Dual-Tracer Positron-Emission Tomography for Identification of Culprit Carotid Plaques and Pathophysiology In Vivo

BACKGROUND: Inflammation and microcalcification are interrelated processes contributing to atherosclerotic plaque vulnerability. Positron-emission tomography can quantify these processes in vivo. This study investigates (1) $^{18}$F-fluorodeoxyglucose (FDG) and $^{18}$F-sodium fluoride (NaF) uptake in culprit versus nonculprit carotid atheroma, (2) spatial distributions of uptake, and (3) how macrocalcification affects this relationship.

METHODS: Individuals with acute ischemic stroke with ipsilateral carotid stenosis of $\geq 50\%$ underwent FDG-positron-emission tomography and NaF-positron-emission tomography. Tracer uptake was quantified using maximum tissue-to-background ratios ($TBR_{\text{max}}$) and macrocalcification quantified using Agatston scoring.

RESULTS: In 26 individuals, median most diseased segment $TBR_{\text{max}}$ (interquartile range) was higher in culprit than in nonculprit atheroma for both FDG (2.08 [0.52] versus 1.89 [0.40]; $P<0.001$) and NaF (2.68 [0.63] versus 2.39 [1.02]; $P<0.001$). However, whole vessel $TBR_{\text{max}}$ was higher in culprit arteries for FDG (1.92 [0.41] versus 1.71 [0.31]; $P<0.001$) but not NaF (1.85 [0.28] versus 1.79 [0.60]; $P=0.10$). NaF uptake was concentrated at carotid bifurcations, while FDG was distributed evenly throughout arteries. Correlations between FDG and NaF $TBR_{\text{max}}$ differed between bifurcations with low macrocalcification ($r_{s}=0.38$; $P<0.001$) versus high macrocalcification ($r_{s}=0.59$; $P<0.001$).

CONCLUSIONS: This is the first study to demonstrate increased uptake of both FDG and NaF in culprit carotid plaques, with discrete distributions of pathophysiology influencing vulnerability in vivo. These findings have implications for our understanding of the natural history of the disease and for the clinical assessment and management of carotid atherosclerosis.
Inflammation and microcalcification—calcium deposits smaller than 50 μm—are related but distinct pathophysiological processes within a carotid atherosclerotic plaque. The consequent enzymatic and mechanical effects on the plaque fibrous cap may trigger the rupture of a vulnerable plaque and precipitate thromboembolic stroke. Yet despite the importance of these interrelated processes in plaque vulnerability, our ability to detect them and understand their relationship in vivo remains limited. This is the first dual-tracer positron-emission tomography study demonstrating uptake of both 18F-fluorodeoxyglucose and 18F-sodium fluoride is increased in culprit plaques, indicating inflammation and microcalcification, respectively, and that these processes are spatially distinct in vivo. This uptake was independent of the degree of stenosis. These results suggest that microcalcification is a focal process located at the bifurcation, most likely in response to biomechanical factors, while inflammation is a diffuse process. This dual-tracer approach demonstrates the complexity of plaque biology and the need to assess, as well as manage, different pathophysiological processes for comprehensive evaluation of plaque vulnerability. The findings also have important methodological implications; indicating that comprehensive evaluation of plaque pathophysiology also requires consideration of microcalcification. Finally, these results have implications for our understanding of the natural history of the disease and for the clinical assessment and management of carotid atherosclerosis, including a potential role of microcalcification as a therapeutic target. Understanding these interrelated processes in vivo, as well as the use of positron-emission tomography to detect subtle changes in pathophysiology, has important implications for anti-inflammatory approaches to atherosclerotic plaque stabilization and the reduction of clinical events.

**CLINICAL PERSPECTIVE**

Carotid atherosclerosis is a major cause of ischemic stroke. Rupture of the atherosclerotic plaque, and subsequent thromboembolic events, is triggered by a combination of enzymatic and mechanical factors. Despite the importance of these interrelated processes in plaque vulnerability, our ability to detect them and understand their relationship in vivo remains limited. This is the first dual-tracer positron-emission tomography study demonstrating uptake of both 18F-fluorodeoxyglucose and 18F-sodium fluoride is increased in culprit plaques, indicating inflammation and microcalcification, respectively, and that these processes are spatially distinct in vivo. This uptake was independent of the degree of stenosis. These results suggest that microcalcification is a focal process located at the bifurcation, most likely in response to biomechanical factors, while inflammation is a diffuse process. This dual-tracer approach demonstrates the complexity of plaque biology and the need to assess, as well as manage, different pathophysiological processes for comprehensive evaluation of plaque vulnerability. The findings also have important methodological implications; indicating that comprehensive evaluation of plaque pathophysiology also requires consideration of microcalcification. Finally, these results have implications for our understanding of the natural history of the disease and for the clinical assessment and management of carotid atherosclerosis, including a potential role of microcalcification as a therapeutic target. Understanding these interrelated processes in vivo, as well as the use of positron-emission tomography to detect subtle changes in pathophysiology, has important implications for anti-inflammatory approaches to atherosclerotic plaque stabilization and the reduction of clinical events.

Inflammation and microcalcification—that are upregulated by metalloproteinases—collagenases that degrade the triple-helical fibrous cap—that are upregulated by inflammation.3 Furthermore, the proinflammatory environment promotes osteogenic transformation of vascular smooth muscle cells, which are derived from the same pluripotent mesenchymal cell as osteoblasts.4 Differentiation towards an osteoblastic phenotype results in upregulation of osteogenic-associated proteins, which are secreted into the extracellular matrix and bind calcium salts with high affinity.5 Subsequent microcalcification is both a consequence of inflammation and a trigger to propagate further inflammation and may either contribute to plaque rupture through mechanical disruption of the fibrous cap or coalesce to form protective macrocalcification.6 In thin fibrous caps, tissue stress may increase up to 5-fold in the presence of concentrated microcalcification, while tissue stress in regions with no microcalcification generally falls below the threshold believed to be necessary for rupture.1,7

A solution for detecting these pathophysiological processes noninvasively is positron-emission tomography (PET). Uptake of 18F-fluorodeoxyglucose (FDG), a radionuclide analogue of glucose that accumulates intracellularly in proportion to cellular demand for glucose, is increased in culprit compared with nonculprit plaques,8 correlates with histological macrophage density,9 and is associated with high-risk morphological plaque features.10 It is also related to clinical sequelae, with increased FDG uptake associated with an increased risk of recurrent cerebrovascular events independent of the degree of luminal stenosis.11 FDG-PET studies have supported inflammation within atherosclerosis being a diffuse process, with FDG uptake correlating between neighbouring arterial regions.12

More recently, 18F-sodium fluoride (NaF) has been used to identify sites of active microcalcification in atherosclerotic plaques, where radiolabelled fluoride is exchanged for the hydroxyl group in hydroxyapatite to form fluorapatite.13 Ex vivo NaF-PET of excised carotid plaques has validated the use of the tracer, with NaF exhibiting strong, specific binding to areas of vascular microcalcification.14 Increased NaF uptake has been identified in both high-risk and culprit coronary plaques15,16 as well as carotid lesions.17,18 NaF uptake is increased in morphologically high-risk but unruptured plaques, further suggesting NaF uptake reflects the microcalcification process rather than simply increased surface area for binding after plaque rupture.15,16

There have been few vascular dual-tracer FDG- and NaF-PET studies to date. In an asymptomatic oncological cohort, only 6.5% of arterial lesions showed concomitant tracer uptake.19 In symptomatic carotid disease, NaF uptake was higher in culprit versus asymptomatic atheroma, though FDG did not differ significantly.18 findings at odds with most published vascular FDG-PET studies.
This study addressed this uncertainty by investigating the ability of FDG- and NaF-PET/computed tomography (CT) to identify culprit plaque (defined as the symptomatic plaque relating to the individual's clinical symptoms) in a cohort of magnetic resonance imaging–confirmed ischemic strokes. Furthermore, the study used tracer uptake to investigate the relative spatial distributions of inflammation and microcalcification within atherosclerosis in vivo. Finally, we assessed how macrocalcification affected the relationship between FDG and NaF uptake within the plaque.

METHODS

Study Eligibility and Recruitment

The data that support the findings of this study are available from the corresponding author upon reasonable request. The ICARUSS (Imaging Carotid Atherosclerosis in the Recovery and Understanding of Stroke Severity) Study prospectively recruited individuals presenting with first-ever ischemic stroke within the previous 7 days with ipsilateral common or internal carotid artery stenosis of ≥50% on CT angiography or ultrasound Doppler at Addenbrooke's Hospital, Cambridge, United Kingdom. All individuals had magnetic resonance imaging–confirmed ischemic strokes using diffusion-weighted imaging. Individuals presenting with a transient ischemic attack were not recruited. Carotid artery stenosis was measured using the North American Symptomatic Carotid Endarterectomy Trial criteria, and plaque characteristics (echogenicity and regularity) were recorded. Baseline cardiovascular risk factors and stroke severity were collected at time of stroke. Individuals with atrial fibrillation were excluded. All participants provided written informed consent. The study was approved by a national research ethics committee (Nottingham One Research Ethics Committee, 14/EM/0128).

PET/CT Imaging

FDG- and NaF-PET/CT imaging was performed using a GE Discovery 690 (GE Medical Systems Ltd, Hatfield, United Kingdom) with 64-slice CT on 2 separate visits within 14 days of ischemic stroke. Participants were injected intravenously with a target dose of 250 MBq of FDG, followed by a 90-minute uptake time. A silence protocol (minimal vocalization, small sips of water only) was adopted during this period to reduce physiological tracer uptake in structures neighbouring the carotid arteries. Participants fasted for 6 hours before injection. Blood glucose concentrations were confirmed as ≤7.0 mmol/L before injection. Participants with diabetes mellitus took oral anti-diabetic medications as usual, but insulin was omitted within 4 hours before imaging, in line with previous methodology.

For the NaF-PET/CT, participants were injected intravenously with a target of 125 MBq of NaF, followed by a 60-minute uptake time. A CT carotid angiogram was performed concurrently. Carotid endarterectomy was not delayed by research imaging.

Radiotracer Uptake Quantification

For both NaF-PET/CT and FDG-PET/CT, co-registered images were resampled to 3-mm slice thickness and regions of interest (ROIs) drawn manually on fused PET/CT images along the common carotid and internal carotid artery to encompass the region 0.9 cm proximal and 3 cm distal to the carotid bifurcation, as per previous methodology. Tracer uptake within ROIs was measured using maximum standardized uptake values (SUV$_{\text{max}}$); the maximum tracer uptake within a ROI adjusted for injected dose and patient weight. To correct for blood pool activity, arterial SUV$_{\text{max}}$ was adjusted for venous SUV—the mean uptake of the mid-luminal ROIs in the jugular vein over 5 contiguous 3 mm slices—to give the maximum target-to-background ratio (TBR$_{\text{max}}$). TBR$_{\text{max}}$ was measured in prespecified regions of culprit and nonculprit carotid arteries to assess most diseased segment (MDS) and whole vessel (WV) uptake. The MDS considers the most diseased 6 mm of the artery based on tracer uptake, representing the mean of the TBR$_{\text{max}}$ of the ROIs in 3 contiguous axial slices where the central ROI has the highest uptake within the artery. The WV was the median of tracer uptake across all 14 axial slices of the artery. Median was used due to a nonparametric distribution of tracer uptake along the length of the artery.

To assess the carotid bifurcation as the focus of disease, tracer uptake within the bifurcation (defined as the median tracer uptake in the region within 2 slices either side of the bifurcation; slice 0) was compared with the nonbifurcation arterial uptake (defined as the median uptake in the remaining 9 slices outside of the bifurcation).

PET imaging data sets were analyzed using Osirix (version 5.7.1, OsiriX Imaging Software, Geneva, Switzerland). Image analysis of the whole cohort was performed only after all imaging had been completed. The primary reader (Dr Evans) performed readings independently and blinded to clinical information and symptomatic artery. All participants had bilateral carotid atherosclerosis, and study quantification of the degree of stenosis was performed after assessment of tracer uptake. Experienced readers performed reproducibility and quality assurance by repeating ROIs in 20% of the FDG-PET/CTs (Dr Tarkin) and NaF-PET/CTs (M.M. Chowdhury).

Macrocalcification Scoring

Macrocalcification was measured using methods proposed by Agatston et al, scoring calcification as a product of weighted density and area. Carotid artery calcium scores (CACS) were measured from unenhanced CT carotid angiograms using the Calcium Scoring Plugin (version 1.0) in Osirix (version 5.7.1, OsiriX Imaging Software, Geneva, Switzerland). The detection threshold for calcification was set at 130 HU.

Biomarkers

A 4.9 mL sample of venous blood was drawn at the time of FDG-PET/CT for hsCRP (high sensitivity C-reactive protein) as a marker of inflammation.

Clinical Outcomes

Participants were reviewed at 6 months for recurrent neurovascular events (by Drs Evans or Warburton), blinded to PET imaging.
readings. In cases of clinical uncertainty, the participant was reviewed by an independent neurovascular specialist.

Statistical Analysis
Continuous data was tested for normality using the Shapiro-Wilk method. Parametric data was reported as mean±SD and nonparametric data reported as median and interquartile range (IQR). In unpaired groups, parametric and nonparametric data were compared using t testing or Wilcoxon rank-sum testing, respectively. Culprit and nonculprit arteries in the same individual were compared using equivalent paired testing. Comparison of bifurcation versus nonbifurcation regions within the same artery also used paired testing. Correlations were tested using 2-tailed Spearman ρ correlation (nonparametric or ordinal data) or Pearson correlation coefficient (parametric data).

MDS TBR max was compared across stenosis categories (1%–29%, 30%–49%, 50%–69%, 70%–89%, and 90%–99%) in both symptomatic and asymptomatic arteries using Kruskal-Wallis 1-way ANOVA testing.

The cutoff for statistical significance was set at 5%. Data was analyzed using R (version 3.6.1, 2019, R Foundation for Statistical Computing, Vienna, Austria).

All authors had full access to all the data in the study and take responsibility for its integrity and the data analysis.

RESULTS
Study Population
Of 31 participants recruited to the ICARUSS study, 27 underwent dual FDG- and NaF-PET/CT (of the 4 participants who did not undergo scanning; 2 became too unwell to continue in the study, 1 completed only FDG imaging because of expedited carotid surgery, and 1 was unable to complete imaging due to claustrophobia). Twenty-six had imaging suitable for analysis (1 participant had uncorrectable spill-over artifact in the asymptomatic carotid artery on FDG-PET/CT). All participants had bilateral carotid atherosclerosis. All individuals were reviewed for symptomatic carotid endarterectomy (factors influencing the decision not to operate were reviewed by an independent neurovascular specialist. In cases of clinical uncertainty, the participant was reviewed by an independent neurovascular specialist). There was no significant difference when comparing culprit versus nonculprit arteries using WV TBR max. In contrast, FDG uptake was significantly higher in the culprit artery for both MDS and WV TBR max (Table 2). A similar pattern was seen with equivalent SUV max readings.

| Table 1. Clinical characteristics of study cohort. |
|-----------------------------|
| Mean age, y               | 74.8 (SD, 9.7) |
| Men                        | 18 (69.2%)    |
| Median BMI                 | 26 (IQR, 3.9) |
| Smoking history (current/ex-smokers) | 17 (65.4%) |
| Diabetes mellitus          | 4 (15.4%)     |
| Hypertension               | 17 (65.4%)    |
| Statin before index event  | 9 (34.6%)     |
| Antplatelet before index event | 8 (30.8%) |
| Cardiovascular history (previous ischemic heart disease or myocardial infarction) | 8 (30.8%) |
| Median NIHSS               | 4.5 (IQR, 10.75) |
| Thrombolyzed               | 6 (23.1%)     |
| Modal degree of symptomatic stenosis | 70%–89% |
| Modal degree of asymptomatic stenosis | 30%–50% |

BMI indicates body mass index; and NIHSS, National Institutes of Health Stroke Scale.

There was no significant relationship between the degree of stenosis and NaF-PET/CT MDS TBR max (P=0.91) or FDG-PET/CT MDS TBR max (P=0.91). Median (IQR) TBR max did not differ between regular versus irregular plaques for NaF (2.77 [0.40] versus 2.27 [0.92]; P=0.01) or FDG (2.04 [0.56] versus 1.81 [0.70]; P=0.11). Differences in TBR max between echoluent/predominantly echoluent and echogenic/predominantly echogenic plaques approached statistical significance for mean FDG (2.39±0.79 versus 2.00±0.53; P=0.13) and median NaF (2.27 [0.67] versus 2.68 [0.97]; P=0.09).

In our sample, a FDG MDS TBR max of 2.03 had sensitivity of 65.4% and specificity of 76.9% to identify culprit plaque (area under curve, 0.71). For NaF, a MDS TBR max of 2.65 had 53.9% sensitivity and 69.2% specificity to detect culprit plaque (area under curve, 0.62).

There was a significant difference in plaque median (IQR) TBR max between those taking statins versus not before the index stroke for NaF (2.77 [0.40] versus 2.27 [0.92]; P<0.01) and FDG (1.82 [0.53] versus 2.04 [0.45]; P=0.04).

Spatial Distribution of Tracer Uptake
The median TBR max at each slice in culprit and nonculprit arteries demonstrated different spatial distributions in tracer uptake: FDG uptake was diffuse along the length of the artery, while NaF uptake peaked around the point of the carotid bifurcation (in both symptomatic and asymptomatic arteries, with attenuated responses in the nonculprit artery; Figures 1 and 2).
Figure 1. Tracer uptake in symptomatic disease.
A, Axial computed tomography (CT), B) axial fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT, (C) axial FDG-PET, (D) axial CT angiogram, (E) axial sodium fluoride (NaF)-PET/CT, (F) axial NaF-PET showing a symptomatic right carotid artery (purple arrow) and an asymptomatic left carotid artery (green arrow), (G) sagittal FDG-PET/CT showing diffuse uptake in the symptomatic carotid (white arrows), (H) sagittal NaF-PET showing focal uptake in the symptomatic carotid (black arrow).
To assess statistically the bifurcation as a focus of disease, tracer uptake was compared against that in nonbifurcation regions. Median NaF TBR\textsubscript{max} (IQR) in the bifurcation region was significantly higher than nonbifurcation regions (2.19 [0.81] versus 1.71 [0.38]; \(P<0.001\)). In contrast, there was no significant difference for median FDG TBR\textsubscript{max} in the bifurcation versus the nonbifurcation region (1.67 [0.47] versus 1.61 [0.41]; \(P=0.65\)).

### Macrocalcification

The median culprit artery CACS was 367 (IQR, 625), and median nonculprit artery CACS was 324.5 (IQR, 501; \(P=0.36\)). Within the 52 carotid bifurcations, the range of CACS was 0 to 916.

### Influence of Macrocalcification on the Relationship Between Inflammation and Microcalcification

There was a weak correlation between FDG and NaF TBR\textsubscript{max} across all carotid bifurcations (\(r_s=0.18\); \(P<0.01\)) before considering macrocalcification. However, adjustment for macrocalcification (dichotomizing bifurcations based on the range of CACS; low: 0–450, high: ≥451) revealed different patterns of relationship. Median NaF TBR\textsubscript{max} (IQR) was higher in high CACS bifurcations (2.56 [1.14]) than low CACS bifurcations (2.13 [0.66]; \(P=0.03\)). In contrast, the opposite pattern was seen with FDG, with median TBR\textsubscript{max} (IQR) lower in high CACS bifurcations (1.46 [0.47]) versus low CACS bifurcations (1.69 [0.36]; \(P=0.04\)). The strength of the association between FDG and NaF TBR\textsubscript{max} within bifurcations varied with degree of macrocalcification: low calcification (\(r_s=0.38\); \(P<0.001\)) and high calcification (\(r_s=0.59\); \(P<0.001\); Figure 3).

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#### Table 2. Tracer Uptake in Culprit and Nonculprit Carotid Arteries

|          | Culprit Carotid Artery | Nonculprit Carotid Artery | Significance |
|----------|------------------------|---------------------------|--------------|
| NaF      |                        |                           |              |
| MDS TBR\textsubscript{max} |                      |                           |              |
| Median (IQR) | 2.68 (0.63) | 2.39 (1.02) | \(P<0.001\) |
| Mean (SD)  | 2.85 (1.15)    | 2.34 (0.64)    | \(P<0.01\)  |
| WV TBR\textsubscript{max} |                      |                           |              |
| Median (IQR) | 1.85 (0.28) | 1.79 (0.60) | \(P=0.10\)  |
| Mean (SD)  | 1.92 (0.44)    | 1.83 (0.49)    | \(P=0.08\)  |
| FDG       |                        |                           |              |
| MDS TBR\textsubscript{max} |                      |                           |              |
| Median (IQR) | 2.08 (0.52) | 1.89 (0.40) | \(P<0.001\) |
| Mean (SD)  | 2.24 (0.65)    | 1.88 (0.41)    | \(P<0.001\) |
| WV TBR\textsubscript{max} |                      |                           |              |
| Median (IQR) | 1.89 (0.40) | 1.71 (0.36) | \(P<0.001\) |
| Mean (SD)  | 1.92 (0.41)    | 1.71 (0.31)    | \(P<0.001\) |

FDG indicates fluorodeoxyglucose; MDS, most diseased segment; NaF, sodium fluoride; TBR\textsubscript{max}, maximum tissue-to-background ratio; and WV, whole vessel.
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Association With hsCRP
In culprit atheroma, hsCRP concentration was moderately correlated with FDG MDS TBR_{max} (r_s=0.53; P=0.01) but not NaF MDS TBR_{max} (r_s=0.01, P=0.96).

Clinical Outcomes
Three individuals had recurrent neurovascular events (1 ischemic attack and 2 further ischemic strokes), all of whom did not undergo endarterectomy. Culprit plaque MDS TBR_{max} did not differ between individuals with recurrence compared with those without recurrence in those not undergoing endarterectomy (FDG: 2.32±0.76 versus 1.97±0.65, P=0.40; NaF: 2.74±0.78 versus 2.78±0.77, P=0.93).

DISCUSSION
In this study of individuals with magnetic resonance imaging–confirmed strokes across a range of stroke severities, we show the utility of dual-tracer PET using FDG- and NaF-PET to provide comprehensive plaque assessment through detecting inflammation and microcalcification respectively in culprit carotid plaques. The results indicate inflammation, microcalcification, and macrocalcification represent 3 related but spatially distinct pathophysiological processes within atherosclerosis in vivo. Microcalcification represents a focal process at the bifurcation while inflammation is a more diffuse process, with the relationship between tracer uptake stronger in areas of increased macrocalcification.

Dual-Tracer Detection of Culprit Plaque
This study is the first to show an increase in both NaF-PET and FDG-PET uptake in culprit versus nonculprit carotid atheroma. Although these tracers have been found individually to identify culprit and vulnerable plaque, previous dual-tracer studies found only NaF-PET (but not FDG-PET) to be significantly higher in symptomatic carotid plaques 18 or not significantly different for both tracers in a smaller study. 25 This dual-tracer approach demonstrates the complexity of plaque biology and, when put in context with the aforementioned results, the need to assess different pathophysiological processes for comprehensive evaluation of plaque vulnerability. Differences in cohorts between studies may influence the results. Our cohort included a wide range of stroke severities (National Institutes of Health Stroke Scale scores) than previous studies and is arguably more representative of a typical stroke population seen in clinical practice. Furthermore, we had no function-based exclusion criteria, whereas other studies have excluded individuals with a modified Rankin Scale score above 3. 18 Our cohort also appeared to have more extensive vascular disease, displaying an ≈2-fold higher calcium burden in symptomatic arteries than that reported by Vesey et al. 18 Finally, the relatively small sample in each study may contribute to the variation in findings. Meta-analysis or replication in a larger sample would be advantageous.
**Spatial Distribution of Pathophysiology**

Our results indicate FDG and NaF uptake have 2 distinct spatial distributions. The finding for NaF that only MDS TBRmax and not WV TBRmax was significantly higher in the culprit artery suggests a focal pattern of disease. This was illustrated by the peak in NaF spatial uptake around the carotid bifurcation and further supported by significantly higher uptake in the bifurcation versus the rest of the artery. In contrast, FDG demonstrates a diffuse pattern of uptake as demonstrated by significantly higher MDS and WV TBRmax in the culprit artery, and no significant difference between bifurcation uptake versus the rest of the artery.

Our findings are consistent with previous FDG studies that indicate that atherosclerosis is a diffuse inflammatory disease. This has implications for systemic anti-inflammatory therapy in atherosclerosis. This pattern also supports microcalcification as an active process and not simply an automatic consequence of inflammation. This focus of microcalcification and its location suggests a biomechanical element, given that microcalcification appears to occur at the point where the artery is exposed to the highest stress. The bifurcation experiences ≥9 to 14x the circumferential stress of the distal common carotid artery focused over a small area. Shear stress may also play a role; nonlaminar shear stress has been implicated in promoting vascular smooth muscle cell differentiation into the synthetic phenotype.

**Physiological Relationship Between Inflammation, Microcalcification, and Macrocalcification**

The inverse relationship between CACS and FDG uptake in the bifurcation supports the negative association observed in neurovascularly asymptomatic cohorts and that only a minority of plaques with macrocalcification exhibited FDG uptake. In contrast, a range of associations have been reported between NaF uptake and macrocalcification. In asymptomatic atherosclerosis, different studies have reported no difference in carotid TBRs between calcified versus noncalcified segments, reduced plaque TBR with increasing semiquantitative measures of macrocalcification, and a weak positive association with calcification score. In a mix of symptomatic disease and asymptomatic controls, Vesey et al reported a correlation between plaque SUVmean and Agatston score of r=0.72. Dweck et al reported a significant correlation between NaF uptake and coronary Agatston scores (r=0.65) and extensive overlap between NaF uptake and macrocalcification, though there were disparate areas with only 59% of patients with extensive CAC scores (>1000) showing significant NaF uptake. Differences in the extent of macrocalcification may explain differences in the correlation between NaF uptake and calcium burden, as a substantial amount of calcification lies within the calcified plaque. NaF, which binds only to the surface of macrocalcification, cannot permeate into the crystalline mass of large macrocalcifications.

When considering the bifurcation as the focus of disease, our results indicate that the strength of the association between FDG and NaF uptake increases with increasing macrocalcification. These findings are consistent with findings in asymptomatic plaques, where there was no correlation between FDG and NaF TBRmax in noncalcified lesions. However, the same study found an association in mildly calcified (analogous to our low calcification bifurcations) and severely calcified plaques despite lower tracer uptake.

These findings likely reflect the natural history of plaque progression. Inflammation appears to precede mineralization in vivo, before macrophage-induced osteogenic differentiation of vascular smooth muscle cells that initiate mineralization, compounded by macrophage apoptosis resulting in calcium deposition in smooth muscle cells. Our results indicate tracer uptake, and relationship to each other, is influenced by the presence of macrocalcification, and likely reflects the age of the plaque. This has implications not only for understanding the natural history of plaque progression in humans in vivo but also for ongoing vascular PET research, as macrocalcification has not always been considered when measuring tracer uptake. Our findings suggest that comprehensive evaluation of plaque pathophysiology requires not only dual-tracer assessment but also consideration of how tracer uptake and interpretation is influenced by the degree of arterial macrocalcification.

**Associations Between hsCRP and Tracer Uptake**

Our results are consistent with previous studies reporting CRP is produced by smooth muscle cells in active atherosclerotic disease but not in end-stage plaques with heavy calcification. Hence, CRP appears associated with inflammation as measured by FDG but not with microcalcification as measured by NaF.

**Limitations and Future Work**

Our pilot study is limited in size but is comparable in size to other vascular PET studies performed acutely in symptomatic patients. The high sensitivity of PET enables detection of subtle physiological changes, allowing statistically significant differences to be detected despite these small sample sizes. However, validation of our findings through replication in...
a larger cohort or meta-analysis of existing studies would be advantageous.

Replication of our spatial distribution findings in an asymptomatic cohort would support the diffuse nature of the inflammation prerupture rather than as a consequence of plaque rupture. In our study, all participants had experienced acute stroke that, along with other acute syndromes such as myocardial infarction, may increase systemic inflammation and hence influence FDG uptake in both culprit and nonculprit plaques.26

In this study, we used contralateral carotid artery ath-eroma as a comparator. As atherosclerosis was found bilaterally in carotid arteries in all participants, and that tracer uptake was unaffected by the degree of steno-sis, we feel that this provides an appropriate comparato-r as per previous studies.18,21,36 Variation in uptake between individuals (being influenced by vascular risk factors and other interpersonal variation) means that it has proven difficult to establish an absolute value for plaque stability. Robust comparison would require a large sample size, ideally in a prospective study, or meta-analysis of studies with harmonized methodology. In contrast, comparing culprit and nonculprit plaques within the same individual takes account of variation in risk factors between participants, with each individual acting as their own control. As plaque vulnerability is systemic, comparison against the non-culprit contralateral plaque (representing this systemic vulnerability) allows subtle but important differences in tracer uptake to be detected, highlighting the additional tracer uptake specifically in the culprit plaque and illustrating important pathophysiological changes. Additionally, this may also have potential clinical implications in instances of tandem carotid lesions in the same neurovascular territory, where the technique may provide a more nuanced impression over which lesion represents the culprit plaque.

This study did not examine histology, but a number of high quality preclinical and clinical validation studies have demonstrated the relationship between plaque histopathology and tracer uptake as discussed above.9,14,18,37

Although highly sensitive, FDG uptake is nonspecific. The emergence of newer radiotracers with improved specificity for inflammatory cells, such as 68Ga-DOTATATE ([1,4,7,10-tetraazacyclododecane-N,N'N''N'''-tetraacetic acid]-D-Phe1, Tyr3-octreotate), could help elucidate the spatial distribution of atheromatous inflammation.21

Although PET is unlikely to be used in routine clinical use, it provides an effective outcome measure in proof-of-principle studies to understand pathophysiology that underpins larger randomized clinical trials using clinical outcome data. Whether microcalcification predicts recurrent cerebrovascular events in carotid disease or can serve as a novel therapeutic target remains to be proven. NaF-PET is likely to remain the mainstay for assessment of microcalcification given the high sensitivity of PET and the ability to assess physiological responses to intervention, whereas magnetic resonance imaging is disadvantaged by its limited ability to detect microcalcification owing to deposits falling below the spatial resolution of most scanners.

**Implications**

The diffuse nature of inflammation in atherosclerosis has implications for the use of systemic anti-inflammatory therapies for secondary prevention in atherosclerotic disease.36 Furthermore, the results suggest that while focal surgical intervention with carotid endarterectomy may be advantageous for treating focal microcalcification in the vulnerable plaque, it may not address the systemic inflammation and diffuse atheroma vulnerability. Finally, these results suggest the potential of microcalcification as a novel therapeutic target for plaque stabilization.

**Conclusions**

This is the first dual-tracer PET study to demonstrate that both FDG and NaF uptake is increased in culprit carotid atheroma, reflecting increased inflammation and microcalcification found within these plaques. Our dual-tracer approach reveals important differences in the spatial distributions of these pathological processes in vivo and a potential role of microcalcification as a therapeutic target. These findings have implications for our understanding of the natural history of the disease and for the clinical assessment and management of carotid atherosclerosis.

**ARTICLE INFORMATION**

Received June 13, 2019; accepted January 10, 2020.

**Correspondence**

Nicholas Evans, PhD, Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Hills Rd, Cambridge CB2 0QQ, United Kingdom. Email ne214@cam.ac.uk

**Affiliations**

Department of Clinical Neurosciences (N.R.E., E.A.W.), Department of Medicine (N.R.E., I.M.T., E.P.V.L., J.H.F.R.), and Division of Vascular Surgery (M.M.C., P.A.C.), University of Cambridge, Cambridge, United Kingdom.

**Sources of Funding**

Dr Evans is supported by a Research Training Fellowship from The Dunhill Medical Trust (RTP440114). Dr Tarkin is supported by the Wellcome Trust (211100/21/182, 104492/20/147) and the National Institute for Health Research (NIHR). M.M. Chowdhury is supported by Royal College of Surgeons of England and British Heart Foundation (BHF) fellowships (FS/16/29/31957). Dr Rudd is
supported by the NIHR Cambridge Biomedical Centre, BHF, Wellcome Trust, and Higher Education Funding Council for England. Dr Warburton is supported by the NIHR Cambridge Biomedical Centre.

Disclosures
None.

REFERENCES
1. Maldañado N, Kelly-Arnold A, Vengreynuk Y, Laurimer R, Virmani R, Cardoso L, Weinbaum S. A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture. Am J Physiol Heart Circ Physiol. 2012;303:H619–H628. doi: 10.1152/ajpheart.00036.2012.

2. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340:115–126. doi: 10.1056/NEJM199901133400102.

3. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. Circ Res. 1994;75:181–189. doi: 10.1161/01. RES.75.1.181.

4. Hruska KA, Mathew S, Saab G. Bone morphogenetic proteins in vascular calcification. Circ Res. 2005;97:105–114. doi: 10.1161/01.RES.0000017571.53833.6c.

5. Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, Schinke RW, Cardoso L, Weinbaum S. A mechanistic analysis of the role of microcalcification by (18)F-sodium fluoride positron emission tomography. Lancet. 2014;383:705–713. doi: 10.1016/S0140-6736(13)67547-7.

6. Kitagawa T, Yamaamoto H, Toshimitsu S, Sasaki K, Seno A, Kubo Y, Tatsugami F, Awai K, Hirokawa Y, Khara Y. 18F-sodium fluoride positron emission tomography for identification of ruptured and high-risk coronary atherosclerotic plaques: a prospective clinical trial. Lancet. 2014;383:705–713. doi: 10.1016/S0140-6736(13)67547-7.

7. Cheng GC, Loree HM, Kamm RD, Fishbein MC, Lee RT. Distribution of carotid plaque inflammation in patients. J Am Coll Cardiol. 2012;59:1539–1548. doi: 10.1016/j.jacc.2011.12.037.

8. Rudd JH, Warburton EA, Bennett MR, et al. Identifying active vascular calcification, risk factors, and biomarkers: a prospective fluorodeoxyglucose positron-emission-tomography study. J Nucl Med. 2011;52:1020–1027. doi: 10.2967/jnumed.111.087452.

9. Barnett HM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, Ferguson GG, Fox AJ, Rankin RN, Hachinski VC, Wiebers DO, et al; North American Symptomatic Carotid Endarterectomy Trial Collaborators. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. N Engl J Med. 1991;325:445–453. doi: 10.1056/NEJM199105133250701.

10. Tarkin JM, Joshi FR, Evans NR, Chowdhury MM, Figg NL, Shah AV, Starks LT, Martin-Garrido A, Manavaki R, Yu E, et al. Detection of atherosclerotic inflammation by 68Ga-DOTATATE PET compared to 18F-FDG PET imaging. J Am Coll Cardiol. 2017;69:1774–1791. doi: 10.1016/j.jacc.2017.01.060.

11. Dweck MR, Chow MW, Joshi NV, Williams MC, Jones C, Fletcher AM, Richardson H, White A, McKillop G, van Beek EJ, et al. Coronary arterial 18F-sodium fluoride uptake: a novel marker of plaque biology. J Am Coll Cardiol. 2012;59:1539–1548. doi: 10.1016/j.jacc.2011.12.037.

12. Fayad ZA, Mani V, Woodward M, Kallend D, Abt M, Burgess T, Fuster V, Ballantyne CM, Stein EA, Tardif JC, et al; dal-PLAQe Investigators. Safety and efficacy of dalcetrapib on atherosclerotic disease using novel non-invasive multimodality imaging (dal-PLAQe): a randomised clinical trial. Lancet. 2011;378:1547–1559. doi: 10.1016/S0140-6736(11)61383-4.

13. Agatston AS, Janowitz WR, Hildner FJ, Zerhouni EA, Diamond DA, Muller NL, Virmani R, et al. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol. 1990;15:827–832. doi: 10.1016/0735-1097(90)90282-t.

14. Quirce R, Martinez-Rodriguez I, Banzo I, Jiménez-Bonilla J, Martínez-Amarador N, Ibáñez-Salazar-Bravo S, López-Defiló JJ, Jiménez-Alonso M, Revilla MA, Carril JM. New insight of functional molecular imaging into the atheroma biology: 18F-NaF and 18F-FDG in symptomatic and asymptomatic carotid plaques after recent CVA. Preliminary Results. Clin Physiol Funct Imaging. 2016;36:499–503. doi: 10.1111/cpi.12254.

15. Joshi NV, Vesey AT, Williams MC, Shah AS, Calvert PA, Craighead FH, Yeoh SE, Wallace W, Salter D, Fletcher AM, et al. 18F-fluoride positron emission tomography for identification of ruptured and high-risk coronary atherosclerotic plaques: a prospective clinical trial. Lancet. 2014;383:705–713. doi: 10.1016/S0140-6736(13)67547-7.

16. Cockier MS, Spence JD, Hammond R, Wills RA, deKemp RA, Lum C, Ad-eekoo A, Yaffe MJ, Leung E, Hill A, et al; Canadian Atherosclerosis Imaging Network (CAIN). [18F]-NaF PET/CT identifies active calcification in carotid plaque. JACC Cardiovasc Imaging. 2017;10:486–488. doi: 10.1016/j.jcmg.2016.03.005.

17. Vesey AT, Jenkins WS, Irlke A, Moss A, Sng G, Forioyte RO, Clark T, Roberts G, Fletcher A, Lucarelli C, et al. 18F-fluoride and 18F-fluorodeoxyglucose positron emission tomography after transient ischemic attack or minor ischemic stroke: Case-Control Study. Circ Cardiovasc Imaging. 2017;10:004976. doi: 10.1161/CIRCIMAGING.116.004976.

18. Derlin T, Toth Z, Papp L, Wisotzki C, Apostolova I, Habermann CR, Mester J, Kütmann S. Correlation of inflammation assessed by [18F]-FDG PET, active mineral deposition assessed by [18F]-fluoride PET, and vascular calcification in atherosclerotic plaque: a dual-tracer PET/CT study. J Nucl Med. 2011;52:1020–1027. doi: 10.2967/jnumed.111.087452.

19. Barnett HM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, Ferguson GG, Fox AJ, Rankin RN, Hachinski VC, Wiebers DO, et al; North American Symptomatic Carotid Endarterectomy Trial Collaborators. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. N Engl J Med. 1991;325:445–453. doi: 10.1056/NEJM199105133250701.
31. Fiz F, Morbelli S, Piccardo A, Bauckneht M, Ferrarazzo G, Pestarino E, Cabria M, Democrito A, Riondato M, Villavecchia G, et al. $^{18}$F-NaF uptake by atherosclerotic plaque on PET/CT imaging: inverse correlation between calcification density and mineral metabolic activity. *J Nucl Med*. 2015;56:1019–1023. doi: 10.2967/jnumed.115.154229

32. Derlin T, Wisotzki C, Richter U, Apostolova I, Bannas P, Weber C, Mester J, Klutmann S. In vivo imaging of mineral deposition in carotid plaque using $^{18}$F-sodium fluoride PET/CT: correlation with atherogenic risk factors. *J Nucl Med*. 2011;52:362–368. doi: 10.2967/jnumed.110.081208

33. Li X, Heber D, Cal-Gonzalez J, Karanikas G, Mayerhoefer ME, Rasul S, Beitzke D, Zhang X, Agis H, Mitterhauser M, et al. Association between osteogenesis and inflammation during the progression of calcified plaque evaluated by $^{18}$F-fluoride and $^{18}$F-FDG. *J Nucl Med*. 2017;58:968–974. doi: 10.2967/jnumed.116.182790

34. Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weisberg PL. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res*. 2000;87:1055–1062. doi: 10.1161/01.res.87.11.1055

35. Jabs WJ, Theissing E, Nitschke M, Bechtel JF, Duchrow M, Mohamed S, Jahrbeck B, Sievers HH, Steinhoff J, Bartels C. Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. *Circulation*. 2003;108:1428–1431. doi: 10.1161/01.CIR.0000121843.3176.91

36. Joshi FR, Manavaki R, Fryer TD, Figg NL, Sluimer JC, Aigbirhio FI, Davenport AP, Kirkpatrick PJ, Warburton EA, Rudd JH. Vascular imaging with $^{18}$F-fluorodeoxyglucose positron emission tomography is influenced by hypoxia. *J Am Coll Cardiol*. 2017;69:1873–1874. doi: 10.1016/j.jacc.2017.01.050

37. Liu J, Kerwin WS, Caldwell JH, Ferguson MS, Hippe D, Alessio AM, Martinez-Malo V, Pimentel K, Miyaoaka RS, Kohler TR, et al. High resolution FDG-microPET of carotid atherosclerosis: plaque components underlying enhanced FDG uptake. *Int J Cardiovasc Imaging*. 2016;32:145–152. doi: 10.1007/s10554-015-0739-2

38. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Forseca F, Nicolau J, Koenig W, Anker SD, et al; CANTOS Trial Group. Anti-inflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017;377:1119–1131. doi: 10.1056/NEJMoa1707914