Prediction of arteriovenous fistula stenosis and failure via in vivo inflammation and fibrin molecular imaging

Dialysis Access is the “lifeline” for 1.5 million end-stage renal disease (ESRD) patients across the world. Effective hemodialysis treatment requires reliable large caliber vascular access. The preferred hemodialysis access is an arteriovenous fistula (AVF) because of its lower infection rate, lower hospitalization rate and relatively longer longevity compared to grafts. However, the primary failure rate of AVF approaches 50% with neointimal hyperplasia (NH) in the juxta-anastomotic region as a major cause of AVF failure. Currently, no clinical diagnostic modality can reliably predict early AVF failure and no medical treatments can improve juxta-anastomotic stenosis in AVF. Intriguingly, studies have showed inflamed and pathologically altered endothelium plays a vital role in neointimal hyperplasia. Yet the mechanisms by which pathological endothelium drives neointimal hyperplasia and thrombosis remain poorly understood, especially in vivo. The ability to quantitatively image pathological endothelium after arteriovenous fistula (AVF) creation could provide clinically relevant, vital new insights into AVF failure. We have recently shown in a murine end-to-side jugular vein to carotid artery AVF model with carotid artery mobilization that pathological endothelium can be imaged in vivo, and can predict arterial inflow neointimal hyperplasia, using a cell-targeted fluorescent nanoparticle (CLIO) and intravital microscopy (IVM). However, the prior AVF model does not recapitulate venous side disease that more often drives human AVF failure. Therefore, in this study, we will investigate the ability to detect venous outflow inflammation by harnessing a recently described jugular vein mobilization murine AVF model by multi-target IVM. We will then test whether the degree of venous side inflammation will predict subsequent venous neointimal hyperplasia. Lastly, as a translational step, we will perform noninvasive MRI of venous-side pathological endothelium labeled by CLIO. These studies will establish an in vivo translational imaging roadmap to predict venous-side AVF failure, and potentially identify subjects likely to benefit from early intervention.

Specific Aims

Aim 1: Assess the in vivo time course of endothelial injury and fibrinogenesis post AVF creation in wild type C57BL/6J mice using an end-to-side external jugular vein to carotid artery anastomosis AVF model.

Hypothesis 1: Endothelial injury and fibrinogenesis in AVF venous juxta-anastomosis can be simultaneously imaged and quantified in vivo, and will evolve more intensely near the AVF anastomosis.

Aim 1.1: Develop and validate an end-to-side external jugular vein and carotid artery anastomosis AVF model in C57BL/6J mice.

Aim 1.2: Map and quantify endothelial injury and fibrinogenesis post-AVF creation in vivo at day 7, 14 or 21 using fluorescent cross-linked iron oxide (CLIO) and FTP-11 fibrin probe by multi-target intravital microscopy (IVM).

Aim 1.3: Perform survival serial IVM imaging of AVF to quantify the evolution of endothelial injury and fibrinogenesis simultaneously in vivo. Imaging timepoints will be determined based on the results of Aim 1.1 and 1.2. Correlative fluorescence microscopy and histological assessments will be performed after sacrifice.

Aim 2: Determine whether early endothelial injury and fibrinogenesis can predict subsequent venous juxta-anastomotic neointimal hyperplasia in AVF. Hypothesis 2: Endothelial damage leads to fibrin and platelets deposition, which drives smooth muscle cells proliferation.

Perform survival intravital microscopy imaging to quantify endothelial injury and fibrinogenesis in AVF mice at day 7 and quantify venous juxta-anastomotic neointimal hyperplasia at day 14 or 21 by histology (Hematoxylin and eosin stain, and Verhoeff-van Gieson Stain).

Aim 3: Determine if venous side AVF inflammation can be imaged noninvasively using cellular magnetic resonance imaging (MRI) by CLIO-labeled pathological endothelium. Hypothesis 3: CLIO is detectable by MRI and will enable noninvasive detection of venous-side pathological endothelium in AVF.

Overall impact: These studies will provide valuable new in vivo insights into endothelial injury and fibrinogenesis processes that drive venous juxta-anastomotic stenosis in AVF. These studies will further provide a foundation for future in vivo AVF imaging and therapeutic investigations (genetic, pharmacologic) aiming to reduce AVF juxta-anastomotic stenosis formation. In addition, as both MRI molecular imaging system and molecular imaging agents are emerging into clinic, the current molecular imaging strategies may provide a roadmap for future translational imaging studies of patients with AVF.
Significance and Innovation

The prevalence of ESRD increases annually due to the epidemic diabetes mellitus and aging of the population. Hemodialysis remains the most common treatment modality for ESRD patients. The preferred access for hemodialysis patients is arteriovenous fistula (AVF) because of its relatively lower complications and longer longevity. (1, 2) Unfortunately, the primary failure rate of AVF is 50%. Early AVF failure is characterized by juxta-anastomotic stenosis, which often occurs in the venous segment. (7) Human pathology has revealed that neointimal hyperplasia is the culprit of juxta-anastomotic stenosis. (3) Currently, clinical surveillance methods do not predict access maturation and dysfunction reliable, and do not provide biological features such as endothelial injury or fibrin deposition that underlying neointimal hyperplasia (NH). (8) Cellular imaging could provide an important role in identifying high-risk failure AVFs, thus offers a window for therapeutic interventions.

The significance of the anticipated results include:

1) New approach to study endothelial injury and fibrinogenesis in AVF in vivo: The anticipated results will provide new insight into how inflamed endothelium modulates neointimal hyperplasia and thrombosis using high resolution IVM. Loss of endothelial integrity from the initial AVF surgical anastomosis, and augmented blood flow, can both lead to loss of anticoagulation inhibitory factors, thrombus formation, smooth muscle cells activation and proliferation. (4) However, conventional imaging methods cannot assess serial pathological endothelial response and fibrinogenesis in vivo. The ability to image venous juxta-anastomotic pathological endothelium has several significant implications, including the ability to interrogate endothelial function post-AVF creation as well as to assess the effects of anti-stenotic therapies targeting dysfunctional endothelium.

2) New insights into how early timepoint endothelial injury and fibrinogenesis modulate venous neointimal hyperplasia: The anticipated results will provide new understanding into how vascular injury and fibrinogenesis drive venous juxta-anastomotic NH in vivo, using survival IVM. The ability to predict NH based on in vivo CLIO-signal and/or FTP-11 intensity has significant implications, including the ability to investigate the mechanisms of NH induced by endothelial damage and fibrin deposition, as well as to assess the effects of therapeutics aiming to decrease NH.

3) Clinical significance of molecular MRI imaging in AVF: In vivo molecular imaging can unveil the vascular injury post-AVF creation in real time, which can provide comprehensive and longitudinal dynamic molecular information, with potential translational opportunities. Noninvasive MRI has been successfully used in human AVF to investigate the hemodynamic changes after AVF creation and the degree of vascular impairment in malfunctional AVF. (9, 10) CLIO is a magnetofluorescent nanoparticle, which can induce signal loss in T2-weighted magnetic resonance images. Clinical magnetic nanoparticles such as ferumoxytol are similar to the CLIO-VT680 nanoparticles proposed in this study, and can also induce MRI signal loss on T2*-weighted images. MRI imaging of venous juxta-anastomotic inflammation in vivo using CLIO in this study can serve as a translational roadmap for noninvasive MRI imaging in human AVF.

Innovations

(1) The development of intravital imaging as a quantitative in vivo imaging approach to comprehensively assess the pathological endothelial injury and fibrinogenesis post-AVF creation is an innovative research project. Most of the pathology insights of AVF failure have been obtained ex vivo, which is static and a single time point snapshot. In contrast, IVM offers in vivo detection of endothelial injury and serial imaging, which provides comprehensive, longitudinal dynamic molecular readouts of the entire AVF anastomosis at high-resolution.

(2) In vivo AVF imaging using this new jugular vein mobilization animal model to investigate venous juxta-anastomotic stenosis is a novel approach, which recapitulates human AVF pathology and has greater translational application.

(3) The concept of serial in vivo imaging of endothelial injury and fibrinogenesis by cell-targeted fluorescence nanoparticle at two time points is an innovative approach. It will provide longitudinal in vivo biological insights into the vascular injury post AVF creation.

(4) The concept of early time point molecular imaging to identify high-risk failure AVF is a novel concept for early AVF failure detection. New medical or interventional therapies can be tested with this novel imaging method to accelerate clinical translation.
Research strategy

Specific Aim 1. Assess the in vivo time course of endothelial injury and fibrinogenesis post AVF creation in wild type C57BL/6J mice using an end-to-side external jugular vein to carotid artery anastomosis AVF model. Hypothesis: Endothelial injury and fibrinogenesis in AVF venous juxta-anastomosis can be simultaneously and serially imaged and quantified in vivo. The degree of injury and fibrinogenesis will progress over time and be highest nearest the anastomosis.

Rationale for the choice of end-to-side external jugular vein to carotid artery anastomosis AVF model: A prior study showed this new AVF model recapitulates human AVF neointimal hyperplasia. NH was observed in the venous outflow by day 14 post-AVF creation.(6) Compared to the other animal models, the murine AVF is more efficient and cost-effective.

Rationale for investigating endothelial injury post AVF creation: Endothelial injury following surgery, and altered shear stress activates smooth muscle cells (SMC) and drives NH. The degree of NH correlates with the area of endothelial denudation.(11) Endothelial cell implantation reduces vascular restenosis in animal models.(12)

Rationale for investigating fibrin deposition post AVF creation: After endothelial injury, fibrin and platelets are deposited and activated, which activates leukocytes and SMCs.(13) Focal fibrin deposition is proportional to the depth and extent of the arterial wall injury.(14, 15)

Rationale for using intravital microscopy (IVM): IVM is widely used in vascular research including inflammation evaluation and thrombosis formation.(16) Applying molecular imaging to understand in vivo AVF malfunction will enable monitoring of biological process of AVF dynamically and at higher, cellular-level resolution.

Rationale for imaging timepoints: Prior studies showed that the patency of murine AVFs at day 7, 14 and 28 are 88%, 90%, and 50%.(6) Using IVM, we will image at the timepoints of day 7, 14 and 21 following AVF creation. These time points will allow us to validate the IVM methodology using correlative fluorescence microscopy and histological analyses.

Preliminary data – Neointimal hyperplasia (Fig.1). An end-to-side external jugular vein to carotid artery AVF was created in C57BL/6J mice (n=3). Neointimal hyperplasia was observed in the venous outflow at day 14 post-AVF creation, consistent with the results of other recent investigations.(6)

Preliminary data – Pathological endothelium is illuminated by CLIO (Fig.2): In our prior study, AVFs were created by end-to-side carotid artery to jugular vein anastomosis. Mice were injected with the cell-targeted fluorescent nanoparticle CLIO-VT680 (10mg Fe/kg) 24 hours before sacrifice. Ex vivo immunofluorescence and transmission electron microscopy (TEM) of day 14 AVF tissue revealed CLIO deposition in pathological endothelium.(5)

Preliminary data – IVM imaging of AVF (Fig.3): IVM of CLIO activity was performed in mice 14 days post-AVF surgery after CLIO-VT680 injection (n=2). Collagen in the vessel wall was identified by second harmonic generation. Pathological endothelium was illuminated by near-infrared fluorescence emission from CLIO-VT680. CLIO-VT680 signal was identified in the venous outflow on day 14 post-AVF creation. Sham operated contralateral carotid artery exhibited minimal in vivo CLIO signals.

Preliminary data- Fibrin deposition in AVF (Fig.4): Molecular imaging of fibrin using FTP11–CyAm7 was previously validated in murine deep venous thrombosis in our lab.(17) To determine if fibrin deposition in murine AVF could be detected, on day 14 post AVF creation, FTP11 was injected 1 hour prior to sacrifice (n=3). Ex vivo fluorescence microscopy imaging revealed fibrin deposition in the endothelium.

Fig 1. A. AVF was created in C57BL/6J mice by end to side anastomosis between external jugular vein and ipsilateral carotid artery. EJV: external jugular vein; Dash box: anastomotic zone. B.Carstairs stain of day 14 resected AVF showed NH in the venous outflow.

Fig 2. Immunofluorescence microscopy demonstrated CLIO colocalized with CD31 positive endothelial cells (A). TEM showed CLIO nanoparticles were inside the endothelial cells and between the endothelium and basement membrane (B). (From Cui et al. ATVB 2015 in press)

Fig 3. IVM of day 14 AVF demonstrated CLIO deposition in AVF venous outflow in both coronal (A) and axial (B) view. Blue: second harmonic generation signal from arterial wall collagen. Red: CLIO
**Experimental Design.** Flowchart (Fig 5). Approval from the MGH Institutional Animal Care and Use Committee will be obtained for all experiments.

Mice end-to-side external jugular vein to carotid artery AVF. AVF will be created by an end-to-side anastomosis of the right external jugular vein to carotid artery. The left side jugular vein will receive sham surgery and served as control. Mice will be euthanized at day 7, 14 or 21 post-AVF creation. We will harvest the AVF and control vessels for ex vivo pathology analyses.

*In vivo* molecular imaging agents CLIO and FTP11: Mice will receive an IV dose of CLIO-VT680 24 hours beforehand (MGH, 10mg Fe/kg, ex/em 635/695). CLIO allows *in vivo* IVM imaging of pathological endothelium in AVF as shown in our preliminary results. To image fibrinogenesis post-AVF creation, mice will receive an IV dose of fibrin-targeted FTP11-VT680 1 hour beforehand (150nmol/kg). FTP11-VT680 targets fibrin in murine venous thrombi *in vivo* as shown in our lab. (17)

*IVM* imaging of vascular injury and fibrinogenesis in mice on day 7, 14 or 21 post-AVF creation. After CLIO or FTP11 injection, mice will be imaged with a custom-built confocal intravital microscope. (18) Fluorescein-labeled dextran (Sigma Chemical, St. Louis, MO), CLIO (MGH) and FTP-11 (MGH) will be imaged as shown in our prior studies. (5, 17)

Serial IVM imaging of AVF to quantify endothelial injury and fibrinogenesis *in vivo*. After day 7 IVM imaging of endothelial injury by CLIO-VT680, mice will be recovered and monitored closely. Mice will undergo repeat IVM with CLIO-AF555 injection 7 days after the first imaging. Endothelial injury will again be assessed via IVM. Similar procedure will be performed to assess fibrinogenesis using FTP11-AF555.

Simultaneously imaging of vascular injury and fibrinogenesis *in vivo*: An imaging time point will be selected based on Aim1.1 and 1.2. IVM imaging will be performed after CLIO-AF555 and FTP11-VT680 injection. Both vascular injury and fibrinogenesis signals will be imaged simultaneously.

IVM imaging processing and analysis: IVM datasets will be collected as coronal z-stacks, which will be re-sliced into the axial plane. Region of interests will be identified as the area of positive CLIO signals inside the vessel wall, delineated by SHG collagen signals. Target to background ratio (TBR) will be calculated. All image analysis will be performed using NIH ImageJ software.

*Ex vivo* imaging analyses: Fluorescence microscopy. Fresh sections embedded in OCT (atheroma) will be sectioned and imaged as previously using an epifluorescence microscope (Eclipse 90i, Nikon), Histology and immunohistochemistry. H&E, Carstairs’, Van Gieson’s, and immunohistochemistry for endothelial cells, macrophages, neutrophils and SMCs will be performed as demonstrated previously in preliminary data. (5)

**Expected results:** Endothelial injury and fibrinogenesis post-AVF creation can be imaged serially *in vivo* using molecular imaging by IVM. Endothelial injury and fibrinogenesis will preferentially localize to the venous limb of the AVF *in vivo* particularly near the anastomosis and will increase over time. IVM findings will be confirmed by fluorescence microscopy and histological analyses.

**Sample size:** All sample size calculations utilize 80% power and significance of p<0.05. We hypothesize that the endothelial injury TBR of AVF will be 50% higher than the control side. We expect a 20% increase in fibrinogenesis in the AVF compared to the control side. A total of 24 mice will be sufficient to detect the differences between the AVF and the control vessel.

Alternative approaches and pitfalls: Murine AVF model: If sufficient neointimal hyperplasia is not achieved (unlikely based on prelim. data) we will extend the study from 21 days to 42 days to generate greater NH formation. IVM: If low TBR signals limit *in vivo* imaging, we will consider doubling the dose of FTP11. If simultaneously imaging of endothelial injury and fibrinogenesis is not achievable due to signal bleaching in each channel, we will change the conjugated fluorophore.
Aim 2: Determine whether early endothelial injury and fibrinogenesis can predict subsequent venous juxta-anastomotic neointimal hyperplasia in AVF. Hypothesis 2: Endothelial damage leads to fibrin and platelet deposition, which drives smooth muscle cells proliferation and NH.

Rationale for investigating NH: The most common cause of AVF failure is neointimal hyperplasia and thrombosis.(7) At present, scant data is available regarding in vivo biological mechanisms underlying NH.

Rationale for investigating the relationship between endothelial injury, fibrinogenesis and NH: The degree of NH correlates with the area of endothelial denudation.(11) Endothelial cell implantation reduces vascular restenosis in animal models.(12) Fibrin deposition is proportional to the injury of the endothelium. Fibrin deposition is greater in restenotic stent compared to non-restenotic stents.(19)

Preliminary data In our prior study, AVF was created by end-to-side jugular vein and carotid artery anastomosis. The signal intensity of pathological endothelium illuminated by CLIO in the arterial segment decreased as the distance away from the anastomosis. Furthermore, the in vivo intensity of pathological endothelium predicted subsequent site-specific inflow neointimal hyperplasia.(5)

Experimental Design.

Survival IVM imaging of AVF to quantify endothelial injury and fibrinogenesis in vivo and subsequent NH at later time point: Endothelial injury and fibrinogenesis will be imaged in vivo by IVM at day 7 post-AVF creation after CLIO-AF555 and FTP11-VT680 injection. Mice will be recovered and closely monitored. At day 14 or 21 post-AVF creation, mice will be sacrificed. Neointimal hyperplasia area will be quantified by histology.

Expected results: The intensity of in vivo endothelial injury and fibrinogenesis by IVM at early time point can predict subsequent venous outflow neointimal hyperplasia.

Sample size: A total of 12 mice will be required to study the correlation between the intensity of endothelial injury, fibrinogenesis and neointimal hyperplasia (power = 80%, α=0.05).

Alternative approaches and pitfalls: If day 7 imaging is difficult to obtain due to suboptimal survival, we will perform IVM imaging at day 14.

Aim 3: Determine if venous side AVF inflammation can be imaged noninvasively using cellular MRI by CLIO-labeled pathological endothelium. Hypothesis 3: CLIO is detectable by MRI and enables noninvasive detection of venous-side pathological endothelium in AVF.

Rationale for imaging CLIO signals by MRI: CLIO nanoparticles are derived from ultrasmall superparamagnetic iron oxide nanoparticles that can induce signal loss in T2-weighted MRI.(20)

Rationale for using MRI: High-resolution MRI has been shown to accurately imaging biological processes of inflammation and mechanical forces in atherosclerotic lesions.(20) Prior studies showed that MRI could provide information regarding the degree of vascular injury in human AVF.(9)

Preliminary data (Fig.6) – Our prior study showed the capability to image pathological endothelium ex vivo by MRI using CLIO nanoparticle. In mice that received the magnetofluorescent CLIO-VT680 nanoparticles, T2-weighted rapid acquisition with refocused echoes (RARE) images demonstrated signal hypointensity around the AVF anastomosis compared to control animals.(5)

Experimental Design.

Seven days after AVF creation, mice will undergo in vivo and ex vivo MRI imaging. CLIO will be injected 24 hours prior to imaging at a dose of 10mgFe/kg. In vivo AVF MRI will be performed by 7.0T high resolution MRI scanner (Burker, Billerica, MA). Mice will then be sacrificed and AVF tissues will be resected. Resected AVF tissues will be imaged ex vivo by MRI scanner. RARE T2 images will be obtained and images will be analyzed with Image J software (V1.cc, NIH).

Expected results: T2-weighted MRI will detect CLIO-deposition noninvasively in the venous limb of the AVF circuit, and provides a noninvasive readout of venous-side pathological endothelium.

Sample size: We expected a 20% lower MRI signal on T2-weighted images in AVF compared to the contralateral side, a total of 6 mice will be required to detect significant differences (power = 80%, α=0.05).

Alternative approaches and pitfalls: If the contrast of MRI is insufficient, we will increase the dose of CLIO. If the resolution of 7.0T MRI is poor, we will use 9.4T MRI instead. If the differentiation between signal loss caused by CLIO and native low signal in tissue is problematic, we will use GRASP (GRe Acquisition for Superparamagnetic Particles) to generate a positive signal enhancement.(21)
References


Facilities and Resources

Personnel

Research Technologist - Adam Mauskapf BS: Runs the large and small animal research; has experience in micro and macro-surgeries, anesthesia monitor and catheterization techniques.

Post-doctoral Fellow - Chase Kessinger PhD: Applies in vivo molecular imaging including intravital microscopy, FDG-PET and MRI in cardiovascular disease animal models.

Post-doctoral Fellow - Eric Osborn MD PhD: Performs large animal catheter-based intravascular molecular imaging studies evaluating stent inflammation and healing as well as the characterization of clinical translatable atherosclerotic probes.

Equipment

Intravital confocal fluorescence microscopy and second harmonic generation imaging system. Intravital studies of murine AVF with CLIO and FTP 11 fibrin agent will be performed with custom-built video-rate (30 frames/s), multimodality imaging system that is designed specifically for live animal imaging and cell tracking. The system was built and is housed in the Advanced Microscopy Center at MGH and is located in the investigator’s building.

Fluorescence reflectance imaging (FRI) system (KODAK Image Station 4000MM Pro Imaging System, Carestream Health). Housed in the investigator’s laboratory space, includes a 2048 x 2048 pixel cooled CCD camera, 9 excitation filters (390 nm, 430 nm, 470 nm, 530 nm, 550 nm, 610 nm, 630 nm, 710 nm, and 730 nm), automated 4-position emission filter wheel, 4 wide angle emission filters (535 nm, 600 nm, 700 nm, and 790 nm).

Epifluorescence microscope (Nikon 90i Eclipse, Japan): Housed in the PI’s laboratory space. The microscope utilizes a xenon illuminator with a built-in motorized focus & motorized x-y stage. The microscope is equipped with 4x, 10x, 20x, and 40x Plan Apo short working distance objectives, with filter cubes designed to resolve DAPI, FITC, AF555, Texas Red, Cy 5.5, VT680, Cy7, and ICG fluorescent agents. It is also equipped with an Andor iXON+ EMCCD Detector 512x512 back-illuminated CCD camera coupled to a PC running NIS-Elements Software (Nikon). Live focus capability is available under both transillumination and fluorescence with 100 msec refresh rate.

Confocal microscope (Olympus FV1000-MPE): Housed in MGH Wellman Photopathology Core laboratories. The microscope is able to detect in confocal or multiphoton mode. It consists of a spectral scanner system with spectrometer lasers Spectra Physics Mai-Tai DeepSee Red/IR laser tunable 690-1020 nm; a 3-line Argon Laser (458/488/515 nm); a combinational red laser diode (LD559/635 nm); a violet laser diode (LD405 nm); and NIR diode (LP750nm).

Laboratory space and Resources

Advanced Microscopy Center. This facility is located in the Dr. Jaffer’s laboratory building (Simches Research facility) and houses multiple intravital microscopy (IVM) center for confocal and nonlinear microscopy, including 1) a multimodal microscope in which up to three of the following imaging modalities can be realized simultaneously: confocal reflectance microscopy, fluorescence by single- or two-photon excitation, second harmonic generation, and coherent anti-Stokes Raman spectroscopy (CARS). Images are acquired at video rate, which allows real-time monitoring of fast events in the living tissue; and 2) An in vivo flow cytometer, based on confocal design that can provide real-time detection and quantitative information on fluorescently labeled cells while in circulation in a live animal model.

The proposed project would take place in a richly collaborative environment where experts and resources in intravascular and small-animal optical imaging, engineering, large animal surgery including rabbits and swine, and vascular biology are immediately available. The experiments will be performed in 1300 square feet of laboratory space assigned to Dr. Jaffer within the Cardiovascular Research Center (CVRC) and Cardiology Division at the Massachusetts General Hospital. This research facility is optimized for large animal work with a modern cardiac catheterization laboratory for preclinical experimentation (see below), multimodality image analysis, catheter imaging system engineering and optimization, and correlative studies using cellular and molecular biology techniques to study vascular biology.