholesterol absorption (campesterol/cholesterol and sitosterol/cholesterol ratio) remained unaltered (figure 3).
Markers of cholesterol synthesis and absorption in 20 patients before and six weeks after TCZ. Lathosterol/cholesterol ratio, a marker of whole-body cholesterol synthesis, remained unchanged after TCZ. Campesterol/cholesterol as well as sitosterol/cholesterol ratio, both reflecting intestinal cholesterol absorption, also remained unchanged. (*= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$ using Wilcoxon signed ranks test, bars indicate mean, error bars indicate SD)

Panel A: Mean plasma triglycerides (TG) after fatload at week 0, 1 and 6 after TCZ treatment. TG peak in the curves is postponed 6 weeks after TCZ from three hours after fatload to four hours. Panel B: Incremental area under the curve (IAUC) of mean plasma triglycerides (TG) after fatload at week 0, 1 and 6 after TCZ treatment. Six weeks after TCZ, postprandial TG IAUC was 62% higher than baseline (B).

(**= $P = 0.002$ using Wilcoxon signed ranks test, bars indicate mean, error bars indicate SD)
Postprandial TG also increased markedly following TCZ, reflected by a 62% increase in the IAUC of TG after an oral fatload (P=0.002; figure 4). The post-prandial TG-peak was postponed from three to four hours after oral fat ingestion, indicative of perturbed triglyceride clearance after TCZ. Whereas fasting ApoB48 level after TCZ increased by 76% compared to baseline (P=0.003), post-prandial ApoB48 levels displayed a marked further increase after TCZ treatment by 119% (P=<0.001; table 2). The increase in TC, LDLc, HDLc, TG, ApoA-I, ApoB, pre- and postprandial ApoB48 did not correlate to the decrease in CRP, ESR, SAA or DAS28 (data not shown).

In the luciferase reported LDL receptor promoter essay, a significant increase of LDLr expression is observed in HepG2 cells incubated with IL-6 compared to the pGL2-LDLr controls (P<0.001; Figure 5). Incubation with IL-6 + TCZ significantly decreases LDLr expression (P<0.001), whereas IL-6 + Adalimumab does not decrease LDLr expression.

**DISCUSSION**

TCZ administration in RA patients reduces disease activity as reflected by a reduction in DAS28 score and indices of systemic inflammation. Concomitantly, TCZ is associated with marked increases in LDLc, HDLc as well as in both fasting and postprandial TG levels. Since intestinal cholesterol absorption and whole-body cholesterol synthesis...
did not change, these changes are likely to reflect decreased clearance. In support, TCZ was found to reduce hepatic LDLr expression, which is expected to result in LDLc increase as well as an increase in TG- rich lipoproteins (TRL). In addition, the marked increase in (non-)fasting TG also points towards an impaired LPL mediated lipolysis following TCZ. The lipid changes did not correlate with changes in inflammatory activity.

**TCZ treatment and TG metabolism**

The most prominent change in lipid metabolism following TCZ concerns an increase in triglyceride-rich lipoproteins (TRL). Based on the lipid analyses in the present study, this effect most likely involves a combination of attenuated lipolysis of TG in TRL's and decreased hepatic removal of TRL. In support of attenuated lipolysis, we find higher fasting TG levels and a larger mean VLDL particle size with increased TG-content. Moreover, the marked increase in postprandial TG levels, in postprandial apoB48 concentration as well as a delayed TG-peak from 3 to 4 hours after the oral fat load lend further support to an attenuated lipolysis following TCZ. Although TCZ treatment has previously not been associated with changes in lipolysis, increased IL-6 activity has been shown to stimulate LPL-mediated lipolysis of TG [26].

Besides lipolysis, plasma TG levels are also determined by TG and TRL-remnant removal by the liver [27,28]. Following lipolysis of TG by LPL, the ensuing TRL-remnants are taken up by the liver via the LDL receptor, LDL-related protein and HSPG-dependent clearance path [28]. The observed increase in fasting apoB48 levels, indicative of prolonged presence of chylomicron (remnants) points towards decreased hepatic removal of chylomicrons rather than attenuated lipolysis of TG.[27] The latter may also contribute to the marked increase in TG following an oral fat load [29,30]. Our *in vitro* analyses support the involvement of the LDLr, which also contributes to the clearance of TRL's. TCZ decreases the partly IL-6 dependent expression of the LDLr. In line, Gierens *et al* also reported a dose-dependent relation between increasing IL-6 concentrations and increased LDLr promoter expression [19]. We corroborate the relation between IL-6 and LDLr promoter expression, while providing additional evidence showing that TCZ, in contrast to TNF inhibition with adalimumab, markedly decreases LDLr expression. In RA patients, a TCZ mediated decrease in LDLr expression can be expected to contribute to increased TRL levels by reduced hepatic TRL removal [28].

Whereas we and other groups report a marked increase in TG levels following IL-6 receptor blockade [8,10–13,17], no correlation was observed between the IL-6 SNP (rs7529229), associated with impaired IL-6 signaling, and TG levels in a study comprising more than 100.000 individuals [31]. Similarly, TNF inhibition in RA also leads to only modest changes in lipid profiles, comprising an increase in total cholesterol by 2-7% with even lower changes in TG levels [32,33]. Collectively, these findings imply that the increase in TG is a TCZ- specific effect, of which the exact pathophysiology remains to be established.
TCZ treatment and LDL metabolism

The increase in mean LDLc without a significant increase in LDL TG content most likely reflects increased formation of LDLc particles from VLDLc particles [34]. An increase in VLDLc, the predecessor of LDLc, leads to increased LDLc via metabolism through IDL [35]. As previously discussed, the direct inhibition of LDLr expression by TCZ may have contributed to increased levels of LDLc after TCZ treatment. As indicated by the unaffected lathosterol/TC and sitosterol/TC ratios, whole-body cholesterol synthesis and intestinal cholesterol absorption, respectively, are unlikely to have contributed to the LDLc increase.

TCZ treatment and HDL metabolism

Before TCZ treatment, HDLc was significantly lower in patients with RA compared to healthy controls, which is compatible with HDL as an inverse acute-phase reactant in RA [36]. The return-to-normal of HDLc following potent anti-inflammatory interventions, such as TCZ, has been reported in other infectious diseases as well [37]. Indeed, comparable increases in HDLc have been reported following TNF inhibition in RA [38].

The effect of TCZ on cardiovascular risk

The increase in TC and LDLc exceed the mean levels in matched controls. Since TC and LDLc are independent risk factors for cardiovascular morbidity and mortality, the effect of TCZ on the lipid profile may bear direct relevance to the CV-risk of patients receiving TCZ [39]. In line, two out of three cardiovascular risk indices point towards an increased CV risk [40]. In contrast, TCZ treatment also results in a profound reduction of the inflammatory activity. A pro-inflammatory state has been associated with increased CV event rates. In fact, two recent genetic association studies provided compelling evidence for a causal role of inflammatory activation in CV-risk, showing that decreased IL-6 signaling due to genetic causes were significantly associated with lower concentrations of inflammatory disease markers as well a marked reduction in CV-event rates [31,41]. In line, removal of the pro-inflammatory state has been shown to resolve inflammation-associated CV-risk [42,43].

Collectively, these counterbalancing effects of increased atherogenic indices with lowered inflammatory burden underline the need for prospective, randomized clinical trial data to identify the net effect of TCZ on CV-morbidity in patients with chronic inflammatory diseases.

Study limitations

Each of the 20 RA patients in our study served as its own control to optimally identify the individual changes in lipid profiles. Since our data suggest that changes in lipoproteins are not related to changes in inflammatory activity, the impact of TCZ in healthy volunteers would have added to this concept. Unfortunately, ethical approval
was not obtained for healthy volunteers. Second, we only followed patients for 6 weeks of TCZ treatment. Other studies reported normalization of lipid profiles in RA patients after 24 months of treatment [32,44]. In line, in patients with RA treated with TCZ at a lower dose of 4mg/kg, recovery of TC and LDLc towards initial levels was observed between the 14th and the 24th week after treatment initiation, without RA disease deterioration [17]. Third, we evaluated LDLr expression using in vitro assays on hepG2 cells, rather than in liver biopsies from patients receiving TCZ. The latter is, however, not compatible with institutional review board regulations for human research within our institute.

ACKNOWLEDGEMENTS

We would like to thank all patients, the research nurses and consultant nurses of the Department of Clinical Immunology and Rheumatology, A.W.M. Schimmel, J. Peter and M.E. Doorenspleet for their excellent technical assistance and Mark Kok and Koen Vos for recruiting patients at their outpatient clinic. The LDL receptor promoter plasmid was kindly provided by Prof. Wilhelm Krone (Cologne, Germany).

REFERENCES


LOCALLY INCREASED HDL AS THERAPY FOR ARTERIAL WALL INFLAMMATION
ABSTRACT
The risk for cardiovascular disease (CVD) is inversely correlated with high density lipoprotein cholesterol (HDL-C) plasma levels. These plasma HDL-C levels are influenced by the activity of a number of enzymes and receptors and therefore variations in the genes encoding for these proteins may consequently result in an altered CVD risk.

Identification of such pivotal players in HDL-C metabolism that are also strongly associated with CVD risk is crucial for the materialization of novel therapeutic modalities.

A large deal of knowledge has been obtained by studies involving families with extreme HDL phenotypes based on specific to molecular defects. In fact, thus far monogenetic defects have been described in the genes coding for ApoA1, ABCA1, CETP, LIPG, PLTP and LCAT. Despite the fact that the total numbers of carriers of such mutations is rather small, much can be gained by extensively studying the metabolic and vascular consequences of these mutations. Surrogate markers for atherosclerosis have proven to be useful to overcome this sample size limitation and have been widely exploited to study families with decreased or increased HDL-C levels in order to correlate HDL-C phenotypes to atherosclerotic burden in cases and controls.

Apart from such extreme phenotype approaches, novel population based genome-wide association studies (GWAS) have been used to decipher the link between genetic loci and HDL-C levels, and the identification of novel HDL-C related genes is eagerly awaited.

These might be instrumental in the ongoing fight against atherosclerosis.
INTRODUCTION

Despite revolutionary advances allowing early detection and aggressive therapeutic intervention, atherosclerosis remains a major cause of mortality and morbidity in the industrialized world [1]. Through large epidemiological surveys, a number of key risk factors for atherosclerosis have been identified. A low plasma level of high density lipoprotein cholesterol (HDL-C) has consistently been shown to be an independent risk factor [2], which has led to the awareness that low HDL-c levels should be taken into account while assessing CVD risk in patients. As a result, current guidelines from the Adult Treatment Panel III emphasize targeting primarily low density lipoprotein cholesterol (LDL-C), secondarily non HDL-cholesterol (HDL-C) and then HDL-C [3].

Given the aforementioned relationship between HDL-c levels and CVD risk, the development of pharmacotherapeutical agents designed to raise HDL-C has attracted a lot of attention.

Observational studies have shown that each 1-mg/dL decrease in plasma HDL-C concentration is associated with 2% to 3% increased risk in CVD. In addition, each 1-mg/dL increase is associated with a 6% lower risk of coronary death, independent of LDL-C level [4,5]. Most of the approaches, however, are rather non-specific; they do not only result in an HDL-C increase but also affect other lipoproteins, creating a hurdle to really adequately appreciate the precise impact of HDL increase on CVD risk. Current strategies involve fibric acid derivatives (fibrates), and nicotinic acids or its derivatives.

In addition to these since long prescribed agents, novel targets to elevate HDL-C are warranted.

The study of single gene mutations that trigger robust alterations in HDL-C plasma concentrations provides an opportunity for defining important players in HDL metabolism. Such novel discovered proteins can then be considered as potential targets for therapeutic intervention to decrease the atherosclerotic burden.

Based on recent literature, 6 genes can be considered as ‘established HDL genes’. These human genes encode proteins that directly affect HDL synthesis, maturation, conversion and catabolism and, as a consequence, directly affect plasma HDL-cholesterol levels.

The genes encoding apolipoprotein AI (apo-AI), lecithin cholesterol acyltransferase (LCAT) and ATP binding cassette protein AI (ABCAI) are key players in HDL-synthesis, illustrated by the fact that complete deficiency of these proteins leads to extremely low plasma HDL-C levels. In contrast, lack of endothelial lipase (LIPG) and cholesteryl ester transfer protein (CETP) is associated with accumulation of mature HDL in the circulation [6]. Phospholipid transfer protein (PLTP) deficiency leads to absence of HDL remodeling, resulting in deficiency and accumulation of specific HDL sub fractions [7].

In figure 1 the role of these proteins in HDL metabolism is illustrated.

We will also briefly discuss the wide array of novel genes that were identified in Whole Genome Association Studies addressing loci implicated in HDL-C levels.
Apolipoprotein AI (apo-AI) is the major structural apolipoprotein of the HDL particle. It is present on all HDL particles and accounts for approximately 70% of its protein content and 30% of the HDL mass [8]. Apo-AI is the key acceptor of cholesterol, and as such promotes cholesterol efflux from tissues to be transported to the liver for excretion, a process widely known as “reverse cholesterol transport”. Moreover, apo-AI is a crucial cofactor for LCAT activity, resulting in maturation of the HDL particle. Mutations in apo-AI are a frequent cause of hypoalphalipoproteinemia (HA) and apparently have a great impact on cardiovascular risk [9].

Apo-AI mutations and endpoints

Major function-disrupting mutations in the apo-AI gene have been identified, including gene disruptions, nonsense mutations, frame shifts, missense mutations, chromosomal aberrations or deletions; and these are invariably associated with decreased HDL-C levels [10].
Of particular interest is apo-AI_{Milano}, which is characterized by an arginine to cysteine substitution at position 173. Apo-AI_{Milano} exerts special in-vitro anti-atherogenic and antithrombotic activities and possesses greater activity than normal apo-AI in promoting cholesterol efflux from cells [11], and protecting LDL from oxidation [12]. Carriers of this rare mutation exhibit hypertriglyceridemia (HTG) with reduced HDL-C and apo-AI levels [13]. In the Limone sul Garda Study, carotid intima media thickening (IMT) was measured in 21 carriers of apo-AI_{Milano} and compared with controls and individuals with familial hypoalphalipoproteinemia (FHA). Carriers of apo-AI_{Milano} and controls did not differ in terms of mean IMT, suggesting that low HDL-C levels due to the Apo-AI_{Milano} variant are not associated with increased progression of atherosclerosis. By contrast, FHA individuals were characterized by significantly increased arterial wall thickness [14].

The risk of coronary artery disease (CAD) was also studied in heterozygous carriers of another apo-AI mutation (L178P) by comparing the IMT thickness in affected with non-affected family members. Heterozygous carriers of the apo-AI (L178P) mutation showed a 50% reduction in apo-AI levels and HDL-c levels compared to controls. IMT plotted against age showed that arterial wall thickness progression was significantly increased in carriers compared with controls, exemplifying the increased CAD risk [15].

A variety of other mutations, L159P, apo-AI_{Sasebo}, L144R were found in patients with HDL deficiency, but association with CAD has never clearly been demonstrated, predominantly due to the small number of individuals studied in each kindred, presence of associated deficiencies of other apolipoproteins and abnormal circulating Apo-AI variants [16–18].

**Apo-AI as therapeutic target**

Selectively increasing apo-AI levels is thought to be one of the most preferable outcomes to induce potent CAD risk reduction, based on the finding that apo-AI levels correlates more strongly with CAD risk, compared to other HDL-related parameters [9]. Several therapeutic strategies aimed at increasing Apo-AI are currently under investigation, comprising administration of human Apo-AI with or without mutation, synthetic Apo-AI or mimetic peptides and Apo-AI increasing small molecules such as the product recently manufactured by Resverlogix [19].

Clinical studies showed that a single human Apo-AI infusion results in a lasting increase of HDL-C levels which persisted for several days [20]. In addition, HDL infusion resulted in an increase of fecal sterol excretion in patients with familial hypercholesterolemia [21,22], supporting the paradigm that HDL is a rate limiting step in reverse cholesterol transport.

The sequential study addressing the effect of apo-AI infusion on atherosclerosis was performed by Nissen and co-workers. In their placebo-controlled study, weekly intravenous administration of recombinant apo-AI_{Milano}/phospholipid complexes (ETC-216) was shown to induce a significant regression of coronary atherosclerosis measured by IVUS (intravascular ultrasound) [23].
More recently, the ERASE study reported the effect of 4 infusions of 40 mg/kg human Apo-AI containing synthetic HDL (sHDL) on atheroma volume was also assessed by IVUS. The infusions of reconstituted HDL did not result in significant reductions in percentage change in atheroma volume compared to saline treated patients. The quantitative coronary angiography results, however, did reveal an improvement in patients having received HDL. At a higher dose (80mg/kg of apo-AI) infusions were associated with a high incidence of liver function abnormalities [24].

Based on these promising results, a lot of efforts have been put into the construction of safe and effective apo-AI mimetic peptides. The human apo-AI molecule consists of 243 amino-acids. The artificial apo-AI molecules containing short chains of amino acids from the apo-AI molecule have been shown to possess some of the beneficial effects of Apo-AI and therefore can be considered as potential alternatives for recombinant or purified human apo-AI. Examples of such peptides are D-4F and L37pA, which both were shown to exert beneficial effects on atherosclerosis progression and inflammation in experimental models [25].

The D-4F peptide increased pre-HDL formation, increases cholesterol efflux, and reduces lipoprotein oxidation, as enumerated in a recent review [26]. A single oral dose of the D-4F peptide proved to be safe and well tolerated, but has low bioavailability in non-fasting conditions [27].

A small molecule, RVX-208 produced by Resverlogix (resverlogix.com) holds great promise as a novel therapy to induce an increased HDL-C level. It facilitates endogenous apo-AI production and was shown to result in an 11% increase of apo-AI and increased HDL functionality (but the definition of functionality and the way to measure this phenomenon is still a matter of debate) after 7 days of administration [19,28]. To our knowledge has the exact mechanism underlying this result has not been published thus far. More studies are expected, and especially those that are set out to study the vascular consequences of RVX-208 are of great interest.

Besides increasing the lipoprotein fraction apo-AI, phospholipids have also been evaluated as potential therapeutic strategy. In a human study, 16 normolipidemic subjects received a derivative of soy lecithin, phosphatidylinositol, which resulted in a consistent increase of HDL-C as well as apo-AI levels [29]. Unfortunately, little is known about the exact effects and further studies to address the impact of phospholipid modulation on HDL metabolism and atherosclerosis are eagerly awaited.

**ABCAI**

The ATP-binding cassette transporter AI (ABCAI) gene encodes for the key protein regulating the efflux of lipids from peripheral cells (including foam cells) to apo-AI. These lipids are transported to the liver for excretion in a process termed reverse cholesterol transport [30]. Topological analysis reveals that ABCAI consists of 2 large extracellular loops, which may form the apo-AI-binding-target where lipids
are exchanged. At least 73 mutations have been described in the ABCAI gene, including 44 missense and 18 nonsense mutations and 11 insertions and deletions. Homozygosity or compound heterozygosity for loss-of-function mutations in the ABCAI gene lead to Tangier disease (TD), characterized by undetectable low HDL-C levels. Heterozygous carriers of ABCAI mutations typically show half-normal HDL-C levels [31].

**ABCAI Mutations and endpoints**

Whether ABCAI dysfunction results in an increased risk of ischemic heart disease (IHD) remains a matter of debate. The majority of ABCAI mutations are associated with HDL-C levels approximately 50% of control [32]. Mutations associated with greater than 50% of control HDL-C levels, like T929I, A947V, R1680W and W590S are in theory not complete loss-of-function mutations, but retain some activity. A number of mutations are associated with less than 50% of control HDL-C, specifically M1091T, G1216V and truncation mutations R2144X, R282X and R909X.

It is of note that ABCAI is not only a pivotal for HDL-C quantity, but also for quality, and the latter might be of greater value in terms of vascular risk attributed to ABCA1 mutations [33,34].

In their study, van Dam and coworkers showed that heterozygosity for ABCAI mutations was associated with increased progression of arterial thickening, ascertained by IMT. Regression analysis of the data from this study indicated that a 50% increase in ABCAI-mediated cholesterol efflux would result in a 30% increase in HDL-C concentrations and that this could translate into a 35% to 50% reduction in the risk of CAD [35].

**ABCAI as therapeutic target**

The most promising mechanism to raise ABCAI is via liver X receptor (LXR) agonists. The expression of ABCAI and ABCG1 in macrophages is regulated predominantly by the nuclear receptors LXRα and LXRβ [36]. LXRs are activated endogenously by oxysterols, which are generated enzymatically from cholesterol [37]. Both oxysterols as well as synthetic LXR agonists upregulate ABCAI and ABCG1 transcription in macrophages, thus leading to an increased cholesterol efflux. In animal studies, LXR agonists promote RCT and reduce atherosclerosis, which might partially be attributed to the effect on inflammatory signaling in macrophages [38]. However, the majority of the LXR agonists have been shown to cause hepatic steatosis and hypertriglyceridemia. Tissue specific upregulation of LXR modulators are therefore warranted [39].

The role of FXR in the lipid metabolism is complicated, as FXR-activation downregulates expression of Apo-AI and LIPG (see below) and upregulates expression of PLTP (see below) [39].
CETP

Cholesteryl ester transport protein (CETP) plays a role in HDL-C metabolism by transporting cholesteryl esters (CE) from HDL particles to apolipoprotein B-containing particles, in exchange for triglycerides [40]. CETP is a hydrophobic glycoprotein secreted primarily from the liver and adipose tissue and it circulates mainly bound to HDL. As a regulator of cholesterol flux through the RCT system, CETP can be regarded as both proatherogenic as well as an antiatherogenic moiety. By shifting CE from HDL to LDL and VLDL, CETP induces a reduction of RCT via the HDL route. Additional proatherogenic effects of CETP activity may include a reduction in overall HDL levels, potentially reducing cellular cholesterol efflux from the arterial wall, and an increase in atherogenic LDL levels. However, the potentially proatherogenic activities of CETP may, to a large extent, be neutralized by an increase in indirect RCT via the LDL/ hepatic LDL receptor route. Clearly, whether the net effect of CETP activity is pro- or antiatherogenic is currently being addressed in ongoing phase III studies using CETP inhibitors without direct toxic effects as was unambiguously demonstrated for Torcetrapib [41].

CETP mutations and endpoints

In 1985, Koizumi and coworkers described a family suffering from CETP deficiency, resulting in elevated HDL levels and decreased CHD [42]. Ever since, many efforts have been undertaken to create CETP inhibitors.

A homozygous mutation in Japanese individuals with FHA G+1A/In14, results in immeasurable CETP activity and these individuals exhibit markedly raised HDL-C levels [43]. A more common mutation in CETP, D422G, leaves little CETP activity, resulting in less pronounced increased HDL-C levels [44]. Apart from its effect on HDL-C level, CETP also has been found to alter the functionality of the HDL particle [45]. It goes without saying that this phenomenon influences the effect of CETP modulation on CHD risk. While addressing the role of CETP deficiency on CAD risk, controversial findings have been described. In the Honolulu Heart Study it was shown that humans of Japanese descent, who were heterozygous for the D442G CETP gene mutation, had a 50% increased risk of CHD compared with men who had similar HDL levels, but no CETP gene mutations [46]. However, a prospective analysis of 7-year data from the Honolulu Heart Study showed a trend towards a lower incidence of cardiovascular events in subjects with heterozygous CETP gene mutations compared with those without a mutation [47]. In line with the protective role of low CETP activity due to mutations was the finding described by Moriyama and coworkers, that Japanese individuals with either the G+1A/In14 or the D442G mutation showed a very low CHD risk, with no significant difference between these carrier groups [48].

Another way to address the role of CETP in a given population is to study the consequences of single nucleotide polymorphisms (SNPs). In a recent study, carriers of
the Taq1B (located in intron 1 of the CETP gene) were shown to have raised HDL-C levels and decreased CAD risk [49]. The later published REGRESS study concluded that high levels of HDL cholesterol do not necessarily protect against coronary artery disease when found in subjects with combined CETP- and HL-lowering gene variants, of which Taq1B was of major influence [50].

Another well studied SNP is the I405V CETP SNP. In a study of Hawaiian men of Japanese ancestry, plasma CETP concentrations were reduced and HDL-C levels increased in those who were homozygous for the I405V allele compared with those who were heterozygous or homozygous for the I405V allele. In this study, men who were homozygous for the I405V allele and who had high triglyceride levels had a higher prevalence of CHD [51].

In spite of controversial findings in individual SNP studies, a recent meta-analysis unambiguously demonstrated that 3 Common CETP gene variants (Taq1B, I405V, and −629C>A) were consistently associated with a decreased CETP concentrations, modestly increased HDL-C and apo-AI levels and weakly decreased triglycerides and coronary risk. Data were insufficient for informative per-allele estimates in relation to 3 uncommon CETP (D442G, −631C>A, and R451Q). However, they were associated with mean differences in HDL-C of 13.4% , −0.7% and −8.8%, respectively, compared with controls [52].

**CETP as therapeutic target**

Following these beneficial findings, several CETP inhibitors were developed, comprising Torcetrapib, JTT-705 (Dalcetrapib) and Anacetrapib. Clinical studies with Torcetrapib showed significant increases in HDL-C [53]. Unfortunately, the development of Torcetrapib was discontinued abruptly, as a result of a series of the unexpected findings in ILLUMINATE. Also, surrogate marker studies with Torcetrapib such as ILLUSTRATE, RADIANCE-1 and RADIANCE-2 produced disappointing results. These studies invariably revealed lack of improvement in the surrogate cardiovascular marker (IVUS, IMT) in line with an excess of deaths in those subjects receiving Torcetrapib. This could in part be attributed to a Torcetrapib induced increase in mean systolic blood pressure (SBP) of 4.5 mmHg [54]. In vitro studies and animal models resolved that the Torcetrapib mediated raise in blood pressure is caused by raised aldosterone and corticoid production. This increased corticoid release is directly caused by Torcetrapib-induced intracellular calcium increase, and is independent of the inhibitory effect on CETP of Torcetrapib [55]. The blood pressure effect was not diminished in the presence of adrenoceptor, angiotensin II or endothelin receptor antagonists, and Torcetrapib has no contractile effect on vascular smooth muscle [56]. Dalcetrapib efficacy and safety has been tested, where highest tested dose of 900 mg showed 37% decrease of CETP activity from baseline, an increase of HDL-C levels by 34% and 7% LDL-C decrease compared to baseline levels [57]. For this compound, no increase in blood pressure and/or hormonal parameters have been reported to date [58].
Anacetrapib, a new CETP inhibitor, is claimed to be even more potent than Dalcetrapib and Torcetrapib. In a recent phase 3 study, Anacetrapib was generally well tolerated, with no discernable effect on SBP [59].

In conclusion, the activity of CETP is known to relate to HDL-C levels. However, the precise role of CETP in atherogenesis and CHD risk in humans is not well understood, but is likely to be dependent on a combination of metabolic, genetic, and environmental factors. Data from CETP-deficient subjects have suggested that therapeutic inhibition of CETP may be advantageous in raising HDL-C levels and that this may lead to reductions in CVD risk. Yet, the final answer awaits the outcome of ongoing phase III trials with CETP inhibitors that have not been shown to influence blood pressure.

**LIPG**

Endothelial lipase (EL) is a member of the triglyceride lipase family of proteins that also includes lipoprotein lipase (LPL) and hepatic lipase (HL). EL's nomenclature was based on its presence on the surface of endothelial cells, which distinguishes EL from HL and LPL. EL is characterized by its conserved catalytic triad with heparin and triglyceride binding sites and cysteine residues [60]. Whereas EL hydrolyzes HDL most efficiently of all the lipoprotein fractions, it primarily hydrolyses phospholipids. As the different subfractions of HDL have pro- and anti-inflammatory effects, EL has been shown to play a role in modulating lipoprotein metabolism in proinflammatory states, such as atherosclerosis [61]. From animal models it has been established that expression of LIPG, the gene coding for EL, profoundly affects HDL-C levels; inhibition or deletion of EL in mice increases HDL-C levels [62].

**LIPG mutations and endpoints**

At least 11 genetic variations have been studied in humans. The most frequently studied mutation in the population is Thr111Le. Various studies of the effects of the Thr111Le mutation describe increased HDL levels, whereas this association could not be corroborated by others [63]. Edmondson concluded in a group of 3,845 participants, that Thr111Le is not associated with HDL-C levels, and that this variant does not result in an altered lipolytic activity [64].

A second, less common mutation in LIPG is Asn396Ser, which was found to be significantly associated with increased HDL-C. Quantification of the consequences of the Asn396Ser mutation on CVD-risk was performed in the Framingham Heart Study using IMT measurement. These analyses showed no significant difference, emphasizing a small role of EL in atherosclerosis in humans. However, this study was underpowered.

**EL as therapeutic target**

To the best of our knowledge, pharmacological agents directly influencing EL are currently not available.
PLTP

Phospholipid transfer protein is a non-specific lipid transfer protein which plays an important role in HDL metabolism. Its regulatory role is achieved via the main functions, transfer and exchange of phospholipids between lipoproteins, and remodeling of the HDL particle. This process is controlled by at least three different factors: the HDL apolipoprotein/protein composition, the core lipid composition of HDL and the phospholipid transfer activity of PLTP [7]. In contrast to CETP, release of lipid-poor Apo-AI was detected during HDL conversion or remodeling by PLTP [65]. PLTP is circulating in a catalytically active and non-active form; activity measurement is therefore more relevant than mass measurement.

The role of PLTP in human atherosclerosis remains controversial, illustrated by different studies that suggested that low PLTP activity leads to HDL-C increase, while high PLTP activity leads to decrease of HDL-C [7]. While a recent study shows that low PLTP activity is a marker for atherosclerosis [66], several studies show that high PLTP activity is related to increased risk of atherosclerosis [67–69]. One of these studies showed that high plasma PLTP activity is related to fatal and nonfatal cardiovascular events in CAD patients, independent of other markers such as Hs-CRP or HDL-C [68].

PLTP mutations and endpoints

Few mutations or variants are studied in literature. A recent studied variant of the PLTP gene (rs2294213) is associated with an increased level of HDL-C in healthy subjects and PLTP has therefore been suggested to be a potential target for pharmacological intervention [70].

Another study found 2 heterozygous nonsynonymous variants in a selected group of patients with low HDL-C levels; S107Y and R459Q. The results suggest that the S107Y mutant has normal PLTP function, but the R459Q mutant has a significant reduction in PLTP activity, which could contribute to an impaired HDL metabolism [71].

PLTP as therapeutic target

An attempt to target the PLTP promoter was not successful. The PLTP promoter contains recognition motifs for the peroxisome proliferator-activated receptor (PPAR), which is suggested to mediate the down regulation of PLTP expression in a fibrate treated cell culture model, but in vivo results did not result in a change in PLTP activity [72,73].

Currently, no specific PLTP targeted treatment is available.

LCAT

Lecithin cholesterol acyltransferase (LCAT) is a plasma enzyme which catalyses the transfer of a preferentially unesterified fatty acid from the sn-2 position of phosphatidylcholine to the 3β-hydroxy group of cholesterol, and thereby produces
lysophosphatidylcholine and a cholesteryl ester. LCAT preferentially acts on lipids transported by HDL but also on ApoB containing particles. LCAT is a glycoprotein synthesized by the liver and secreted into the circulation. Classical LCAT deficiency is caused by a broad spectrum of missense and nonsense mutations which lead to an altered synthesis or secretion of the protein, or affect its catalytic activity [74]. This will cause defective maturation of small HDL into larger, spherical, cholesteryl ester-enriched HDL and hence lower HDL-C levels. This effect is not counteracted by upregulation of the unaffected LCAT allele in heterozygotes or by other key players in the HDL metabolism [9].

**LCAT mutations and endpoints**

Mutations in the human LCAT gene underlie two clinical phenotypes, familial LCAT deficiency (FLD) and Fish-eye disease (FED). Both diseases are inherited in an autosomal recessive manner and are characterized by the occurrence of corneal opacification as clinical hallmarks. Both patients with FLD and FED present with low levels of HDL-C. In FLD, LCAT is completely inactive, whereas in FED loss of activity is restricted to HDL-cholesterol only and it retains its activity toward LDL-cholesterol. Heterozygous carriers of LCAT mutations do not suffer from corneal clouding. The majority of these carriers present with low HDL-C levels [75].

Studies evaluating the relation between CAD risk and LCAT mutations have provided conflicting results. In 2005 the first surrogate endpoint study in LCAT mutation carriers was reported. The 47 individuals with low HDL-C, elevated triglycerides and Hs-CRP in plasma on average showed significantly increased IMT. These results suggest that normal LCAT function is a principal factor in the protection against atherosclerotic vascular disease [9].

Polymorphisms in the LCAT gene have also been associated with hypoalphalipoproteinemia, but its effect on CAD risk has not been fully elucidated [76].

**LCAT as therapeutic target**

The most direct LCAT-targeted therapy is injection of recombinant enzyme or of encapsulated LCAT-secreting cells into subjects with familial and acquired LCAT deficiency (Low JK, Thesis 2010, University College London, unpublished). These injections in the peritoneal cavity of a LCAT knocked-out mouse showed favorable effects, but a success of LCAT increasing strategies in humans has not been reported. For developing recombinant human LCAT and testing its effect in animal models, Alphacore Pharma received a NIH-grant [77]. In search of other potential strategies, a recent study from Amar and coworkers in monkeys showed successful over expression of human LCAT using an adenovirus. The increased LCAT level led to raised HDL-C levels, which supports their conclusion that LCAT remains a potential drug target for reducing atherosclerosis [78].
Polygenic defects

Ever since the different human genome projects have provided a wealth of information on the human genetic code, many genome-wide association studies (GWAS) have been performed. In lipoprotein metabolism, these DNA sequence variants represent an index of lifelong exposure to altered lipoprotein levels, whereas plasma measurements can vary considerably [79]. In this context, the presence of a genetic variant may add predictive value of this genotype to the risk of CVD. The potential of such genetic testing is limited by the fact that each sequence variant that has been discovered to date, explains only a modest fraction of the variance in lipid levels [80].

The power of the GWAS analyses improved when genotype scoring was introduced. In each subject, unfavorable alleles carried by that subject for each SNP is numbered. Subjects with specific genotype scores are collapsed into one group. In this way, a significant improvement in risk classification could be established, compared with models that did not use genotype scoring. Unfortunately, genotype scoring does not improve the accuracy of the clinical risk prediction, but does improve the clinical reclassification for individual subjects, independent of the other covariates [80].

Using GWAS and large-scale replication, 30 loci have been mapped that contribute to variation in lipoprotein concentrations in humans [81]. These loci consist of genes shown to affect lipoprotein metabolism in humans. Each variant conferred a modest effect on HDL-level, and all identified variants together accounted for only about 5–9% of the variation in the HDL-level [81,82].

Kathiresan and co-authors showed that a panel of SNP’s was associated with increased cardiovascular risk in a multivariable analysis. The genotype score was constructed for each subject by counting the number of unfavorable alleles, the potential for each subject ranged from 0 to 18. As compared with subjects with a genotype score of 11 or more had an increased risk of cardiovascular events by a factor of 1.63 (P=0.001) [80].

The suggested next step in GWAS is that specific regions of the genome have to be fully sequenced, so that lipid genotypes with allelic dosage score may allow for early identification and treatment for at-risk individuals to prevent atherosclerosis [81].

SUMMARY

HDL-C levels are mainly determined by a combination of genetics and environmental exposure. Monogenetic defects resulting in either extremely low or high HDL-c levels are instrumental in studying the result of life-long exposure to altered protein levels. By thorough assessment of the CAD risk attributed to this exposure, one could identify targets for therapy that would hypothetically not only result in increased HDL-C levels, but, more importantly, also a decrease in atherosclerosis.

Based on this knowledge, several novel compounds have reached different levels of clinical applicability. Unfortunately, HDL-C increasing therapy has suffered from a major set back by the devastating results obtained in the studies of CETP inhibition.
Novel therapies are in the pipeline and the studies will provide further insight in the intriguing and continuous debate about the potential outcome of HDL increasing therapy.

REFERENCES


EFFECT OF ANTI-APOA-I ANTIBODY-COATING OF STENTS ON NEOINTIMA FORMATION IN A RABBIT BALLOON-INJURY MODEL
ABSTRACT

Background and aims: Since high-density lipoprotein (HDL) has pro-endothelial and anti-thrombotic effects, a HDL recruiting stent may prevent restenosis. In the present study we address the functional characteristics of an apolipoprotein A-I (ApoA-I) antibody coating in vitro. Subsequently, we tested its biological performance applied on stents in vivo in rabbits.

Materials and Methods: The impact of anti ApoA-I- versus apoB-antibody coated stainless steel discs were evaluated in vitro for endothelial cell adhesion, thrombin generation and platelet adhesion. In vivo, response to injury in the iliac artery of New Zealand white rabbits was used as read out comparing apoA-I-coated versus bare metal stents.

Results: ApoA-I antibody coated metal discs showed increased endothelial cell adhesion and proliferation and decreased thrombin generation and platelet adhesion, compared to control discs. In vivo, no difference was observed between ApoA-I and BMS stents in lumen stenosis (23.3±13.8% versus 23.3±11.3%, p=0.77) or intima surface area (0.81±0.62 mm² vs 0.84±0.55 mm², p=0.85). Immunohistochemistry also revealed no differences in cell proliferation, fibrin deposition, inflammation and endothelialization.

Conclusion: ApoA-I antibody coating has potent pro-endothelial and anti-thrombotic effects in vitro, but failed to enhance stent performance in a balloon injury rabbit model in vivo.
INTRODUCTION

The introduction of stents has increased the success of percutaneous coronary interventions (PCI) by reducing coronary artery restenosis rates [1]. This advantage, however, comes at a price. Bare metal stents (BMS) are more prone to in-stent restenosis (ISR) and their pro-thrombotic capacity may yield occlusion rates of up to 24% in the absence of pharmacological treatment [2]. Drug-eluting stents (DES) have proven highly effective through local delivery of antiproliferative drugs [1,3], albeit the imminent threat of late stent thrombosis (LST) has hampered their success. Thus, a comparative meta-analysis revealed that long-term cardiac death rates for BMS and DES were not significantly different [4]. These results highlight the need for novel stents with better long-term performance compared to both BMS and DES.

We hypothesized that stent performance could be enhanced by coating the stent struts with anti-apolipoprotein A-I (ApoA-I) antibodies, aimed to attract high-density lipoprotein (HDL) to the stent. Indeed, HDL has been shown to carry a wide array of anti-inflammatory and anti-proliferative proteins [5], which collectively have the capacity to prevent intimal hyperplasia [6–8]. In addition, HDL has also been shown to promote recruitment of endothelial progenitor cells, thereby promoting restoration of an intact endothelial monolayer covering the stent struts as well as the injured artery wall [9]. The physiological role of the endothelium is expected to reduce the inflammatory response and decrease coagulation activation with subsequent thrombus formation [10,11].

In the present study, we evaluated the efficacy and therapeutic potential of anti-ApoA-I antibody coating on metal discs in vitro and bare metal stents in vivo. First, we addressed whether anti-ApoA-I coating improved the vascular homeostasis by optimizing endothelial cell adhesion and proliferation and decreasing thrombogenicity in vitro, compared to a bare-metal surface. Subsequently, we compared neointimal hyperplasia in a balloon-injury model in rabbits in vivo using an anti-ApoA-I coated versus bare-metal stent.

METHODS

In vitro model

The anti-human monoclonal ApoA-I antibody, ApoB100 antibody and isotype control IgG antibody were covalently coupled to stainless steel discs (5 mm diameter; double-sided). The surfaces with immobilized ApoA-I antibody (Clone 2F1, Ottawa Heart Institute Research Corporation, Ottawa, Canada) [12] were treated with human HDL (Sigma-Aldrich, Zwijndrecht, The Netherlands) or oxidized (ox)-HDL (0.2 mg/ml). Oxidized lipoproteins were obtained by dialysis of 0.8 mg/ml solutions of HDL or LDL against 5 μM Cu SO4 for 20 hours and using Slide-A-Lyzer with MWCO of 3,500 (Thermo Fisher, Etten-Leur, The Netherlands) [13]. The surfaces with ApoB antibody...
(Clone 1D1, Ottawa Heart Institute Research Corporation) were incubated with human LDL (Sigma-Aldrich, Zwijndrecht, The Netherlands) or ox-LDL (0.2 mg/ml), while the surfaces with the isotype control IgG antibody were treated with a mixture of HDL and LDL or ox-HDL and ox-LDL (0.2 mg/ml). Ox-HDL, LDL and ox-LDL were used as negative control.

Human microvascular endothelial cells (HMEC-1; obtained from The Breakthrough Breast Cancer Research Center, London, England) were grown in MDCB-131 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 μg/ml hydrocortisone, 10ng/ml recombinant h-EGF, and antibiotics (100U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) [14]. In order to determine proliferation of HMEC-1 on the different surfaces, metal discs were incubated for 1 hour with HDL, LDL, or a 1:1 mixture of both. After washing, HMEC-1 cells were deposited on the discs and allowed to adhere for 1 hour at 37 °C. After addition of medium, the discs were incubated for 1, 2 or 4 days. Subsequently, the discs were rinsed and frozen at -80°C. The number of adhered cells to the metal surfaces was determined using the CyQuant kit (Life Technologies, Breda, The Netherlands)[15].

In order to quantify HMEC-1 adhesion, pre-incubated metal discs were put in a sterile 2.0 ml tube and incubated with 1.5x10^5 cells in 0.8 ml MDCB-131 medium for 20 hours at 37 °C under rotation. After rinsing, discs were stored at -80°C. The number of adhered cells was determined using the CyQuant kit. Thrombin generation was determined in a static set-up [16]. (described in detail in the supplemental materials)

Platelet adhesion was determined using PRP that was prepared as described above. Metal discs were pre-incubated with HDL, LDL, or a HDL/LDL mixture and incubated with PRP for 1 hour at 37 °C under continuous stirring at 150 rpm. Subsequently, the discs were washed with phosphate buffered saline (PBS), and the number of adhered platelets was determined using the CytoTox kit (Promega, Leiden, The Netherlands) [17]. The modified surfaces were incubated with native or oxidized versions of HDL or LDL and treated with PRP under identical conditions as described above. Oxidized HDL and LDL were used to rule out the effect of oxidative modification on platelet activation [18]. Platelet activation was studied by fixing platelets adhered to modified surfaces with cold 2.5 % glutaraldehyde in PBS. After careful washing with PBS, the samples were dehydrated with an ethanol series followed by incubation in hexamethyldisilazane (Aldrich, Zwijndrecht, The Netherlands) in order to achieve rapid drying [19]. Subsequently the samples were sputter coated with gold and observed using a SEM (Philips XL30 Scanning Electron Microscope, Philips, Eindhoven, The Netherlands). Photographs of randomly chosen areas were taken, and the morphology of the platelets was recorded according to the method described by Cooper et al [20].

**Stent coating**

The anti–human monoclonal ApoA-I antibody was covalently coupled to struts of a 9mm x 3mm R-stent evolution 2 (OrbusNeich, US) at highest surface density (100%)
using a proprietary multistep process (Ssens, The Netherlands). The R-stent has 316L stainless steel struts with dimensions of 0.09mm x 0.10mm, which are smooth and electropolished, and achieve 17% vessel wall coverage when implanted in the artery. The stent has an open, double helical design with recoil of less than 4% after implantation. The ApoA-I antibody coating was similar to the 100% density ApoA-I coated disks. An untreated R-stent was used as BMS comparator.

**Rabbit model**

The study protocol was reviewed and approved by the Institutional Animal Care and Research Committee at the Academic Medical Center, Amsterdam, The Netherlands (protocol number DCA101095) and conforms to the Directive 2010/63/EU of the European Parliament. We used 15 female New Zealand White rabbits (3.0 - 3.5kg), which were treated with acetylsalicylic acid 38mg/day started 5 days prior to stent implantation. The rabbit model of balloon-induced artery injury is an established model to test intravascular stent devices since it is characterized by fast development of intima hyperplasia (described in detail in the supplemental materials) [21–23]. In short, after induction of anaesthesia, stents were placed via cannulation of the left carotid artery, followed by endothelial denudation by pulling an inflated balloon through the iliac arteries. Directly thereafter, ApoA-I coated versus BMS stents were pair wise implanted in both endothelial denudated iliac arteries. During the experiment, rabbits had free access to food (AB-diets, high fibre complete diet).

At 28 days after stenting, the rabbits were anesthetized. Via the abdominal cavity, the retroperitoneal infrarenal aorta was cannulated with an 18G cannula and after intravenous injection of heparin 200IU/kg, angiography of the iliac arteries was performed. Subsequently, the animals were euthanized and the iliac arteries were flushed with saline, followed by perfusion-fixation using Neutral Buffered Formalin (NBF). The stented arteries were taken over a distance from the aorta-iliac bifurcation to one cm distal of the stent, fixated in NBF for 24 hours and subjected to the tissue embedding procedure.

After dehydration, the harvested arteries were stepwise embedded in MMA/BMA mix in a 1:1 ratio and allowed to harden for 24 hours under a vacuum at 4°C. Using a microtome with diamond knife, 10 µm thick transversal sections were cut from 3 regions (proximal, middle and distal) of the stented arteries and 2 peri-stent regions proximal and distal to the stented artery. The middle region (region 3) is defined as 30 sections taken from the exact centre of the stent. The proximal and distal regions (regions 2 and 4) are defined as the 30 sections cut at a distance from 100µm from the stent edge, inside the stent, inwards. The 2 peri-stent regions (regions 1 and 5) are defined as the 30 sections cut at a distance from 100µm from the stent edge, outside the stent, outwards. Sections were stretched on a 60°C bath of 40% acetone and adhered to glass slides using 70% Ethanol overnight at 60°C under mechanical pressure.
Three randomly selected sections of the 5 regions (Figure 1 in supplemental materials) were used for staining of the arterial laminae. Sections were stained using Haematoxilin-eosin staining, Lawson staining and immunohistochemical stainings detecting α-smooth muscle actin (α-SMA), Ki-67, rabbit macrophage-specific monoclonal antibody, von Willebrand factor and fibrin. Details about these stainings and morphometrical analysis are described in detail in the supplemental materials.

![Graph A](image1.png)

**Figure 1.** HMEC-1 cell growth (A) on coated disks
HMEC-1 cell growth on ApoA-I-coated discs in 100%, 10% and 1% density, incubated in HDL after 1, 2 and 4 days, compared to similarly incubated uncoated discs (control). Error bars indicate Standard Deviation (SD) of mean (n=3). (*=P<0.05)

![Graph B](image2.png)

**Figure 1.** HMEC-1 cell adhesion (B) on disks – HMEC-1 cell adhesion on ApoA-I-, ApoB- or Isotype-coated discs with 100% and 10% antibody density, incubated for 4 days. Error bars indicate Standard Deviation (SD) of mean (n = 4). (*=P<0.05)
Immunohistochemical analysis was performed by 2 separate reviewers, who were both blinded for type of stent coating. The mean score per stent was based on the average score of 2 randomly selected slides taken from both the proximal and distal stented area. Cell proliferation, inflammation, fibrin deposition and endothelialisation of the stented artery were scored (described in detail in the supplemental materials) [24–27]. One rabbit was sacrificed after 28 days to perform scanning electron microscopy (SEM) on the implanted stents. For this purpose, the stented segment of the iliac artery was fixed in 2% glutaraldehyde and processed for SEM imaging.

Statistical analysis
Statistics were performed using IBM SPSS statistic (version 19) and graphs were constructed using Prism Graphpad (version 5). Based on previous studies utilizing this bilateral vascular injury in rabbits for a side-to-side comparison of two different stent modalities, we determined that for a difference of 25% in lumen stenosis with a standard deviation of 30% within one animal the minimum number of rabbits needed was 12 (power of 0.80 and a type I error probability of <0.05). Data are expressed as mean ± SD. Significance of differences in in vitro tests was tested using one-way ANOVA. Comparisons of histological findings between BMS-stent and ApoA-I-coated stent were made by the Wilcoxon signed ranks test. Comparisons of immunohistochemistry results were made by Wilcoxon signed ranks test. A probability value of p<0.05 was considered significant.

RESULTS

In vitro studies
HMEC-1 cell growth and adhesion
After 4 days of incubation, the number of HMEC-1 cells on the anti-ApoA-I antibody coated surfaces was significantly higher compared to the isotype-antibody control, independent from antibody concentration (10% and 100%) (Figure 1A; p<0.05). There was an increased proliferation of HMEC-1 cells after 4 days on the surfaces with the highest density of anti-ApoA-I antibody, compared to those with lower densities (p<0.05).

The anti-ApoA-I antibody coated surface with the highest antibody density was associated with a higher HMEC-1 adhesion under dynamic conditions as compared to the isotype controls (p<0.05; Figure 1B). No effect on HMEC-1 adhesion was observed for the surfaces with the lowest anti-ApoA-I and anti-ApoB antibody density.

Thrombin generation
Figure 2 shows that the anti-ApoA-I antibody coated surfaces that were incubated with HMEC-1 caused a significant prolongation in the thrombin generation time when compared to uncoated surfaces (p<0.01). In addition, lowered peak thrombin and
the total amount of thrombin produced (p<0.05) was observed as compared to the isotype control. These anticoagulant effects increased with a higher density of the anti-ApoA-I antibody. In contrast, anti-ApoB antibody covered discs had no impact on the thrombin generation.

Platelet adhesion and activation
Platelet adhesion to the coated discs was clearly reduced on anti-ApoA-I antibody coated surfaces, particularly at a high density, as compared to the ApoB and isotype control (p<0.05; Figure 3A). Similarly, the ApoB antibody coated surface at high density showed reduced platelet adhesion compared to the isotype control (p<0.05). To further elucidate the implications of this latter finding we studied the influence of the oxidation state of these lipoproteins, and in particular oxidized LDL, on the morphology and activation of the adhered platelets using scanning electron microscopy (Figure 3B). Only in the presence of oxidized LDL we noted significantly increased adhered platelets activation.

In vivo model
All rabbits (n=15; weight 3.3kg ± 0.2 kg) were successfully stented, no peri-procedural complications occurred. Two rabbits were euthanized prematurely because of gastrointestinal discomfort and significant (>10%) weight loss at day 11 and day 19 after stenting. At 28 days, the stented iliac arteries of all remaining rabbits were patent.
Figure 3. Platelet adhesion on coated disks
Panel A: Platelet adhesion on coated disks. Platelet adhesion: SEM analysis of adhered platelets on surfaces coated with ApoA-I-, ApoB- or isotype antibody after corresponding pre-treatment. The number of adhered platelets are given as the mean ± SD (n=3). (*=P<0.05)
Panel B: The morphology of the adhered platelets was divided into 4 different classes representing various degrees of activation, ranging from “round” (weak), “dendritic” (intermediate), “spread/dendritic” (strong) to “spread” (very strong). The data are presented as the percentage of platelets exhibiting the indicated morphology. Data error bars are the standard deviation of the mean (n=3).
Morphometric analysis and scanning electron microscopy analysis

The Lawson-stained sections were assessed for mean lumen stenosis, intima surface and IM-ratio. As shown in panel A1 of Figure 4, anti-ApoA-I coated stents with a mean lumen stenosis of 23.3% showed stenosis rates similar to the BMS stent \((p=0.77)\). Comparison of the corresponding regions between the two stent types indicated no significant difference (panel A2). This degree of in-stent stenosis is comparable to the magnitude of stenosis observed by other groups using a similar rabbit model [28–30]. In addition, mean intima surfaces in ApoA-I coated and bare metal stents were 0.81 and 0.84 mm\(^2\) respectively \((p=0.85;\) panel B1). Consistent with these findings, mean IM-ratios in both stents (3.0 vs. 2.7 for ApoA-I vs. BMS, \(p=0.28\)) were not different (panel C1). With respect to intima surface and IM-ratio, corresponding regions were comparable between the two stent types (panel B2 and C2).

We did observe a trend towards decreased RAM-11 positive regions in the vicinity of struts with the anti-ApoA-I coated stent (Figure 5; \(p=0.056\)). The number of KI-67-positive (proliferating) cells did not differ between the two stents \((P=0.673)\). No difference was observed in fibrin deposition between the two stent types \((p=0.187)\). Next to the Von Willebrand staining reflecting endothelialization, the en face analysis of endothelium using SEM (Figure 6) shows that in both stent types, endothelialization pattern is comparable. Immunohistochemical analysis of Von Willebrand stained slides showed a trend to an increased grade of endothelialization in the ApoA-I coated stents compared to BMS \((p=0.072)\).

DISCUSSION

The present study shows that an anti-ApoA-I antibody coated surface, saturated with HDL, improves endothelial cell adhesion and proliferation with a concomitant decrease in thrombin generation and platelet adhesion in vitro. These beneficial features in vitro did, however, not translate into improved stent performance in vivo in a rabbit model of iliac artery balloon injury. The discrepancy between the in vitro and in vivo effect of anti-ApoA-I antibody coating may reflect insufficient availability of ApoA1 near the stent struts or limited capacity of HDL to attenuate the vascular injury response in an injury model of intimal hyperplasia.

In vitro experiments

The improved endothelial cell adhesion and proliferation in our in vitro experiments using anti-ApoA-I coated metal discs endorse the results from earlier experimental studies on the protective functions of HDL. HDL has been shown to exert a protective effect on the endothelium by preventing apoptosis and promoting migration of endothelial cells in in vitro models [31–34]. In humans, HDL has consistently been shown to exert a beneficial effect on abnormal vascular reactivity [35,36]. The concomitant
Figure 4. Morphometric analysis
Lumen stenosis (A1 and A2), intima surface (B1 and B2) and IM-ratio (C1 and C2) of anti ApoA-I coated stent and BMS stent 28 days after implantation. Bars indicate mean value per section with error bars indicating the standard deviation (SD). Three left panels (A1, B1 and C1) show results of five stent regions together. No significant differences were observed between the two stent types. Three right panels (A2, B2 and C2) show results of individual stent regions in the two stent types. No significant differences were observed between the corresponding regions in the two stent types.
Figure 5. Immunohistochemical analysis
Results of immunohistochemical analysis (top) with corresponding representative examples of the immunohistochemical staining (20x objective). KI-67 (first panels), Fibrin (second panel), RAM11 (third panel) and VWF (fourth panel) are shown. Bars indicate mean score or count with error bars indicating the standard deviation (SD). Proliferation (KI-67), fibrin deposition, macrophage infiltration (RAM11) and endothelialization (VWF) were not significantly different between the two stent types.
antithrombotic effects of ApoA-I in our *in vitro* experiments correspond to previous studies reporting antithrombotic effects of HDL. Thus, Fleisher *et al* showed that human HDL stimulates endothelial cell prostacyclin synthesis *in vitro* [37], whereas ApoA-I-Milano resulted in decreased thrombus formation in a rat model [10]. In humans, low HDL has also been recognized as a risk factor for venous thrombo-embolism [38,39]. Mechanistically, HDL has been shown to serve as a carrier of a wide array of proteins affecting the innate immune system and proteolytic cascades [40]. In line, systemic infusion of HDL has been shown to reduce vessel wall inflammation [41,42]. Collectively, our *in vitro* results lend further support to the concept that the presence of ApoA-I on the stent struts may contribute to a better stent performance *in vivo*.

**Rabbit experiments**

Anti ApoA-I-coated stents were not capable of decreasing the vascular response in a rabbit model of iliac balloon artery injury. Similar degree of stenosis, cell proliferation and endothelialisation were observed between ApoA-I antibody coated stents and the BMS. The apparent discrepancy between the beneficial effects *in vitro* and the absence of a beneficial effect *in vivo* may have several explanations.

First, since the stent is fenestrated, the majority of the arterial wall area is not covered by the stent struts. In contrast to coatings such as sirolimus and paclitaxel that are delivered into the local environment, the ApoA-I/HDL complex is tightly bound at the surface of the stent strut. The spatial distance between the HDL particles in relation to the lesion area between the struts may have undermined a potentially beneficial effect. In support, there was a trend towards a decreased number of inflammatory cells in the anti ApoA-I-coated stents, as well as a trend to a higher degree of endothelialisation.

Second, increased oxidative modification of fixated HDL combined with overgrowth of stent struts by intimal hyperplasia may reduce the bio-availability and potentially beneficial effects of HDL [43]. The latter may even imply that the struts become devoid of HDL delivered from the blood. Attempts to visualize the presence of HDL on stent struts using immunohistochemistry, however, failed due to the plastic embedding of the tissue [44].

**Study limitations**

Despite that inflammatory, proliferative and thrombotic stimuli within the first two weeks after stent implantation are immense [43], the stent harvest after 28 days may have been too early to detect clinically significant differences between the two stents if in-stent restenosis occurs after longer periods of observation. Therefore, longer observation periods may yet reveal superior performance of the ApoA-I coated stent. Another drawback of the study involves the lack of information regarding the antibody binding-place availability and its effects on circulating HDL recruitment within the stented arterial wall. (illustrated in detail in supplemental Figure 1)
Clinical implications

Relevance of HDL mediated protection beyond reverse cholesterol transport has been widely acknowledged, whereas its impact on cardiovascular outcome remains to be proven. Positive functional effects are confirmed by our in vitro studies with anti-

Figure 6. Scanning electron microscopy overview
SEM images of a 28-day BMS (upper half) and anti-ApoA-I coated stent (lower half) implanted in the rabbit common iliac artery. High magnification details of stent strut coverage are shown of three randomly chosen spots, next to the low magnification overview in the centre. Complete endothelial lining of the lumen is shown in all high-magnification details, with similar endothelial cell aspect.
ApoA-I antibody coated metallic surfaces. The attempt to translate these results to a (pre)clinical setting has, however, failed. Whereas strategies aimed at increasing local ApoA-I concentration may still prove to be beneficial for long-term stent patency, we were unable to provide in vivo support for the use of anti-ApoA-I antibody coated stents to reduce intimal hyperplasia.

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REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

**Thrombin generation test**

Blood was collected from healthy donors in vacuum tubes containing 3.2 % sodium citrate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Platelet-rich-plasma (PRP) was prepared by centrifugation for 15 minutes at 180g, and kept at 37 ºC. The different surfaces were pre-incubated with HDL or LDL. Discs were placed in a 96-well plate and 0.2 ml recalcified PRP, containing 400 μM fluorogenic substrate and 20 mM CaCl₂ (final concentration) was added. This fluorogenic thrombin specific substrate, Z-Gly-Gly-Arg-AMC (Bachem Ag., Bubendorf, Switzerland), allows for the determination of thrombin generation by measuring the production of fluorescent product aminomethylcumarin (excitation 368 nm, emission 460 nm). The reaction was performed at 37 ºC for 90 minutes. The concentration of thrombin is directly proportional to the amount of fluorescent product formed over time.[45] The lag time, also called thrombin-generation time, is appointed as the time until 2 nM thrombin was formed in the PRP. Peak height and time to peak are also extracted from curves.

**Rabbit stent implantation model**

The rabbits were anesthetized using a cocktail of Ketamine 35mg/kg and Xylazine 5mg/kg, followed by isoflurane gas anaesthesia. Blood was withdrawn and heparinised in heparin gas tubes (BD Vacutainer, BD, Plymouth, UK). After intravenous administration of heparin 100IU/kg, the left common carotid artery was cannulated with a 5F sheath introducer (Avanti +, Cordis, Fremont, USA). Under fluoroscopic guidance (Hexabrix 300; Philips BV, Eindhoven, The Netherlands) bilateral iliac artery injury was performed using an inflated 3.0 x 10mm balloon catheter (Orbus Neich, Hoevelaken, The Netherlands; 1.2:1.0 diameter ratio compared with normal vessel, mostly achieved at 10 atm) which was pulled through the external iliac artery (EIA) and deflated before removal. Subsequently, a BMS or anti- ApoA-I-coated stent was randomly placed at either side, by inflating the delivery balloon 1mm distal to the internal iliac branch, which is held for 10 seconds at a pressure of 9 atm. Stent position and patency was checked using fluoroscopy.

**Histological staining**

Sections were deplastified for 10 minutes in 100% acetone and rehydrated in series of ethanol. Sections were stained in Lawson (Klinipath, Duiven, The Netherlands) solution in order to selectively stain elastin for 60 minutes and differentiated in 100% ethanol. Sections were rinsed in tap water whereupon they were dehydrated and covered with coverslips and Pertex (Leica, Rijswijk, The Netherlands). Similarly, Haematoxylin-eosin (HE) staining (Klinipath, Duiven, The Netherlands) on one section from each region was performed to examine cell organisation and density. HE staining was also used to assess nucleus number in specific regions for immunohistochemical
analyses. Of all stented arteries, a section from the second and fourth region was used for immunohistochemical staining. Semi quantitative analysis was performed on these sections immunostained with an α-smooth muscle actin (α-SMA) specific monoclonal antibody (Dako, Heverlee, Belgium; clone 1A4, dilution 1:500), to determine differences in proliferation of smooth muscle cells. Complementarily, a monoclonal antibody against Ki-67 (Dako, Heverlee, Belgium; dilution 1:50) was used to score cell proliferation. For scoring inflammation grade, the rabbit macrophage-specific monoclonal antibody (RAM11, Dako, Heverlee, Belgium; dilution 1:50) was used, together with a fibrin specific antibody (America Diagnostica, Lexington, USA; dilution 1:50) to perform a fibrin deposition score. Deposited fibrin is a substrate for neutrophil adhesion in a damaged vessel wall. A fifth, monoclonal antibody against von Willebrand Factor (Abcam, Cambridge, UK; dilution 1:30) was used on sections of the stented arteries to stain endothelial cells, facilitating the ability of scoring endothelial coverage of the vessel’s lumen. All primary antibodies were detected using an horseradish peroxidise (HRP) conjugated secondary antibody (Southern Biotech, Birmingham, USA; dilution 1:100) which was visualized with 3,3’-diaminobenzidine (DAB; Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands).

**Morphometric analysis**

Lawson stained sections were used for morphometric analysis using a light microscope (Axiophot, Zeiss, Sliedrecht, The Netherlands) with a 1.25x or 2.5x objective and histology quantification software (QWin Leica, Rijswijk, The Netherlands). Picture colours were converted to 8-bit grey values, with the use of a saturation threshold, outer elastic lamina (OEL), inner elastic lamina (IEL) and lumen outline were recognized. Stent struts and surrounding shrink-artefacts were outlined and summed and called stent strut surface. Lumen surface was measured as content of lumen outline. Intima surface was calculated by deducting lumen surface and summed strut surface from surface inside IEL. Media surface was calculated by deducting the surface inside IEL from the surface inside OEL. Intima-media (IM-) ratio was calculated by dividing intima surface by media surface. Lumen stenosis was calculated by taking the proportion of surface within IEL which was occupied by intima and strut surface with respect to total IEL surface. Maximal intima surface was measured by arbitrarily measuring the tunica intima at its thickest part viewed from the centre of the lumen. Mean surfaces of three sections within a region were calculated.

**Immunohistochemistry and the scoring systems**

Proliferation was scored by dividing the Ki-67 positive cells by the total number of cells in the HE stained coupes. The degree of endothelialisation was scored by determining the number of von Willebrand positive cells and was categorized as 0-50% and 50-100%.
The inflammation score was determined by dividing the number of struts that stained positive for RAM11, by the total number of struts. A fibrin deposition score was based on dividing the number of struts that stained positive for fibrin staining by the total strut count in fibrin stained sections.

**Scanning electron microscopy**

To further evaluate stent endothelialisation, stents (n=1 per condition) were cut in a sagittal fashion and incubated with 2% aqueous osmium tetroxide for 45 min, dehydrated in a graded ethanol series and then critical point dried in liquid CO$_2$ using the Balzers CPD-010 (Balzers Instruments, Liechtenstein). Stent halves were mounted on aluminium stubs (Ted Pella Inc, Redding, USA) and sputter coated with gold/palladium using the Polaron E5100 SEM coating system (Thermo VG Scientific, Waltham, USA). Endothelial facing layer was examined on a scanning electron microscope at 15kV and at magnifications ranging from 400 to 4000x.

**Supplementary Figure 1.** hypothetic view on mechanism

Schematic overview of the predefined stent regions (lower left) and a transversal section of a stented artery (upper left) The details (A-C) of an anti ApoA-I antibody coated strut compared with the strut of a bare metal stent (BMS; D-F) provide an overview of the hypothesis: anti ApoA-I antibody coated stents are implanted in the pre-injured artery (A). The coated struts attract HDL cholesterol by binding ApoA-I (B). The presence of ApoA-I and HDL prevent restenosis and promotes restoration of the endothelial layer (C). Restenosis occurs in bare metal stents (BMS), which after implantation in the artery (D) does specifically attract ApoA-I and HDL (E) and becomes overgrown by proliferating vascular smooth muscle cells, effectuating stenosis.
SUPERIOR IN VIVO COMPATIBILITY OF HYDROPHILIC POLYMER COATED PROSTHETIC VASCULAR GRAFTS

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ABSTRACT

Purpose: To compare protein adsorption, cell adhesion and graft patency of hydrophilic versus hydrophobic polymer coated prosthetic vascular grafts. We hypothesize that in vivo compatibility of hydrophilic polymer coated prosthetic vascular grafts is superior to in vivo compatibility of hydrophobic grafts.

Methods: Pairwise side-to-side common carotid artery interposition grafting was performed in 8 female landrace goats (mean weight 55kg). Protein adsorption was assessed using Western Blot in 2 hydrophilic and 2 hydrophobic grafts, harvested after 3 days. Graft patency was monitored during 28 days in 6 goats with continuous wave Doppler ultrasonography. Adherence of endothelial cells, leukocytes and platelets was determined with ELISA and compared between the two graft types after 28 days.

Results: After 3 days, more ApoA-I, albumin and VEGF and less fibrin adsorbed to hydrophilic grafts. After 28 days, compared to hydrophobic grafts, higher numbers of endothelial cells were present on hydrophilic grafts ($p=0.016$), and less thrombocytes and leukocytes ($p=0.012$ and 0.024, respectively). Two out of 8 hydrophobic grafts lost patency, while none of the hydrophilic grafts failed ($p=0.157$).

Conclusions: Hydrophilic polymer coated vascular grafts have superior in vivo compatibility when compared to hydrophobic grafts as characterized by reduced platelet and leukocyte adherence as well as higher endothelialisation.
INTRODUCTION

Over the past decades, the need for peripheral bypass grafts as well as arteriovenous fistulas (AVFs) for hemodialysis (HD) has increased dramatically. For all interventions in vascular surgery, the use of native blood vessels instead of synthetic grafts is preferred, given that both primary and secondary patency rates of the former are superior and native grafts have also been associated with a survival advantage [1–4]. However, there is a decreased availability of high-quality native blood vessels, due to the increasing age and vascular comorbidity in the general population affecting native donor vein quality [5,6]. Additionally, auto-transplantable vessel availability may be reduced due to use of arteries and veins in previous coronary or peripheral revascularization procedures. Consequently, the use of synthetic grafts increases steadily. Despite initial high blood flow rates following placement, synthetic grafts are frequently faced with the problem of continuous flow rate decline due to neo-intimal proliferation, pro-coagulant state and, eventually, occlusion [7]. Whereas the exact pathophysiology remains complex, parts of the adverse effects have been attributed to the absence of endothelial coverage leading to pro-thrombotic and pro-inflammatory effects [7].

In an effort to overcome lacking endothelial coverage, several strategies have been employed to improve endothelialisation. The ultimate surface adaption of synthetic materials seems pre-seeding of the graft with endothelial cells prior to implantation. However, such an approach is extremely expensive and without impressive patency improvements [8], partly due to poor retention of seeded cells.

Synthetic grafts are usually made from polyethylene terephthalate (PET) or polytetrafluorethylene (PTFE), which are both hydrophobic materials [9]. Recently, we challenged the dogma that hydrophilic surfaces are protein repellent by providing evidence that a hydrophilic coating attracts many proteins and protein particles that promote human microvascular endothelial cell (HMEC-1) proliferation on biomaterial surfaces in vitro [10–12]. Among these protein particles, high-density lipoprotein (HDL) appeared to be critical for dictating this HMEC-1 proliferation [13]. HDL and its primary protein constituent ApoA-I, exert vasculoprotective functions via different molecular pathways comprising anti-inflammatory, anti-thrombotic and anti-apoptotic effects [14–16]. In addition, both ApoA-I and HDL have been shown to enhance vascular function by promoting endothelial integrity [17]. Therefore, the use of a hydrophilic copolymer coating could be a promising method to improve patency rates of synthetic vascular grafts.

In the present study, we compared a hydrophobic copolymer-coated vascular graft with a hydrophilic copolymer-coated vascular graft in a goat model of arterial interposition grafting. The primary objective of this study was to compare protein adsorption and recruitment of leukocytes, platelets and endothelial cells to the surface of the prosthetic grafts. The secondary objective was to assess patency rates.
METHODS

Production of vascular grafts

The hydrophobic copolymer coating consists of 10% 1-Vinyl-2-pyrrolidinone (NVP) and 90% n-Butyl-methacrylate (BMA); the hydrophilic coating consists of 10% BMA with 90% NVP. Details about copolymer production and prosthetic graft coating have been reported previously [18] and are provided in supplemental material. In short, a stainless steel coil is coated with either hydrophilic copolymer coating or the hydrophobic equivalent, and is encapsulated in a Tygon sleeve to suture the common carotid artery on (see figure 1).

Goat model for arterial prosthetic graft implantation

The study protocol was reviewed and approved by the Institutional Animal Care and Research Committee at the Academic Medical Center, Amsterdam, The Netherlands. The goat carotid artery interposition graft model was chosen because of the adequate length and diameter of the artery matches with the graft's dimensions and the model has been described before [19]. Details about the graft implantation are described in the supplemental material. In eight anesthetized female Dutch landrace goats, a hydrophilic and a hydrophobic coated graft are side-to-side implanted as interposition in the common carotid artery. Perioperative heparin was added to continuous administered acetylsalicylic acid to prevent thrombosis.

Prosthetic graft patency monitoring

On day 1, 2, 3, 7, 11, 14, 18, 21, 25, and 28 post-surgery, graft patency as reflected by pulsatile flow in the common carotid artery was assessed manually by use of continuous wave Doppler ultrasound at the proximal and distal site of the prosthetic graft.

Prosthetic graft harvesting

Two randomly chosen goats were sacrificed at three days after prosthetic graft implantation. The remaining 6 goats were sacrificed at 28 days after implantation. Before sacrifice, anaesthesia was initiated similarly to anaesthetic procedures at implantation. After Doppler ultrasound, patency monitoring of the common carotid artery, the 8 cm grafted section was prepared for prosthetic graft explantation. 200IU/kg heparin was injected intravenously to prevent acute thrombosis and both prosthetic grafts were explanted after marking proximal and distal in situ position.

The grafts were excised together with 1 cm of adjacent artery on both sides in order to include the graft's anastomosis region in analyses. Immediately after explantation, the grafts were gently flushed and rinsed using 100 mL 0.9% NaCl with 200 IU heparin added to prevent thrombosis. Patency loss was confirmed by the inability to flush obliterated grafts. Grafts were incubated for preservation in Normal Saline for a maximum of 60 minutes before further processing.
Prosthetic graft sectioning and processing

Prosthetic grafts, harvested after three and 28 days, were sectioned into five pieces using a diamond belt saw (Figure 1). After dehydration in ethanol, the harvested pieces of the first, third, and fifth regions (corresponding with the proximal, middle, and distal section) of the prosthetic graft were fixated in neutral buffered formalin (NBF) for 24 hours. Fixated grafts then were embedded stepwise in methylmethacrylate and n-butylmethacrylate (MMA/BMA) mix in a 1:1 ratio and allowed to harden for 24 hours at a vacuum at 4°C. The intended diamond knifed microtome sectioning failed due to the large metal content. Diamond saw microtome sectioning failed due to the metallic coil breaking out of the sections, taking debris of coating and cells with it. Direct staining on the embedded tissue “en bloc” was also unsuccessful due to loss of contrast in imaging the tissue. The highly important three of five regions thus were lost.
for histological and morphometric analysis, including the anastomosis regions where the artery is attached to the prosthetic graft.

**Protein elution from graft surface**

The second and fourth regions of the grafts, harvested after three days, were used for western blot analysis of adsorbed proteins. Adsorbed proteins after three days were eluded from the grafts surface using an elution buffer, consisting of 50 mM tris(hydroxymethyl)methylamine (Tris), 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% sodium dodecyl sulfate SDS in H$_2$O. Graft pieces were incubated in elution buffer for 45 minutes at 4ºC. Debris of cells was pelleted by centrifugation the eluate at 3000rpm at room temperature for 15 minutes, the supernatant was isolated and to purify, proteins were precipitated by adding 2:1 of ice-cold acetone for 10 minutes on ice. Precipitate was pelleted by centrifugation at 20000 g at 4ºC for 10 minutes. After decanting the supernatant, the pellet was allowed to dry and re-suspended in analysis buffer, consisting of 50 mM Tris, 2 mM EDTA and 50mM natriumchloride (NaCl) in H$_2$O.

**Western blot protein analysis**

To confirm the presence of specific pro-endothelial proteins on the second and fourth graft sections (Figure 1) of grafts harvested after three days, and to compare the amounts between eluates of the hydrophobic and hydrophilic coating, standard western blot analysis was performed. Details about the detection of albumin, fibrinogen, vascular endothelial growth factor and apo A-I are described in the supplemental material.

**Enzyme-linked immunosorbent assay**

Second and fourth graft sections (Figure 1) of the grafts harvested after 28 days were used to determine cell deposition on the grafts luminal surface using an enzyme-linked immunosorbent assay (ELISA) resembling analysis. Endothelial cells, thrombocytes and leucocytes were traced with antibodies against von Willebrand Factor (vWF), CD61 and CD11a respectively. Subsequently, the amount of bound antibodies was detected using a concentration dependent colour changing reagent. Details about this ELISA are described in the supplemental material.

**Scanning electron microscopy analysis of graft sections**

After performing the ELISA-like experiment, second and fourth graft sections (Figure 1) of the grafts harvested after 28 days were used to visualize cell adherence to the grafts and to compare the surface of implanted grafts with non-implanted grafts. Graft sections were washed extensively with PBS after the ELISA-like experiment. The pieces of coil were dehydrated in an ethanol and incubated with hexamethyldisilazane to allow for fast air-drying without loss of structure [20]. The samples were sputter-
coated with gold under constant rotation (108 auto/SE, Cressington Sci. Instruments, Watford, UK). Micrographs were made using a Philips XL30 SEM under an appropriate sample angle (20 to 45 degrees), looking into the coil at this angle.

**Statistical analysis and sample size calculation**

A non-parametric paired comparison between thrombocytes, leucocytes and endothelium adhered to the hydrophobic and hydrophilic coating was performed using a Wilcoxon signed-rank test. Patency comparisons between the hydrophobic and hydrophilic coated graft was performed using a log-rank test. Data will be presented as mean values with standard deviation added with error-bars.

**RESULTS**

**Graft patency results**

In eight goats, vascular prostheses were successfully implanted in both common carotid arteries (CCA). In one of the two goats that were sacrificed three days after implantation of the grafts, one hydrophobic graft had lost patency. In one of the six goats that were sacrificed after 28 days, another hydrophobic graft had lost patency. During weekly intermediate Doppler ultrasound monitoring of graft patency, no loss of patency was observed. Thus, two hydrophobic coated grafts lost patency compared to none of the hydrophilic coated grafts ($p=0.157$).

**Western blot analysis on eluate from grafts harvested after three days**

After three days, different proteins adsorbed to the grafts surface, depending on the hydrophilicity (Figure 2). As usual, albumin showed multi-band patterns on western blot, while fibrinogen, VEGF and ApoA-1 gave single bands. Fibrinogen was more abundant on the surface of the hydrophobic coating, as were immunoglobulin fragments. Both proteins were hardly detectable on the hydrophilic surface. Together with ApoA-1, larger amounts of albumin and VEGF were detected on the surface of the hydrophilic coated grafts after three days.

**ELISA analysis of cells on grafts explanted after 28 days**

We observed large differences in cell adsorption after 28 days on the hydrophobic and hydrophilic surfaces of the grafts (Figure 3). Higher amounts of CD11a and CD61 were detected on the surface of the hydrophobic graft ($p=0.012$ and 0.024 respectively). Concomitantly, a lower number of thrombocytes and leukocytes adhered to the surface hydrophilic coated grafts after 28 days, compared to the hydrophobic coated grafts. In line, many more vWF positive cells were detected on the hydrophilic surfaces.
Figure 2. Western blot analysis of the eluate of the hydrophobic and hydrophilic graft, harvested 3 days after implantation. Standard molecular weights are indicated on the left in kilodalton (kD).

Figure 3. Thrombocyte, leukocyte and endothelial cell amounts on hydrophobic and hydrophilic coated grafts, harvested after 28 days. Data are presented as mean arbitrary values with standard deviation (SD).

(p= 0.016), indicating more adhering endothelial cells at the hydrophilic surface than at the hydrophobic coated graft.

SEM analysis of luminal surface of explanted after 28 days
The luminal surface of the grafts explanted after 28 days contained deposition of endothelial cells, thrombus and leucocytes (figure 4). Endothelial cells were visible especially on the hydrophilic graft surface, whereas thrombus formation and leukocyte
Figure 4. Scanning Electron Micrographs (SEM) of interior surface of hydrophobic graft section (left 2 columns) and surface of hydrophobic graft section (right 2 columns). Non-implanted graft sections are visualized in the upper row. Details of grafts explanted after 28 days are shown in the lower 3 rows. Endothelial cells are especially visible on the hydrophilic graft surface, whereas on the hydrophobic surface thrombus as well as leukocyte deposition and adherence is visible. Scale bars indicate size in micro meters (µm).

deposition and adherence were visible especially on the hydrophobic graft surface. The coating on surface of the non-implanted grafts was smooth.

DISCUSSION

In the present study, we demonstrate that in a goat model of arterial interposition grafting, vascular grafts with a hydrophobic coating result in indifferent patency rate when compared to grafts with a hydrophilic coating. Indeed, in vitro, cell- and blood-compatibility were superior on the hydrophilic coating, especially after pre-incubation with human plasma. The adsorbed plasma proteins on the hydrophilic coating, which turned out to be mainly HDL and some albumin, strongly increased endothelial cell growth and thrombo-resistance [13].
At three days after graft implantation in goats, hydrophilic coated grafts contained increased concentrations of ApoA-I and VEGF, whereas the hydrophobic coated grafts contained increased amounts of fibrinogen. After 28 days, the hydrophilic grafts were characterized by an increased number of endothelial cells, with reduced amounts of leukocytes and thrombocytes as compared to the hydrophobic coated grafts. These data imply that hydrophilic coated grafts may be less prone to patency loss than hydrophobic coated grafts.

Protein adsorption after three days
The presence of albumin, fibrinogen, VEGF and ApoA-I, is consistent with other findings describing protein adsorption on polymers facing whole blood. Albumin and fibrinogen are frequently described as proteins adsorbing on surfaces within seconds after contact with whole blood. Typically, the type and concentrations of proteins adsorbing to the surface of a graft after contact with whole blood depends on the polymer type and its physical properties [6,21].

In our analysis, albumin is the most prominent protein that differs in concentration between the eluates of the hydrophobic and hydrophilic coating. Albumin comprises up to 70% of human plasma proteins [22]. Albumin binding is generally considered to improve the functional profile of vascular grafts since it reduces the acute inflammatory response to the prosthetic material [22–25].

In contrast, fibrinogen was present in higher amounts in the hydrophobic eluate. Fibrinogen is the third most common plasma component and appears to play a major role in the coagulatory- and associated inflammatory response. Denaturation of fibrinogen after binding to a surface leads to induction of the coagulation cascade as well as adherence of white blood cells, which correlates strongly with the degree of acute inflammatory response [26–28].

The adsorption of VEGF on the hydrophilic coated grafts might contribute to a reduction of the inflammatory response since the role of VEGF stimulating graft endothelialisation has been suggested [29–31]. Whereas VEGF increase was relatively limited in hydrophilic grafts, ApoA-I was specifically adsorbed to the hydrophilic coated grafts. This is in line with our previous in vitro studies [13]. The retention of an increased level of albumin on the hydrophilic surface is somewhat different from the in vitro results. This may be the result of interaction with full blood, or the flow rate may have influenced adsorption and desorption rates of proteins to the coating. Overall, the hydrophilic coating adsorbed higher levels of vasculo-protective and endothelialisation-stimulating proteins.

Analysis of cells on graft after 28 days
The higher amount of endothelial cells on the hydrophilic coated graft after 28 days seems to be crucial for the grafts long-term performance. Indeed, the endothelium
acts as a first line of defence against vascular disturbances. The antithrombotic and anti-proliferative effects of the endothelial cells abidingly prevent thrombotic occlusions and occlusions due to intima hyperplasia of prosthetic grafts [32]. In line with these endothelial effects, the lower platelet and leukocyte count seems to be a logic counterpart of the increased presence of endothelial cells. Compared to the hydrophobic coated graft, the higher endothelial cell count and lower thrombocyte and leukocyte detection can be translated into better biocompatibility with lower inflammation and thrombogenicity of the hydrophilic graft.

ApoA-I links our in vitro findings [13] with the results of our present study. ApoA-I adsorbs specifically to the surface of the hydrophilic coated graft and exerts vasculoprotective functions. By inhibiting inflammation, locally increased ApoA-I can contribute to preventing intimal hyperplasia [33–35]. By promoting endothelial function and regenerative capacity, an intact endothelial monolayer may rapidly cover the luminal surface of the graft, thus preventing activation of the coagulation cascade and subsequent thrombus formation [36]. The presence of ApoA-I or the full lipoprotein HDL on the graft surface could also prevent thrombus formation via inhibition of platelet aggregation and activation of the protein C system [37,38].

The hypothesis that hydrophilic coatings specifically attract ApoA-I that mediates a pro-endothelial effect in vivo, has never been evaluated before. Locally increased ApoA-I, delivered intramurally to prevent in-stent thrombosis, significantly inhibited luminal narrowing in the porcine stent model [39]. In mice, carotid artery re-endothelialisation after endothelial denudation was promoted by HDL [40]. In humans, systemic elevation of ApoA-I and HDL-cholesterol is associated with anti-inflammatory and anti-thrombotic effects [41]. The in vitro analysis of our hydrophilic coating demonstrated that ApoA-I adsorbs to the surface, and that the presence of ApoA-I is associated with decreased thrombus formation and increased endothelial cell adherence [13]. The result of our current in vivo analysis confirms these observations.

**Study limitations**

Due to the coil’s high metal content, we were unable to perform thorough histological and morphometric analyses. The number of compared grafts was insufficient to confirm a statistical difference in patency rate between the two graft coatings. However, by western blotting and ELISA analysis, we provided support for the hypothesis that ApoA1 adsorption is potentially associated with increased endothelialisation and improved patency of the hydrophilic grafts. The polymer coated metallic wires were useful study objects to demonstrate the performance of hydrophilic coatings and the role of plasma protein adsorption. However the polymer coated metallic coil obviously does not fulfil clinical requirements. In the near future, we will investigate the performance of hydrophilic polymer coatings on different base materials that do possess the required flexibility.
CONCLUSIONS

Our hydrophilic polymer coated prosthetic vascular graft showed superior in vivo compatibility in goats when compared to hydrophobic polymer coated grafts. Most likely, the adsorption of endothelial-protective proteins such as ApoA-I and VEGF has facilitated the enhanced endothelialisation of the hydrophilic coating, which may promote improved patency rates in vivo. These data call for further research in humans to evaluate in vivo behaviour of hydrophilic graft coatings.

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REFERENCES


SUMMARY AND GENERAL DISCUSSION
This thesis starts with a general introduction of arterial wall inflammation in chapter 1. The use of Magnetic Resonance Imaging (MRI) to quantify arterial wall characteristics is described in part 1, chapter 2, 3 and 4. The additional value of FDG-PET/CT in arterial wall inflammation imaging is discussed in chapter 4, 5 and 6 (part 2). In the final part of the thesis (chapter 7, 8 and 9), high-density lipoprotein (HDL) is tested as therapeutic target after reviewing effects of genetic changes in HDL or proximate working proteins.

PART 1: MRI TO QUANTIFY ARTERIAL WALL CHARACTERISTICS

The use of MRI in carotid artery wall imaging has become an established surrogate marker to determine cardiovascular disease (CVD) risk. It allows non-invasive, non-ionizing and safe imaging in all stages of atherosclerosis. The increase in field strength from 1.5 Tesla to 3 Tesla scanning showed an increase in signal to noise ratio (SNR) [1,2] and increased or comparable contrast to noise ratio (CNR) [3]. However, this did not result in increased reproducibility of carotid artery wall dimension measurements. In chapter 2 we found prove that increase spatial resolution in 3 Tesla scanning improves reproducibility of carotid artery wall dimension measurements in patients with advanced atherosclerotic carotid disease. These results encourage the use of 3T MRI with high resolution for fast and robust imaging of the carotid artery wall as surrogate marker for approach CVD-risk. Noteworthy, the increase in field strength from 3 Tesla to 7 Tesla has shown not to further increase the dimension measurement reproducibility [4].

Carotid artery wall scanning using MRI also allows plaque component identification which correlates well with gold-standard histology [5]. These MRI identified plaque components allow identification and classification of atherosclerotic lesions [6]. Increasing field strength allows identification of stable or instable atherosclerotic plaques by characterizing the plaque content [7–9]. In chapter 3 we show that the increase in spatial does not increase reproducibility of carotid artery plaque component identification and measurement in patients with advanced atherosclerotic carotid disease. Although reproducibility in our low resolution group was already very good, increasing the field strength may yield even higher reproducibility and allow automated identification of plaque components [4], although first in vivo application of 7 Tesla imaging showed disappointing correlation between histological findings and carotid artery wall contrast [10].

In chapter 4, 1.5 Tesla MRI scanning of the carotid artery wall in patients with or without type 2 diabetes mellitus (DM2) was performed to measure efficacy of guideline-based therapy on carotid artery wall dimensions in a 2 year follow-up study. Especially DM2-patients with high body mass index (BMI) contributed to the reduction in carotid artery wall dimensions in DM2 patients, whereas non-DM2 patients showed
stable wall dimensions after 2 year follow-up compared to baseline. Indeed, high BMI has been widely associated with increased cardiovascular mortality and morbidity in both DM2- and non-DM patients [11]; high BMI is associated with stronger progression in intima media thickness (IMT) [12]. In line with our results, high BMI is also associated with superior treatment response [13] and a decrease in BMI results in a decrease in coronary atherosclerosis, especially in metabolic syndrome [14]. We therefore hypothesize that increased inflammatory activity in the carotid artery wall in DM2 patients allows attenuation with especially statins and anti-hypertensive medication next to lifestyle changes.

PART 2: FDG-PET/CT TO QUANTIFY ARTERIAL WALL INFLAMMATION

Whereas MRI imaging allows fast, high-resolution and highly reproducible quantification of arterial wall dimensions and components (chapter 3 and 4), 18F-fluordeoxyglucose positron emission tomography with computed tomography (FDG-PET/CT) is increasingly applied to quantify arterial wall inflammation as a surrogate marker for CVD [15,16]. To promote and increase the optimal use of FDG-PET/CT in CVD research, we confirmed measurement reliability and reproducibility in chapter 5. In order to increase comparability between studies, we propose standardized analysis of specific arterial segments. The reference ranges we defined in these conditions allows comparison of mean arterial FDG uptake in a study group with mean arterial wall FDG uptake in control subjects, subjects with a history of CVD or a group with intermediate risk for CVD according to the Framingham CHD risk-algorithm. Since 95% confidence intervals of these groups overlap, classification of individual subjects and thus application of FDG-PET/CT is unsuitable for use in daily clinic.

In chapter 6 we used MRI to measure carotid artery wall dimensions and FDG-PET/CT to discriminate the arterial wall inflammatory activity between patients with rheumatoid arthritis (RA) in remission with or without dependency of the use of a biological, anti-TNF \( \alpha \) therapy. No structural differences were detected between patients with or without biological dependency to remain in remission. Surprisingly, higher inflammatory activity was present in biological-dependent remissive RA patients. Their higher disease intensity and duration may have caused joint destruction beyond a certain point, causing irreversible epigenetic changes switching on inflammatory pathways [17]. Despite effective ‘RA activity decrease’ in subjects using anti-TNF therapy, active inflammation appears to persist at least in the arterial wall, implying a remaining CVD risk increase. These observations urge the need for potent CVD prevention in RA, particularly in those patients with a persistent need for biologics to maintain a remissive state. In chapter 7 we illustrate the complexity of the search for new anti-RA drugs. Tocilizumab, an interleukin-6 (IL-6) receptor blocking antibody, appears to have stronger anti-inflammatory properties that may cure the
persistent inflammatory activity in biological dependent remissive RA-patients, but the compound has undesirable side-effects affecting the lipid metabolism. Fasting plasma levels of LDL-cholesterol, HDL-cholesterol and triglycerides increased within one week after tocilizumab treatment initiation. Post-prandial triglyceride levels increased also, accompanied with a strong increase in apolipoprotein B48 levels. The combination of these findings, after ruling out cholesterol synthesis defects, pointed toward a hepatic chylomicron (remnant) removal. A decrease in hepatic LDL-receptor expression, a principal clearance pathway, is held responsible for the tremendous effects of tocilizumab on the lipoprotein metabolism. Because anti-TNF therapy only mildly increased lipid levels [18,19] and genetic IL-6 signaling defects were not associated with such pro-atherogenic lipid changes [20], increase in chylomicron (remnant) levels, presumably caused by decreased LDL-receptor expression, presumably is a tocilizumab-specific effect rather than a consequence of blocking the IL-6 receptor signaling pathway.

PART 3: LOCALLY INCREASED HDL AS THERAPY FOR ARTERIAL WALL INFLAMMATION

High-density lipoprotein cholesterol (HDL-c) which mainly consists of Apolipoprotein A1 (ApoA-I), phospholipids, cholesterol and triglycerides, is inversely correlated with CVD risk [21–23]. In chapter 8, Mendelian disorders affecting ApoA-I or HDL-c metabolism are discussed. The genes encoding ApoA-I, lecithin cholesterol acyltransferase (LCAT) and ATP binding cassette protein AI (ABCAI) are key players in HDL-synthesis, illustrated by the fact that complete deficiency of these proteins leads to extremely low plasma HDLc levels. In contrast, lack of endothelial lipase (LIPG) and cholesteryl ester transfer protein (CETP) is associated with accumulation of mature HDL in the circulation [24]. Despite low prevalence of these disorders requiring imaging as surrogate endpoint marker for CVD risk, extremely low levels of HDL-c were associated with increased CVD risk.

CVD risk association of genetic analysis of single nucleotide polymorphisms (SNP) and common variants was awaited when chapter 8 was written, but to date, no associations between common variants and coronary heart disease (CHD) were found [25,26]. Even the association between plasma level HDL-c increase and a decrease in CVD events has not been proven robustly because HDL-c increasing therapies also cause low-density lipoprotein reduction [27]. Especially CETP inhibitors, the most potent HDL-c increasing agent available, cause significant LDL-reduction, thereby confounding a potential effect of HDL on CVD event reduction. Besides, because HDL particle size, density and composition varies, not HDL-c plasma levels itself, but HDL function appears to be key in CVD risk reduction [28] supported by the observation that HDL mediated cholesterol efflux is inversely associated with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease [29] and
the incidence of cardiovascular events [30], independent from HDL-c plasma levels. As a consequence, studies that aim on systemically increasing ApoA-I concentrations by infusions of lipid-poor ApoA-I with stabilizing phospholipids, regarded as nascent HDL-particle, are expected to increase plasma accepted capacity as well as improve HDL function [31,32] and therefore are essential in confirming the role of HDL in CVD risk prevention. However, to date also the data on ApoA-I mimetic infusion has revealed equivocal results (CHI SQUARE trial [33], ERASE trial [34], SAMBA trial (Kootte et al; EAS 2014 session), MODE (Smits et al; EAS 2014 session; NCT01412034).

An alternative to systemic infusion of ApoA-I mimetics is to profit from local presence of apoA-I near the site of the damaged artery wall, allowing for the beneficial effects of apoA-I on inflammation, coagulation and endothelial integrity. In order to achieve maximum benefit of ApoA-I at the injured site of the coronary artery, an anti-ApoA-I antibody coating was developed and tested in vitro and in vivo in a rabbit model, applied on the surface of coronary artery stents, as described in chapter 9. We hypothesized that these stents would prevent in-stent restenosis better than bare metal stents (BMS), but without the long-term dual anti-platelet aggregation therapy to prevent acute or late-onset in-stent thrombosis which is obligatory after drug-eluting stent implantation. In our preclinical study, despite a density dependent decreased endothelial cell adhesion and growth and decreased thrombin generation on or at anti-ApoA-I antibody coated discs compared with uncoated disks, restenosis in anti-ApoA-I antibody coated stents implanted in the pre-injured iliac artery of a rabbit was indifferent compared with BMS, 28 days after implantation. The fixed antibody in the coating is supposed to bind ApoA-I to the stent surface and thereby improve biocompatibility, may have failed due to the fenestrated surface of stents covering the injured artery. Intima hyperplasia at approximately 80% of the uncoated area between stent struts may rapidly have overgrown the struts, which loss contact with plasma which acts as the ApoA-I supplying reservoir. Moreover, the anti-ApoA-I antibody bound ApoA-I may become inactive after being active for a certain period, but thereafter it may idly occupy the antibody binding site without further protective function.

An alternative approach for locally increasing HDL near the artery wall is derived from data from Knetsch et al, who showed that in contrast to hydrophobic coatings that are usually applied in arterial interposition prostheses, HDL adsorbs to a hydrophilic co-polymer coating in very high concentrations [35]. In vitro analysis revealed that presence of HDL contributes to higher endothelial cell coverage and decreased thrombus formation compared to hydrophobic ones [35,36]. In chapter 10, in vivo performance was tested by implanting prosthetic hydrophilic coated prosthetic grafts as arterial interposition in the common carotid artery of 8 goats in a side-to-side comparison with hydrophobic coated grafts. We showed that hydrophilic coated grafts, 3 days after implantations, show higher ApoA-I adsorption combined with lower fibrinogen compared with hydrophobic coated grafts. After 28 days, none of the hydrophilic coated grafts were occluded, but 2 grafts were occluded with the
hydrophobic coating. Higher thrombocyte and leukocyte count in the hydrophobic coated graft and higher endothelial cell count in the hydrophilic coated graft provides a mechanistically link between how HDL adsorbing hydrophilic coated vascular grafts have higher patency compared with hydrophobic ones.

Our data contribute and agree with previous human data on HDL functions in maintaining arterial patency by preventing acute occlusion via improving nitrous oxide mediated endothelial function [37–39], promoting endothelial progenitor cells [40], and preventing platelet activation [41]. Vessel wall inflammation decreases in presence of high concentrations of HDL by increased cholesterol efflux [42,43], decreased leukocyte adhesion and decreased cytokine release [44]. Non-human data in addition confirmed a central role for HDL in improving plaque stability by decreasing oxidative stress and apoptosis within the vessel wall [45–47].

FUTURE PERSPECTIVES

Imaging modalities in CVD risk estimation and treatment allocation

Recent imaging studies have established the increasing value of arterial wall plaque composition [48,49] and functional properties [50,51] of the inflamed arterial wall on CV event risk. In the present thesis we show that accuracy and reproducibility of MRI (structural) and PET/CT imaging (functional) in atherosclerotic vascular disease has been confirmed. Instead of focusing on risk factors, systemic biomarkers (CRP, LDLc) and ultrasound techniques (Duplex ultrasound), these imaging strategies may allow estimation of a patients individual CV event risk in daily practice, especially in secondary prevention. Technical innovation may lead to a one-stop-shop technique, allowing for rapid, high-throughput evaluation of atherosclerotic disease. For the success and cost-effectiveness of tailored therapy enabled by individual risk estimation, I expect that the proof of the pudding will be in the eating.

Anti-inflammatory therapies in CVD patients

Cardiovascular diseases are the result of exposure to a composition of environmental factors combined with genetic predisposition. Until to date, CVD prevention and treatment mainly consisted of eliminating environmental factors. Next to lifestyle changes including dietary changes and smoking cessation, hypertension treatment and lipid lowering was focus of research and treatment. Especially lipid lowering strategies are regarded effective but despite all effort, only 25% of CVD was prevented. This thesis supports the commence of a new era of focusing on arterial wall inflammation as the product of environmental and genetic factors. As demonstrated in part 2 of the thesis, arterial wall inflammation imaging enters the standard arsenal of a clinician in individual CVD research. This diagnostic leap forward demands the use of therapeutic agents which are already available, to interrupt the inflammatory cascade in CVD. In chronic inflammatory diseases like ankylosing spondylitis, psoriasis and mostly in
rheumatoid arthritis, biologicals are related to a decrease cardiovascular morbidity and mortality [52]. Clinical endpoint studies aiming on the cardio protective effects of biologicals without presence of chronic systemic inflammatory diseases have to be initiated. The pivotal pathway of interleukin-6 receptor blockade has been associated with coronary heart disease using genetic and biomarker data in a meta-analysis [53], but the only available compound interfering with this pathway disturbs hepatic chylomicron (remnant) clearance (chapter 7). Nanoparticle delivery of compounds to the specific therapeutic site of interest may overcome side effects (Van Wijk et al, manuscript in preparation; NTR2936).

**HDL increasing strategies**

Although ApoA-I and HDL increasing strategies show disappointing results on cardiovascular endpoints, many studies report beneficial effects of apoA-I and HDL on reverse cholesterol transport and other mechanisms, including inflammation, endothelial function and coagulation. Especially increasing ApoA-I and HDL near the inflamed artery wall is potential. Instead of coating a stent surface with the ApoA-I antibody coating, appliance of the coating on a solid and continuous surface of intravascular catheters, pacemaker leads and grafts may demonstrate the superior in vivo compatibility compared with other synthetic surfaces.

**REFERENCES**


ADDENDUM
DEEL 1: Het gebruik van de MRI-scanner ter kwantificatie van slagaderlijke vaatwand karakteristieken

Kwantificatie van slagaderlijke vaatwand karakteristieken met behulp van de MRI-scanner heeft de laatste jaren een vlucht genomen. Het meten van de vaatwanddikte en -compositie wordt binnen wetenschappelijk onderzoek steeds meer gebruikt om binnen een omschreven groep het risico op hart- en vaatziekten in te schatten. In de zoektocht naar nog betere methoden, blijkt dat de toename van magnetisch veldsterkte van de scanner niet leidt tot een betere reproduceerbaarheid van de metingen. In hoofdstuk 2 tonen we aan dat in een groep patiënten met een ernstig verdikte vaatwand van de halsslagader door aderverkalking, het verhogen van de scanresolutie wél tot een betere reproduceerbaarheid van de metingen leidt. Dit geldt niet voor de identificatie van componenten van de verdikte vaatwand, zoals beschreven in hoofdstuk 3. Het herkennen van de vaatwand-componenten heeft als doel om inzicht te krijgen in gevoeligheid voor deze zogenaamde vaatwandplaque om open te scheuren waarbij de inhoud van de vaatwandplaque verderop in het bloevat een afsluiting veroorzaakt door vorming van een bloedprop (trombus). Deze vaatafsluiting uit zich als een hart- of herseninfarct.

In hoofdstuk 4 wordt aangetoond dat de middels MRI-scanner gemeten vaatwanddikte van de halsslagader na twee jaar afneemt bij patiënten met type 2 diabetes mellitus, als zij strikt volgens de richtlijn worden behandeld. Dit in tegenstelling tot een vergelijkbare groep zonder type 2 diabetes mellitus, waar geen verschil in vaatwanddikte werd gemeten. De body-mass-index (BMI) bleek de belangrijkste voorspeller van afname van vaatwanddikte bij de patiënten met type 2 diabetes mellitus.
Deel 2: Het gebruik van de FDG-PET/CT scanner ter kwantificatie van slagaderlijke vaatwand ontsteking

In navolging van de MRI-scanner is de FDG-PET/CT scanner waarschijnlijk nog beter in staat om het risico op hart- en vaatziekten van een omschreven groep mensen in te schatten. In tegenstelling tot de MRI-scanner, die alleen meting van de vaatwanddikte en -compositie toestaat, is het met de FDG-PET/CT scanner mogelijk om directe ontstekingsactiviteit in de slagaderlijke vaatwand te meten. Met deze methode zou het mogelijk moeten zijn om het risico op hart- en vaatziekten per individu in te schatten voor na behandeling. In hoofdstuk 5 bevestigen we de betrouwbaarheid en reproduceerbaarheid van de metingen door toepassing van een gestandaardiseerd protocol. Gebruikmakend van dit protocol zijn in dit hoofdstuk tevens referentiewaarden voor patiëntengroepen met een verschillend risico op hart- en vaatziekten gedefinieerd. De spreiding in de meting per individu is te groot om een betrouwbare schatting van het risico op hart- en vaatziekten per individu te geven.

In hoofdstuk 7 wordt de kracht van het gebruik van de FDG-PET/CT scanner getoond. In patiënten met reumatoïde artritis die wel of niet afhankelijk van een biological zijn om de ziekteactiviteit op een laag niveau te houden, blijkt de MRI-scanner geen onderscheid te vinden in de vaatwanddikte van de halsslagader. Met de FDG-PET/CT scanner wordt tussen deze groepen wel degelijk een verschil gezien in ontsteking van deze delen van de halsslagader, wat een persistend verhoogd risico op hart- en vaatziekten inhoudt voor mensen die krachtigere therapie nodig hebben.

De complexiteit van de behandeling van reumatoïde artritis wordt beschreven in hoofdstuk 8, waar naast effectieve vermindering van de ziekteactiviteit als gevolg van het gebruik van een interleukine-6 receptor antagonist, ook een dyslipidemie ontstaat. Uit metingen van de lipoproteïne fracties voor en na inname van een vetrijk dieet blijkt dat de interleukine-6 receptor antagonist de klaring van chylomicronen en remnant partikels remt, meest waarschijnlijk door verminderde LDL-receptor expressie op de hepatocyt. In vitro onderzoek bevestigt het remmend effect van de interleukine-6 receptor antagonist op de LDL-receptor expressie. Ondanks het sterke ontstekingsremmende effect bestaat er via een andere route dus een bijwerking die het risico op hart- en vaatziekten verhoogt.

Deel 3: Lokaal verhoogd HDL als therapie voor slagaderlijke vaatwand ontsteking

Hoofdstuk 8 is een literatuur review waarin de gunstige effecten van high-density lipoprotein (HDL, ook wel het gunstige cholesterol genoemd) op het risico op hart- en vaatziekten wordt beschreven door te kijken naar de uiting van genetische aandoeningen die de werking van HDL of de bij het HDL-metabolisme betrokken
Deze gunstige effecten van HDL-cholesterol hebben geïnspireerd om in hoofdstuk 9 te kijken wat het effect is van met anti-ApoA-I antilichaam beklede stents op de in-stent restenose ten opzichte van een niet-beklede metalen stent. Omdat ApoA-I het sleuteleiwit is aan de oppervlakte van HDL, verhoogt de bekleding in in vitro experimenten door lokale toename van HDL, de hechting en groei van endotheelcellen en remt het de vorming van stolsels op het oppervlak. In een proefdiermodel laat deze experimentele stentbekleding na 28 dagen geen voordeel zien ten opzichte van de niet beklede variant. De beperkte bedekking van het vaatwandoppervlak bij gebruik van een stent zou de sleutel kunnen zijn tot de oorzaak van het falen van deze stentbekleding. In hoofdstuk 10 wordt middels een andere techniek het HDL cholesterol in van de vaatwand verhoogd. Uit eerder onderzoek blijkt dat een hydrofiele bekleding van vaatwandprothesen veel HDL aantrekt, in tegenstelling tot de hydrofobe variant die in de praktijk het meest gebruikt wordt. De toepassing van een hydrofiele bedekking van een halsslagaderprothese laat in een proefdiermodel een voordeel zien in doorgankelijkheid na 28 dagen ten opzichte van een hydrofobe bekleding. Er blijkt en zich dan ook op de hydrofiele bekleding vaatwandprothese meer endotheelcellen en minder leukocyten en trombocyten te hebben afgezet, wat verantwoordelijk gehouden kan worden voor het gunstige resultaat. Onderzoek naar de afzetting van eiwit op de vaatwandprothese toont een hoge afzetting van ApoA-I op de hydrofiele bekleding, hetgeen causaal wordt geacht voor de het gunstige afzettingsprofiel van de cellen.

Dit proefschrift sluit af met hoofdstuk 11, een Engelstalige samenvatting en discussie.
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