Novel visualization techniques towards identification of atherosclerotic patients at risk
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 13-01-2020
Folate receptor-β imaging using 99mTc-folate to explore distribution of polarized macrophage populations in human atherosclerotic plaque

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J Nucl Med. (accepted)
Abstract

OBJECTIVES In atherosclerotic plaques the risk of rupture is increased at sites of macrophage accumulation. Activated macrophages express folate receptor-β (FR-β) which can be targeted by folate coupled to radioactive ligands to visualize vulnerability. The aim of this study is to explore presence of activated macrophages in human atherosclerotic plaques by $^{99m}$Tc-folate imaging and to evaluate whether this technique can discriminate between a M1-like and M2-like macrophage phenotype.

METHODS Carotid endarterectomy specimens of 20 patients were incubated with $^{99m}$Tc-folate, imaged using µSPECT, and divided in 3mm slices. Mean accumulation was calculated per slice and distribution of M1-like and M2-like macrophages per slice was quantified by immunohistochemical staining for CD86 and iNOS for M1, and CD163 and FR-β for M2 macrophages. Monocytes from healthy donors were differentiated towards M1-like or M2-like phenotype by in vitro culturing. mRNA levels of specific M1- and M2-markers were measured by RT-PCR and expression of FR-β, CD86, and CD163 by flow cytometry.

RESULTS There was a heterogeneous accumulation of $^{99m}$Tc-folate in plaques (median 2.45 (0.77-6.40) MBq/g). Slices with highest $^{99m}$Tc-folate accumulation of each plaque showed significantly more expression of FR-β and CD163, compared to slices with lowest $^{99m}$Tc-folate accumulation which showed significantly more expression of iNOS. In in vitro polarized macrophages, mRNA expression of FR-β, mannose receptor, IL-10 and MMP-9 was significantly increased in M2-like macrophages compared to M1-like macrophages. On receptor level CD86 was shown to be over expressed on M1-like macrophages while FR-β and CD163 where over expressed on M2-like macrophages measured by flow cytometry.

CONCLUSION In areas of high $^{99m}$Tc-folate accumulation, more M2-like macrophages were present compared to areas with low accumulation. It is anticipated that $^{99m}$Tc-folate imaging using SPECT as a marker for M2-like macrophages in atherosclerosis might be a good indicator for plaque vulnerability.

Keywords Carotid artery; atherosclerotic plaque; vulnerability; folate receptor-β imaging; M2-like macrophages.
Introduction

Cardiovascular atherosclerotic disease is the main cause of death in the Western world (1). Clinical events such as myocardial infarction and ischemic cerebrovascular accident are directly related to the risk of atherosclerotic plaque rupture. Histological analysis of a vulnerable plaque suggests the importance of necrotic core size, extent of inflammation, and angiogenesis, lipid accumulation, and fibrous cap thickness (2). Furthermore, in plaque vulnerability matrix metalloproteinase’s (MMPs), reactive oxygen species, inflammatory cytokines and various growth factors released by activated macrophages play important roles (3). Patients with symptoms and carotid artery stenosis >70% or those asymptomatic with carotid artery stenosis of 80-90%, are deemed suitable for endarterectomy thereby significantly reducing the risk of subsequent major cerebrovascular accident or death (4). Imaging techniques currently used to visualize carotid artery stenosis include duplex ultrasound, computed tomography angiography (CTA) and angiography (5). However, these techniques focus on anatomic features of the plaque and barely give any information on molecular and cellular processes. They therefore cannot distinguish between stable and unstable plaques. As plaque composition rather than stenosis is important in detecting an unstable plaque, new imaging modalities are needed (5).

2-deoxy-2-[18F]fluoro-D-glucose positron emission tomography ([18F]FDG-PET) can be used to identify vulnerable plaques in atherosclerotic disease, since FDG accumulates in activated macrophages. FDG-microPET accumulation correlated well with macrophage content in a rabbit model, while in vivo quantification did not support this finding, possibly due to high accumulation in surrounding tissue (6). Folate receptor β (FR-β) is present on activated macrophages, but not on resting macrophages or other immune cells. For that reason it might be a good indicator for inflammation in the human atherosclerotic carotid plaque, as previously shown by imaging carotid artery specimens with fluorescence labeled folate (7). Furthermore, Ayala-López et al. localized activated macrophages in apolipoprotein E knockout mice fed a western diet using a 99mTc-folate labeled compound (8).

A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (9). The M1 macrophage is thought to have pro-inflammatory properties and polarization in vitro is driven by interferon gamma and low concentration lipopolysacharride. Macrophages are driven towards the M2 type when the environment includes IL-4 and IL-10 (10). However, polarization of macrophages within a plaque is determined by the local micro-environment present in the atherosclerotic lesion and is thought to be rather more complex than the often used M1/M2 paradigm (11). As MMPs trigger plaque rupture, a M2-like macrophage type might be important as a direct cause of plaque vulnerability by producing tissue degrading MMPs (12).

Therefore, the major goal of this pilot study was to explore the distribution of macrophage...
subtypes in atherosclerotic plaques and the FR-β expression on the respective macrophage subtypes. In addition, the potential of technetium labeled folate as marker for atherosclerotic carotid plaque vulnerability was explored. To achieve this goal, the $^{99m}$Tc-folate signal was compared with FR-β staining using specimens with a positive imaging ratio (accumulation slice/total plaque accumulation>1) to validate the folate technetium compound. Additionally, macrophages were differentiated and polarized in vitro into M1-like and M2-like macrophages to investigate expression of FR-β and known M1 and M2 markers.

**Materials and Methods**

**Study Design**

Between August 2011 and May 2012, a total of 20 carotid specimens were obtained by means of carotid endarterectomy (CEA) of the carotid bifurcation using standard techniques (13). Patients underwent open carotid surgery at the University Medical Center Groningen (UMCG) and were symptomatic (i.e. with a history of recent cerebrovascular accident (CVA), transient ischaemic attack (TIA) or amaurosis fugax). They presented with a stenosis of the common carotid artery of 70-99% as detected by duplex ultrasound. Additionally, asymptomatic patients with a stenosis of 80-99%, found by routine control were eligible for surgical treatment. Risk factors such as hyperlipidemia, hypertension, smoking status, obesity (body mass index, BMI) and diabetes mellitus were recorded. Hyperlipidemia and hypertension was defined as described before by our group (7). For validation of macrophage markers six healthy volunteers where included without known cardiovascular disease or risk factors. The study was approved by the Institutional Review Board (IRB) of the UMCG and informed consent was obtained from all patients and healthy volunteers.

**Carotid Endarterectomy Sample Collection and Timepath of Study**

The carotid samples collected during CEA, were immediately transported in a phosphate buffer on ice to the U-SPECT-II system (MILabs, Utrecht, The Netherlands), with a mean transport time of 18±6 minutes. The optimal incubation time and concentration of $^{99m}$Tc-folate was determined. To validate the $^{99m}$Tc-folate signal, immunohistochemistry was used for determining M1-like and M2-like macrophage levels in regions with high and low $^{99m}$Tc-folate accumulation.

**$^{99m}$Tc-folate Labeling and Imaging of the Plaque using μSPECT**

To determine the exact location where the $^{99m}$Tc-folate accumulation had taken place within the plaque, ex vivo imaging was performed. A $^{99m}$Tc-labeled imaging agent consisting of the vitamin folic acid was conjugated to a chelating agent with specificity for $^{99m}$Tc was used as follows; 1mL of Sodium Pertechnetate $^{99m}$Tc injection (100 MBq) was injected into a
shielded vial. The vial was gently swirled for 30 seconds to dissolve the lyophilized powder and placed into a lead shield and incubated at 100 °C for 18 minutes. Subsequently, the ⁹⁹ᵐTc-folate vial was cooled for 15 minutes protected from light, and the radiochemical purity of ⁹⁹ᵐTc-folate was determined by ITLC using saline as the eluent. The radiochemical purity must be >90% to pass acceptance criteria. The carotid specimens were weighed and incubated for one hour in 5mL of a ⁹⁹ᵐTc-folate containing solution at room temperature. After washing ⁹⁹ᵐTc-folate accumulation was measured using the dose calibrator. The specimens were put into a micro single-photon emission computed tomography (μSPECT) scanner, near the center of field of view (FOV) and scanned for one hour (as determined by serial dilution). μSPECT images were corrected for scatter and reconstructed applying an interactive reconstruction algorithm (OSEM 2D). After incubation and scanning, all specimens were divided in equal slices of 3mm. For every slice the mean accumulation was calculated as counts/voxel of Region of Interest (ROI) versus total dose calibrator activity per gram (total plaque weight), expressed as MBq/g. Images acquired were processed using Amide software (14).

**Immunohistochemistry and Validation of ⁹⁹ᵐTc-folate Signal**

After determining ⁹⁹ᵐTc-folate accumulation of every slice, slices of plaques of 10 patients were embedded in paraffin, and sections of 4 μm were made. Macrophages were identified by incubation with monoclonal mouse anti-human CD68 (1:50; mo876 clone PG-M1 DAKO, Glostrup, Denmark). For detection of M1 macrophages, rabbit anti-human iNOS and rabbit anti-human antibody CD86 (ab15323 and ab53004 respectively, Abcam, Cambridge, UK) were used. To show M2-like macrophage distribution mouse anti-human CD163 (a widely used M2 macrophage marker and mainly found in advanced human lesions near intraplaque haemorrhage areas (15), ab74604, Abcam, Cambridge, UK) and biotinylated sheep anti-human FR-β antibody were used (BAF 5697, R&D systems, Minneapolis, USA). Also, FR-β and CD163 were used for double staining to show co-localization of M2-like macrophages and FR-β expression. Appropriate secondary antibodies labeled with horseradish peroxidase (HRP), and alkaline phosphatase (AF) for double staining was used. Color reaction was developed using DAB (Dako) staining with chromogen (fast red (Sigma) for double staining with AF), and sections were counterstained with hematoxylin. All sections were stored digitally after examination using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074-01, Hamamatsu Photonics K.K., Japan) and quantified (percentage of positive cells / total cells) with software of ImageScope Viewer (V11.2.0.780 Aperio, e-Pathology Solution, CA, USA). For validation, slices with the lowest mean accumulation of the ⁹⁹ᵐTc-folate signal in imaging where compared with the slices with the highest mean accumulation in every carotid plaque separately (both compared to mean accumulation in the total plaque in counts/voxel).
CHAPTER 7

In Vitro Differentiation and Polarization of M1-like and M2-like Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from 6 healthy donors by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, monocytes were allowed to adhere to culture plates. The adherent cells were maintained for 5 days in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% filtered fetal calf serum (FCS). 20 ng/ml macrophage colony stimulating factor (M-CSF, R&D systems) was added to the cell culture for differentiation into macrophages. Macrophages were directed towards M1-like and M2-like phenotypes by use of 48 hours stimulation with 100 U/ml interferon gamma (PeproTech, USA) and 1 ng/ml lipopolysacharride (Sigma, Germany) or 20 ng/ml IL-4 and 10ng/ml IL-10 (PeproTech), respectively.

Flow Cytometry of M1-like and M2-like Macrophages

In vitro differentiated M1-like and M2-like macrophages were stained with biotinylated antihuman FR-β antibody (R&D systems) to explore protein expression. Streptavidine–allophycocyanine (APC, Biolegend, San Diego, CA, USA) was used as secondary antibody. Anti-CD86-FITC (BD Bioscience, San Jose, CA, USA) and anti-CD163-PE (Biolegend) were used to distinguish expression of these markers on macrophage subtypes. These were measured using flow cytometry.

RNA Isolation, cDNA Synthesis and qRT-PCR

RNA was extracted from M1-like and M2-like macrophages as described before (7). For measurement of mRNA (messenger ribonucleic acid) 1 μl cDNA sample was added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). Investigated genes were: GAPDH, IL-6, TNF, Toll-like receptor (TLR) 2 and 4, FR-β (folate receptor 2 or β), mannose receptor (CD206 or MR), IL-10 and MMP-9 (matrix metalloproteinase 9). Ct (threshold cycle) values were determined using the software program SDS 2.4 (Applied Biosystems). Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula; Relative gene expression = 2^−ΔCt (ΔCt = Ct gene of interest – Ct GAPDH).

Statistics

Values are presented as mean ± standard deviation or median (range), unless stated otherwise. For correlations, Pearson’s and Spearman’s correlation coefficients were used when appropriate. A two-tailed, paired student T-test was used for parametric distributions. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).
Results

Patient Characteristics
A total of 12 men and eight women with a mean age of 68.0 ± 8.4 years were included. Patient characteristics are shown in Table 1.

Table 1. Baseline characteristics and risk factors for atherosclerosis

<table>
<thead>
<tr>
<th>Patients (n=20)</th>
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<tbody>
<tr>
<td>Men, n (%)</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Symptomatic, n (%)</td>
</tr>
<tr>
<td>Transient ischemic attack (TIA), n (%)</td>
</tr>
<tr>
<td>Cerebro vascular accident (CVA), n (%)</td>
</tr>
<tr>
<td>Amaurosis fugax, n (%)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
</tr>
<tr>
<td>&gt; 1 pack a day, n (%)</td>
</tr>
<tr>
<td>≤ 1 pack a day, n (%)</td>
</tr>
<tr>
<td>None, smoked in last 10 years (%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
</tr>
<tr>
<td>Controlled with single drug, n (%)</td>
</tr>
<tr>
<td>Controlled with 2 drugs, n (%)</td>
</tr>
<tr>
<td>Requires &gt; 2 drugs or uncontrolled, n (%)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
</tr>
<tr>
<td>Use of lipid-lowering drugs, n (%)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
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</tbody>
</table>

* Two patients had diabetes controlled by diet or oral agents, one patient was on insulin. Data are expressed as mean ± standard deviation; percentages between brackets.

Imaging of the Plaque using μSPECT
Imaging of 20 atherosclerotic plaques using μSPECT showed $^{99m}$Tc-folate accumulation occurred in specific areas of the plaques, with a median total activity of 2.45 (0.77-6.40) MBq/g measured in a dose calibrator (figure 1). After scanning all specimens were divided in equal slices and ratio per slice was calculated (accumulation slice/total plaque accumulation). There were 5 ± 1 slices per atherosclerotic carotid plaque. $^{99m}$Tc-folate accumulation was heterogeneously distributed throughout plaques (slices with highest $^{99m}$Tc-folate ratio: 1.28 (1.03-2.24) and slices with the lowest $^{99m}$Tc-folate ratio: 0.8 (0.18-0.99)).
Figure 1. Transversal (left), coronal (middle), and sagittal (right) sections of microSPECT image of an atherosclerotic carotid plaque specimen after $^{99m}$Tc-folate incubation showing heterogeneous accumulation. The transversal image shows that area of higher accumulation (red) is confined within the vessel wall.

**Immunohistochemistry and Validation of $^{99m}$Tc-folate Signal**
Plaque slices of 10 patients were stained using FR-β to validate the $^{99m}$Tc-folate signal, as well as for CD68 (pan macrophages), CD86 and iNOS (M1 macrophage markers) and CD163 (M2 macrophage marker). In figure 2A representative staining is shown. All stainings were quantified with Imagescope software and expressed as percentage of positive cells per total cells. Slices with a positive $^{99m}$Tc-folate imaging ratio (accumulation slice/total plaque accumulation >1) where positively correlated to FR-β staining (P =0.006, figure 2B).
Figure 2. Immunohistochemical staining of 10 atherosclerotic plaques. (A) Staining of CD68, CD86, CD163, FR-β and iNOS is depicted in a slice with the highest accumulation of $^{99m}$Tc-folate in a plaque. (B) Positive $^{99m}$Tc-folate slices (accumulation slice / accumulation total plaque >1) showed a positive correlation with FR-β staining ($r=0.46$, $P=0.006$).

Figure 3 shows that FR-β staining in slices with the highest $^{99m}$Tc-folate accumulation of each plaque was higher compared to slices with lowest accumulation ($P=0.042$). The same was shown for CD163 staining ($P=0.036$). iNOS staining, on the contrary, showed a significantly higher percentage of positive cells in the slices with lowest accumulation $^{99m}$Tc-folate compared to slices with highest accumulation ($P=0.043$). However, staining of CD68 and CD86 was not significantly different in slices with highest accumulation $^{99m}$Tc-folate compared to slices with lowest accumulation ($P=0.43$ and $P=0.44$ respectively). Furthermore, CD163 co-localized with FR-β expression in a carotid plaque, as can be seen in a representative picture (Figure 3B).
**Figure 3.**

(A) Quantification of stainings (left) Slices with highest 99mTc-folate accumulation (closed dots) showed significantly more FR-β expression compared to slices with lowest 99mTc-folate accumulation (open dots, P = 0.043) (middle) Slices with highest 99mTc-folate accumulation (closed dots) showed significantly more CD163 staining compared to slices with lowest 99mTc-folate accumulation (open dots, P = 0.036). (Right) Slices with lowest 99mTc-folate accumulation (open dots) showed significantly more iNOS staining compared to slices with highest 99mTc-folate accumulation (closed dots, P = 0.043) (B) Double staining of CD163 and FR-β to show co-localization of M2-like macrophages and FR-β expression.

**Expression of Macrophage Markers by Flow Cytometry**

To validate FR-β, CD86 and CD163 as macrophage subtype markers, their expression on macrophage subpopulations was determined using flow cytometry (representative examples in figure 4). FR-β is expressed on 31% of M1-like macrophages and on 72% of M2-like macrophages. In M1-like macrophages, 93% (60-97) of cells were positive for CD86, while 34% (5-45) was positive for CD163. On the contrary, M2-like macrophages showed a high expression of CD163 (86% (56-95)) while CD86 expression was lower (27% (10-40)), both measured in 10 healthy volunteers.
Figure 4. Representative flow cytometric analysis of M1-like and M2-like macrophage population from *in vitro* polarized macrophages, derived from PBMCs from a healthy control. (A) FR-β is expressed on 31% of M1-like macrophages and on 72% of M2-like macrophages (red line). (B) CD86 is expressed on 93% of M1-like macrophages and on 27% of M2-like macrophages (red line). (C) CD163 is expressed on 34% on M1-like macrophages and on 86% of M2-like macrophages (red line). Isotypes in black in all graphs.
**In Vitro mRNA Expression of M1-like and M2-like Markers**

mRNA levels of GAPDH, IL-6, TNF, TLR-2 and -4, FR-β, MR, IL-10 and MMP-9 were determined in M1-like and M2-like differentiated macrophages in duplicate from 6 healthy volunteers. mRNA expression of FR-β, MR, IL-10 and MMP-9 was increased in M2-like macrophages compared to M1-like macrophages (P=0.09, P=0.04, P=0.06 and P=0.004, respectively, figure 5). On the contrary, TNF and TLR-2 mRNA levels were significantly higher in M1-like macrophages compared to those in M2-like macrophages (see figure 6, P=0.002 for both). No significant difference could be found in mRNA levels of IL-6 and TLR-4 between M1-like and M2-like macrophages.

**Figure 5.**

![Figure 5](image)

Figure 5. Relative expression of mRNA levels to GAPDH in 6 healthy volunteers. Quantitative reverse transcription polymerase chain reaction revealed a higher relative expression in M2-like macrophages (black dots) compared to M1-like macrophages (open dots) in (A) FR-β (P=0.09), (B) Mannose receptor (P=0.04), (C) IL-10 (P=0.06) and (D) MMP-9 (P=0.004).
Figure 6. Relative expression of mRNA levels to GAPDH in 6 healthy volunteers measured in duplo showing a higher relative expression in M1-like macrophages (open dots) compared to M2-like macrophages (black dots) in (A) TNF ($P=0.002$) and (B) TLR-2 ($P=0.002$)

**Discussion**

This is the first study to show feasibility using $^{99m}$Tc-folate to image human atherosclerotic carotid plaques ex vivo. Slices with the highest $^{99m}$Tc-folate accumulation showed significantly more FR-β and CD163 expression compared to slices with lowest $^{99m}$Tc-folate accumulation, and significantly less expression of iNOS. Therefore, FR-β positive macrophages were found to be primarily M2-like polarized macrophages, which might be pro-atherogenic, due to production of matrix degrading enzymes.

A number of research groups succeeded in specifying macrophage subsets expressing the folate receptor on tumor-associated M2 macrophages (16), and on M2 macrophages expressed in experimental allergic asthma in mice (17). Furthermore, FR-β targeted immunotoxin treatment resulted in reduction of macrophages and plaque progression (18). However, by our knowledge this is the first study to show an overexpression of FR-β on M2-like macrophages in atherosclerosis. Nevertheless, identification of macrophage subtypes and determination of their role in the atherosclerotic process is still a major challenge.

The M1 and M2 macrophage subtypes are observed in atherosclerotic lesions previously (19,20). Shaikh et al. found a higher proportion of M1 pro-inflammatory macrophages and a reduced proportion of M2-cells in symptomatic carotid lesions (21). The discrepancy
to our study could be due to the fact that the former study compared the ratio of macrophage subtypes in the carotid artery to those in vascular femoral plaques. In an experimental atherosclerotic ApoE KO mice model, lesion progression was correlated with the dominance of M1 (arginase (Arg) II+ over the M2 (Arg I+) macrophage subtype (22). This could be due to the proportion of M1 and M2 macrophages depending on stadium of plaque formation in the APOE model. In recent published studies other M2 markers were found to be related to worse cardiovascular outcome. In sections derived from subjects enrolled in the Cardiovascular Pathology Institute Sudden Coronary Death Registry, stable plaques revealed very few M2-like macrophages (made visible by targeting the Mannose Receptor) compared to unstable plaques where the M2-like macrophage subpopulation was abundant (23). Furthermore, patients with a 2.4 fold increased cardiovascular risk compared to patients with a low ratio, were found to have a high MMP-12/CD68 ratio (24). MMP-12 was found to be correlated with MMP-9 positive macrophage subpopulations in a mouse model for obesity (25). Stöger et al found a correlation between areas of intraplaque hemorrhage and CD163 staining in human atherosclerosis. During development of plaques both M1 and M2 cells accumulated, and the fibrous caps of lesions showed no significant differences between subsets. In contrast, vascular adventitial tissue displayed a pronounced M2 activation profile (26). These different studies support our hypothesis that accumulation of M2-like macrophages might be indicative of plaque vulnerability, which can be imaged by 99mTc-folate imaging.

Visualizing folate receptors has been performed before using optical fluorescent contrast agent (FITC) labeled to folate by our group (7). Other groups also used optical imaging for the identification of tumor processes, as several solid tumors express folate receptor α, demonstrated in ovarian cancer (27) and in head- and neck carcinoma expressing folate receptor-β (28). However, applications for non-invasive optical imaging of fluorescent signals by the use of fluorophores such as FITC could be of less clinical value because of the limited penetration depth of only a few millimeters. This could be extended to a few centimeters using near infrared (NIR) probes, but not ideal for the use in coronary arteries for instance. 99mTc-folate has a good tissue penetration and a relatively short half life. Furthermore its radion labeling procedure is simple and it has not shown toxicity or immunogenicity (8).

For in vivo folate receptor imaging, the SPECT modality could be used; Ayala Lopez et al. tested 99mTc-folate accumulation in ApoE knockout mice on Western chow diet, and showed a significantly greater accumulation in atherosclerotic lesions than mice on normal chow (8). In humans 111In-DTPA-folate and 99mTc-EC20-folate have been tested in clinical trials as radiotracer for imaging of cancer (FR-α) (29,30). Nevertheless, Winkel et al. showed 111In-EC0800 (a radiolabelled folate compound) detects plaque but was not able to differentiate between a stable and a vulnerable atherosclerotic carotid plaque in a shear stress induced ApoE knockout mouse model (31). However, in this model macrophage content is not different between the oscillatory shear stress region (stable plaque) and the lowered shear
stress region (vulnerable plaque) (32). Also PET tracers have been developed recently, of which 3'-Aza-2'-[\(^{18}\)F]fluorofolic acid (33) has been shown to selectively target FR-\(\beta\) positive macrophages in atherosclerotic plaques (34). No determination of differential folate accumulation in the respective M1-like and M2-like subpopulation was performed in this study. [\(^{18}\)F]fluoro-PEG-folate has been used in a rat model of arthritis to image (sub)clinical arthritis (35). For imaging of plaque inflammation 2-deoxy-2-[\(^{18}\)F]fluoro-D-mannose ([\(^{18}\)F]FDM), has been developed recently, supporting a higher accumulation in macrophages compared to [\(^{18}\)F]FDG (23).

A limitation of our study is that division of macrophages solely as M1 or M2 subtypes may be too simple. As Wolfs et al. suggests the role of the local micro-environment makes macrophage polarization in the atherosclerotic tissue more complex than the typically described M1 and M2 macrophages distribution (11). So, not only cytokine environment, but also foam cell formation and CXCL4 (chemokine ligand 4, forming M4 macrophages), among other factors, play major roles. This could be an explanation for the fact that in Figure 2A a CD68+ population of macrophages in the plaque does not stain for the M1/M2 markers or FR-\(\beta\). Further studies to investigate the release of matrix metalloproteinases by macrophage subtypes may give more insight. Also, due to the inclusion of a low number of asymptomatic patients, no analyses between the ratio of M1-like and M2-like macrophages in symptomatic and asymptomatic disease could be made. More research is necessary to explore this issue.

In conclusion, this is the first study showing a positive correlation between accumulation of a \(^{99m}\)Tc-folate compound in atherosclerotic plaques and FR-\(\beta\) expression on M2-like macrophages. Furthermore we characterized macrophage phenotypes \textit{ex vivo} and \textit{in vitro} by a panel of macrophage subtype markers. This not only enables the specific analysis of the proportion of macrophage subtypes within a plaque, but also opens ways to measure M2-like macrophage populations \textit{ex vivo}. More insight in macrophage subtypes and behavior in atherosclerotic plaques may give a better understanding of the pathogenesis related to the vulnerability of atherosclerotic plaques.

\textbf{Acknowledgements}

We thank Fleur Schaper, Gerda Horst and Gerda de Vries for measuring macrophage markers using flow cytometry, Irene Zwarts for measuring macrophage markers using RT-PCR, and Berber Doornbos for performing immunohistochemical stainings.
References
