Targeting of vascular cell adhesion molecule-1 by $^{18}$F-labelled nanobodies for PET/CT imaging of inflamed atherosclerotic plaques

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Aims

Positron emission tomography–computed tomography (PET-CT) is a highly sensitive clinical molecular imaging modality to study atherosclerotic plaque biology. Therefore, we sought to develop a new PET tracer, targeting vascular cell adhesion molecule (VCAM)-1 and validate it in a murine atherosclerotic model as a potential agent to detect atherosclerotic plaque inflammation.

Methods and results

The anti-VCAM-1 nanobody (Nb) (cAbVCAM-1–5) was radiolabelled with Fluorine-18 ($^{18}$F), with a radiochemical purity of $\geq 98\%$. In vitro cell-binding studies showed specific binding of the tracer to VCAM-1 expressing cells. In vivo PET/CT imaging of ApoE$^{-/-}$ mice fed a Western diet or control mice was performed at 2h30 post-injection of $[^{18}$F]$\cdot$FB-cAbVCAM-1–5 or $^{18}$F-control Nb. Additionally, plaque uptake in different aorta segments was evaluated ex vivo based on extent of atherosclerosis. Atherosclerotic lesions in the aortic arch of ApoE$^{-/-}$ mice, injected with $[^{18}$F]$\cdot$FB-anti-VCAM-1 Nb, were successfully identified using PET/CT imaging, while background signal was observed in the control groups. These results were confirmed by ex vivo analyses where uptake of $[^{18}$F]$\cdot$FB-cAbVCAM-1–5 in atherosclerotic lesions was significantly higher compared with control groups. Moreover, uptake increased with the increasing extent of atherosclerosis (Score 0: $0.68 \pm 0.10$, Score 1: $1.18 \pm 0.36$, Score 2: $1.49 \pm 0.37$, Score 3: $1.48 \pm 0.38\%\text{ID/g}$, Spearman’s $r^2 = 0.675$, $P < 0.0001$). High lesion-to-heart, lesion-to-blood, and lesion-to-control vessel ratios were obtained (12.4 $\pm 0.4$, 3.3 $\pm 0.4$, and 3.1 $\pm 0.6$, respectively).

Conclusion

The $[^{18}$F]$\cdot$FB-anti-VCAM-1 Nb, cross-reactive for both mouse and human VCAM-1, allows non-invasive PET/CT imaging of VCAM-1 expression in atherosclerotic plaques in a murine model and may represent an attractive tool for imaging vulnerable atherosclerotic plaques in patients.

Keywords

atherosclerosis • VCAM-1 • nanobody • molecular imaging • PET/CT

Introduction

Many data support a crucial role of inflammation in the development and progression of atherosclerosis. Moreover, inflammation is involved in plaque destabilization and promotes thrombus formation. Indeed, post-mortem studies, related to acute coronary events, reveal extensive infiltration of inflammatory cells in culprit lesions. Vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells, plays an important role in the disease progression by attracting inflammatory cells (monocytes and T-lymphocytes) to the developing lesion. In advanced lesions, VCAM-1 is also expressed at the level of neovessels and may reflect the ongoing inflammation
within the plaque. These properties make VCAM-1 an attractive target for the molecular imaging of inflammation in atherosclerosis.

Nanobodies (Nbs) are the smallest antigen-binding fragments (12–15 kDa) that are derived from heavy-chain-only antibodies naturally occurring in camels. Their small size, nanomolar-range affinities, high specificity, and fast blood clearance make them appealing ligands for molecular imaging approaches and this has been shown for several experimental disease models. Moreover, a Phase I clinical trial demonstrated the applicability of Nb-based tracers in humans.

Nuclear imaging techniques are the modalities of choice for molecular imaging because of their high sensitivity. We have recently reported the use of a technetium-99 m (99mTc)-labelled nanobody (cAbBCII-10) for 20 min at room temperature, and the labelled cAbBCII-10 was incubated during 1.5 h at 37°C. To demonstrate the specific uptake of the Nb, additional in vitro binding studies using the mouse endothelial cell line bEND5 (ECACC). These cells were stimulated with 10 ng/mL tumour necrosis factor-alpha (TNF-α) for 18 h to induce VCAM-1 expression. Five nanomolars of [18F]FB-cAbVCAM-1–5 was incubated during 1.5 h at 37°C. After removal of unbound tracer, the bound fraction was collected and counted in a gamma-counter (Canberra-Packard, Downers Grove, IL, USA). Non-stimulated cells were used as negative control. Competition binding studies with a 300-fold molar excess of unlabelled Nb were performed to show the specificity of the binding. Experiments were performed in triplicate. The results were normalized to the TNF-α-negative condition.

In vitro assessment of functionality and specificity

The functionality of [18F]FB-anti-VCAM-1 Nb was assessed by cell-binding studies using the mouse endothelial cell line bEND5 (ECACC). These cells were stimulated with 10 ng/mL tumour necrosis factor-alpha (TNF-α) for 18 h to induce VCAM-1 expression. Five nanomolars of [18F]FB-cAbVCAM-1–5 was incubated during 1.5 h at 37°C. After removal of unbound tracer, the bound fraction was collected and counted in a gamma-counter (Canberra-Packard, Downers Grove, IL, USA). Non-stimulated cells were used as negative control. Competition binding studies with a 300-fold molar excess of unlabelled Nb were performed to show the specificity of the binding. Experiments were performed in triplicate. The results were normalized to the TNF-α-negative condition.

Animal model

The animal study protocol was approved by the ethical committee for animal research of the Vrije Universiteit Brussel. Female (5-week-old) ApoE−/− mice (Charles-River, L’Arbresle, France) were fed a Western diet (D12108C, Research Diets, New Brunswick, NJ, USA) for 21–25 weeks to induce atherosclerotic lesions. Lesions develop all along the aorta, but are most prevalent in the region of the ascending aorta and aortic arch. Female healthy control C57Bl/6 mice (Charles-River) were used as control mice and remained on a standard chow diet.

Ex vivo biodistribution and atherosclerotic lesion uptake

ApoE−/− mice (n = 6) and C57Bl/6 (n = 6) control mice were injected intravenously with [18F]FB-cAbVCAM-1–5 Nb (5–10 µg/7.1 ± 1.7 MBq). To demonstrate the specific uptake of the Nb, additional ApoE−/− mice were injected with [18F]FB-labeled non-targeting control Nb ([18F]FB-cAbBCII-10) (n = 6) or with 70-fold molar excess of unlabelled cAbVCAM-1–5 Nb (n = 6). Mice were sacrificed at 3 h post-injection. Organs and tissues of interest were dissected, weighed and counted against a standard of known activity. Tissue or organ uptake was calculated and expressed as percentage of injected dose per gram (%ID/g), corrected for decay. Additional biodistribution data in C57Bl/6 mice at earlier time points are added as Supplementary data.

To assess atherosclerotic lesion uptake, the aorta was dissected from aortic root to iliac bifurcation and was cut into 8–10 segments. The segments were analysed on a microscope and a lesion-extension score was given according to plaque content: (Score 0) no lesion, (Score 1) lesion covering up to 30% of the segment, (Score 2) lesions covering 30–75% of the segment, and (Score 3) lesions extending over the whole segment. Each segment was then weighed and counted for radioactivity in the gamma-counter. Lesion-to-blood and lesion-to-heart ratios were also determined. The ‘lesion’ and ‘control lesion’ were defined according to plaque content: (Score 0) no lesion, (Score 1) lesion covering up to 30% of the segment, (Score 2) lesions covering 30–75% of the segment, and (Score 3) lesions extending over the whole segment length (Score 3) and aorta segment with no atherosclerosis development (Score 0), respectively.

Methods

18F-Radiolabelling of cAbVCAM-1–5 nanobody

Nanobodies recognizing both mouse and human VCAM-1 homologues have been described previously. Herein, a lead compound, cAbVCAM-1–5, was selected as best candidate for further studies. N-Succinimidyl-4-[18F]fluorobenzoate ([18F]-SFB) synthesis consisted of a three-step, one-pot reaction by solid-phase extraction (SPE) purification as described previously. The [18F]-SFB synthesis was incorporated into a commercially available synthetic module (SynthERA® module, IBA Molecular, Belgium). Coupling of dry [18F]-SFB with cAbVCAM-1–5 Nb was performed in borate buffer (0.1 M, pH 8.5, 300 µL, 18.5 nmol Nb) for 20 min at room temperature, and the labelling mixture was purified using a PD-10 column (GE Healthcare, Belgium). The radiochemical identity and purity were assessed by size-exclusion chromatography (SEC, Superdex 75, 5/150 GL, GE Healthcare, PBS 0.3 mL min−1) and by reverse-phase high-performance liquid chromatography (RP-HPLC). For the latter, a polystyrene divinylbenzene copolymer reversed-phase column (PLRP-S 300 Å, 5 µm, 250/4 mm, Agilent) was used applying the following gradient (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile): 0–5 min 25% B, 5–7 min 25–34% B, 7–10 min 34–100% B, and 10–25 min 100% B at a flow rate of 1 mL min−1. To evaluate the stability in vitro, [18F]FB-cAbVCAM-1–5 Nb was incubated in phosphate-buffered saline (PBS) or human serum for 3 h and analysed by RP-HPLC or SEC. Additionally, urine and serum samples were obtained after intravenous injection of [18F]FB-cAbVCAM-1–5 and analysed by SEC.
PET/CT imaging

In vivo imaging was performed on a FLEX Triumph II triple-modality system (TriFoil Imaging, Chatsworth, CA 91311, USA). Four groups of animals were scanned (n = 3 per group): C57Bl/6 control mice, ApoE<sup>-/-</sup> mice, ApoE<sup>-/-</sup> mice injected with non-targeting control Nb, and ApoE<sup>-/-</sup> mice injected with 70-fold molar excess of unlabelled Nb. One hundred and fifty minutes after tracer injection (4.68 ± 2.45 MBq), the animals were placed under general anaesthesia using 2–5% isoflurane, and a 30-min PET scan was acquired. The PET images were reconstructed into a 200 × 200 × 63 matrix by a 2D maximum likelihood expectation maximization (MLEM) algorithm (LabPET Version 1.12.1, TriFoil Imaging, Chatsworth, CA, USA) using 60 iterations and a voxel size of 0.5 × 0.5 × 1.175 mm<sup>3</sup> (x, y, z). Each PET scan was followed by a CT scan using the following acquisition parameters: 256 projections, detector pixel size 50 μm, tube voltage 75 kV, tube current 500 μA, and field of view 90 mm. CT images were analytically reconstructed using the filtered back projection reconstruction software of the scanner (Cobra Version 7.3.4, Exxim Computing Corporation, Pleasanton, CA, USA) into a 256 × 256 × 512 matrix with 200 μm isotropic voxel size. Each resultant CT image was inherently co-registered with the corresponding PET scan.

Image analysis was performed using AMIDE imaging software. For quantification of the PET signal, regions of interest (ROIs) were drawn at the level of the ascending aorta and aortic arch (region with most extensive atherosclerotic lesion development) based on anatomical CT imaging. Mean standardized uptake values (SUV) were used to compare aortic uptake.

Statistical analysis

Data are expressed as mean ± standard deviation. Variables were tested for homogeneity of variance by the Levene test. Comparisons between groups were performed using the one-way ANOVA tests with corrections for multiple comparisons. Correlations between variables were evaluated using the Spearman correlation coefficient. A P-value of <0.05 was considered significant. Statistical analysis was done using SPSS Statistics software (version 22.0.0, IBM Company, Chicago, IL, USA).

Results

<sup>18</sup>F -Radiolabelling of cAbVCAM-1–5 nanobody

As direct <sup>18</sup>F-fluorination methods of proteins are difficult, the first step in <sup>18</sup>F-radiolabelling of cAbVCAM-1–5 Nb was the synthesis of the prosthetic group [<sup>18</sup>F]-SFB. [<sup>18</sup>F]-SFB was radiosynthesized and purified with 55 ± 5% yield and a radiochemical purity of >95%. After the radiolabelling reaction with cAbVCAM-1–5 Nb and purification, [<sup>18</sup>F]FB-anti-VCAM-1 Nb was obtained with a radiochemical purity of >99% (Figure 1A and B). The chemical identity of [<sup>18</sup>F]FB-cAbVCAMb1–5 was confirmed by comparison of the gamma-trace with the UV-profile of the non-radioactive cAbVCAM-1–5 Nb on RP-HPLC. The radiolabelled compound was stable in PBS over a period of at least 3 h with >98% intact [<sup>18</sup>F]FB-cAbVCAM-1–5 Nb (Figure 1C). Both in vitro and in vivo, the <sup>18</sup>F-labelled Nb showed minimal binding to serum proteins and was not degraded (Figure 1D and E). Conversely, [<sup>18</sup>F]FB-cAbVCAM-1–5 was metabolized in the kidneys, and metabolites are excreted in the urine (Figure 1F).

In vitro assessment of functionality and specificity

To assess the functionality and specificity of [<sup>18</sup>F]FB-cAbVCAM-1–5, cell-binding studies on VCAM-1 expressing bEND5 cells and VCAM-1-negative bEND5 cells were performed. To demonstrate that the binding was receptor-specific, a 300-fold excess of unlabelled cAbVCAM-1–5 Nb was added to VCAM-1-positive cells. The results showed that the binding of [<sup>18</sup>F]FB-cAbVCAM-1–5 was receptor-mediated and could be inhibited by receptor saturation (Figure 2).

Biodistribution

A summary of the biodistribution data for [<sup>18</sup>F]FB-cAbVCAM-1–5 in ApoE<sup>-/-</sup> and C57Bl/6 mice at 3 h post-injection is presented in Table 1. These data show that <sup>18</sup>F-labelled Nbs were rapidly cleared from circulation due to their fast renal clearance. An uptake of <0.5% ID/g was observed in all other tissues and organs, except for the spleen (1.76 ± 0.76 and 1.88 ± 0.34%ID/g in ApoE<sup>-/-</sup> and C57Bl/6, respectively), an organ known to express VCAM-1 constitutively. This splenic uptake was not observed in the presence of an excess of cold cAbVCAM-1–5 or for [<sup>18</sup>F]FB-cAbBCII10.

Uptake in atherosclerotic lesions

In ApoE<sup>-/-</sup> mice, [<sup>18</sup>F]FB-cAbVCAM-1–5 uptake in excised aorta segments with Score 1 (1.18 ± 0.36%ID/g), Score 2 (1.49 ± 0.37%ID/g) and 3 (1.61 ± 0.41%ID/g) was significantly higher compared with segments with Score 0 (0.68 ± 0.16%ID/g, P < 0.001), compared with the uptake in aorta segments of control mice (0.52 ± 0.20%ID/g, P < 0.0001), and compared with the uptake of the non-targeting [<sup>18</sup>F]FB-cAbBCII10 Nb in segments of all scores (Score 3: 0.33 ± 0.09%ID/g, P < 0.0001) (Figure 3). Co-injection of a 70-fold excess of unlabelled cAbVCAM-1–5 Nb in ApoE<sup>-/-</sup> mice resulted in a significant decrease in aortic uptake to baseline level (Score 3: 0.78 ± 0.27%ID/g, P = 0.001) (Figure 3). Moreover, [<sup>18</sup>F]FB-cAbVCAM-1–5 uptake in aorta segments of ApoE<sup>-/-</sup> mice correlated with the lesion-extension index (Spearman’s r<sup>2</sup> = 0.675, P < 0.0001). Fast blood clearance and low myocardial uptake of the Nb resulted in high lesion-to-blood, lesion-to-heart, and lesion-to-control vessel ratios (3.3 ± 0.4, 12.4 ± 0.4, and 3.1 ± 0.6, respectively).

PET/CT imaging

In accordance with the ex vivo biodistribution data, PET/CT images revealed for both <sup>18</sup>F-labelled Nbs high signals in the kidneys and the bladder. Signals in other organs and tissues were very low. Analysis of transversal, coronal, and sagittal PET/CT images showed accumulation of [<sup>18</sup>F]FB-cAbVCAM-1–5 at the level of ascending aorta and aortic arch of ApoE<sup>-/-</sup> mice, the region where most of the segments are scored as 3, while only background signals were observed for the control groups (Figure 4A). Hence, quantification of the mean SUV in this region revealed this difference was significant (P < 0.0001) (Figure 4B). Lesions along the abdominal aorta could not be identified due to the high renal activity.
In the present study, we have described the development and validation of a new PET tracer specific for VCAM-1, and we demonstrated the non-invasive PET/CT imaging of VCAM-1 expression in atherosclerotic plaques in a murine model. This may represent an attractive tool for clinical translation of imaging inflamed atherosclerotic plaques in patients.

Recently, we have reported the selection of the lead compound cAbVCAM-1–5 with nanomolar affinity for mouse and human VCAM-1 based on the screening of 10 Nbs derived from heavy-chain-only antibodies raised in an immunized dromedary.10

**Figure 1** Evaluation of radiochemical purity, stability and metabolization of the $[^{18}F]$FB-cAbVCAM-1–5 tracer by RP-HPLC analysis (A and C) and SEC analysis (B and D–F). (A and B) $[^{18}F]$FB-cAbVCAM-1–5 elutes as a single peak without the presence of free $^{18}$F or unreacted $^{18}$F-SFB [gamma-trace, $t_R = 12.5$ (RP-HPLC) and 8 min (SEC)]. (C and D) In vitro stability of $[^{18}F]$FB-cAbVCAM-1–5 after 3 h, respectively, in PBS (>98%) and serum (>98%). (E) In vivo stability in serum 5 min after injection. (F) Urine sample 30 min after injection showing metabolization of $[^{18}F]$FB-cAbVCAM-1–5. (Blue: Absorbance at 280 nm representing serum proteins, black: radioactive signal.)
This compound was labelled with $^{99m}Tc$ for SPECT imaging of atherosclerotic lesions in ApoE $^{-/-}$ mice and it was shown that the uptake of $^{99m}Tc$-cAbVCAM-1–5 correlated with the level of VCAM-1 expression in these lesions. Moreover, SPECT/CT imaging with this tracer was used to monitor the anti-inflammatory effects of statins. In the current study, cAbVCAM-1–5 Nb was radiolabelled with $^{18}F$, often referred to as the ‘radionuclide of choice’ for PET imaging. Compared with other imaging modalities, PET is currently the preferred clinical modality because it is the most sensitive and quantitative clinical molecular imaging technology. This feature is particularly important for the detection of molecular markers of atherosclerotic plaques where targets are commonly small and sparse.

Essential characteristics of a radiotracer for clinical translation are purity and stability, high specificity and affinity for the target, and fast blood clearance to reach high target-to-background ratios. $^{18}F$-radiolabelled cAbVCAM-1–5 Nb was obtained with high radiometal purity and remained stable and functional, both in vitro and in vivo. Atherosclerotic lesions located in the region of the ascending aorta and aortic arch of ApoE $^{-/-}$ mice were specifically identified by PET/CT imaging. To assure minimal background values in tissues such as blood and myocardium, imaging was performed at 2–3 h after injection, a time frame feasible for $^{18}F$-labelled probes. These findings make the $[^{18}F]FB$-cAbVCAM-1–5 Nb a quite attractive tracer for human imaging.

Nanobodies labelled with radiometals such as $^{99m}Tc$ typically show intense kidney retention in the proximal tubuli of the kidney.

**Table 1** Ex-vivo biodistribution data of $[^{18}F]FB$-cAbVCAM-1–5 and $[^{18}F]FB$-cAbBCII-10 Nb 3 h after injection in C57Bl/6 and ApoE $^{-/-}$ mice

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<tr>
<td>Blood</td>
<td>0.48 ± 0.20</td>
<td>0.41 ± 0.03</td>
<td>0.52 ± 0.17</td>
<td>0.31 ± 0.16</td>
<td>NS</td>
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<tr>
<td>Liver</td>
<td>0.39 ± 0.16 $^{a,b}$</td>
<td>0.28 ± 0.03</td>
<td>0.22 ± 0.06</td>
<td>0.13 ± 0.03</td>
<td>$^{a}P = 0.048$</td>
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<td>Spleen</td>
<td>1.76 ± 0.76 $^{a,b}$</td>
<td>1.88 ± 0.34</td>
<td>0.47 ± 0.06</td>
<td>0.11 ± 0.03</td>
<td>$^{a}P = 0.009$ $^{b}P = 0.003$</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.17 ± 0.07</td>
<td>0.14 ± 0.13</td>
<td>0.16 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Kidneys</td>
<td>27.84 ± 12.83 $^{a,b}$</td>
<td>3.33 ± 0.74</td>
<td>21.52 ± 6.3</td>
<td>40.64 ± 15.90</td>
<td>$^{a}P &lt; 0.0001$ $^{b}P = 0.042$</td>
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<tr>
<td>Stomach</td>
<td>0.22 ± 0.12</td>
<td>0.13 ± 0.03</td>
<td>0.18 ± 0.07</td>
<td>0.25 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Small intestines</td>
<td>0.21 ± 0.11</td>
<td>0.19 ± 0.05</td>
<td>0.14 ± 0.04</td>
<td>0.14 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Large intestines</td>
<td>0.17 ± 0.07</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Heart</td>
<td>0.13 ± 0.04</td>
<td>0.18 ± 0.05</td>
<td>0.13 ± 0.03</td>
<td>0.09 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.44 ± 0.30 $^{b}$</td>
<td>0.60 ± 0.18</td>
<td>0.39 ± 0.11</td>
<td>0.20 ± 0.07</td>
<td>$^{b}P = 0.038$</td>
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<tr>
<td>Thymus</td>
<td>0.28 ± 0.08 $^{a,b,c}$</td>
<td>0.19 ± 0.05</td>
<td>0.17 ± 0.07</td>
<td>0.11 ± 0.03</td>
<td>$^{c}P = 0.021$ $^{a}P = 0.004$ $^{b}P &lt; 0.0001$</td>
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<tr>
<td>Muscle</td>
<td>0.08 ± 0.05</td>
<td>0.07 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.06 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Bone</td>
<td>0.19 ± 0.07 $^{b}$</td>
<td>0.22 ± 0.06</td>
<td>0.16 ± 0.06</td>
<td>0.13 ± 0.06</td>
<td>$^{b}P = 0.04$</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0.27 ± 0.10 $^{b}$</td>
<td>0.19 ± 0.06</td>
<td>0.16 ± 0.06</td>
<td>0.13 ± 0.06</td>
<td>$^{a}P = 0.016$ $^{b}P = 0.003$</td>
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<tr>
<td>Salivary gland</td>
<td>0.16 ± 0.10</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Fat</td>
<td>0.08 ± 0.05</td>
<td>0.08 ± 0.05</td>
<td>0.12 ± 0.04</td>
<td>0.07 ± 0.06</td>
<td>NS</td>
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Data are expressed as mean %ID/g ± SD.

$^{a}P$-cAbVCAM-1–5 in ApoE $^{-/-}$ vs. $^{18}F$-cAbVCAM-1–5/ blocking in ApoE $^{-/-}$.

$^{a}P$-cAbVCAM-1–5 in ApoE $^{-/-}$ vs. $^{18}F$-cAbBCII-10 in ApoE $^{-/-}$.

$^{a}P$-cAbVCAM-1–5 in ApoE $^{-/-}$ vs. $^{18}F$-cAbVCAM-1–5 in C57Bl/6.
cortex by a mechanism that is at least partially mediated by the megalin-receptor system. Remarkably, the kidney retention of $^{[18F]}$FB-cAbVCAM-1–5 was much lower compared with $^{99mTc}$cAbVCAM-1–5 Nb (27.84 ± 12.83 vs. 222 ± 12%ID/g). This can be explained by renal metabolization of the $^{18F}$-labelled tracer and excretion of the metabolites via the urine. Furthermore, a higher uptake of $^{99mTc}$cAbVCAM-1–5 Nb is observed in organs with constitutive VCAM-1 expression such as spleen, thymus, lymph nodes, and bone marrow, compared with $^{[18F]}$FB-cAbVCAM-1–5 [e.g. spleen (1.76 ± 0.76 vs. 9.2 ± 1.0%ID/g), thymus (0.28 ± 0.08 vs. 1.7 ± 0.1%ID/g)]. We speculate that these differences are partly due to different specific activities of the tracers next to different biophysical and chemical properties of the radiolabelled compounds in vivo. Nevertheless, comparable lesion-to-blood and lesion-to-heart ratios were observed.

The abundant presence of macrophages is a hallmark feature of potential vulnerable plaques. Over the past decade, there has been increasing interest in the development of molecular imaging methods capable of imaging the extent of plaque inflammation that could potentially provide powerful predictive information on future cardiovascular events. VCAM-1 expression has been most preferentially studied because of its function to recruit inflammatory cells to the arterial intima and because its expression pattern could reflect the degree of plaque inflammation. Indeed, several molecular imaging strategies targeting VCAM-1 in the context of atherosclerosis have been demonstrated using ultrasound, magnetic resonance, optical or nuclear imaging. To this purpose, Nahrendorf et al. have previously developed a multimeric peptide-based tracer, $^{18F}$-4V, for PET/CT imaging of VCAM-1 expression. Comparable results with the present study were obtained in terms of lesion-to-normal vessels, lesion-to-blood, and lesion-to-myocardium ratios.

Besides the anti-VCAM-1 Nb presented in this study, many other Nb-based tracers have been developed and validated in preclinical models for the imaging of a variety of specific disease-related biomarkers. They have been successfully labelled with different radio-isotopes ($^{68Ga}$, $^{99mTc}$, $^{18F}$, $^{111In}$), near infrared dyes, microbubbles, or gadolinium vesicles allowing their use for multiple imaging modalities such as SPECT, PET, optical imaging, ultrasound, or MRI. The Nb technology represents thus a versatile tool for molecular imaging with great potential for clinical applications. Hence, a Phase I clinical trial has been finalized successfully regarding the application of a $^{68Ga}$-labelled HER2-specific Nb as PET tracer to screen breast cancer patients for HER2 expression. Moreover, preparations for a Phase I clinical trial with cAbVCAM-1–5 Nb have already been initiated as the current tracer is being produced complying with Good Manufacturing Practice (GMP) requirements.

In clinical research $^{18F}$-FDG has been widely used for imaging of plaque inflammation in carotid lesions. However, coronary artery imaging remains extremely challenging due to high myocardial background that is generally greater than any signal originating from a plaque. The low myocardial uptake of $^{[18F]}$FB-cAbVCAM-1–5 Nb with a high lesion-to-myocardium ratio renders it a promising tracer for potential molecular imaging of coronary atherosclerosis in patients. Indeed, the feasibility of coronary molecular imaging in patients using PET/CT has already been shown using $^{18F}$-sodium fluoride ($^{18F}$-NaF) as a tracer of active microcalcification in atherosclerotic plaques or $^{18F}$-FLT, a thymine analogue accumulating in proliferating cells. However, further studies are needed to evaluate the impact of these findings on patient management and treatment.

**Study limitations**

The present approach of PET/CT imaging of VCAM-1 expression requests some considerations. The majority of thrombotic events in culprit lesions are caused by plaque rupture. These plaques show typically intensive infiltration of inflammatory cells. However,
20–30% of coronary thrombi evolve from plaques that do not have a large lipid core or significant inflammation but have superficial endothelial erosions. This subset of high-risk plaques might be missed by the current method.

In this study, we were able to visualize plaque inflammation in a murine model of atherosclerosis. However, atherosclerotic lesions of murine models differ from human pathology. \[^{[18F]}\text{FB-cAbVCAM-1–5 Nb}\] imaging of plaque inflammation in human coronary and carotid arteries remains to be further investigated.

**Conclusion**

The present study demonstrates a sensitive and specific method for non-invasive PET/CT imaging of VCAM-1 expression within atherosclerotic lesions, which may provide important information to characterize atherosclerotic plaque inflammation. This preclinical validation study holds the potential to be translated in patients. Further clinical studies are needed to put this technology a step forward in our quest for imaging potential vulnerable plaques.

**Supplementary data**

Supplementary data are available at *European Heart Journal – Cardiovascular Imaging* online.

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