



Ultrasound Molecular Imaging: Principles and Applications in Cardiovascular Medicine

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Abstract

Purpose of Review Non-invasive molecular imaging is currently used as a research technique to better understand disease pathophysiology. There are also many potential clinical applications where molecular imaging may provide unique information that allows either earlier or more definitive diagnosis, or can guide precision medicine-based decisions on therapy. Contrast-enhanced ultrasound (CEU) with targeted microbubble contrast agents is one such technique that has been developed that has the unique properties of providing rapid information and revealing information only on events that occur within the vascular space. **Recent Findings** CEU molecular probes have been developed for a wide variety of disease states including atherosclerosis, vascular inflammation, thrombosis, tumor neovascularization, and ischemic injury. While the technique has not yet been adapted to clinical use, it has been used to reveal pathological processes, to identify new therapeutic targets, and to test the efficacy of novel treatments. **Summary** This review will explore the physical basis for CEU molecular imaging, its strengths and limitations compared to other molecular imaging modalities, and the pre-clinical translational research experience.

Keywords Contrast-enhanced ultrasound · Molecular imaging · Targeted ultrasound · Microbubbles

Introduction

Over the past decade, there has been rapid development in molecular imaging techniques that are able to non-invasively assess molecular or cellular phenotype in animal models of disease and in humans. These techniques are advantageous in that they provide both spatial and temporal information on complex disease processes at the molecular level, and can be

combined with traditional measurements provided by imaging of structure, function, and flow. Molecular imaging techniques can be applied in research settings to study cardiovascular disease pathophysiology and test novel therapies, as well as in clinical settings to detect pathological processes, risk-stratify patients, and guide precision treatment (Fig. 1) [1].

Methods for molecular imaging are diverse and span all forms of medical imaging including radionuclide, optical, magnetic resonance imaging (MRI), and ultrasound [2]. The general approaches to imaging a molecular profile in situ are diverse (Table 1) and rely primarily on the detector capabilities [1, 3]. One of the most common approaches is to use contrast agents that are functionalized to reveal a targeted molecular process. Some simple examples of this are the radiolabeling of antibodies or antibody fragments for radionuclide imaging of a disease process [4], or taking advantage of a metabolic process that uses a particular substrate such as the positron emission tomography (PET) imaging of the consumption rate of ¹⁸F-fluorodeoxyglucose (FDG), which enters the glycolysis pathway and becomes trapped in cells at a rate proportional to cellular glucose uptake [5]. However, given the expanding range of options in both probes and detectors, it is essential to carefully consider many of the factors that govern whether a technique is well suited to a specific clinical or research application.

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Clinical Applications

Early diagnosis of disease
More definitive diagnosis of disease
Selection of most appropriate therapy
Monitor disease progression
Assess response to therapy

Response to new therapy
Optimizing therapeutic strategies
Off-target effects of new therapies

Define pathophysiology
Assess temporal patterns of expression
Assess spatial patterns of expression
Dose response for new therapies
Use as surrogate endpoint

Research Applications

Fig. 1 Schematic illustrating some of the major clinical and investigational roles of molecular imaging in medicine and science

Contrast-enhanced ultrasound (CEU) molecular imaging is a technique that relies on the ultrasound detection of encapsulated gas microbubbles (MBs) or other acoustically active micro- or nanoparticles that are retained in tissue on the basis of their ability to bind to molecules or cells of interest. CEU has several unique characteristics that differentiate it from other molecular imaging techniques. It is able to rapidly (within 5–10 min) obtain non-invasive measures of disease and can be done with portable and relatively inexpensive imaging technology, meaning that bedside molecular imaging is possible. From a research perspective, the rapid imaging protocols and the ability to “null” the signal from the agent provide an opportunity to examine multiple molecular processes in a short period of time. As a purely intravascular probe [6], MBs are an ideal tool for non-invasive interrogation of the endothelial-blood pool interface, but they are poorly suited for examining either extravascular or intracellular events. This review will

focus on some of the principles underlying CEU molecular imaging and will highlight recent advancements in the application of targeted CEU to cardiovascular disease.

Principles of CEU Molecular Imaging

Contrast Agent Properties

CEU imaging relies on receiving signal generated by encapsulated gas MBs when exposed to ultrasound [7]. In the pressure fluctuations of an ultrasound field, MBs undergo volumetric oscillation. Steady expansion and contraction (stable cavitation) occurs at low pressure which can be non-linear with regard to the relationship between pressure and volume [8, 9]. Destruction of microbubbles can occur from exaggerated oscillation at high pressures, known as inertial cavitation [10]. Both inertial cavitation and non-linear stable cavitation result in emission of broad band ultrasound signals and ultrasound energy peaks at harmonic frequencies, thereby producing a unique acoustic signature that can be detected and isolated from background tissue signal [11–13]. Conventional MB agents used in humans are between 1 and 5 μm in diameter, have a core composed of a high-molecular weight inert gas such as perfluorocarbons or sulfur hexafluoride, and have a shell composed of lipids or proteins such as albumin. A wide variety of other acoustically active experimental ultrasound contrast agents has been explored, but are out of the scope of this review [14]. MB agents are smaller than erythrocytes and have a microvascular rheology similar to that of erythrocytes [6, 15]. Accordingly, they normally pass unimpeded through capillaries, which is important for both safety considerations and their use as flow tracers. Lipid MBs also often contain surface “coats” composed of biocompatible polymers such as polyethylene glycol (PEG), which prevent their interaction with cells and plasma proteins [16–18].

After intravenous injection, MBs remain in the vascular space, thereby opacifying the blood pool on ultrasound imaging. In clinical cardiovascular medicine, CEU is most often used to opacify the left ventricular (LV) cavity on echocardiography in order to better evaluate global or regional function,

Table 1 General approaches for in vivo molecular imaging

Strategy	Contrast	Modality
Tracer retention by ligand-receptor binding	Y	RN, US, CT, MR, OI
Cellular retention from metabolic machinery	Y	RN, US, MR, OI
Tracer activation by targeted enzymatic process	Y	OI
Endogenous signal characteristics	N	MR

N, No; *Y*, Yes; *CT*, computed tomography; *MR*, magnetic resonance imaging; *OI*, optical imaging; *RN*, radionuclide imaging; *US*, ultrasound

LV dimensions, and the presence of intracavitary masses such as thrombi [19]. Myocardial contrast echocardiography (MCE) for evaluation of regional myocardial perfusion can also be performed with conventional MB agents, during which blood flow is quantified by measuring the rate and extent of replenishment of MBs within the coronary circulation after a destructive ultrasound pulse [20, 21].

Molecular imaging with CEU is most commonly performed by imaging targeted MBs that have been retained by attachment to specific molecular processes within the vascular compartment. The retention of MBs can occur by two distinct approaches. Non-specific retention of MBs, by their binding to endothelial cells or to adherent and activated leukocytes, occurs through the endogenous ability of these cells to bind to MB shell constituents. There are many pathways involved in this binding, but the process that has been best defined is opsonization, whereby serum complement proteins bind to the MB surface and are then recognized by complement receptors on leukocytes or on endothelial cells [22, 23]. Choice of shell composition, charge, and the presence of PEG all play key roles in determining the degree of MB attachment to leukocytes and endothelial cells, as well as the rate of their removal from the reticuloendothelial system [17, 24, 25]. These non-specific interactions with MBs can be leveraged for detecting pathological processes that heighten opsonization or complement-mediated attachment, as is the case with MBs with phosphatidylserine shells, which are known to preferentially bind in regions of ischemic myocardium [26].

An alternate and more specific strategy for targeting of MBs occurs via direct binding of a targeting ligand on the MB surface, often an antibody, peptide, or glycoprotein [27, 28]. Targeting ligands are attached to the MB surface via a variety of approaches, including biotin-streptavidin and direct covalent links. Conjugation is often at the end of the flexible PEG tethers in order to improve stoichiometry and reduce necessary bond force for retention [29]. In general, in excess of 25,000 ligands can be conjugated to each “multivalent” MB [30], and it is even possible to place several different ligands on each MB which may enhance their attachment efficiency [31].

Specificity of an MB probe for a target molecule is determined by factors such as off-target binding, specificity of the targeting ligand to the molecule of interest, bond kinetics, surface density of the targeting ligand, orientation of targeting ligands, and other factors shown in Fig. 2 [32]. Even with a highly specific probe, it is important to consider target molecule characteristics such as regional and temporal expression patterns, relevance of the target molecule to the disease pathway of interest, and bond kinetics under varying shear conditions.

In Vivo Protocol for Targeted CEU

There are several approaches used to detect targeted MB retention in tissue with ultrasound. Since circulating MBs are

rapidly cleared by the reticuloendothelial system, it is possible to simply inject MBs as a venous bolus injection, then measure signal intensity for tracer retained in tissue 5–10 min later, after the majority of freely circulating MBs have been cleared from the blood pool. The efficacy of this approach is further enhanced by protocols to eliminate the signal attributable to any remaining freely circulating MBs [27]. An alternative and somewhat more complex method is to use transfer kinetics to evaluate the retention fraction (Fig. 3). With this method, video intensity is measured constantly after an intravenous injection which allows deconvolution of the two curves: one that represents freely circulating tracer which rises and decays and the one curve representing tracer that is retained which rises and plateaus [33]. This latter method is particularly helpful in research settings where tissue perfusion, which influences the number of microbubbles entering into tissue, changes substantially over time or between conditions.

Strengths and Limitations of CEU Molecular Imaging

Whether using molecular imaging as a research tool or for a clinical application, the selection of the most appropriate modality for any given application requires one to consider the relative advantages and disadvantages for each approach. For molecular imaging approaches that use targeted contrast agents, one important consideration is the biodistribution of the targeted probe relative to the target pathway. A limitation of CEU molecular imaging is that the imaging probes are restricted to the vascular compartment. Hence, CEU is not an ideal method for finding abnormal cell types within atherosclerotic plaque or for detecting abnormal matrix composition in certain myocardial diseases. On the other hand, in conditions where it is desirable to limit the evaluation to endothelial or intraluminal phenotype (e.g., transplant vasculopathy, thrombosis), techniques that employ purely intravascular probes may offer advantage in terms of specificity. There are other key differences between small molecule contrast agents and larger multivalent particle-based agents. The former generally bind in a 1:1 ratio to a target receptor or are retained by virtue of a metabolic process and are well suited to precisely quantifying the degree of expression or enzymatic activity [34]. For the latter, many bond formations have to occur for retention, resulting in a threshold-effect with regard to target molecular expression before attachment is seen, and a greater influence of vascular shear [35]. However, particle-based agents provide a form of biomimicry where their behavior can provide a readout of the molecular environment that influences cellular or platelet adhesion.

There are also practical considerations in terms of detector performance. In general, techniques such as MRI and CT are considered to have the highest spatial resolution, while

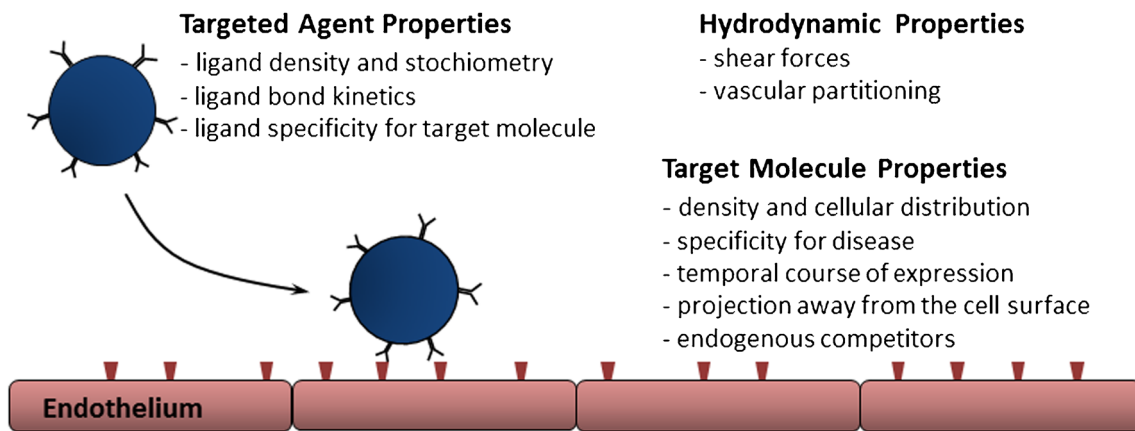


Fig. 2 Major determinants of targeted microbubble retention in areas of disease. Factors that influence microbubble retention are separated into contrast agent variables, target molecular variables, and hydrodynamic properties

radionuclide imaging (PET, SPECT) tends to have the highest sensitivity and dynamic range for detecting tracer. Accordingly, one must consider whether it is more important to spatially localize an event or to be able to detect very low levels of abnormal molecular expression. In general, CEU molecular imaging offers a balance in that it provides moderate spatial resolution and sensitivity, and is suitable for applications where a balance of both traits is needed. If it is necessary to co-localize regional molecular imaging signal with anatomical features, CEU has the advantage of near-simultaneous structural and molecular imaging, while MR and hybrid PET-CT or SPECT-CT have the advantage of providing more detailed views of smaller anatomical features.

Temporal resolution is also essential to consider when selecting the most appropriate molecular imaging modality. CEU molecular imaging can be performed with rapid

acquisition times of less than 10 min, while commonly used PET/SPECT and MR protocols require anywhere from just under an hour to a day between contrast injection and imaging [36–38]. Additionally, rapid clearance of MBs from the blood pool allows multiple different targeted contrast agents to be administered sequentially.

There are certain practical issues other than speed that are important when considering different molecular imaging modalities, particularly if their use is intended as an approach for disease screening in large populations or for rapid detection in common diseases (e.g., myocardial ischemia, atherosclerosis). Safety is one key issue. CEU contrast agents have an excellent safety profile, with serious pseudoanaphylactic reactions occurring in only 1 in 10,000 for conventional contrast agents [39–42]. CEU molecular imaging also does not require ionizing radiation. Other advantages include ease of use, portability of ultrasound which allows for bedside diagnosis, and low cost for both equipment and production of contrast agents.

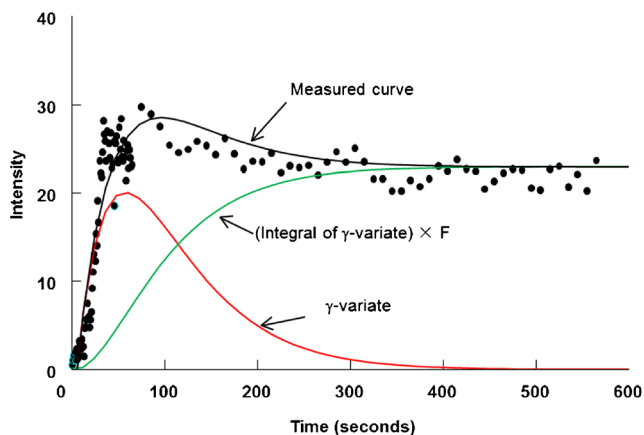


Fig. 3 Kinetic model for calculating retention fraction of microbubbles. Continuous measurement of contrast agent in a tissue is measured. Persistent elevation of signal represents agent that remains in tissue rather than is cleared from blood pool. The measured curve can then be deconvolved into two separate curves: (1) a transit curve for the population that transits freely through the tissue without retention and is cleared from blood pool after about 300–400 s (fit in this case to a γ -variate function) and (2) the fraction (F) of agent that is retained which can be fit to an integral of the transit function (γ -variate)

Applications of Myocardial CEU Molecular Imaging

Ischemic Memory Imaging

In patients with acute coronary syndrome (ACS), the clinical diagnosis generally relies on clinical history, laboratory evaluation, and electrocardiogram (ECG). Unfortunately, many patients do not have classic angina symptoms [43], and many of those with ACS do not have diagnostic changes on the ECG [44]. Moreover, in those with unstable angina, ECG changes and even wall motion abnormalities detectable on point-of-care echocardiography can resolve before the initial evaluation by a health care provider. While high-sensitivity troponins have a high sensitivity for detecting non-ST-elevation MI [45], their performance in unstable angina is less certain, and they often lack specificity in certain populations [46]. Methods for rapidly evaluating both the

presence and the spatial extent of recently resolved myocardial ischemia have a high likelihood of improving the quality and efficiency of clinical care in those presenting with symptoms suspicious for ACS.

CEU molecular imaging is capable of assessing ongoing or recent, but resolved, myocardial ischemia at the patient bedside and provides a spatial readout of the affected area. Ischemic memory imaging has been achieved both with MBs bearing targeting ligands for the endothelial selectin family of adhesion molecules and with phosphatidylserine-containing MBs (PS-MB) (Fig. 4) [26•, 47, 48]. Targeting of selectins on the endothelial surface, which has been validated in murine and non-human primate models of myocardial infarction [48, 49], is based on early and late endothelial response to ischemia. In the first minutes following ischemia, P-selectin stored in endothelial Weibel-Palade bodies is mobilized to the cell surface where it can remain for many hours, whereas E-selectin expression requires approximately 1 h to be expressed due to its dependence on transcriptional upregulation [50]. Agents targeted to selectins are thereby retained in the microcirculation of the post-ischemic risk area, even in the absence of any significant necrosis, for hours after resolution of hypoperfusion. Alternatively, ischemic memory imaging can be performed without a targeting ligand using PS-MBs, as validated in murine and canine models of resolved myocardial ischemia [26•]. The selective retention of phosphatidylserine MBs in areas of recent ischemia is mediated in part through opsonization and binding to complement

receptors. The simplicity of this approach will likely accelerate its clinical translation.

Detection of Transplant Rejection and Myocarditis

Orthotopic heart transplantation is an important therapeutic option for eligible candidates with severely symptomatic heart failure. However, there have been a relatively flat number of allografts available for transplant in the USA each year. Approximately one-quarter of all allografts have evidence for rejection in the first year [51]. This figure together with the problem of sampling error for detecting rejection by endomyocardial biopsy has resulted in a need for better surveillance. Molecular imaging for this application would require a technique that ideally could be done rapidly and without repetitive exposure to ionizing radiation. The ability to detect transplant rejection has been demonstrated in a rat model using MBs targeted to ICAM-1 [52]. This study was predicated on the notion that rejection involved not only a T lymphocytic response to myocytes but also to the allograft endothelial cells resulting in endothelial activation and adhesion molecule expression. More recently, CEU molecular imaging has been used to directly assess T lymphocytes in a model of orthotopic heart transplantation [53•]. This study relied on MBs bearing a CD3-targeted antibody that allowed them to assess lymphocytes in the process of intravascular recruitment.

The pathophysiology of myocarditis involves many common pathways with heart transplant rejection, and the most important of which involves recruitment of cells involved in

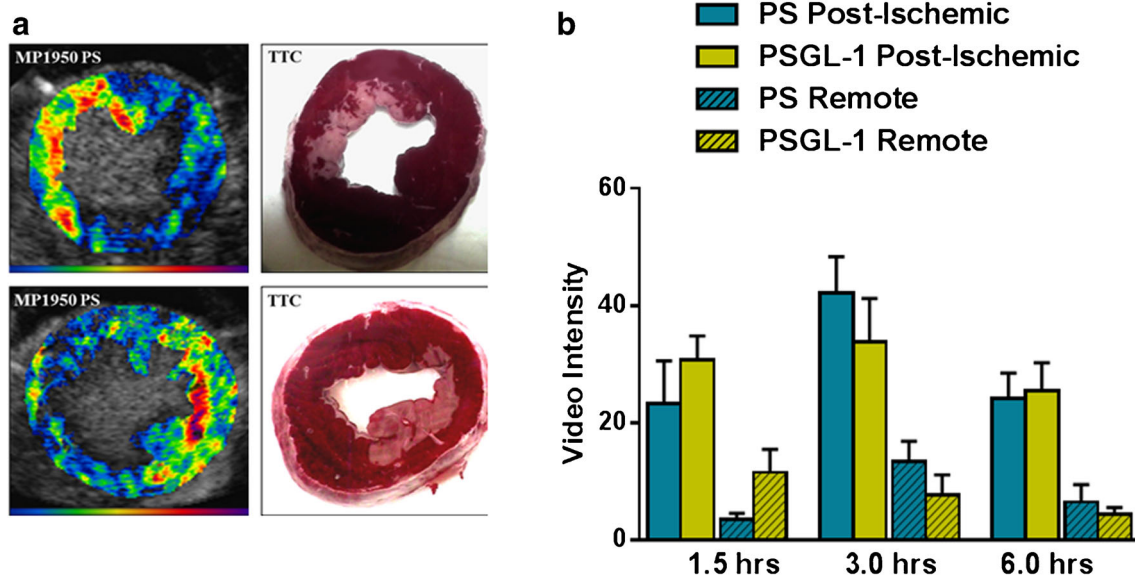


Fig. 4 Molecular imaging of ischemic memory. **a** Examples of CEU molecular imaging with MB-PS (left panels) and triphenyltetrazolium chloride (TTC) staining (right panels) in a canine model of ischemia reperfusion of the LAD (top panels) or left circumflex coronary artery (bottom panels). Images demonstrate contrast enhancement that encompasses the entire risk area which extends beyond the region of infarction. The color-coded scale for CEU is provided at the bottom (modified from Christiansen JP et al. *Circulation* 2002

Apr 16;105(15):1764–7) [75]. **b** Graph illustrating signal enhancement on CEU molecular imaging from a murine model of brief ischemia-reperfusion injury without infarction. Signal enhancement in the post-ischemic risk area was greater than in the remote area and was similar for MB-PS and P-selectin-targeted MBs and persisted for > 6 h after ischemic injury. PS phosphatidylserine, PSGL-1 p-selectin glycoprotein ligand-1 (modified from Mott B, et al. *Cardiovasc Imaging*. 2016;9(8):937–46, with permission from Elsevier) [26•]

both adaptive and innate immunity. Accordingly, CEU with MBs targeted to selectins, CD4, and phagocytic cells have been used in a rodent model of myocarditis and shown to detect not only severe fulminant disease but also more moderate involvement as well [54•]. Because of the rapid and quantitative nature of CEU, and its ability to provide information immediately to the clinician, there is hope that it may provide unique diagnostic opportunities for all of these forms of myocardial inflammation in the future.

Atherosclerosis

Atherosclerosis is a process that develops over decades and involves vascular inflammation, including expression of endothelial cell adhesion molecules (ECAMs) and secondary recruitment of leukocytes and platelets [55, 56]. Often, atherosclerosis is clinically silent for decades of plaque progression. Although biomarkers and other techniques have been developed for risk-stratifying patients [57, 58], many patients with severe atherosclerotic disease will not have major risk factors and many will experience MI as their first manifestation of disease [59]. Often, patients are diagnosed only once disease progression has reached the point of critical stenosis, which results in angina [60]. The ability to non-invasively detect the early molecular signatures of aggressive atherosclerosis may provide opportunity for early stratification to therapies that can arrest disease development.

CEU is just one of many molecular imaging techniques that have been used to detect atherosclerotic plaque growth in the early stages of disease. CEU probes targeted against ECAMs including selectins, ICAM-1, and VCAM-1 have been shown to detect early and late stage atherosclerosis in rodent models

of atherosclerosis [61–65]. In non-human primates on a Western diet, CEU molecular imaging of VCAM-1 and P-selectin has been shown to detect the very earliest stages of endothelial activation prior to any changes in intima media thickness (Fig. 5) [66]. Recently, CEU molecular imaging of endothelial phenotype has provided *in vivo* evidence for the role of platelet-endothelial interactions in early disease progression [67, 68]. This process involves abnormal regulation of endothelial-associated ultra-large multimers of VWF that mediate platelet attachment through ligation of platelet GPIIb α and increase atherosclerotic progression [56]. While all of these probes have been developed for early clinical detection of disease, they have also played an important role in identifying new therapeutic targets and for assessing the effects of either new or established therapies [63, 69, 70•].

Thrombus Detection

There are many potential applications for a technique that can detect the presence of thrombus in cardiovascular disease. Some of the most common include need to diagnosis acute venous or peripheral arterial thrombus, detection of high-risk carotid or coronary arterial plaque, detection of left atrial or left ventricular thrombus, and detection of microvascular thrombosis in post-MI no-reflow phenomenon. Molecular imaging of thrombus has been performed with a variety of targeted ultrasound contrast agents [71]. The targeting moiety employed depends on the intended role of molecular imaging. MBs have been targeted by surface conjugation of ligands that recognize the platelet GPIIb/IIIa receptor, fibrin, or tissue factor [28, 72–74]. As mentioned above, MBs have also been targeted to platelet GPIIb α and VWF using peptide ligands for each. The relative value of each

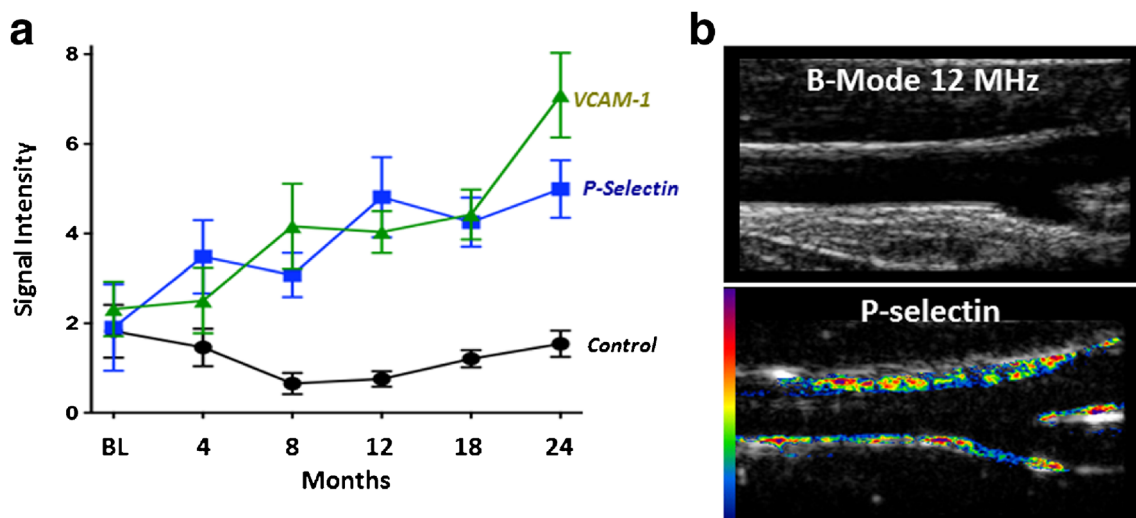


Fig. 5 Molecular imaging data from the carotid artery of rhesus macaques after starting a Western-style high-fat diet. **a** CEU molecular imaging signal for control non-targeted MBs and MBs targeted to VCAM-1 or P-selectin from the carotid arteries at baseline (BL) and every 4 months after starting Western. **b** Non-contrast 2-D image of the

carotid artery at the bifurcation (top) and a background-subtracted color-coded image obtained after injecting P-selectin-targeted MBs demonstrating enhancement of the vascular endothelium (bottom) (modified from Chadderdon S et al., *Circulation* 2014;129(4):471–8) [66]

of these agents depends on (1) whether the thrombotic process of interest is platelet-rich or fibrin-rich; (2) the shear forces in the chamber/vessel involved; and (3) whether the process involves disruption of the endothelial barrier or not. An additional consideration is whether or not MBs need to outcompete endogenous ligands for the intended target. For example, MBs need to outcompete fibrinogen for GPIIb/IIIa, but little competition exists for GPIb α .

Conclusions

CEU molecular imaging is a versatile technique that has many potential applications in clinical cardiovascular medicine, and is already being used as a valuable research tool to understand cellular biology in disease. The technique is well suited to evaluating pathophysiologic events that occur at the interface between the blood pool and the vessel wall. Accordingly, the most common cardiovascular applications have involved imaging of endothelial activation, endothelial adhesion molecule expression, thrombus formation, and interactions between leukocytes or platelets and the vascular endothelium. However, clinical adoption will rely on both the development and testing of human-ready targeted contrast agents, and demonstration that CEU molecular imaging provides incremental value to conventional paradigms of care.

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Compliance with Ethical Standards

Conflict of Interest Eran Brown grants from American Heart Association (Grant: 18PRE33960532).

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