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In vivo SPECT/CT molecular imaging of LFA-1 in atherosclerotic inflammatory development with radiolabeled DANBIRT

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BY

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M.D., Medical Surgeon

THESIS

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DEDICATION

This thesis is dedicated to the living memory of Hector Javier Mota Martinez "Neno"; your legend will live with me forever.

To my best friends, Borre, Luigi and Fredy, thank you for the many years of enriched support and for never stopped believing in me, for always supporting failures and successes. To both of my parents and brother, who gave me immeasurable support over the years and for invaluable encouragement in every step of my career.

And finally but most importantly to my gorgeous wife Nahomi, and beautiful and happy children Oliver and Robin, your love is the greatest gift of all and I will always be proud of being part of your life. I love you with all of my heart.

I have a special dedication for anyone that ever doubted me personally or professionally because I make the everyday goal to prove that concept of me to be horribly incorrect.

Thank you all.

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ABSTRACT

The objective of this thesis project is to assess inflammatory leukocyte presence

and accumulation in vascular atherosclerotic plaque using [^{111}In] In-DANBIRT as

a non-invasive diagnostic imaging tool. ApoE$^{-/-}$mice were fed either normal or

high fat diet (HFD) for 8 weeks. Sprague Dawley male rats were exposed to

filtered air and ozone for 4 hours. SPECT/CT imaging was performed at baseline,

4 and 8 weeks. 3D autoradiography was performed after 8 weeks. Leukocyte

isolation methods were performed for neutrophil and peripheral blood

mononuclear cell (PBMC) separations post-incubation with [^{68}Ga] Ga-DANBIRT

and [^{111}In] In-DANBIRT. Whole blood isolation correlated these findings showing

increased specific uptake by neutrophils in the ozone-exposed group.

Radioisotopic dilution methods evidence a saturation of binding sites in isolated

neutrophil samples starting at a 10-fold dilution from initial concentration of [^{111}In]

In-DANBIRT. Longitudinal molecular imaging evidenced an increased uptake in

HFD fed mice, consistent with increased immune response to vascular injury in

cardiovascular and immune tissues. Histologic analysis illustrated atherosclerotic

plaque disease progression and increased vascular lesion area in HFD fed mice.

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PREFACE

I will start by pointing out that my path in cardiovascular research started by a determining personal moment when I lost a very close and loved uncle due to atherosclerotic disease complications. As a physician there was a direct sense of personal blame due to not being able to be there to save his life. I became immediately drawn into this thesis project because of the implication of having a transcendental impact in modern medicine. The implications of this thesis project have a real value as translational research and could lead the way in a major breakthrough in molecular imaging.

Evolution has made it very clear that in order to survive we have to adapt and enhance our survival ability. In the context of health, we as humans have to rely in biotechnological advance to extend the chance of survival. Because disease pathogenesis varies exponentially in every patient, the effort of modern medicine is to improve continuously. Many of the capabilities for understanding pathogenesis impact the ability to prevent and diagnose disease as early as possible. In some cases this is achievable, but in many others the course of a disease is dictated by fatal outcomes. With great power comes great responsibility and in molecular imaging the power of such diagnostic systems are limited by patient's safety. There are many comprehensive approaches to identify areas that are prone to atherosclerotic plaque rupture and subsequent complications.

The rationale relies on the vulnerable stage of atheroma development in which such intense immune response can be identified using molecular imaging.

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The specific aim is to validate the capability of [^{111}In] In-DANBIRT as an in vivo imaging tool for atherosclerosis' inflammatory progression in the developmental stages of plaque instability.

I have hypothesized that using a Single Photon Emission Computerized Tomography (SPECT) imaging system I am able to discriminate [^{111}In] In-DANBIRT uptake in areas prone to vascular atherosclerosis development. As of today, there are no reliable noninvasive in vivo imaging tools for such purpose, taking the importance of findings to a next level.

Extent of hypothesized results impact beyond the scope of this project and apply to multiple disease models such as infection, inflammation, cancer (leukemia, lymphoma and other hematopoietic diseases) and most diseases affected by an immune modulation. Drug delivery systems and imaging diagnostic technology are required to be able to translate preclinical medical advantages.

There is a stronger commitment in modern medicine to discover new technology rather than to find better uses of available biotechnological resources. Restructuring and repurposing(Oprea, Bauman, Bologa, Buranda et al., 2011) are terms that are overlooked and limited as secondary efforts most of the time. The most important asset of nuclear medicine and molecular imaging is the ability to investigate a system without the system knowing. Having such ability has many aspects to the argument but there is great potential for obtaining high specificity using such modalities. Metabolic pathways and physiological processes can become altered by working in such low pharmacologic levels, relying on high affinity binding to the target molecule. The interaction of

radioactivity using high performance imaging systems has been implemented since ancient times were it was seen as a complicated method rather than an indispensible diagnostic tool. Even today, most nuclear medicine procedures are remained as last resources and believed to have limited potential as gold standards. Believing that there is an increased potential in the chemical restructure and rescuing of [^{111}In] In-DANBIRT guided me to pursue this thesis project with the firm conviction it is a promising molecular imaging probe.

Chapter 1. Introduction

Cardiovascular disease and atherosclerosis epidemiology

Cardiovascular disease is among the leading causes of death in the modern world(Coste, 2001); around 70% of cardiovascular complications are related to atherosclerosis, especially in small vessels(Herrington, Lacey, Sherliker, Armitage et al., 2016; Naruko, Ueda, Haze, van der Wal et al., 2002). Coronary arteries are the most frequently affected vessels and cause the worst outcomes(Abedinzadeh, Pedram, Sadeghian, Nodushan et al., 2015). Although the number of coronary atherosclerosis-related emergency room visits have decreased from 2006 to 2011 in the US(Skinner, Blanchard, & Elixhauser, 2006), there is still a substantial number of patients that develop complications directly related to an atherosclerotic vascular condition.

Statistics from NHLBI highlight that in 2010 cardiovascular disease caused 788,000 deaths or 32% of all deaths in the US. Such numbers reflect a global pandemic scenario, considering that such numbers are close to or higher in low-income countries like Mexico(Rodriguez-Saldana, Rodriguez-Flores, Cantu-Brito, & Aguirre-Garcia, 2014). As such, preventive therapies should be a major priority in biomedical research(Herrington, Lacey, Sherliker, Armitage et al., 2016). Knowing that cardiovascular risks vary between each individual, the relationship from one person to its environment is without a doubt an important evolutionary phenomenon till this day.

Most of the clinical implications of atherosclerosis development depend in the affected vessel, as mentioned. Arterial vessels like the carotids, supply most

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of the blood flow to the brain, neck and adjacent structures to the heart. Such vital functions are affected by atherosclerosis by remodeling the vessel wall lumen, thereby reducing blood flow or increasing shear turbulence. Serious complications like stroke, heart attacks and unstable angina arise from such etiologies and can be life-threatening.

Risks for developing cardiovascular disease are related to lifestyle, genetic predisposition, environmental exposure, modifiable and non-modifiable risk factors. Risk factors are important to guide diagnosis, while improving therapeutics based on risk stratification, improving true value of risk prediction. Most diagnostic tools are able to identify atherosclerosis-associated incidental findings like aortic curvature calcification in X-Rays and Computerized Tomography, hypercholesterolemia in screening blood test and physical evaluation that can reveal signs and symptoms of decreased blood flow in certain anatomical regions. Every human being responds differently, the importance in this statement is that while modern medicine agrees that there are compelling risk factors for cardiovascular disease, many others remain to be stratified and can represent a challenge to diagnose in a timely manner.

Atherosclerosis is a chronic cardiovascular disease, adversely influenced by increased serum lipid levels (cholesterol, triglycerides(Durrington, 1998)) especially in concentrations of small lipoprotein particles (LDL, VLDL), which can accumulate inside the intima of injured vessels. The process is characterized as an early stage with the pathognomonic formation of "foam cells", which are macrophages that release cytokines and phagocytize large amounts of

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intraplaque lipids(Brown, Shelness, & Rudel, 2007). Accumulation of these cells has been identified in the early-stage lesion of atheroma.

An immune response proliferates from an increase in local and systemic cytokines, increasing the amount of leukocyte recruitment that interacts with activated endothelial cells and translocate into the vessel wall. As cellularity in the lesion increases, partial or complete vessel lumen narrowing becomes the single most important feature capable of eliciting specific disease signs and symptoms. Until such remodeling has developed, identifying patients at high risk of adverse cardiac events is extremely difficult. Other complications involve atherothrombotic events, embolism, peripheral artery disease and many others. Atherosclerosis progression involves unique characteristic developmental stages, significant towards development of ideal diagnostic and therapeutic applications. I will expand on this matter in the next section.

Atherosclerotic plaque vulnerability and instability

The inflammatory process that happens in the atherosclerotic plaque changes along disease progression and can make the plaque "vulnerable", or "unstable", and then in many cases prone to rupture, ulceration and/or hemorrhage. At late stages, complications associated with atheroma in the aorta, coronary and carotid arteries are often irreversible and or life threatening.

The Merriam-Webster dictionary (June 2016) describes "vulnerable" as *open to attack or damage*, making it easier to understand the concept of identifying a harmful process along disease progression. If the evolution of a

disease follows its natural pathological course, there will be stages in the disease were the aggressor becomes partially or completely a victim and at this point "vulnerability" comes into play. The exact time and process to evaluate in such specific timeline is the main question, where the idea of having a diagnostic tool that confers anytime in vivo assessment would be of great value.

Atherosclerotic plaque is typically defined in evolutionary progression trough stages of stability in which the immune components of such process differ. As plaque progresses, the innate immune response is not able to control the inflammation and the adaptive immunity is then needed. Plaque features evolve from an initial small lesion and endothelial dysfunction into a full blown occluding mass in major arterial vessels. Many of the components of an acute and chronic immune response inside and adjacent to the plaque overlap, both coexist but there has to be something else that triggers a plaque to quickly become unstable and rupture.

Acute phase inflammation is, to some extent, a quicker and more damaging process than a chronic inflammation. Components of a plaque such as calcification, remodeling, vascular smooth muscle cell proliferation and slow progression are more characteristic of a chronic stable plaque. Lipid rich necrotic core, intraplaque hemorrhage and thin fibrous cap are more specific of an unstable plaque. The description of such specific feature would be subject of a more detailed review(Burtea, Ballet, Laurent, Rousseaux et al., 2012; Seneviratne, Hulsmans, Holvoet, & Monaco, 2013).

Local and systemic infection has also been directly related to development of atherosclerosis in arterial vessels(Arshinov & Maslova, 2010). Immunodepressed patients also exhibit susceptibility to development atherosclerosis(Campbell & Rosenfeld, 2015), arising to the ability of cytokines and acute phase reactants as promoters of such accelerated process. Such studies imply that a plaque is normally a chronic subclinical process that is triggered to become unstable by an external stimulus that gives the necessary push for the development of "vulnerability". Infection and inflammation are tightly bound and the presence of one will precipitate the presence of the other (Arshinov & Maslova, 2010). The concept of an atherosclerotic plaque becoming infected could explain many sides of the discussion about what causes a plaque to become vulnerable and rupture (Campbell & Rosenfeld, 2015). Recent finding would agree that the role of polymorphonucleated leukocytes inside the plaque is the answer to this long standing question (van Leeuwen, Gijbels, Duijvestijn, Smook et al., 2008).

Animal models of atherosclerosis

Atheroma development is a process that involves the body's own immunological response as disease progresses; the extent of successful vascular remodeling and disease control varies extensively among patients(Imanaka-Yoshida, 2012). Such variability complicates the reduction of fatal outcomes by appropriate disease prevention and diagnosis.

Many diseases turn the attention into prevention or aggressive therapy, but some etiologies may not clearly benefit from either of those strategies, because of specific epidemiologic distinctions(Coste, 2001). Many of the risk factors for atheroma development include exogenous modifiable factors that could normally be changed or eliminated (Alavantic & Djuric, 2006), but the variability of an extensive identifiable number of risk sometimes makes it hard to appreciate and assess in a patient with a relatively normal medical history and with little to no risk factors. Animal models of atherosclerotic disease are inducible and have a strong correlation to human conditions that have distinctive genetic alterations(Gaut & Heinecke, 2001).

Humans share many mutations with animal models especially with rodents in which specific transgenic mutations guide atherosclerosis development in such biological models. Apo E lipoprotein and low-density lipoprotein receptor (Ldlr) (Silvola, Laitinen, Sipila, Laine et al., 2011) are the most important mice models that development spontaneous atherosclerotic lesions because of dyslipidemia (Y. J. Choi, Roberts, Wang, Geaney et al., 2012). Such mutations have inducible components that are advantageous for scientific research(von Rossum, Enns, Shi, MacEwan et al., 2014). Atherosclerotic plaque characteristics in a mouse model become evident over a different time course of disease development(Fenning & Wilensky, 2014; Nakata & Maeda, 2002).

Leukocyte Function Associated Antigen-1 (LFA-1)

The integrin composed of a heterodimeric structure of CD11a/CD18, because of cluster of differentiation also known as LFA-1 is expressed strictly in leukocytes and is mostly found in mature circulating cells.

LFA-1 mediates leukocyte adhesion, rolling and transmigration (Chigaev & Sklar, 2012a) by a direct complex formed with the intracellular adhesion molecule 1 (ICAM-1) expressed in endothelial and dendritic cells(Collins, Velji, Guevara, Hicks et al., 2000). Endothelial activation and vascular permeability directly affect the recruitment of leukocytes to site of inflammation (Chigaev & Sklar, 2012b), in this case atherosclerotic plaque.

Three stable configurations of LFA-1 have been identified by low affinity "bent", intermediate-affinity and high-affinity state(Giagulli, Scarpini, Ottoboni, Narumiya et al., 2004) upregulated by CXCR2 and PSGL inside out signaling. The regulation of such receptor has been identified in the process of antigen presenting cell interaction with major immune cell subtypes(Larson, Davis, Bologa, Semenuk et al., 2005). T cells are especially effective at upregulating through the T cell receptor the number and high affinity state (Chigaev, Smagley, Haynes, Ursu et al., 2015) of LFA-1 receptors expressed in cell surface(Y. Wang, Shibuya, Yamashita, Shirakawa et al., 2008). LFA-1 as many other adhesion molecules work in the transport of such cells to site of action and in conjunction makes an efficient way for leukocytes to be recruited.

Neutrophils are important in the immediate innate response of the immunity that is also guided by LFA-1 activation, guided by intracellular co-

activators such as Talin-1 and Kindlin-3(Lefort & Ley, 2012). LFA-1 is then an ideal target with the desired specificity to illustrate the inflammatory process in atherosclerotic process with also important application in many other diseases with an important inflammatory process (Yee, 2000).

BIRT 377 and DANBIRT

The small molecule BIRT 377 is an enantiomer (molecular weight of 442G) allosteric inhibitor of LFA-1. BIRT 377 has been shown to inhibit the low bent affinity state in the I-domain of the alpha chain of CD11a(Kelly, 2015). Many attempts have been made to exploit such effects in therapeutic blood malignancies. This is the case of BIRT, which was developed to target leukemia and lymphoma treatments (Chigaev, Smagley, Zhang, Waller et al., 2011). Unfortunately, at pharmacologic therapeutic levels the drug exhibited a poor safety profile with lung toxicity and was not used in clinical trials.

DOTA(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) functions as a bifunctional chelator to DANBIRT structure: DOTA-Alkylamino-NorBIRT (DANBIRT), which is a chemically restructured radioligand capable of binding to LFA-1 antigen expressed by leukocytes (molecular weight of 885.6G). DANBIRT has been shown to bind to human and murine hematopoietic cells that express LFA-1 with a dissociation constant (Kd) of 135nM and 186nM respectively(Rahul B. Poria, 2006). DANBIRT chemical structure allows the ability to radiolabel such small molecule via chelation: [Radiometal]-DOTA-1-(4-aminobutyl)-5-(4-bromobenzyl)-3-(3,5-dichlorophenyl)-5-methylimidazolidine-2,4-dione. DANBIRT

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value is conferred due to its non-ionic intravenous binding form and its molecular weight, which confers rapid distribution, LFA-1 binding and excretion. The value of [^{111}In] In-DANBIRT is further enhanced by the restricted expression of LFA-1 exclusively in leukocytes (Weitz-Schmidt, Welzenbach, Dawson, & Kallen, 2004)

Translational molecular imaging

Nuclear medicine comes to mind when we have to trace a physiological response of a tissue without a pharmacological response or detectable effect. The diagnostic and therapeutic advantages of nuclear medicine are believed to overcome standardized clinical procedures in specific diseases, just by implying that we can investigate a system without having a response. Working with invasive procedures can help increase efficiency and improve outcomes of this disease but invasive is not always a favorable method. Diagnostic nuclear medicine only accounts for 1.9% of all diagnostic tests performed in developed countries, with an even lower number of applications in therapeutic nuclear medicine.

The body will try to reach homeostasis by any means possible, by regulating any damaging or harmful process that could deteriorate equilibrium. The extent of the regulation can also affect the "vulnerable" stage of a disease and decrease the impact and relevance of important diagnostic imaging tests. Medical manipulation limit is reached whenever a positive outcome is exceeded by severe complications like thromboembolism, plaque rupture or co-infection.

Risk stratification in cardiovascular diseases helps dictate how aggressive we can be in therapeutic strategies.

There are limited commercially available radiopharmaceuticals that can be employed for the purpose of specifically identifying inflammation in life-threating situations. Gallium and Indium are two relevant radioisotopes used because of availability, desired decay emissions and effective labeling methods working with DANBIRT. ^{67}Ga and ^{111}In both have half-lives close to 3 days, decay by electron capture and are cyclotron-produced; Gallium and Indium have been used in diagnostic imaging of blood malignancies and for radioimmunodiagnosis by labeling antibodies respectively. ^{67}Ga was historically used in nuclear medicine as a SPECT agent to diagnose Hodgkin's disease, lymphoma and inflammatory disease especially in cases of unknown fever. ^{111}In oxyquinolone (oxine) is another important clinical radiopharmaceutical used for radiolabeling of autologous leukocytes but has limitations because of the process of extracting and isolating patient's leukocytes for radiolabeling before being re administered to the patient. The sensitivity of such test is limited to soft tissue infections and its relevance in nuclear medicine is mostly exceeded by other tests.

Some other imaging tests come to mind such as angiograph that has high target sensitivity and direct implication in diagnostic and therapeutic cardiology, but is invasive to name some of the limitations. There is currently no commercially available similar imaging diagnostic test with the potential and capabilities of [^{111}In] In-DANBIRT.

The purpose of this study is to validate [^{111}In] In-DANBIRT as a non-invasive diagnostic tool using SPECT/CT imaging for inflammation assessment in vulnerable atherosclerotic plaque in a mouse model.

Hypothesis: [^{111}In] In-DANBIRT is an in vivo non-invasive SPECT/CT imaging probe targeting the expression of LFA-1 in the inflammatory process of the developing atherosclerotic plaque.

Chapter 2. "LFA-1 targeting with [^{111}In] In-DANBIRT: in vivo SPECT/CT imaging of Inflammation in Atheroma Development"

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INTRODUCTION

Atherosclerosis is a chronic cardiovascular disease common in patients exhibiting hypercholesterolemia(Arai, Hirose, Yamamura, Kimura et al., 2002). The number of adults with diagnosed heart disease has been estimated to be 26.6 million (11.3%) in the US(Blackwell DL, 2012), with more than half of those patients associated and potentially causal atherosclerotic disease. Prevention and early diagnosis of atherosclerotic disease complications is a top priority in modern medicine(Bykov, Chernyshev, Vartazaryan, & Lobastov, 2015), as early lifestyle and medical interventions can slow the rate of atheroma development and avert adverse cardiovascular sequelae. Early stage diagnosis is challenging, however; symptoms often become clinically evident only at late stages where complete prevention is no longer viable(Jie Sun, Niranjan Balu, Daniel S. Hippe, Li Dong et al., 2013). As vascular disease progresses, the inflammatory and remodeling process also evolves, and the plaque can become unstable or vulnerable to erosion or rupture, which in turn initiates life-threatening thrombotic outcomes. The penultimate pathological event, plaque rupture, is identified due to thrombogenic processes and immune cell infiltration(S. Y. Choi & Mintz, 2010). Histological changes in a vulnerable plaque such as thin fibrous cap, intraplaque hemorrhage and/or a lipid-rich necrotic chore (LRNC) are present in ~80% of ruptured plaques, but such manifestations require a prolonged and variable time for progression(ten Kate, Sijbrands, Valkema, ten Cate et al., 2010).

Cardiovascular inflammation(Hansson, Robertson, & Soderberg-Naucler, 2006) and remodeling due to atherogenic progression(Imanaka-Yoshida, 2012) have

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also been identified as leading causes of plaque instability, despite incomplete understanding of these complex process(Nakata & Maeda, 2002). The Apolipoprotein-E deficient (ApoE$^{-/-}$) mouse model on HFD exhibits rapid development of atheromatous plaques(Johnson, Carson, Williams, Karanam et al., 2005), with features and stages that mirror those of the human disease(Sasaki, Kuzuya, Nakamura, Cheng et al., 2006). Early stages of atherosclerosis typically include altered homeostasis and activation of the vascular endothelium, typified by a loss of nitric oxide generation and increased expression of chemokines and adhesion molecules (Dimastromatteo, Broisat, Perret, Ahmadi et al., 2013), which is evident in the ApoE$^{-/-}$ model(O'Brien, 1993). The role of T cells and monocytes recruited to the plaques and perivascular regions have been studied extensively in the vascular immune response, but the ability to identify intraplaque presence and extent of specific leukocyte subtypes is mostly limited to invasive and/or post-mortem or post-resection imaging(Sadeghi, 2006; Sadeghi, Glover, Lanza, Fayad et al., 2010; Willem J. M. Mulder, 2014). The role of intraplaque neutrophil accumulation has been recently identified as a focus point in atherosclerotic plaque vulnerability(van Leeuwen, Gijbels, Duijvestijn, Smook et al., 2008). Elevation of typical biomarkers for disease progression is exploited by molecular imaging(Hong, Gona, Fontes, Oyama et al., 2013), which is limited at the same time because of the low specificity of current diagnostic tools.

DANBIRT was developed by chemical repurposing of BIRT 377, which is a specific therapeutic agent for leukemia and lymphoma, as it targets LFA-1

expressed on both B and T-cells(Horst, Radaszkiewicz, Hooftman-den Otter, Pieters et al., 1991). Developed targeted ligand, DANBIRT, is a small non-ionic compound that acts as an allosteric inhibitor of LFA1(Yee, 2000). The importance of LFA-1 is critical for the initiation and impulse of a vascular immune response to injury(Kelly, 2015). LFA-1 is involved in specific interaction with Intracellular Adhesion Molecule-1 (ICAM-1) in endothelial cells by which transmigration is achieved(Shaw, Ma, Kim, Rao et al., 2004). Radiolabeling DANBIRT using [111]In (Figure 1) allows co-localization of the radiopharmaceutical in cardiovascular and immune tissues(Silvola, Laitinen, Sipila, Laine et al., 2011).
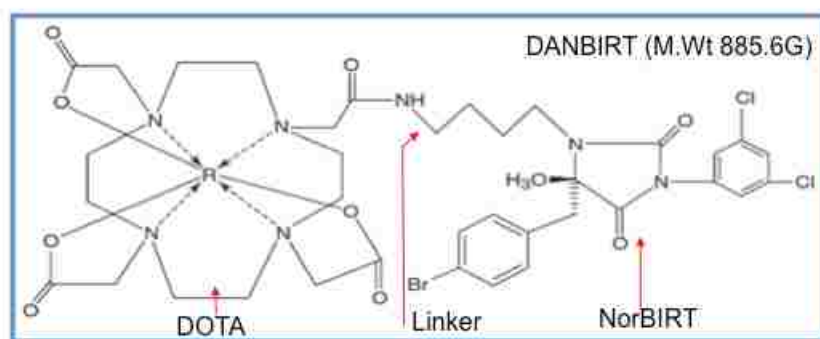
Figure 1. DANBIRT chemical structure and molecular weight.

The aim of this study is to assess the inflammatory process in atheroma development within cardiovascular tissues, by assessing leukocyte presence and accumulation in vascular atherosclerotic plaque in an ApoE$^{-/-}$ mouse model as a preclinical validation for the use of [^{111}In] In-DANBIRT as a non-invasive diagnostic imaging tool.

MATERIALS AND METHODS

Animals

Male Apo-E$^{-/-}$ mice on a C57BL/6 background (Taconic Laboratories, Indianapolis, IN) aged 6 weeks were housed two per cage and allowed to acclimate over the course of one week after delivery. Mice were fed either a normal chow diet or HFD (Harlan-Teklad, TD.88137: 1.5g/kg of cholesterol and 42% kcal from fat) for 8 weeks; food and water were provided ad libitum. Food was changed every 3 days and stored at -20°C and thawed before administration to the mice. For isolated blood distribution studies, male Sprague Dawley rats (Taconic Laboratories, Indianapolis, IN) aged 6-8 weeks were allowed 1 week of acclimation following delivery. Rats were housed two per cage and given food and water ad libitum. Both rats and mice were maintained on a 12h light:dark schedule in AAALAC-approved facilities, and euthanized via cardiac exsanguination while under deep anesthesia with Isofluorane (Piramidal Healthcare, NDC 66794-093-25). The UNM Institutional Animal Care and Use Committee (IACUC) approved all procedures performed. Animal experimental design is summarized in Table 1.

ApoE$^{-/-}$ mice with C57BL/6 background, n:16			
	Normal Diet	High Fat Diet (HFC)	Total
SPECT/CT, Bio distribution	4	4	8
Serum lipids	4	4	8
Total	8	8	16

Sprague Dawley male rats, n:6		
	Filtered Air	Ozone (1ppm)
Ozone vs Filtered Air 4-hr Exposure	3	3

ApoE$^{-/-}$ mice with C57BL/6 background, n:8		
	Normal Diet	High Fat Diet
3D Autoradiography	4	4

Table 1. [^{111}In] In-DANBIRT animal study design. ApoE$^{-/-}$ mice were exposed

for 8 weeks to either normal chow or high fat chow for experiment samples.

Serum lipid levels and sub particle analysis

Mice were euthanized after 8 weeks of normal or HFD; whole blood was collected by direct cardiac puncture using a 24-gauge needle coated with 100IU of Heparin (Sagent, WH0127N). Blood was centrifuged for 15 minutes at 1000g at 4^oC, serum was isolated from other blood components and transferred to individual Eppendorf tubes with no added anticoagulant and frozen at -20^oC. Serum was analyzed for total cholesterol and triglyceride level, as well as size dependent sub-particle quantification (Skylight biotech, Liposearch).

Radiolabeling of DOTA-Alkylamino-NorBIRT (DANBIRT) with ^{111}In and ^{68}Ga

DANBIRT was radiolabeled using either ^{68}Ga or ^{111}In with a determined specific activity of 625mCi/pM and a concentration of 1µg:1µL. Indium chloride was obtained from General Electric Radiopharmaceutical Department (GE Healthcare, INS.1PA) and ^{68}Ga was eluted from an Eckert and Ziegler ^{67}Ge/^{68}Ga generator using 0.01M HCl. The pH was assessed for acidic concentration of radionuclide solution using a BAKER-pHIX color-fixed indicator stick (J.T. Baker, 4394-01) as well as activity and volume. The volume of stock solution necessary for desired labeling activity is calculated, buffer is added to the solution before adding DANBIRT. Amount of DANBIRT needed for every 4mCi of ^{111}In or ^{68}Ga for radiolabeling was determined to be 6µg of DANBIRT (M.W. 886.5 G/mole). The reaction vial is buffered using a pH range of 4.0 - 4.5 with addition of 4mM ammonium acetate (J.T. Baker, 0599-08) for ^{111}In and a pH of 4.5 for ^{68}Ga. The reaction vial was heated in a hot block at 100^oC for 35 minutes, vortexing every 10 minutes. Quality assurance was performed with Instant thin layer

chromatography (ITLC) and High Pressure Liquid Chromatography (HPLC) following methods by Poria et al (Rahul B. Poria, 2006).

In Vitro Stability of Radiolabeled DANBIRT

Incubation of [^{68}Ga] Ga-DANBIRT with Fetal Bovine Serum (FBS) (Atlanta Biologicals, S11150) and 0.9% saline solution was performed in vitro stability analysis. Triplicate samples were drawn from radiolabeled stock and kept incubated at 37°C until time of analysis. ITLC was performed once at each time point and HPLC assessment was performed in triplicates at baseline, 5, 10, 30, 60, 120 and 240 minutes of incubation. The mean incorporation yield and radiochemical purity were calculated from sample results. Following methods by Poria et al (Rahul B. Poria, 2006) methods were modified for use in a Ultra-Performance Liquid Chromatography (UPLC) using an Acquity UPLC BEH C-18 1.7µm column (2.1x50mm) (Waters Corporation, 186002350). UPLC gradient with flow rate is described in Table 2.

	Time (min)	% Solvent B	Flow (ml/min)
1	0.00	50.0	0.800
2	0.15	50.0	0.800
3	3.00	60.0	0.800
4	5.00	80.0	0.800
5	7.00	100.0	0.800
6	7.50	100.0	0.800
7	8.00	0.0	0.800
8	8.50	0.0	0.800
9	8.60	50.0	0.800
10	9.00	50.0	0.800

Table 2. UPLC method gradient with flow rate

Biodistribution of [^{111}In] In-DANBIRT

Upon completion of 8 weeks on normal or HFD, mice were euthanized 3 hours post-injection of 700µCi of [^{111}In] In-DANBIRT via tail vein; organs were harvested for weight assessment and [^{111}In] In-DANBIRT uptake using an Automatic Wizard 2 Gamma counter (Perkin Elmer). Organs collected were: tail, whole blood, aorta (from aortic arch to thoracic aorta), carotids (from aortic arch to bifurcation), heart, liver, muscle and adipose tissue.

Whole blood was collected with direct cardiac puncture of right ventricule using a 24-gauge needle of right ventricle with a heparinized syringe and centrifuged for 15 minutes at 1000g at 4°C. Serum, erythrocytes (RBC) and leukocytes (WBC) containing isolated samples were collected and measured for [^{111}In] In-DANBIRT uptake using a gamma counter. Uptake was decay-corrected and percent-injected activity per gram (%IA/gr) was determined for each mouse.

Ozone Exposure and Blood Component Distribution

Rats were exposed to either filtered air (FA) or ozone (1.0 ppm) for 4 hours using an OREC silent arc discharge ozone generator (Osmonics, Phoenix, AZ); ozone concentrations were continuously monitored over the course of 4 hours. Exposures took place in a chamber without bedding to minimize ozone scrubbing and to ensure consistent nasal exposures. Whole blood was collected 24 hours post-exposure. Histopaque two-step gradient (Sigma Aldrich, 10771) neutrophil isolation protocol was performed following manufacturer's methods; modified protocol was included for PBMC isolation. Hetastarch at 5% (B. Braun, L6511) was added to whole blood before running isolation protocols. Cell samples were

washed and suspended in 1% Gelatin in PBS at a pH of 7.2. Final volumes of neutrophil and PBMC samples were increased to 1mL. Final samples were identified as serum, RBC, neutrophils and PBMCs and incubated for 1 hour with [^{68}Ga] Ga-DANBIRT (specific activity of 625mCi/pM). Sample smears were performed and stained with Wright-Giemsa horizontal staining protocol according to manufacturer instructions (Sigma Aldrich, SLBN4704V). Cell morphology, differentials and sample purity were assessed using a light microscope at 40x objective (Olympus BX51).

Radioisotopic Dilution Methods

Radioisotopic dilution methods were performed with log-fold serial dilutions at concentrations of 1:1, 1:10, 1:100 and 1:1000 radiolabeled DANBIRT. Rat whole blood was collected and incubated for 10 minutes with [^{111}In] In-DANBIRT. Leukocyte isolation was performed for neutrophil and PBMC separations post-incubation with radiolabeled DANBIRT (specific activity 625mCi/pM). Cytospin 2 (Shandon 2,59900002) with double-funnel and white filter (Simport, 930941126) was used for clustered cell differentials identification methods. Cytospin samples were spread in Superfrost Plus slides (VWR, 48311-703) and fixed with 5% methanol. Wright-Giemsa horizontal staining protocol was performed according to manufacturer's package insert instructions (Sigma Aldrich, SLBN4704V). Cell morphology, differentials and sample purity were assessed using a light microscope at 40x objective (Olympus BX51).

3hr [¹¹¹In] In-DANBIRT SPECT/CT imaging and analysis

Animals were imaged at 0, 4 and 8-weeks following 700µCi via tail vein injection of [^{111}In] In-DANBIRT. Imaging was performed using 45 minute acquisitions on a NanoSPECT/CT® dedicated small-animal imaging system (Bioscan, Inc. Washington, DC) with specific topogram and SPECT parameters (Table 3) while animals were in deep anesthesia with isofluorane (Piramidal Healthcare, NDC 66794-093-25). Regions of Interests (ROI) were determined and drawn using the best quality image phantom from reconstructed CT (Table 3) to determine muscle, adipose, heart, aortic arch and descending aorta organs using VivoQuant 2.00 (inviCRO, Boston, MA). The ROI were extrapolated from phantom scan image onto every image and adjusted according to specific morphologic parameters. Concentration and sum per volume normalized to muscle was determined to identify accurate measurements and eliminate ROI signal interference from adjacent tissues. Activity was decay corrected and compared between and among dietary groups.

Topogram/CT				SPECT	
Scan Range (mm)	152-237	Projections	180	Projections	32
Tube voltage (kVp)	65	Pitch	1.5	Time/Projection	38
Exposure time (ms)	1.000	Acquisition time (min:sec)	6:00	Acquisition time (min:sec)	~45:00
AOR line	0.0	Aperture	1:4		

Table 3. SPECT/CT imaging parameters.

24-hour [^{111}In] In-DANBIRT 3D autoradiography and image analysis

Mice were euthanized by CO_2 method after 8 weeks of dietary assessment. After removal of limbs, ears, tail and whiskers carcasses were individually frozen in a hexane/dry ice bath and embedded vertically in a mold by adding an aqueous solution of 5% carboxymethylcellulose sodium salt (CMC) (C5678, Sigma, St. Louis Mo) in a hexane/dry ice bath. Block was stored at least 12 hours at -20°C prior to removal from mold and mounting on a specimen stage in the cryomacrotome (Leica CM3600 X, Leica Biosystems, Nussloch, Germany). Multiple 1/8-inch holes were drilled in the CMC block adjacent to the carcasses and filled with a solution of black India ink and ^{14}C (1µCi/mL) to provide registration marks to allow linking the white light block images with the autoradiography images. The carcasses were cut in 50µm sections in the vertical plane, starting from below the ear location. Prior to cutting each section, a digital photo was taken (Canon EOS 70D Focal Length of 35mm, Melville, NY) of the block surface. Every 10 slices, a section was transferred to 2.5 mm Label Guard Protection Tape (Scotch 3M 821, DeKalb, IL) and dehydrated in the cryomacrotome for a minimum of 24 hours. After a total of 55 sections, autoradiography was taken and accompanied by approximately 600 white light photos. Cryosections were removed and mounted on black cardboard along with a set of calibration standards prepared from the radiolabeled DANBIRT, serially diluted in 1% CMC, and sectioned from a separate block. Dehydrated sections were covered with 1.4 µm Isotope Imaging Film (FlushTec, Hemet, CA) and placed on Fujifilm Imaging Plates (BAS-IP SR 2025, GE Healthcare, Piscataway,

NJ) and exposed for 24 hours in a lead chamber (Raytest, Straubenhardt, Germany) with 0.04" lead sheeting between each screen cassette. Screens were imaged on a Phosphorous Imager Typhoon FLA 7000 (GE Healthcare, Piscataway, NJ). Autoradiography and white light files were compiled and analyzed using VivoQuant 2.00 (inviCRO, Boston, MA). Determined ROI were drawn using the best quality image phantom from reconstructed CT (Table 3) for the thymus, muscle, carotids and aortic arch. The ROI were extrapolated from phantom scan into every image and adjusted according to specific morphologic parameters.

Histologic analysis

ApoE$^{-/-}$ mouse hearts were collected, perfused and snap frozen in liquid nitrogen and stored at -80°C. The upper third of the heart with the aortic outflow tract were mounted in optimal cutting temperature (OCT) compound for sectioning. Cryosectioning was performed on OCT heart blocks using a Leica CM 3050S Cryostat at -20°C for collection of 10μm sections from the subaortic leaflet region. For Oil Red O staining (Sigma-Aldrich, O0625, SLBP5248V), slides were left at room temperature for 10 minutes and incubated with Oil Red O working solution and counterstained with Mayer's hematoxylin (Sigma-Aldrich, SLBPG176V) following manufacturer's staining protocol. Surface lesion area and arterial vessel circumference were quantified using Olympus cellSens Standard 1.13 to calculate percentage of atheroma lesion area by arterial wall circumference. Quality control assessment was performed to compare intensity of Oil Red O staining between groups.

Immunohistochemistry

The OCT embedded frozen sections (10µm) were fixed in methanol for 10 minutes at -20°C. After washing in PBS, the sections were incubated with fluorescein isothiocyanate (FITC) conjugated rat anti mouse CD11a (SouthernBiotech S1555-02) antibody (1:250) for 2 hours in a humidifier chamber. Sections were counterstained with DAPI (1:1000 dilution), imaged at 10x and 63x using a Fluorescence Microscope (Zeiss AxioObserver-Hamamatsu Flash4.0 sCMOS Monochrome Camera), image reconstruction was performed under FITC (490 Ex, 525Em) channel. Image processing was performed using Olympus cellSens Standard 1.13.

Statistical Methods

All statistics were computed using two-tailed Student's t-tests, or one-way and 2-way factorial ANOVA. Tukey's post-hoc test and Sidak's correction test were used for multiple comparisons. Resulting p-values <0.05 were considered significant. The results from 3D autoradiography results were compared as a mean difference in quantitative uptake reflected as %IA/gr normalized to muscle of radiolabeled DANBIRT because of limitations in compatibility of ^{111}In radioactive standards in this pilot study. GraphPad Prism 6.0 software for mac (GraphPad Software, Inc.) was used for all statistical analyses.

RESULTS

Radiolabeling and in vitro characterization of [^{111}In] In-DANBIRT

DANBIRT was efficiently labeled for experiments using ^{111}In and ^{68}Ga, achieving high *in vitro* stability. Assessment reflected >99% incorporation yield by ITLC and >99% radiochemical purity by HPLC analysis. Triplicate serial samples of radiolabeled DANBIRT incubated with fetal bovine serum (FBS) and 0.9% saline solution showed >95% mean radiochemical purity using UPLC method and >97% mean incorporation yield using ITLC method. Stability of [^{111}In] In-DANBIRT was maintained throughout 4 hours of incubation (Figure 2).

***Ex vivo* biodistribution of [^{111}In] In-DANBIRT post tail vein injection**

From biodistribution analysis of [^{111}In] In-DANBIRT, liver samples showed high uptake when compared to other tissues regardless of dietary treatment. Liver inspection after dissection revealed a pale white color with fibrous consistency in all HFD fed mice but not evident in normal diet fed mice, suggestive of steatosis. Serum samples also showed a high uptake compared to other blood components, similar only of those levels from liver samples. Cardiovascular tissues (heart, aorta, carotids) reflected higher uptake in animals that were fed a HFD ($p < 0.05$).
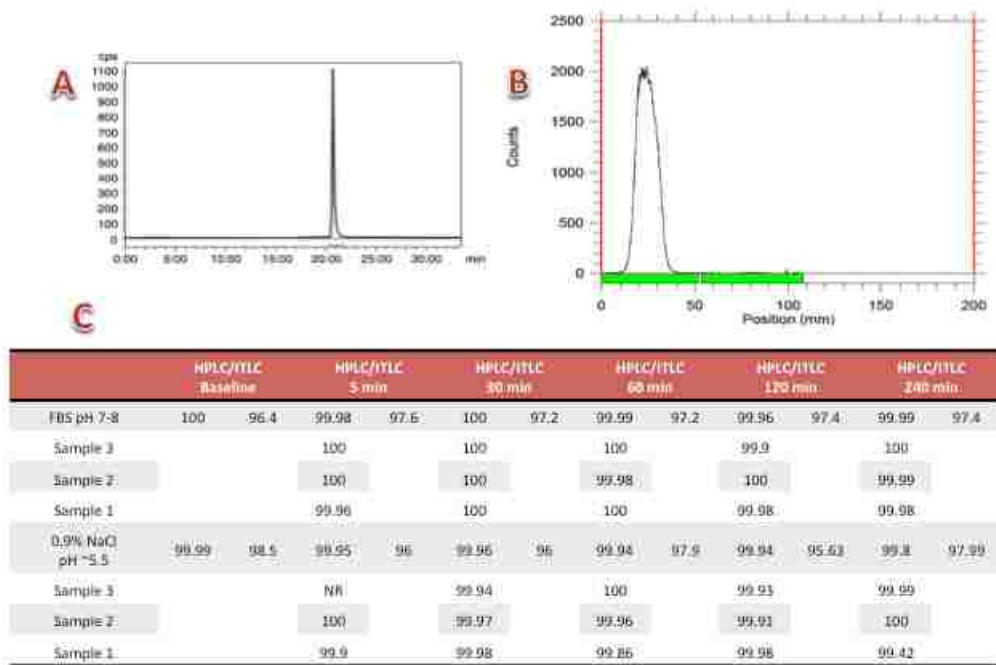
Figure 2. Radiolabeled DANBIRT in vitro stability. Representative HPLC (A) and ITLC (B) results were performed to assess in vitro stability after radiolabeling protocol for ^{68}Ga was completed. (C) Incubation in Fetal Bovine Serum (FBS) and 0.9% Saline solution showed >95% mean radiochemical purity and incorporation yield in triplicate samples at baseline, 5 minutes, 30 minutes, 60 minutes, 120 minutes and 240 minutes of incubation.

	HPLC/ITLC Baseline		HPLC/ITLC 5 min		HPLC/ITLC 30 min		HPLC/ITLC 60 min		HPLC/ITLC 120 min		HPLC/ITLC 240 min	
FBS pH 7-8	100	96.4	99.98	97.6	100	97.2	99.99	97.2	99.96	97.4	99.99	97.4
Sample 3			100		100		100		99.9		100	
Sample 2			100		100		99.98		100		99.99	
Sample 1			99.96		100		100		99.98		99.98	
0.9% NaCl pH ~5.5	99.99	98.5	99.95	96	99.96	96	99.94	97.9	99.94	95.63	99.8	97.99
Sample 3			NR		99.94		100		99.93		99.99	
Sample 2			100		99.97		99.96		99.91		100	
Sample 1			99.9		99.98		99.86		99.98		99.42	

Similar findings were found in RBC and WBC samples with increased uptake in HFD fed mice compared to normal diet fed mice (p<0.05) (Figure 3). Distribution of [^{111}In] In-DANBIRT showed an increase in %IA/gr of tissue in animals that were fed a HFD in comparison to animals fed a normal fed diet (p<0.05).

Metabolic effects in ApoE$^{-/-}$ mouse model after 8 weeks of HFD

Biological model was guided by mice ApoE deficiency that were placed on a HFD, resulting in an increased percent weight gain per week compared to animals in a normal diet (p<0.05) (Figure 4). Serum lipid levels and subparticle analysis showed that total cholesterol levels were higher in HFD fed mice, and higher levels of total triglycerides in normal diet fed mice; VLDL-Cholesterol and HDL-Cholesterol were also statistically different between dietary groups (p<0.05) (Figure 5). These results confirm the dietary effects in this model.

Histologic and immunohistochemical analysis of aortic atherosclerosis development

Histologic analysis reflected an accumulation of inflammatory tissue in the subaortic leaflet region of ApoE$^{-/-}$ mice. Histologic analysis of OCT frozen cryosections showed a higher percentage of atherosclerotic lesion area in relationship to vessel wall area in HFD fed mice compared to normal chow fed mice (p<0.05) which were correlated by Oil Red O staining for lipid accumulation. Oil red O staining quality control and assessment also reflected higher intensity of tissue staining area in HFD fed mice compared to normal fed mice (Figure 6). Immunohistochemistry was performed using anti-CD11a (LFA-1) antibody to identify qualitative atherosclerotic intraplaque CD11a+ leukocytes (Figure 7).
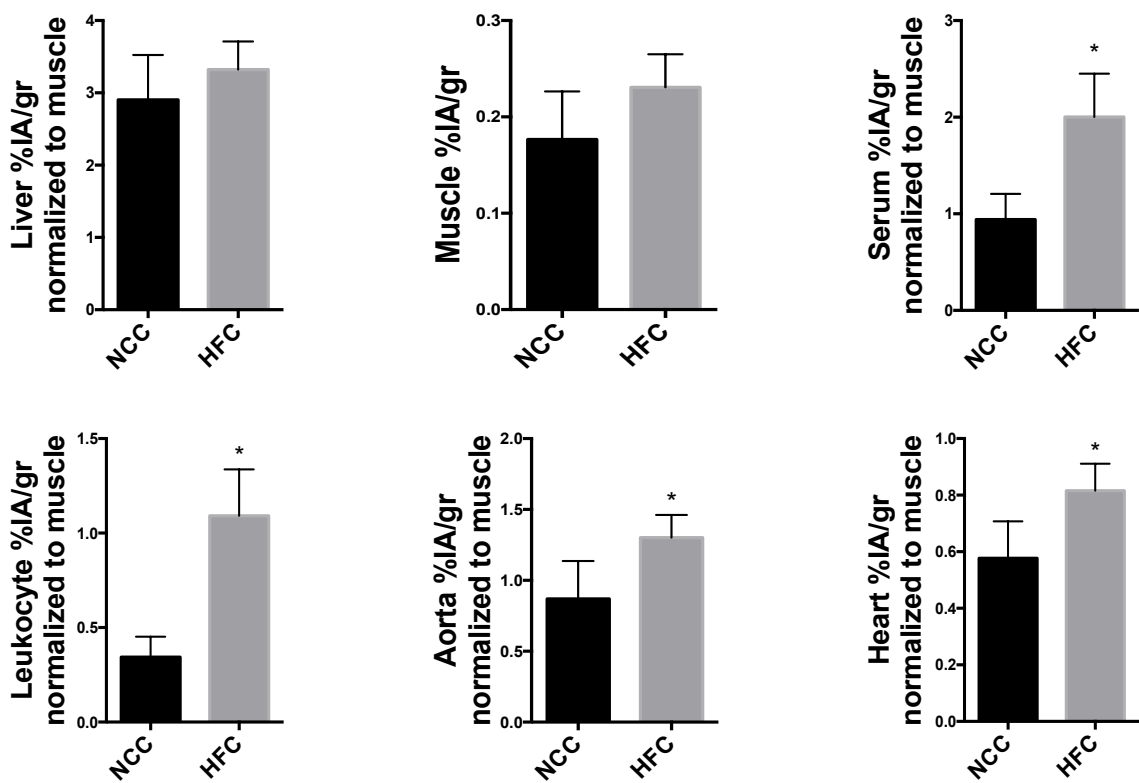
Figure 3. Biodistribution analysis of [^{111}In] In-DANBIRT on ApoE$^{-/-}$ mice after 8 weeks of dietary assessment. Muscle, heart, aorta, serum, WBC and liver were harvested and uptake was quantified. Results showed higher uptake in cardiovascular tissues and leukocytes in mice that were fed a HFD (p<0.05).
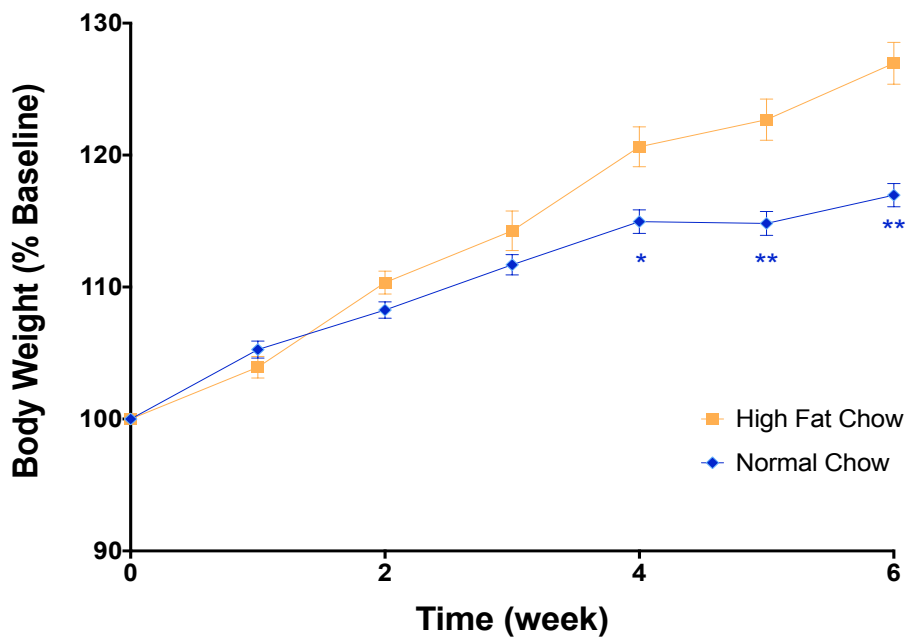
Figure 4. Percentage weight gain per week from baseline weight of ApoE^{-/-} mice after 8 weeks of dietary assessment. Percentage of baseline body weight increase compared at weekly time points represented as weeks per dietary group.
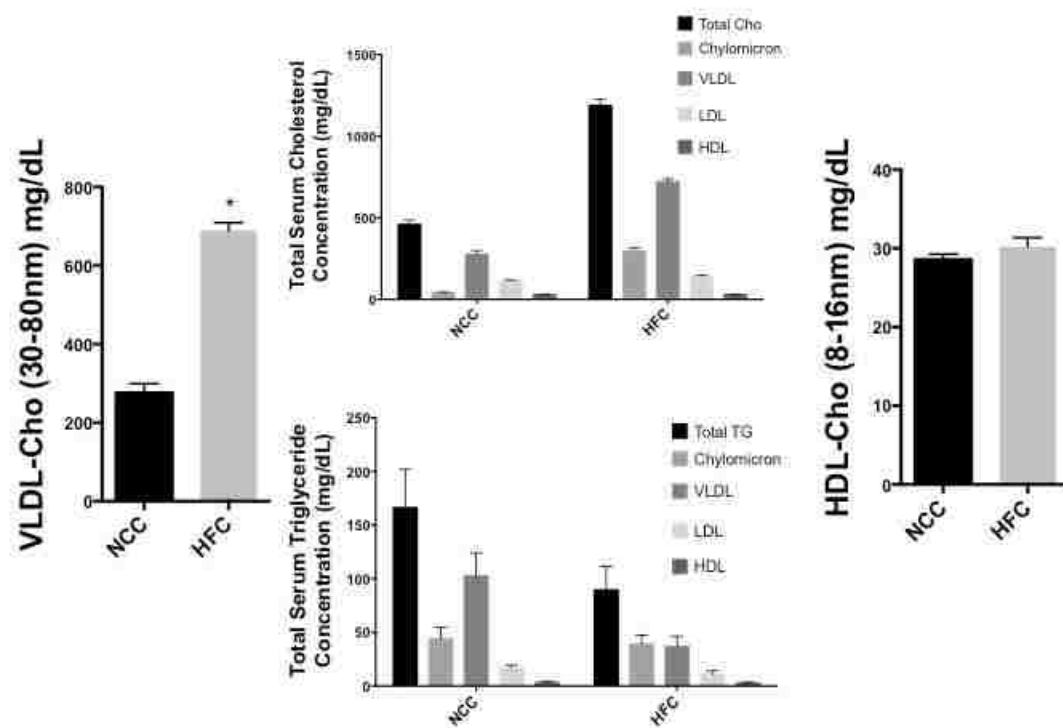
Figure 5. Serum total cholesterol and triglyceride level quantification in ApoE$^{-/-}$ mice per dietary group. Total cholesterol, total triglyceride, VLDL, HDL, LDL and chylomicron quantification are expressed as concentration in milligrams per deciliter (mg/dL) and in nanometers (nm) for particle size.
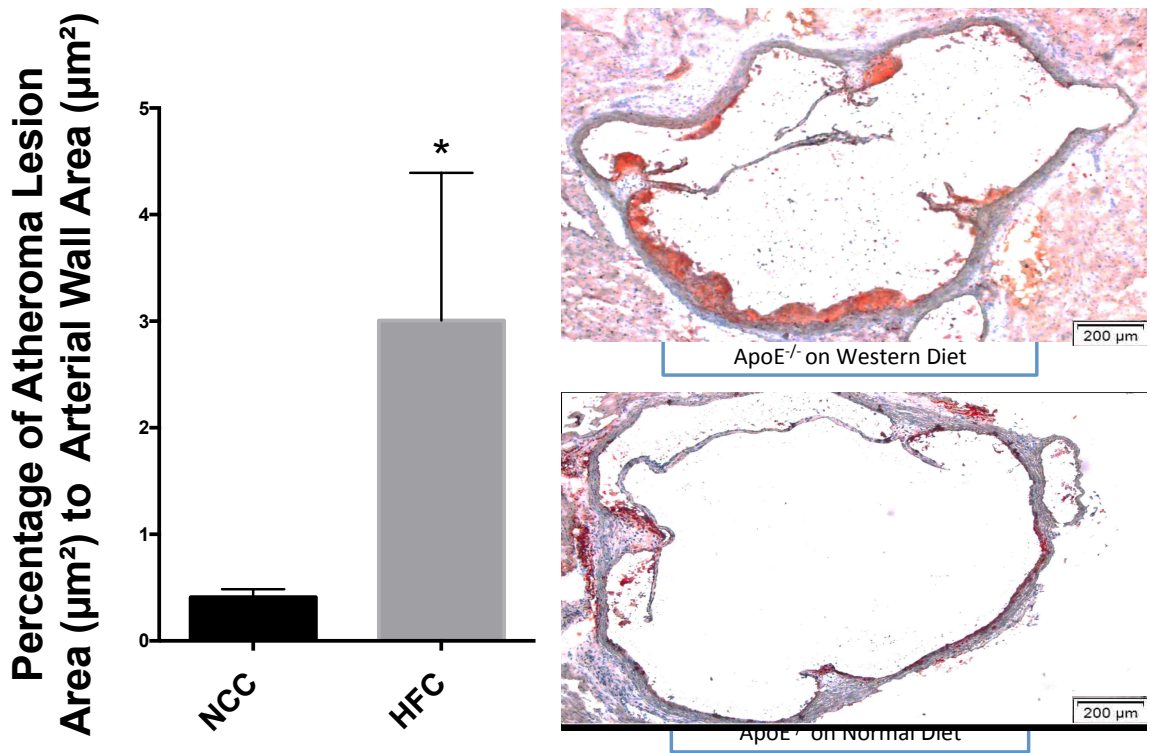
Figure 6. Histologic analysis of OCT frozen subaortic leaflet atherosclerotic lesions. Oil red O staining of histologic sections of OCT frozen subaortic leaflet atherosclerotic lesions under a 10x objective, represented as percentage of atheroma lesion area to arterial vessel wall area (μm^2) per dietary group.
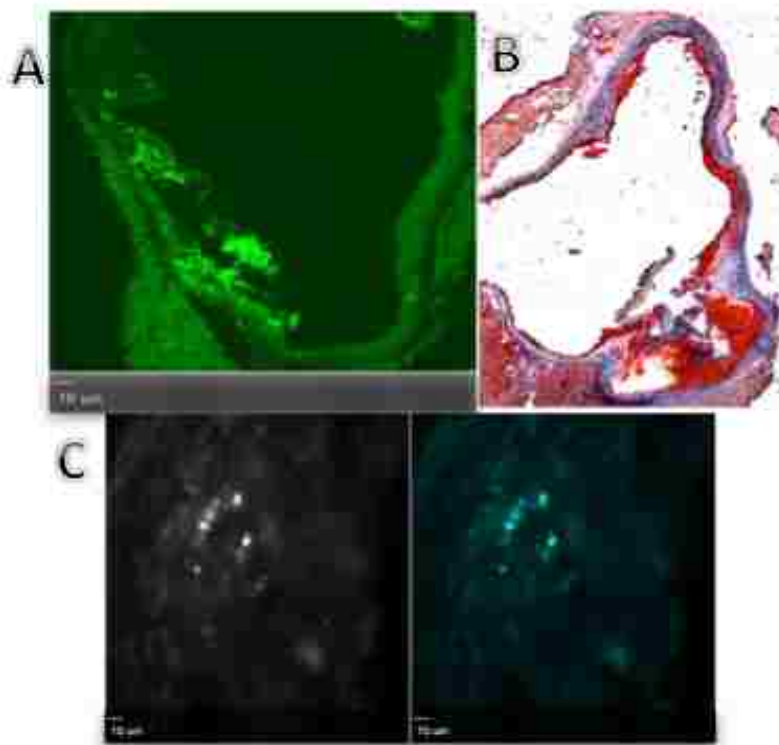
Figure 7. Immunohistochemistry of OCT frozen subaortic leaflet atherosclerotic lesions. (A) FITC conjugated rat anti-mouse CD11a (10x objective) (B) compared to Oil Red O staining of consequent slides in HFD fed animals (10x objective). (C) Subaortic vessel wall leaflet atherosclerotic lesions stained with FITC conjugated rat anti-mouse CD11a (63x objective w/immersion oil)

LFA-1-specific targeting and competitive leukocyte binding with radiolabeled DANBIRT

Leukocyte isolation methods successfully identified purity of samples, with predominance of immature neutrophils (~59%) and PBMCs (~53%) in ozone-exposed rats (used to induce a circulating neutrophilia, as previously described(Paffett, Zychowski, Sheppard, Robertson et al., 2015)) with no other relevant morphologic cell changes. Data showed an increased uptake of [^{68}Ga] Ga-DANBIRT in neutrophils (*p=0.008) (Figure 8), with a decreased uptake in PBMCs post-ozone exposure.

Log-fold dilution concentrations of [^{111}In] In-DANBIRT identified a competition for LFA-1 receptor binding in target leukocytes. Incrementing radiolabeled DANBIRT concentration resulted in competitive binding, identifying a saturation of binding sites in neutrophils starting at a 1:10 log-fold serial dilution concentration of [^{111}In] In-DANBIRT (Figure 9). A pattern of 50% decreased initial uptake after each log fold dilution concentration was evidenced in serum, RBC and PBMC samples. PBMCs at different dilution concentrations did not evidence any saturation levels or reflected a tendency when compared to neutrophil sample (p<0.05).

Longitudinal molecular imaging of cardiovascular LFA-1 presence with [^{111}In] In-DANBIRT

The 3hr [^{111}In] In-DANBIRT SPECT/CT imaging showed a longitudinal increased uptake in heart, aortic arch and descending aorta ROI concentration quantification in the 4th and 8th week time points in HFD fed mice (Figure 10). The 24hr [^{111}In] In-DANBIRT 3D autoradiography allowed a functional and more

advanced anatomical assessment of the presence of inflammatory LFA-1 in immune and cardiovascular tissues, an essential finding to characterize the biological model. Mice fed exhibited an increase in carotids and thymus's concentration per volume normalized to muscle in HFD fed mice compared to normal diet fed mice (Figure 11).
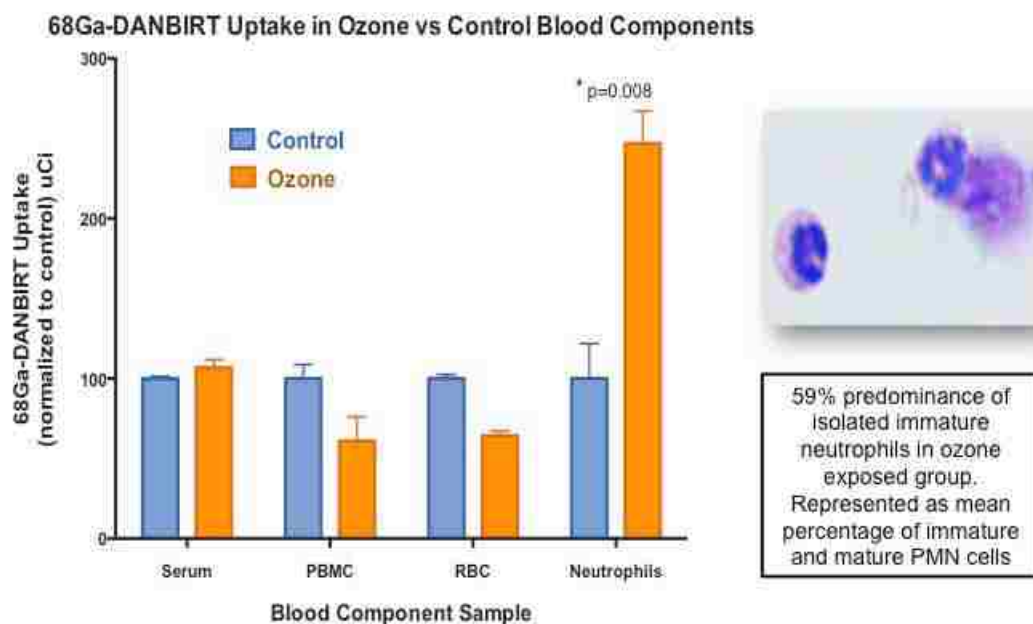
Figure 8. Radiolabeled DANBIRT uptake in isolated blood components post 4-hour exposure to ozone. Data showed an increased uptake of [^{68}Ga] Ga-DANBIRT in neutrophils post 4-hour ozone exposure (*p=0.008) with a decrease in PBMC and serum uptake. Cytospin slides were stained using Wright-Giemsa horizontal method for differential analysis of isolated sample purity under 100x objective w/immersion oil with a light microscope.
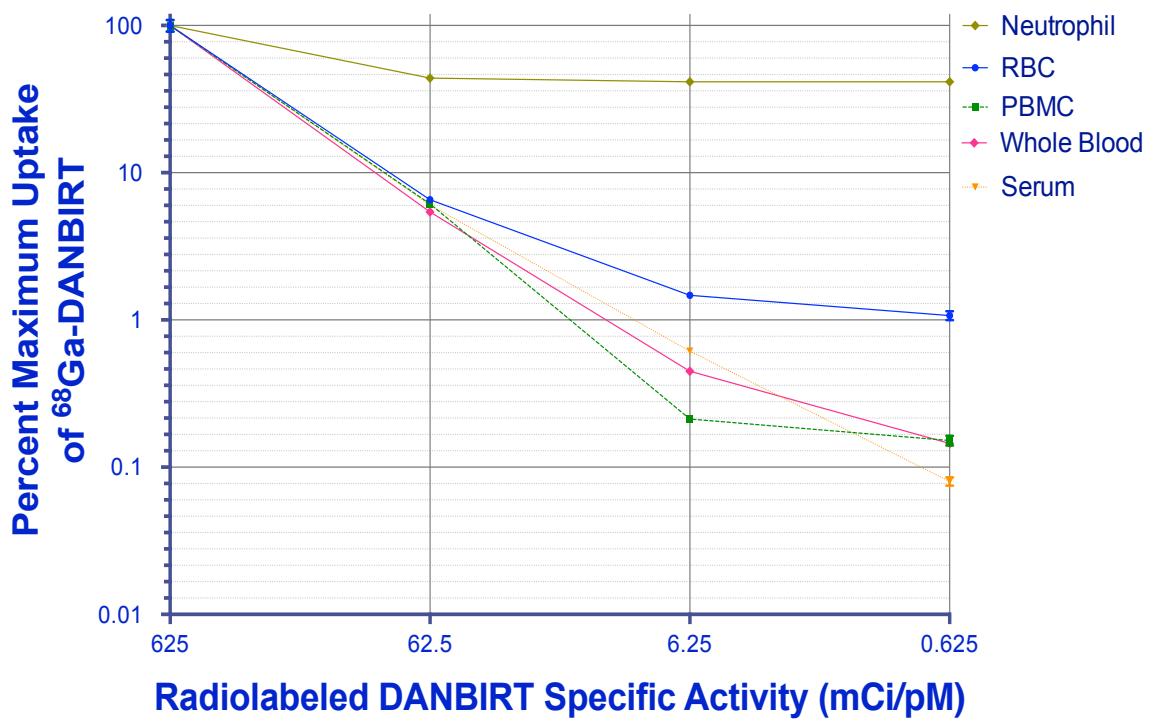
Figure 9. Radiolabeled DANBIRT competitive binding assays. Radioisotopic dilution methods were performed using an initial specific activity of 625mCi/pM and consequent serial log-fold dilution concentrations. Saturation of isolated neutrophils as of percent of maximum uptake was observed starting at 62.5mCi/pM (1:10X log fold dilution concentration) specific activity of radiolabeled DANBIRT.
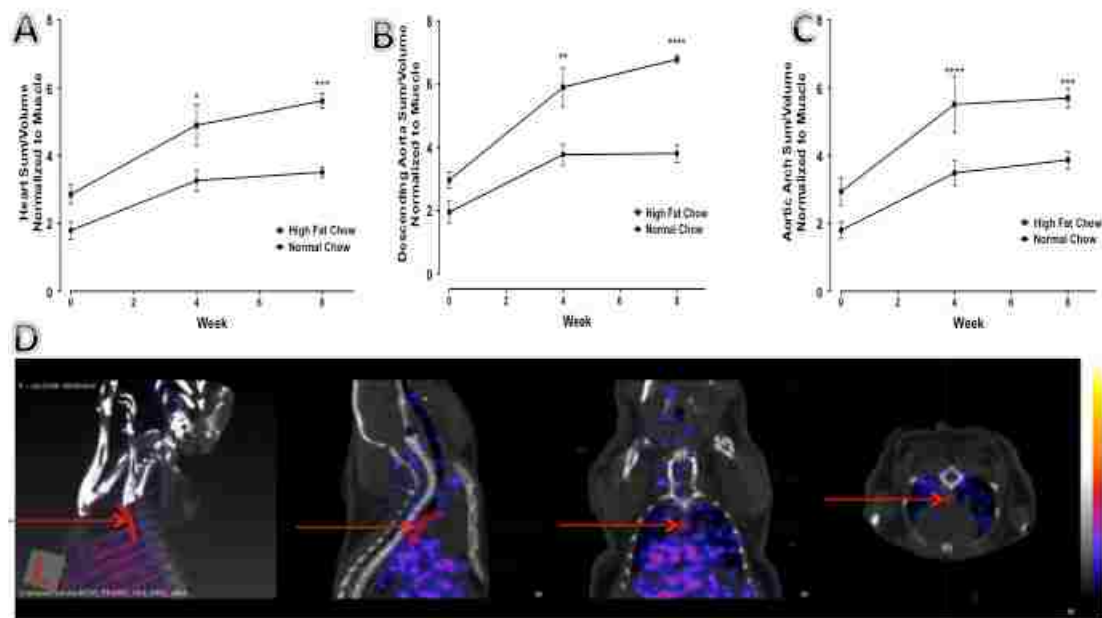
Figure 10. 3hr [¹¹¹In] In-DANBIRT SPECT/CT imaging. (A, B, C) Increased

longitudinal uptake of [¹¹¹In] In-DANBIRT in cardiovascular areas prone to

vascular atherosclerosis development (heart, descending aorta, aortic arch)

reflecting a longitudinal increase per time point (week) shown by SPECT/CT

imaging. (D) ROI were drawn for muscle, descending aorta, aortic arch and heart

using VivoQuant (inviCRO) from phantom scan and extrapolated in each subject.

Red arrows point to ROI of descending aorta.

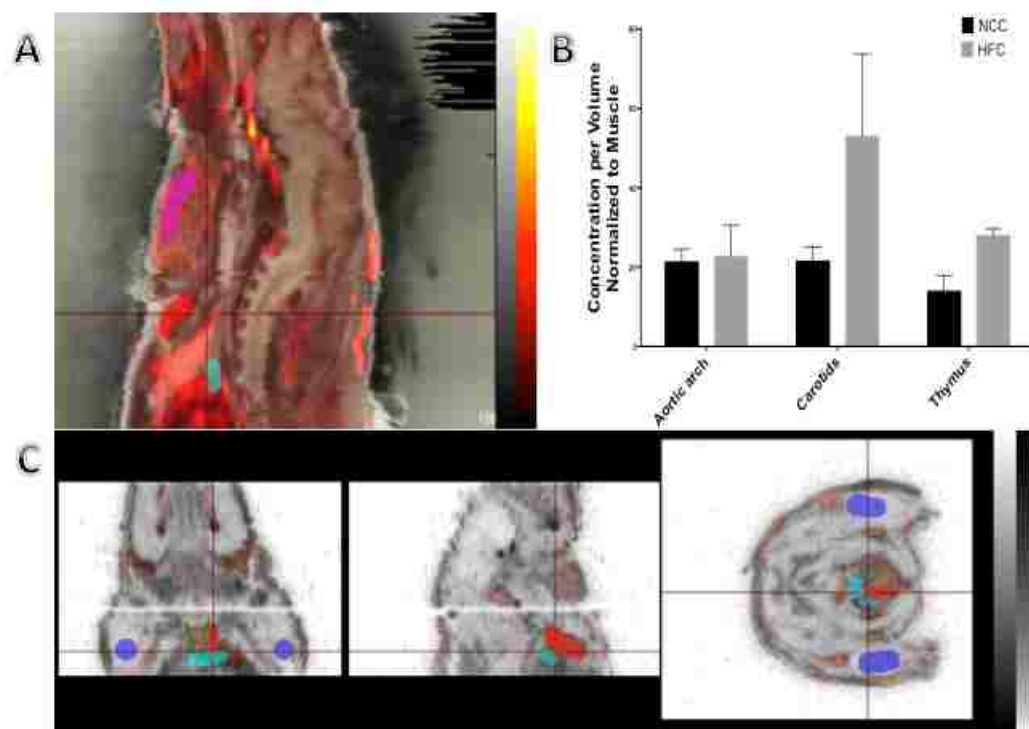**Figure 11. 24hr [^{111}In] In-DANBIRT 3D autoradiography.** (A,C) ROI were drawn for muscle (blue), common carotids (green), aortic arch (cyan) and thymus (red) using VivoQuant from phantom scan and extrapolated in each subject, reflecting concentration per volume normalized to muscle. (B) Increased uptake of [^{111}In] In-DANBIRT in common carotids and thymus in HFD fed mice compared to normal diet fed mice.

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DISCUSSION

The purpose of this study was to determine the value of [^{111}In] In-DANBIRT as a non-invasive *in vivo* imaging tool for LFA-1 expression in leukocytes in the inflammatory process of atheroma development by longitudinal SPECT/CT molecular imaging. Results showed that we were able to target the increased LFA-1 expression in neutrophils under an acute systemic immune injury model (i.e., ozone exposure), used analogously to early inflammatory developmental stage of vascular atherosclerotic lesions. Longitudinally-increased uptake in cardiovascular tissues, shown by SPECT/CT, illustrates the translational component and value in a chronic model. *In vivo* assessment of atherosclerotic plaque vulnerability is a clear need in cardiovascular research. Biotechnological approaches designed to identify, assess, and potentially eliminate fatal repercussions of atheroma development could have great benefits to patients(Herrington, Lacey, Sherliker, Armitage et al., 2016). Longitudinal *in vivo* imaging of plaque inflammatory processes is a very promising concept to advance understanding and reduce cardiovascular related complications(Burtea, Ballet, Laurent, Rousseaux et al., 2012).

The selective expression of LFA-1 in leukocytes and DANBIRT's small size contribute to a theoretically ideal imaging probe of inflammatory activity inside of the plaque. LFA-1/ICAM-1 complex is important in almost every vascular disease because of its role in leukocyte recruitment and transmigration(Collins, Velji, Guevara, Hicks et al., 2000). T-cell activation and migration to sites of inflammation is guided predominantly by LFA-1/ICAM-1 interaction and Signal-

2(Anderson & Siahaan, 2003), making the allosteric inhibition of LFA-1 an effective way to target intraplaque inflammation(Rahul B. Poria, 2006). Recent papers address the functionality and sensitivity of LFA-1, understanding that modulation of this integrin will impact immune progression of disease(X. J. Wang, Xu, Zhang, Krishnamurthy et al., 2010). Atherosclerosis development is dependent on the severity of the immune response at sites of vascular injury, along with the ability to resolve such inflammation (Vestweber, 2007). Inflammatory components such as leukocytes have major roles in plaque vulnerability and instability, impacting barrier integrity, releasing peptidases that facilitate remodeling, and generating pro-inflammatory chemokines. Local and systemic immune response appears to guide cardiovascular tissue uptake (Tabdanov, Gondarenko, Hone, & Kam, 2011), while illustrating the potential for enhancing SPECT/CT interpretative results in a more sensitive and specific system.

Numerous *in vivo* imaging strategies are being assessed for effective diagnosis of atherosclerotic plaque vulnerability. Many other studies have characterizes the unique plaque characteristics, such as calcium presence and morphology, without fully addressing the role of neutrophil accumulation in the plaque. Work performed with DANBIRT and the detection of vascular LFA-1 levels stands as an attempt to complement other imaging approaches. Novel approaches utilizing enhanced hybrid imaging systems that target metabolic activity inside of the plaque to assess glucose uptake in such tissues may further add to a battery of plaque characteristics that can be more precisely linked to adverse pathological

outcomes (Riou, Broisat, Dimastromatteo, Pons et al., 2009). Understanding the role of neutrophils in plaque vulnerability is important(van Leeuwen, Gijbels, Duijvestijn, Smook et al., 2008), because of the correlation between plaque instability and the presence of neutrophil-derived acute immune response markers.

From results we know that radioisotopic dilution methods helped us achieve a concentration in which the observe competitive binding(Perales, 2015) illustrated receptor saturation in neutrophils. Saturation in these samples correlates to findings in the biodistribution and post-ozone exposure data, confirming the role of [^{111}In] In-DANBIRT for radiolabeling neutrophils at a proven specific activity and concentration. The increased volume from serum and RBC samples did not reflect nonspecific binding in these experiments, but did explain the concept of saturation of LFA-1 binding sites in neutrophils. Another important characteristic of ^{111}In (Indium chloride, chemical presentation provided by GE Healthcare) is that it has high uptake in the liver and lungs because of binding interaction with transferrin and lactoferrin(Otsuki, Brunetti, Owens, Finn et al., 1989) illustrating initial biodistribution high uptake levels in the liver. These studies effectively demonstrated radiolabeling of DANBIRT using ^{68}Ga (short-lived PET radioisotope) and ^{111}In (longer-lived SPECT radioisotope) demonstrating high stability throughout 4 hours of incubation in FBS and 0.9% NaCl. Through in vitro stability studies and effective labeling methods it is possible to safely administer [^{111}In] In-DANBIRT intravenously as a stable radioligand probe.

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The biodistribution of [^{111}In] In-DANBIRT showed a high uptake in the liver, serum and RBCs, which identified an issue with the binding potential was postulated as nonspecific uptake due to the mass effect of the radioligand specific activity. Adipose tissue and muscle were identified as potential low uptake tissues. Tissues surrounding the vessel lamina adventitia have been recently identified as sites of accumulation of inflammatory cells in ApoE$^{-/-}$ mice on a normal diet(Moos, John, Grabner, Nossmann et al., 2005). This effect was not clearly identified in the studies due to the limits of spatial resolution of the SPECT imaging system. However, adipose tissue was identified as lacking significantly different uptake compared to the tissue designated as background (muscle). These biodistribution results to hypothesize three specific scenarios: no binding, aspecific binding or free, unlabeled drug. *In vitro* stability studies showed that at 4 hours post incubation in FBS and 0.9% NaCl, the radiolabeled DANBIRT was stabile, eliminating the possibility of free unlabeled drug/ligand post administration. By blood component isolation and post-ozone environmental exposure, the target cells were visualized in a proven animal model correlating with local and systemic acute immune response reflected parallel results from an innate immune response in the targeted cells(Ramot, Kodavanti, Kissling, Ledbetter et al., 2015).

Molecular imaging shows a longitudinally-increased uptake in cardiovascular tissues in mice fed a HFD, which correlates to atherosclerotic development because of the presence of shear stress areas in principal arterial vessels(Katakami, 2016). Functional and morphological assessment of [^{111}In] In-

DANBIRT guides the translational component of probe development.

The limitations of these studies included reduced statistical power of the animal sample, although this was lessened due to the longitudinal collection of repeated measures data by SPECT/CT. Many mouse models use an ApoE$^{-/-}$ model of atherosclerosis development because of the resultant hypercholesterolemia and spontaneous presentation of plaque starting at 3 months of age(Sasaki, Kuzuya, Nakamura, Cheng et al., 2006). The HFD increases the amount of plaques by 14 weeks of age(Meng, Ma, Yan, Chen et al., 2016). Although deficiency of the LDL receptor will also cause high levels of lipids and some spontaneous diet-dependent plaques, the extent and slow progression of Ldlr deficiency makes the ApoE$^{-/-}$ model a desireable choice. The use of the wild type control strain (C57BL/6) was not considered as these mice fail to develop any observable vascular pathology.   In a translatable approach, humans with hereditary hyperlipidemia have associated mutations of Ldlr and/or ApoE, which has been shown to increase the susceptibility to developing atherosclerosis(Mata, Alonso, Ruiz-Garcia, Diaz-Diaz et al., 2014). Human atherosclerotic plaques resemble the ApoE$^{-/-}$ mouse model but, the Ldlr deficient mouse model develop a closer resemblance in the lipidemic panel in humans(Abd El-Aziz & Mohamed, 2016).

From these results, the development of early stage atheroma is includes acute inflammatory components. Histologic and immunohistochemical analysis supports the findings by characterizing the early development of the atherosclerotic model. The increased areas of atherosclerotic lesions and lipid accumulation correspond to results found in the HFD-fed animals. [^{111}In] In-

DANBIRT is a promising radioligand for identification of the presence of LFA-1 in circulating leukocytes. Accumulation of immune cells in atherosclerotic plaque lesions mediates disease progression and vulnerability. Vascular atherosclerotic lesions in areas prone to atheroma development can be characterized by radioligand uptake. Thus, these findings support future investigation of a more advanced vascular disease model that will validate [^{111}In] In-DANBIRT as a diagnostic tool for assessment of inflammation in cardiovascular injury models.

CONCLUSIONS

Characterization of the biologic model identified the early stage development of atherosclerotic plaque in cardiovascular tissues with an acute inflammatory component. The longitudinally increase in [^{111}In] In-DANBIRT uptake in cardiovascular tissues in mice fed a HFD was consistent with histopathologically-observed advancement of aortic lesions. Neutrophil and PBMC isolation methods demonstrated selective and competitive LFA-1 antigen receptor binding in leukocytes. Radiosotopic dilution and receptor-binding assays evidence a mass effect in neutrophils with radiolabeled DANBIRT at a specific activity of <62.5mCi/pM. These correlative results demonstrate competitive and specific LFA-1 antigen receptor binding to targeted leukocytes.

This study validates the potential of [^{111}In] In-DANBIRT to competitively bind leukocytes, especially neutrophils, while illustrating the presence in cardiovascular and immune tissues identified in a systemic inflammatory response following exposure to inhaled ozone. Recent publications corroborate BIRT377 as a potential drug for clinical applications in high-risk

populations(Kelly, 2015; Rahul B. Poria, 2006). Further studies are needed to determine the value of this radioligand probe as an *in vivo* non-invasive imaging tool in an enhanced chronic vascular injury model exhibiting predominance of adaptive immunity components.

ACKNOWLEDGEMENTS

Chapter 3. Discussion and conclusion

I will start the discussion by stating the principal aim of this study: Validate the use of [^{111}In] In-DANBIRT as a novel in vivo non-invasive SPECT/CT molecular imaging tool in the development of atherosclerotic inflammatory plaque. I have read the aims over and over again to try to comprehend how such an important diagnostic tool is not available, and the answer is clear, because it is a very difficult task to accomplish(Dietrich, Berndorff, Heinrich, Hucko et al., 2015). There are many sides to the chemical repurposing of DANBIRT, there has been groundwork done with DANBIRT that actually showed that it is able to bind to bone marrow hematologic precursors in human and murine cells. In conjunction with my committee, I was able to come up with the most direct way to illustrate the value of [^{111}In] In-DANBIRT using nuclear medicine assays, metabolic assessment, environmental exposure and toxicology in a specific atherosclerosis approach.

Appreciating that atherosclerosis is a disease that changes and evolves is the first step to effectively prevent and diagnose its progression(Herrington, Lacey, Sherliker, Armitage et al., 2016). The immune component of atherosclerosis shifts dramatically in a short amount of time, challenging the body's adaptability. The idea that our own immune cells play a key role in atherosclerotic plaque rupture and complications is another important concept. I employed all efforts to try to comprehend the behavior of the radioligand [^{111}In] In-DANBIRT, and to illustrate the potential for its implementation in different models of acute immune injury.

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Radiolabeled DANBIRT has been used in the past in a chronic exposure to Mixed Vehicle Exhaust (MVE) emissions in young (2 month) and old (18 month) mice (Mumaw, Levesque, McGraw, Robertson et al., 2016), The lung and heart uptake of DANBIRT increased, suggesting a local and systemic inflammation in young animals exposed to MVEs. This was correlated by bronchoalveolar lavage fluid results showing an increase in total cell and neutrophil quantification in young but not old mice after MVE exposure. Such results highlight the biological model of chronic exposure as a mean to assess inflammatory modulation over time in animals that have different levels of immune coherent systems. Under these conditions, it is comprehensible that the assessment of immune modulation is essential to the local and systemic inflammatory signaling in cardiovascular diseases (Santarpino, Caroleo, Onorati, Dimastromatteo et al., 2009).

For many years, environmental exposure research has been one of the leading fields of focus and will continue to be so because of the inadequate control for hazardous environmental exposures (Last, Gohil, Mathrani, & Kenyon, 2005; Ramot, Kodavanti, Kissling, Ledbetter et al., 2015). Evaluation of cardiovascular effects of environmental exposure is easily translatable to multiple research areas in which the inflammatory components of such biologic models of disease are easily demonstrated (Sun, Liu, Xu, Ying et al., 2013). LFA-1 targeting of [^{111}In] In-DANBIRT was also evidenced under an ozone exposure assay and competitive binding assays to isolated blood components. Environmental exposure played a key role to illustrate the potential value of this imaging probe.

Limitations of the Study

Atherosclerosis is a complicated disease in which demonstrating the correct development of the mouse model is a long and tedious task. I believe the most important limitation for the thesis project was being underpowered. Many of the thesis related experiments ideally would have had a larger sample size, thereby reducing variance as much as possible. Even though these studies were somewhat underpowered, the analysis was rigorously performed increasing the amount of assays and experiments that I was able to complete. Having multiple, repeated measurements in mice, there is high variability from the analysis that directly affect the study design The animal model of atherosclerosis is difficult to manage because of the constant monitoring of food intake and adjustments plus the involvement of competition in mice cages for dominance and other secondary adverse effects from ApoE deficiency.

Other limitations included DANBIRT determined specific activity for imaging at low target dose because of proven a proven mass effect, when working with radioactivity and small molecules, the specificity of the radioligand to target LFA-1 has to be high. Effective radiolabeling and stability effect intravenous administration and logically the analysis of image data. Rapid biodistribution and clearance may also interfere with the natural process of leukocyte migration and may only target the circulating pool of mature available leukocytes. The hypothesis was focused in identifying the localization of such immune cells inside of the plaque to illustrate the longitudinal approach of the imaging modality(Issekutz, 1995). I had to demonstrate that because I used an in

vivo model, the in vitro assessment was correlated to the findings while characterizing an effective evolution of the disease model.

The ApoE$^{-/-}$ mouse model of atherosclerosis has been studied extensively, although most of the assessment performed is focused on other characteristic components of a vulnerable plaque(Freitas, Campana, Pozzan, Brandao et al., 2015). Spontaneous plaque development in ApoE deficient mice has been described in the literature (Hu, Ma, Huang, Mao et al., 2013) but, it does not always respond to interventions in the same way (Zalewski & Macphee, 2005). A larger animal model of disease, such as a rat, may allow more complete assessment of specific morphologic parameters with a SPECT/CT system.

Initially, co-administration of intravenous contrast agents (gold nanoparticles with high macrophage uptake, and iodine based contrast agents to enhance SPECT/CT imaging. Macrophage uptake of contrast agents confounded the use of such agents because it could potentially interfere from signal acquired from inside the plaque and disrupt from areas that might have specific macrophage subsets. Such similar problems were implied with the use of gold nanoparticles and they were also not used for imaging studies. Iodine-based contrast agents were tried repeatedly to illustrate the value of enhanced vascular identification. However, because of the effects on heart rate and cardiac output, these efforts were unsuccessful in the ApoE$^{-/-}$ mouse model.

The contrast agent was not being observed in the blood pool even at multiple time points but was present in the kidneys even seconds after administration. I did increase the aperture for the SPECT imaging scans to try to

enhance results and have better quality images that illustrate the potential of the probe. Phantom scans from reconstructed CT was performed to correctly extrapolate the use of analytic software to localize ROIs in cardiovascular tissues without the need for a contrast agent (Vemulapalli, Metzler, Akabani, Petry et al., 2007). I also took many measures to try to decrease the variability between the mouse model; biased interpretation of longitudinal molecular imaging intra- and inter-animal error was eliminated by randomized and blind interpretation of image data from each mouse. Most of the experiments had samples run in duplicates or triplicates to improve the statistical analysis.

There would have to be substantial evidence to prove the concept of radiolabeled DANBIRT as a fully translatable imaging diagnostic tool. The concept has legitimate value, the characterization of the intravenously administered probe showed great results but, there is still a much needed implementation of DANBIRT in a Positron Emission Tomography (PET) imaging system that can translate to clinical imaging. If the effective specific activity of [^{68}Ga] Ga-DANBIRT is proven to be high enough to find similar results in a specific chronic vascular disease model that can increase the spatial and temporal resolution, then I believe we would be in the verge of a new molecular imaging diagnostic tool for the assessment of cardiovascular disease and risk of cardiothrombotic events in clinical patients.

Implications for Future Research

There is still work to be done to validate the translatable value of [^{111}In] In-

DANBIRT, but we are in the right path. Findings from this thesis project and work done from past students from the radiopharmaceutical sciences program at the University of New Mexico Health Sciences Center (Rahul B. Poria, 2006) support investigation of a more advanced vascular disease model that will help validate $[^{111}In]$ In-DANBIRT as a diagnostic tool for assessment of inflammation in cardiovascular injury models. The continuously changing inflammatory components of atherosclerosis development changes the value and/or limitations of $[^{111}In]$ In-DANBIRT(Henderson, Hobbs, Mathies, & Hogg, 2003). Because of the intrinsic reliability of performing nuclear medicine procedures in patients, it is ideal for a radiopharmaceutical to have high reproducibility and diagnostic sensitivity in multiple applications.

Whether the value of $[^{111}In]$ In-DANBIRT relies on high specificity or sensitivity, we need to establish its value in multiple scenarios. Radiopharmaceuticals should have multiple applications in identifying at risk patients and having a high positive and negative predictive value, with the potential for improvement. There is a great need in nuclear medicine and molecular imaging for a diagnostic tool that is able to discriminate the inflammatory component of a disease in vivo and longitudinally (Gallino, Stuber, Crea, Falk et al., 2012). It is very difficult for a clinician to evaluate the extent of inflammation in specific tissues without performing additional diagnostic tests.

Disease models of inflammatory bowel disease, cancer, asthma, lupus related vasculitis, endocarditis and many other diseases with strong immune and inflammatory components would benefit from in vivo (Zhao, Yao, Deng, Ju et al.,

2010) assessment using [^{111}In] In-DANBIRT, evaluating its imaging capabilities in pre clinical scenarios were longitudinal assessment of immune modulation can correctly evidence the expression of LFA-1 in specific tissues.

The mouse model was identified as a stage 2 atheromatous vascular lesion, but it is important to test the value of [^{111}In] In-DANBIRT in more vulnerable severe plaques. There is a recent concept of development of a shear stress-related advanced atherosclerotic vascular lesions (Lee, Choi, Seo, & Yeom, 2015) with the use of a microsurgery for carotid cuff placement performed with a plastic "cast" around a unilateral carotid artery (Kuhlmann, Cuhlmann, Hoppe, Krams et al., 2012). The surgery is performed on ApoE$^{-/-}$ mice that are placed on a HFD for 5 weeks after surgery. Mice develop prominent plaques that can be observed by physical evaluation after carotid resection (Katakami, 2016). This model identifies potential vascular zones that are susceptible to atherosclerotic development and also to rupture due to low and high shear stress to certain areas around the cast placement. Unstable plaque characteristics such as: intraplaque hemorrhage, neovascularization (vasa vasorum) and increased intraplaque immune cell activity that will develop from this model will enhance result from the longitudinal increased uptake of radiolabeled DANBIRT in atherosclerotic lesions.

Using DANBIRT as a diagnostic tool to accurately distinguish areas with significant radioligand uptake (Dimastromatteo, Riou, Ahmadi, Pons et al., 2010) due to the presence of immune inflammatory components may improve diagnosis and staging of cardiovascular disease, which, in turn may reduce or

eliminate the need for unnecessary diagnostic surgery, improper diagnostic testing, increase the specificity of invasive procedures and reduce overall cost. Pediatric and elderly patients are examples of patients for whom a thorough physical evaluation may not provide an adequate assessment of a patient's health. The ability of a diagnostic test is to complement the differential diagnostics that have been determined by a physician (Riou, Broisat, Dimastromatteo, Pons et al., 2009).

Molecular imaging is an ideal companion for a clinician to reduce diagnostic uncertainty that might have been present at the time of medical evaluation. Routine nuclear medicine imaging tests are performed in outpatient settings with a small number of patients having tests performed as inpatient(Rybicki, Udelson, Peacock, Goldhaber et al., 2016). Molecular imaging I spossible because of the lack of interference with the body's natural biochemical processes while allowing assess pathology. This last statement may be the single most important feature to look for when designing or repurposing a radiopharmaceutical for the ideal imaging system.

I used SPECT/CT imaging with [^{111}In] In-DANBIRT, being able to efficiently radiolabel DANBIRT with a shorter half-life radioisotope like ^{68}Ga with high in vitro stability shown by characterization. Ideally, we would like to use [^{68}Ga] Ga-DANBIRT not as a SPECT agent but as a PET imaging probe because of its dual value in both imaging platforms. This will be something to look forward and make DANBIRT a validated probe for imaging inflammatory disease.

SPECT/CT imaging confers a similar temporal and spatial resolution (Keenan, Pugliese, & Davies, 2014) to PET/CT in small-animal imaging. However, in clinical imaging, the larger body-mass of patients requires attenuation correction, making PET imaging the most researched imaging modality(Perez-Medina, Binderup, Lobatto, Tang et al., 2016). Fluorodeoxyglucose (FDG) is a PET imaging radiopharmaceutical that identifies glucose metabolism in tissues with the ability to discriminate between low, moderate and high degree uptake areas(Golestani, Mirfeizi, Zeebregts, Westra et al., 2015). Almost every tissue uses glucose in a both physiologic and pathological pathway, illustrating the high sensitivity but low specificity of FDG especially in cardiovascular disease.

Because of the limitations of SPECT, the advantages of hybrid imaging PET/CT systems would not be greater in terms of the imaging system itself. Functional imaging of small animals such as mice and rats eliminates the need for attenuation correction and other resolution compensations between SPECT/CT and PET/CT imaging(Garrigue, Giacomino, Bucci, Muzio et al., 2016).

Conclusions

Results from this thesis project showed that ApoE$^{-/-}$ mice on a high fat "Western" diet exhibit increased cardiovascular and immune tissue uptake of [^{111}In] In-DANBIRT after 4 weeks of HFD with a longitudinal increase after 8 weeks of HFD compared to mice on a normal diet. 3hr SPECT/CT and 24hr 3D

autoradiography imaging illustrated a consistent pattern of increased uptake in cardiovascular (aortic arch and descending aorta) (Schramm, Menger, Schaefers, & Thorlacius, 2004) and immune tissues in HFD fed mice, evidencing a positive correlation to the metabolic changes in the biological model that identifies sites of accumulation of immune cells with a systemic and local response. Isolated whole blood components evidenced increased specific uptake in neutrophils after exposure to ozone, indicating that radiolabeled DANBIRT is able to accurately evidence the acute immune response in the animal model by targeting increased expression of LFA-1 in neutrophils (Block, Stadtmann, Riad, Rossaint et al., 2016).

In vivo imaging of LFA-1 expression correlates with vascular lesions from subaortic leaflet cryosections, while illustrating the increased lipid content in the development of atherosclerosis in mice fed a HFD. [^{111}In] In-DANBIRT is a promising radioligand for targeting the presence of LFA-1 in circulating mature leukocytes, specifically targeting neutrophil increase in the acute immune response in atherosclerotic plaque due to vascular injury (Nathan, 2006). Accumulation of immune cells in atherosclerotic plaque lesions mediates disease progression and vulnerability, were the aortic arch and descending aorta are most prone to atheroma development can be characterized by the increased uptake of [^{111}In] In-DANBIRT(Phinikaridou, Andia, Shah, & Botnar, 2012).

This research project has been a fantastic opportunity to integrate one of the greatest passions I have in my life, translatable research. I have been able to explore many areas of cardiovascular and immunopathologic research in

atherosclerosis while implementing a novel approach to an ancient problem. It is fundamental in modern medicine to outreach and collaborate between different areas of biomedical research. The field is positively impacted whenever young investigators are encouraged to try novel approaches to known problems in health. The extent of these results will benefit future research to be conducted with DANBIRT and will make room for better and transcendental work in my career. I am grateful for everyone that had a part in this research project and that made an impact in my research career.

Appendices

3D blocking autoradiography was performed to demonstrate the anatomical and functional biodistribution of [^{111}In] In-DANBIRT in wild type mice, blocked (pre-administered with 1,000X IV dose of cold DANBIRT) and unblocked C57BL6 male mice (n=3 per group) 24 hours post injection of 100μCi of [^{111}In] In-DANBIRT. Image processing of autoradiography images was performed to allow and analysis by region of interest (ROI) determination in relationship to concentration per volume; values were decay corrected and normalized to muscle uptake; percentage injected dose per gram of tissue (%ID/gr) was calculated from ROI quantification.
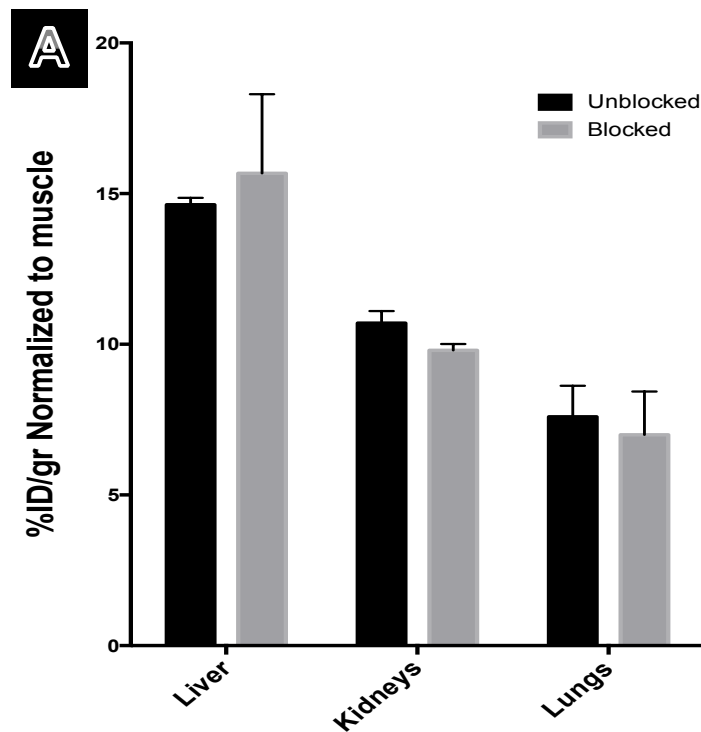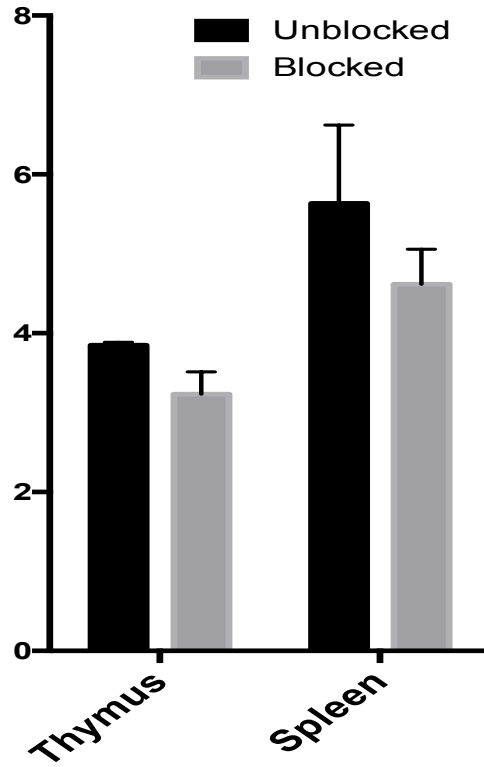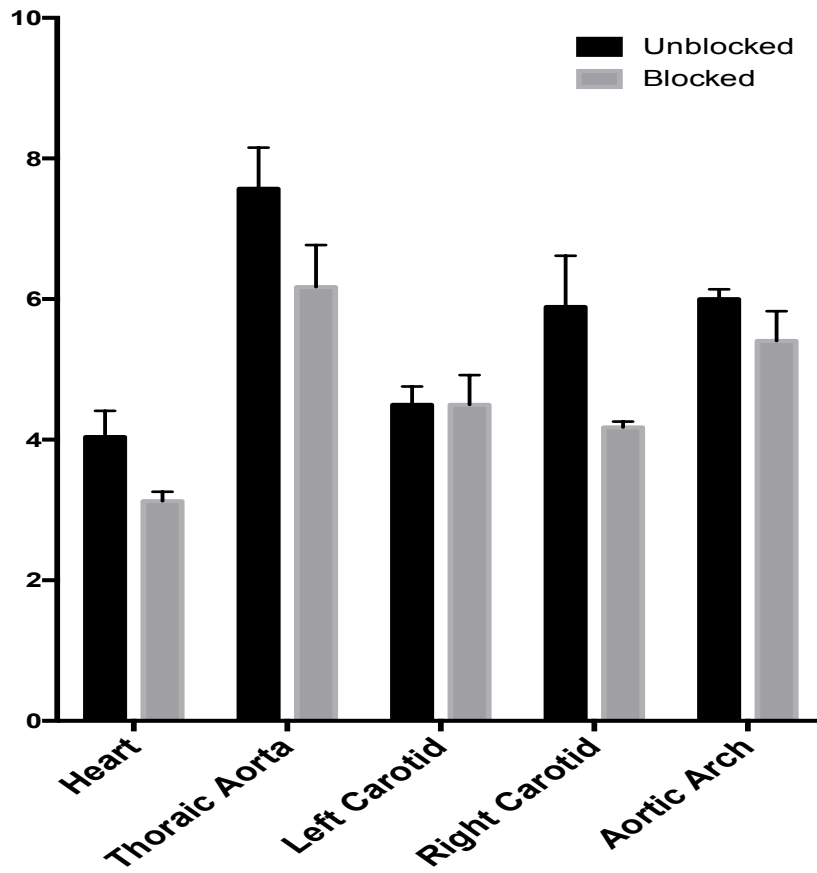
There were no significant differences in the lungs or liver uptake between groups with a slight increased uptake in the unblocked kidneys (A), correlating to longitudinal molecular imaging and biodistribution results from past studies with [^{111}In] In-DANBIRT. High uptake in unblocked immune-competent lymphoid (thymus, spleen) (B) and cardiovascular tissues (heart, thoracic aorta, right carotid, aortic arch) (C), directly illustrate high specificity in LFA-1 expressing resident and circulating leukocytes, targeted in vivo using [^{111}In] In-DANBIRT.

3D blocking autoradiography showed the functional and anatomical biodistribution of [^{111}In] In-DANBIRT in cardiovascular (heart, aortic arch, descending aorta, carotids) and immune tissues in unblocked mice indicating specificity for LFA-1 expression in target tissues.

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