Chapter 3

$^{18}$F-sodium fluoride positron emission tomography assessed microcalcifications in symptomatic and asymptomatic human carotid plaques

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ABSTRACT

Background: $^{18}$F-sodium fluoride ($^{18}$F-NaF) positron emission tomography (PET) has been shown to target microcalcifications. We compared ex vivo microPET assessed $^{18}$F-NaF uptake between symptomatic and asymptomatic human carotid plaques. Furthermore, we compared $^{18}$F-NaF uptake with calcification visualized on high-resolution microcomputed tomography (CT).

Methods: Carotid plaques from patients undergoing carotid endarterectomy were collected and incubated in $49.4\pm7.2$ Mbq $^{18}$F-NaF and scanned using a microPET and a microCT scan. The average PET assessed $^{18}$F-NaF uptake was quantified and expressed as percentage of the incubation dose per gram (\%Inc/g). $^{18}$F-NaF PET volumes of interest ([VOI], $\geq$50\% of the maximum $^{18}$F-NaF uptake) on were compared with CT calcification VOI (Hounsfield Unit $\geq$1000).

Results: 23 carotid plaques (17 symptomatic, 6 asymptomatic) from 23 patients (median age 72 years, interquartile range [IQR] 61-75, 85\% male) were included. The average $^{18}$F-NaF uptake in symptomatic carotid plaques was comparable with the uptake in asymptomatic carotid plaques (median 2.32\%Inc/g [IQR 1.98-2.81] vs. median 2.35\%Inc/g [IQR 1.77-3.00], $P=0.916$). Only a median of 10 \% (IQR 4-25) of the CT calcification VOI showed increased $^{18}$F-NAF uptake, while merely a median of 35 \% (IQR 6-42) of $^{18}$F-NaF PET VOI was assigned as calcification on a CT scan.

Conclusion: $^{18}$F-NaF PET may represent a different stage in the calcification process than CT. We observed a similar PET assessed $^{18}$F-NaF uptake and pattern in symptomatic and asymptomatic plaques of high risk patients, indicating that this method may be of more value in earlier stages of carotid artery stenosis development.
INTRODUCTION

Surgical removal of atherosclerotic plaques from the carotid artery highly reduces the risk of future stroke in symptomatic patients with ≥70% stenosis. However, most of these patients will not have a new event when treated with best medical therapy. Furthermore, the role of surgery in moderate symptomatic stenosis (50-69%) and asymptomatic stenosis is under debate. Therefore, taking into account the potential risk for surgical complications, the selection of patients who will benefit most from surgery is challenging.

In order to improve risk stratification, research has been focused on the identification of plaque at risk for rupture, so-called vulnerable plaques. Currently, plaque thickness and intraplaque processes, such as inflammation and microcalcification, are seen as important contributors to vulnerability. These processes have become targets of various molecular imaging techniques, as they potentially allow non-invasive risk stratification of individual patients with carotid artery stenosis.

Recently, several studies have shown the feasibility of 18F-sodium fluoride (18F-NaF) positron emission tomography (PET) for imaging of atherosclerotic plaques. 18F-NaF predominantly binds to areas of microcalcification within the plaque. Appearance of microcalcifications indicates the active formation of calcification and is associated with plaque vulnerability. In contrast, established calcifications are seen as atherosclerotic end stage products and are associated with plaque stability.

It has been suggested that 18F-NaF may additionally be a useful marker for plaque vulnerability. Indeed, a clinical study by Joshi et al. showed that ruptured and high-risk coronary plaques have a significantly higher 18F-NaF uptake than low-risk coronary plaques. However, data on 18F-NaF uptake in carotid plaques is limited and its usefulness for the prediction of future stroke is unclear. Additionally, limited data has been published on the relation between active microcalcifications and established calcifications in human carotid plaques.

The primary objective of this study is to compare ex vivo microPET assessed 18F-NaF uptake between symptomatic and asymptomatic carotid plaques, using non-macrocalcified renal arteries from healthy kidney donors as controls. The secondary objective is to compare the distribution of 18F-NaF uptake on microPET with calcification visualized on a high-resolution microcomputed tomography (microCT) in carotid plaques.
MATERIALS AND METHODS

Study subjects
Carotid plaques were collected from patients who underwent carotid endarterectomy (CEA) at the Department of Surgery (Division of Vascular Surgery) of the University Medical Center Groningen (UMCG), between July 2015 and March 2016. Indication for CEA was decided by a surgeon expert panel and was based on the presence of symptomatic stenosis (≥50%) or asymptomatic stenosis (≥70%) of the internal carotid artery, according to internal guidelines. One patient with <50% stenosis was selected for CEA because of an irregular aspect of the plaque surface.

In order to increase the reliability of our measurements, we used renal artery specimens from healthy kidney donors as negative controls. The specimens were obtained during living donor nephrectomy.

Clinical and demographic data from the included patients were collected from medical records. In the symptomatic group, medication use and history of cardiovascular diseases prior to the recent event were registered. The study was reviewed by the ethics committee of the UMCG (METc 2015/258). All patients gave written informed consent.

Study procedure
Immediately after excision, carotid plaques and renal artery specimens were placed into phosphate buffered saline (PBS) and kept on ice. Both were incubated for one hour in 49.4±7.2 MBq 18F-NaF in 20 mL. After incubation, the plaques and renal arteries were carefully rinsed 5 times with 10 mL PBS. Then, tissue samples were weighed and microPET and microCT scans were performed. After the imaging procedure, the carotid plaques were cut transversely into segments of 3-4 millimeters. The renal arteries had a maximum size of 5 millimeters and therefore no cross-sections were made. The segments were embedded in paraffin for histological analysis.

Production of 18F-NaF
18F-NaF was produced by passing a solution of 18F-fluoride in water over a quaternary methyl ammonium (QMA) light anion exchange cartridge (Waters Chromatography B.V., Etten-Leur, The Netherlands). After washing the QMA with water, 18F-fluoride was eluted with saline and passed over a sterile Millex GS 0.22 µm filter (Millipore B.V., Amsterdam, The Netherlands). The radiochemical purity for all runs was >95%.

PET and CT acquisition
Carotid plaques and renal arteries were positioned into a microPET scanner (MicroPET Focus 220, Siemens Medical Solutions USA, Knoxville, TN, USA), and an emission scan of 30 minutes was performed. After the PET scan was finished, the bed of the PET scanner was
transferred to a microCT scanner (Inveon CT, Siemens Medical Solutions USA, Knoxville, TN, USA) without moving or touching the tissue samples. The CT exposure settings were 50 keV and 500 µAs, and a 100-ms exposure time for 360 projections during one 360° rotation.

The PET scans were reconstructed into a single frame of 30 minutes, using OSEM2D (4 iterations and 16 subsets), after being normalized and corrected for attenuation and decay of radioactivity. The CT images were reconstructed with the Feldkamp algorithm.

**Histological staining**

To validate our data, von Kossa and alizarin red staining for calcification were performed on five paraffin-embedded segments of five different carotid plaques. These segments were selected based on a high accumulation of $^{18}$F-NaF on the corresponding PET images. The two renal arteries with the highest $^{18}$F-NaF uptake were selected for staining as well. For a detailed description of the staining procedure, see Supplemental 1.

**Data analysis**

The PET and CT images were automatically registered using PMOD 3.7 (PMOD Technologies LLC, Zürich, Switzerland). The registration was visually inspected and manually corrected when necessary. For quantification of the average $^{18}$F-NaF uptake, three-dimensional volumes of interest (VOIs) were drawn around the whole tissue samples. The uptake (in kBq/cc) was corrected for weight of the specimen and the incubation dose, and expressed as percentage uptake of total incubation dose per gram of tissue (%Inc/g). It was assumed that 1 cubic centimeter equals 1 gram of tissue.

VOIs were also automatically drawn around $^{18}$F-NaF PET areas with a threshold of ≥50% of the maximum $^{18}$F-NaF uptake and assigned as $^{18}$F-NaF PET VOI. VOIs were automatically drawn around CT areas with a Hounsfield Unit (HU) ≥1000 and assigned as CT calcification VOI. The threshold of 50% of the maximum uptake value was chosen in order to select the volume with the highest $^{18}$F-NaF uptake, and thereby minimize the bias of a partial volume effect. The HU of 1000 was based on the CT scan of a phantom with various known calcium hydroxyapatite densities, whereby a lower threshold was chosen in order to not miss any calcification. To determine the overlap between the $^{18}$F-NaF PET VOIs and CT calcification VOIs, an intersection VOI was automatically drawn. Then, the CT calcification area (HU≥1000) within the $^{18}$F-NaF PET VOI was measured and expressed as a percentage of the $^{18}$F-NaF PET VOI; and the other way around: $^{18}$F-NaF PET uptake area (≥50% of maximum $^{18}$F-NaF uptake) within the CT calcification VOI was measured and expressed as a percentage of the CT calcification VOI.
Statistical analysis

Descriptive data are presented as frequencies (percentage), median (interquartile range) or mean ± SD. Based on the distribution of data (tested by normal probability plots), differences between data were analyzed with non-parametric tests. For continuous data the Mann-Whitney U test (two groups) or the Kruskal-Wallis test (≥two groups) was used. Categorical data were analyzed with the Chi Square test. A Spearman Correlation was used to test the association between continuous data. A two-sided \( P<0.05 \) was considered statistically significant. Statistical analyses were performed using SPSS for Windows (version 23.0).

RESULTS

Patient characteristics

We included 23 carotid plaques (17 symptomatic and 6 asymptomatic) from 23 patients (median age 72 years, interquartile range [IQR] 61-75, 85% male) who had undergone CEA, and 15 renal artery specimen from healthy kidney donors. The demographic and clinical characteristics were comparable between patients with symptomatic and asymptomatic plaques (Table 1). Only BMI was higher in the asymptomatic group (\( P=0.020 \)). The mean time between the cerebrovascular event (stroke, TIA or amaurosis fugax) and CEA in the symptomatic group was 21±14 days. All patients with asymptomatic plaques had a history of stroke related to the contralateral carotid artery. The healthy donors (renal arteries) were younger than CEA patients (\( P=0.001 \)) and had no history of cardiovascular disease.

Visual assessment of PET and CT images

PET images of all 23 plaques, showed a heterogeneous \(^{18}\text{F-NaF} \) uptake distribution and clear hotspots (Figure 1). The registered PET and CT images (\( n=16 \)) showed a discordant pattern between CT assessed calcification and \(^{18}\text{F-NaF} \) PET assessed calcification. In all plaques, \(^{18}\text{F-NaF} \) uptake was seen in regions without calcifications visualized on CT scan. The \(^{18}\text{F-NaF} \) uptake in the renal arteries was only visible when a low \(^{18}\text{F-NaF} \) uptake threshold was chosen compared with the carotid plaques, and no calcification was visible on the CT (\( n=8 \)).

\(^{18}\text{F-NaF} \) uptake in symptomatic and asymptomatic plaques

The average \(^{18}\text{F-NaF} \) uptake was similar in symptomatic and asymptomatic carotid plaques (median 2.32 %Inc/g [IQR1.98-2.81] vs. median 2.35 %Inc/g [IQR 1.77-3.00], \( P=0.916 \)), while the uptake in carotid plaques was significantly higher than in renal arteries (median 2.32 %Inc/g [IQR1.86-2.80] vs. median 0.44 %Inc/g [IQR 0.18-0.68], \( P<0.001 \)) (Figure 2).
Table 1 | Clinical characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic plaques (n=17)</th>
<th>Asymptomatic plaques (n=6)</th>
<th>Renal arteries (n=15)</th>
</tr>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>72 (64-76)</td>
<td>71 (55-72)</td>
<td>55 (41-63)</td>
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<tr>
<td><strong>Male gender</strong></td>
<td>14 (82)</td>
<td>5 (83)</td>
<td>5 (33)</td>
</tr>
<tr>
<td><strong>Stenosis degree (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70-99</td>
<td>14 (82)</td>
<td>6 (100)</td>
<td>–</td>
</tr>
<tr>
<td>50-69</td>
<td>2 (12)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>&lt;50</td>
<td>1 (6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Presenting symptoms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>9 (53)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TIA</td>
<td>7 (41)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>1 (6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Cardiovascular history</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>3 (18)</td>
<td>3 (50)</td>
<td>–</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>4 (24)</td>
<td>6 (100)</td>
<td>–</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>4 (24)</td>
<td>2 (33)</td>
<td>–</td>
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<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>1 (6)</td>
<td>3 (50)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Current smoker</strong></td>
<td>6 (34)</td>
<td>2 (33)</td>
<td>6 (40)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>25 (23-30)</td>
<td>31 (29-31)</td>
<td>26 (24-28)</td>
</tr>
<tr>
<td><strong>SBP (mm Hg)</strong></td>
<td>139 (132-150)</td>
<td>144 (127-173)</td>
<td>134 (127-148)</td>
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<tr>
<td><strong>DBP (mm Hg)</strong></td>
<td>76 (60-83)</td>
<td>73 (68-78)</td>
<td>76 (70-83)</td>
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<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td>4.4 (3.5-6.1)</td>
<td>4.2 (3.4-10)</td>
<td>5.5 (4.8-6.2)</td>
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<td><strong>LDL cholesterol (mmol/L)</strong></td>
<td>3 (2.2-3.8)</td>
<td>2.5 (2.0-8.2)</td>
<td>3.1 (2.7-4.1)</td>
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<td>9 (53)</td>
<td>3 (50)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Statins</td>
<td>7 (41)</td>
<td>6 (100)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Antiplatelet therapy</td>
<td>5 (29)</td>
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</tr>
<tr>
<td>Anticoagulation</td>
<td>3 (18)</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Data are expressed as number (%) or median (interquartile range)

* in symptomatic plaques: other than current event

** in symptomatic plaques: medication use prior to the recent cerebrovascular event

TIA=transient ischemic attack; BMI=body mass index; SBP=systolic blood pressure; DBP=diastolic blood pressure; LDL=low-density lipoprotein.

Comparison of 18F-NaF PET VOIs with CT calcification VOIs

The median 18F-NaF PET VOI of the carotid plaques was 41 mm³ (IQR 20-74), consisting of median 6% (IQR 3-10) of the total plaque volume (Table 2). CT calcification areas were measured in median 35% (IQR 6-42) of the 18F-NaF PET VOI.
<table>
<thead>
<tr>
<th>No.</th>
<th>18F-NaF PET VOI</th>
<th>CT calcification VOI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (mm³)</td>
<td>18F-NaF PET VOI of total plaque volume (%)</td>
</tr>
<tr>
<td>1</td>
<td>74</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>6</td>
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<tr>
<td>3</td>
<td>130</td>
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<tr>
<td>4</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>7a</td>
<td>46</td>
<td>9</td>
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<td>8</td>
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<td>15</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>28</td>
<td>4</td>
</tr>
</tbody>
</table>

* in this carotid plaque, no calcifications could be identified on CT scan

 b asymptomatic carotid plaque

PET, positron emission tomography; 18F-NaF, 18F-sodium Fluoride; %Inc/g, percentage uptake of total incubation dose per gram; CT, computed tomography; HU, Hounsfield Units.

18F-NaF PET VOI; ≥50% of maximum 18F-NaF uptake; CT calcification VOI (HU ≥ 1000)
The median CT calcification VOI was 149 mm$^3$ (IQR 16-290), consisting of median 16% (IQR 3-27) of the total plaque volume. $^{18}$F-NaF uptake areas were measured in 10% (IQR 4-25) of the CT calcification VOI.

The median averaged HU of CT calcification VOI was median 3258 HU (IQR 2465-3616). In renal arteries no CT calcification VOI could be detected; median HU of total renal artery volume was -470 HU (IQR -530 – -390).

**Figure 1** | Human carotid plaque after carotid endarterectomy (A). Sagittal view of ex vivo positron emission image (PET) showing a heterogeneous distribution of $^{18}$F-sodium Fluoride ($^{18}$F-NaF) uptake with a clear hotspot (red) (B). Sagittal view of corresponding computed tomography (CT) images (C). Fused images showing different distributions of microcalcification ($^{18}$F-NaF PET) and established calcification on high resolution CT (D).

**Figure 2** | $^{18}$F-NaF uptake, as measure of microcalcification, in symptomatic and asymptomatic human carotid plaques. Renal arteries of healthy kidney donors are used as negative controls. Data is expressed as percentage $^{18}$F-NaF uptake of the total incubation dose per gram of tissue (%Inc/g). Horizontal line represents the median.
No significant association was found between $^{18}$F-NaF uptake of the plaque (%Inc/g) and the CT calcification VOI ($R=0.382$, $P=0.144$), although $^{18}$F-NaF uptake seems to increase when CT calcification VOI increases in most plaques (Figure 3). When the two plaques with minimal CT calcification VOI and a high $^{18}$F-NaF uptake (>3.0 %Inc/g), the outliers, were excluded the association became significant ($R=0.680$, $P=0.008$).

![Figure 3](image_url) | Relation between PET assessed average $^{18}$F-NaF uptake and CT calcification VOI (mm³) in human carotid plaques. Data is expressed as percentage $^{18}$F-NaF uptake of the total incubation dose per gram of tissue (%Inc/g).

**Histological staining**

The von Kossa and alizarin red staining showed calcification deposits in all selected segments of the carotid plaques. No calcifications were identified in the segments of the renal arteries (Supplemental Figure 1).

**DISCUSSION**

The present study investigated *ex vivo* $^{18}$F-NaF uptake, in symptomatic and asymptomatic human carotid plaques. We hypothesized that $^{18}$F-NaF uptake in symptomatic plaques was higher than in asymptomatic carotid plaques, based on previous results in coronary plaques, and the concept of microcalcification and plaque rupture. Our results, however, demonstrate comparable $^{18}$F-NaF uptake in symptomatic and asymptomatic carotid plaques. Interestingly, we found that $^{18}$F-NaF uptake was present in regions without evidence of
calcification visualized on a CT scan. Furthermore, most of the CT calcification VOI had low overlap with $^{18}$F-NaF uptake, indicating that both techniques represent a different stage of calcification.

Recently, Vesey et al. showed that \textit{in-vivo} $^{18}$F-NaF uptake was higher in symptomatic carotid artery stenosis than in the contralateral asymptomatic stenosis in 18 patients with recent CVA ($\log_{10}$ standardized uptake value, mean 0.29±0.10 versus 0.23±0.11 respectively, $P<0.001$).\textsuperscript{22} These findings are consistent with the results of a clinical study of Quirce et al., in nine patients, where $^{18}$F-NaF uptake reported as mean target-to-background ratio was higher in symptomatic plaques (2.12±0.44) than in contralateral asymptomatic plaques (1.85±0.46, $P=0.220$).\textsuperscript{21} Age and sex distribution were comparable between these two studies and our study. Only Vesey et al. provided information about the cardiovascular history and other cardiovascular risk factors of the included patients. The prevalence of smoking and diabetes mellitus was similar. Furthermore, more than 50% of the patients had other manifestations of atherosclerosis, as in our study. Since the included patients in both studies were comparable, how can these contradictive results can be explained?

First, although Vesey \textit{et al.} did find a significant difference between $^{18}$F-NaF uptake in symptomatic and contralateral asymptomatic plaques, the differences were small and a substantial overlap between the uptake values in both groups was present, as was in the study of Quirce \textit{et al.} Furthermore, the $^{18}$F-NaF uptake between symptomatic patients and control patients differed to a larger extent (delta 0.17 SUV\textsubscript{mean}) than the difference between symptomatic and asymptomatic uptake (delta 0.07 SUV\textsubscript{mean}).\textsuperscript{22} Therefore, no absolute cut off value for the diagnosis of symptomatic plaques based on $^{18}$F-NaF uptake can be determined, only when compared with control patients there is a relevant difference. This is in line with the results of our study because $^{18}$F-NaF uptake between symptomatic and asymptomatic carotid plaques was comparable while the uptake in plaques was significantly higher than in control renal arteries.

Second, there may be differences in the degree of stenosis of the carotid arteries between patients in the aforementioned studies and our patients. Vesey \textit{et al.} found that $^{18}$F-NaF uptake was related to the degree of stenosis on CT, but they did not report the stenosis degree in the separate groups, neither did Quirce \textit{et al.} In our study, the stenosis degree in both, asymptomatic and the symptomatic plaques, was high. This could explain the similar $^{18}$F-NaF uptake.

$^{18}$F-NaF activity was increased in areas without calcification on CT and most of the CT calcification VOI showed minimal $^{18}$F-NaF uptake. This supports the idea that microcalcification, as visualized with $^{18}$F-NaF PET, and established calcification visualized on CT may reflect different stages of the calcification processes in atherosclerotic plaques.\textsuperscript{22,26,30} Established calcification is a well-known marker of total plaque burden and is strongly associated with the risk for cardiovascular events.\textsuperscript{31} However, the amount of established calcification, as detected by CT, only provides information about the processes in the past
and not about the actual biological activity of the plaque.\textsuperscript{32} Moreover, larger and denser areas of calcification may even stabilize the plaque.\textsuperscript{33} This has, for example, been suggested by Shalaan et al., who found a higher CT assessed calcification volume in asymptomatic than in symptomatic carotid plaques.\textsuperscript{34} The average percentage of plaque volume that was calcified was comparable with our study. Unfortunately, in our study CT assessed calcification volume could not be compared between symptomatic and asymptomatic plaques, due to limited availability of CT images in the asymptomatic group (n=1) because of image reconstruction failures.

Increased $^{18}$F-NaF uptake was related to the calcium volume on CT in the majority of the carotid plaques. This is probably caused by binding of $^{18}$F-NaF at the surface of the calcifications.\textsuperscript{13} However, a few plaques with low calcium volume had a high $^{18}$F-NaF uptake and vice versa. This suggests $^{18}$F-NaF accumulation in areas without any evidence of calcification, or at least no calcification with a size above the detection limit of the microCT scan.\textsuperscript{13} These observations indicate that $^{18}$F-NaF imaging can detect biologically active plaques, before they can be visualized on CT. This implies that $^{18}$F-NaF imaging may be useful in evaluating disease progression, as was further shown in patients with aortic stenosis, where baseline $^{18}$F-NaF uptake correlated well with the calcium progression after one year.\textsuperscript{35} Especially, $^{18}$F-NaF uptake in areas without established calcification on CT was the best predictor of calcium progression.

Furthermore, Derlin et al.\textsuperscript{36} found a positive correlation was between $^{18}$F-NaF uptake in the carotid arteries and, age and various cardiovascular risk factors in 269 patients with no history of stroke.\textsuperscript{36} Derlin et al. included a heterogeneous population, consisting of patients with low or minimal cardiovascular risk as well. In contrast, we included only patients with already a history of cardiovascular disease and, therefore, at an high-risk for a cardiovascular event. These findings further highlight the possibility of $^{18}$F-NaF imaging to identify patients at high-risk for cardiovascular disease in a low-risk population. In addition, the finding that $^{18}$F-NaF uptake in carotid plaques exceeded that of controls (renal arteries) and no calcification was visible in renal arteries on microCT and with histological staining, add evidence to the hypothesis that the presence of microcalcification identified by $^{18}$F-NaF are a feature of atherosclerosis.

Strengths of our study are the inclusion of a control group, and the scanning of calcium phantoms in order to accurately determine the calcium threshold. Furthermore, as far as we know, this is one of the first studies that compared calcification identified by $^{18}$F-NaF PET imaging with calcification visualized on microCT to gain more insight in development of atherosclerosis and imaging possibilities.

Our study has some limitations. First, the tracer uptake could be underestimated due to partial volume effects, as with every imaging study. Second, the number of asymptomatic plaques was small, although more samples would probably not have led to different conclusions, given the similar distribution of $^{18}$F-NaF uptake in both groups. Third, CT images
of 16 plaques (one asymptomatic) out of 23 were available for analysis due to practical and technical issues.

In conclusion, this study showed that the calcification patterns on $^{18}$F-NaF PET images and CT images are different. Clearly, $^{18}$F-NaF PET visualizes a different stage of the calcification process than CT. $^{18}$F-NaF uptake in carotid plaques exceeded the uptake in non-calcified renal arteries, but was comparable between symptomatic and asymptomatic carotid plaques, potentially due to the advanced nature of atherosclerotic disease in our patients. Therefore, we conclude that $^{18}$F-NaF has the potential to identify carotid plaques with active calcification. Further prospective studies on $^{18}$F-NaF uptake and symptomatology are required to assess the predictive and diagnostic value of $^{18}$F-NaF imaging in patients with early stage atherosclerosis.

Conflicts of interest
The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions
SAdB, HH, HHB, RHJAS, CJZ and DJM conceived and designed the study. SAdB, MR, HH, RAP, CJZ, MHdB, SAdB, HH, PWK and JLH collected the data. JD, MR, SAdB and HH analyzed the data. HH and SAdB conducted statistical analysis; JD, PWK and DJM helped with the interpretation of the results. HH wrote the first draft of the manuscript in close collaboration with SAdB and DJM. All authors have read, critically revised the manuscript and agree to the manuscript as written.
REFERENCES


Supplemental File 1
Staining procedure
Carotid plaque and renal artery segments were cut in sections of 5 µm, deparaffinised with xylene, and rehydrated with ethanol and demineralized water. Calcifications were then identified with Alizarin Red staining and von Kossa staining. In brief, sections were incubated in 2% Alizarin Red for five minutes at room temperature. After incubation, sections were dipped 20 times in 1:1 acetone:xylene, followed by 100% xylene. Then, the sections were rinsed with ethanol and dried.

For the Von Kossa staining, the sections were incubated in 1% silver nitrate solution and exposed to sunlight for 30 minutes. Then, sections were rinsed with demineralized water, and 3% thiosulfate was added for five minutes. After the sections were rinsed again, Nuclear Fast Red counterstain was added for three minutes, after which the sections were washed with ethanol and dried.

Digital images of the stained sections were made using the NanoZoomer Digital Pathology Scanner (Hamamatsu Photonics K.K., Japan).

Supplemental Figure 1 | PET images and calcification staining of carotid plaques. (A) Sagittal and (B) transversal ¹⁸F-NaF PET images of a human carotid plaque, showing a hotspot (red). (C) Alazarin Red staining (red spots) and (D) Von Kossa staining (brown spots) of corresponding transversal slices, both showing calcifications (Originally x 7).
Supplemental Figure 2 | PET images and calcification staining of renal arteries (controls).

(A) Transversal $^{18}$F-NaF PET image of a human renal artery, used as negative control. $^{18}$F-NaF PET assessed uptake was very low and no clear hotspots could be identified. (B) Alazarin Red staining and (C) Von Kossa staining of corresponding transversal slices, both showing no calcification (Originally x 7).