

Biomarkers of atherosclerosis and the potential of MRI for the diagnosis of vulnerable plaque

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Abstract Atherosclerosis is a chronic inflammatory vascular disease. As it is an inflammation process, many cellular and molecular events are involved at each step of the progression of atherosclerosis from an early fatty streak lesion to a highly dangerous rupture-prone plaque. Magnetic resonance imaging (MRI) is a well-established diagnostic tool for many kinds of chronic inflammation in various systems and organs, and recent improvements in spatial resolution and contrast strategies make it a promising technique for the characterization of inflammatory vessel walls. The first part of this review will briefly introduce the main cellular and molecular processes involved in atherosclerotic lesions; the second part will focus on the use of high-resolution MRI and present-generation contrast agents for plaque characterization; and the third part will present some recent and ongoing cellular and molecular MRI studies of atherosclerosis.

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Abbreviations

CAM	Cell adhesion molecule (ICAM, VCAM, Selectins)
CD44	Lymphocyte integrin, interaction with extracellular matrix (hyaluronan)
CR3 or 4	Complement receptors for fragments 3 and 4 expressed on monocytes
CRP	C-reactive protein
GP Ib-IX-V	Glycoprotein complex (=GP Ib α , GP Ib β , GP IX and GP V), P selectin ligand
ICAM	Intercellular adhesion molecule, LFA-1 ligand
IFN- α	Interferon-alpha
IL	Interleukin
LFA-1	Lymphocyte function-associated antigen-1 (immunoglobulin)
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
oxLDL	Oxidized low-density lipoprotein
PCAM	Platelet/endothelial cell adhesion molecule
ROS	Reactive oxygen species
sCD40L	Soluble CD40 ligand
TNF- α	Tumor necrosis factor-alpha
VCAM	Vascular cell adhesion molecule, VLA-4 ligand
VLA-4	Very late antigen-4, integrin

Introduction

Magnetic resonance imaging (MRI) has continuously developed, enhancing image quality and thereby providing more information on the physiopathological status of biological

tissue. The unique advantages of MR for tissue characterization lie in its inherent contrast flexibility, combining different sequences and various contrast media. For atherosclerosis, recent advances in coil and acquisition chain technologies in current clinical scanners have increased spatial resolution, enabling carotid plaque to be imaged with a high signal-to-noise ratio in patients. Carotid plaque is characterized by MRI using multicontrast acquisition with or without nonspecific contrast agent injection. However, for the diagnosis of vulnerable plaque, new contrast agents dedicated to molecular imaging and *in vivo* biological targeting are required. The question that remains unresolved is the choice of biological target to achieve this goal.

Histopathologically, atherosclerosis is characterized by a progressive accumulation of lipids, cells (smooth-muscle cells, macrophages, and T-lymphocytes) and extracellular matrix in the arterial wall. The progression of atherosclerosis from an initial fatty streak to the complex advanced lesion has been described using Stary's six-stage histological classification [1]. It is now established that molecular and cellular inflammatory processes are involved at each step of the pathology [2–4]. The initial events comprise disrupted endothelial homeostasis, characterized by four elements: adhesiveness, permeability, proliferation, and thrombogenesis. The initial steps of inflammation are thus clearly described at the interface between blood and vessel wall. The multiple molecular and cellular events explaining subsequent progression are often viewed from the inside of the vessel wall, except for the late event when a clear rupture happens at the wall/blood interface, accompanied by thrombus formation. As these cellular and molecular processes are also found in numerous other human disorders [5–8], any new diagnostic technology for atherosclerosis would also have a wider impact for the molecular imaging of chronic inflammatory diseases. The increasing number of original articles and reviews demonstrates that atherosclerosis and inflammation are important new challenges for MRI. In the present review, after a short description of the essential biological events, we will focus on the potential diagnostic contribution of MRI and MRI markers at each stage of the atherosclerotic disease and for detecting a vulnerable lesion ahead of rupture.

Atherosclerosis: the molecular and cellular processes involved in an inflammatory disease

Blood-side events

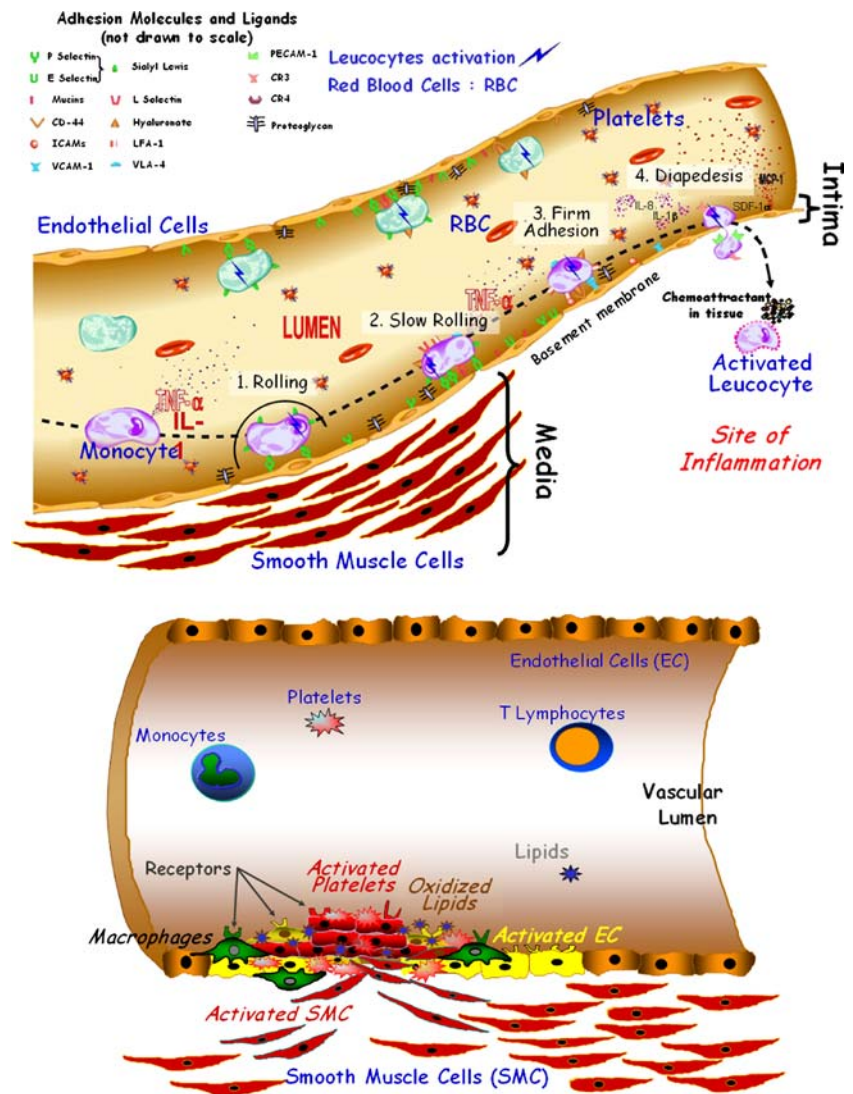
Activation of vascular endothelial cells and circulating blood cells together with an increased plasma concentration of inflammatory mediators is characteristic of ongoing inflammatory processes during the progression of atherosclerosis. Some circulating markers of inflammation [e.g., C-reactive

protein (CRP), cell adhesion molecules (CAM), monocyte chemoattractant protein (MCP-1), tumor necrosis factor- α (TNF α), interleukins (IL), soluble CD40 ligand (sCD40L), or von Willebrand factor] are currently undergoing clinical trials in patients suffering from cardiovascular diseases [5–14].

Upon activation, circulating white blood cells express adhesion glycoproteins at their surface (Fig. 1). Activated platelets also play a key role as a link between inflammation, thrombosis, and atherogenesis [15, 16]. Like leukocytes, platelets can roll on the surface of inflamed endothelium. This process is mediated by endothelial P-selectin and a counter-receptor for P-selectin [17–19]. This glycoprotein is part of the GPIb-IX-V complex, which mediates platelet adhesion to exposed subendothelial von Willebrand factor at injury sites [20]. Its involvement in platelet adhesion to both activated endothelium and exposed subendothelium makes GPIb α , one of the four polypeptides of the complex, an attractive model for the design of mimetic compounds [21]. Activated endothelial cells express CAMs, which favor the recruitment of leukocytes into the arterial wall [22]. The main receptors expressed in the vascular components (Fig. 1) are divided into five groups [23]:

1. **Integrins:** integrins are heterodimers of $\alpha\alpha$ subunits and $\alpha\beta$ subunits. The integrin family has subfamilies, designated as $\beta 1$ through $\beta 8$. The most widely studied subfamilies are $\beta 1$ [CD29 or very late activation (VLA) members], $\beta 2$ (leukocyte integrins such as CD11a/CD18, CD11b/CD18, CD11c/CD18, and $\alpha\text{d}\beta 2$), $\beta 3$ (CD61 and CD51/CD61 or $\alpha\text{v}\beta 3$ integrin or vitronectin receptor), and $\beta 7$ ($\alpha 4\beta 7$ and $\alpha\text{E}\beta 7$). A particular integrin may recognize several proteins.
2. **Immunoglobulins:** the immunoglobulin superfamily includes leukocyte function antigen-2 (LFA-2 or CD2), leukocyte function antigen-3 (LFA-3 or CD58), intercellular adhesion molecules (ICAMs), vascular adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), and mucosal addressing cell adhesion molecule-1 (MAdCAM-1). The binding sites are different for each of these cell-adhesion molecules.
3. **Cadherins:** cadherins are major cell–cell adhesion molecules. The most extensively studied are N-cadherin, E-cadherin, P-cadherin, and NgCAM. One unique feature of the cadherin family is homophilic binding, i.e., cadherins bind identical cadherins on other cells.
4. **Selectins:** the selectin family (CD62) includes E-selectin, P-selectin, and L-selectin. Selectins are well-known examples of proteins interacting with saccharide structures. Selectins are type-1 glycoproteins that share common structural determinants. P-selectin (CD62P, PADGEM, GMP-140) and E-selectin (CD62E, ELAM-1)

Fig. 1 Adhesion molecules and ligands involved in the initial phases of vascular inflammation (a), and cell responses in the vessel wall during inflammation and plaque formation (b). The initial inflammation cascade is shown with monocyte activation and secretion of $\text{TNF-}\alpha$ and IL-1 , followed by pathways involving monocytes, T-lymphocytes, endothelial cells, and platelets (a). Endothelial cells are then activated and favor adherence of activated platelets and leukocytes (b). Smooth muscle cells are activated and changed their phenotype. Plaque formation is also characterized by the presence of oxidized lipids, monocyte migration, and homing of macrophages, with the final interrelationship between inflammation and thrombosis (b)



are inducible receptors expressed on activated endothelial cells and/or platelets and binding to glycoconjugates on most leukocytes [24]. L-selectin (CD62L, previously known as MEL-14 antigen or LECAM-1) is constitutively expressed on circulating lymphocytes, neutrophils, monocytes, and platelets, and recognizes specific ligands on endothelial cells.

- 5. Proteoglycans:** the proteoglycans encompass a large group of core proteins with attached sulfated glycosaminoglycans (GAG). The specificity of their interactions with vascular components is related to the nature and distribution of the GAG chains [25].

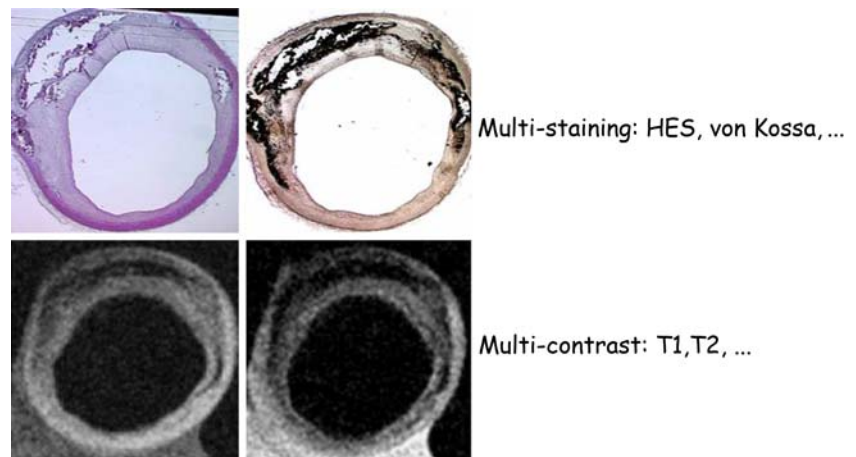
Thus, arterial wall dysfunction is modulated by interaction between blood elements (circulating leukocytes, platelets, and microparticles derived from platelets or damaged endothelial cells), circulating or local bioactive molecules, cellular and molecular arterial wall components, and the

lesion's microenvironment (blood flow, wall shear stress, and thrombogenic elements). The goal is then to identify lesions at risk of acute event, i.e., rupture and thrombosis.

Inside the wall inflammation cascade

Precursor signs of atherogenesis are early phenotypic modulations of intimal smooth-muscle cells and cellular activation of the vessel wall by oxidized lipids (oxLDL) and reactive oxygen species (ROS) (Fig. 1). Endothelial cells and macrophages are mainly involved in these early processes (Fig. 1). For later evolution, pathological examination has demonstrated that plaques with thin ulceration-prone fibrous caps with inflammatory characteristics are responsible for fatal acute thrombosis. This finding has led to the concept of *vulnerable* lesions, independently of stenosis degree and plaque volume [2, 26–28].

Fig. 2 Establishing a new standard for plaque characterization, from histology to MRI (ex vivo MRI of a calcified lesion in a Watanabe rabbit). *Top row* standard Hematoxylin, Eosin and Saffron (HES) histology and Von Kossa staining for calcification. *Bottom row* Ex vivo multicontrast MR images (T1 and T2 spin echo). No signal for calcified tissue is seen with either sequence (adapted from Chaabane et al. [116])



Macrophages and foam cells (lipid-laden macrophages originating from monocytes or from smooth-muscle cells) are seen as the central actors in the vicious circle of the establishment of atherosclerosis, but many other cells are also involved in the process. Cell recruitment from the blood stream is followed by migration of cells such as T cells and mast cells from the immune system. In the vessel wall, smooth-muscle cells are activated to a proliferating and secretory phenotype (Fig. 1). A cascade of signaling and processes such as activation, proliferation, angiogenesis, enzymatic reactions, and apoptosis causes evolution to move towards the formation of a complex plaque.

The following events are linked to atherosclerotic plaque evolution:

- activation of an inflammatory process;
- oxidative stress and production of reactive oxygen species (ROS);
- modified phenotypes (activation, proliferation);
- apoptosis;
- angiogenesis;
- thrombogenesis.

These processes involve many cellular and molecular agents. The frequently cited inflammatory mediators or markers (both circulating and tissue bound) are naturally considered as targets for diagnostic and therapeutic approaches [5, 6, 14, 26, 28–32].

The presence of key players in circulating blood, at the blood/tissue interface or inside the vessel wall is leading to a new concept of vulnerability. Vulnerable plaques are rupture-prone lesions with a prothrombotic microenvironment. A vulnerable patient is a patient at risk of an acute vascular event, presenting a combination of local and systemic risk factors. Clinical biomarkers and molecular imaging of key identified targets will help guide indications and treatment of vulnerable plaque [2, 5, 9, 14, 28].

MRI techniques for vessel wall imaging: the role of contrast agents in plaque characterization

For any imaging modality, the first step is to visualize the vessel wall in vivo. This is technically challenging as it requires both high spatial resolution and a high signal-to-noise ratio with a minimal acquisition time. Historically, attempts to characterize atherosclerotic plaque components were made ex vivo, to determine whether the contrast versatility of MRI was helpful. From these initial studies, it was found necessary to associate multiple sequences—i.e., T1, T2, proton density and diffusion-weighted sequences—to obtain useful information about plaque composition (Fig. 2).

MR characteristics of plaque components

Recent reviews have described plaque components examined by MRI in experimental and clinical studies. Lipid and necrotic components, fibrous tissue with a dense or loose matrix and extracellular lipids, fibrocellular tissue, calcification with cholesterol crystals, and fresh and old thrombi have been extensively explored [32–41]. Calcification is unequivocally characterized by absence of signal on MR sequences (Fig. 2), dense fibrosis by a high signal on T2, lipid-necrotic core by a low signal on T2 and a variable signal on T1, and fresh thrombus by a high signal on T1. Many studies are now focusing on characterizing the fibrous cap—thickness, presence of fissures or inflammation sites—as elements for the assessment of lesion vulnerability or instability. MR contrast agents are very useful here, to delineate the fibrous cap and ulcerations and enhance inflamed tissues [42–47].

In vivo high-resolution MRI techniques in clinical and experimental settings

In vivo techniques involve high-resolution sequences with the best compromise for temporal resolution. Until recently,

spin echo sequences were considered the most useful, despite their relatively long acquisition time.

In humans, most clinical plaque imaging studies have been performed on the carotids. Carotid imaging in patients involves four prerequisites:

- optimized coil for a small field of view (carotid coil);
- carefully adjusted coil and patient position, to ensure patient comfort;
- multicontrast high-resolution sequences in minimal acquisition time;
- electrocardiograph (ECG) synchronization and bright and black blood acquisitions, with swallowing and respiratory motion under control.

Imaging with 3 T or higher magnetic fields is beneficial in terms of increased signal-to-noise ratio [48], but limitations such as specific absorption rate (SAR) deposition and optimal coil design still need to be fully explored.

Experimentally, high resolution is even more crucial in animal models of atherosclerosis such as hypercholesterolemic rabbits or genetically modified mice. In these studies, the main focus of research is seldom plaque component identification but rather physiopathological exploration by molecular targeting and bioengineering, with MRI being used in a multimodal exploration context [49].

Role of clinically available contrast agents

Plaque permeability and neovascularization are explored with MRI via conventional nonspecific contrast agents [43,

46,47,50]. Either a compromise low-resolution sequence for dynamic acquisition or a single postcontrast time point with a spatial resolution T1 sequence can be used. Nonspecific contrast agents diffuse rapidly in the fibrous cap, enabling measurement of thickness and assessment of reactivity [42,44–46]. In MR acquisition using T1 agents, a sufficiently robust T1 sequence, meeting all the requirements of in vivo acquisition [black blood, ECG synchronization, stable T1 weighting, exclusion of respiratory artifacts, multislice two-(2D) or three-dimensional (3D) imaging], remains to be obtained [51–53]. The main studies using gadolinium chelates have dealt with vasa vasorum in the adventitia and angiogenesis. Kinetic models are then applied to evaluate parameters such as blood volume and permeability index [46,50]. The principal limitations of kinetic modeling are those encountered for tumoral angiogenesis measurement, i.e., interstitial diffusion of nonspecific contrast agents, and the nonlinear relationship between MR signal and contrast agent concentration. The additional difficulty for kinetic studies in the vessel wall is to combine strong and fast T1-weighted MR acquisition, high spatial resolution, a reference blood curve kinetic, and blood and fat signal suppression.

Two main issues are awaiting the arrival on the market of new blood pool agents [54]. First, blood pharmacokinetics is far more appropriate for the study of plaque permeability and neovascularization [55] (Fig. 3). Second, the capacity to shorten either tissue T1, T2 or T2* is extended by using macromolecular platforms or nanoparticles [28, 31,56–58]. The efficiency of a paramagnetic MR contrast agent is described by its relaxivities r_1 and r_2 , which are the constants of proportionality between contrast agent concen-

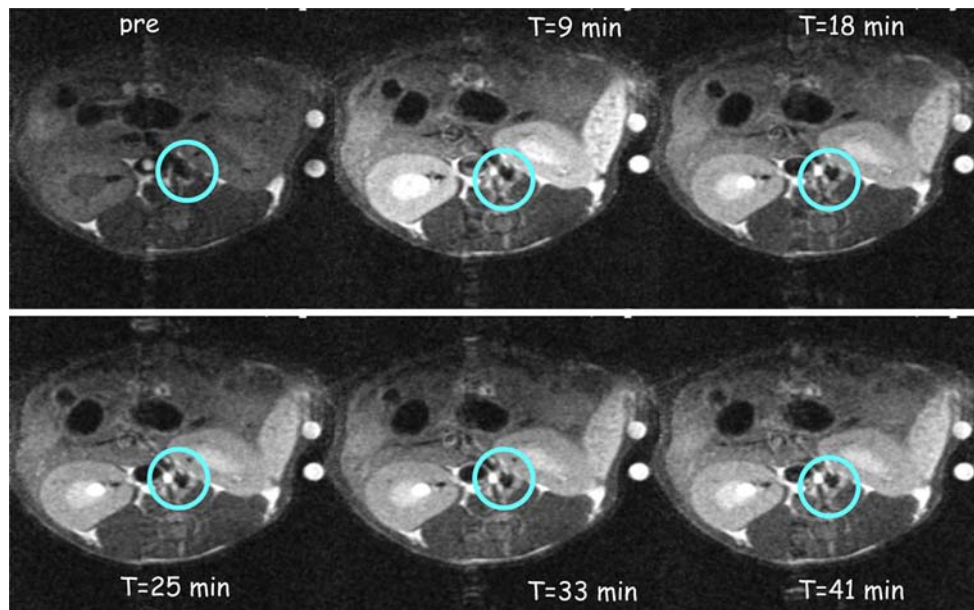
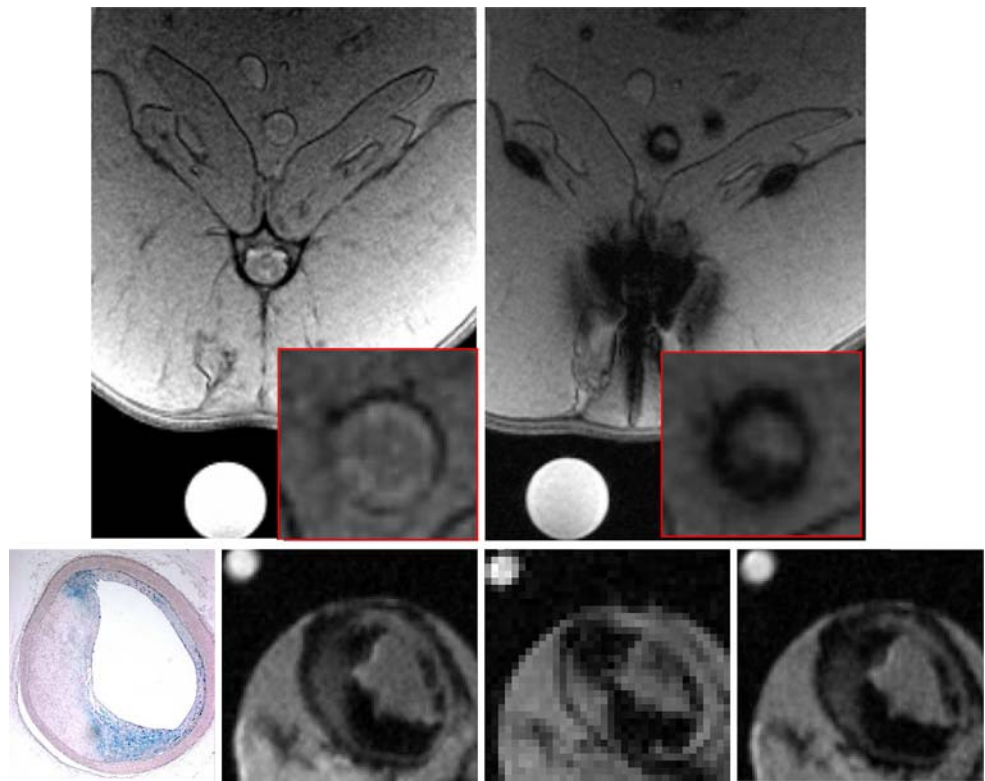


Fig. 3 Dynamic contrast enhancement in the abdominal ApoE^{-/-} mouse aorta with a macromolecular paramagnetic blood pool agent (from Chaabane et al. [55], with permission)

Fig. 4 Inflammation imaging with iron oxide particles: susceptibility-induced signal loss in a cholesterol-fed rabbit after denudation and balloon angioplasty of the infra-renal abdominal aorta (adapted from M. Sigovan et al., Magn Reson Med, submitted). *Top row* In vivo gradient echo imaging (pre- and post-contrast). *Bottom row* Histology with Perls staining shows iron in blue at the shoulder of the lesion. Ex vivo multicontrast images (from left to right proton density, gradient echo, and T2 spin echo) show signal loss at the same location



tration and change in longitudinal or transversal relaxation rate, expressed in $s^{-1} \text{mM}^{-1}$ per gadolinium or iron unit. With a nonspecific gadolinium contrast agent, r_1 efficiency is rather low ($3\text{--}5 \text{ s}^{-1} \text{mM}^{-1}$) at a 1.5 T clinical magnetic field. Moreover, commercial gadolinium complexes are composed of a single gadolinium entity, whereas blood pool agents may contain several tens of contrast ions. The ratio between r_2 and r_1 determines whether a contrast agent can be better used for contrast-enhanced T1-weighted or T2/T2*-weighted imaging. The gadolinium-based contrast agents (see example in Fig. 3) are generally employed as T1 agents, generating positive contrast (bright spot), as their r_2/r_1 ratio is low (typically between 1.1 and 2), whereas iron oxide particles are more often viewed as T2* or susceptibility agents with dark spots (Fig. 4).

Ultrasmall iron oxide particles (USPIO) have versatile behavior, depending on their coating and the size of the iron crystal. They are used either as T1 agents for MR angiography applications or as T2/T2* agents for cell-labeling purposes [31, 54, 59–62]. When injected into the blood stream, iron oxide particles encounter phagocytosis by cells of the reticulo-endothelial system. In atherosclerosis, small or ultras-small iron oxide particles (SPIO or USPIO) have been evaluated both experimentally [61–70] and clinically [71–75] as markers of activated monocytes/macrophages, using gradient echo sequences and the T2* effect. Intraplaque iron particle deposition is visualized as susceptibility-induced signal loss [64]. Contrast changes are related to macrophage iron

uptake inside the plaque. However, the intensity of contrast changes depends on many critical factors, i.e., the animal model (genetically modified mice, Watanabe rabbits, or New Zealand rabbits with endothelial denudation and fat diet), and the MRI protocol (USPIO dose and characteristics, MR sequences and imaging window), as discussed in dedicated papers [67, 76].

Studies using iron oxide nanoparticles as markers of activated macrophages in various inflammatory contexts such as stroke or multiple sclerosis have shown that signal loss is a combination of iron particle uptake by macrophages and nonspecific diffusion of nanoparticles as a consequence of increased permeability and neovascularization in inflamed tissue [77]. Discriminating T1, T2, and T2* effects is then thought to help the determination of iron location in the extracellular space (T1 effect) and its cellular internalization (T2* effect) [61]. The combination of both should allow follow-up of iron uptake and cell trafficking after iron uptake. Specificity could also be improved by modified coatings or by conjugating the particles with specific ligands (see the next section). Thus, ongoing USPIO research aims at improving the specificity and quantification of iron nanoparticles:

1. by combining T2/T2* and T1 effects on the MR signal using different MR sequences to improve iron location and quantification [61, 77]
2. by developing MR sequences to generate positive signal from susceptibility effects [78, 79]

3. by functionalizing USPIOs to target the inflammatory process [28,49]
4. by performing direct tracking of iron or fluorescently labeled monocytes by a combination of optical and MR techniques [80].

Cellular and molecular targeting of atherosclerosis by MRI markers

MRI markers can be designed to target either cellular players at the blood/vessel interface or deeper inside the vessel wall, or molecular components identified as key biomarkers (i.e., soluble markers circulating in blood or in the interstitium, or molecules expressed at the surface of activated cells). The main targets and protocols are summarized in Table 1.

In a first approach, nonspecific contrast agents or blood pool contrast agents with dynamic contrast enhancement protocols can be used to evaluate inflammatory consequences such as increased permeability and neovascularization [45, 50, 55, 81, 82] (Fig. 3).

Cellular MRI in atherosclerosis is mainly represented by monocyte/macrophage imaging. Macrophages were the first targeted cells to be explored by MRI for two reasons: they have a central role in the inflammatory process, and their MR labeling by iron oxide particles is well known [64, 71–73]. They have been characterized in various experimental animal models (Watanabe rabbit or New Zealand rabbit, fed with a fatty diet after endothelial injury). After iron oxide uptake by macrophages, a susceptibility effect and a focal signal loss is induced, collocated with inflammation on histopathology (Fig. 4). Ongoing studies are exploring the influence of the pharmacokinetic behavior and surface coating of the particles (starch, dextran, polyethylene glycol, anionic charges, etc.) on increasing macrophage uptake and MR response specificity, thereby decreasing the nonspecific signal from the passive diffusion of iron oxide [31, 62, 66–69]. The fundamental requirements to go on to large-scale clinical trials with USPIOs are: first, to improve specificity and quantification of iron-induced signal changes in the vessel wall; second, to design standardized protocols on clinical scanners at 1.5 and 3 T; and lastly, to document late iron particle biotransformations in inflammatory tissue and their consequences for longitudinal intraplaque signal intensity monitoring [67, 76].

For molecular imaging, targeting can address the main events of atherosclerosis evolution, i.e., inflammation and angiogenesis, oxidative stress, proliferation and apoptosis, and thrombosis (Table 1).

Inflammation and angiogenesis targeting

The main inflammation targets are cell adhesion molecules (CAM) (Fig. 1). MR markers have been designed to target

immunoglobulins, such as VCAM-1 [49, 83], the selectin family, such as E selectins [84–87] and integrins (alpha- ν beta-3) [88–90]. As the expression of adhesion molecules on activated endothelial cells has been extensively explored in biology and biochemistry, targeting specificity is generally tested on these cells. For VCAM-1, a very small iron oxide particle (CLIO) is used as a contrast carrier, combined with a mimetic of VLA4 (Fig. 1), the main ligand of VCAM-1. A fluorochrome is added on the iron particles to enable dual optical and MR detection [49, 83]. Targeting efficiency was demonstrated by an extensive in vitro study [83], confirmed in vivo in mice and improved by the phage display technique [49, 91] (Fig. 5). For E-selectin, two targeting strategies have been employed, one by adding a monoclonal antibody fragment to the CLIO particles [84], and the second by grafting a mimetic of the E-selectin ligand onto a gadolinium complex [85]. The specificity of the new markers was evaluated in vitro [84, 85], ex vivo and in vivo in animal models [86, 87]. In addition, a strategy using a mimic of a P-selectin ligand was recently evaluated in mice by our own team to target plaque inflammation [92].

Alpha- ν beta-3 integrin was targeted by addition of the RGD peptide or RGD mimetic on either perfluorocarbon Gd platforms [89, 90] or iron oxide particles [93, 94]. The general applications were neovascularization in tumors and angiogenesis [94], but the strategy was also applied to plaque inflammation diagnosis with the exciting new option of targeted drug delivery [88, 89].

Oxidative stress

As vessel-wall accumulation of lipids triggers oxidative stress, lipophilic or LDL particle markers are of particular interest for plaque imaging. One study investigated specific binding to LDL via the LDL receptor interaction: a lipophilic gadolinium-based agent with an additional fluorophore was synthesized to label LDL particles in vitro and to track labeled LDL in vivo [95]. Other lipophilic gadolinium agents such as gadofluorine [96] or gadolinium micelles [82] were applied to plaque imaging in rabbits and mice, and gadolinium liposomes [81] and functionalized HDL particles [97] to plaque imaging in mice. Gadolinium micelles have been successfully immunolabeled using the biotin–avidin–biotin bridge technique to target the macrophage scavenger receptor [98].

Oxidative stress is also under investigation by targeting enzymes such as matrix metalloproteinase [99] or myeloperoxidase [100].

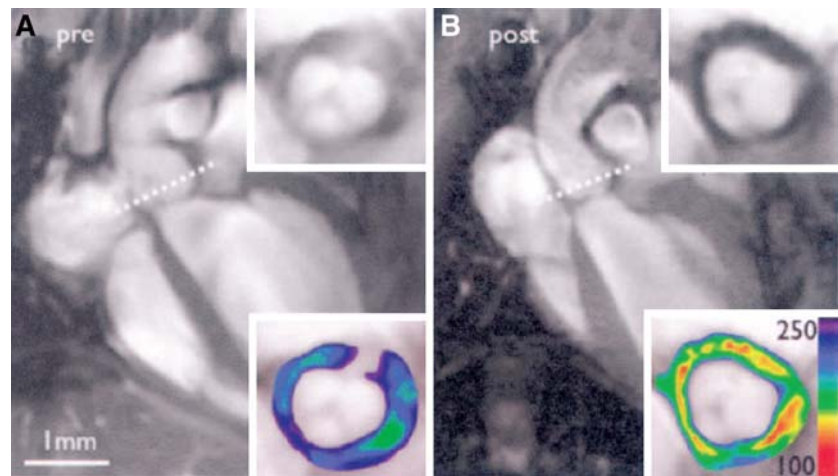
Proliferation and apoptosis

Smooth-muscle cell proliferation is involved in the response to vessel wall injury (Fig. 1). For proliferation, two options have already been tried on cell cultures, one for molecular

Table 1 Targets of the main pathological processes assessed using specific MR markers and their associated MR protocols

Pathological processes	MR markers	Target	MR methodology, species	References
Inflammation (angiogenesis)	Gd Chelates	Nonspecific: permeability and leakage	Clinical, dynamic T1, increased permeability and vascularity	[42,46,50]
	Blood pool agents (Gd liposomes, Gd macromolecules)	Nonspecific: permeability and leakage	T1, animals (rabbits, mice), increased permeability and vascularity	[55,81]
Inflammation	Iron oxide particles (SPIO, USPIO)	Activated macrophages	Ex vivo T2* (SPIO)	[71]
	Iron oxide particles (mimetic Ab VLA4+fluorochrome)	Adhesion molecules	In vivo T2* (USPIO), rabbits	[64,66,68,69,115]
	Gd platforms (biomimetic carboxymethyl-dextran-Gd)	Adhesion molecules	Clinical, T2* (aorta, carotids)	[65,72–75]
	Gd platforms (perfluorocarbon emulsion + peptidomimetic vibronectin antagonist = RGD)	Integrins (α vs β 3)	VCAM-1, T1/T2* ex vivo, in vivo (mice)	[49,83,91]
Oxidative stress	Gd micelles	Macrophage scavenger receptor	Selectins, T1, in vivo (mice)	[92]
	Theranostics nanoparticles (multifunctional USPIO)	Macrophages	In vivo rabbit aorta (fat diet), T1	[89,90]
	In vitro labeling (iron particles, Indium111)	Macrophages	Mice	[98,117]
	LDL labeled (Gd chelates + fluorochrome)	Monocytes/macrophages	Mice	[118]
	Lipophilic Gd chelates, HDL or lipid-based Gd nanoparticles	Lipid distribution	Mice	[80,119,120]
	Functionalized Gd chelate	Lipid distribution	In vitro LDL labeling	[95]
	Serotonin Gd chelate (SMART)	MMPs	Gadofluorine, micelles or liposomes, rabbit aorta, T1	[81,82,96,97]
	Gd platforms (perfluorocarbon emulsion + Biotin)	Myeloperoxidase	In vivo T1, mice	[99,121]
	Iron oxide particles (mimetic Ab VLA4 + fluorochrome)	SMC	T1 upon enzyme activation, in vitro	[100]
	Iron oxide particles (CD62E Ab fragment)	Tissue factor (via TF-Ab-avidin)	In vitro, cultured SMC, 19F imaging	[101]
Angiogenesis	Functionnalized Gd complexes	Activated endothelial cells	T1/T2 relaxivities (=f(B0))	[56]
	Gd platforms (perfluorocarbon emulsion + Peptidomimetic vibronectin antagonist)	E selectin	In vivo, ApoE mice, T2	[49,91]
Apoptosis	Iron oxide particles (CLIO-Annexin V + Cy55)	E selectin	Culture cells	[84]
	Lipid-based Gd or Fe (+quantum dots)	E selectin	Culture cells, mice (brain)	[85,87]
	No contrast agents (natural iron derivatives)	Integrins (α v β 3),	In vivo rabbit aorta (fat diet), T1	[89]
Thrombogenesis	Functionnalized Gd complexes (EP-, EPIX)	Annexin V	In vivo rabbit aorta (+drug), T1	[88]
	Gd platforms (perfluorocarbon emulsion + Biotin)	Annexin V	Cells expressing phosphatidylserine, mice, T2	[102]
Thrombogenesis	Functionnalized Gd complexes (EP-, EPIX)	Thrombus	Cells expressing phosphatidylserine, T1/T2	[103,104]
	Gd platforms (perfluorocarbon emulsion + Biotin)	Fibrin	Specific MR sequences 5	[10,33,34]
Thrombogenesis	Functionnalized Gd complexes (EP-, EPIX)	Fibrin	Fibrin, T1 sequences, in vivo (rabbits, swine)	[106–108]
	Gd platforms (perfluorocarbon emulsion + Biotin)	Fibrin	Fibrin (via Fibrin-Ab-avidin), T1 and 19F	[109,110,122]

Fig. 5 Molecular imaging of inflammation in the mouse aortic sinus. Cardiac and respiratory gated MRI before and after injection of iron oxide nanoparticles targeted to the VCAM-1 adhesion molecule. The high specificity of the probe was obtained by grafting a peptide identified by phage display technique (from Nahrendorf et al. [91], with permission)



imaging only and the second with addition of local drug delivery [56, 101]. The target was tissue factor, a transmembrane glycoprotein present at the surface of activated cells. It is responsible for the initial phases of thrombosis and is also involved in proliferation and angiogenesis. The marker was designed to target proliferating smooth-muscle cells expressing tissue factor [56].

Apoptosis is a key process in many malignant progressions, such as cancer, chronic inflammation, and atherosclerosis. The membrane translocation of the apoptosis marker phosphatidylserine (PS) was targeted in two ways: the design of fluorescent CLIO particles with annexin V [102] and the synthesis of annexin V-conjugated gadolinium or iron liposome containing quantum dots for dual MRI and optical detection [103, 104].

Thrombosis

The final process is thrombosis, which can be detected either by natural MR contrast of methemoglobin [34, 105, 106] or by specific fibrin markers [106–110]. For fibrin marker validation, proof of concept and validation steps include imaging of fibrin-labeled clots, in vitro experiments, and in vivo administration [107, 109, 110]. Perfluorocarbon particles enabled enhanced specificity by additional ^{19}F MRI [110].

Each new MR marker validation involves the following steps: (1) establishing the relevance of the chosen strategy in terms of targeting and specificity of the newly synthesized compound; (2) MRI detection of the marker and the limit of sensitivity; (3) collocation of the marker with the target, confirmed by experimentally enhanced target expression or by in vitro and in vivo competition protocols; and (4) in vivo validation after a systemic injection with careful examination of pharmacokinetic profile and discrimination from non-specific enhancement (for gadolinium) or background noise (for iron particles). The first three phases are shared by any

sensor and have been handled with optical probes in confocal microscopy. This is one reason for associating the gadolinium or iron marker with a fluorophore in the development of new MR markers. The second reason is the lack of sensitivity with MRI. For gadolinium contrast agents, the limit for one gadolinium unit is usually in the micromolar range. Sensitivity can be increased to the nanomolar or even the picomolar range by increasing the relaxivity per gadolinium unit (high-relaxivity agents) and by formulation with a high payload of gadolinium per binding molecule (macromolecules, gadolinium nanoparticles, or gadolinium liposomes) [56]. Another way is to choose a target with a large number of binding sites, such as fibrin [108]. For iron oxide particles, the susceptibility effect is more powerful than the paramagnetic effect and iron particles are already loaded with multiple irons, with an in vitro detection limit in the nano- to picomolar range. However, in vivo detection with iron oxide particles requires careful protocols, as a dark spot is more difficult to interpret than a bright spot. Thus, the specificity of in vivo iron oxide imaging is MR-sequence-dependent and new detection strategies are still under intense investigation [78, 79].

Limitations to the clinical use of Gd- and iron-based platforms for molecular imaging in atherosclerosis

From the increasing amount of experimental data on atherosclerosis and targeted MR contrast agents, several limitations can be identified.

First, for gadolinium-based markers, nonspecific uptake due to increased inflammatory tissue permeability makes a dynamic follow-up from 1 to 24 h post-injection mandatory, to highlight differences between nonspecific and specific enhancement. The poor sensitivity of MRI is also an important limitation when using gadolinium agents. An optimal balance has to be found between the number of gadolinium units per macromolecule (i.e., >100), the stability

of the gadolinium complex, a high relaxivity per gadolinium unit and conserved macromolecule r1 relaxivity at high magnetic fields. The poor sensitivity also limits the choice of target, which needs to have a high expression level in the lesion.

The steric hindrance of these gadolinium platforms (nanoparticles >100 nm) determines specific routes for bio-distribution and clearance, and sets limits to extracellular targets. Moreover, clinical applications will be subject to safety concerns, i.e., liver uptake and Gd complex stability, which will have to be determined as a function of formulation and biodistribution. For clinical applications, cheap and easy chemistry will also be a major concern for synthesis scale-up.

The limitations with iron oxide particles lie mainly in their negative effect on the signal and in the lack of quantification. On gradient echo, small signal loss at short TE is difficult to discriminate from artifacts: a long TE gives larger susceptibility effects but with poor image quality and location. In animal compared to human studies, larger USPIO doses are injected (typically, 45 $\mu\text{mol Fe/kg}$ in humans versus 200–1000 $\mu\text{mol Fe/kg}$ in animals). The interspecies variability of USPIOs' blood half-life and pharmacokinetics, which are also dose dependent (partly by saturation of the reticulo-endothelial system), makes it difficult to extrapolate experimental results to clinical findings. By testing different USPIO preparations, it has been shown that prolonged blood residence facilitates penetration into deep compartments such as atherosclerotic lesions; but a long delay between injection and the imaging window is then a major issue for clinical application. The time after injection to allow the signal from blood to decrease as well as the nonspecific distribution by permeability gives an acquisition window that may be delayed by as long as 5–7 days after USPIO administration. Lastly, for clinical applications, long-term follow-up of iron nanoparticle biotransformation, and of the fate of iron-labeled cells, will be required to fully understand their behavior in inflammatory tissue.

The ultimate goal for clinical diagnosis is to characterize vulnerable plaque by molecular imaging. Plaque rupture is a major cause of atherothrombosis; unfortunately there is no animal model for plaque rupture with consequences such as sudden death, brain or myocardial infarction. Plaque rupture has been reported in the innominate artery of ApoE KO mice, but the interval between thrombus formation and thrombolysis is much shorter than in humans, making it very difficult to observe [111–114]; also, the thrombus is very small in mice compared to humans. Defining vulnerable plaque in animal models has to take account of these kinetic factors, and plaque vulnerability biomarkers also need to adapt to such interspecies differences. In the literature, the ApoE KO mouse is the most common animal model of atherosclerosis, often using a Western diet for up to 6 months to study inflammation and the

key processes involved in plaque vulnerability. Another animal model is the Watanabe rabbit, which also has to be fed a high-fat diet to increase plaque inflammation. For vessel wall inflammation in the rabbit, the alternative is a surgically induced endothelial lesion, together with a high-fat diet. Depending on the animal model used, very different results are observed, as shown with USPIOs [63–66, 68–70, 115].

Conclusion

The present review deals with the interplay between the complex vascular biology involved in plaque formation, inflammation and atherothrombosis and the potential role of MR contrast agents in this interplay. We focused on inflammation and adhesion molecules to illustrate their high potential for molecular imaging with MRI. They can be injected directly, both to target elements in the blood stream, as adhesion molecules are present both at the surface of immune cells and platelets, and to characterize the vessel wall, as they are highly expressed on activated endothelium and plaque components. Moreover, a growing body of evidence shows their importance in advanced lesions as a link between inflammation and immune response on the one hand and thrombosis on the other. The triggering sequence leading to plaque rupture is still a matter of debate. In humans, the definition of vulnerable plaque is moving towards a more integrated vision of the *vulnerable patient*. In animal models, plaque rupture is rarely seen, and moreover shows interspecies differences that have to be taken into account in defining plaque vulnerability. Bringing molecular imaging and vascular biology together will provide new information on dynamic processes, such as inflammation and thrombogenesis.

Due to its contrast flexibility and high spatial resolution, MRI is a promising new technique for the characterization of inflammation in atherosclerosis. MRI of intraplaque macrophage activity using iron oxide particles is already under clinical investigation. Processes such as oxidative stress, proliferation, apoptosis, and thrombogenesis have recently been investigated by MRI. A fibrin marker for the diagnosis of thrombosis is under development for clinical trials. Newly designed gadolinium or iron oxide MR markers for molecular imaging have targeted integrins, immunoglobulins, and selectins to characterize vessel wall inflammation and angiogenesis, but still have certain limitations for application in large-scale clinical trials

In conclusion, molecular MRI of atherosclerosis is still in its infancy, and numerous targets and imaging strategies remain to be explored in close collaboration with molecular biologists. Information needs to be gathered from multiple modalities and experimental models to deal with the different facets of this complex chronic vascular disease.

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