18F-NaF Uptake Is a Marker of Active Calcification and Disease

Progression in Patients with Aortic Stenosis

Dweck et al: Validation of 18F-NaF Activity in AS

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DOI: 10.1161/CIRCIMAGING.113.001508

Subject Codes: Diagnostic testing:[32] Nuclear cardiology and PET, Diagnostic testing:[124] Cardiovascular imaging agents/techniques
Abstract

**Background**—18F-Sodium fluoride (18F-NaF) and 18F-fluorodeoxyglucose (18F-FDG) are promising novel biomarkers of disease activity in aortic stenosis. We compared 18F-NaF and 18F-FDG uptake with histological characterization of the aortic valve and assessed whether they predicted disease progression.

**Methods and Results**—Thirty patients with aortic stenosis underwent combined positron emission and computed tomography (PET/CT) using 18F-NaF and 18F-FDG radiotracers. In 12 patients undergoing aortic valve replacement surgery (10 for each tracer), radiotracer uptake (mean TBR) was compared to CD68 (inflammation), alkaline phosphatase and osteocalcin (calcification) immunohistochemistry of the excised valve. In 18 patients (6 aortic sclerosis; 5 mild, 7 moderate), aortic valve CT calcium scoring was performed at baseline and after 1 year. Aortic valve 18F-NaF uptake correlated with both alkaline phosphatase (r=0.65, P=0.04) and osteocalcin (r=0.68, P=0.03) immunohistochemistry. There was no significant correlation between 18F-FDG uptake and CD68 staining (r=0.43, P=0.22). After 1 year, aortic valve calcification increased from 314 (193-540) to 365 (207-934) Agatston units (P<0.01). Baseline 18F-NaF uptake correlated closely with the change in calcium score (r=0.66, P<0.01) and this improved further (r= 0.75, P<0.01) when 18F-NaF uptake overlying CT-defined macrocalcification was excluded. No significant correlation was noted between valvular 18F-FDG uptake and change in calcium score (r=−0.11, P=0.66).

**Conclusions**—18F-NaF uptake identifies active tissue calcification and predicts disease progression in patients with calcific aortic stenosis.

**Clinical Trial Registration**—URL: http://www.clinicaltrials.gov. Unique identifier: NCT01358513.

**Key Words:** aortic stenosis; positron emission tomography; computed tomography; inflammation; calcification; 18F-FDG; 18F-NaF
The mechanisms underlying aortic stenosis remain incompletely understood and the accurate prediction of disease progression remains a challenge. Calcification and inflammation are believed to play key pathophysiological roles. Indeed, the amount of established calcium in the valve correlates with disease severity and predicts future adverse cardiovascular events. Whilst computed tomography and echocardiography can provide measures of established valvular calcification, they cannot directly assess ongoing calcification activity, which is considered to be the main driver of disease progression.

Recent reports have investigated two positron emission tomography (PET) radiotracers, 18F-sodium fluoride (18F-NaF) and 18F-fluorodeoxyglucose (18F-FDG), as measures of calcification activity and inflammation respectively in the aortic valve, coronary arteries and major vessels. 18F-FDG PET has become a widely used tool for the assessment of inflammation in the aorta and carotid arteries with uptake correlating with macrophage burden. Several studies have investigated its uptake in aortic stenosis although histological validation is lacking. 18F-NaF has been used as a bone tracer for over 40 years, displaying increased activity in conditions associated with increased bone metabolism such as Paget’s disease. In bone, it is believed to bind and then incorporate into exposed hydroxyapatite crystals, via an exchange mechanism with hydroxyl groups to form fluoroapatite. Given that hydroxyapatite is also a key structural component of calcification in the aortic valve and vascular atheroma, it is presumed that similar mechanisms explain its accumulation in these tissues. However, this remains hypothetical. The principal aims of the present study were therefore to validate the use of 18F-NaF and 18F-FDG in aortic stenosis by comparing in vivo radiotracer uptake with immunohistochemistry of calcification and inflammation in excised valvular tissue, and to investigate whether either of these agents predicts disease progression at 1 year.
Methods

Patient Populations

Two cohorts of patients with aortic stenosis were recruited into this study: (i) 12 patients undergoing valve replacement surgery, and (ii) 18 patients with asymptomatic disease under surveillance at the Edinburgh Heart Centre. The latter cohort were randomly selected for repeat scanning from a larger, previously described population who underwent baseline PET imaging.4, 5, 10

All patients were aged >50 years and exclusion criteria included a normal aortic valve, insulin-dependent diabetes mellitus, end-stage renal failure, life expectancy of <2 years, and metastatic malignancy. Patients with severe AS were excluded from the cohort of patients under surveillance because of the potential for disease progression and symptom development before the follow up 1-year scan. The study was performed in accordance with the Declaration of Helsinki and after local research ethics committee approval. All patients provided written informed consent before participating.

Baseline Assessment

All patients underwent full clinical assessment at baseline and aortic stenosis severity was assessed using Doppler and two-dimensional echocardiography by means of the peak transvalvular velocity, mean gradient and aortic valve area according to American Heart Association/American College of Cardiology guidelines.11 Aortic sclerosis was defined as thickening of the aortic valve cusps in the absence of accelerated flow (<2 m/s) through the valve. Combined positron emission and computed tomography (PET/CT) scans of the aortic valve were performed using a hybrid scanner (Biograph mCT, Siemens Medical Systems, Erlangen, Germany) 60 min after administration of 125 MBq of 18F-NaF.
Subsequently a second PET/CT scan was performed using the same hybrid scanner 90 min following administration of 200 MBq of 18F-FDG. Glucose is a major energy source of the myocardium, so that intense 18F-FDG uptake frequently occurs, spilling over and contaminating the signal in the valve. We attempted to reduce myocardial uptake by asking patients to avoid carbohydrates for 24 hours prior to their 18F-FDG scan, thereby switching the myocardium from glucose to free fatty-acid metabolism. Myocardial 18F-FDG uptake was assessed within regions of interest placed in the basal septum of the left ventricle and classified as being adequately suppressed if mean SUV values were <5.0. An ECG-gated breath-hold CT scan (non-contrast enhanced, 40 mA/rot [CareDose], 100 kV) was performed for calculation of the aortic valve calcium score using dedicated analysis software (VScore, Vital Images, Minnetonka, USA) on axial scans. Particular care was taken to differentiate valvular calcium from that in the aortic root and mitral valve annulus. At one-year follow up, patients in the surveillance cohort underwent repeat clinical assessment and CT calcium scoring using the same protocol.

Quantification of Aortic Valve PET activity

18F-NaF and 18F-FDG uptake in the aortic valve was quantified using an Osirix workstation (OsiriX version 3.5.1 64-bit; OsiriX Imaging Software, Geneva, Switzerland) as reported previously. Briefly, fused PET-CT images were re-orientated into the plane of the valve and circular regions of interest (ROIs) drawn on adjacent 3-mm slices until the entire valve had been examined. For 18F-NaF, ROIs were placed around the perimeter of the valve whilst excluding the aortic root (whole-valve technique). In order to reduce the potential for myocardial 18F-FDG activity contaminating the aortic valve signal, ROIs for this tracer were drawn in the center of the valve as previously described (center-valve technique). Within these ROIs, mean standard uptake values (SUV) were calculated for
each slice, averaged and corrected for blood pool activity to provide mean tissue-to-background ratios (TBRs). Mean TBRs were selected prospectively for subsequent comparisons with histology and disease progression as this measure was felt to best represent tracer uptake across the valve as a whole.

**Distribution of 18F-NaF in the aortic valve relative to calcium scoring**

We undertook a voxel-by-voxel analysis comparing the distribution of calcium on CT with 18F-NaF uptake. Regions of interest were drawn around the valve, and each voxel was assessed for the presence of calcium (>130 HU) and increased 18F-NaF uptake (TBR max >1.97, based upon the highest uptake in the control cohort of our previous study) using dedicated software MATLAB® (Mathworks inc., Massachusetts USA). We hypothesized that regions of completely novel calcium development might have an even more important impact upon disease progression and we therefore calculated the percentage of the valve with increased radiotracer uptake in the absence of underlying calcium on CT (% of PET positive but CT negative pixels).

**Histological Assessment**

In the patients undergoing aortic valve replacement, the aortic valve was removed at the time of operation with care taken to preserve the integrity of the valve architecture.

Samples were then fixed in 4% paraformaldehyde for 24 hours. Plaques were decalcified in ethylenediaminetetraacetatic acid for 10 days, embedded in paraffin and 5-μm sections prepared. Immunohistochemical staining for osteocalcin (anti-human mouse mAb ab13418, Abcam), CD68 (anti-human mouse clone PG-M1 m0876, DAKO) and tissue non-specific alkaline phosphatase (TNAP; anti-human rabbit pAb CAT#LF PA50004, Abfrontier) was then undertaken following heat-induced epitope retrieval (HIER) using a Citrate Buffer pH 6 (Novocastra Leica microsystems) in a decloaking chamber. Osteocalcin staining required
no HIER. Sections were stained using a Leica Vision Biosystems Bond x immunostaining robot. After blocking in peroxide for 10 min, sections were incubated with the specific anti-human antibodies for 2 hours at room temperature at the following dilutions: osteocalcin 1:200, tissue non-specific alkaline phosphatase 1:100 and CD-68 1:100. All incubation steps were followed by washing in TBS/Tween. Sections for osteocalcin and CD68 were incubated for 15 min with pre-polymer/post primary followed by 15 min with polymer (HRP) for all antibodies prior to DAB (3,3'-diaminobenzidine) visualization and haematoxylin counterstain. Sections were dehydrated in graded ethanol, cleared in xylene before cover slipping in Pertex.

Images were taken on a Zeiss Axioskop2 fitted with an Axiocam MRc digital camera using Axiovision software. Tissue cross-sectional area on each section was manually delineated using Image Pro Plus 5 (Rockville, MD, USA). Immunohistochemical staining for osteocalcin and TNAP was identified by visual assessment and quantified using automated color-based segmentation by a trained observer blinded to the PET data. Staining was expressed as a percentage of the total valve area. Macrophage infiltration using CD68 was assessed using a similar approach but with an object size set threshold applied at 20 x 10 pixels, to limit counting to cell-sized objects. The density of cell staining in the valve tissue was expressed as cells per mm². This technique was also utilized to identify cellular staining for TNAP and osteocalcin.

Reproducibility Studies

Interobserver reproducibility of the immunohistochemical data was investigated. Tissue staining with alkaline phosphatase, CD68 and osteocalcin was quantified in 5 valves independently by two trained observers (WSAJ, ATV).
**Autoradiography**

Clinical PET systems have limited resolution. In order to gain further information about the precise localization of the 18F-NaF signal in aortic valve tissue, we undertook autoradiography. Non-decalcified valvular tissue was rapidly cooled in dry ice and then sectioned at 7-μm thickness using a cryostat (CM1520 Wetzlar, Germany). Sections for autoradiography were mounted on Superfrost slides (Gerhard Menzel, Braunschweig, Germany) before treatment with spray fixative. Sections were bathed in a solution of 18F-NaF at a concentration close to in-vivo imaging concentrations (1 kBq/mL) for 60 min and then rinsed with PBS. A freshly blanked phosphor screen was then placed over the slides and an overnight exposure undertaken. The screen was then read using a FujiFilm FLA-5100 Fluorescent Image Analyser (Raytek Scientific Limited, Sheffield, UK). Sections adjacent to those used for autoradiography were stained for elemental phosphate (i.e. calcium orthophosphate) using Von Kossa’s stain, and following surface decalcification in situ with Von Ebner’s solution, for TNAP and osteocalcin. Sections were then manually registered and examined for co-localization with 18F-NaF signal.

**Statistical Methods**

Continuous variables were assessed for normality both visually and using the D’Agostino-Pearson test. Variables were expressed as either mean ± standard deviation or median with interquartile ranges subject to whether they approximated a normal distribution. Categorical data were presented as n (%). The 95% normal range for differences between sets of immunohistochemical measurements (the limits of agreement) were estimated using Bland-Altman analysis by multiplying the standard deviation of the mean difference by 1.96.13 Intra-class correlation coefficients with 95% confidence intervals were calculated for inter-observer variation. Baseline and follow-up calcium scores approximated a normal distribution, and were compared using a paired t-test. However, despite attempts at data transformation, the changes in
calcium scores were not normally distributed and correlations with CT progression data were assessed using Spearman’s correlation and linear regression analysis. We acknowledge the limitations in using linear regression in the context of a non-normal distribution. A two-sided P<0.05 was regarded as statistically significant. Statistical analysis was performed with the use of Graph Pad Prism version 6.0 (GraphPad Software Inc, California USA).

Results

Histology Cohort

Twelve patients with symptomatic aortic stenosis were recruited into the histology cohort (8 male, age 76±6, peak aortic valve velocity 4.6±0.9 m/s). Patients underwent PET scanning a median of 92 days prior to surgical aortic valve replacement. Eight patients received both 18F-NaF and 18F-FDG PET scans. Additionally, two had a single 18F-NaF scan whilst two more had a single 18F-FDG scan. Thus 10 valves were available for the histological validation of each tracer. No patient suffered a significant peri-operative complication (Table 1). Effective myocardial suppression of 18F-FDG activity was achieved in 40% (median myocardial SUV 5.4, IQR 1.9-10.4).

Immunohistochemistry and Autoradiography

All valve samples displayed positive cellular staining for TNAP (225 cells/mm² valve tissue; IQR 143-328), osteocalcin (130 cells/mm² valve tissue, IQR 85-274) and CD68 (172 cells/mm² valve tissue; IQR 73-271) (Figure 1). Extensive TNAP and osteocalcin staining was also observed in the extracellular matrix, occupying approximately a sixth of the valve area sampled (17±5% and 17±7% respectively).
On autoradiography, 18F-NaF uptake was observed to co-localise closely with staining for structural calcium phosphate, TNAP and osteocalcin (Figure 1). However signal was also clearly apparent in areas free of macroscopically visible calcium thus highlighting the sensitivity of 18F-NaF in the detection of newly evolving calcification.

Reproducibility of Immunohistochemistry

Interobserver reproducibility was good for the quantification of osteocalcin and TNAP staining as well as CD68 cell counting. All observations were characterised by an absence of fixed or proportional biases, narrow limits of agreement (-13.4-9.3%, -8.0-5.0% and -7.9-9.6% respectively) and ICC values of 0.90 (0.35-0.99), 0.88 (0.60-0.97) and 0.99 (0.99-1.00) respectively (Table 2).

Correlation with radiotracer uptake

There was a good correlation between in vivo valvular 18F-NaF uptake and both alkaline phosphatase (r=0.65 (95% confidence interval: 0.03-0.90), P=0.04) and osteocalcin (r=0.68 (0.10-0.91), P=0.03; Figure 2) staining of the excised tissue. By comparison there was no association between 18F-FDG uptake and CD68 staining in the valve (r=0.43 P=0.22).

Disease Progression

Of the 18 patients (age 75±6 years, 17 male, peak aortic-jet velocity 2.6±0.9 m/s) reassessed at a median interval of 386 days (Table 1), 6 had aortic sclerosis, and 7 had mild and 5 moderate aortic stenosis. Effective myocardial suppression of 18F-FDG uptake was achieved in 66% (median myocardial SUV 3.6, IQR 2.0 – 5.4).
A correlation was observed between baseline aortic valve calcium scores on CT and 18F-NaF activity on PET (r=0.74 (0.42-0.90), P<0.001). However, as described previously the pattern of 18F-NaF uptake was distinct from the distribution of established calcium.4, 5, 14 Indeed 18F-NaF uptake in the absence of underlying calcium occupied a median of 8.3% (IQR 1.6-23.4) of the total valve area, emphasizing that 18F-NaF provides distinct and complementary information to CT calcium scoring (Figure 1).

At one year, aortic valve calcium scores increased from 314 (193-540) to 365 (207-934) AU (P<0.01). Interestingly these regions of novel calcium developed in much the same distribution as the observed baseline 18F-NaF uptake (Figure 3A-B). Indeed we observed an excellent correlation between baseline valvular 18F-NaF PET uptake and the change in calcium score after 1 year (r=0.66 (0.27-0.86), P=0.003; Figure 3C). This was similar to that observed for the current gold-standard method of prediction: the baseline calcium score (r=0.58 (0.15-0.82), P=0.01; Figure 3D) and improved further when only increased 18F-NaF uptake in the absence of underlying CT macrocalcification was considered (r= 0.75 (0.42-0.90), P=0.01). No statistically significant correlation was observed between 18F-FDG uptake and the subsequent change in CT calcium score (r=-0.11 (-0.56-0.39), P=0.66; Figure 3E).

**Discussion**

We provide the first preliminary evidence that valvular 18F-NaF uptake acts as a marker of calcification activity in patients with aortic stenosis. Not only did uptake values demonstrate a correlation with histological markers of active calcification (TNAP and osteocalcin) but they were also a good predictor of the subsequent progression in aortic valve CT calcium scores at 1 year. In contrast, 18F-FDG uptake did not correlate with CD68 staining on histology nor the progression in calcium scores. Our data indicate that 18F-NaF holds promise as a biomarker of disease activity in patients with aortic stenosis.
The pathophysiology of aortic stenosis is incompletely understood, delaying the development of biomarkers and effective medical therapies. Calcification and inflammation are thought to play a key pathological role,\textsuperscript{1} so that non-invasive markers of their activity are of interest in better understanding the etiology of this condition as well as in predicting disease progression.

Recent studies have investigated 18F-NaF PET as a marker of vascular calcification in aortic stenosis\textsuperscript{3} and atherosclerosis affecting the aorta,\textsuperscript{7} and coronary\textsuperscript{5,6} and carotid arteries.\textsuperscript{15} However this is the first study to provide histological validation of 18F-NaF uptake in vascular tissue. In bone, 18F-NaF is believed to incorporate onto the surface of hydroxyapatite crystal.\textsuperscript{14} Given that hydroxyapatite is also a key component of vascular calcification, it too has been the presumed radiotracer target in aortic stenosis and atherosclerosis. This hypothesis is supported by our autoradiography and immunohistochemical data, demonstrating a good correlation between 18F-NaF activity and osteocalcin staining: a well-recognized osteogenic protein that itself binds to hydroxyapatite.

Given that 18F-NaF binds to a structural component of vascular calcification, why then does it not simply label all regions of macrocalcification identified by CT? Indeed it is common for regions of dense calcium on CT to show no 18F-NaF uptake. This phenomenon is likely related to the available surface area of exposed hydroxyapatite crystal to which the 18F-fluoride ion can adsorb and the inactivity of established areas of calcification. 18F-NaF uptake is much greater at sites of evolving powdery microcalcification than established regions of field calcification in which the core of hydroxyapatite is internalized and therefore hidden from the 18F-NaF tracer. Thus 18F-NaF binds more readily to regions of developing calcium and acts as a marker of calcification activity providing distinct information to calcium scoring. In contrast, the latter quantifies regions of established macroscopic calcium in the valve but cannot inform whether the
process of calcification is quiescent or active. Again this hypothesis is supported by our data. We have demonstrated a strong correlation between \textit{in vivo} 18F-NaF uptake and staining for one of the key enzymes regulating mineralization: tissue non-specific alkaline phosphatase. This enzyme is expressed in the early stages of new calcium formation and is known to work by breaking down pyrophosphate: a potent inhibitor of mineralization.\textsuperscript{16} Furthermore, as one would expect from a measure of activity, baseline 18F-NaF uptake closely correlated with the subsequent change in calcium score at 1 year. Indeed 18F-NaF uptake performed as well as the current gold standard method of prediction, the degree of established calcium in the valve at baseline.\textsuperscript{2, 3} However, larger studies are now required to compare these two techniques and whilst calcium scoring may be easier to obtain, changes in the 18F-NaF PET signal are likely to occur more quickly, making it a more attractive technique with which to assess the early and more immediate effects of novel treatment strategies.

Interestingly the pattern of 18F-NaF uptake may be important, with 18F-NaF uptake remote from established macrocalcification on CT offering the best prediction of calcium score progression in our cohort. The spatial resolution of PET/CT is \textasciitilde 4 mm, and we acknowledge that the voxel-by-voxel analysis used to establish this observation is at the limit of resolution for PET imaging. Nevertheless the strong correlation with progression is of interest and indicates that further investigation of the spatial distribution of 18F-NaF uptake is warranted.

The results for valvular 18F-FDG imaging were somewhat disappointing and surprising given previous data suggesting an important role for inflammation in aortic stenosis.\textsuperscript{17} Whilst correlations between 18F-FDG uptake and macrophage burden have previously been demonstrated in regions of aortic and carotid atheroma,\textsuperscript{8} we were unable to replicate this with respect to the valve. There are several explanations for this discrepancy. The first is the close...
proximity of the valve to the myocardium. As discussed, avid uptake of 18F-FDG by the left ventricular myocardium can spill over into the aortic valve contaminating its signal. Unfortunately even despite the stringent dietary restrictions and center-valve analysis technique, it remains possible that myocardial contamination occurred, confounding the correlation with CD68 immunohistochemistry. Indeed poor myocardial suppression was achieved in the histology group perhaps reflecting their advanced disease and symptomatic status. Alternative methods have been utilized to reduce further this myocardial uptake, including administration of heparin\textsuperscript{18} and a high-fat drink prior to scanning.\textsuperscript{19} However these make the practicalities of scanning more difficult and are yet to show a clear advantage over dietary restrictions. An alternative explanation for the poor correlation with histology is that the aortic valve 18F-FDG signal relates to uptake by non-macrophage cell types within the valve, such as osteoblasts, or is governed by external factors such as hypoxia.\textsuperscript{20} In this scenario, one might still expect 18F-FDG to predict disease progression but once again this was not evident in our cohort. It would therefore appear that 18F-FDG holds less potential as a predictor of disease progression than 18F-NaF, although it remains possible that longer periods of follow-up are required to detect such an association. Indeed on occasion we also observed 18F-NaF activity that did not translate into a detectable change in calcium score at 1 year. Aortic stenosis is a slowly developing condition, so that it is likely to take time for relatively low levels of 18F-NaF or 18F-FDG uptake to translate into new areas of macrocalcification detectable on CT imaging. Larger studies with longer follow up are therefore required to address this issue, to confirm our preliminary data and to assess whether 18F-NaF PET can predict disease progression with respect to echocardiographic parameters of valvular stenosis.
Conclusion

In conclusion, we provide the first preliminary data to support 18F-NaF as a marker of valve calcification activity in aortic stenosis and as a potential method for predicting disease progression.

Acknowledgements

The study was funded by the British Heart Foundation (PG/12/8/29371). MRD, WSAJ and DEN are supported by the British Heart Foundation (CH/09/002, FS/12/84/29814, SS/CH/09/002/2636, FS/10/026). The work of JHFR is supported by the Higher Education Funding Council for England, the British Heart Foundation and the Cambridge National Institute for Health Research Biomedical Research Centre. EJRB is supported by the Scottish Imaging Network—a Platform of Scientific Excellence. The Wellcome Trust Clinical Research Facility and the Clinical Research Imaging Centre are supported by NHS Research Scotland (NRS) through NHS Lothian. We acknowledge the support of staff at the Edinburgh Heart Centre at the Royal Infirmary of Edinburgh, the radiography and radiochemistry staff of the Clinical Research Imaging Centre, and the histology staff at the Queens Medical Research Institute.

Sources of Funding

This work and Dr Marc Dweck were supported by a fellowship and project grant from the British Heart Foundation (FS/10/026 & PG/12/8/29371). Dr William Jenkins was supported by a British Heart Foundation scholarship scheme and fellowship grant (SS/CH/09/002/2636 & FS/12/84/29814).

Disclosures

None.
References


Table 1. Baseline Characteristics of Progression Cohort

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<td>Aortic Valve area (cm²)</td>
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<td>Mean Gradient (mmHg)</td>
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<td>Aortic Valve Calcium Score (AU)</td>
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<td>124 (117-127)</td>
<td>123 (117-128)</td>
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Categorical displayed as total number (percentage).
Median (inter quartile range).
CT - Computed tomography, 18F-NaF – 18F-sodium fluoride, 18F-FDG – 18F-fluorodeoxyglucose
Table 2. Histology Cohort Data

**Baseline Characteristics**

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<td>Ischaemic Heart Disease</td>
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<td>Cigarette Smoking</td>
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<td>Diabetes</td>
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<td>Serum Creatinine (μmol/L)</td>
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<td>Peak aortic valve velocity (m/s)</td>
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<td>Aortic Valve area (cm²)</td>
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<td>Mean Gradient (mmHg)</td>
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</tr>
<tr>
<td>Aortic sclerosis</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mild aortic stenosis</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Moderate aortic stenosis</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Severe aortic stenosis</td>
<td>9 (75%)</td>
</tr>
<tr>
<td>Time between 18F-NaF scan &amp; AVR (days)</td>
<td>92 (24-345)</td>
</tr>
<tr>
<td>Time between 18F-FDG scan &amp; AVR (days)</td>
<td>96 (23 – 331)</td>
</tr>
<tr>
<td>18F-FDG dose injected (MBq)</td>
<td>200 (193-209)</td>
</tr>
<tr>
<td>18F-NaF dose injected (MBq)</td>
<td>129 (119-132)</td>
</tr>
</tbody>
</table>

**In vivo aortic valve PET Data**

<table>
<thead>
<tr>
<th>PET Data</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>18F-NaF Uptake (mean TBR)</td>
<td>2.15 (1.98-2.48)</td>
</tr>
<tr>
<td>18F-FDG Uptake (mean TBR)</td>
<td>1.40 (1.31-1.76)</td>
</tr>
</tbody>
</table>

**Histology**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Osteocalcin</th>
<th>TNAP</th>
<th>CD-68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean valve area analysed (mm²)</td>
<td>234±152</td>
<td>253±116</td>
<td>190±86</td>
</tr>
<tr>
<td>% staining of the valve</td>
<td>17±7</td>
<td>17±5</td>
<td>n/a</td>
</tr>
<tr>
<td>Positive cellular staining (cells/mm²)</td>
<td>130 (85-274)</td>
<td>225 (143-328)</td>
<td>172 (73-271)</td>
</tr>
</tbody>
</table>

**Inter-observer Reproducibility**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Osteocalcin</th>
<th>TNAP</th>
<th>CD-68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Difference</td>
<td>-2.1%</td>
<td>-1.5%</td>
<td>0.8%</td>
</tr>
<tr>
<td>Limits of Agreement</td>
<td>-13.4-9.3%</td>
<td>-8.0-5.0%</td>
<td>-7.9-9.6%</td>
</tr>
<tr>
<td>ICC</td>
<td>0.88 (0.60-0.97)</td>
<td>0.90 (0.35-0.99)</td>
<td>0.99(0.99-1.00)</td>
</tr>
</tbody>
</table>
Categorical data is displayed as n (%). Normally distributed data displayed as mean±SD. Non-normally distributed data as median (inter quartile range).

AVR – aortic valve replacement, 18F-NaF – 18F-sodium fluoride, 18F-FDG – 18F-fluorodeoxyglucose.

Inter-class correlation coefficients (ICC) as value (95% confidence interval).
Figure Legends

Figure 1. Histology and 18F-NaF autoradiography of excised aortic valve tissue from patients with aortic stenosis.

A-F; Fixed, decalcified and paraffin embedded aortic valve tissue after exposure to tissue non-specific alkaline phosphatase (TNAP), osteocalcin and CD68 antibodies. Images A-C display widespread positive staining for TNAP, osteocalcin and CD68 (x4 magnification) in the extracellular matrix (ECM), which is also observed on an individual cellular level (D-F, x20 magnification) respectively.

G-I; Three adjacent and consecutive aortic valve leaflet sections displaying positive immunohistochemical staining for osteocalcin (I, x4 magnification) that co-localizes to areas of maximal 18F-NaF uptake on autoradiography (H). These likely represent areas of ongoing calcification activity, which extend beyond the areas of established calcium identified in black by Von Kossa’s stain (G, x4 magnification).

Figure 2. Correlations between in vivo aortic valve PET activity and histological markers of calcification and inflammation.

A) 18F-NaF vs. tissue non-specific alkaline phosphatase (TNAP). A good correlation was observed between the percentage aortic valve tissue staining for TNAP and the valvular 18F-NaF activity (mean tissue to background ratio, TBR); r=0.65, P=0.04.

B) 18F-NaF vs. osteocalcin. Again a strong correlation was observed between the percentage surface area of the valve stained with osteocalcin and the aortic valve 18F-NaF PET activity (mean TBR); r=0.68, P=0.03.

C) 18F-FDG vs CD68. A poor correlation was observed between CD68 staining on immunohistochemistry and 18F-FDG PET activity in the aortic valve (mean TBR); r=-0.43, P=0.22.
**Figure 3. Change in aortic valve CT calcium score and 18F-NaF PET activity after 1 year**

A-B; Co-axial short axis views of the aortic valve from 2 patients with mild aortic stenosis (top and bottom rows). On baseline CT scans (left) established regions of macro calcification appear white. Baseline fused 18F-NaF PET and CT scans (middle) show intense 18F-NaF uptake (red, yellow regions) both overlying and adjacent to existing calcium deposits on the CT. 1 year follow up CT scans (right) demonstrate increased calcium accumulation in much the same distribution as the baseline PET activity.

C-E; Predictors of progression in aortic valve calcium score. An excellent correlation was observed between baseline 18F-NaF activity in the aortic valve and the subsequent change in calcium score at 1 year $r=0.66, P<0.01$ (A). This matched the current gold standard predictor of disease progression the baseline calcium score $r=0.58, P<0.01$ (B). By contrast there was a poor correlation with 18F-FDG activity in the valve $r=0.11, P=0.66$ (C).
TNAP

% Surface area of the valve stained

Valve $^{18}$F-NaF Activity (Mean TBR)

$r=0.85$
$p=0.04$

Osteocalcin

% Surface area of the valve stained

Valve $^{18}$F-NaF Activity (Mean TBR)

$r=0.88$
$p=0.03$

CD-68

CD68 positive cells/ mm$^2$

Valve $^{18}$F-FDG Activity (Mean TBR)

$r=-0.43$
$p=0.22$
18F-NaF Uptake Is a Marker of Active Calcification and Disease Progression in Patients with Aortic Stenosis


_Circ Cardiovasc Imaging_. published online February 7, 2014;

_Circulation: Cardiovascular Imaging_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-9651. Online ISSN: 1942-0080

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