Imaging Macrophage and Hematopoietic Progenitor Proliferation in Atherosclerosis


Rationale: Local plaque macrophage proliferation and monocyte production in hematopoietic organs promote progression of atherosclerosis. Therefore, noninvasive imaging of proliferation could serve as a biomarker and monitor therapeutic intervention.

Objective: To explore 18F-FLT positron emission tomography–computed tomography imaging of cell proliferation in atherosclerosis.

Methods and Results: 18F-FLT positron emission tomography–computed tomography was performed in mice, rabbits, and humans with atherosclerosis. In apolipoprotein E knock out mice, increased 18F-FLT signal was observed in atherosclerotic lesions, spleen, and bone marrow (standardized uptake values wild-type versus apolipoprotein E knock out mice, 0.05±0.01 versus 0.17±0.01, P<0.05 in aorta; 0.13±0.01 versus 0.28±0.02, P<0.05 in bone marrow; 0.06±0.01 versus 0.22±0.01, P<0.05 in spleen), corroborated by ex vivo scintillation counting and autoradiography. Flow cytometry confirmed significantly higher proliferation of macrophages in aortic lesions and hematopoietic stem and progenitor cells in the spleen and bone marrow in these mice. In addition, 18F-FLT plaque signal correlated with the duration of high cholesterol diet (r²=0.33, P<0.05). Aortic 18F-FLT uptake was reduced when cell proliferation was suppressed with fluorouracil in apolipoprotein E knock out mice (P<0.05). In rabbits, inflamed atherosclerotic vasculature with the highest 18F-fluorodeoxyglucose uptake enriched 18F-FLT. In patients with atherosclerosis, 18F-FLT signal significantly increased in the inflamed carotid artery and in the aorta.

Conclusions: 18F-FLT positron emission tomography imaging may serve as an imaging biomarker for cell proliferation in plaque and hematopoietic activity in individuals with atherosclerosis. (Circ. Res. 2015;117:835-845. DOI: 10.1161/CIRCRESAHA.115.307024.)

Key Words: atherosclerosis ■ 18F-fluorothymidine ■ imaging ■ inflammation ■ macrophage ■ positron emission tomography ■ proliferation

Inflammatory monocytes and macrophages are innate immune cells that promote the growth and complication of atherosclerotic lesions. Once recruited to the arterial wall, mononuclear phagocytes can ingest lipoproteins. Often, the cells produce proinflammatory mediators and differentiate into foam cells. Activated macrophages also elaborate proteases that weaken the plaque’s extracellular matrix.1,2 In early-stage atherosclerosis in mice, most plaque macrophages are direct progeny of recruited blood monocytes that originate in the bone marrow and spleen. In advanced disease, monocyte-derived macrophages proliferate locally, a process that contributes dominantly to the cell population in mature plaques.3 Hence, in early and in late disease stages, cellular proliferation pivotally promotes expansion of both the systemic and local

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myeloid cell pools. By extension, proliferation likely also drives disease progression. Hematopoietic cell proliferation, either remote in bone marrow and spleen or locally in plaque, may thus represent a therapeutic target.

Commonly, we rely on ex vivo cell cycle analysis to measure cellular proliferation. These assays have limited use for assessing proliferative tissue activity in patients with atherosclerosis because the tissues of interest are not readily available for biopsy. With few exceptions, we are currently limited to investigating circulating leukocytes. However, these cells do not proliferate, and monocyte numbers in blood may not faithfully reflect proliferation in plaque or hematopoietic organs. In a hypothetical scenario, plaque macrophage proliferation may be high, despite normal blood monocyte levels, resulting in inflamed vascular lesions that are prone to complications. An imaging biomarker that reports on proliferating cells would expand diagnostic capabilities to disease-relevant tissues and aid development of emerging cardiovascular immuno-modulatory drugs.

Imaging of cancer cells has explored the use of such a biomarker.\(^{18}\)\(^{18}\)F-FLT, a positron emission tomography (PET) isotope-labeled thymidine analog, enriches in proliferating cells. Phosphorylation traps the agent intracellularly. Thymine is one of the 4 bases needed for DNA replication that occurs before cell division. The small molecule PET agent \(^{18}\)F-FLT has undergone clinical exploration for assessing proliferation, and its retention correlates with the histological cell cycle marker Ki67 in tumor cells, especially in brain, lung, and breast cancer.\(^6\) In oncology, \(^{18}\)F-FLT PET imaging has emerged as a promising strategy to monitor treatment response for some cancers that rely more on the cell proliferation, an additional cohort of ApoE\(^{-/-}\) mice was treated with \(^{18}\)F-FLT at the dose of 150 \(\mu\)g/g IP 12 hours before imaging). To investigate whether the \(^{18}\)F-FLT uptake in atherosclerosis reflects cell proliferation, an additional cohort of ApoE\(^{-/-}\) mice was treated with \(^{18}\)F-FLT at the dose of 150 \(\mu\)g/g IP 12 hours before imaging.

Mice
Female ApoE\(^{-/-}\) and wild-type C57BL/6 mice (10–12 weeks old) were purchased from Jackson Laboratories. Atherosclerosis was induced by feeding high-cholesterol diet to ApoE\(^{-/-}\) mice ranging from 10 to 28 weeks. In wild-type mice, modulation of hematopoietic progenitor cell proliferation in bone marrow and the spleen was induced by injecting lipopolysaccharide (10 \(\mu\)g/mouse IP on 3 and 1 days before imaging) and \(^{5}\)FU (150 \(\mu\)g/g IP 12 hours before imaging). For cell cycling analysis by flow cytometry, BrdU (DB Biosciences), an antibody-recognizable analog of thymidine, was injected IP in these ApoE\(^{-/-}\) mice immediately after \(^{18}\)F-FLT injection. All procedures in mice were approved by the Institutional Animal Care and Use Committee Subcommittee on Research Animal Care, Massachusetts General Hospital, Charlestown, MA.

Rabbits
Atherosclerosis was induced in 4 male New Zealand White rabbits by a combination of high-cholesterol diet and double balloon injury of the abdominal aorta.\(^5\) Rabbits were imaged 6 months after diet initiation. Four control rabbits received normal diet. All procedures in rabbits were approved by the IACUC at Mount Sinai Hospital, New York City, NY.

Small Animal PET–Computed Tomography Imaging and Image Analysis
\(^{18}\)F-FLT was injected via tail vein 120 minutes before in vivo imaging with an average dose of 22.4±1.07 MBq. This dose and imaging time point were chosen based on published biodistribution data and because mice have high circulating thymidine levels that compete with the PET agent for uptake into proliferating cells.\(^7\) Mice were imaged with an Inveon small-animal PET-computed tomography (CT) scanner (Siemens Medical Solutions, Inc, Malvern, PA). CT was performed before PET, acquiring 360 cone beam projections (source power 80 keV and current 500 \(\mu\)A). During CT acquisition, iodine contrast was infused via the tail vein at a rate of 35 \(\mu\)L/min. For quantitative analysis, 1 to 3 regions of interest were drawn manually in the aortic root, the spleen, and the spine of each animal, guided by CT images. After in vivo imaging, mice were euthanized and the direct \(\gamma\) counting was performed on the aortic root, the spleen, and a femur. The data are presented as percent injected dose per gram of tissue (%IDGT).

Rabbit Imaging
Animals were subjected to high-resolution 3-dimensional T2-weighted magnetic resonance imaging with a 7-tesla scanner (Magnetom 7T, Siemens Healthcare, Erlangen, Germany). To establish \(^{18}\)F-FLT’s pharmacokinetics, animals were injected with \(\approx\)111 MBq, and blood was drawn at 2, 5, 15, 30, 60, 120, and 240 minutes. Radioactivity was measured by a Wizard 2470 Perkin Elmer
automatic gamma counter. Values were calculated as %IDGT. All 8 rabbits (average weight 3.47±0.37 kg) underwent 2 PET/CT scans on 2 different days. The first PET/CT was performed after injection of 18F-FDG, whereas the second PET/CT was performed after the injection of 18F-FLT. For both imaging sessions, rabbits were fasted for 4 hours before the isotope injection (water was provided at libitum). Before injection, rabbits were appropriately restrained, and the isotope was injected through a catheter placed in a marginal ear vein. The average injected dose of FDG was 190.3±9.9 MBq (55.2±5.0 MBq/kg), whereas the average injected dose of FLT was 199.2±29.1 MBq (57.5±8.0 MBq/kg). Rabbits were imaged ≈200 minutes after isotope injection (18F-FDG, 197±5.50 minutes; 18F-FLT, 198.9±10.5 minutes). Approximately 15 minutes before imaging, rabbits were anesthetized using an injection of ketamine (35 mg/kg) and xylazine (5 mg/kg), and the bladder was emptied. Anesthesia was maintained during imaging using 1% isofluorane breathing anesthesia. PET/CT was performed using a clinical scanner (Siemens Biograph mCT, PET/CT, Siemens, Erlangen, Germany), in 3D mode, using 1 bed position, for 60 minutes; 18F-FDG, 197.5±5.5 minutes; 18F-FLT, 198.9±10.5 minutes). Approximately 15 minutes before imaging, rabbits were anesthetized using an injection of ketamine (35 mg/kg) and xylazine (5 mg/kg), and the bladder was emptied. Anesthesia was maintained during imaging using 1% isofluorane breathing anesthesia. PET/CT was performed using a clinical scanner (Siemens Biograph mCT, PET/CT, Siemens, Erlangen, Germany), in 3D mode, using 1 bed position, for 60 minutes. Images were acquired from the thoracic aorta down to the iliac bifurcation. A noncontrast enhanced CT was also performed for attenuation correction of the PET images and to identify the aorta.

### Human 18F-FDG-PET/CT and 18F-FLT-PET/CT

Twenty patients with paired 18F-FDG-PET/CT and 18F-FLT-PET/CT scans performed between December 2011 and March 2013 were retrospectively included. Ten patients with high risk of cardiovascular disease (atherosclerotic group) and 10 patients with low risk (control group) were enrolled based on their Framingham Score (Table). The study was performed in accordance with the Helsinki Declaration and approved by the regional scientific ethical committee (H-3-2011-092). All patients were diagnosed with neuroendocrine tumors, and written informed consent was obtained from all participants. Patients underwent an 18F-FDG-PET/CT and an 18F-FLT-PET/CT within 2 weeks of each other performed on a Siemens Biograph 40 or 64 PET/CT scanner (Siemens Medical Systems, Erlangen, Germany). Patients were instructed to fast at least 6 hours before tracer injection, and static images were acquired 1 hour postinjection of either 18F-FDG or 18F-FLT. Reconstructed images were analyzed using the OsiriX Lite open-source software (Pixmeo). Image analysis was done in a blinded fashion on anonymized scans. Tracer uptake was quantified as maximum and mean standardized uptake values (SUV max and SUV mean). Regions of interest were drawn on all slices of the ascending aorta and at least 10 consecutive slices of the carotid artery. Averaged SUV max and SUV mean were then calculated for each patient in the 2 target regions based on SUV max and SUV mean values from all regions of interest in the respective region. To correlate the uptake of 18F-FDG and 18F-FLT on a subregional level, matched slices of 18F-FDG and 18F-FLT scans were analyzed side-by-side in the ascending aorta.

### Results

#### Arterial Uptake of 18F-FLT Increases in ApoE−/− Mice

We began by exploring the uptake of 18F-FLT in the thoracic aorta of ApoE−/− mice that consumed a high-fat diet. In these mice, the aortic root harbors plaques inhabited by proliferating macrophages. ApoE−/− but not wild-type mice had areas of increased PET signal that colocalized with the aortic root and the ascending aorta on CT angiography (Figure 1A). The PET-derived SUV measured in the aortic root in ApoE−/− mice significantly exceeded that in wild-type mice (wild-type controls versus ApoE−/− mice, 0.053±0.010 versus 0.169±0.013, P<0.05; Figure 1B). We further observed signal in the thoracic vertebrae and the sternum in both cohorts, a finding that stimulated further exploration. Ex vivo data obtained by scintillation counting (%IDGT, wild-type controls versus ApoE−/− mice, 0.214±0.015 versus 0.376±0.037, P<0.05; Figure 1C) and autoradiography (Figure 1D) confirmed higher activity in the aortae excised from ApoE−/− mice, and peak signal colocalized with Oil Red O stained atherosclerotic lesions. As reported previously, the aortic root contained numerous Ki67+ CD68+ macrophages (Figure 1E).3 Macrophages contributed 73.5±2.8% of all proliferating Ki67+ cells in the aortic wall. We found significantly less proliferating endothelial cells,
smooth muscle cells, and T lymphocytes in the atherosclerotic aortic root (Figure 1E and 1F). The fraction of proliferating macrophages was quantified in digested whole aortae by flow cytometric analysis. Significantly more macrophages were in the active S/G2/M phases of the cell cycle in aortae harvested from ApoE−/− mice (Figure 1G and 1H). Previously, we had described that local macrophage proliferation increases in mature atherosclerotic lesions.3 In line with these data, we found a positive correlation between scintillation counts in aortae of mice injected with 18F-FLT and the duration of high cholesterol diet (HCD) measured in wild-type (HCD=0) and ApoE−/− aortae (HCD>0). Data are mean±SEM. *P<0.05, Student t test.

18F-FLT Signal in Hematopoietic Organs of ApoE−/− Mice

The thymidine analog 18F-FLT enriches in organs with high proliferative rates, including the hematopoietic bone marrow.5 Intrigued by our observation of high PET signal in the sternum and vertebrae and because the hematopoietic system supplies monocytes to inflamed atherosclerotic plaque, we systematically compared 18F-FLT uptake in the bone marrow and spleen of ApoE−/− mice to wild-type controls (Figure 3A). In vivo SUV in bone marrow rose in mice with atherosclerosis (wild-type controls versus ApoE−/− mice, 0.132±0.013 versus 0.276±0.020, P<0.05; Figure 3B)
as did activity detected by ex vivo scintillation counting (%IDGT of the femur, wild-type controls versus ApoE−/− mice, 0.735±0.053 versus 1.261±0.114, P<0.05; Figure 3C). These observations could result from higher thymidine uptake, DNA synthesis, and proliferation of HSPC. Indeed, flow cytometric analysis paralleled the in vivo PET data.

Figure 2. Fluorouracil (5-FU) treatment reduced 18F-FLT uptake in murine atherosclerotic plaque. A, Positron emission tomography–computed tomography (PET-CT) images show reduced 18F-FLT uptake in atherosclerotic lesions (arrow) of ascending aorta in an apolipoprotein E knock out (ApoE−/−) mouse after 5-FU injection compared with the control ApoE−/− mouse with PBS injection. Arrow heads indicate sternum and vertebrae. B and C, Bar graphs show in vivo standardized uptake values (SUV; WT, n=9; ApoE−/−, n=5) and ex vivo percent injected dose per gram of tissue in the aorta (WT, n=10; ApoE−/−, n=6). Flow cytometry plots (D) and quantitative data (E) show significantly lower proliferation of macrophages in aortae after 5-FU treatment (WT, n=4; ApoE−/−, n=6). Data are mean±SEM. *P<0.05, Student t test.

Figure 3. Increased uptake of 18F-FLT in bone marrow and spleen of apolipoprotein E knock out (ApoE−/−) mice. A, In vivo positron emission tomography–computed tomography (PET-CT) shows higher uptake of 18F-FLT in the spine of an ApoE−/− when compared with a wild-type mouse (WT). In vivo standardized uptake values (SUV; WT, n=5; ApoE−/−, n=13; B) and ex vivo percent injected dose per gram of tissue (%IDGT; WT, n=5; ApoE−/−, n=15; C) in bone marrow. Flow cytometry plots (D) and quantitative cell cycle (E) analysis of lineage− Sca-1+ c-Kit+ (LSK) cells in bone marrow of ApoE−/− and wild-type mice (WT, n=12; ApoE−/−, n=10). F, Representative in vivo PET-CT images of spleen. In vivo SUV (WT, n=5; ApoE−/−, n=13; G) and ex vivo scintillation counting of spleens from ApoE−/− and wild-type mice (WT, n=5; ApoE−/−, n=15; H). Flow cytometry plots (I) and quantitative cell cycle analysis (J) of LSK cells in spleens of ApoE−/− and wild-type mice (WT, n=8; ApoE−/−, n=10). Data are presented as mean±SEM. *P<0.05, Student t test.
because more lineage− Sca-1+ c-Kit+ (LSK) progenitor cells were in the S/G2/M phase of the cell cycle (Figure 3D and 3E). Examination of the spleen yielded a similar pattern: both in vivo PET signal (SUV, wild-type controls versus ApoE−/− mice, 0.059±0.007 versus 0.224±0.013, \( P < 0.05 \); Figure 3F and 3G), and ex vivo scintillation counting of the organ (%IDGT, wild-type controls versus ApoE−/− mice, 0.455±0.065 versus 1.345±0.134, \( P < 0.05 \); Figure 3H) revealed higher 18F-FLT uptake. As in the marrow, the higher tracer uptake paralleled increased cycling of LSK in the spleen of ApoE−/− mice (Figure 3I and 3J). These data indicate that 18F-FLT reflects increased hematopoietic activity in the marrow and spleen of mice with atherosclerosis, in accordance with the previous description of increased bone marrow hematopoiesis in atherosclerotic mice\(^{12} \) and the reactivation of extramedullary, that is, splenic hematopoiesis in atherosclerosis.\(^{13} \)

**Hematopoietic Activation With a TLR Ligand Increases 18F-FLT Signal**

Hematopoiesis provides billions of blood cells every day and increases the production of leukocytes according to numerous stimuli. For instance, HSPCs express TLR4 on their surface, and TLR4 ligation increases proliferation, differentiation, and output of mature cells, including monocytes.\(^{14} \) We therefore hypothesized that the changes observed in hematopoietic organs of mice with atherosclerosis could be reproduced with lipopolysaccharide, a bacterial TLR4 ligand that strongly stimulates hematopoiesis.\(^{14} \) Wild-type mice that received lipopolysaccharide showed increased bone marrow uptake of 18F-FLT (Figure 4A and 4B) that paralleled results of ex vivo scintillation counting (Figure 4C). In accordance with the observed uptake of 18F-FLT, a higher fraction of LSK was in the active cell cycle phase after lipopolysaccharide injection (Figure 4D and 4E). TLR4 ligands induce HSPC migration from the bone marrow to the spleen, where the cells seed extramedullary niches. Indeed, splenic uptake of 18F-FLT was increased, as observed by in vivo imaging (Figure 4F and 4G) and ex vivo scintillation counting (Figure 4H). In parallel, the number of cycling LSK increased dramatically in spleens of mice that received lipopolysaccharide (Figure 4I and 4J).

**Dampening of Hematopoiesis With 5-FU Decreases 18F-FLT Signal**

Next we explored whether 18F-FLT signal might also monitor a decrease of hematopoietic activity. To test this hypothesis, we treated wild-type mice with 5-FU, a clinical drug that is frequently used in hematology research to ablate cycling stem cells. Previously, 5-FU reduced 18F-FLT signal in tumor-bearing mice.\(^{15} \) Treatment with 5-FU reduced bone marrow...
uptake of 18F-FLT measured in vivo (Figure 5A and 5B) and ex vivo (Figure 5C). As expected, 5-FU decreased proliferation of bone marrow LSK (Figure 5D and 5E). The spleen showed a similar pattern: 5-FU treatment reduced 18F-FLT signal (Figure 5G and 5H) and the frequency of proliferating LSK in parallel (Figure 5I and 5J).

In Vitro 18F-FLT Uptake
To better understand cellular 18F-FLT uptake, we pursued in vitro incubation of the tracer in primary murine cells. Peritoneal macrophages were isolated by negative magnetic bead selection and either treated with 5-FU or not. Scintillation counting revealed that macrophages that were not treated with 5-FU showed higher uptake of the PET tracer (Figure 6A). Fluorescence-activated cell sorter analysis of these samples reported decreased staining for the proliferation marker Ki67 (Figure 6B and 6C). Further, we used positive magnetic bead selection of murine bone marrow to enrich for c-kit+ progenitor cells and Ly6G+ neutrophils. Incubation of these cells with 18F-FLT was followed by scintillation counting of these samples reported decreased staining for the proliferation marker Ki67 (Figure 6B and 6C). Further, we used positive magnetic bead selection of murine bone marrow to enrich for c-kit+ progenitor cells and Ly6G+ neutrophils. Incubation of these cells with 18F-FLT was followed by scintillation counting. We detected higher uptake of 18F-FLT into c-kit+ cells (Figure 6D), and flow cytometry documented their higher proliferative activity when compared with Ly6G+ cells (Figure 6E and 6F). Of note, these data do not confer specificity of the PET tracer to progenitor cells but rather indicate that proliferating cells, including c-kit+ progenitor cells, have a higher propensity to take up the imaging agent.

18F-FLT PET in Rabbits With Atherosclerosis
To study 18F-FLT plaque uptake in a different species, we subjected 4 rabbits with atherosclerosis and balloon injury and 4 control rabbits without atherosclerosis to PET/CT imaging. Magnetic resonance imaging confirmed that the treatment induced robust atherosclerotic lesions in the infrarenal aorta (Figure 7A). Rabbits first underwent 18F-FDG PET imaging, which showed a heterogeneous pattern of inflammatory activity (Figure 7B). As expected, the 18F-FDG signal was significantly higher in the aortic segments of rabbits with atherosclerosis (SUV mean controls, 0.20±0.002; atherosclerosis cohort, 0.58±0.02, \(P<0.0001\)). Immunohistochemistry of antirabbit macrophage (RAM11) staining demonstrated abundant macrophages in the atherosclerosis of these rabbits (Figure 7C). Previous correlation of 18F-FDG to histology in atherosclerotic rabbits and in humans showed that 18F-FDG PET signal increases with higher macrophage plaque burden. Within 48 hours of the 18F-FDG PET, rabbits were rescanned after injection of 18F-FLT, enabling a comparison of both PET agents. Pharmacokinetic experiments revealed a blood half-life of 20.36±3.54 minutes for 18F-FLT in atherosclerotic rabbits and an optimal injection-imaging sequence of 200 minutes. The aortic 18F-FLT signal was higher in rabbits with atherosclerosis when compared with control rabbits without atherosclerosis (Figure 7D and 7E). We used the 18F-FDG data to classify 3 aortic regions in rabbits with atherosclerosis: low (SUV<0.4), intermediate (0.4–0.6), and high-grade...
showed increased vascular uptake of both PET agents in aging (Figure 8B and 8C). Analysis of the ascending aorta frequency of Ki67+ macrophages by flow cytometry. B and dot plots of flow cytometry (C) show minutes. Bar graph (D) and dot plots (E) of flow cytometry showing frequency of cells in S/G2/M phase for Ly6G+ versus c-kit+ cells. Experiments were done in triplicates. Data are mean±SEM. *P<0.05, Student t test.

**Figure 6.** 18F-FLT uptake into myeloid and progenitor cells. A, Gamma counting of fluorouracil (5-FU) treated and nontreated control peritoneal macrophages after 18F-FLT incubation for 60 minutes. Bar graph (B) and dot plots of flow cytometry (C) show frequency of Ki67+ macrophages by flow cytometry. D, Gamma counting of bone marrow c-kit+ and Ly6G+ cells after 18F-FLT incubation for 60 minutes. Bar graph (E) and dot plots (F) of flow cytometry showing frequency of cells in S/G2/M phase for Ly6G+ versus c-kit+ cells. Experiments were done in triplicates. Data are mean±SEM. *P<0.05, Student t test.

inflammation (SUV>0.6). The vascular territories that were classified as high-grade inflammation showed the highest 18F-FLT signal (Figure 7F and 7G). Linear regression analysis in aortic segments of rabbits with atherosclerosis revealed that 18F-FLT and 18F-FDG signal were weakly positively correlated in rabbits, linear regression analysis in aortic segments showed a weak positive correlation between 18F-FLT and 18F-FDG uptake (Figure 8 L).

**18F-FLT PET in Humans With Atherosclerosis**

To assess the potential of 18F-FLT for PET imaging of proliferation in human atherosclerotic plaque, we performed a retrospective analysis of 10 patients with high risk of cardiovascular disease (atherosclerosis group) and 10 patients with a low cardiovascular risk profile (control group) that were originally imaged because of malignant disease and stratified by Framingham Scoring (Table). We found that patients with high cardiovascular risk showed intense calcification, indicative of atherosclerotic lesions (Figure 8A). Patients had undergone both, 18F-FDG and 18F-FLT PET imaging (Figure 8B and 8C). Analysis of the ascending aorta (Figure 8D–8G) and the carotid artery (Figure 8H–8K) showed increased vascular uptake of both PET agents in the individuals with high cardiovascular risk. As observed in rabbits, linear regression analysis in aortic segments showed a weak positive correlation between 18F-FLT and 18F-FDG uptake (Figure 8 L).

**Discussion**

Why are we interested in imaging macrophage proliferation? In inflamed tissue, these cells have a short to intermediate life span, ranging from hours to weeks. In mice with atherosclerosis, the entire plaque macrophage population turns over in 1 month. Provision of mononuclear phagocytes influences atherosclerosis progression and plaque characteristics. Depleting macrophages and monocytes or inhibiting their recruitment to plaque reproducibly diminishes atherosclerosis in animals. Although several interventions may influence macrophage proliferation, noninvasive imaging reporting on this process could markedly facilitate drug development by informing dose selection and providing an early biomarker of effective targeting. Oncology and neurodegenerative research adopted such companion-imaging strategies for rapid, noninvasive feedback on the targeted pathway or molecule in small numbers of patients. Such information would inform study design and the triage of candidates to advance into large-scale trials, enabling a nimble drug development strategy.

Tissue-resident macrophages proliferate everywhere. All major organ systems, including the vasculature, heart, and brain, host a network of noninflammatory stromal macrophages. These cells have as of yet unclear functions, but they may promote tissue homeostasis, defense, and regeneration. We found that most healthy organs had low 18F-FLT baseline uptake, likely reflecting the low proliferative rate of stromal cells and tissue macrophages. The bone marrow, intestine, kidney, and liver furnish notable exceptions, as reported previously. These organs have high baseline proliferative activity or participate in the excretion of 18F-FLT.

HSPCs increase proliferative activity in the bone marrow in atherosclerosis after myocardial infarction and after stroke. Circulating danger signals may also directly alert HSPCs to remote organ ischemic or other injury. Compromised reverse cholesterol transport induces increased HSPC activity, linking metabolism to monocyte production. Monocyte numbers in blood then rise with progressing hyperlipidemia and associate with poor prognosis. Cardiovascular disease also reactivates splenic leukocyte production. Gradually in mice with atherosclerosis, and rapidly after ischemia, HSPCs seed the splenic red pulp and expand monocyte production. Splenic HSPC retention relies on vascular cell adhesion molecule-1 expression by CD169+ macrophages. GM-CSF, M-CSF, and interleukin-3 mediate splenic myelopoiesis. Indeed, increased splenic and bone marrow 18F-FDG uptake associates with higher cardiovascular event rates in patients. The decisive contribution of local proliferation to the enlarged macrophage pool in atherosclerotic lesions engendered interest in this process. In mice with mature lesions, genetic deficiency of scavenger receptor A reduces incorporation of the thymidine analog BrdU into lesional macrophages.
These pathways may have contributed to the increased $^{18}$F-FLT PET signal observed in the present study, and their exploration as therapeutic targets will benefit from an imaging biomarker, such as $^{18}$F-FLT PET.

Rabbits with atherosclerosis displayed increased $^{18}$F-FLT signal in vascular territories with high $^{18}$F-FDG uptake. However, the correlation between both tracers in aortic segments of atherosclerotic rabbits and patients was weak. $^{18}$F-FDG is trapped inside cells with high glucose uptake and correlates with macrophage number in the atherosclerotic plaque in rabbits and humans. $^{16,17}$ Macrophage accumulation depends on increased proliferation and on monocyte recruitment. The observation of matching tracer uptake in some aortic segments supports that $^{18}$F-FLT enriches in plaques with high macrophage proliferation because cell growth and proliferation are energy-intensive processes. In distinction to $^{18}$F-FLT, $^{18}$F-FDG is not specific for proliferating cells. Thus, some aortic segments high in $^{18}$F-FDG may have a high glucose uptake but low proliferating rates. Taken together, $^{18}$F-FLT may be a useful to study macrophage supply while reducing proliferation of hematopoietic cells. Myocardial uptake of $^{18}$F-FDG can interfere with coronary artery imaging, a limitation that does not occur with $^{18}$F-FLT.

As indicated by its use for imaging proliferating cancer cells, $^{18}$F-FLT is not specific for macrophages or hematopoietic progenitor cells. Parenchymal cells that proliferate in plaque or hematopoietic organs may contribute to the observed signal, including endothelial and smooth muscle cells. This is a significant limitation of $^{18}$F-FLT PET imaging, which is perhaps counterbalanced by its clinical availability. Our histological data imply, however, that most Ki67+ plaque cells were macrophages. A strategy to increase specificity for macrophages could rely on multimodal PET/magnetic resonance imaging, in which $^{18}$F-FLT PET could be combined with iron oxide macrophage magnetic resonance imaging. $^{18}$F-FLT signal colocalization with T2* MR signal decrease may confer increased cellular specificity. $^{18}$F-FLT has not shown adverse effects in clinical imaging trials, paving the way for a prospective trial that correlates $^{18}$F-FLT signal in plaque and hematopoietic organs with clinical outcome.

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Figure 8. $^{18}$F-FLT and $^{18}$F-FDG positron emission tomography–computed tomography (PET-CT) in humans with atherosclerosis. 

A, Sagittal image revealing extensive calcification in the aorta and carotid artery (arrows). $^{18}$F-FDG (B) and $^{18}$F-FLT (C) images demonstrate uptake of the PET tracers in the vessel wall of the aortic arch (arrows). D–G, In the ascending aorta, $^{18}$F-FDG and $^{18}$F-FLT uptake was significantly higher in the atherosclerotic patients when compared with low-risk subjects. H–K, In the common carotid artery, both $^{18}$F-FDG and $^{18}$F-FLT uptake was significantly higher in atherosclerotic patients. *$P$<0.05, Student t test. L, Linear regression of $^{18}$F-FDG and $^{18}$F-FLT standardized uptake values (SUV) max in aortic segments.
Disclosures

None.

References


Novelty and Significance

What Is Known?

• Macrophages promote disease progression in atherosclerosis.
• The turnover of macrophages is rapid, making it interesting to study their supply.

What New Information Does This Article Contribute?

• 18F-FLT, a thymidine analog that avidly incorporates into proliferating cells, enriches in atherosclerotic plaque of mice, rabbits, and human patients.
• Although other stromal cells contribute, the majority of proliferating cells in atherosclerotic lesions are macrophages.
• 18F-FLT positron emission tomography (PET)-CT imaging reports increased activity in the hematopoietic organs of mice with atherosclerosis.

• 18F-FLT plaque uptake increases with the duration of diet exposure in apolipoprotein E knock out mice.
• Originally developed for imaging proliferation in malignant disease, this PET imaging method may allow monitoring the expansion of hematopoietic cells in the setting of atherosclerosis.

Myeloid cells are key protagonists in the progression of atherosclerotic disease. In inflammation, their life span is considerably shortened, which motivated the field’s recently emerging interest in the production of these cells. The small molecule positron emission tomography tracer 18F-FLT is an analog to the DNA building block thymidine. Just like bromodeoxyuridine, another thymidine analog used to measure cell proliferation, it incorporates into cells that are actively cycling. Our data suggest that 18F-FLT positron emission tomography-CT imaging may be a clinically available method to study hematopoietic activity in patients with atherosclerosis. This could be of particular value in testing novel antiatherosclerotic therapies designed to diminish macrophage infiltration into inflammatory atherosclerotic plaques.
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Supplemental Material

Imaging macrophage and hematopoietic progenitor proliferation in atherosclerosis

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Detailed Methods

Synthesis of PET agent. $^{18}$F-FLT was synthesized by modifying a previously published procedure$^{1,2}$. Briefly, no-carrier-added $^{18}$F-fluoride ion was produced by 17-MeV proton bombardment of 95% $^{18}$O-enriched water via $^{18}$O (p,n) $^{18}$F nuclear reaction. This aqueous $^{18}$F-fluoride ion (~111,000 MBq) was treated with potassium carbonate and Kryptofix 2.2.2. (Aldrich Chemical Co.). Water was evaporated by azeotropic distillation with acetonitrile. The dried K$^{18}$F/Kryptofix residue was reacted with the FLT precursor (5'-O-benzoyl-2,3'-anhydrothymidine) and then hydrolyzed with 0.1% NaOH. The crude $^{18}$F-labeled product was purified by semi-preparative high-performance liquid chromatography (HPLC) (Phenomenex Luna column (250 X 10 mm, 10 µ C18 100 Å); 92% 10 mM phosphate buffer/8% ethanol; flow rate, 6.0 mL/min) to give 5550–7400 MBq (~7.5%–10% decay corrected radiochemical yield) of chemically and radiochemically pure $^{18}$F-FLT. The product was made isotonic with sodium chloride and sterilized by passing through a 0.2 µm Pediatric Filter into a sterile vial. The final product was sterile and pyrogen free. The purity of the final drug product was confirmed by analytical HPLC (Phenomenex Luna column (250 X 4.6 mm, 5 µ C18 100Å); 10% ethanol in water; flow rate, 1.5 mL/min; 267nm UV and radioactivity detection; specific activity, $\sim$7400 MBq/µmol and radiochemical purity >99%).

Mice. Female apolipoprotein E knock out (ApoE$^{-/-}$) and wild-type C57BL/6 mice (10–12 weeks old) were purchased from Jackson Laboratories. Atherosclerosis was induced by feeding high cholesterol diet to ApoE$^{-/-}$ mice ranging from 10 to 28 weeks. Wild-type mice were fed normal chow. In wild-type mice, modulation of hematopoietic progenitor cell proliferation in bone marrow and the spleen were induced by injecting lipopolysaccharide (LPS, 10 µg/mouse i.p. on three and one day before imaging) and fluorouracil (5-FU, 150 µg/g i.p. 12 hours before imaging). Wild-type mice given PBS served as controls. All procedures in mice were approved by the Institutional Animal Care and Use Committee (IACUC) Subcommittee on Research Animal Care (SRAC), Massachusetts General Hospital, Charlestown, MA.

Rabbits. Atherosclerosis was induced in 4 New Zealand White (NZW) male rabbits by a combination of high-cholesterol diet and double balloon injury of the abdominal aorta$^{3}$. Rabbits were imaged 6 months after diet initiation. 4 NZW non-atherosclerotic male rabbits were used as controls. All procedures in rabbits were approved by the IACUC at Mount Sinai Hospital, New York City, NY.

Small animal positron emission tomography (PET)-computed tomography (CT) imaging and image analysis. $^{18}$F-FLT was injected via tail vein 120 min before in vivo imaging with an average dose of 22.24±1.07MBq. This dose and imaging time point were chosen based on published biodistribution data and because mice have high circulating thymidine levels that compete with the PET agent for uptake into proliferating cells$^{4}$. Because FLT has a relatively long retention in blood, mice were hydrated by giving 400µl PBS i.p. prior to FLT injection. In addition, 4.5mg/Kg Furosemide (Hospira Inc., Lake Forest, IL) was given i.p. after FLT injection. Mice were imaged with an Inveon small-animal PET-CT scanner (Siemens Medical Solutions, Inc., Malvern, Pennsylvania). CT was performed prior to PET, acquiring 360 cone beam projections (source power 80 keV and current 500 µA). The CT contrast agent Isovue370 was infused via tail vein at 50µl/min. Static PET was acquired for 45 min. During image acquisition, mice were anesthetized by using 1-2% isoflurane mixed with oxygen. A high-resolution Fourier rebinning algorithm and the ordered subset expectation maximization (OSEM) method were used for image reconstruction. Image voxel sizes are 0.11 x 0.11 x 0.11 mm and 0.83 x 0.83x 0.83 mm for CT and PET, respectively. For quantitative analysis, regions of interest (ROIs) were drawn manually in the aortic root, the spleen and the spine of each animal, guided by CT images. Standardized uptake values (SUV) were defined as the ratio of the tissue radioactivity concentration c(t) (Bq/g) at the time point of imaging t, and the injected activity (in Bq, decay
corrected for the time point of imaging t) divided by the body weight (g). After in vivo imaging, mice were sacrificed and the direct γ counting was performed on the aortic root, the spleen and a femur using a gamma counter (1480 Wizard 3”, PerkinElmer, Boston, Mass). The data are presented as percent injected dose per gram of tissue (%IDGT).

**Autoradiography and oil red O staining of aortae.** After 18F-FLT injections, aortae from both wild-type and ApoE/− mice were excised and exposed for autoradiography on the PhosphorImager (SI, Molecular Dynamics, Sunnyvale, Calif) for 24 hours. Subsequently, aortae were briefly incubated with 60% 2-propanol and stained with 0.5% Oil Red O solution for 15 min at room temperature. After rinsing in 60% 2-propanol, aortae were washed repeatedly in PBS. Photographs of aortae were taken by using a digital camera with a micron lens (Sony).

**Rabbit imaging.** Animals were subjected to high-resolution 3 dimensional T2-weighted magnetic resonance imaging (MRI) with a 7-tesla scanner (Magnetom 7T, Siemens Healthcare, Erlangen, Germany). To establish 18F-FLT’s pharmacokinetics, animals were injected with ~111MBq and blood was drawn at 2, 5, 15, 30, 60, 120 and 240 min. Radioactivity was measured by a Wizard 2470 Perkin Elmer automatic gamma counter. Values were calculated as %IDGT. All 8 rabbits (average weight 3.47 +/- 0.37 Kg) underwent two PET/CT scans, on 2 different days. The first PET/CT was performed after injection of 18F-FDG, while the second PET/CT was performed after the injection of 18F-FLT. For both imaging sessions rabbits were fasted for 4 hours before the isotope injection (water was provided at libitum). Before injection rabbits were appropriately restrained, and the isotope was injected through a catheter placed in a marginal ear vein. The average injected dose of FDG was 190.3 +/- 9.9 MBq (55.2 +/- 5.0 MBq/Kg), while the average injected dose of FLT was 199.2 +/- 29.1 MBq (57.5 +/- 8.0 MBq/Kg).

Rabbits were imaged approximately 200 minutes after isotope injection (FDG: 197.5 +/- 5.5 minutes; FLT: 198.9 +/- 10.5 minutes). Approximately 15 minutes before imaging, rabbits were anesthetized using an injection of ketamine (35mg/kg) and xylazine (5mg/kg), and the bladder was emptied. Anesthesia was maintained during imaging using 1% isofluorane breathing anesthesia. PET/CT was performed using a clinical scanner (Siemens Biograph mCT, PET/CT, Siemens, Erlangen, Germany), in 3D mode, using one bed position, for 60 minutes. Images were acquired from the thoracic aorta, down to the iliac bifurcation. A non contrast enhanced CT was also performed for attenuation correction of the PET images, and to identify the aorta during image analysis.

Following acquisition, images were transferred to a workstation for image analysis. Both PET and CT images were under-sampled to a resolution of 3 mm in the longitudinal direction, with respect to the scanner reconstruction (1 mm). PET images were reconstructed using an OSEM +PSF+TOF algorithm (Ordered Subset Expectation Maximization with Point Spread Function and Time Of Flight), using 3 iterations and 21 subsets. The iliac and renal arteries were identified on CT images and used as anatomical landmarks. On axial CT slices, circular ROIs were drawn to encompass the aorta, from the left renal artery to the iliac bifurcation using Osirix (http://www.osirix-viewer.com). Care was taken to exclude slices where aortic uptake was contaminated by bowel or bladder uptake. ROIs were then copied to the co-registered PET images and isotope concentration values (Bq/ml) were converted to SUV. Both mean and maximum SUV within aortic ROIs was calculated. Following, FDG SUV mean and maximum values were divided into 3 groups using a custom made Matlab program (www.mathworks.com). For SUV mean the groups were: 1) SUV between 0 and 0.4; 2) SUV between 0.4 and 0.6; SUV higher than 0.6. For SUV maximum the groups were: 1) SUV between 0 and 0.6; 2) SUV between 0.6 and 0.9; 3) SUV higher than 0.9. FLT slices corresponding to these FDG cut-offs were identified. FDG and FLT SUV mean and maximum values were correlated slice-by-slice starting from the left renal artery, identified in both scans as anatomical landmark.

**Flow cytometry.** Aortae of ApoE/− and wild-type control mice were digested in enzyme mixture. Single-cell suspensions of the digested aorta were stained with the following antibodies against
lineage markers including B220 (RA3-6B2), NK1.1 (PK136), Ly-6G (1A8), Ter119 (TER-119), CD90 (53-2.1), CD49b (DX5), as well as antibodies against markers CD45.2 (104), CD11b (M1/70), Ly6C (AL-21) and F4/80 (BM8). Macrophages were identified as Lineage− CD11b+ F4/80+Ly-6C−. For hematopoietic progenitor cells analysis on spleens and bone marrow from femurs, single-cell suspensions of these organs were stained with biotin conjugated antibodies against lineage markers including B220 (RA3-6B2), CD4 (GK1.5), CD8α (53-6.7), NK1.1 (PK136), CD11b (M1/70), CD11c (N418), Gr-1 (RB6-8C5), Ter119 (TER-119) and IL7Rα (SB/199) followed by streptavidin Pacific OrangeTM or APC/Cy7 conjugates, and antibodies against c-Kit (2B8) and Sca-1 (D7). LSK cells were defined as Lineage− c-Kit+ Sca-1+. For cell cycle analysis, the above described cells stained for macrophage and LSK cell surface markers were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer’s instructions. Cells were then stained with anti-Ki67 antibody (SolA15) or isotype control. All antibodies used in this study were purchased from eBioscience, BioLegend or BD Biosciences. After washing, 1µl of DAPI (FxCycleTM Violet stain, Life Technologies) was added to samples. Cells from animals injected with BrdU before imaging were fixed and permeabilized with the BrdU FACS kit (BD Biosciences) according to manufacturer’s instructions. Cells were stained with anti-BrdU antibody. Data were acquired using LSRII Flow Cytometer (BD) and were analyzed with FlowJo software (Tree Star).

**Histology mice.** After γ counting, aortic roots from ApoE−/− and wild-type mice were embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetek) for sectioning and staining. Monoclonal antibodies against CD68 (FA11, AbD Serotec), CD31 (MEC13.3, BD Biosciences), α-SMA (ab5694, abcam), CD3 (17A2, BD Biosciences) and anti-Ki67 (ab15580, abcam and SolA15, eBioscience) were used to detect macrophages, endothelium cells, smooth muscle cells, T cells and proliferation marker Ki67, respectively. DAPI was applied to detect nuclei. Quantification of proliferative cells in atherosclerotic aortae was performed on high-power-field (HPF) microscopic images of the immunofluorescent staining. We counted the Ki67+CD68+ and Ki67+ cells within each HPF (1-3 HPF/aortic root). The percentage of Ki67+CD68+ cells within all Ki67+ cells from the same HPF was calculated. The number of Ki67+CD31+, Ki67+α-SMA+ and Ki67+CD3+ cells were also counted on HFDs located in the similar area of the adjacent tissue slices. The result was presented as mean ± SEM of the number of cells.

**In vitro uptake of 18F-FLT.** For in vitro experiments, single cells suspensions of bone marrow mononuclear cells were obtained by flushing long bones (femur and tibia) from C57Bl/6 mice. Bone marrow stem and progenitor cells were enriched by staining marrow cells with anti-C-kit-PE antibody (2B8; eBioscience), followed by anti-PE microbeads and then isolation using LS magnetic columns according to manufacturer’s instructions (Miltenyi Biotec). Ly6G+ cells from bone marrow (neutrophils) were first stained with anti-Ly6G-PE (1A8; BD Biosciences) and then retrieved with the same procedure. For peritoneal macrophage isolation, cells from the peritoneal cavity were retrieved by peritoneal lavage using cold PBS. Cells were then stained with an anti-PE cocktail antibody including B220 (RA3-6B2; BD Biosciences), NK1.1(PK136; BioLegend), Thy1.2 (53-2.1; BD Biosciences), Ter119 (TER-119; BD Biosciences) and anti-Ly6G-PE (1A8; BD Biosciences), followed by incubation with anti-PE microbeads. Using negative selection in LS columns, macrophages were eluted. Viable cells counts were determined and 2 million cells in 250µl FACS buffer were transferred to 1.5ml Eppendorf tubes. C-kit+ (n=3 tubes) and Ly6G+ (n=3 tubes) bone marrow mononuclear cells were incubated with 5µCi 18F-FLT for 60min on ice. Peritoneal macrophages with and without 5FU treatment were incubated with 185kBq 18F-FLT for 60 min at 37°C. Three tubes of peritoneal macrophages were subjected to 5-FU treatment (500µM for 60 min at 37°C) before 18F-FLT incubation. The other three tubes of peritoneal macrophages were incubated only with FACS buffer for the same period of time at the same temperature prior to 18F-FLT incubation. Cells were washed three times with 1000µl FACS buffer and transferred to new tubes after 18F-FLT incubation. The cellular 18F activity was measured with a gamma counter (1480 Wizard 3”, PerkinElmer, Boston, Mass).
**Histology rabbits.** Within a week of the last imaging session, atherosclerotic rabbits were euthanized using an intra-venous injection of sodium pentobarbital (100 mg/Kg). Following sacrifice, rabbits were perfused with 500ml of 0.9% sodium chloride. The aorta was excised. Five 5 mm thick samples from the aorta were cut at regular intervals from the left renal artery to the iliac bifurcation. All specimens were embedded in OCT compound and stored at -80 Celsius. Immunohistochemistry staining using mouse anti-rabbit macrophage (RAM11, Dako) was performed on the aorta sections.

**Human FDG-PET/CT and FLT-PET/CT.** Twenty patients (16 men and 4 women) with paired FLT-PET/CT and FDG-PET/CT scans performed between December 2011 and March 2013 were retrospectively enrolled in the study according to risk assessment for cardiovascular disease. Ten patients with high risk of cardiovascular disease (atherosclerotic group) and 10 patients with low risk (control group) were enrolled according to Framingham Scoring (Table 1). The study was performed in accordance with the Helsinki Declaration and approved by the regional scientific ethical committee (H-3-2011-092). All patients were diagnosed with neuroendocrine tumors and written informed consent was obtained from all participants. All patients had an FDG-PET/CT and an FLT-PET/CT scan within 2 weeks of each other performed on a Siemens Biograph 40 or 64 PET/CT scanner (Siemens Medical Systems, Erlangen, Germany). Unless contra-indicated, the CT scan in combination with the FLT-PET was a contrast-enhanced CT scan of diagnostic quality. To minimize radiation burden, the CT scan of the FDG-PET was a low-dose CT (40 mAs). Patients were instructed to fast at least 6 hours prior to tracer injection and static images were acquired 1 hour post-injection of either \(^{18}\text{F}-\text{FDG}\) or \(^{18}\text{F}-\text{FLT}\). Reconstructed images were analyzed using the OsiriX Lite open-source software (Pixmeo). Image analysis was performed in a blinded fashion on anonymized scans. We cannot exclude however, that the nature of arterial calcifications might have made the reader aware of the disease burden. Tracer uptake was quantified as maximum and mean Standardized Uptake Values (SUV max and SUV mean). To evaluate if global uptake of \(^{18}\text{F}-\text{FLT}\) and \(^{18}\text{F}-\text{FDG}\) differed between atherosclerosis and control subjects, ROIs were drawn on all slices of the ascending aorta and at least 10 consecutive slices of the carotid artery. Averaged SUV max and SUV mean were then calculated for each patient in the 2 target regions (carotid artery and ascending aorta) based on SUV max and SUV mean values from all ROIs in the region. To correlate the uptake of \(^{18}\text{F}-\text{FDG}\) and \(^{18}\text{F}-\text{FLT}\) on a sub-regional level, matched slices of \(^{18}\text{F}-\text{FDG}\) and \(^{18}\text{F}-\text{FLT}\) scans were analyzed side-by-side in the ascending aorta. Due to differences in patient alignment between the 2 scans, no slice-by-slice comparison was made in the carotid artery region.

**Statistical analysis.** Results are reported as mean ± standard error of mean. Statistical analysis was performed with use of GraphPad Prism 6 software (GraphPad Software, Inc.). Normal distribution of variables was tested using the Kolmogorov-Smirnov-test or the D’Agostino-Pearson omnibus normality test. Data were analyzed by parametric tests if normal distribution was detected. An unpaired student t-test was applied for two-group comparisons. If more than two groups were compared, an ANOVA correction was used. If data were non-normally distributed, differences were evaluated using an unpaired, nonparametric Mann-Whitney test. Comparisons of \(^{18}\text{F}-\text{FLT}\) SUV mean and max corresponding to low, medium and high \(^{18}\text{F}-\text{FDG}\) SUV mean and max cutoffs in rabbits were evaluated using the non parametric Kruskal-Wallis test. With a total of 20 patients in the two groups, a sigma of 0.2 and a difference between groups of 19%, a power > 95% was obtained for the human study. A linear regression model was used to correlate matched \(^{18}\text{F}-\text{FDG}\) and \(^{18}\text{F}-\text{FLT}\) uptake in atherosclerotic rabbits and humans. Significance level in all tests was 0.05.
References