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Molecular imaging of the extracellular matrix in the context of atherosclerosis

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Abstract

This review summarizes the current status of molecular imaging of the extracellular matrix (ECM) in the context of atherosclerosis. Apart from cellular components, the ECM of the atherosclerotic plaque plays a relevant role during the initiation of atherosclerosis and its' subsequent progression. Important structural and signaling components of the ECM include elastin, collagen and fibrin. However, the ECM not only plays a structural role in the arterial wall but also interacts with different cell types and has important biological signaling functions. Molecular imaging of the ECM has emerged as a new diagnostic tool to characterize biological aspects of atherosclerotic plaques, which cannot be characterized by current clinically established imaging techniques, such as x-ray angiography. Different types of molecular probes can be detected in vivo by imaging modalities such as magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT). The modality specific signaling component of the molecular probe provides information about its spatial location and local concentration. The successful introduction of molecular imaging into clinical practice and guidelines could open new pathways for an earlier detection of disease processes and a better understanding of the disease state on a biological level. Quantitative in vivo molecular parameters could also contribute to the development and evaluation of novel cardiovascular therapeutic interventions and the assessment of response to treatment.

1. Introduction

Atherosclerosis is a major contributor to the group of cardiovascular diseases (CVD). Clinical manifestations of atherosclerosis, such as acute myocardial infarction (MI) and stroke, remain major causes of morbidity and mortality worldwide, even in spite of the growing awareness of the disease in recent years [1]. A substantial rise in the incidence of cardiovascular diseases is expected over the next decades due to an aging western population and an increase in risk factors such as obesity, diabetes and hypercholesterolemia [2, 3]. The last decades of research in the field of atherosclerosis revealed novel molecular pathways and new mechanisms causing complications in human atherosclerotic plaques [4-7]. The growing understanding of the pathogenesis of atherosclerosis has opened up new perspectives for molecular imaging. This review summarizes the state of the art of molecular imaging with a specific focus on the extracellular matrix (ECM) in atherosclerotic vessel wall disease. The ECM is highly abundant in almost all biological tissues. It mainly consists of different kinds of collagen and elastin fibers, which represent the highest expressed extracellular matrix proteins [8], interspersed with glycosaminoglycans and proteoglycans [9]. The ECM is also the most abundant component of the normal arterial wall and the atherosclerotic plaque, including its fibrous cap. A dynamic balance between the synthesis and breakdown of ECM proteins controls its available amount and influences the progression of atherosclerotic disease. Within the matrix of atherosclerotic plaques, smooth muscle cells (SMCs), fibroblasts and to a lesser extent macrophages synthesize and excrete different kinds of ECM proteins. In contrast, ECM-degrading enzymes such as metalloproteinases, cathepsins, serine proteases, chymase and tryptase are expressed by macrophages, SMCs, mast cells and T-lymphocytes [10]. These enzymes have a strong proteolytic activity, especially in vulnerable or unstable atherosclerotic lesions [11, 12]. They the great ECM components which leads to a fragmentation of the ECM and a decrease of the available amount of these extracellular proteins. This leads to a thinning of the plaque's fibrous cap. Therefore, ECM degradation can make plaque regions more prone

to disruption and predispose instability [13]. From a biological standpoint, the transition from a stable to an unstable or vulnerable atherosclerotic plaque is the consequence of complex interactions between various different molecular components of the plaque.

Molecular imaging is emerging as a non-invasive method for the characterization of these pathological processes at the molecular and cellular level. It enables the direct *in vivo* visualization of biological processes and aims to elucidate the interactions between these processes during the initiation and progression of disease by applying different imaging techniques in combination with molecular probes [14-16]. In contrast to conventional clinical imaging methods such as x-ray angiography, molecular imaging aims at visualizing and quantifying underlying pathological molecular mechanisms, rather than imaging the resulting anatomical/morphological consequences, such as the degree of stenosis [17, 18].

2. The role of extracellular matrix components during the development of atherosclerosis

2.1 Role of elastin

Elastin is one of the dominant proteins of the ECM. It is found mainly in the media of the “healthy” arterial wall [19]. It contributes to up to 50% of the dry weight of arteries [20]. In veins it is expressed in substantially lower concentrations. Elastin plays a key structural role in maintaining the integrity of the arterial wall. It significantly contributes to the tensile strength of large and small arteries, enabling them to sustain permanent mechanical stress from arterial pulsation and intravascular pressure. Elastin is primarily expressed by SMCs. The production starts with the synthesis and secretion of the soluble precursor tropoelastin [8]. In the next step, tropoelastin is cross-linked and aligned into long elastin polymers that organize into rings of elastic lamellae in the arterial wall. The degree of elastin cross-linking is directly related to the tensile strength of the extracellular matrix in the arterial wall [21]. Elastin’s abundance and its high expression in the matrix of atherosclerotic plaque makes this protein a promising molecular target [22, 23]. These properties make elastin especially useful as a biomarker for magnetic resonance imaging (MRI), which is associated with a lower sensitivity for probe detection compared to e.g. positron emission tomography (PET). However MRI enables imaging with a higher spatial resolution and soft tissue contrast. Imaging with high spatial resolution is important for the visualization of morphological and biological changes within the thin atherosclerotic arterial wall. Apart from its’ structural role, elastin has important biological signaling and regulatory functions during arterial development. It controls proliferation of for example proinflammatory cells in the vascular wall [24]. Different biological and biophysical triggers initiate the increased elastogenesis in the matrix of plaques. [25, 26]. This leads to an increase in the relative amount of elastin during plaque development [8, 19, 27]. The relative composition of the matrix is relevant for plaque progression and the differentiation from stable to unstable/vulnerable plaques.

Different factors lead to a degradation and fragmentation of elastin. Elastic fibers are targeted and degraded by various matrix metalloproteinases (MMPs), especially subtypes MMP 2 and MMP 9 [28]. Under physiologic conditions MMPs are already expressed in latent forms. Their activation is triggered by an injury of the arterial wall or by proinflammatory processes [29, 30]. The reduction of elasticity of the tunica media is a result of reduced elastin expression and alterations in elastin cross-linking and elastolysis. These processes lead to further vascular damage and therefore represent a predispositional factor for the progression of atherosclerosis [31, 32]. The visualization of quantitative changes in elastin expression in the matrix enables an improved *in vivo* characterization of plaques. This is highly relevant as human stable and unstable/vulnerable plaque types can be discriminated based on their relative elastic fiber composition [8].

2.2 Role of collagen

Collagen describes a family of proteins with at least 19 genetically distinct subtypes [33]. Six of these collagen subtypes (subtype I, III, IV, V, VI and VIII) are expressed in the arterial system [34]. Collagen subtypes I and III are highly expressed and are found mainly in the ECM of plaques [35]. Approximately two-thirds of the total amount of collagen is made up of subtype I [36]. In advanced atherosclerotic plaques, subtype V collagen is also highly expressed in the ECM [37]. In the fibrous cap regions of plaques, pronounced subtype IV collagen depositions can be measured [35, 37, 38]. Following vascular injury and during the progression of atherosclerosis, subtype VIII collagen, a short chain collagen, is highly expressed. The accumulation of collagen is characteristic for atherosclerotic plaques and it is estimated that the different forms of collagens can comprise up to 60% of the overall protein in the atherosclerotic plaque matrix [39]. Comparable to elastic fibers, collagen also provides

tensile strength to the fibrous cap thereby stabilizing the plaque and reducing the probability of plaque rupture [40]. The overall synthesis of the different types of collagen is increased dramatically after the initiation of atherosclerosis in experimental models and human plaques [41-45]. A strong interaction between collagen, SMCs and macrophages can be observed within the matrix of atherosclerotic lesions [42, 46, 47]. During atherogenesis, SMCs transition from a contractile to a synthetic phenotype and begin to deposit different ECM proteins including the different subtypes of collagen [48]. There are also critical events that increase the activity of MMPs, mainly MMP 1, 8, 13 [49-51], which cleave collagen monomers in the plaque's matrix into fragments [52]. MMPs secreted by macrophages and SMCs in the fibrous cap degrade collagens and render plaques susceptible to rupture [49, 53-55]. A substantial fragmentation of collagen fibers and decrease in the collagen density is found in the fibrous cap of vulnerable human plaques [56, 57].

2.3 Role of fibrin

Fibrin is an extracellular protein and an important matrix component during the development of atherosclerotic plaque [58]. It is less abundant compared to elastin and collagen, and in contrast to both it does not represent a relevant component of the normal/healthy arterial wall. Fibrin plays an important role in the coagulation cascade and the formation of thrombi after plaque rupture. However, it is also an important protein in the ECM during plaque development [63, 64]. Fibrin fibers are associated with the majority of cells relevant for plaque development, as these cells act as procoagulants [59]. Fibrin also accumulates in the extracellular matrix as a result of an increased endothelial permeability during plaque progression with the influx of blood-derived components in early and advanced lesions [60-62]. Fibrin influences the development of plaques by different further processes. Fibrin drives

the development of atherosclerotic plaques by contributing to the total amount of the extracellular matrix [65]. It also serves as an adhesion protein, supporting the capture and stabilization of platelets and pro-inflammatory cells from the circulation [66, 67]. Additionally, fibrin in the ECM supports the proliferation and migration of pro-inflammatory cells and SMCs [68, 69]. The relative amount of fibrin in the ECM increases in late stage plaques and high amounts can be found in late stage necrotic cores [64, 70]. Therefore, fibrin could represent a valuable biomarker for the characterization of the necrotic core and the discrimination of preatheroma from atheroma [64, 70].

2.4 Role of matrix metalloproteinases

Matrix metalloproteinases are zinc-dependent ECM degrading proteinases which play a central role in the different stages of plaque development [28]. They can be secreted by almost all cells present within the plaque's matrix including SMCs, endothelial cells and macrophages [71]. As most cells within the matrix express receptors for ECM proteins, cellular function is indirectly influenced by MMPs and their effect on ECM proteins [72]. The various subtypes of MMPs are thought to have different roles during plaque development, some mainly have pro-atherogenic properties (MMP 2, 9), others mainly have anti-atherogenic properties (MMP 1, 3), and some have been suggested to have no relevant effect on atherosclerotic plaque development (MMP 7, 12, 13) [73]. Each type of MMP has specific target substrates, such as collagenase (MMP-1, -8, -13 and 18), gelatinase, stromelysin and matrilysin [74]. The activity of MMPs is inhibited by a family of antagonists, the so-called tissue inhibitors of MMPs (TIMPs) [30, 75]. An uncontrolled increase in activity of MMPs has been implicated in matrix remodeling as it results in tissue damage and functional alterations [76]. It has been shown that MMPs play a crucial role in plaque cap thinning and

weakening [30]. The balance between MMPs and TIMPs is central to plaque development, stabilization and destabilization. Different studies have shown that the increased expression MMP 2 and MMP 9 is associated with the thinning of the fibrous cap and the destabilization of plaques [28, 77].

2.5 Role of proteoglycans, glycoproteins and lipoproteins

Proteoglycans are especially important in the initial stages of plaque development, the hallmark is their accumulation in intimal lesions [78]. They also provide tensile strength and stability in more mature plaques [79]. Proteoglycans consist of a protein core bound covalently to one or more glycosaminoglycans (GAGs). These core proteins have important structural and biologic properties, including the binding of collagen and the interaction with cytokines [80, 81]. Additionally, GAGs are relevant for the development of plaque including the modulation of platelet aggregation, anticoagulation and formation of complexes with low density lipoproteins (LDLs) [82, 83]. This is especially important as the early influx of LDLs into the arterial wall is an initiating event in plaque development. Overall, the most common proteoglycans in the artery wall are decorin, biglycan, perlecan, versican, and syndecan [84]. Versican is the most abundant ECM proteoglycan which accumulates in human atherosclerotic lesions [85, 86]. Regarding quantitative expression, it is directly followed by biglycan and decorin [87]. In this context, the intima of atherosclerosis-prone arteries was shown to be rich in biglycan and versican, while the intima of atherosclerosis-resistant arteries was shown to be rich in decorin [88]. Different studies have shown that structural changes in GAG chains are an important early proatherogenic step that results in an increased affinity of proteoglycans for pro-atherogenic lipoproteins, e.g. LDL [89].

2.6 Role of lipids, lipoproteins and the necrotic core

One of the earliest events in plaque development is the subendothelial accumulation of fatty streaks of lipids [90]. These early atherosclerotic lesions or fatty streaks consist of up to 77 % of cholesterol esters [91]. The intimal thickening is characterized by lipid pools rich in hyaluron and proteoglycans [90]. Compared to other parts of the plaque, lipid pools lack SMCs. This is most likely a result of the apoptotic cell death of SMCs [92]. The congregation of lipids in lesions, combined with chronic oxidative stress, local hypoxia, and limited perfusion of nutrients, leads to the formation and enlargement of the lipid-rich necrotic core [93]. Additionally, infiltrating macrophages which ingest lipids, contribute to the progression of the lipid pool into a necrotic core [90]. The free cholesterol content of the necrotic core increases in later stages of plaque development [94]. Breakdown products of red blood cell (RBCs) are also important for the formation of a necrotic core, as RBC membranes are enriched with cholesterol [95]. Lipoproteins also play a role in the development of atherosclerotic plaques and consist of variable amounts of four main elements: cholesterol, triglycerides, phospholipids and specific proteins called apoproteins. Lipoprotein particles are differentiated into five major classes, based on their hydrated density, size and relative content of cholesterol and triglycerides: chylomicrons, very-low-density-lipoproteins (VLDL), intermediate-density-lipoproteins (IDL), low-density-lipoproteins (LDL), and high-density-lipoproteins (HDL) [96]. The interaction between LDL and proteoglycans enhances the aggregation and modification of LDL [97]. Structurally altered lipoproteins trigger the transformation from contractile to proliferative SMCs [97]. The overexpression of ECM proteoglycans enhances lipoprotein trapping, which represents an early and central process during plaque development [98].

3. Molecular imaging of the extracellular matrix

3.1 Magnetic resonance imaging

MRI allows the noninvasive characterization of the relative plaque composition, the integrity of the fibrous cap [99] and the visualization of the arterial wall, including the quantification of plaque burden. This can be achieved by multiparametric imaging protocols in combination with MR probes to generate contrast between plaque structures, such as the necrotic core and the fibrous cap [100, 101]. Most MRI probes with a clinical approval are derived from paramagnetic complexes, such as paramagnetic gadolinium (Gd) based substances or iron oxide nanoparticles [102-104]. Based on recent developments in molecular imaging research, different categories of molecular imaging probes were developed [105-107]. Molecular gadolinium-based probes, especially small molecular weight probes, are composed of a chelated gadolinium in combination with a target specific component. Molecular iron oxide based MRI nanoparticles are typically composed of different parts: (I) nanoparticle cores for signal enhancement; (II) water dispersible shells to improve biocompatibility and to prevent non-specific binding and (III) surface coating for stabilization and targeting (e.g. dextran) [108]. Iron-oxide-based magnetic nanoparticles are available in variable sizes from nano-sized ultrasmall (20–50 nm) superparamagnetic particles of iron oxide (USPIO), superparamagnetic (60–250 nm) particles of iron oxide (SPIO) and micro-sized (0.9–4.5 μm) particles of iron oxide (MPIO). These types of particles offer different features for molecular imaging [109].

Gd-based probes cause a bright or positive signal effect, as a result of the shortening of the T1 relaxation time [110]. In contrast, iron oxide based nanoparticles cause a strong dark negative signal effect, as a result of the shortening of the T2/T2* relaxation time [111]. Iron oxide based probes have a stronger effect on the T2/T2* relaxation time and can therefore be detected with a higher sensitivity compared to gadolinium based agents in T1 sequences. Advantages of MRI, compared to modalities such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), include that it is a radiation free

technique, therefore imaging can be repeated multiple times without associated risks. This is especially relevant in a clinical setting that often requires frequent follow-up investigations. Additionally, MRI allows the detection and visualization of molecular probes with a high spatial and temporal resolution. In contrast to PET, which requires an additional computed tomography (CT) scan for the anatomical localization of the probe, MRI also permits the generation of anatomical images, as well as the native characterization of atherosclerotic plaque components with a high soft tissue contrast based on local tissue properties [112]. MRI is a relatively time-consuming technique, which is a limitation compared to e.g. CT [112]. Compared to nuclear techniques like PET and SPECT sensitivity for the detection of molecular probes is lower with MRI.

3.1.1 Molecular MRI targeting elastin

Different histopathological studies have shown that elastin represents a key part of the matrix during the development of atherosclerosis [8, 19, 113]. Recently, a novel elastin specific probe has been developed and introduced. The specificity of this probe for elastin was shown in different *ex vivo* and *in vivo* settings, including the use of electron microscopy to demonstrate the colocalization of the probe with elastic fibers [22, 114, 115]. The elastin-specific MR probe was used for the first time *in vivo* to quantify plaque burden and the relative elastin content of the matrix during the progression of atherosclerosis in an apolipoprotein E-deficient mouse (apoE^{-/-}) model [22] (**Figure 1**). In this study, *in vivo* plaque burden measurements were assessed with delayed-enhancement MR imaging after administration of the elastin-specific probe and were shown to be in agreement with *ex vivo* plaque burden measurements at histologic examination. Additionally, the relative elastin content in the matrix could be quantified *in vivo* and could represent a novel marker to

differentiate between stable and unstable plaques. In a further study, the elastin-specific probe was able to detect plaques that would rupture after pharmacologic triggering with high sensitivity and specificity [116]. It was shown, that the high SNR (signal-to-noise ratio) and CNR (contrast-to-noise ratio) generated by the elastin specific probe in combination with its highly abundant molecular target, enables a reliable and fast imaging of the aortic and coronary vessel wall for the characterization of atherosclerotic plaques in a clinical imaging setup in a large animal model [117].

3.1.2 Molecular MRI targeting collagen

The different subtypes of collagen represent important components of the atherosclerotic plaque matrix. For the *in vivo* visualization of collagen, a gadolinium-based probe was recently introduced in the context of myocardial infarction [118]. Caravan et al. developed this collagen specific MR probe. It consists of three Gd-DTPAs linked to a collagen type I specific peptide [119]. The high *in vivo* collagen type I expression makes this a promising target for molecular MR imaging. This is especially relevant as MRI is associated with a lower sensitivity for the detection of molecular probes compared to SPECT and PET [120]. In a comparable approach, Chen et al. used high density lipoprotein (HDL) based nanoparticles conjugated with the introduced collagen-specific peptide for *in vivo* targeting of collagen type I in atherosclerotic plaques. In this study, collagen-specific HDL nanoparticles were successfully used to visualize the collagen content *in vivo* and to monitor the stabilization of human atherosclerotic plaques following therapeutic intervention [121]. The collagen content during plaque regression correlated with the *in vivo* MR signal using EP3533-HDL [121] (**Figure 2**). Using a different approach, Sanders et al. applied Gd-containing liposomes functionalized with the collagen-binding protein CNA35. They demonstrated that these

nanoparticles enable the visualization of collagen type I in the extracellular matrix of atherosclerotic plaques [122]. Fluorescently labeled CNA35 was used to confirm the targeting of collagen in *ex vivo* samples by immunohistology [123].

3.1.3 Molecular MRI targeting fibrin

Fibrin plays an important role in the extracellular matrix during plaque progression. It is also central to thrombus formation after plaque rupture with the exposure of matrix collagen to the blood stream. This process triggers the aggregation of platelets. Using MRI it is more challenging to image fibrin, compared to elastin and collagen, as it is less abundant within the atherosclerotic plaque. Yu et al. first introduced a fibrin-targeted probe, demonstrating its specificity by *ex vivo* measurements on human thrombi from atherosclerotic arteries [124]. The probe was based on a lipid-encapsulated perfluorocarbon nanoprobe with Gd-DTPA complexes in its surface [124]. In this study, it was determined to which extent this fibrin-targeted probe enables the visualization of fibrin-rich human clots [124]. In a further experimental *in vivo* study, Flacke et al. used a perfluorocarbon nanoprobe and reported the visualization of fibrin rich thrombi and clots in fissures of atherosclerotic lesions [15]. It was suggested that these nanoprobe could enable the prediction of myocardial infarctions and strokes in patients at risk [15]. Fibrin has also been visualized using MRI in combination with targeted ultra-small paramagnetic iron oxide (USPIO) particles in rabbits [125]. In a different study, Sirol et al. demonstrated the successful *in vivo* thrombus detection using a gadolinium based fibrin-targeted MR probe in an experimental model [126]. In a further study, Botnar et al. visualized thrombi using a gadolinium-labeled small molecular weight fibrin-binding peptide (EP-1873) in an experimental model [127]. This small molecular weight gadolinium based probe was one of the few molecular MR probes translated into patient studies, thereby

demonstrating the successful translation of a targeted molecular gadolinium based MR probe into the clinical setting. This probe was subsequently used for the successful detection and characterization of aortic, carotid, and cardiac thrombi in patients in a clinical setting [128] (**Figure 3**).

3.1.4 Molecular MRI targeting matrix metalloproteinases

In different previous studies MMPs were associated with the degradation of the fibrous cap, leading to an increase risk of rupture and the subsequent development of thrombi [11, 53, 54, 129, 130][131]. *In vivo* imaging of MMP activity could be useful for the monitoring of changes in the atherosclerotic plaque's matrix and the identification of instable plaques [132]. The identification of patients with instable plaques could also help to guide and monitor preventive therapies [132]. Lancelot et al. investigated the probe P947, which specifically targets several MMPs, for *in vivo* molecular MR imaging [133] (**Figure 4**). P947 is a molecular MR probe in which a gadoteratemeglumine moiety is covalently bound to a peptide that specifically binds MMP 2, 3 and 9 at the enzymatic active site [131, 133]. In a different study, Hua et al. evaluated a fluorescent-labeled activatable cell penetrating peptid (ACPP), cleaved by matrix metalloproteinases (MMPs) in a rabbit model. This targeted ACPP probe enabled the discrimination of ruptured and stable atherosclerotic plaques in an experimental study [135].

3.2 Positron emission tomography (PET)

The nuclear imaging technique PET (positron emission tomography) is clinically used to image and quantify radiotracers *in vivo* [136]. Radiotracer for PET emit 511 keV photons, which results from the annihilation of an electron and positron. The two 511-keV resulting photons move in opposite (approximately 180 degree) directions which allows their spatial localization along the line of response. The advantage of PET is its high sensitivity for the visualization of radiolabeled probes, compared to MRI [112]. The main limitations are the relatively low spatial resolution, the associated radiation exposure, the overall high costs and limited clinical availability [112]. The glucose analog fluorine-18-fluorodeoxyglucose (18F-FDG) is the most frequently used radiotracer, which competes with free glucose for transport into cells. Within the cell, 18F-FDG is trapped metabolically after phosphorylation. In the context of atherosclerosis, the uptake of 18F-FDG is thought to reflect the activity of macrophages and other proinflammatory cells in the matrix of metabolically active plaques [137, 138].

Focal microcalcifications are a different promising molecular target in the extracellular matrix that can be detected and quantified by PET. These microcalcifications occur early in response to intraplaque inflammatory processes. PET allows the detection of these micro-calcifications much earlier compared to CT. 18F-sodium-fluoride (18F-NaF) is the radiotracer which associates with these focal micro-calcifications by exchanging fluoride-ions with hydroxyl-ions on hydroxyapatite-crystals. Different studies have shown that this radiotracer can be used to detect and characterize advanced coronary lesions [139, 140] (**Figure 5**). In patients with symptomatic carotid artery disease 18F-NaF was shown to bind in areas with an accumulation of hydroxyapatite [141]. An important aspect of 18F-NaF PET is that there is only minor uptake in the myocardium, which makes this tracer especially well-suited for imaging of atherosclerotic plaques in the coronary system. In a recent study, greater 18F-NaF uptake was measured in vulnerable coronary plaques compared to stable plaques [139]. Based on these

first observations, ^{18}F -NaF could represent a promising molecular PET probe for the differentiation of unstable and stable atherosclerotic plaque.

Regarding imaging of the ECM component collagen, Schulz et al. applied the ^{124}I -platelet-collagen-receptor-glycoprotein (GP) VI to visualize and quantify collagen type I and II [142] (**Figure 6**). It was demonstrated that this radiotracer allows the visualization of collagen rich components of atherosclerotic plaque.

3.3 Single photon emission computed tomography (SPECT)

Compared to PET, SPECT systems are more widely available and SPECT tracers usually have a longer half-life compared to PET agents, which makes the handling of the radiotracer easier in a clinical setting. A limitation of SPECT, compared to PET, is the lower spatial resolution and the lower sensitivity for radiotracer detection [112]. In the context of the extracellular matrix mainly experimental SPECT studies have been performed. In one study, the broad-spectrum MMP inhibitor CGS-27023A was used to develop the radioligand [^{123}I]-HO-CGS 27023A, for the direct evaluation of the activity of MMPs. Using this probe, MMP activity was successfully imaged by scintigraphy in MMP-rich vascular lesions in apoE^{-/-} mice. *In vivo* measurements showed a strong correlation with the high MMP activity *ex vivo* [143]. In a different study, $^{99\text{m}}\text{Tc}$ labeled monoclonal antibodies specific for membrane-bound MMPs ([$^{99\text{m}}\text{Tc}$]anti-MT1-MMP mAb) showed an increased accumulation in unstable lesions (grade IV atheroma) in a rabbit model [144]. Regional $^{99\text{m}}\text{Tc}$ -MT1-MMP mAb correlated positively with MT1-MMP expression [144]. Applying a different approach, a technetium-99m-labeled MMP inhibitor was tested in a rabbit model of atherosclerosis. Uptake in plaques correlated with the histologically verified high expression of MMP-2 and MMP-9 [145]. Using this probe, the response to a lipid-lowering therapy with statins was

successfully quantified [145]. In a further study, Zhang et al used the radiotracer ^{111}In -RP782, which is specific for a broad spectrum of activated MMPs [146]. It was shown that this probe allows the quantification of the hyperplastic atherosclerotic process in vascular remodeling and in apoE^{-/-} mouse model.

5. Summary and clinical perspective

The development of atherosclerotic vessel wall disease is the result of complex interactions between different molecular components of the atherosclerotic plaque. Molecular imaging is emerging as a promising non-invasive method to characterize these pathological components at the molecular level. It enables the direct *in vivo* visualization of biological processes with the overall aim to improve our understanding of the dynamic interactions between biological components during the progression of disease by applying different imaging techniques in combination with molecular probes [147]. In this context, the ECM is a promising molecular target, as its' different components are important during the distinct developmental stages of atherosclerotic plaque. In the preclinical setting, molecular imaging contributes to our understanding of basic pathophysiological mechanisms during the development of atherosclerosis with the specific visualization of biological targets. Additionally, it enhances the proof of concept of novel therapeutic interventions with pharmacologic agents [148]. In the clinical setting, the overall aim of molecular imaging is to develop a safe and economic method for the sensitive and specific detection of plaques vulnerable to rupture and associated thrombosis [136, 149]. Additionally, molecular imaging has the potential to enable an early assessment of response to therapy and to contribute to the evaluation of novel cardiovascular therapeutic interventions [148].

Molecular imaging still has hurdles to overcome before it can achieve a wide clinical utility. Even though molecular MRI allows imaging with high spatial/temporal resolution and advanced motion compensation, it still suffers from a relatively low sensitivity for the detection of molecular probes. PET/CT on the other hand is associated with high costs, limited availability, the exposure to ionizing radiation and limited spatial resolution, which is a important for imaging atherosclerotic lesions in the coronary system. The recent introduction of combined simultaneous PET/MRI systems will help to overcome these limitations.

In the future, it will be important for both molecular MRI and PET to demonstrate their potential in large prospective multicenter studies and to prove the clinical and prognostic value of novel molecular markers in a clinical setting. In the future, the development of novel MRI and PET probes and the ongoing improvement of the sensitivity for the detection of molecular probes will advance the clinical *in vivo* characterization of atherosclerosis and our understanding of the development of cardiovascular diseases.

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Figure Legends:

Figure 1: Assessment of atherosclerotic plaque burden with an elastin-specific MR probe.

A: Chemical structure of ESMA and *in vivo* binding characteristics using high-resolution DE-MRI. A strong enhancement after administration of ESMA is detectable in the atherosclerotic vessel wall in an ApoE^{-/-} mouse model. B: Biodistribution analysis of ESMA. C: Ex vivo characterization of the binding of ESMA to elastic fibers using transmission electron microscopy. Typical gadolinium (Gd) spectra could be measured in areas colocalizing with elastic fibers (bottom right). D: In vivo comparison between CNRs derived from DE-MRI scans after injection of Gd-ESMA on day 1. On day 2, pre-injection of non-paramagnetic La-ESMA resulted in a marked decrease of CNR after ESMA injection, demonstrating the specificity of ESMA to its target. E: Comparison of average percent plaque media volume (PAMV), calculated from morphometric measurement on high-resolution DE images after ESMA injection. A significant increase in PAMV could be observed after 8 and 12 weeks of HFD. Statin treatment led to a significant decrease in PAMV compared to 12 weeks HFD (n=8 per group). ESMA: elastin-specific MR agent, HFD: high fat diet, TOF: time-of-flight. Scale bar: black 100 μm , white 250 μm . Adapted from Makowski et al. [22].

Figure 2: High density lipoprotein based nanoparticles conjugated with a collagen-specific peptide for *in vivo* MR imaging of atherosclerotic plaques.

A: MR images of abdominal atherosclerotic plaques pre- and 24h post-injection of HDL, EP3533-HDL, and EP3612-HDL at baseline (yellow-bars) and after 28 days (green-bars) in an experimental mouse model. EP3533-HDL, which shows a strong association with collagen, demonstrated a significantly increased *in vivo* enhancement in aortic vessel walls compared with HDL alone and the nonspecific control EP3612-HDL. B: Corresponding

normalized enhancement ratios (NERw) and C: change of contrast-to-noise ratios (ΔCNRw). The arrows point to the aortic wall. * $p < 0.05$. Adapted from Chen et al. [121].

Figure 3: Molecular MR imaging of fibrin rich thrombi in the carotid artery of patients

A: Molecular MR imaging of a fibrin rich thrombus in the carotid artery in a patient with a TIA. Signal enhancement from the fibrin specific molecular probe at the clot surface can be appreciated on contrast enhanced images (image on the right, arrow and magnification shown as an inset). On unenhanced sequences minor signal enhancement of the arterial wall is also seen (image on the left, arrowhead), which may represent intraplaque hemorrhage. **B:** Descending thoracic aorta in a patient with a fibrin rich clots. High local signal enhancement from the fibrin specific probe allows the visualization of the fibrin rich clot (arrow). **C:** Pre-contrast T2-weighted imaging reveals a relevant wall thickening at the level of the thrombus. Adapted from Spuentrup et al. [128].

Figure 4: MMP-specific MR probe for the characterization of atherosclerotic plaque.

In vivo MRI of an ApoE^{-/-} mouse model before (arrow) and following P947 injection (arrowhead). The molecular MR probe specifically binds MMP 2, 3 and 9 at the enzymatic active site. Following administration, contrast-enhancement can be appreciated in aortic plaque as demonstrated on the inset images. The demarcation of the morphology of plaques following probe administration is substantially improved (arrowhead). The bottom-right panel represents the matched histopathological slides. Adapted from Lancelot et al. [133].

Figure 5: 18F-NaF PET-CT for the identification of high-risk coronary atherosclerotic plaque in a prospective clinical trial.

A: Proximal stenosis of the left anterior descending artery on invasive x-ray coronary angiography (red arrow). B: Intense 18F-NaF uptake at the site of the culprit plaque on PET-CT (red arrow). C: Corresponding 18F-FDG image showing no uptake in this part of the vessel wall. However, significant myocardial FDG uptake overlapping with the coronary artery (yellow arrow) and uptake within the esophagus (blue arrow). D: Culprit lesion in the left anterior descending artery (red arrow) and bystander non-culprit lesion in the circumflex artery (white arrow) on coronary angiography. Both lesions were stented during the admission. E: The culprit lesion demonstrate increased 18F-NaF uptake on PET-CT following coronary intervention. F: Corresponding 18F-FDG PET-CT showing no uptake at the bystander stented lesion. G: Showed non-obstructive disease in the right coronary artery. H: Corresponding PET-CT scan showed increased 18F-NaF uptake in the mid-right coronary artery (positive lesion, red line) and a region without increased uptake in the proximal vessel (negative lesion, yellow line). I: Radiofrequency intravascular ultrasound (IVUS) demonstrates that the 18F-NaF negative plaque is mainly composed of fibrous and fibrofatty tissue (green) with confluent calcium. J: The 18F-NaF positive plaque shows high-risk features such as a large necrotic core (red) and microcalcification (white). Adapted from Joshi et al. [139].

Figure 6: PET imaging using 124I-platelet-collagen-receptor-glycoprotein (GP) VI for the visualization of collagen type I and II in atherosclerotic plaque.

A: PET images of ApoE^{-/-} and wild type mice acquired 24 hours following administration of 124I-GPVI, which binds to collagen rich components of atherosclerotic lesions. Quantitative PET measurements show a significant increase in binding in the aortic arch of atherosclerotic

ApoE^{-/-} mice compared to wild type mice. B: Sudan III staining of the aortic arch of ApoE^{-/-} and wild type mice with corresponding autoradiography confirming increased radioactive uptake in plaques. Adapted from Schulz et al. [142].

Figures:

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