Section 1.0 General Information

TITLE: AN EXPLORATORY, PHASE II, OPEN LABEL, SINGLE-CENTER, NON-RANDOMIZED STUDY OF [F-18] RGD-K5 POSITRON EMISSION TOMOGRAPHY (PET) IN PARTICIPANTS WITH CAROTID ARTERY STENOSIS; K5-C200

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Study Support Provided By: NHLBI-NIH
(NATIONAL INSTITUTES OF HEALTH)

Section 2.0 Background information

Atherosclerotic vascular disease is the primary cause of heart attacks and stroke. The rupture and downstream embolization of unstable plaque in the carotid artery can result in stroke or transient ischemic attack (TIA). Stroke is the fourth leading cause of mortality in the United States. In addition, rupture of unstable atherosclerotic plaque in the coronary artery results in myocardial infarction (MI), the major cause of death in the United States. A noninvasive imaging test that can detect unstable plaque is critical for identification of high-risk patients who would benefit from aggressive medical therapy, carotid artery stenting or percutaneous coronary intervention. Among patients with carotid artery stenosis, a significant number of them undergo carotid endarterectomy (CEA) or stenting purely based on the presence of plaque causing ≥70% stenosis even though they may have never had a stroke or TIA [1, 2]. **A non-invasive imaging test that can accurately identify unstable plaque may prevent unnecessary surgical and endovascular procedures and could potentially reduce cardiovascular mortality.**

Current State of the Art for Noninvasive Detection of Unstable Plaque

CTA: Carotid plaque is usually detected by Doppler ultrasound and alternatively by CTA and magnetic resonance imaging (MRI) [3]. CTA can identify high-risk plaque features such as spotty calcification, plaque ulceration and enriched lipid composition [4]. However, dense calcification can mask high-risk plaque features on CTA.

MRI: Morphologic features associated with unstable plaque such as a necrotic lipid core and intraplaque hemorrhage can be imaged with MRI [5-10]. Even still, MRI is not widely used for detection of unstable plaque due to the technical challenges associated with obtaining high resolution images in smaller arteries [11]. Ultrasmall paramagnetic nanoparticles (USPIO) have been used in clinical studies to bind to vulnerable plaque based on the high density of macrophages; however, there have been no clinical trials of USPIO for routine identification of vulnerable plaque in the United States [12, 13].

18F-Sodium fluoride (NaF): NaF has been shown to bind to culprit lesions in the coronary artery of patients who have suffered a myocardial infarction and in carotid artery plaque in patients who have suffered a stroke or TIA [14, 15]. However, there are no prospective studies demonstrating the ability of NaF to predict plaque rupture at this time.

18F-labeled deoxyglucose (FDG): FDG is the only molecular imaging agent that has been used for positron emission tomography (PET) of atherosclerotic plaque in large clinical studies [16, 17]. In 2002, Rudd et al. showed that FDG uptake was preferentially increased in carotid artery plaque of patients with symptoms of stroke or TIA compared to asymptomatic patients. Plaque removed from patients with high carotid FDG uptake was enriched in macrophages and was

characterized by a large necrotic core. This study concluded that FDG uptake may be a marker of plaque vulnerability; however, there are no studies that have tested the benefit of FDG PET imaging of plaque for clinical decision-making. FDG is extracted by a number of metabolically active cellular targets cells including macrophages, lymphocytes and smooth muscle cells found in plaque. A plaque imaging agent with selective affinity for macrophages and neovascular endothelial cells might significantly improve our ability to identify vulnerable plaque compared to FDG. Molecular imaging agents that target proteins that are presumably enriched in plaque such as matrix metalloproteases and annexinVa have been used in animal models of atherosclerosis; however, clinical translation of these imaging tracers is still in progress [18].

Most importantly, there is lack of data in support of clinical decision making based on the presence of high-risk plaque-features-using CTA, MRI, FDG-PET and NaF-PET in individuals with plaque that causes <70% luminal stenosis.

Need for Alternate Tracers for Plaque Imaging

There is an unmet clinical need for a plaque imaging agent that preferentially binds to cells and proteins that are present in unstable plaque and will thereby identify patients at risk for stroke or MI. Vulnerable plaque is characterized by high inflammatory activity and an overabundance of macrophages and neovessels. An imaging agent that selectively binds to macrophages and (or) neovascular endothelial cells may allow identification of inflamed vulnerable plaque. Such a-specific-tracer will provide higher lesion-to-background ratio and will improve clinical accuracy in identifying vulnerable plaque.

18F-flotegatide: 18F-flotegatide, a PET imaging agent with high specificity for αVβIII and α5β1 integrin present on macrophages and neovascular endothelial cells may offer the opportunity to detect vulnerable plaque.

The objective of this proposal is to evaluate the use of 18F-flotegatide to noninvasively detect unstable plaque in clinical subjects. I propose to accomplish this objective by first testing the hypothesis that 18F-flotegatide can bind to atherosclerotic plaque with morphologic, histologic and clinical features of vulnerability using PET-MR imaging in patients with carotid atherosclerosis.

INNOVATION

Advantages of using 18F-Flotegatide for the identification of vulnerable plaque

Integrins are heterodimeric glycoproteins composed of α and β subunits. They are key molecules involved in angiogenesis and mediate cell locomotion through the extracellular matrix [19, 20]. The integrin αVβIII plays an important role in the development and progression of atherosclerotic plaque and is considered to facilitate inflammatory cell recruitment to the damaged vessel wall and angiogenesis [20-23]. The α5β1 integrin is known to be involved in macrophage recruitment and angiogenesis in atherosclerotic plaque [24-26]. There is prominent expression of αVβIII on the endothelial cell surface of adventitial vasa vasorum and plaque neovessels [27]. Integrin αVβIII and α5β1 are also expressed by macrophages in atherosclerotic plaque [26, 28, 29]. Until recently, the most promising tracer for non-invasive detection of αVβIII expression in humans using PET has been 18F-Galacto-RGD [30, 31]. On the basis of the αVβIII receptor binding, the glycosylated cyclic tripeptide, 18F-Galacto-RGD demonstrates high affinity and selectivity for the receptor in vivo [32, 33]. Recently, 18F-Galacto-RGD was shown to bind to carotid artery plaque in a small group of patients with atherosclerosis [34]. **However, the long labeling time (4h) and complex chemistry required for the synthesis of 18F-Galacto-RGD makes it less amenable for routine clinical applications** [30]. Our

proposal to use 18F-Flotegatide (18F-RGD) for clinical plaque imaging in patients with carotid atherosclerosis is innovative for the following reasons:

1. The relatively short labeling time (75min) achieved through the use of click chemistry for its synthesis in comparison to the synthesis of 18F-GalactoRGD makes 18F-flotegatide an attractive agent for large scale clinical investigation.
2. Blood and background clearance is faster due to the triazole, a by-product of the click reaction. The plasma clearance half time is 12min. This in turn provides higher target to background ratio (TBR).
3. The synthesis of 18F-flotegatide is automated (unlike other RGD-tracers), which makes production of this tracer manufacturing-friendly and adaptable to various synthesis platforms at different clinical sites.
4. The high affinity of 18F-flotegatide for both αVβIII and α5β1 (Kd of 7.9nM for αVβIII and 26.5nM for α5β1 measured by inverse surface plasmon resonance) increases the number of potential cell surface targets for binding of 18F-flotegatide in atherosclerotic plaque. This translates to an improved signal to background ratio of 18F-flotegatide for in-vivo PET imaging relative to other integrin targeted agents.
5. We propose to use PET-MR instead of PET-CTA for the following reasons:
   (a) MR offers superior tissue characterization for assessment of plaque vulnerability compared to CTA.
   (b) PET-CT and CTA are often performed independently and accurate co-registration can be limited. The use of PET-MR allows simultaneous MR and PET imaging and allows accurate co-registration of plaque by MRI with the PET uptake of 18F-flotegatide.
6. Carotid plaque is removed surgically by CEA in patients with stroke, TIA or significant luminal obstruction. This offers a unique opportunity to directly compare observations from noninvasive imaging to histologic and immunohistochemical characterization of the same plaque after endarterectomy.
7. A significant number of patients with carotid atheroma are sent for carotid stenting with optical coherence tomography (OCT) to assess plaque composition at the time of stenting. In-vivo information from OCT can be correlated to the tracer uptake and MRI plaque features.
8. Our study is conducted by an interdisciplinary, highly synergistic team comprised of a cardiologist (PI), neurologist, vascular surgeon, PET-radiochemist, PET and MR physicists and radiologists.

Based on our preliminary data with PET-CTA, we strongly believe we will be able to reproducibly detect significant 18F-flotegatide uptake in plaque from symptomatic patients. Ultimately, demonstrating preferential 18F-flotegatide uptake in symptomatic patients will significantly impact the way in which patients with carotid plaque (at risk for stroke) are treated and it may prevent unnecessary surgical and endovascular procedures in this population.

Overall Study Design Background: The rupture of unstable plaque in the carotid artery is one of the most common causes of stroke. Plaque rupture is followed by activation of the thrombotic cascade and formation of an intravascular thrombus. Downstream embolization of all or part of the thrombus causes ischemia of brain tissue thus precipitating a stroke. Smaller emboli that originate from unstable plaque may result in TIA.

Currently Accepted Model for Testing New Tracers for Imaging Vulnerable Plaque: PET imaging studies that investigated the use of FDG, and NaF for detection of unstable carotid plaque have used stroke as the clinical surrogate marker for presence of unstable plaque [15, 17]. Although, patients were imaged with FDG or NaF several days after plaque rupture had occurred, these studies still showed tracer uptake in the remaining carotid plaque. We propose to use a similar approach to test our hypothesis that 18F-flotegatide is extracted by atherosclerotic plaque (**Specific Aim I**). We also propose to determine if plaque removed from patients who exhibit 18F-flotegatide uptake by PET exhibits MRI features and histologic markers of vulnerability (**Specific Aim II**).

Rationale: Carotid artery plaque serves as an ideal model system for testing molecular imaging agents designed to bind to biological targets in unstable plaque. The carotid artery wall is perfused by vasa vasorum similar to the aorta and coronary arteries making observations made in the carotid artery broadly applicable to clinically important vascular beds in the human body [35-37]. Carotid artery plaque can be localized and imaged by multiple noninvasive techniques including ultrasound, CTA and MRI. The relatively large size of carotid plaque lends itself to better visualization of intraplaque targets using imaging techniques including PET.

Section 3.0 Trial objectives and purpose

Primary Objectives:

Aim 1: To validate that 18F-flotegatide may be extracted and differentially retained by carotid artery plaque in patients with clinical symptoms of stroke or TIA (vulnerable plaque) compared to asymptomatic control patients with stable plaque using PET-MR imaging. *Our hypothesis is that noninvasive PET imaging of 18F-flotegatide uptake can clinically distinguish unstable plaque from stable plaque.*

- We propose to validate our preliminary results (that were obtained using PET-CT and CTA) by demonstrating that atherosclerotic plaque can be detected by PET-MRI using 18F-flotegatide.
- We propose to accomplish this aim by performing in-vivo PET imaging of patients with unstable carotid plaque (within 96h of a stroke or TIA). We propose to quantify plaque uptake of 18F-flotegatide relative to 18F-flotegatide uptake in segments of the carotid vessel wall that are free of atherosclerosis.
- We also propose to test our hypothesis that 18F-flotegatide is preferentially retained by unstable plaque and may be used to distinguish unstable plaque from stable plaque.
- We propose to achieve this by comparing 18F-flotegatide uptake in patients who have been free of antecedent stroke or TIA (asymptomatic-control group) to 18F-flotegatide uptake of patients with unstable plaque with recent stroke or TIA.

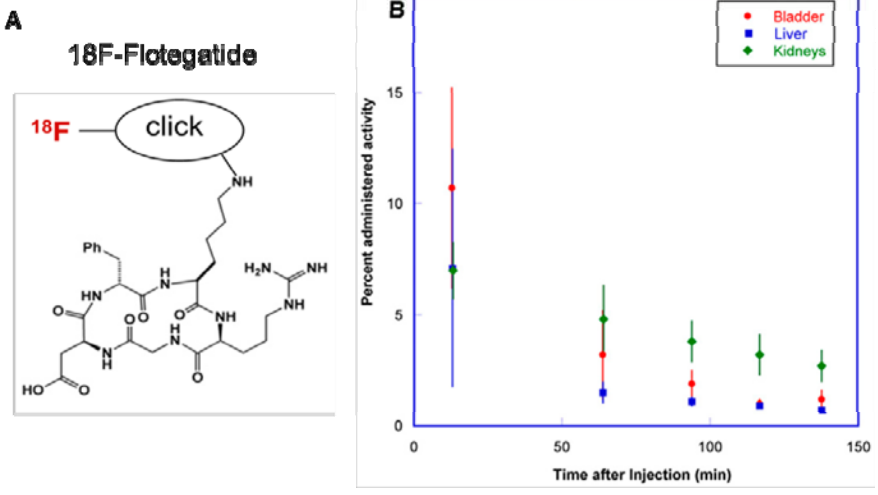
• In the section "Aim 1. Preliminary results", we demonstrate the relative increase in 18F-flotegatide uptake by vulnerable atherosclerotic plaque in the carotid artery in 3 patients with stroke compared to 2 asymptomatic patients with similar degree of carotid artery stenosis.

Aim 2a and 2b:
To test our hypothesis that 18F-flotegatide uptake is reflected by histologic markers of plaque vulnerability and mediated by an integrin-targeted mechanism. To achieve this goal, we propose to characterize the extent of macrophage infiltration and neovascularization in atherosclerotic plaque removed during endarterectomy and its association with 18F-flotegatide uptake.

Background:

Aim 1: *Macrophages and Neovessels as Markers for Vulnerable Plaque*
Rupture of the fibrous cap on the plaque surface and accelerated intravascular thrombus formation in response to plaque rupture is the primary event that precedes stroke, TIA and myocardial infarction. Atherosclerotic plaque develops in response to entry of LDL into the intimal layer of the vessel wall and endothelial injury. In response to this injury, monocytes are recruited into the vessel wall and differentiate into macrophages, whose primary role is the phagocytosis of modified LDL [38-40]. The inflammatory cascade initiated by the phagocytosis of oxidized LDL by macrophages stimulates angiogenesis and the formation of intraplaque neovessels allows the entry of red blood cells (RBCs), leukocytes, lipids and oxidized lipoproteins [35, 41]. Phagocytosis of RBCs further stimulates recruitment of new macrophages. Macrophage apoptosis and the release of matrix metalloproteases destabilizes the extracellular matrix and is thought to cause plaque rupture [39, 42]. **Intuitively, a noninvasive imaging technique that can detect plaque hemorrhage, neovascularization, and macrophage infiltration would enable identification of plaque that is vulnerable and may provide a means of distinguishing unstable plaque from stable plaque.**

Figure 2. Structure of 18F-flotegatide and tissue specific activity of 18F-flotegatide



Mean % administered activity and SD for Bladder (RED), Liver (BLUE) and kidneys (GREEN) determined on basis of four 18F-flotegatide PET emission scans in human volunteers, as function of time after injection.

Imaging of Neovessels and Macrophages with Integrin Targeted Imaging Agents

Due to the relative enrichment of macrophages and neovessels in unstable atherosclerotic plaque, they have been used as surrogates for noninvasive detection of atherosclerotic plaque. The expression of

αVβIII integrin on the cell surface of macrophages, smooth muscle cells and neovascular endothelial cells has offered a convenient target for designing molecular imaging agents with improved specificity for unstable atherosclerotic plaque. Multiple ligands can bind to αVβIII integrin in an Arg-Gly-Asp (RGD)- dependent manner and the high affinity binding of this tripeptide sequence has been exploited for MRI and for PET imaging. PET tracers designed for *in vivo* imaging of integrin expression utilize the R-G-D peptide motif, usually within a cyclic peptide structure. Examples of current tracers include 18F-Galactosyl-RGD [43] a cyclic pentapeptide, which enables integrin imaging [34, 44, 45]. Scientists at Siemens Medical Solutions, Culver City, CA have synthesized 18F-flotegatide which has a number of advantages over other RGD-based imaging agents [46]. 18F-flotegatide is easily labeled through click chemistry, without requiring protecting groups, by the reaction of 18F-fluoropentyne and an azide precursor in the presence of *in situ* generated Cu(I) (**Figure 2(A))** with a synthesis time of 75min [47]. 18F- flotegatide is metabolically stable for 2 hours and exhibits favorable biodistribution and clearance properties characterized by fast blood washout (plasma clearance half time of 12 min) and favorable renal excretion [48] (**Figure 2(B**)). In oncologic studies, intra-tumor tracer distribution of 18F-flotegatide matches the patterns of integrin and CD31 expression [47]. **We hypothesized that 18F-flotegatide may function as an ideal molecular imaging agent for noninvasive detection of unstable plaque since macrophages and neovascular endothelial cells express integrin αVβIII and α5β1 and vulnerable plaque is characterized by neovascularization and macrophage infiltration.**

MRI for anatomic localization of plaque and PET-MR of 18F-flotegatide uptake
In order to anatomically localize the plaque, we propose to perform MRI for visualization of vulnerable plaque characteristics and to localize the plaque along with time of flight (TOF) Magnetic resonance angiography (MRA) of the carotid artery for quantification of stenosis severity. PET images will be co-registered with MRI and 18F-flotegatide uptake will be compared to plaque features suggestive of vulnerability including presence of necrotic lipid core and intraplaque hemorrhage. MRI is especially robust for visualization of inflammation and neovascularization with the use of specialized pulse sequences and contrast agents. Enhancement of atherosclerotic plaque with Gadolinium (Gd) based contrast agent, Gd-diethylenetriaminepentaacetic acid (DTPA) has been used to differentiate between the fibrous cap and the lipid core [49, 50]. Gd enhancement has been shown to correspond to zones of neovascularization and inflammation [9, 51]. The extent of plaque neovascularization can also be measured by dynamic contrast enhancement (DCE) MRI [57]. The ability to detect intraplaque hemorrhage, lipid rich core and neovascularization illustrate the superiority of MRI based plaque imaging over CTA. Furthermore, MRI is not associated with ionizing radiation and is therefore safer than CTA and thus lends itself to serial imaging in high risk patients. We propose to use MRI in our plaque imaging studies to both localize atherosclerotic plaque in the carotid artery and to identify high risk morphologic plaque features such as presence of a lipid rich core and a thin fibrous cap.

Aim 2a and 2b:
Macrophage density and neovascularization as surrogate markers of unstable plaque
In clinical studies of atherosclerotic plaque imaging with radiolabeled molecular imaging agents, macrophage infiltration and neovascularization have been used as the gold standard among histologic biomarkers to adjudicate plaque vulnerability [14, 15, 17, 34]. In an early plaque imaging study with FDG, Rudd et al. used ex-vivo incubation of plaque to demonstrate that carotid plaque uptake of FDG was confined to macrophage- rich areas of plaque [17].

This has been used as evidence that FDG uptake is specific for plaque with histologic features consistent with vulnerability [17]. The important role played by macrophages in causing plaque rupture has been documented in histologic examination of carotid artery plaque. In plaque removed during endarterectomy, macrophage density was increased in patients with symptoms of stroke or TIA compared to presumably stable plaque removed from asymptomatic patients [55, 56]. The strong association between an increase in plaque microvessel density, plaque progression and plaque vulnerability has been demonstrated in ex-vivo studies [39]. Plaque collected during endarterectomy from patients with antecedent stroke contained more staining for CD31, an endothelial cell marker when compared to plaque from asymptomatic patients [57] Histologic studies show that dysmorphic neovessels are seen more often in patients with symptomatic carotid artery disease [58]. **We propose to use a similar approach to demonstrate that plaque with increased 18F-flotegatide uptake is highly enriched for one or several histologic biomarkers of plaque vulnerability including inflammation and angiogenesis.**

A positive correlation between 18F-flotegatide extent and enrichment for necrotic lipid-rich zones, increased macrophage density and (or) neovascularization would support to our hypothesis that uptake of this radiotracer reflects plaque vulnerability at the histologic level. Most importantly, we will perform ex-vivo imaging with a portion of plaque from 3 symptomatic patients to evaluate 18F-flotegatide binding in the presence or absence of unlabeled RGD peptide. The goal of this experiment is to demonstrate that 18F-flotegatide uptake occurs through an integrin mediated mechanism. Absence of ex-vivo 18F-flotegatide binding to plaque in the presence of unlabeled RGD peptide would suggest that 18F-flotegatide uptake is mediated by a specific integrin-RGD interaction.

Experimental Approach

Aim 1.

18F-flotegatide will be synthesized as described previously and PET imaging of the carotid arteries will be performed with MR based attenuation correction. 18F-flotegatide uptake will be quantified using commercially available software and plaque uptake of 18F-flotegatide by the carotid artery plaque implicated in the stroke will be compared to the contralateral carotid artery or other healthy reference vessel. 18F-flotegatide uptake of carotid plaque will also be compared between patients with stroke and asymptomatic patients. 18F-flotegatide uptake of carotid plaque will be compared to MRI based plaque features of vulnerability.

Chemical formulation and synthesis of 18F-Flotegatide and dosage for PET imaging

Pent-4-yn-1-yl 4-methylbenzenesulfonate (pentyne tosylate) will be reacted with anhydrous ^{18}F-fluoride in the presence of Kryptofix® 2.2.2 and K_2CO_3 in MeCN at 110°C with the resulting ^{18}F-fluoropentyne distilling into a collection vial for the click reaction [52]. The collection vial contains Flotegatide azide precursor, Cu(I) (generated by the *in situ* reduction of $CuSO_4$ with sodium ascorbate), and tris[(1-benzyl-1H-1,2,3-triazol-4- yl)methyl]amine (TBTA) in aqueous ethanol/acetonitrile [53]. TBTA will be removed during the semi-preparative RP-HPLC step and is not detected in the final dose. After reacting for 10 to 30 minutes at room temperature, the crude reaction mixture will be transferred to an intermediate HPLC loading vial containing water for dilution prior to semi-preparative HPLC purification. After purification of the crude reaction mixture by semi-preparative RP-HPLC (acetonitrile:aqueous TFA), the product will be reformulated via C18 cartridge reconstitution as a solution in a maximum of 10% EtOH:water. **The total process time is 75 minutes, i.e.**

less than one half-life. The final drug product formulated in a hydroalcoholic vehicle containing up to 10% ethanol will be administered intravenously at a target imaging dose of 10mCi per patient. Even at the lowest specific activity allowed-under the IND-for injection (0.4Ci/μmol), the mass is only 27μg.

Aim 2a.

Collection of plaque and processing for histologic studies

Carotid artery containing the plaque (specimen) from 3 symptomatic and 3 asymptomatic patients will be obtained at the time of endarterectomy and labeled with sutures to identify cranio-caudal orientation. The specimen will be divided into three segments (cranial, middle and caudal). This will allow us to identify any particular slice on axial PET images with high 18F-flotegatide uptake (based on SUV_{max}) as located in a specific segment of the specimen. We propose to pick the slice corresponding to the segment (cranial, middle or caudal) with the highest SUV_{max}. This segment of the specimen containing the plaque will be excised and placed in formalin for processing prior to immunohistochemical studies **(Aim 2a. Preliminary Results Figure 8)**.

Histological Evaluation of Plaque Composition

Plaque will be placed into 10% Neutral Buffered Formalin for 24 hours after which the samples will be decalcified (Cal-Rite, Richard-Allen Scientific, Kalamazoo, MI). After fixation and decalcification transferred to 70% Ethanol until processing. 5 μm thick sections will be taken at 1 mm steps. Plaque samples will be processed using the Leica Peloris with a xylene free processing protocol and embedded in paraffin. Sections, 5 microns thick, will be made with a Leica microtome and placed on positively charged slides. Sections will be stained using H&E and Movat's pentachrome staining. H&E staining will identify nuclei (black), calcium (purple), and hemorrhage(magenta) in contrast to the general pink staining of other tissues. The Movat pentachrome can distinguish fibrous regions (dark yellow-green) from lipid rich zones (turquoise). The prior presence of cholesterol appears as "cleft" voids after processing. Immunohistochemistry (IHC) will be performed on a Benchmark XT (Ventana Medical Systems Inc, Tucson, AZ) automated stainer. Slides will be deparaffinized and blocked with a hydrogen peroxide solution. Antigen retrieval will be performed using Cell Conditioner 1 (Ventana, 950-124) and a Tris/borate/EDTA buffer, pH 8.0-8.5 @ 95C for 60 minutes. Slides will be incubated with primary antibody for approximately 32 minutes @ 37C followed by incubation with secondary antibody, a biotinylated Mouse/Rabbit mixture. The secondary antibody and the HRP-based detection are part of the i-View DAB detection kit (Ventana, 790-091). The slides will be counterstained with Hematoxylin II (Ventana, 790-2208) and Bluing Solution (Ventana, 760-2037). Tissue sections will be imaged in their entirety at 20X magnification (0.5um/pixel) using a Leica SCN 400FL Slide Scanner (Leica Microsystems, GmbH, Wetzlar, Germany). Antibody staining will be measured using ImagePro Plus Analysis software (Media Cybernetics, Inc., Rockville, MD). The program's Color Segmentation tool will identify regions of HRP staining (brown) for IHC and fibrous regions (dark yellow-green) or lipid rich zones (turquoise) for MOVATS in each tissue section. The area of stained tissue will be compared to a measurement of the total tissue area to give the "percent area stained." CD31 (JC70), ventana, 760-4178 and CD68 (KP-1), Ventana, 790-2931 will be used to detect and quantify macrophage infiltration and endothelial cell density respectively. Smooth muscle proliferation will be identified and quantified using αSMA antibodyDako, M0851. In order to

correlate 18F- flotegatide uptake with integrin expression, we will use an Integrin αVβIII antibody from R&D Systems, MAB3050 and α5β1 integrin antibody from Bioss, bs-2016R.

Comparison of Immunohistochemical and Movat Pentachrome Staining with 18F-flotegatide Uptake

IHC staining will be quantified as the percent area that is stained with the particular antibody for the marker of interest (CD68, macrophage; CD31, neovessel endothelium; αSMA, smooth muscle and Integrin αVβIII and α5β1). Percent area of lipid rich necrotic core and fibrous zones will be quantified from Movats staining. Correlation between the TBR of the segment with the highest 18F-flotegatide uptake on PET imaging for each individual patient to the percent area with lipid, fibrous elements, macrophage staining, endothelial staining and smooth muscle staining of the tissue sections from this segment will be evaluated in all patients (**Aim 2. Preliminary Results Figure 8 and 9**). We will also quantify and compare staining in patients with symptoms (unstable plaque) vs asymptomatic patients (stable plaque) (**Aim 2a. Preliminary Results Figure 10**).

Aim 2b.

Ex-vivo Competition Study to Demonstrate Integrin Mediated Binding of 18F-flotegatide

A 5mm segment of plaque from 3 symptomatic patients will be collected for assessment of 18F-flotegatide uptake ex-vivo. This plaque segment will be embedded in optimum cutting temperature compound (Tissue- Plus, Fisher Scientific), snap-frozen in liquid nitrogen and stored at –70°C. Samples will be sectioned using a cryostat (Leica Microsystems, Wetzlar, Germany) and placed on electrically charged glass slides. 20 μm sections will be warmed to room temperature (RT), preincubated for 5 min in PBS buffer, and incubated with 10 nM 18F-flotegatide (specific activity 2.5Ci/μmol) in PBS buffer for 30 min at RT. Every alternate section from each plaque will be pre-treated with 10 mmol of unlabeled cyclic RGD (Siemens) prior to incubation with 18F- flotegatide. After incubation, the slides will be washed twice for 5 min with PBS, rinsed in cold water, air dried, and placed on an autoradiography imaging plate (Fuji Imaging Plate BAS-MS2040, Fuji Photo Film Co., Ltd., Japan). After overnight exposure, 18F activity in the imaging plates will be measured with Fuji Analyzer FLA- 2000. After co-registration of autoradiography and H&E-stained histological images of the sections, count density (PSL/mm2) of plaques will be quantified. 18F activity (PSL/mm2) in the absence of unlabeled competing tracer will be compared to 18F activity in the presence of competing tracer.

Expected Results

Aim 1.

1. A detectable increase in the (uptake) TBR of 18F-flotegatide in atherosclerotic plaque compared to the unaffected arterial wall.
2. An increase in TBR (18F-flotegatide uptake) in plaque that is implicated in the stroke or TIA compared to plaque that may be present in the contralateral carotid artery in symptomatic patients.
3. Most importantly, a detectable increase in atherosclerotic plaque uptake (TBR) of 18F-flotegatide in patients with stroke or TIA compared to 18F-flotegatide uptake in plaque in patients without symptoms of stroke or TIA.
4. A positive correlation between volume of lipid rich necrotic core and 18F-flotegatide uptake and a negative or lack of correlation between thickness of fibrous cap and 18F-flotegatide uptake.

Aim 2a and 2b.

1. Percent area of lipid rich necrotic core quantified from MOVATS staining may be positively correlated with TBR of the 18F-flotegatide uptake on PET imaging whereas, percent area stained for fibrous elements is expected to exhibit a negative correlation with 18F-flotegatide uptake.
2. We expect % area of plaque with CD68 (macrophage infiltration) and (or) CD31 staining (endothelial cell) to be higher among patients with increased 18F-flotegatide uptake relative to plaque from patients with lower levels of 18F-flotegatide uptake detected by PET imaging (**Figure 9**). Staining for α-smooth muscle actin may be increased in stable plaque with reduced 18F-flotegatide uptake. We also expect to find a strong correlation between area of staining with integrins αVβIII, α5β1 and 18F-flotegatide uptake.
2. We expect to find % area with macrophage staining and neovascular endothelial cell staining to be greater in plaque from patients with symptoms of stroke or TIA compared to plaque from those who are asymptomatic (**Figure 10**).
3. Ex-vivo binding studies: we expect 18F activity (PSL/mm2) of plaque sections measured by (autoradiography) in the absence of unlabeled RGD to be significantly greater than 18F activity in the presence of competing unlabeled RGD (Similar to **Figure 11**).

Feasibility

Aim 1.

Tracer Synthesis and PET Imaging: The Cleveland Clinic Department of Nuclear Medicine has a radiochemistry laboratory for the synthesis of novel PET tracers for research. The radiochemistry laboratory has synthesized several batches of 18F-flotegatide for imaging over the last 3 years (**see Aim 1. Preliminary Results**). In our preliminary studies, the average isolated yield of 18F-Flotegatide was 26.9% ± 10.3% with specific activity of 2.6 +/- 1.4 Ci/umol. We have injected this tracer in 5 patients without any adverse effects. Siemens has allowed us to use the tracer and has expressed support for this project (see letter from Siemens in the original RO1 application). We have injected 18F-flotegatide in 5 patients without any adverse effects. Siemens has allowed us to use the tracer and has expressed support for this project (see letter from Siemens). Furthermore, the PI has previously held an IND from the FDA for use of this tracer in human subjects for carotid plaque imaging and will be reapplying for an IND from Cedars Sinai Medical Center. Our preliminary results in **Table 1**, show an increased uptake of the radiotracer by carotid plaque that is associated with a recent stroke. In these same patients, plaque in the contralateral carotid artery which was not associated with stroke (and may therefore represent a relatively stable plaque) did not exhibit significant 18F-flotegatide uptake (**Table 1**). We also observed that plaque associated with a stroke (unstable plaque) exhibited a higher uptake of 18F-flotegatide relative to stable plaque (asymptomatic patients) (**Aim 1. Preliminary Results Table 1 and Figure 5**).

PET-MR: PET-MR is routinely performed in our imaging laboratory (**Aim 1. Preliminary Results Figure 4)**. We have the technical expertise of Dr. Zhaoyang Fan at Cedars Sinai Medical Center (see letter attached in original RO1 application) and he will be able to help us with pulse sequences and quantitative image analysis.

Aim 2a and 2b.

We have been able to stain carotid artery sections successfully with CD68 and CD31 (**Figure 8 and 10**). We have been able to detect prominent staining with CD68 and CD31 in plaque

from a patient who underwent CEA 8 weeks following a stroke (**Figure 8**). We are currently optimizing staining conditions for α-SMA, αVβIII and α5β1 antibodies.

Furthermore, we show an association between uptake of 18F-flotegatide and macrophage density in 5 patients who underwent plaque imaging with 18F-flotegatide (**Figure 9).** We have also demonstrated that atherosclerotic plaque uptake of 18F-flotegatide occurs via an RGD-specific mechanism in a mouse model of atherosclerosis (**Figure 11).** We expect to find similar specificity of 18F-flotegatide binding in human plaque samples.

Limitations and Alternative Strategies

Aim 1.

Radiotracer Synthesis, PET-MR and Plaque Uptake: We have synthesized and labeled several batches of 18F-flotegatide and we do not expect to encounter any technical difficulties in the synthesis of 18F-flotegatide. We have technical support from Siemens for trouble shooting if any issues relating to the synthesis of 18F-flotegatide were to arise. High affinity binding of purified 18F-flotegatide to αVβIII and α5β1 has been confirmed in-vitro with inverse surface plasmon resonance [53, 54]. Documentation of radiotracer purity from our synthesis will also be verified by the FDA (IND to be obtained). In the unlikely event of difficulties in performing PET-MR, we will be able to seamlessly switch to PET-CT imaging for detection of 18F-flotegatide uptake. We have 3 PET/CT scanners located in the Department of Nuclear Medicine. Our preliminary data in **Figure 5 and 6** and **Table 1** indicates that we are likely to be successful in demonstrating that atherosclerotic plaque extracts 18F-flotegatide and that uptake of 18F-flotegatide is increased in unstable plaque compared to stable plaque. Since we are using clinical criteria to define plaque vulnerability, there is the possibility that we may be misclassifying some patients. We may be able to include LDL and C-reactive protein (CRP) levels in our analysis and consider matching symptomatic and asymptomatic patients based on these serum markers (classified as normal vs abnormal). This will allow for comparisons to be made between symptomatic and asymptomatic patients with a similar inflammatory and lipid profile and minimize potential confounding effects.

Aim 2a and 2b.

Macrophages and neovascular endothelial cells are considered gold standard histologic biomarkers for adjudication of unstable plaque in clinical studies [12, 13, 17, 50, 56, 59]. Based on our preclinical study of 18F-flotegatide uptake in a mouse model of atherosclerosis and our preliminary data from 5 patients with carotid atheroma, there is a high likelihood of detecting an association between macrophage density and 18F- flotegatide uptake. We also plan to compare 18F-flotegatide uptake to histologic plaque features such as area of fibrous elements and lipid core. This will serve as an alternate method by which we can confirm that 18F- flotegatide uptake is associated with features of plaque vulnerability. To maximize sampling of macrophages, endothelial cells and smooth muscle cells, we propose to obtain multiple sections from the plaque segment with the highest 18F-flotegatide uptake. We have optimized the conditions for immunohistochemical staining of CD68 and CD31. For our proposed studies, we will use commercially available antibodies to α-SMA and αVβIII that have been used by other investigators with formalin fixed, paraffin embedded plaque samples. The histology core is well equipped and has expertise for optimization of staining conditions and has access to other vendors of antibodies if this becomes necessary.

Preliminary results

Aim 1.

Carotid MRI for plaque characterization (**Figure 4**) and 18F-flotegatide PET-CT in 5 patients (3, with stroke due to carotid plaque rupture) with. Preliminary results confirm uptake of 18F-flotegatide by atherosclerotic plaque in the carotid artery (**Figures 5** and **6)** and increased uptake of 18F-flotegatide by plaque in patients with stroke compared to asymptomatic patients (**Figure 5** and **Table 1**).

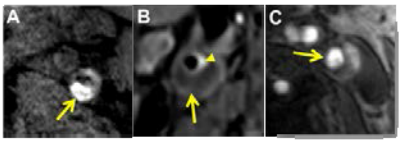


Figure 4. MRI of Carotid Plaque
from a patient with 75% stenosis of the left internal carotid artery.
Panel A: MP-RAGE image; bright area represents plaque hemorrhage
Panel B: Contrast enhanced T1 weighted image; region of low intensity represents necrotic core.
Panel C: Contrast filled vessel lumen with eccentric plaque

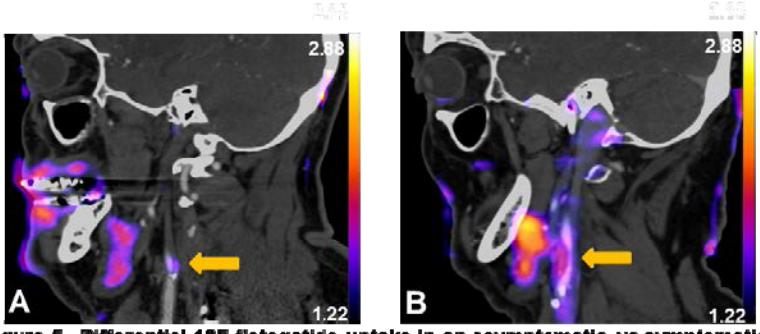


Figure 5. Differential 18F-flotegatide uptake in an asymptomatic vs symptomatic patient.
Panel A: asymptomatic patient with carotid plaque and Panel B: patient with recent stroke. Yellow arrows represent plaque visualized by CTA and co-registered with PET-CT performed 2h after injection of 18F-flotegatide. Images are represented on the same SUV color scale, max(2.88) and min(1.22). Plaque uptake of 18F-flotegatide is higher in the symptomatic patient compared to the asymptomatic patient.

Table 1. 18F-flotegatide uptake (TBR) in carotid artery of interest vs contralateral carotid artery in 3 patients with stroke and 2 asymptomatic patients who underwent CEA

Symptomatic Patients	Stroke Related Unstable Plaque TBR (% stenosis)	Contralateral Plaque TBR (% stenosis)
1	2.18 (100%)	1.79 (60%)
2	2.1 (80%)	1.68 (70%)
3	2.02 (90%)	1.88 (60%)

Asymptomatic Patients	Asymptomatic Stable Plaque TBR (% stenosis)	Contralateral Plaque TBR (% stenosis)
4	1.57 (75%)	1.24 (30%)
5	1.91 (90%)	1.82 (60%)

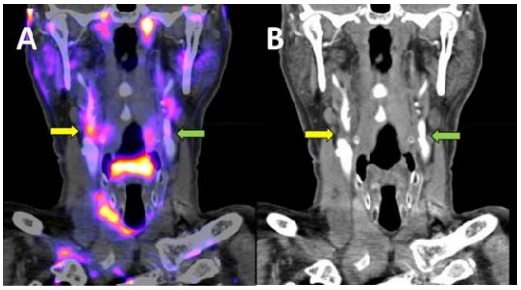


Figure 6. 18F-flotegatide uptake by right internal carotid artery plaque
PET-CT coregistered with a CTA (panel A) and a CTA (panel B) in a patient with nearly complete stenosis in the right internal carotid artery and stroke 8 weeks before the scan.
Plaque causing the stroke (yellow arrow) and the contralateral carotid artery mild plaque (green arrow). Panel A: prominent uptake of 18F-flotegatide in the same region as the plaque (yellow arrow) with significantly low uptake in the contralateral carotid artery (green arrow).

Aim 2a and 2b.

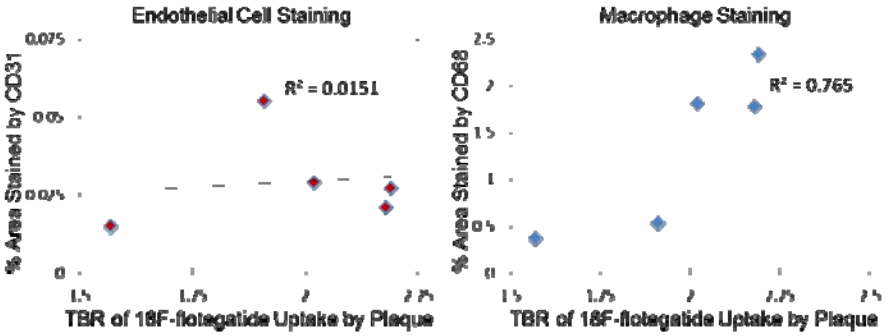


Figure 9. Correlation between markers of plaque vulnerability and 18F-flotegatide uptake % Area stained by antibodies for macrophages and endothelial cells in sections prepared from a segment of the plaque with the highest 18F-flotegatide uptake (TBR) was quantified. There was strong correlation between flotegatide uptake and macrophage staining. R2=0.765 and p=0.05.

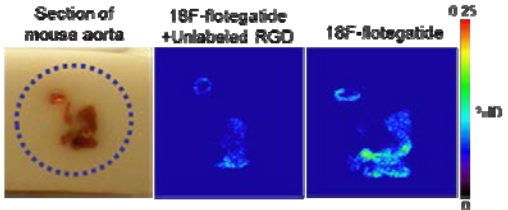


Figure 11. Specificity of 18F-flotegatide uptake. Autoradiograph of a section of mouse aorta with atherosclerotic plaque from APOE-KO mouse incubated in 18F-flotegatide in the presence or absence of unlabeled RGD. 18F-flotegatide uptake is reduced in the presence of unlabeled RGD.

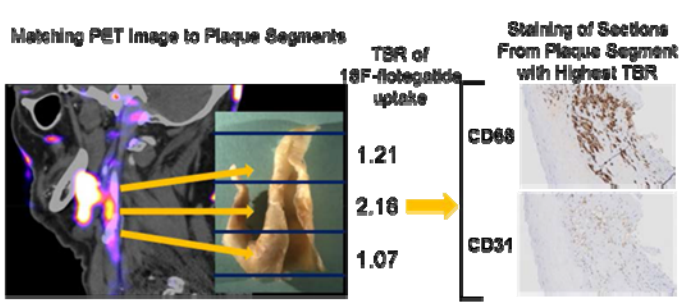


Figure 8. Identification of plaque segment with highest TBR and immunostaining of tissue sections from that segment for macrophage and neovascular endothelial cells (markers of plaque vulnerability).

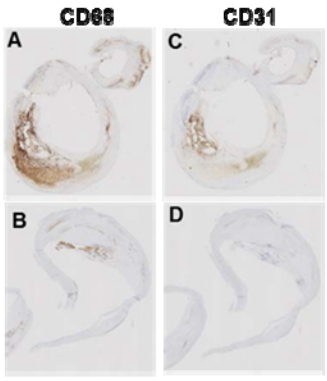


Figure 10. Macrophage and neovascular endothelial staining of carotid plaque in patients with stroke (unstable plaque) and asymptomatic patients(stable plaque). Dark brown areas in Panel A and B: CD68 staining (macrophage marker); Panels C and D: CD31 staining (endothelial cell marker). 5µm sections of carotid plaque were removed during CEA. 18F-flotegatide in unstable plaque (panels A and C) was increased (TBR of 2.18) compared to stable plaque (panels B and D) (TBR 1.57).

TIMELINE (ALL AIMS)

	0-4mo	4-8mo	8-12mo
Optimization of tracer synthesis, PET-MRI and immunohistochemical staining			
SA1 PET-MRI of patients with carotid plaque			
SA2 Histologic studies of plaque			
SA2 Ex-vivo binding studies			
Data Analysis			

FUTURE DIRECTIONS

Our long-term goal is to translate the use of 18F-flotegatide for identification of vulnerable plaque in individuals with carotid atherosclerosis. The R56 bridge award will enable us to optimize PET-MRI of carotid plaque with 18F-flotegatide, demonstrate that 18F-flotegatide uptake in carotid plaque can be imaged with PET-MRI and will establish that flotegatide uptake in carotid plaque is integrin mediated. Results from the studies proposed in this R56 application will be used as preliminary data (demonstrating the feasibility of using 18F-flotegatide for imaging unstable plaque with PET-MRI) in an RO1 application. In the future RO1 application

we propose to test our hypothesis that 18F-flotegatide may be preferentially extracted and retained by carotid artery plaque in patients with clinical symptoms of stroke or TIA (vulnerable plaque) compared to asymptomatic control patients (stable plaque) in a larger cohort of 20 symptomatic patients and 20 asymptomatic controls.

Section 4.0 Trial Design

APPROACH

PET-MR of Carotid Plaque

PET-Protocol:

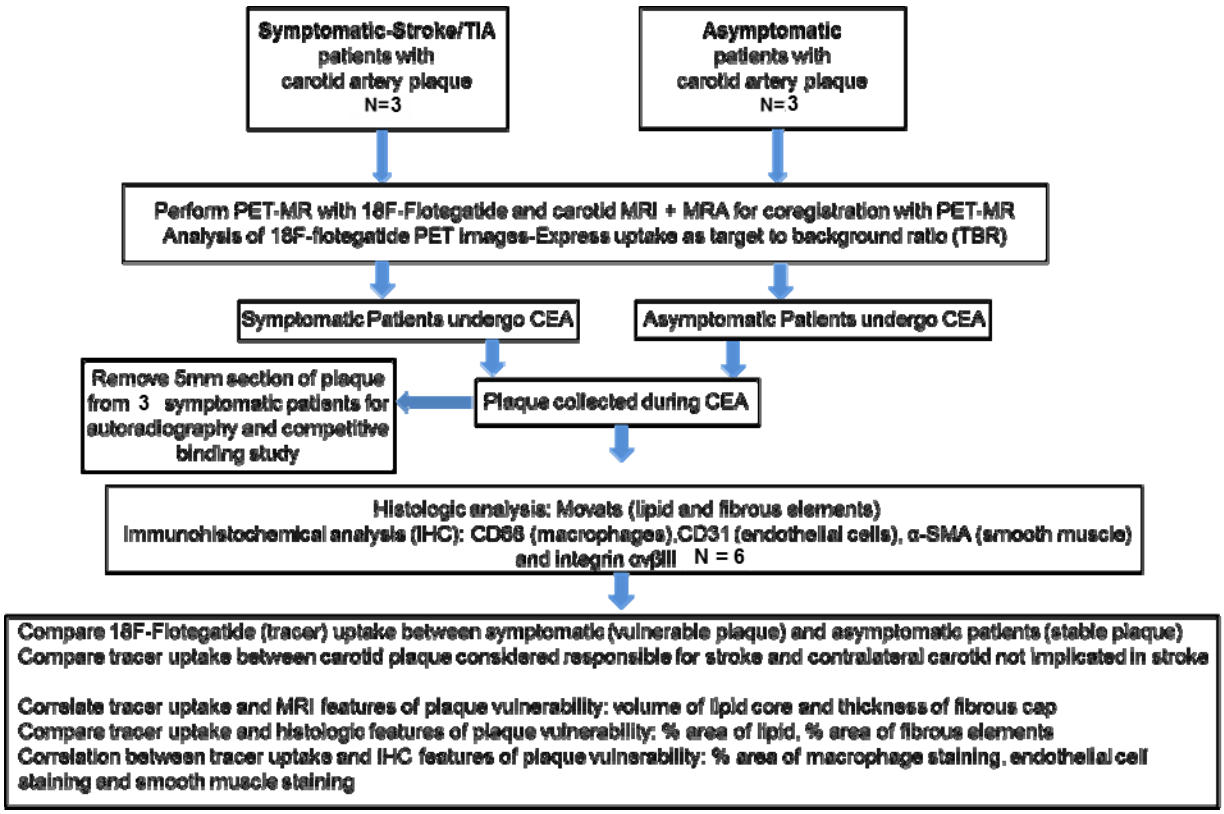
A total of 6 patients (3 with stroke or TIA and 3 asymptomatic) will undergo noninvasive PET-MR imaging (Imaging protocol is described below) after injection of 18F-Flotegatide. Our preliminary studies show that there is maximal plaque to blood pool contrast at 2h after injection of 18F-flotegatide**.** A dose of 10-12.5 milliCurie (370MBq-462.5MBq) of 18F-flotegatide will be injected intravenously and PET-MR imaging will be performed 2 hours (+/- 30 mins) after injection on a Siemens Biograph mMR (Siemens Healthcare Erlangen, Germany) using a dedicated head and neck coil (Siemens Healthcare). Scout images will be obtained for localization of the carotid arteries. An mMR standard Dixon water-fat MR sequence will be obtained and segmented (into air, lung tissue, soft tissue and fat) for MR-based PET attenuation correction (MR-AC) [54]. PET acquisition will begin with the start of the Dixon MR sequence in the same bed position. PET acquisition time will be 15 min in three-dimensional list mode. The PET data from the PET-MR scan will be reconstructed using ordered-subsets expectation maximization iterative reconstruction algorithm (OSEM 3D) (6 iterations, 21 subsets, zoom 2.0) with MR based attenuation correction yielding 512 x 512 image slices (voxel size: 0.70 x 0.70 x 2.03 mm). Attenuation maps for correction of the PET data from the PET-MR scanner will be generated on the basis of the Dixon water-fat MR sequence using the postprocessing software of the scanner. Once plaque is localized on the MR images, the corresponding coregistered PET image will be visually evaluated for 18F-flotegatide uptake. The region corresponding to the plaque on the PET image will be analyzed with commercially available software (MIM) to quantify standard uptake value (SUV) and TBR of 18F-flotegatide. MRI of plaque will be examined for markers of vulnerability including, fibrous cap, lipid rich necrotic core and intraplaque hemorrhage.

MRI-MRA for visualization and co-registration of atherosclerotic plaque:

The MRI protocol will consist of high- spatial resolution 3D imaging sequences in order to detect major vulnerable plaque features and minimize partial volume effects due to tortuous carotid arteries. The sequences will be: a) 3D MP-RAGE -a heavily T1- weighted imaging sequence for detection of intraplaque hemorrhage; b) 3D time-of-flight (TOF) for definition of luminal stenosis; and c) pre- and post-contrast T1-weighted 3D turbo spin echo (SPACE) to detect and quantify the lipid-rich necrotic core and fibrous cap. This will be followed by contrast injection (Gadolinium- DTPA, single dose 0.1 mmol/kg bodyweight, injection rate 2-3 cc/sec). Post-contrast SPACE will be acquired 5 minutes post injection. In addition, contrast-enhanced MRA may also be added into the protocol to obtain high- quality MRA. Typical imaging parameters are: 3D MP-RAGE axial acquisition, TR/TE = 15/3.5 msec, FOV = 160x160 mm2, resolution = 0.6x0.6 mm2, slice = 2 mm interpolated to 1 mm, flip angle = 15º, inversion time = 500 msec, turbo factor = 30. 3D TOF axial acquisition, TR/TE = 20/4.6 msec,

FOV = 160x160 mm2, resolution = 0.6x0.6 mm2, slice = 2 mm interpolated to 1 mm, flip angle = 20º. 3D SPACE coronal acquisition, TR/TE = 100/15 msec, FOV = 160x160 mm2, resolution = 0.6x0.6 mm2, slice = 0.6 mm, echo train = 50. Image quality review, processing (multi-planar reconstruction, MPR), and tissue characterization (outlining and size quantification) will be conducted on an image workstation with FDA-approved carotid plaque analysis software, MRI-Plaqueview (VPDiagnostics, Seattle, WA). From each of 3D sequences, contiguous cross-sectional slices that are perpendicular to the common carotid artery will be reconstructed. Intraplaque hemorrhage will be identified on 3D MP-RAGE as a hyper-intense area. Lipid rich necrotic core and fibrous cap will be identified on post-contrast 3D SPACE as hypo-intense and hyper-intense areas, respectively, with pre- contrast SPACE as reference. Vessel wall, lumen, and individual components will be outlined in a semi- automatic fashion. Their volumes will then be reported by the software.

Figure 1. Overall Experimental Scheme



Patients will be enrolled when they present to the neurologist for evaluation of stroke or TIA or when they are seen by vascular surgeon for CEA or an interventional cardiologist for carotid stent (**Figure 1).** All patients will have fasting lipid levels, C- Reactive protein and HgBA1C measured as routine standard of care. Symptomatic patients would be required to have plaque ≥70% in at least one carotid artery that would be implicated as the source of embolus responsible for the stroke. We propose to enroll 3 patients with stroke or TIA for PET imaging. They will undergo PET-MR of the neck (to image the bilateral carotid arteries) with 18F-Flotegatide within 96h of stroke or TIA and a simultaneous MRI and magnetic resonance angiography (MRA) of the carotid arteries for anatomic localization of the plaque (**Specific Aim 1**). In addition to providing an anatomic map for coregistration of the images obtained by PET, MRI

will provide morphologic information about the carotid plaque. Plaque removed during CEA or carotid stenting in all patients (within 7d of carotid PET-MR) will be used for histologic examination (**Specific Aim 2a**). A small portion of the plaque from 3 symptomatic patients will be used for ex-vivo 18F-flotegatide uptake-competition studies to confirm integrin specific binding (**Specific Aim 2b**). We propose to also enroll 3 asymptomatic patients with a luminal stenosis of ≥70% in at least one carotid artery (stable plaque) referred for CEA or carotid stenting. These patients will be age-matched by deciles to symptomatic patients. These patients will undergo PET-MR of the neck (to image the bilateral carotid arteries) with 18F-flotegatide along with an MRI and MRA of their carotid arteries. Plaque removed from asymptomatic patients at the time of CEA or carotid stenting (within 7d of PET-MR) will be used for histologic examination. Exclusion criteria include, stroke due to atrial fibrillation, preexisting carotid stents in the artery of interest, renal dysfunction defined as glomerular filtration rate <40ml/min, allergy to gadolinium based contrast agents, metal implants incompatible with MRI, pregnancy, inability to provide informed consent and age ≤18 years. We propose to enroll a total of 6 patients over a period of 1 year.

Section 5.0. Selection of Subjects

Inclusion Criteria:

1. Patients presenting
   a. to the neurologist for evaluation of stroke or TIA (Symptomatic) with:
      i. Symptomatic patients with plaque ≥70% in at least one carotid artery that would be implicated as the source of embolus responsible for the stroke/TIA.
   b. or to the vascular surgeon for CEA (Asymptomatic) with:
      i. asymptomatic patients with a luminal stenosis of ≥70% in at least one carotid artery (stable plaque) referred for CEA
      ii. age-matched by deciles to symptomatic patients
   c. or to the interventional cardiologist for evaluation and possible carotid stenting with:
      i. asymptomatic patients with a luminal stenosis of ≥50% in at least one carotid artery (stable plaque) referred for carotid stenting
      ii. age-matched by deciles to symptomatic patients

Exclusion criteria:

1. stroke due to atrial fibrillation,
2. preexisting carotid stents in the artery of interest,
3. renal dysfunction defined as glomerular filtration rate <40ml/min,
4. allergy to gadolinium based contrast agents,
5. Volunteers who have had four or more prior previous gadolinium contrast scans
6. metal implants incompatible with MRI or other condition that prohibits MRI,
7. pregnancy,
8. allergy to animal dander or animal-instigated asthma
9. inability to provide informed consent and
10. age ≤18 years.

We propose to enroll a total of 6 patients over a period of 1 year.

Section 6.0 Treatment of Subjects

All eligible subjects will undergo PET-MR of the neck (to image the bilateral carotid arteries) with 18F-Flotegatide within 96h of stroke or TIA and a simultaneous MRI and magnetic resonance angiography (MRA) of the carotid arteries for anatomic localization of the plaque

Plaque removed from asymptomatic patients at the time of CEA or carotid stenting (within 7d of PET-MR) will be used for histologic examination.

Section 7.0 Assessment of Efficacy

Validation of the atherosclerotic plaque detected by PET-MRI using 18F-flotegatide will be completed via in-vivo PET imaging of patients with unstable carotid plaque. Plaque will be quantified by measuring the plaque uptake of 18F-flotegatide relative to 18F-flotegatide uptake in segments of the carotid vessel wall that are free of atherosclerosis.

Section 9.0. Statistics

Image Analysis of plaque using MRI and MRA: Hemorrhage appears bright on MP-RAGE (**Figure 4**, panel A, yellow arrow). On contrast enhanced T1W images, fibrous portions of atherosclerotic plaque appear bright (**Figure 4** Panel B, arrowhead) and necrotic core in the plaque will be identified on the postcontrast T1W image as the area with no or slight contrast enhancement (**Figure 4** panel B yellow arrow). The percent stenosis will be quantified from TOF images (panel C, bright lumen is shown by the yellow arrow). Thickness of the fibrous cap and volume of lipid rich necrotic core will be quantified and plaque features including hemorrhage, and intact vs ruptured fibrous cap will be recorded.

Image Analysis of PET-MR: PET-MR images will be fused with the simultaneously acquired MR images. Coregistration of PET images with MRI will be performed using anatomic landmarks. Plaque is normally present in the carotid bifurcation and the proximal internal carotid artery. Plaque localized on the axial MR images will be correlated with PET signal. Uptake of 18F-flotegatide will be quantified in regions of interest (ROI) including both the vessel wall and the vessel lumen. The standard uptake value (SUV) in each slice position on the axial images will be quantified as SUV_{mean} and SUV_{max} using commercially available software (MIM software Inc, Cleveland, OH). These are derived from the injected dose, time to acquisition to account for decay and patient weight. The SUV_{max} and SUV_{mean} of unaffected segment of the carotid artery as well as the uptake of the tracer in the same level in the contralateral carotid artery will be quantified. SUV_{mean} of the blood pool in the aortic arch will be quantified to derive background uptake. SUV_{max} of the target ROI is divided by the background SUV_{mean} to derive a target to background ratio (TBR). The TBR of 18F-flotegatide in the plaque will be compared to TBR of unaffected vessel wall.

Comparison of morphologic markers of vulnerability with 18F-flotegatide uptake: MRI based plaque features including, volume of lipid core and thickness of fibrous cap will be directly compared to plaque uptake of 18F-flotegatide expressed as TBR from PET-MR images.

Sample Size and Power for Between Group Comparisons

Effect Size	Number per Group	Power (1-β)
1.16	19	80%
1.16	25	90%
1.2	13	80%
1.2	17	90%
1.25	9	80%
1.25	12	90%
1.3	7	80%
1.3	9	90%

Assumes 2-group (Non-paired) design. Level of significance =0.05; Sigma =0.3

Sample Size and Power for Correlation

R	Number of Samples	Power (1-β)
0.5	29	80%
0.5	38	90%
0.6	19	80%
0.6	25	90%
0.7	13	80%
0.7	10	90%
0.8	7	80%
0.8	12	90%

For non-parametric Spearman's rank correlation. Level of significance =0.05

Figure 3. Power Calculations

Statistical Analysis

Aim 1.

The statistical analysis is provided to demonstrate how we propose to analyze data when we are able to test our hypothesis in a larger group of patients as part of our future RO1. 18F-flotegatide uptake will be analyzed as a continuous variable. The thickness of fibrous cap and volume of lipid rich necrotic core will also be treated as continuous variables. We will evaluate the use of a parametric test or a transformation of the data (e.g. log transform) prior to using a parametric test. However, if this is not appropriate due to distributional assumptions etc., we will use a nonparametric Mann Whitney U test with the following **null** hypotheses:

1. 18F-flotegatide uptake by carotid plaque (TBR of plaque) in patients with symptoms of stroke or TIA is similar to 18F-flotegatide uptake by the unaffected vessel wall.
2. 18F-flotegatide uptake by carotid plaque (TBR of plaque) implicated in stroke or TIA in symptomatic patients is similar to 18F-flotegatide uptake by stable plaque in asymptomatic patients.
3. There is no correlation between 18F-flotegatide uptake and area of the fibrous cap or volume of necrotic lipid core quantified by MRI.

With a sample size of 17 per group, we will have 90% power to detect a 1.2-fold difference in the TBR of the

plaque responsible for causing stroke/TIA compared to TBR of unaffected vessel wall (based on level of significance of 0.05 and the standard deviation of 0.3 for TBR measurements). For our correlation studies

between TBR of 18F-flotegatide uptake and MRI based characteristics of vulnerability, a sample size of 19 will

provide 80% power to detect at least moderate association (correlation coefficient of 0.6) at a level of significance of 0.05.

The goal of our proposed studies in this R56 application is to demonstrate that detection of 18F-flotegatide by PET-MRI is feasible and that there is a detectable difference in 18F-flotegatide uptake between unstable and stable atherosclerotic plaque. Even though it may not be statistically significant due to the limited number of patients who will be enrolled during the

1-year time-frame, we will be able to observe qualitative differences in plaque uptake of 18F-flotegatide between symptomatic and asymptomatic patients.

Aim 2a and 2b.

Figure 7. Power Calculations for Estimating Sample Size for Histologic Comparison Studies

Sample Size and Power for Correlation

R	Number of Samples	Power (1-β)
0.5	29	80%
0.5	38	90%
0.6	19	80%
0.6	25	90%
0.7	13	80%
0.7	10	90%
0.8	7	80%
0.8	12	90%

For non-parametric Spearman's rank correlation. Level of significance =0.05

We propose to use a Spearman correlation (a nonparametric test of association) that does not assume linearity or normal distribution of the values of 18F-flotegatide uptake and staining density for macrophages, smooth muscle cells and endothelial cells. An overall sample size of 19, will provide 80% power to detect moderate to strong associations (correlation coefficient of 0.6 or greater) at a level of significance of 0.05 (**Figure 7**). Our plan to enroll 40 patients is significantly greater than the sample size needed for 80% power.

The goals of Aims 2a and 2b in this R56 application are to demonstrate that 18F-flotegatide uptake in carotid plaque is associated with markers of vulnerability and is mediated by integrin-flotegatide interaction. Even though our planned enrollment will not be powered to reach statistical significance for Aim 2a, we will be able to demonstrate qualitative differences in histologic markers of plaque vulnerability between symptomatic and asymptomatic patients. We will also be able to unequivocally demonstrate that 18F-flotegatide uptake in atherosclerotic plaque is mediated by integrin binding with our ex-vivo assay (Aim 2b).

Section 10.0. Quality Control and Quality Assurance

Descriptive summaries will be provided for all primary and secondary end-points. All analyses and reports will be developed per Good Clinical Practices.

Section 11.0 Ethical considerations relating to the trial

This study will be conducted in accordance with Good Clinical Practice (GCP) requirements described in the current revision of International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use (ICH) Guidelines and all applicable regulations, including current United States Code of Federal Regulations (CFR), Title 21, Parts 11, 50, 54, 56, and 312 and Title 45, Part 164. Compliance with these regulations and guidelines also constitutes compliance with the ethical principles described in the current revision of the Declaration of Helsinki. This study will also be carried out in accordance with local legal requirements.

Section 12.0. Data Handling and Recordkeeping

Patients' names will remain confidential and will not be included in the database. Only patient number, patient initials, and birth date will be recorded in the data system. If the patient name appears on any other document collected (e.g., hospital discharge summary), the name will be obliterated before the document is transmitted. All study findings will be stored in paper CRFs. The patients will give explicit permission for representatives of the Sponsor, regulatory authorities, and the IRB/IEC to inspect their medical records to verify the information collected. Patients will be informed that all personal information made available for inspection will be handled in the strictest confidence and in accordance with all state, local, and federal data protection/privacy laws, including, without limitation, the HIPAA. All participants in the study will provide written authorization to disclose private health information either as a part of the written ICF or as a separate authorization form. The authorization will contain all required elements specified by 45 CFR 164 and ICH E6 as applicable, and will contain a waiver of patient access to study-related private health information until the conclusion of the clinical study. The authorization will remain valid and in full force and effect until the first to occur of (1) the expiration of 2 years after the study therapy is approved for the indication being studied, or (2) the expiration of 2 years after the research program is discontinued.

Individual patient medical information obtained during this study is confidential and its disclosure to third parties (other than those mentioned in this section) is strictly prohibited. In addition, medical information obtained during this study may be provided to the patient's personal physician or to other appropriate medical personnel when required in connection with the patient's continued health and welfare.

The study site will maintain a personal patient identification list (patient and treatment numbers with the corresponding patient names) to enable records to be identified.

In accordance with 21 CFR 312.62, an Investigator participating in this study shall retain records, including the CRFs and supporting data including signed and dated consent forms, and medical records, progress notes of the physician, the individual's hospital chart(s), and the nurses' and staff notes.

Section 13.0 FLOWCHART OF PROCEDURES

Procedures	**Visit 1** [3] **Baseline**	**Visit 2 (Pre-Operative PET Scan)**	**Follow-up Phone Call**	**Visit 3 (Surgical Day)**
	(-28 days)	**(-7 to 0 days)**	**(-4 - +3 Days)**	**Day 0**
Standard of Care Procedures: Items, drugs and services that are part of regular care and would be done even if you did not take part in this research study. These will be billed to you and/or your insurance company.				
Medical History Review	X			
Medication Review	X	X	X	
Vital Signs x 3		X		
Physical Exam		X		
Electrocardiogram x 3		X	-	
Pre-op blood tests including Fasting lipid panels, C-reactive protein and HgBA1C1	X[1]	X[1]	-	
Pregnancy test, ***if applicable***		X		
IV (intravenous) line inserted		X		
Carotid Endarterectomy or Stenting of Carotid Artery				X
MRI/MRA Imaging[2]		X		
Research Related Procedures: Items and services done for research purposes only. These will NOT be billed to your insurance company.				
Subject Informed Consent	X			
Inclusion/Exclusion Criteria	X			
PET/MR[2] Imaging with 18-flotegatilde		X		
Adverse Events	As they occur			
Sample of carotid artery plaque removed				X

[1]If labs been collected within 30 days prior to the research imaging tests, no blood will be drawn to check your kidney function.
[2] Within 96 hours of stroke or TIA (if applicable; "Symptomatic" subjects only)
[3] Baseline visit (Visit 1) may be combined with Visit 2.