Noninvasive Assessment of Hypoxia in Rabbit Advanced Atherosclerosis Using $^{18}$F-fluoromisonidazole Positron Emission Tomographic Imaging

Jesus Mateo, PhD; David Izquierdo-Garcia, PhD; Juan J. Badimon, PhD; Zahi A. Fayad, PhD; Valentin Fuster, MD, PhD

**Background**—Hypoxia is an important microenvironmental factor influencing atherosclerosis progression by inducing foam-cell formation, metabolic adaptation of infiltrated macrophages, and plaque neovascularization. Therefore, imaging plaque hypoxia could serve as a marker of lesions at risk.

**Methods and Results**—Advanced aortic atherosclerosis was induced in 18 rabbits by atherogenic diet and double balloon endothelial denudation. Animals underwent $^{18}$F-fluoromisonidazole positron emission tomographic and $^{18}$F-fluorodeoxyglucose positron emission tomographic imaging after 6 to 8 months (atherosclerosis induction) and 12 to 16 months (progression) of diet initiation. Four rabbits fed standard chow served as controls. Radiotracer uptake of the abdominal aorta was measured using standardized uptake values. After imaging, plaque hypoxia (pimonidazole), macrophages (RAM-11), neovessels (CD31), and hypoxia-inducible factor-1α were assessed by immunohistochemistry. $^{18}$F-fluoromisonidazole uptake increased with time on diet (standardized uptake value mean, 0.10±0.01 in nonatherosclerotic animals versus 0.20±0.03 [$P=0.002$] at induction and 0.25±0.03 [$P<0.001$] at progression). Ex vivo positron emission tomographic imaging corroborated the $^{18}$F-fluoromisonidazole uptake by the aorta of atherosclerotic rabbits. $^{18}$F-fluoromisonidazole uptake also augmented in atherosclerotic animals, with an standardized uptake value mean of 0.43±0.02 at induction versus 0.35±0.02 in nonatherosclerotic animals ($P=0.031$) and no further increase at progression. By immunohistochemistry, hypoxia was mainly located in the macrophage-rich areas within the atheromatous core, whereas the macrophages close to the lumen were hypoxia-negative. Intraplaque neovessels were found predominantly in macrophage-rich hypoxic regions (pimonidazole+/hypoxia-inducible factor-1α/RAM-11+).

**Conclusions**—Plaque hypoxia increases with disease progression and is present in macrophage-rich areas associated with neovascularization. $^{18}$F-fluoromisonidazole positron emission tomographic imaging emerges as a new tool for the detection of atherosclerotic lesions. *(Circ Cardiovasc Imaging, 2014;7:312-320.)*

Key Words: atherosclerosis ■ hypoxia ■ neovascularization ■ positron emission tomography

Recent studies indicate that hypoxia, a decrease in oxygen tension within tissues, plays an important role in atherosclerosis progression.1,2 Nourishment of healthy artery walls is achieved by oxygen diffusion from the lumen of the vessel and from adventitial vasa vasorum. As the disease progresses, the arterial wall thickens, and oxygen diffusion into the intima is markedly reduced.3,4 Moreover, the elevated oxygen consumption and high metabolic rate of infiltrated macrophages and foam cells contribute to impair the oxygenation of the vessel wall.5,6 Indeed, advanced atherosclerotic plaques show severely hypoxic regions, particularly those containing macrophage-rich areas, in both humans7 and animal models.8,9 Hypoxia, through the activation of the hypoxia-inducible factor (HIF-1),10 represents an essential signal for plaque growth by inducing critical processes, such as the formation of lipid droplets and 11,12 the activation of a metabolic switch to anaerobic glycolysis,10,13 and increasing the secretion of proinflammatory and angiogenic mediators.1,14 In fact, hypoxia is the most potent stimulus for angiogenesis, predominantly through the activation of the HIF-1/vascular endothelial growth factor (VEGF) pathway,15 and microvessel density has been shown to increase in inflamed lesions.16,17

The recent development of molecular and functional imaging technologies has made it possible to quantify the extent of disease in terms of visualization of some plaque elements related to clinical complications (eg, inflammation,
neovascularization). Among them, positron emission tomography (PET) with \(^{18}\)F-fluorodeoxyglucose (\(^{18}\)F-FDG) has emerged as one of the most promising imaging modalities to assess inflamed (and likely at risk) atherosclerotic plaques because of its predominant uptake by cells with enhanced glucose metabolism, such as plaque macrophages. However, the factors that determine \(^{18}\)F-FDG uptake in atherosclerotic plaques are not fully understood; a recent study has suggested that \(^{18}\)F-FDG uptake in atheroma may be the result of the hypoxic activation of glucose uptake in macrophages rather than mere inflammatory burden. Therefore, given the essential role of hypoxia/HIF-1 in inflammation and neovascularization, hypoxia may represent a novel target for noninvasive imaging of atherosclerotic plaque at risk.

At present, PET is the most suitable method for in vivo noninvasive clinical assessment of hypoxia. \(^{18}\)F-fluoromisonidazole (\(^{18}\)F-FMISO) is one of the leading PET radiotracers for imaging hypoxia, and it has been successfully used to image ischemic stroke, myocardial ischemia, and a wide variety of malignancies. \(^{18}\)F-FMISO is a cell-permeable 2-nitroimidazole derivative that is reduced in vivo by nitroreductases regardless of the intracellular oxygen concentration. Under normal oxygenation conditions, \(^{18}\)F-FMISO is rapidly reoxidized and diffuses out the cells. However, in hypoxic viable cells, \(^{18}\)F-FMISO is further reduced to a more reactive form that binds covalently to intracellular macromolecules and remains in the cells. This biochemical process exhibits a sharp increase when the partial pressure of oxygen drops <10 mm Hg (=1.5% O\(_2\)), which makes it a good marker of hypoxia in the pathological range. Although \(^{18}\)F-FMISO biodistribution properties do not result in high contrast images, they result in images that unambiguously reflect regional partial pressure of oxygen.

The aim of this study was to evaluate the feasibility of \(^{18}\)F-FMISO PET imaging for in vivo detection of hypoxia in advanced lesions in a rabbit atherosclerosis model and to compare it with the more established \(^{18}\)F-FDG PET imaging of inflammation/metabolic activity. Hypoxia was confirmed with pimonidazole, the gold standard immunohistochemical hypoxia marker, and correlated with macrophage content, neovessels count, and the expression of the hypoxia-inducible transcription factor (HIF-1\(\alpha\)).

Methods
A full Methods section is available in the Data Supplement.

Animal Protocol
Aortic atherosclerosis was induced in New Zealand white male rabbits (n=18; mean weight 3.1±0.2 kg; Covance) by a combination of atherogenic diet (0.2% cholesterol, Research Diets Inc) and double balloon endothelial denudation. All procedures were performed under general anesthesia by intramuscular injection of ketamine (30 mg/kg; Fort Dodge Animal Health) and xylazine (5 mg/kg; Bayer Corp). Four rabbits fed standard chow and without balloon denudation served as controls. To evaluate the disease progression, we established 3 longitudinal experimental conditions: control (nonatherosclerotic animals); atherosclerosis induction, at 6 to 8 months after initiation of the atherogenic diet; and atherosclerosis progression, at 12 to 16 months of diet initiation. The study design is summarized in the Figure in the Data Supplement (see Materials in the Data Supplement). The animal protocol was approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai.

\(^{18}\)F-FDG PET Imaging
\(^{18}\)F-FDG PET images were acquired using a combined PET/computed tomography (CT) scanner (Discovery LS, GE Healthcare). A low-dose CT was used for anatomic coregistration and for attenuation correction of the PET data set. Images were acquired 3 hours after injection of 148 to 185 MBq (4–5 mCi) of \(^{18}\)F-FDG. Acquisition was performed for 15 minutes in 2D mode.

\(^{18}\)F-FMISO PET Imaging
\(^{18}\)F-FMISO PET images were acquired with the sequential whole-body PET/MR system Philips Ingenuity TF (Philips Healthcare). PET images were acquired 3 hours after injection of 148 to 185 MBq of \(^{18}\)F-FMISO. Acquisition was performed for 30 minutes in 3D mode.

Magnetic Resonance and CT Imaging
Before the PET imaging sessions, animals were scanned with magnetic resonance imaging (MRI) using the Philips Achieva 3T X-series scanner (Philips Healthcare). T2-weighted turbo spin echo sequences were acquired to provide high-resolution anatomic detail.

Ex Vivo \(^{18}\)F-FMISO PET Imaging
Immediately after the final in vivo \(^{18}\)F-FMISO PET session, 6 animals (1 control and 5 atherosclerotic rabbits at the end of the longitudinal study) were euthanized, and the aortas were harvested for ex vivo \(^{18}\)F-FMISO PET imaging using the PET/MRI scanner described previously. Thereafter, the aortas were processed for histological analysis (see section below).

PET Quantification
PET, MRI, and CT images were automatically coregistered using the Extended Brilliance Workstation (Philips Healthcare). For in vivo PET image analysis, circular regions of interest (ROIs) encompassing the vessel wall were manually drawn on the corresponding axial CT images to cover the whole abdominal aorta using OsiriX Imaging Software. ROIs were quantified using the standardized uptake values (SUV). SUVmean and SUVmax were calculated by averaging ROIs mean and maximum SUVs, respectively, through the entire abdominal aorta. For the ex vivo images, circular ROIs of 1 cm\(^2\) were placed on the T1-weighted sequence used for attenuation correction. Ex vivo image quantification was provided using SUVmean and SUVmax from the ROIs.

Histology and Immunostaining
On the day of the final imaging session, the hypoxia marker pimonidazole hydrochloride (60 mg/kg; Hypoxyprobe Inc) was injected intravenously 2 hours before euthanizing the animals. After imaging, rabbits were euthanized and the aortas were fixed overnight in 10% buffered-formalin. Serial sections (5-μm thick) were obtained and stained with hematoxylin-eosin. Additional serial sections were assessed for hypoxia, macrophages, neovessels, and HIF-1\(\alpha\) using mouse monoclonal antibodies against pimonidazole (MAb1, Hypoxyprobe Inc), anti-rabbit macrophage (clone RAM-11, Dako), anti-C3d1 (clone JC70A, Dako), and anti-HIF-1\(\alpha\) (clone H1α67, Novus Biologicals), respectively.

Statistical Analysis
Data are presented as means±SEM. Analyses were performed using GraphPad Prism (GraphPad Software Inc). The validity of normal distribution assumptions was assessed with the Kolmogorov–Smirnov test. For the in vivo PET data, group differences were tested by either an unpaired \(t\) test with the Welch correction to compare control group versus induction and progression or by a paired \(t\) test to compare paired data from the same animals (induction versus progression). An unpaired \(t\) test was used to compare the histological quantifications. When indicated, Pearson correlations were estimated. \(P<0.05\) was considered statistically significant.
Results

MRI Monitoring of Aortic Atherosclerosis

All the animals underwent an MRI before each PET study for in vivo anatomic monitoring of the disease. T2-weighted MRI showed marked wall thickening of the aortic wall after the atherosclerosis induction period, as compared with the MRI control in nonatherosclerotic animals, and confirmed the development of aortic atherosclerosis with time on diet (Figure 1).

Detection of Hypoxia in Aortic Atherosclerosis by $^{18}$F-FMISO PET

In vivo $^{18}$F-FMISO PET scans demonstrated areas of hypoxia evidenced by a significant increase in the uptake of the radiotracer along the abdominal aorta of the rabbits after the atherosclerosis induction period (average SUVmean of 0.20±0.03, n=16) in comparison with healthy animals (average SUVmean of 0.10±0.01, n=4; P=0.002). Importantly, the uptake of $^{18}$F-FMISO continued to strengthen with time on atherogenic diet until reaching an SUVmean of 0.25±0.03 (n=12) at the end of the feeding period (progression; ≈150% increase over control group; P<0.001). Figure 2 shows representative images of $^{18}$F-FMISO PET/CT scans at the different feeding periods and the radiotracer uptake quantification.

Immediately after the in vivo PET imaging, some animals were euthanized, and their aortas were extracted for ex vivo determination of $^{18}$F-FMISO uptake using PET/MR scanning. As shown in Figure 3, $^{18}$F-FMISO uptake was dramatically increased in the abdominal aortas from atherosclerotic rabbits (SUVmean of 9.3±2.0×10$^{-3}$ at the end of the feeding period) in comparison with negligible uptake in control. Importantly, atherosclerotic plaques showing strong $^{18}$F-FMISO uptake contained intense hypoxic areas on corresponding histological sections, as identified by the presence of hypoxia-specific pimonidazole adducts in the artery wall (Figure 3D).

We also measured $^{18}$F-FDG uptake in parallel to $^{18}$F-FMISO. $^{18}$F-FDG uptake increased significantly in the abdominal aortas of atherosclerotic rabbits, with average SUVmean of 0.43±0.02 (n=16) after the atherosclerosis induction period versus 0.35±0.02 (n=4) in control animals (P=0.031). However, the magnitude of $^{18}$F-FDG uptake did not change by the end of the feeding period, with an average SUVmean of 0.43±0.02 (n=12; P=0.046 versus control). Figure 4 shows representative images of $^{18}$F-FDG PET/CT scans at the different feeding periods and the radiotracer uptake quantification.

We correlated the global uptake of $^{18}$F-FMISO and $^{18}$F-FDG in the abdominal aorta of corresponding animals and time points. We found a poor positive correlation between the 2 PET modalities that did not reach statistical significance, at both induction (r=0.29, P=0.27) and progression (r=0.43, P=0.14; Figure 4D). Examples of studies performed within a few days of each other showing corresponding planes of FDG uptake can be found in Figure 4E.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** In vivo magnetic resonance imaging monitoring of plaque progression. Representative axial T2-weighted magnetic resonance images of the abdominal aorta of a control (A), and an atherosclerotic rabbit scanned at 7 months (B) and at 16 months (C) after diet initiation. Images in B and C correspond to the same animal and at the same anatomic location. Red arrows indicate the abdominal aorta (magnified view provided at the bottom left of each image).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** In vivo $^{18}$F-fluoromisonidazole ($^{18}$F-FMISO) positron emission tomography (PET) of hypoxia. A, Combined PET/computed tomography coronal views (top) of the abdominal aorta illustrating the $^{18}$F-FMISO uptake of 2 atherosclerotic rabbits after 8 months (middle) and 16 months (right) on atherogenic diet, compared with a control animal (left). The bottom shows axial views of the corresponding slices indicated with a green line in the coronal view. Green arrows denote the abdominal aorta (magnified view provided at the bottom of each image). L indicates left; and R, right. B, Dot plot comparing the $^{18}$F-FMISO uptake (standardized uptake values [SUV] mean) of the abdominal aorta of atherosclerotic rabbits versus control animals. Lines represent the mean.
and FMISO images at the level of the abdominal aorta in coronal projection are given in Figure 4C.

Immunohistochemical analysis showed that hypoxia, as shown by pimonidazole staining, was located predominantly in the deep macrophage-rich areas within the atheromatous core of the plaque. Interestingly, the superficial macrophages adjacent to the lumen were negative or much more weakly stained with pimonidazole (Figure 5A). Hypoxia was not detected in noninflamed plaques or in plaques with only superficially infiltrated macrophages, irrespective of the time on diet (Figure 5B).

Similarly to the in vivo findings, the hypoxic area of the abdominal aorta was significant after the atherosclerosis induction period, and that trend continued up to the end of the feeding period, whereas macrophage density was fairly stable after the induction period. Figure 5C summarizes the staining quantification. Also, we found a strong correlation between the hypoxic area (pimonidazole+) and the RAM-11+-macrophage density of the plaque (r=0.87, P<0.0001; Figure 5D).

**Discussion**

Hypoxia has been demonstrated in atherosclerotic arteries using different invasive techniques: directly, with polarographic oxygen microelectrodes; and indirectly, by immunohistochemistry and autoradiography using exogenous hypoxia probes that form stable adducts with intracellular macromolecules under low oxygenation conditions, mainly nitroimidazole derivatives, or by measuring endogenous molecular markers whose expression is associated with hypoxia (HIF-1α, VEGF, hexokinase, glucose transporters). However, these techniques have important limitations, predominantly their invasiveness, but also the limited sampling size and the inability to perform repetitive measurements in patients. Therefore, noninvasive techniques that allow serial imaging of hypoxia in arteries could provide valuable information on disease status, lesions severity, and monitoring of therapeutic interventions.

There is increasing interest in PET for detection of cellular and molecular targets because it is currently the most sensitive and quantitative noninvasive clinical imaging technology. The emergence of new radiotracers has allowed noninvasive assessment of hypoxia, with 18F-FMISO being the most widely investigated and validated radiotracer. In this study, we have demonstrated the feasibility of in vivo PET imaging with 18F-FMISO for noninvasive visualization and quantification of plaque hypoxia in an experimental model of advanced atherosclerosis. We observed significant accumulation in intima) with the time on atherogenic diet, as determined by CD31 immunostaining. Whereas microvessels were essentially absent in the artery wall of healthy animals, we found an average of 50±24 neovessels per section after the atherosclerotic induction period and 80±17 neovessels per section by the end of the feeding period (Figure 6). Neovessels were mainly detected in the media and at the base of the plaque, in the macrophage-rich hypoxic regions of the thickened intima. Also, in the most advanced lesions, we observed plexuses of neovascularization developing in hypoxic areas at the junction between the intima and media (Figure 6C). Remarkably, most of the microvessels found in the tunica media were not associated with hypoxia or macrophages, whereas the neovessels of the intima were typically associated with hypoxic macrophages (pimonidazole+/RAM-11+ regions) or were developing toward hypoxic regions of the thickened intima (Figure 6). Electron microscopy confirmed the presence of microvessels in the intima, predominantly at the base of the plaque, surrounded by cells filled with lipid droplets, mainly macrophages and smooth muscle foam cells (Figure 6D).

Because neoangiogenesis is the major response to hypoxia through the activation of the HIF-1, we also determined the expression of HIF-1α. We found that HIF-1α was expressed by nearly all macrophages present in the plaque regardless of their oxygenation state (pimonidazole+/RAM-11+ and pimonidazole+/RAM-11+ regions), indicative of the expression of HIF-1α by hypoxia-dependent and hypoxia-independent mechanisms. However, neovascularization of the plaque (tunica intima) took place almost exclusively in regions containing hypoxic macrophages (pimonidazole+/HIF-1α+/RAM-11+) and was barely found at sites containing nonhypoxic macrophages, despite the expression of HIF-1α (Figure 7).
the aortas of atherosclerotic animals compared with healthy ones, and the uptake increased over time with atherogenic diet, suggesting hypoxia as a good biomarker of disease progression. The in vivo detection of hypoxia was convincingly corroborated by ex vivo PET imaging of the excised aorta, which showed areas of strong 18F-FMISO accumulation in atherosclerotic aortas, as compared with almost absolute absence of uptake in the normal aorta. Importantly, the specificity of the signal originated from the hot spots in the ex vivo PET scans was validated by subsequent immunohistochemical detection of genuine hypoxia with pimonidazole and comparison with less avid uptake regions of same aortas and with nonatherosclerotic aortic segments. In addition, our histological findings demonstrated that hypoxia is present in deep regions of the plaque, always associated with macrophages. However, not all macrophages present in the plaque were hypoxic, and we found that those macrophages located closer to the arterial lumen were much less or not hypoxic at all. These results are in agreement with previous reports in rabbits and humans, suggesting that enough oxygen can diffuse from the lumen to nourish this shallow macrophage population. Furthermore, previous work has shown ATP and glucose depletion as well as lactate accumulation predominantly in the macrophage-rich core of advanced plaques in rabbits, indicative of increased consumption and reduced supply of O2 and nutrients from the lumen and vasa vasorum to those regions. Whether these 2 macrophage populations, with regard to their oxygenation status, are phenotypically different and have distinct origin and biological functions within the plaque is currently unknown and will need to be explored in future work.

Yet FDG remains as the best characterized tracer available for PET imaging, and it has proven valuable for quantifying metabolic activity/inflammation within atherosclerotic lesions in humans and animal models. Thus, this study also aimed to compare both PET modalities in our model of advanced atherosclerosis. As expected, we observed a significant increase of 18F-FDG uptake by the aortas of atherosclerotic rabbits. However, unlike 18F-FMISO, the uptake of 18F-FDG remained stable after 6 to 8 months on diet, with no further increase in the progression group. This was unpredicted because a previous study in the same animal model had shown increased 18F-FDG uptake by the rabbit aorta throughout the progression of the disease. But several differences between both studies may justify this discrepancy. We scanned 16 rabbits at induction and 12 at progression, in comparison with 8 rabbits at induction and 4 at progression in the previous work, increasing the statistical power of the present study. We also scanned 3 hours after radiotracer injection instead of 30 minutes,
reducing considerably the potential for partial volume error; and we imaged the abdominal aorta instead of the thoracic. Furthermore, our histological findings support the in vivo imaging results, which showed an increase of the hypoxic area of the artery wall from induction to progression (≈60%), whereas macrophage density remained almost unchanged (≈15% increase). Given the clinical implications of this finding, additional experiments will be performed to clarify the stabilization of 18F-FDG uptake in the most advanced stages of the disease.

Importantly, we observed a poor correlation between the global uptake of 18F-FMISO and 18F-FDG by the abdominal aorta of atherosclerotic rabbits, despite the good correlation found between hypoxia (pimonidazole) and macrophages (RAM-11) by immunohistology. On the one hand, we must remind that both techniques were scanned with 2 different PET cameras that provide slightly different sensitivity and image resolution. However, this could only have a small and limited impact on quantification because both cameras were properly calibrated to provide absolute quantification parameters. On the other hand, it must be considered that FDG enters the cells through glucose transporters and accumulates in cells with elevated glucose consumption, but it lacks intrinsic specificity for macrophages. Conversely, the mechanism of FMISO uptake and retention makes it specific for hypoxic cells. In oxygen-starved cells, HIF-1 activates transcription of the genes encoding glucose transporters, hexokinase, and other glycolytic enzymes, increasing glycolysis. Given the link between hypoxia and glycolysis, it could be argued that FDG uptake may detect tissue hypoxia as well. Interestingly, a recent study has shown that hypoxia, but not a proatherogenic inflammatory environment, potently stimulated glucose uptake in human macrophages and foam cells. Moreover, other cell types present in the plaque, such as smooth muscle and endothelial cells, markedly increased glucose uptake in a proinflammatory milieu. Therefore, the authors suggested that FDG accumulation in atherosclerotic lesions could be the result, at least partly, of hypoxia-stimulated glycolysis in macrophages rather than an estimate of inflammatory burden.
Although we cannot discard that possibility, the significant discrepancy between 18F-FMISO and 18F-FDG uptake found here suggests that the 2 tracers do not account for the same biological processes.

It should be underscored that the in vivo 18F-FMISO PET images of the aorta exhibited much lower contrast compared with the surrounding tissue than those obtained using 18F-FDG. This is likely because of slow diffusion of 18F-FMISO into the cells because of the absence of an active uptake mechanism and to the slow clearance of unbound tracer from nonhypoxic areas, resulting in low uptake and reduced image contrast.25,33 Contrarily, FDG exhibits high diffusibility, transmembrane carrier-mediated transport, and fast metabolism that results in enhanced image contrast. Although the modest signal-to-noise ratios provided by 18F-FMISO might pose a drawback for precise local uptake quantification, our study demonstrates that 18F-FMISO PET is capable of detecting significant differences in the oxygenation of large arteries of an animal the size of a rabbit despite the use of clinical scanners. Therefore, these results show promise for translation to human studies.

In atherosclerosis, plaque angiogenesis is linked to lesion progression and instability.17,34,35 We found that in parallel to the increase in plaque hypoxia, there was an increase in microvesSEL density throughout disease progression. Neovascularization largely occurred in deep areas of the thickened intima and in the media, with much lower incidence of capillaries in the proximity of the arterial lumen. Hypoxia, through the induction of HIF-1 and the ensuing expression of VEGF, is the primary stimulus for angiogenesis.15 Recent studies have found a correlation between HIF-1α accumulation and VEGF expression in the plaque, mostly in macrophage-rich areas, in both humans7,31,36 and animal models.9,37 Here, we demonstrate that nearly all

**Figure 6.** Association of neovascularization with hypoxia and inflammation in atherosclerotic plaques. A, Detection of neovessels (CD31, right), hypoxia (pimonidazole, left), and macrophages (RAM-11, middle) in serial sections of abdominal aortas obtained from atherosclerotic rabbits after the induction period (top) and at progression (bottom). Photomicrographs taken at 20× magnification. B, Bar graph showing neovessel quantification, as number of CD31-positive vessels per section, in the atherosclerotic animals. Bars represent mean±SEM. C, Higher power magnification image (100×) showing abundant microvessels (CD31 staining) in the media and at the plaque base of an advanced lesion. D, Transmission electron microscopy detail of capillaries surrounded by lipid-laden foam cells in the media (right) and in the plaque base (left) of an advanced lesion.

**Figure 7.** Plaque neovessels colocalize with hypoxia-inducible factor (HIF)-1α-positive hypoxic macrophages. Representative staining of hypoxia (pimonidazole), HIF-1α, macrophages (RAM-11), neovessels (CD31), and H&E in serial sections of an advanced atherosclerotic lesion showing colocalization of intimal plaque neovessels with HIF-1α-positive macrophage-rich hypoxic areas (pimonidazole+/HIF-1α+/RAM-11+). Photomicrographs taken at 100× magnification. L indicates lumen.
macrophages present in the plaque expressed HIF-1α, irrespective of their oxygenation state. Nevertheless, internal neovascularization was almost restricted to regions containing hypoxic macrophages (pimonidazole/’HIF-1α’/RAM-11’), which is in clear agreement with the previous work by Sluimer et al’ in human atherosclerotic carotid plaques. Importantly, we found that neovessels were scarce at sites with nonhypoxic macrophages, despite the expression of HIF-1α. It is well established that stimuli other than hypoxia can induce HIF-1 in normoxia.38 A variety of atherogenic factors, such as oxidized low-density lipoprotein (oxLDL) and the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are strong inducers of HIF-1 in normoxia.39,40 However, the paucity of neovessels in HIF-1α/pimonidazole/macrophage-rich regions closer to the lumen stresses the need for additional work to define precisely the mechanisms underlying HIF-1 expression in the plaque and its relation to angiogenesis.

In summary, this is the first study to noninvasively demonstrate plaque hypoxia using in vivo 18F-FMISO PET imaging in an experimental model of advanced atherosclerosis. We have shown that hypoxia develops in deep areas of advanced plaques, always associated with macrophages, HIF-1α expression, and with intimal neovascularization. Our results demonstrate that plaque hypoxia increases all through the progression of the disease in this experimental model, as revealed by in vivo detection with 18F-FMISO PET, and immunohistochemical validation with pimonidazole. In contrast to 18F-FMISO, 18F-FDG PET showed stabilization of the inflammatory component of the plaque at an earlier stage of the disease. Thus, given the marked association of hypoxic macrophages and neovascularization, critical hallmarks of atherosclerosis progression, plaque hypoxia may represent a novel target for noninvasive imaging of plaques at risk, potentially allowing early diagnosis and risk prediction of patients with atherosclerosis. Nevertheless, human translation will require further work in preclinical models as well as the assessment of additional hypoxia-specific radiotracers.

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Disclosures
None.

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Supplemental Methods

Animal Protocol

Aortic atherosclerosis was induced in New Zealand white male rabbits (n=18; mean age 3 months; mean weight 3.1±0.2 kg; Covance) by a combination of atherogenic diet (0.2% cholesterol, Research Diets Inc.) and double balloon endothelial denudation of the aorta (2 weeks and 10 weeks after starting the high-cholesterol diet), as previously described1. In brief, aortic balloon de-endothelializations were performed from the aortic arch to the iliac bifurcation with a 4-F Fogarty embolectomy catheter introduced through the iliac artery under fluoroscopic guidance. This approach generates aortic lesions with fibrous- and lipid-rich components similar to those observed in humans. All procedures were performed under general anesthesia by intramuscular injection of ketamine (30 mg/kg; Fort Dodge Animal Health) and xylazine (5 mg/kg; Bayer Corp.). Four rabbits fed standard chow and without balloon denudation served as controls. To evaluate the disease progression we established three longitudinal experimental conditions: control (non-atherosclerotic animals); atherosclerosis induction, at 6 to 8 months after initiation of the atherogenic diet; and atherosclerosis progression, at 12 to 16 months of diet initiation. The study design is summarized in the Supplemental Figure. For each specified experimental condition, animals underwent the following imaging techniques: 18F-FMISO PET, 18F-FDG PET, computed tomography (CT) and magnetic resonance imaging (MRI). In order to cover all imaging techniques per experimental condition, animals underwent 18F-FDG PET/CT and MRI on one session and 18F-FMISO PET/MRI and CT on a second session within less than a week, to allow comparisons under similar conditions (see Imaging...
animals were placed on a half-circle animal bed developed in-house and comprised of 2-mm-thick fiber-glass that immobilized the body of the rabbit in a rigid/stable prone position. The bed was designed to fit inside the MRI knee coil used to provide high-resolution anatomical data for this study. Animals were injected with 148-185 MBq (4-5 mCi) of either $^{18}$F-FMISO (Cardinal Health) or $^{18}$F-FDG (PETNET Solutions Inc.), via the marginal ear vein. During the three hours circulation time, animals were kept in a temperature-controlled room. Prior to imaging, the animals were anesthetized by intramuscular injection of ketamine/xylazine as described before. Anesthesia was maintained during the imaging sessions with isoflurane inhalation via a facemask. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai.

$^{18}$F-FDG PET Imaging

$^{18}$F-FDG PET images were acquired using a combined PET/CT scanner (16-slice multi-detector CT) with no time-of-flight (ToF) capability (Discovery LS, GE Healthcare). A non-contrast low-dose CT was used for anatomic coregistration and for attenuation correction of the PET dataset. Images were acquired 3 hours after injection of 148-185 MBq (4-5 mCi) of $^{18}$F-FDG\(^2\). Acquisition was done in 2D mode during 15 minutes in one single bed position covering the whole abdominal aorta. The resulting images were reconstructed using the OSEM method with 28 subsets and 2 iterations. The final image matrix contained 35 slices of 128 x 128 voxels, with 3.9 x 3.9 x 4.25 mm voxel size, using corrections for normalization, dead time, attenuation, scatter, random coincidences, sensitivity and decay.

$^{18}$F-FMISO PET Imaging

$^{18}$F-FMISO PET images were acquired with the sequential whole-body PET/MR system Philips Ingenuity TF (Philips Healthcare), which combines a standalone time-of-flight PET camera (GEMINI TF) and a MRI Achieva 3T X-series system with an innovative rotating bed that accurately positions the subject inside each gantry\(^3\). Although the scanner does not provide simultaneous MR and PET acquisitions, it
provides built-in accurate positioning and coregistration of MR and PET images obtained sequentially. A specially designed MR T1-weighted sequence that matches the PET dimensions allowed both anatomical detail and attenuation correction, similarly to a low-dose CT image in a standard PET/CT scanner. Details for the attenuation correction method and its performance on this particular animal model have been recently published\(^4\). During PET acquisitions, no additional MR coils were present to ensure no further attenuation from the coil electronics. PET images were acquired 3 hours after injection of 148-185 MBq (4-5 mCi) of \(^{18}\text{F}-\text{FMISO}\)\(^5\). Acquisition was done for 30 minutes in 3D mode, using time-of-flight information standard for this system. Images were reconstructed into a 128 x 128 matrix, containing 90 slices with 4 x 4 x 4 mm voxel size, using a 3D RAMLA reconstruction algorithm with 3 iterations and 33 subsets, using corrections for normalization, dead time, attenuation, scatter, random coincidences, sensitivity and decay.

**MRI and CT Imaging**

Prior to the PET imaging sessions, animals were scanned for MR imaging using the Philips Achieva 3T X-series scanner (Philips Healthcare). T2-weighted turbo spin echo (T2-TSE) sequences were acquired in order to provide with high-resolution anatomical detail. These images allowed us to visualize the progression of the atherosclerotic lesions\(^6\). An 8-channel high-resolution knee coil (Philips Healthcare) was used to image the whole abdominal aorta (from the renal arteries to the iliac bifurcation) in one single bed position. The customized animal bed designed for this study helped to consistently positioning the animal within the coil, minimizing the animal movement between different imaging procedures. Details of the T2-TSE sequence are: 2D acquisition, TE=50 ms, TR=2000 ms, flip angle=90º, echo train length (ETL)=19, number of averages=4, sense encoding, image matrix=560 x 560, number of slices=40 and voxel size=0.2 x 0.2 x 3 mm. The total duration of the sequence was 23 minutes. Additionally, prior to the \(^{18}\text{F}-\text{FMISO}\) PET/MRI sessions, CT images were also acquired in order to complete the whole imaging protocol: PET-MRI-CT. Non-contrast low-dose CT images were acquired using a standalone CT (256-slice multidetector CT) scanner (Philips Brilliance iCT, Philips Healthcare) using the following
parameters: 120 kV, current 300 mA/slice, pitch 0.993, table speed 105.9 mm/s, rotation speed 0.75 s/rot, voxel size 0.625 x 0.625 x 1 mm and final matrix size 512 x 512 x 399. The total scan duration was 3.76 s.

**Ex-vivo $^{18}$F-FMISO PET Imaging**

Immediately after the final *in vivo* $^{18}$F-FMISO PET session, 6 animals (1 control and 5 atherosclerotic rabbits at the end of the longitudinal study) were euthanized and the aorta was harvested for *ex vivo* $^{18}$F-FMISO PET imaging using the PET/MR scanner described previously. To cover the whole aorta (approx. 20 cm), 2 bed positions were scanned (20 minutes per bed position). Images were reconstructed following the same protocol described above (see $^{18}$F-FMISO PET Imaging). The final *ex vivo* images consisted of 132 slices of 128 x 128 voxels and 2 x 2 x 2 mm voxel size. Thereafter, the aortas were processed for histological analysis (see section below).

**PET Quantification**

PET, MRI and CT images were all automatically coregistered using the Extended Brilliance Workstation (Philips Healthcare). We have recently reported the results on the quality and performance of the coregistrations\(^4\). For *in vivo* PET image analysis, circular regions of interest (ROIs) encompassing the vessel wall were manually drawn on the corresponding axial CT images to cover the whole abdominal aorta using OsiriX Imaging Software. In order to minimize the impact of the limited spatial resolution of the scanners (~5 mm), which might lead to value overestimation due to the partial volume effect\(^7\), ROIs suffering from high-uptake neighboring contamination were excluded from the analysis. In most cases, such areas corresponded to the iliac bifurcation, due to the high-uptake of adjacent bladder as a consequence of the excretion of the radiotracer through urine (for both $^{18}$F-FDG and $^{18}$F-FMISO). ROIs were quantified using the standardized uptake values (SUV). SUVmean and SUVmax were calculated by averaging ROIs mean and maximum SUVs respectively, through the entire abdominal aorta. For the *ex vivo* images, circular ROIs of 1 cm\(^2\) covering the whole abdominal aorta were placed on the T1-weighted
sequence used for attenuation correction, as no further CT image was acquired for the \textit{ex vivo} protocol. In a similar fashion to the \textit{in vivo} images, \textit{ex vivo} image quantification was provided using SUVmean and SUVmax from the ROIs of the entire abdominal aorta.

\textbf{Histology and Immunostaining}

On the day of the final imaging session, the hypoxia marker pimonidazole hydrochloride (60 mg/kg; Hypoxyprobe Inc.) was injected intravenously 2 hours before euthanizing the animals. Pimonidazole is reductively activated in hypoxic cells ($pO_2 \leq 10$ mmHg), where the activated intermediate forms stable covalent adducts with thiol groups in proteins. Subsequently, \textit{in vivo} formed pimonidazole adducts can be detected by immunohistochemistry with an antibody specific for hypoxia-derivatives of pimonidazole. Thus, after imaging, rabbits were euthanized and the aorta was perfused with phosphate-buffered saline, gently removed, and fixed overnight in 10% buffered-formalin. The abdominal aorta (from the right renal artery to the iliac bifurcation) was cut into 4-mm segments (20-25 sections/aorta), tagged for orientation, and embedded in paraffin. Serial sections (5-µm thick) were obtained and stained with hematoxylin-eosin. Additional serial sections were assessed for hypoxia, macrophages, neovessels and HIF-1α using mouse monoclonal antibodies against pimonidazole (MAb1, 1:50 dilution, Hypoxyprobe Inc.), anti-rabbit macrophage (clone RAM-11, 1:1000 dilution, Dako), anti-CD31 (clone JC70A, 1:50 dilution, Dako), and anti-HIF-1α (clone H1α67, 1:100 dilution, Novus Biologicals), respectively. Sections incubated in parallel without primary antibody served as negative controls. Biotinylated anti-mouse secondary antibody and biotinylated-horseradish peroxidase-avidin complexes were applied for 30 minutes each by using the Vectastain ABC Elite Kit (Vector Labs.). Peroxidase activity was visualized with diaminobenzidine (Dako). Staining quantification was automatically performed with an in-house software in Matlab (Mathworks), based on the colorimetric properties of the stained areas. Results were given as percentage of total area. For plaque neovessels, identified as tubuloluminal CD31-positive structures using a 40x magnification objective, manual counting was provided slice by slice. We excluded the tunica adventitia from the quantification because it possesses a complex physiological network of blood vessels.
(vasa vasorum) and because we encountered a highly variable quantity of adventitial tissue in these sections. An average value per aorta was calculated by averaging all the stained slices analyzed.

**Electron Microscopy**

Freshly harvested aortic segments for electron microscopy were fixed in 4% glutaraldehyde overnight at 4°C, post-fixed in osmium tetroxide, dehydrated in an ascending series of alcohols, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Images were acquired using a transmission electron microscope (HITACHI H-7650, Japan) linked to a SIA digital camera (Scientific Instruments and Applications) controlled by Maxim CCD software.

**Statistical analysis**

Data are presented as mean±SEM. Analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software Inc.). The validity of normal distribution assumptions was assessed with the Kolmogorov-Smirnov test. For the *in vivo* PET data, group differences were tested by either an unpaired *t* test with the Welch’s correction to compare control group vs. induction and progression, or by a paired *t* test to compare paired data from the same animals (induction vs. progression). An unpaired *t* test was used to compare the histological quantifications. When indicated, Pearson correlations were estimated. *P*<0.05 was considered statistically significant.
**Supplemental Figures**

**Supplemental Figure. Study design.** Atherosclerosis was induced in 18 rabbits by combination of two balloon denudations of the aorta and atherogenic diet (0.2% cholesterol). Four weight-matched animals without balloon injury and fed standard chow served as controls. Two rabbits died during the atherosclerosis induction period and were subsequently not analyzed. After 6 to 8 months of diet initiation, the first imaging scans were performed in all the animals (induction, n=16). At this time, 4 atherosclerotic rabbits were euthanized for histological validations. The remaining rabbits continued under atherogenic diet for additional 6 to 8 months to evaluate the progression of the disease. Then, a second round of imaging scans was performed starting at 12 months from diet initiation until completion of the study at 16 months (progression, n=12). The animals were sacrificed immediately after the last imaging session and tissue analysis was performed.
Supplemental References


