64Cu-DOTATATE PET/MRI for Detection of Activated Macrophages in Carotid Atherosclerotic Plaques

Studies in Patients Undergoing Endarterectomy

Sune Folke Pedersen, Benjamin Vikjær Sandholt, Sune Høgild Keller, Adam Espe Hansen, Andreas Ettrup Clemmensen, Henrik Sillese, Liselotte Højgaard, Rasmus Sejersten Ripa, Andreas Kjær

Objective—A feature of vulnerable atherosclerotic plaques of the carotid artery is high activity and abundance of lesion macrophages. There is consensus that this is of importance for plaque vulnerability, which may lead to clinical events, such as stroke and transient ischemic attack. We used positron emission tomography (PET) and the novel PET ligand [64Cu] [1,4,7,10-tetraazacyclododecane-N,N',N″,N‴-tetraacetic acid]-d-Phe1,Tyr3-octreotate (64Cu-DOTATATE) to specifically target macrophages via the somatostatin receptor subtype-2 in vivo.

Approach and Results—Ten patients underwent simultaneous PET/MRI to measure 64Cu-DOTATATE uptake in carotid artery plaques before carotid endarterectomy. 64Cu-DOTATATE uptake was significantly higher in symptomatic plaque versus the contralateral carotid artery (P < 0.001). Subsequently, a total of 62 plaque segments were assessed for gene expression of selected markers of plaque vulnerability using real-time quantitative polymerase chain reaction. These results were compared with in vivo 64Cu-DOTATATE uptake calculated as the mean standardized uptake value. Univariate analysis of real-time quantitative polymerase chain reaction and PET showed that cluster of differentiation 163 (CD163) and CD68 gene expression correlated significantly but weakly with mean standardized uptake value in scans performed 85 minutes post injection (P < 0.001 and P = 0.015, respectively). Subsequent multivariate analysis showed that CD163 correlated independently with 64Cu-DOTATATE uptake (P = 0.031) whereas CD68 did not contribute significantly to the final model.

Conclusions—The novel PET tracer 64Cu-DOTATATE accumulates in atherosclerotic plaques of the carotid artery. CD163 gene expression correlated independently with 64Cu-DOTATATE uptake measured by real-time quantitative polymerase chain reaction in the final multivariate model, indicating that 64Cu-DOTATATE PET is detecting alternatively activated macrophages. This association could potentially improve noninvasive identification and characterization of vulnerable plaques.

Key Words: atherosclerosis, endarterectomy, carotid □ magnetic resonance imaging □ positron-emission tomography

Carotid atherosclerosis is a major risk factor of stroke and transient ischemic attack. Randomized trials have shown that carotid endarterectomy significantly reduces the risk of recurrent stroke in patients with recent symptoms of transient ischemic attack or stroke and at least 50% stenosis of the relevant carotid artery.1 However, not all patients will benefit from the surgery because some will have stable plaques that are not prone to cause new thromboembolic lesions. In addition, many patients without significant carotid stenosis will experience recurrent stroke and could have benefited from endarterectomy.

A quest for new and more sensitive methods for in vivo identification of vulnerable atherosclerotic plaques is needed. Positron emission tomography (PET) for molecular imaging, typically in conjunction with anatomic imaging with computed tomography (CT), is one promising hybrid modality. The molecular tracer of choice has to date primarily been 2-[18F]-fluoro-2-deoxy-D-glucose (FDG). FDG is a glucose
enrolled in the study before clinically scheduled endarterectomy. One (symptomatic) plaque was recovered in toto from each patient and sectioned in 3-mm slices. This yielded different slice numbers per patient according to the physical size of each plaque specimen and came to a total of 10 plaques and 62 slices in all. Detailed patient characteristics can be seen in Table 1, and patient scan and surgery information are shown in Table 2.

**Simultaneous PET/MRI of Carotid Atherosclerotic Plaques**

All patients underwent MRI for anatomic evaluation of carotid atherosclerosis simultaneously with collection of $^{64}\text{Cu}$-DOTATATE PET emission data. All 10 patients received an early scan and a total of 7 patients completed both an early and a late scan. Evidence of arterial wall thickening was seen both in the internal as well as the external carotid arteries in T1, T2, and PD-weighted imaging. These findings were matched by a pattern of stenotic lumen on time-of-flight–weighted imaging in the affected arteries (Figure 1). Plaque burden was determined using volumetric analysis: mm$^3$ of index lesions (0.5±0.02; n=67) for all patients on a slice-by-slice basis. No correlation between MRI assessment of plaque burden and $^{64}\text{Cu}$-DOTATATE uptake determined by PET was found in univariate analysis ($P=0.116$).

Examples of in vivo combined $^{64}\text{Cu}$-DOTATATE PET/ MR scans demonstrating tracer uptake in plaques of the internal carotid artery can be seen in Figures 2 and 3A. $^{64}\text{Cu}$-DOTATATE uptake was determined by mean standardized uptake value (SUV$_{\text{mean}}$): early scan (1.18±0.03; n=61) and late scan (0.61±0.02; n=45). SUV$_{\text{mean}}$ obtained early was significantly higher than SUV$_{\text{mean}}$ obtained later, and a slice-by-slice comparison showed a mean difference of 43.9% (95% confidence interval, 40.1%–47.8%; n=45; Figure 3B). Also, we found broad limits of agreement...

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics</th>
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<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Age, y, median (IQR)</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
</tr>
<tr>
<td>BMI, kg/m$^2$, median (IQR)</td>
</tr>
<tr>
<td>CRP, mg/L, median (IQR)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
</tr>
<tr>
<td>Never</td>
</tr>
<tr>
<td>Former</td>
</tr>
<tr>
<td>Current</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
</tr>
<tr>
<td>Cholesterol lowering treatment*, n (%)</td>
</tr>
<tr>
<td>Ischemic heart disease, n (%)</td>
</tr>
<tr>
<td>Symptom, n (%)</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
<tr>
<td>Transient ischemic attack</td>
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</tbody>
</table>

*Only 30% were on statins before their index event. BMI indicates body mass index; CRP, C-reactive protein; and IQR, interquartile range.
between early and late PET scans (from 17.3%–70.6% of the early SUVmean). Finally, the SUVmean values were significantly higher in the index lesion compared with the contralateral carotid artery (13.1% higher in index lesions; \( P<0.001 \), paired \( t \) test). Figure 3A shows representative images of \(^{64}\text{Cu-DOTATATE} \) PET/MR scans of the internal carotid artery from a single patient at 2 different transaxial levels with heterogeneous and no uptake of \(^{64}\text{Cu-DOTATATE} \), respectively. Overall \(^{64}\text{Cu-DOTATATE} \) uptake was heterogeneously distributed throughout the plaques. This finding was corroborated with the recovery of a single plaque that was visualized in toto using a preclinical PET/CT system to show the heterogeneity of tracer distribution ex vivo (Figure 4).

**Macrophenage Detection in Carotid Atherosclerotic Plaques by \(^{64}\text{Cu-DOTATATE} \) PET, Immunohistochemistry, and Gene Expression Analysis**

As for \(^{64}\text{Cu-DOTATATE} \) uptake, gene expression analysis from all slices and all patients demonstrated heterogeneity; note that gene expression data are log2 transformed: mean fold change in gene expression for CD163 showed (3.42±0.2; \( n=61 \)), CD68 showed (3.4±0.2; \( n=61 \)), cathepsin K showed (1.1±0.1; \( n=61 \)), interleukin-18 showed (2.6±0.1; \( n=61 \)), matrix metalloproteinase-9 showed (6.2±0.4; \( n=61 \)), and TNF-\( \alpha \) showed (1.4±0.1; \( n=62 \)). Nonparametric testing (Spearman correlation matrix) demonstrated good interplaque correlation and strong statistical significance between the marker of activated macrophages (CD163) and the other molecular markers of plaque vulnerability (Table 3).

We used a mixed model to take into account the possible nonindependence of slices from the same patient. When entering 1 molecular marker (univariate), we found a weak but highly significant correlation between CD163 expression and \(^{64}\text{Cu-DOTATATE} \) uptake (\( P<0.001 \)), as well as between CD68 expression and \(^{64}\text{Cu-DOTATATE} \) uptake (\( P=0.015 \)). When entering both CD68 and CD163 into the mixed model, only CD163 remained significant (\( P=0.031 \)). cathepsin K, interleukin-18, matrix metalloproteinase-9, and TNF-\( \alpha \) were not significant when entered individually into the mixed model (Table 4). We also calculated TBR values to compensate for background activity and performed the same statistical analyses with an identical outcome (data not shown).

A case study was subjected to immunohistochemical analysis. This confirmed the macrophage presence in the vicinity of and within the lipid core deep in the atheromateous plaque shown by specific CD68 and CD163 staining, which concomitantly colocalized with matrix metalloproteinase-9 expression (Figure 5). Furthermore, the presence of both the inflammatory cytokine interleukin-18 and cathepsin K was detected by immunohistochemistry (Figure 5).

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**Table 2. Patient Scan and Surgery Information**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom onset to surgery, d, median (IQR)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>PET scan to surgery, d, median (IQR)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Injection time to PET scan, min, median (IQR)</td>
<td>85 (16)</td>
</tr>
<tr>
<td>Early scan</td>
<td>299 (130)</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range; and PET, positron emission tomography.

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**Figure 1.** Multisequence MRI of the internal carotid artery at 3 different levels: first column; time-of-flight (TOF), second column; T1-weighted turbo-spin echo (T1), third column; T2-weighted turbo-spin echo (T2); and fourth column; proton density-weighted (PD). **Top,** Most caudal transaxial projection demonstrating A. communis (arrow). **Middle,** Intermediate transaxial projection demonstrating C. interna (arrowheads) and C. externa (open arrows). **Bottom,** Most cranial transaxial projection demonstrating C. interna (arrowheads) and C. externa (open arrows). Note the reduced blood flow on TOF in C. externa (middle) and C. interna (bottom). Plaque buildup can be seen in C. externa in T1, T2, and PD (middle) and in C. interna in T1, T2, and PD (bottom).
In this study, we present the first results from simultaneous PET/MRI of human carotid plaques using the novel somatostatin receptor tracer $^{64}$Cu-DOTATATE. We found a highly heterogeneous intrapatient uptake within each atherosclerotic plaque. Interestingly, this uptake was significantly associated with biomarkers of macrophage load (CD68) and macrophage activation (CD163) in univariate analysis. This association seemed primarily driven by CD163 positive (CD163+) macrophages. This finding could have special interest because CD163+ macrophages are known to have an important role in plaques with hemorrhagic zones. In addition, we found evidence that time from tracer injection to PET acquisition is of paramount importance because tracer accumulation decreases almost 50% from our early scan to our late scan after correction for decay. Finally, we found no correlation between plaque burden and $^{64}$Cu-DOTATATE uptake.

In Vivo Imaging

Only a few studies have used somatostatin receptor imaging in atherosclerosis. Both previous clinical studies used $^{68}$Ga-labeled DOTATATE. A preclinical study demonstrated colocalization of $^{68}$Ga-DOTATATE and macrophage-rich plaques by autoradiography in a mouse model. $^{68}$Ga has the advantage of being generator-eluted and thus has no need for an onsite cyclotron. However $^{68}$Ga has high maximum positron energy of 1.899 MeV which translate into a high-positron range in water: 8.2 mm (maximum) and 2.9 mm (mean) and thus diminished spatial resolution. This is of vital importance for imaging small objects like carotid plaques. To circumvent this, we recently introduced $^{64}$Cu-labeled DOTATATE. $^{64}$Cu is a low-energy positron emitter (maximum positron energy, 0.653 MeV) with a positron range in water of 2.9 mm (maximum) and 0.64 mm (mean) that is comparable with that of $^{18}$F ($\approx$1 mm), the isotope used in $^{18}$F-FDG PET. This is an essential advantage of $^{64}$Cu compared with $^{68}$Ga. In addition, $^{64}$Cu has a half-life of 12.7 hours giving the opportunity for delayed imaging. We found higher $^{64}$Cu-DOTATATE uptake in the index vessel compared with the contralateral carotid artery in our population; however, most included patients also had significant plaques in the contralateral vessel. We therefore suggest that it is reasonable to expect an even higher absolute difference in SUV_{mean} values between atherosclerotic versus healthy carotid arteries. Importantly, we found that plaque burden was not correlated with plaque $^{64}$Cu-DOTATATE uptake, indicating that plaque macrophage activity is not associated with plaque size per se. These results emphasize that PET has a promising role in molecular characterization of vulnerable plaques.

Discussion

In Vivo Imaging

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Plaques by providing in vivo information. We used hybrid PET/MRI instead of PET/CT in our study. MRI has superior soft tissue contrast allowing for better delineation of the carotid artery and atherosclerotic plaque when compared with CT. Finally, our PET/MR system acquires the PET and MR simultaneously allowing for perfect alignment between the 2 sets of images, when compared with the sequential acquisition in PET/CT where minor head movements can cause misalignment.

**CD68, CD163, and TNF-α**

CD68 is a class D scavenger receptor and, although not exclusively expressed by macrophages, it is a widely used macrophage marker, which is why we use it as a surrogate measure of macrophage load. Several previous studies have shown a good correlation between CD68 and uptake of the glucose analogue FDG.[14-16] Because FDG can be labeled with a positron emitter, this compound can be used for in vivo imaging. However, the major drawback of this imaging tracer is the low specificity of FDG limiting its use in assessing vulnerability of plaques.

CD163 is a hemoglobin scavenger receptor and macrophage-specific protein. It is upregulated in a subpopulation of alternatively activated M2 macrophages called hemorrhage-associated macrophages that are found in hemorrhagic zones of atherosclerotic plaques.[10,17] A crucial role of hemorrhage-associated macrophages is to clear hemoglobin–haptoglobin complexes directly via the CD163 receptor and reduce oxidative stress, which subsequently mediates anti-inflammatory properties in vulnerable atherosclerotic plaques.[10,18,19] Macrophages expressing CD163 have been detected in atherosclerotic plaques,[20] and the soluble form of CD163 is associated with coronary atherosclerotic burden in the general population.[21] The association between expression of CD163 and in vivo imaging of atherosclerotic plaques has not been investigated previously. However, a study of HIV infected patients found a correlation between plasma concentration of soluble CD163 and FDG uptake in the ascending aorta.[22]

Interestingly, a recent study into M1/M2 polarization of macrophage phenotypes in carotid plaques found that M2 macrophages are present in both symptomatic and asymptomatic plaques, whereas M1 macrophages are exclusive to symptomatic plaques.[23] TNF-α is a cytokine involved in systemic inflammation and an important part of the acute phase reaction. It is produced primarily by activated macrophages in inflammation and considered a principal marker of M1 activation.[24] Little recent work has been done on TNF-α expression in atherosclerotic lesions in humans; however, 1 investigation reported markedly raised (but nonsignificant) TNF-α RNA levels in symptomatic human endarterectomy specimens,[24] and its role in systemic inflammatory disease is inarguable.[25,26]

**Table 3.** Spearman Correlation Coefficient Matrix of Gene Expression in Matched Plaque Slices

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD68</th>
<th>CTSK</th>
<th>IL-18</th>
<th>MMP9</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>0.760</td>
<td>0.517</td>
<td>0.536</td>
<td>0.750</td>
<td>0.511</td>
</tr>
<tr>
<td>P (2-tailed)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Interplaque correlation of gene expression on a slice-by-slice basis after logarithmic transformation (n=61). CD68 indicates cluster of differentiation 68; CD163, cluster of differentiation 163; CTSK, cathepsin K; IL, interleukin; MMP9, matrix metalloproteinase-9; and TNF-α, tumor necrosis factor-α.

**Table 4.** Results of the Linear Mixed Model (AR1) Estimates of Fixed Effects; Univariate and Multivariate Analyses With Mutual Adjustment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>p-value</th>
<th>Estimate</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>log(CD68)</td>
<td>0.056</td>
<td>0.011</td>
<td>0.100</td>
<td>0.014</td>
<td>-0.023</td>
<td>-0.119</td>
<td>0.058</td>
<td>0.503</td>
</tr>
<tr>
<td>log(CD163)</td>
<td>0.076</td>
<td>0.031</td>
<td>0.121</td>
<td>0.001</td>
<td>0.103</td>
<td>0.001</td>
<td>0.197</td>
<td>0.031</td>
</tr>
<tr>
<td>log(MMP9)</td>
<td>0.010</td>
<td>-0.001</td>
<td>0.031</td>
<td>0.308</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>log(IL-18)</td>
<td>0.023</td>
<td>-0.019</td>
<td>0.065</td>
<td>0.279</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>log(CTSK)</td>
<td>0.021</td>
<td>-0.043</td>
<td>0.085</td>
<td>0.517</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>log(TNF-α)</td>
<td>0.022</td>
<td>-0.056</td>
<td>0.100</td>
<td>0.571</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

CD68 indicates cluster of differentiation 68; CD163, cluster of differentiation 163; CTSK, cathepsin K; IL, interleukin; MMP9, matrix metalloproteinase-9; and TNF-α, tumor necrosis factor-α.
Molecular markers of plaque vulnerability (cathepsin K, matrix metalloproteinase-9, and interleukin-18) that were previously found to be associated with FDG-uptake were not associated with 64Cu-DOTATATE uptake, highlighting how the 2 tracers image different biological processes using PET. Our finding substantiate that 64Cu-DOTATATE is correlated primarily with CD163 positive macrophages (CD163+) and only weaker with CD68 positive macrophages (CD68+) making 64Cu-DOTATATE uptake a predictor of hemorrhage-associated macrophages (CD163+) of the hemorrhage-associated phenotype in atherosclerosis needs further elucidation, and future studies will have to show whether 64Cu-DOTATATE has higher specificity than 18F-FDG for imaging vulnerability of plaques.

**Time of Imaging**

We found only a moderate correlation between tracer accumulation at the early versus the later scan, and the significant association between tracer uptake and macrophage markers (CD68 and CD163) disappeared at the late time scan. In every single case, the SUV value decreased from early to late imaging indicating that binding to somatostatin receptor subtype-2 decreases over time. This is in contrast to PET imaging of atherosclerosis using 18F-FDG where imaging should be postponed to 3 hours after injection because FDG uptake in the plaques is either stable or increases, whereas FDG is cleared from the blood leading to improved target-to-background ratios. As FDG, in contrast to 64Cu-DOTATATE, is not bound to membrane-bound receptors but is continuously taken up and trapped by the cells, this discrepancy in optimal timing of imaging is not surprising. It could be speculated that instability of the 64Cu–DOTA complex in vivo and thereby increase in nonspecific background could be an explanation for the lack of correlation at the late time-point. However, because there was no increase in background activity observed, this explanation seems unlikely. Also, we recently published the first clinical PET study using the tracer 64Cu-DOTATATE in patients with neuroendocrine tumor. Here, we scanned patients 1, 3, and 24 hours post injection and did not see large liver accumulation or increase in blood-borne activity indicating that 64Cu-DOTATATE is indeed stable in humans for a long time. Also, as part of the approval by the Danish Health Authorities of 64Cu-DOTATATE, extensive stability studies were undertaken, demonstrating a shelf-life of at least 24 hours. Taken together, we find it most likely that decreasing signal-to-noise ratio over time explained by decrease in signal from receptor binding fully explains why early scans are superior. Based on our results, we...
therefore suggest that PET imaging of atherosclerosis with 64Cu-DOTATATE should be performed early, for example, at 80 minutes after injection.

Limitations
Although our imaging method using 64Cu-DOTATATE seems promising, it is inherently challenged by low tracer uptake and a poor spatial resolution that could limit the usefulness. Nevertheless, the spatial resolution may not be a major obstacle, as it is at the same level as for FDG-PET, which increasingly seems to establish itself as an accepted method for plaque evaluation. However, only large clinical studies, ideally with head-to-head comparison with other methods, can establish the true clinical value of our method and its relation to other methods, for example, FDG-PET.

Conclusions
In conclusion, we demonstrate the uptake of a novel PET tracer, 64Cu-DOTATATE, in human atherosclerotic plaques. We found a correlation between in vivo tracer uptake and ex vivo markers of activated macrophages. This association could potentially improve noninvasive identification of vulnerable plaques.

Acknowledgments
The expertise and technical support of principal researchers Karin Stahr and Jakup Poulsen with positron emission tomography (PET)/MRI procedures and PET reconstructions are highly valued as is the expert statistical advice by Julie Lyng Forman.

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Disclosures
None

References
Significance

Macrophages express the somatostatin receptor subtype-2. By targeting this receptor using the ligand [64Cu][1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid]-d-Phe1,Tyr3-octreotate ($^{64}$Cu-DOTATATE), macrophages can be imaged noninvasively using positron emission tomography (PET). We hypothesized that in vivo $^{64}$Cu-DOTATATE uptake quantified by PET would correlate with ex vivo markers of macrophage infiltration. Atherosclerotic plaques of the internal carotid artery were imaged using $^{64}$Cu-DOTATATE PET. Subsequently, plaques recovered by carotid endarterectomy were analyzed using real-time quantitative polymerase chain reaction to assess molecular markers of macrophage infiltration and activity. We found a correlation between $^{64}$Cu-DOTATATE PET and gene expression of the hemoglobin/haptoglobin scavenger receptor CD163 by macrophages, also considered a marker of macrophage activation. Our work thus demonstrates that $^{64}$Cu-DOTATATE PET can be used to detect a hemorrhage-associated subpopulation of macrophages (CD163+) in vivo in atherosclerotic plaques of the internal carotid arteries. This finding has potential clinical implications for noninvasive identification of vulnerable plaques.
Andreas Kjær

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Supplemental material

Study design
All patients referred to our institution for carotid endarterectomy were screened for inclusion. Inclusion criteria were significant carotid stenosis with recent cerebrovascular symptoms; stroke, amaurosis fugax or TIA. Exclusion criteria were age <50 years, current infection, pregnancy, renal insufficiency, or severe claustrophobia. All patients underwent two simultaneous PET/MR procedures after one injection of a mean of 154 MBq of $^{64}$Cu-DOTATATE. An early PET/MR was performed a median of 85 minutes (range: 56-90 min) after injection and the late PET/MR was performed a median of 299 minutes (range: 265-428 min) after injection. Carotid endarterectomy was performed the following day using standard clinical procedure. The plaque specimens were conserved for later gene expression analysis. The study was approved by the Regional Scientific Ethical Committee (protocol H-4-2012-064) and all patients received oral and written information about the study and signed an informed consent prior to inclusion.

In-vivo PET/MR acquisition
Simultaneous PET/MR was performed on the Biograph mMR¹ (Siemens Healthcare, Erlangen, Germany) using the mMR standard PET compatible dedicated head and neck coil (Siemens Healthcare). Patients were placed in the scanner in the supine position and scout images were obtained for localization of the carotid arteries. An mMR standard Dixon water-fat MR sequence was then recorded and segmented (into air, lung tissue, soft tissue and fat) for MR-based PET attenuation correction². PET acquisition was started along with the Dixon MR sequence. Acquisition time was 10 min in three-dimensional list-mode (scanner default list mode recording) in one bed position. A 3D time-of-flight (TOF) magnetic resonance angiography sequence was then performed to obtain lumen contours. MR parameters for 3D-TOF were: TR/TE=21/3.6 ms, voxel size 0.8x0.5x1.0 mm³. Next, a stack of 14 axial 2D turbo spin echo dark blood slices (thickness 2.2 mm, gap 0.88 mm, field of view 150 mm) with fat suppression and proton density, T1 and T2 weightings was placed using the 3D-TOF angiography. Images were centered at the stenotic area. MR parameters were: T1-weighted: TR/TE=1,060/26 ms, voxel size 0.6x0.6 mm². T2-weighted: TR/TE=2,500/76 ms, voxel size 0.6x0.6 mm². Proton density-weighted: TR/TE=2,400/16 ms, voxel size 0.4x0.4 mm². For detailed MR parameters of 3D TOF, T1, T2 and proton density-weighted sequences see supplementary material. On average, the total examination time was below 30 minutes.

MR quantification
The carotid artery was analyzed slice by slice on transaxial images in every subject guided by T1, T2, TOF and PD images by the same operator. Free-hand regions of interest were drawn to include vessel wall, plaque and lumen. Next vessel lumen was determined in an identical manner and finally the two volumes were subtracted to give a measure of plaque burden (mm³) on a slice by slice basis.

PET image reconstruction
The in vivo PET data was reconstructed using ordered-subsets expectation maximization iterative 3-dimensional reconstruction (OSEM 3D). The reconstruction was done using 6 iterations, 21 subsets and zoom = 2.0 with MR based attenuation correction yielding 127 image slices each 512x512 voxels of size: 0.70x0.70x2.03mm³ which were filtered post-reconstruction with a 2 mm full width half maximum Gaussian filter. The µ-maps for attenuation correction of the PET data were generated on the basis of the Dixon water-fat MR sequence² which is the standard MR attenuation correction for the Biograph mMR scanner. The segmentation and generation of the µ-maps from the Dixon sequence correction was done fully automatically by the scanner software.
**PET quantification**

Anatomical co-registration of MR and PET was carefully checked by matching anatomical landmarks like salivary glands. The carotid artery was analyzed slice by slice in every subject. The PET data was fused with the simultaneously acquired axial MR images (T1, T2, and proton density weighted). A free-hand region of interest (ROI) was drawn to include plaque, vessel wall and lumen starting at the carotid bifurcation proceeding caudally and cranially (not including the external carotid artery) to include the entire plaque. The mean standardized uptake value (SUV$_{\text{mean}}$) corrected for injected dose, patient weight and time to acquisition were calculated for each region of interest. For calculation of target-to-background (TBR) values the, SUV$_{\text{mean}}$ for each slice was normalized to blood pool activity (SUV$_{\text{mean}}$) obtained from a ROI placed in the ipsilateral jugular vein resulting in TBR values for subsequent calculations.

**Ex vivo PET/CT**

For the *ex vivo* acquisition, one extirpated plaque underwent PET/CT imaging 23 hours after injection of $^{64}\text{Cu}$-DOTATATE using a Siemens Inveon MM PET/CT small animal scanner (Siemens Molecular Imaging, Knoxville, USA). The specimen was PET-scanned for 90 minutes with a 350-650 keV energy window and 3,432 ns coincidence window. The PET list mode data was reconstructed using an OP-MAP iterative algorithm, applying 18 iterations and 2 subsets, with a requested resolution of 1.0 mm. The CT scan was done using x-ray voltage and current of 50 kVp and 500 mA, respectively. Projections were acquired over 360 degrees using 720 steps, with an exposure time of 2 seconds and 1 second of settle time. The CCD detector was using 4096 by 4032 pixels and a binning factor of 2. The CT projections were reconstructed into an image using filtered back-projection resulting in an isotropic voxel size of 21.7 µm. The images were co-registered and processed using Siemens Inveon Research Workplace version 4.1 (Siemens Molecular Imaging, Knoxville, USA).

**Tissue RNA extraction**

Tissue conservation and cDNA production for real-time qPCR analysis was performed as earlier described$^{3-5}$. Briefly; plaque tissue secured by carotid endarterectomy was sliced immediately at bedside in the operating theater into 3 mm slices caudally (common carotid artery) and cranially (internal carotid artery) oriented from the vessel bifurcation. A custom made knife with blades spaced evenly at 3 mm intervals was used to ensure consistency of slice thickness. The slices corresponded to the PET/MR imaging modality and were immediately conserved in RNAlater® (cat. # AM7021, Ambion (Europe) Limited, Cambridgeshire, United Kingdom) in 1.5 mL PCR grade Eppendorf tubes for subsequent RNA extraction (n=61 slices). One plaque was conserved *in toto* for *ex vivo* scanning prior to slicing and RNA extraction. Additionally a piece of superior thyroid artery was secured as reference tissue from every patient. Subsequently tissue was stored at 5°C for 24 hours before RNAlater® was drained and the tissue transferred to -80°C storage.

RNA extraction was performed in a specially assigned RNA laboratory and procedures were executed in a fume cupboard. Each tissue slice and reference tissue was freeze fractured *in toto* using a cryoPREP™ impactor (Covaris Europe, Brighton, UK) and TRI Reagent® (cat. # TR118, Molecular Research Center Inc., Cincinnati, USA) was used for RNA isolation in a protocol modified from the manufacturer’s instructions to accommodate freeze fracture: Each sample was transferred from Eppendorf tubes into PCR grade tissue bags (cat. # TT1XT, Covaris Inc., Europe, Brighton, UK) cooled in liquid Nitrogen and freeze fractured using the impactor. The pulverized sample was transferred to fresh 1.5 mL PCR grade Eppendorf tubes and 1000 µL TRI Reagent® added along with 100 µL of BCP (cat. # BP151, Molecular Research Center Inc., Cincinnati, USA). The samples were stirred vigorously using a vortex mixer for 15 seconds and left to incubate at room temperature for 15 minutes. Subsequently the samples were centrifuged at 12,000 g for 15 minutes at 4°C leaving an upper aqueous phase containing RNA, an interphase containing DNA and a lower (red) phase containing protein. The upper phase (450 µL) was carefully transferred to a fresh 1.5 mL Eppendorf tube and an equal volume (450 µL) of isopropanol was added for RNA
precipitation. The samples were mixed gently by hand and left to incubate for 10 minutes at room temperature. Centrifugation at 12,000 g at 4°C for 8 minutes was followed by careful removal of the supernatant leaving an RNA pellet. The pellet was then washed 2 times using 1 mL of RNase-free 75% ethanol alternating with centrifugation at 7,500 g at 4°C for 5 minutes before a final centrifugation at 13,000 g at 4°C for another 5 minutes where the remaining supernatant could be removed and pellet left to air dry for a minimum of 20 minutes. Finally 20 µL of molecular grade water (cat. # 2900136, 5-Prime GmbH, Hamburg, Germany) was added and the RNA reconstituted by vigorous mixing for 15 seconds using a vortex mixer. The RNA was stored at -80°C until further processing.

**RNA quality and concentration**

The RNA quality was assessed using the Agilent 2100 Bioanalyzer and associated reagents (cat. # G2938-80023, Agilent Technologies Inc., Santa Clara, CA, USA) to assign RNA integrity values (RINs). The system utilizes chip-based microfluidic technology providing electrophoretic separation in an automated and reproducible manner of up to 12 samples pr chip. RNA samples (1 µL pr sample) were separated in the channels of the chip according to molecular weight and detected by laser induced fluorescence detection of an added dye. Electropherograms for each sample was constructed for visualization of RNA quality. The “Agilent RNA 6000 Nano Assay Protocol – Edition April 2007” was used for preparing and assigning RIN values to all samples. In preparation for analysis 2 µL of samples and 1.2 µL of ladder (cat. # 5067-1529, Agilent Technologies Inc., Santa Clara, CA, USA) were denatured at 70°C for 2 minutes.

Briefly: RNA 6000 Nano gel matrix (550 µL) was pipetted into a spin filter and centrifuged at 1500 g for 10 minutes at room temperature and divided into 65 µL aliquots. Concentrated RNA 6000 Nano dye was stirred for 10 seconds and 1 µL added to the 65 µL of the RNA 6000 Nano gel matrix. The gel-dye mix was then vigorously stirred using a vortex mixer and subsequently centrifuged at 13,000 g for 10 minutes at room temperature. A 6000 Nano chip (cat. # 5067-1511, Agilent Technologies Inc., Santa Clara, CA, USA) for determination of total eukaryotic RNA was placed in the chip priming station and loaded with gel-dye mix for priming of the chip using the plunger system of the priming station. Once primed, the sample and ladder wells were all loaded with 5 µL of RNA 6000 Nano Marker whereas 1 µL of sample and 1 µL of ladder was added to the wells marked “sample” and “ladder” respectively. The chip was stirred in a custom vortexer for 1 minute at 2400 rpm and the analyses were performed on the Bioanalyzer immediately thereafter.

RNA concentrations from the isolation procedure were measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) prior to dilution for the cDNA synthesis step.

**cDNA synthesis**

Using the AffinityScript™ QPCR cDNA Synthesis Kit (cat. # 600559 Stratagene, La Jolla, CA, USA), 0.3 ng of total RNA was reverse transcribed to cDNA for subsequent real-time qPCR analysis of whole-slice gene expression. In short: A mixture containing 7 µL (0.3 ng) RNA, 10 µL first strand master mix (2x), 2.45 µL Oligo (dT) primer (100 ng/µL), 0.55 µl random primer (100 ng/µL) and 1 µL of Stratascript™ RT/RNase Block Enzyme Mixture were prepared and run on a Mastercycler gradient RT-PCR machine (Eppendorf AG, Hamburg, Germany) utilizing a program of 5 minutes of primer annealing at 25 °C, 15 minutes of cDNA synthesis at 42 °C and finally termination for 5 minutes at 95 °C. Subsequently cDNA was stored at -20 °C until qPCR analysis.

**Quantitative real-time PCR**

Optimal housekeeping genes were identified as described previously. Briefly; we tested both reference and diseased tissue, using a commercially available panel of 12 housekeeping genes (cat. # A101, Version 1.3 TATAA Biocenter AB, Göteborg Sweden) and found 60S acidic ribosomal protein P0 (RPLP, gene ID: NM_001002) and peptidylprolyl isomerase A (PPIA, gene ID:...
NM_021130) to be optimal using the geNorm algorithm embedded in the qBasePLUS software (Biogazelle NV, Zwijnaarde, Belgium). Taqman® based gene expression assays were designed using Beacon Designer™ 7.90 (PREMIER Biosoft, Palo Alto, CA, USA) and analyzed using Mx3000P® or Mx3005P™ real-time PCR systems (Stratagene, La Jolla, CA, USA). Five genes of interest; cluster of differentiation 68 (CD68, gene ID: NM_001251) cluster of differentiation 163 (CD163, gene ID: NM_004244), matrix metalloproteinase 9 (MMP9, gene ID: NM_004994), interleukin 18 (IL-18, gene ID: NM_001562), cathepsin K (CTSK, gene ID: NM_000396) and tumor necrosis factor α (TNFα, gene ID: NM_000594.3) were analyzed. The thermal profile of the TaqMan® assays consisted of an initial denaturation step at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and finally combined annealing and elongation for one minute at 60°C. Gene expression data analysis was performed in qBasePLUS incorporating a modified delta-delta-Cq model (2^\Delta\DeltaCq) and multiple reference genes. Inter-run calibration (three different samples replicated on every plate in each run) was used to eliminate plate-to-plate variation between runs. For assay and analysis details, see Tables I and II. All primers and Taqman® probes were purchased at Sigma (Sigma-Aldrich, St. Louis, MO, USA).

**Histology**

Recovered carotid plaque specimens were fixed in 10% neutrally buffered formalin for 24 hours and subsequently processed and paraffin embedded. A representative plaque was sectioned (4 µm), transferred to slides, deparaffinized and stained with Mayer’s hematoxylin and eosin (cat. # 860213 and 854653, Region H Apothecary, Copenhagen, Denmark) before being dehydrated using ethanol and finally mounted with pertex.

**Immunohistochemistry**

Immunohistochemistry was performed to assess markers of vulnerability using mouse monoclonal antibodies against macrophages (CD68; dilution 1:100, clone PG-M1, Dako, Glostrup, Denmark [cat. # M087629-2] and CD163; dilution 1:100, clone EDHu-1, AbD Serotec, Kidlington, UK [cat. # MCA1853T]), a cytokine (IL-18; dilution 1:75, clone H44, abcam®, Cambridge, UK [cat. # ab140834]) and both monoclonal mouse and rabbit antibodies against markers of proteolysis (CTSK; dilution 1:150, clone ab66237, abcam®, Cambridge, UK [cat. # ab66237] and MMP9; dilution 1:150, clone EP1255Y, abcam®, Cambridge, UK [cat. # ab137867] respectively). Briefly: 4 µm sections was mounted on glass slides and placed at 40°C overnight. Next day, the temperature was increased for one hour to 60°C and the slides deparaffinized in shifts of xylene for 15 minutes followed by multiple shifts of ethanol in decreasing concentrations advancing the sections to demineralized water. Epitope demasking was performed using heat-induced epitope retrieval (HIER) in citrate buffer pH 6.0 for 15 minutes in a microwave oven followed by 30 minutes rest at room temperature and then immersion in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (cat. # P1379-25mL, Sigma-Aldrich, St. Louis, MO, USA) for five minutes. Subsequent reactions all took place at room temperature. After 10 minutes of immersion in PBS, the slides were transferred to humidity chambers where each section was covered with peroxidase blocker (cat.# S2023, Dako, Glostrup, Denmark) for eight minutes and then rinsed with three shifts of PBS every two minutes. The sections were blocked using 2% bovine serum albumin (cat. # A7906-100g, Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes followed by primary antibody diluted in 2% bovine serum albumin for samples and positive control tissue, whereas species-matched FLEX control (cat. # IS600 [rabbit] / IS750 [mouse], Dako, Glostrup, Denmark) was added to negative control samples. The samples were then incubated for one hour followed by rinse in three shifts of PBS every two minutes. Species matched secondary horse-radish peroxidase conjugated antibody was now added to the samples; EnVision FLEX (cat. # K4003 [rabbit] / K4001 [mouse], Dako, Glostrup, Denmark) which were left to incubate for another 40 minutes followed by rinse in two shifts of PBS for five minutes. The samples were developed using 3,3’-Diaminobenzidine (DAB) for 10 minutes (cat. # K3468, Dako, Glostrup, Denmark) followed by rinse in three shifts of PBS. The samples were transferred to soak in demineralized water for five minutes, counterstained by two
minutes of Mayer’s hematoxylin, soaked for five minutes in demineralized water, before being dehydrated in ethanol and finally mounted using pertex.

Slides were scanned using an Axio Scan.Z1 slide scanner (Carl Zeiss Microscopy GmbH, Jena, Germany) and subsequent image preparation was performed using a combination of Photoshop CS6 (Adobe Systems, San Jose, CA, USA) and Microsoft Powerpoint 2010 (Microsoft Corporation, Redmond, WA, USA).

**Statistical analysis**

Data was analyzed using a linear mixed model adjusting for serial correlation (AR1) predefined in repeated measurements from the same patient. Effects of explanatory variables (CD163, CD68, CTSK, IL-18, MMP9 and TNFα) were tested in univariate analyses. Significant explanatory variables (CD163, CD68) were thereafter included in a multivariate analysis. Gene expression and MR data are presented as mean ± SEM. Analyses were performed using SPSS 20 (IBM Corporation, Armonk, New York, USA) while Graphpad Prism 5 (Graphpad software Inc, La Jolla, CA, US) was used for illustrations. Normal distribution assumptions were tested using the Kolmogorov-Smirnov test. Furthermore in vivo PET data and gene expression analyses were compared using the Spearman correlation coefficient as appropriate. Early and late PET data were compared using paired samples t-test and Bland Altman statistics. Logarithmic transformation (log_2) of the gene expression data was applied to obtain normal distribution. P<0.05 was considered statistically significant.
References


3D-TOF:SIEMENS: fl_tof

**Routine**
Slab group 1
Slabs 2
Dist. factor -19.23 %
Position L0.7 A26.1 F0.1
Orientation Transversal
Phase enc. dir. R >> L
Rotation 90.00 deg
Phase oversampling 0 %
Slice oversampling 23.1 %
Slices Per Slab 52
FoV read 200 mm
FoV phase 75.0 %
Slice thickness 1.00 mm
TR 21 ms
TE 3.60 ms
Averages 1
Concatenations 2
Filter Distortion Corr.(2D), Elliptical filter
Coil elements HEA;HEP;NEA;NEP

**Contrast**
TD 0.000 ms
MTC Off
Flip Angle 25 deg
Fat suppr. None
Water suppr. None

Averaging mode Short term
Reconstruction Magnitude Measurements 1

**Resolution**
Base resolution 384
Phase resolution 65 %
Slice resolution 63 %
Phase partial Fourier Off
Slice partial Fourier 6/8
Interpolation Off

PAT mode GRAPPA
Acce. factor PE 2
Ref. lines PE 24
Acce. factor 3D 1
Matrix Coil Mode Auto (Triple)
Reference scan mode Integrated

Image Filter Off
Distortion Corr. On
Mode 2D
Unfiltered images On
Prescan Normalize Off
Normalize Off
B1 filter Off
Raw filter Off
Elliptical filter On
Mode Inplane
POCS Off
**Geometry**
Multi-slice mode Sequential
Series Descending

---

Special sat. Tracking H
Gap 10 mm
Thickness 40 mm

---

Set-n-Go Protocol Off
Table Position H
Table Position 0 mm
Inline Composing Off

**Sequence**
Introduction On
Dimension 3D
Elliptical scanning Off
Asymmetric echo Allowed
Contrasts 1
Bandwidth 250 Hz/Px
Flow comp. Yes

---

Gradient mode Fast
RF spoiling On
651/+
T1-weighted:

SIEMENS: tse
Routine
Slice group 1
Slices 14
Dist. factor 40 %
Position Isocenter
Orientation Transversal
Phase enc. dir. R >> L
Rotation 90.00 deg
Phase oversampling 0 %
FoV read 150 mm
FoV phase 100.0 %
Slice thickness 2.2 mm
TR 1060 ms
TE 26 ms
Averages 2
Concatenations 2
Filter Distortion Corr.(2D), Elliptical filter
Coil elements HEA;HEP;NEA;NEP
Contrast
TD 0.0 ms
MTC Off
Magn. preparation None
Flip Angle 180 deg
Fat suppr. SPAIR
Fat sat. mode Strong
Water suppr. None
Restore magn. Off

Averaging mode Short term
Reconstruction Magnitude
Measurements 1
Multiple series Each measurement
Resolution
Base resolution 256
Phase resolution 100 %
Phase partial Fourier Off
Trajectory Cartesian
Interpolation Off

PAT mode None
Matrix Coil Mode Auto (CP)

Image Filter Off
Distortion Corr. On
Mode 2D
Unfiltered images On
Prescan Normalize Off
Normalize Off
B1 filter Off
Raw filter Off
Elliptical filter On
Mode Inplane

**Geometry**
Multi-slice mode Interleaved
Series Interleaved

---

Special sat. Parallel F/H
Gap 10 mm
Thickness 50 mm

---

Set-n-Go Protocol Off
Table Position H
Table Position 0 mm
Inline Composing Off

---

Tim CT mode Off

**Sequence**
Introduction Off
Dimension 2D
Compensate T2 decay Off
Reduce Motion Sens. Off
Contrasts 1
Bandwidth 130 Hz/Px
Flow comp. No
Allowed delay 5 s
Echo spacing 13.1 ms

---

Define Turbo factor
Turbo factor 7
Echo trains per slice 37
RF pulse type Normal
Gradient mode Fast
T2-weighted:

SIEMENS: tse

Routine
Slice group 1
Slices 14
Dist. factor 40 %
Position Isocenter
Orientation Transversal
Phase enc. dir. R >> L
Rotation 90.00 deg
Phase oversampling 0 %
FoV read 150 mm
FoV phase 100.0 %
Slice thickness 2.2 mm
TR 2500 ms
TE 76 ms
Averages 2
Concatenations 2
Filter Distortion Corr.(2D), Elliptical filter
Coil elements HEA;HEP;NEA;NEP

Contrast
TD 0.0 ms
MTC Off
Magn. preparation None
Flip Angle 120 deg
Fat suppr. SPAIR
Fat sat. mode Strong
Water suppr. None
Restore magn. Off

Averaging mode Long term
Reconstruction Magnitude
Measurements 1
Multiple series Each measurement

Resolution
Base resolution 256
Phase resolution 100 %
Phase partial Fourier Off
Trajectory Cartesian
Interpolation Off

PAT mode None
Matrix Coil Mode Auto (CP)

Image Filter Off
Distortion Corr. On
Mode 2D
Unfiltered images On
Prescan Normalize Off
Normalize Off
B1 filter Off
Raw filter Off
Elliptical filter On
Mode Inplane

**Geometry**
Multi-slice mode Interleaved
Series Interleaved

----------

Special sat. Parallel F/H
Gap 10 mm
Thickness 50 mm

----------

Set-n-Go Protocol Off
Table Position H
Table Position 0 mm
Inline Composing Off

----------

Tim CT mode Off

**Sequence**
Introduction Off
Dimension 2D
Compensate T2 decay Off
Reduce Motion Sens. Off
Contrasts 1
Bandwidth 133 Hz/Px
Flow comp. No
Allowed delay 5 s
Echo spacing 15.1 ms

----------

Define Turbo factor
Turbo factor 15
Echo trains per slice 18
RF pulse type Normal
Gradient mode Fast
Proton density-weighted:

SIEMENS: tse

**Routine**
Slice group 1
Slices 14
Dist. factor 40 %
Position Isocenter
Orientation Transversal
Phase enc. dir. R >> L
Rotation 90.00 deg
Phase oversampling 0 %
FoV read 150 mm
FoV phase 100.0 %
Slice thickness 2.2 mm
TR 2400 ms
TE 16 ms
Averages 2
Concatenations 2
Filter Distortion Corr.(2D), Elliptical filter
Coil elements HEA;HEP;NEA;NEP

**Contrast**
TD 0.0 ms
MTC Off
Magn. preparation None
Flip Angle 120 deg
Fat suppr. SPAIR
Fat sat. mode Strong
Water suppr. None
Restore magn. Off

Averaging mode Long term
Reconstruction Magnitude
Measurements 1
Multiple series Each measurement

**Resolution**
Base resolution 384
Phase resolution 100 %
Phase partial Fourier Off
Trajectory Cartesian
Interpolation Off

PAT mode None
Matrix Coil Mode Auto (CP)

Image Filter Off
Distortion Corr. On
Mode 2D
Unfiltered images On
Prescan Normalize Off
Normalize Off
B1 filter Off
Raw filter Off
Elliptical filter On
Mode Inplane

**Geometry**
Multi-slice mode Interleaved
Series Interleaved

----------------------------------------

Special sat. Parallel F/H
Gap 10 mm
Thickness 50 mm

----------------------------------------

Set-n-Go Protocol Off
Table Position H
Table Position 0 mm
Inline Composing Off

----------------------------------------

Tim CT mode Off

**Sequence**
Introduction Off
Dimension 2D
Compensate T2 decay Off
Reduce Motion Sens. Off
Contrasts 1
Bandwidth 133 Hz/Px
Flow comp. No
Allowed delay 30 s
Echo spacing 16.1 ms

----------------------------------------

Define Turbo factor
Turbo factor 15
Echo trains per slice 26
RF pulse type Normal
Gradient mode Fast
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<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>5' fluorophore</th>
<th>Probe (5'-3')</th>
<th>3' quencher</th>
<th>Amplicon length (bp)</th>
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<tbody>
<tr>
<td>CD68</td>
<td>GGTGGAGTACAATGTGTC</td>
<td>GCTGGTGAAAGAATGATG</td>
<td>FAM</td>
<td>ACGCAGCAGATGGAGATC</td>
<td>BHQ1</td>
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<tr>
<td>IL-18</td>
<td>GCCCTCTATTGAAGATATGAC</td>
<td>CACAGGAGAGGTGAAAA</td>
<td>Cy5</td>
<td>TTCACAGAGATAGTTACATGC</td>
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<td>CD163</td>
<td>CCTGTGTACTATGAAGAAAA</td>
<td>CAGAGAGAGTCCGAATC</td>
<td>HEX</td>
<td>TCACATGCTTCATTCAACAG</td>
<td>BHQ1</td>
<td>146</td>
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<tr>
<td>MMP9</td>
<td>TCCAGTACGAGAAGAAGAG</td>
<td>CAGATGGCTCATAGTGCAC</td>
<td>FAM</td>
<td>CTGCCAGGAGGCTTACT</td>
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<td>113</td>
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<tr>
<td>CTSK</td>
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<td>GGTGTCAGTACATCCTC</td>
<td>Cy5</td>
<td>TTCACAGAGATAGTTACATGC</td>
<td>BHQ2</td>
<td>100</td>
</tr>
<tr>
<td>PPIA</td>
<td>GGGTGTTCACAGATCTTTG</td>
<td>ACCTCTAAATGAGATAGAACC</td>
<td>FAM</td>
<td>AACCTCAATTTGGCTCCTGCTT</td>
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<td>Cy5</td>
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<td>TNFα</td>
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<td>FAM</td>
<td>TGGCCCAATCCCTTTATTACCC</td>
<td>BHQ1</td>
<td>150</td>
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</table>

BHQ = black hole quencher; CD68 = cluster of differentiation 68; CD163 = cluster of differentiation 163; CTSK = cathepsin K; Cy5 = cyanine; FAM = Fluorescein; HEX = hexachlorofluorescein; IL-18 = interleukin 18; MMP9 = matrix metalloproteinase 9; PPIA = peptidylprolyl isomerase A; RPLP = ribosomal protein large P; TNFα = tumor necrosis factor α.
<table>
<thead>
<tr>
<th>Gene</th>
<th>FP concentration (nM)</th>
<th>RP concentration (nM)</th>
<th>Probe concentration (nM)</th>
<th>Filter settings</th>
</tr>
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<td>CD68</td>
<td>300</td>
<td>300</td>
<td>150</td>
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<td>IL-18</td>
<td>300</td>
<td>600</td>
<td>200</td>
<td>Cy5 x 2</td>
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<tr>
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<td>300</td>
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<tr>
<td>TNFα</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>FAM x 2</td>
</tr>
</tbody>
</table>

CD68 = cluster of differentiation 68; CD163 = cluster of differentiation 163; CTSK = cathepsin K; Cy5 = cyanine; FAM = fluorescein; FP = forward primer; HEX = hexachlorofluorescein; IL-18 = interleukin 18; MMP9 = matrix metalloproteinase 9; PPIA = peptidylprolyl isomerase A; RP = reverse primer; RPLP = 60S acidic ribosomal protein P0; TNFα = tumor necrosis factor α.