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## Multidimensional Contribution of Matrix Metalloproteinases to Atherosclerotic Plaque Vulnerability: Multiple Mechanisms of Inhibition to Promote Stability

Jean Marie Ruddy<sup>a,c</sup>, John S. Ikonomidis<sup>b,c</sup>, Jeffrey A. Jones<sup>b,c</sup>

<sup>a</sup>Division of Vascular Surgery, Department of Surgery, Medical University of South Carolina, Charleston, S.C., USA

<sup>b</sup>Division of Cardiothoracic Surgery, Department of Surgery, Medical University of South Carolina, Charleston, S.C., USA

<sup>c</sup>Division of Cardiothoracic Research, Department of Surgery, Medical University of South Carolina, Charleston, S.C., USA

### Abstract

The prevalence of atherosclerotic disease continues to increase, and despite significant reductions in major cardiovascular events with current medical interventions, an additional therapeutic window exists. Atherosclerotic plaque growth is a complex integration of cholesterol penetration, inflammatory cell infiltration, vascular smooth muscle cell (VSMC) migration, and neovascular invasion. A family of matrix-degrading proteases, the matrix metalloproteinases (MMPs), contributes to all phases of vascular remodeling. The contribution of specific MMPs to endothelial cell integrity and VSMC migration in atherosclerotic lesion initiation and progression has been confirmed by the increased expression of these proteases in plasma and plaque specimens. Endogenous blockade of MMPs by the tissue inhibitors of metalloproteinases (TIMPs) may attenuate proteolysis in some regions, but the progression of matrix degeneration suggests that MMPs predominate in atherosclerotic plaque, precipitating vulnerability. Plaque neovascularization also contributes to instability and, coupling the known role of MMPs in angiogenesis to that of atherosclerotic plaque growth, interest in targeting MMPs to facilitate plaque stabilization continues to accumulate. This article aims to review the contributions of MMPs and TIMPs to atherosclerotic plaque expansion, neovascularization, and rupture vulnerability with an interest in promoting targeted therapies to improve plaque stabilization and decrease the risk of major cardiovascular events.

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Dr. Jean Marie Ruddy, Division of Vascular Surgery, Department of Surgery, Medical University of South Carolina, 114 Doughty Street, MSC 295, Charleston, SC 29425 (USA), [ruddy@muscc.edu](mailto:ruddy@muscc.edu).

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## Keywords

Atherosclerosis; Plaque rupture; Matrix metalloproteinases; Angiogenesis

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## Introduction

Atherosclerotic plaque deposition occurs in all major vascular beds and is a primary source of morbidity in the USA [1]. While chronic stenosis is often well tolerated due to compensation by collateral vessels, life-threatening consequences may result from plaque rupture with thrombus formation, vessel occlusion, or embolization. Depending on the location, this pathological phenomenon is manifested clinically as a myocardial infarction, limb ischemia, or acute stroke [2]. Medical management of atherosclerosis with antiplatelet agents and statins may decrease the incidence and severity of events, but additional therapies directed at modification of plaque biology may attenuate growth and promote stability. Recent research has improved the understanding of progression from fatty streaks to mature atheroma with particular attention to inflammatory cell infiltration, vascular smooth muscle cell (VSMC) migration, extracellular matrix (ECM) remodeling, and plaque neovascularization [3, 4]. All of these processes rely on the production and activation of a subset of enzymes from the family of matrix metalloproteinases (MMPs) [5]. These peptidases have been well studied in various aspects of vascular remodeling [6], but the contribution of MMPs to atherosclerotic plaque neovascularization and rupture vulnerability, with potential targeted therapies to achieve MMP inhibition to promote plaque stabilization, will be the focus of this review.

## Atherosclerosis

A detailed discussion of atherosclerotic plaque deposition is beyond the scope of this article, but key components of the pathophysiology will be introduced to identify pertinent cells, cytokines, growth factors, and enzymes. Endothelial cells (ECs) may become dysfunctional due to pathologic forces such as hypertension, hyperlipidemia, or smoking, and the presence of oxidized low-density lipoprotein (ox-LDL) in the subintimal space can promote the release of chemokines such as monocyte chemoattractant protein-1 [7]. Expression of cell surface adhesion molecules are also upregulated during the initiating phases of fatty streak formation [7]. Adherence and transmigration of monocytes and T lymphocytes into the intima leads to the release of additional cytokines and growth factors that can stimulate VSMC outgrowth from the media [8]. The VSMC phenotypic switch necessary for migration also supports proliferation and synthetic function [9]. Intraplaque cellular expansion of VSMCs and macrophages progresses to apoptotic degeneration and the formation of a necrotic core covered by a thin fibrous cap of newly synthesized matrix components, a mature atheroma [10]. These latter migration and remodeling processes require matrix degeneration that is mediated by VSMC- and macrophage-derived MMP activation and secretion [11]. In early plaque deposition, outward remodeling of the vessel can preserve the lumen area, but this adaptation is frequently overwhelmed by progressive disease and localized vascular stenosis. The stability of these plaques has been attributed to thick fibrous caps [4], while vulnerable plaques are characterized histologically to include a

thin cap, with a high macrophage:VSMC ratio, and a large necrotic lipid core [12]. Increased mechanical strain is endured at the shoulder regions of the plaque, a region also noted to have high concentrations of synthetically active macrophages, and contributes to the risk of plaque rupture [13]. Subsequent exposure of thrombogenic substrates and rapid local thrombus formation can then precipitate the clinical consequences of myocardial infarction, acute limb ischemia, or stroke [14]. Understanding the spatial and temporal activity of MMPs in plaque progression and vulnerability may allow targeted therapies to attenuate this unstable vascular remodeling (fig. 1).

## Matrix Metalloproteinases

Regulated activity of the greater than 24 members of the MMP family of zinc-containing proteases allows deconstruction and reorganization of the vascular ECM as well as controlled release of several ECM-embedded cytokines and growth factors. MMPs are categorized into six groups based on substrate specificity, including collagenases (MMP-1, 8, 13, 18), gelatinases (MMP-2, 9), stromelysins (MMP-3, 10, 11), matrilysins (MMP-7, 26), membrane-type (MT1-, 2-, 3-, 4-, 5-, 6-MMP, also known as MMP-14, 15, 16, 17, 24, 25), and others (MMP-12, 19, 21, 23, 28) [15]. Carefully orchestrated control of MMP activity is vital to healthy vessel maintenance and investigation into the consequences of proteolytic imbalance can provide significant insight into the progression of aberrant vascular remodeling [16]. Synthesis may be controlled at the transcriptional, posttranscriptional, and posttranslational levels. Once translated, MMPs are released into the interstitium in a proform that allows zymogen activation to serve as a key mechanism of proteolytic activity management [17]. In addition, the MT-MMPs are bound to the cell membrane and thereby limited to pericellular activity and regulated membrane trafficking. An additional point of regulation relies on interactions with the endogenous tissue inhibitors of metalloproteinases (TIMP-1, 2, 3, 4) [18]. Members from each MMP subfamily are represented in atherosclerotic remodeling, providing several potential targets for therapeutic engineering but also displaying the complexity of this pathophysiologic process and suggesting that interruption of a single participant may be inadequate (table 1) [19].

## Collagenases

Fibrillar type I, II, and III collagens, major components of the atheroma fibrous cap, can be cleaved by the collagenases, and overexpression by macrophages, VSMCs, and ECs has been documented in mature atherosclerotic plaques [20, 21]. More than the inherent cells, it has been postulated that the inflammatory infiltrate plays a primary role in MMP production to breakdown local ECM and alter cap morphology [22]. The localization of MMP-1 to areas of high circumferential stress and of MMP-1, 8, and 13 to the frequently ruptured plaque shoulders supports the critical role of collagenases in fibrous cap thinning and vulnerability [23-25]. Largely outweighing the increased TIMP-1 identified in acute carotid plaque specimens as well as late atheromatous restenotic lesions, amplified MMP-1 expression has demonstrated a critical contribution of this enzyme, and collagen processing in general, to plaque remodeling [26, 27]. In fact, in a study utilizing extensive histologic and molecular examination of over 50 carotid endarterectomy specimens, only MMP-1 transcript levels were associated with a thin fibrous cap, a potential sign of plaque instability

[28]. A specific collagen cleavage-site antibody has further confirmed the activity of macrophage-derived interstitial collagenases MMP-1 and 13, as well as the neutrophil collagenase MMP-8, in inflamed atheromatous plaques [25, 29]. Intraplaque rupture is a commonly encountered histopathologic abnormality with significant clinical manifestations, particularly in the carotid arterial system, and macrophages at the perimeter of the lipid core were shown to have increased MMP-1 expression that was linearly related to the size of the intraplaque hemorrhage, suggesting that characteristics of plaque instability are augmented by MMP-1 overactivity [23]. Exploring this hypothesis in ApoE knockout mice with macrophage-specific overexpression of MMP-1, however, demonstrated smaller and less mature atheromas and posed the question of how the collagenases contribute to ECM remodeling, such as activity differentiation during plaque initiation, growth, late expansion, or instability [30]. Nevertheless, inhibition of MMP-1 activity should be explored as a means of promoting plaque stability in humans.

MMP-13 has mostly been identified in conjunction with MMP-1, but also offers a significant collagenolytic impact. When concurrently knocked out with ApoE, mice had no change in plaque size but a significant increase in volume and organization of type I collagen fibers, proposing a destabilizing role for MMP-13 [31]. The contribution of neutrophils to atherogenesis continues to be defined and is currently characterized as an early atherogenic inflammatory mediator to assist in monocyte recruitment and contribute to the production of MMP-8 [32, 33]. Alternatively, MMP-8 has been localized to neutrophils in regions of neovascularization and intraplaque hemorrhage within advanced carotid plaques that demonstrate additional evidence of vulnerability (large lipid core and high macrophage count), suggesting a contribution of extravasated neutrophils and/or MMP-8 to the rupture risk in these areas [34]. Macrophages are an additional source of MMP-8 in carotid lesions, however, with association of this protease to symptomatic lesions and histologic signs of plaque vulnerability [35, 36]. Moreover, ApoE knockout mice with a concurrent lack of MMP-8 had smaller aortic lesions with fewer macrophages and higher collagen content, supporting a role for MMP-8 in plaque vulnerability [37].

## Gelatinases

Denatured or degraded products of collagen, referred to collectively as gelatin, and type IV collagen are frequent substrates for MMP-2 and MMP-9, along with elastin, aggrecan, fibronectin, laminin, proteoglycans, and several nonstructural components of the ECM [15]. Gelatinases can provide positive feedback mechanisms to local matrix remodeling by activating pro-MMP-1, pro-MMP-2, pro-MMP-9, and pro-MMP-13 as well as by modulating the activation of sequestered inflammatory cytokines such as TGF- $\beta$ 1, IL-1 $\beta$ , and TNF- $\alpha$  [5, 38-40]. Similar to collagenases, gelatinase activity may be clustered at the shoulder regions of mature atheromas [20, 41], but alternative regional cellular contributions are pertinent to early fatty streak progression [14]. VSMCs constitutively express the inactive pro-MMP-2, but inflammatory stimulation will promote phenotypic modulation to support the secretion of all components necessary for zymographically active MMP-2 and MMP-9, enzymes vital to the ECM degradation necessary for VSMC proliferation and migration [42]. Soluble cytokines such as C-reactive protein (CRP) as well as T-lymphocyte-bound CD40L have also demonstrated a targeted increase in MMP-2 expression in VSMCs

[43, 44]. MMP-9 is highly expressed by macrophages [45], and production can be further amplified by exposure to ox-LDL in early atheromas [46]. Neither classical nor alternative macrophage activation effectively enhances MMP-2 production, however, suggesting that VSMCs may be the primary source of MMP-2 in an atheroma [47].

Specifically within atherosclerotic plaques, VSMC migration is imperative for fibrous cap formation, and there are contradictory accounts of the contribution of gelatinases to cap stability. Migration of VSMCs is severely compromised by MMP-2 deficiency [48, 49], and the increased enzymatic activity of MMP-2 identified in fibrous carotid plaques containing a larger proportion of VSMCs suggests an association with stability in this lesion [32]. When MMP-2 was concomitantly knocked out in ApoE-deficient mice, smaller plaques with fewer VSMCs were noted in the murine aorta, implying that MMP-2 is necessary for plaque growth but could also support stability through VSMC migration [49]. Some evidence suggests a role for MMP-9 in modifying plaque growth and promoting collagen organization by VSMCs to potentially enhance plaque stability [50, 51], but MMP-9 colocalization with macrophages and MMP-8 in unstable carotid plaques supports a destabilizing effect in this highly inflammatory, and frequently clinically symptomatic, atheromatous plaque phenotype [32, 52]. Reductions in aortic plaque burden, macrophage infiltration, and collagen content were observed in ApoE/MMP-9 double knockout mice [53]; however, a separate study with mice of a similar genetic background resulted in more progressive plaque deposition with reduced VSMC density and suspected plaque instability in the brachiocephalic artery [50], demonstrating a potential regional component to MMP-9 contributions to atheroma growth. Additional variation among studies may be attributed to a temporal contribution of MMP-9 to plaque morphology. For instance, in an interesting study utilizing bone marrow transplantation among ApoE/MMP-9 single and double knockout mice, resident arterial cells (VSMCs and ECs) were identified as primary producers of MMP-9 in the early stages of murine brachiocephalic artery atherosclerotic plaque deposition versus late production by infiltrating macrophages [54]. Moreover, late overexpression of MMP-9 in ApoE knockout mice specifically supported intraplaque hemorrhage [55]. In the setting of plaque rupture, the impact of MMP-9 is further perplexing since it can minimize platelet aggregation and degrade fibrin, potentially protecting against thrombus formation, and retroviral overexpression of MMP-9 in ApoE knockout mice failed to show a significant increase in disruption [56-58]. Targeted inhibition of gelatinases, therefore, may be beneficial but individually insufficient to prevent signs of plaque destabilization.

## Stromelysins

Stromelysins act upon a broad range of proteins, but primary substrates for MMP-3, 10, and 11 include nonfibrillar collagens, elastin, fibronectin, proteoglycans, and several nonstructural ECM proteins such as E-cadherin, L-selectin, IL-1 $\beta$ , TNF- $\alpha$ , and pro-MMP-1, 7, 8, and 9 [15]. MMP-11 demonstrates unique characteristics including secretion in an active form and primary substrate specificity for serine protease inhibitors (serpins) [59]. MMP-3 is not produced by healthy vessels, but was one of the first MMPs identified in atherosclerotic plaques, and its expression has been localized to macrophages as well as lymphocytes and activated VSMCs [42, 45, 60, 61]. MMP-3 expression can be induced by IL-1 $\beta$  and platelet-derived growth factor (PDGF) [62]. Release of ECM-bound inflammatory

mediators along with the activation of other MMPs suggests a positive feedback mechanism with MMP-3 promoting plaque remodeling. Interestingly, studies of MMP-3/ApoE double knockout mice have shown greater plaque growth with increased macrophage and decreased VSMC composition, signifying impaired VSMC migration and an unstable phenotype in the absence of MMP-3 [50, 63]. The subsequently demonstrated reliance of VSMC migration on MMP-3 activation of MMP-9 likely contributes to this instability and emphasizes the apparent stabilizing effects of MMP-3 [64]. Additional clinical evidence supporting a protective role for MMP-3 includes the impact of the 6A, less transcriptionally active, MMP-3 promoter polymorphism that results in decreased MMP-3 in these patients and rapid progression of atherosclerotic disease [65, 66].

While the specific contribution of MMP-10 to atheroma pathophysiology has not been studied as extensively as MMP-3 due to its association with inflammatory markers such as CRP, serum MMP-10 levels have been gathered for prognostic implications and will be discussed separately [67]. In mature carotid plaques, however, elevated MMP-10 production was quantified in regions heavily populated with macrophages [68]. Likewise, tracking the immune mediator CD40L in atherosclerotic plaque cellular constituents allowed for the identification of augmented expression of MMP-11 in mature atherosclerotic plaques while no MMP-11 was visualized in healthy arteries or in fatty streaks [69]. This spatiotemporal expression of MMP-10 and 11 suggests a role for these stromelysins to influence plaque progression with ongoing inflammatory stimulation.

## Matrilysin

MMP-7 has substrate specificity for type IV collagen, elastin, laminin, and proteoglycan as well as several nonstructural ECM components such as N-cadherin, plasminogen, pro-MMP-2, 7, 8, and TNF- $\alpha$  [15]. Of particular note is the high affinity binding domain for versican, a plentiful component of atherosclerotic plaques [70]. Localized production of MMP-7 by fully activated macrophages has been identified at the interface between the lipid core and fibrous cap, a divergence from the pattern of MMP-1, 3, and 9 secretion at plaque shoulders [70]. Proteolytic activity in this region may weaken apposition of the cap and predispose to plaque rupture, a scenario supported by the elevated MMP-7 plasma levels identified in patients with symptomatic carotid lesions [71]. Transcriptional activation of MMP-7 has been documented in hypoxic macrophages and the relevance of this physiologic adaptation to the regional production in atheromatous plaques remains to be determined [72]; however, a relationship to the initiation of angiogenesis ought to be considered. Attempts to delineate the specific MMP-7 contribution to atherosclerotic plaque progression through hypercholesterolemic knockout mice have identified increased VSMC infiltration in the absence of MMP-7 but no alterations in plaque growth or accepted characteristics of stability [50]. The larger contribution of MMP-7 to fibrous cap instability may be related to the initiation of VSMC apoptosis via cleavage of the cell-cell junction protein N-cadherin [73].



## Membrane Type

The MT-MMPs are integral membrane proteins with catalytic domains on the cell surface and contribute to the modification of the pericellular matrix environment [22]. Primary substrates include fibrillar collagens (I, II, and III), aggrecan, fibronectin, proteoglycans, and nonstructural ECM proteins including integrins, pro-MMP-2 and 13, and pro-TNF- $\alpha$  [15]. MT1-MMP is constitutively expressed by VSMCs in healthy vessels with additional expression in atherosclerotic plaques [74]. The induction of MT1-MMP in ECs following exposure to ox-LDL or TNF- $\alpha$  may indicate a mechanism for early degradation of the intima to facilitate inflammatory cell infiltration [75]. In addition to a role in monocyte penetration through the endothelium [76], MT1-MMP has been shown to promote VSMC migration and proliferation by mechanical degradation of adhesion points to the basement membrane [77]. Overexpression of MT1-MMP has also been documented at rupture-prone areas of atherosclerotic plaques [74, 75, 78, 79]. Potentially the most important contribution of MT1-MMP in atherosclerotic plaque progression, however, is the concomitant increased activation of pericellular MMP-2 [80]. Mice in which MT1-MMP has been knocked out display a severe dysmorphism and typically die within 3 weeks of birth. As such, these mice are unable to be studied in adulthood, significantly limiting the opportunities to isolate and define the role of this MMP in atherosclerosis [81]. Bone marrow transplantation from MT1-MMP knockout mice into radiated ApoE knockout mice, however, has demonstrated increased collagen content with no change in plaque burden, supporting a primarily collagenolytic role for MT1-MMP [82, 83].

## Other MMPs

Within this group of miscellaneous MMPs, the most pertinent contribution to atherosclerotic plaque growth and potential rupture has been documented for MMP-12, also known as macrophage metalloelastase [15, 84]. In addition to elastin, major substrates include type IV collagen, fibronectin, plasminogen, and pro-MMP-2 and 3 [15, 85]. The indispensable contribution of this protease to basement membrane degradation has implications for macrophage infiltration in response to inflammatory mediators [86-88]. Interestingly, increased synthesis and secretion of MMP-12 has been localized to mature macrophages at the border zone between the lipid core and fibrous cap, a region where ECM degradation may predispose to rupture [70]. This spatial distribution supports the elevated MMP-12 expression documented in ruptured, symptomatic carotid plaque specimens, and patients with elevated MMP-12 levels within the extracted plaques had a higher risk of cardiovascular complications perioperatively [28, 89]. Moreover, genome-wide analysis identified an overexpression of MMP-12 in patients with large artery atherosclerosis as their stroke etiology [90]. Temporal analysis of MMP-12 production in rabbit atherosclerotic plaque has indicated a lack of MMP-12 in healthy vessels with marked upregulation in progressive lesions, again supporting the association with mature macrophages and rupture morphology [91]. ApoE/MMP-12 double knockout mice had no alteration of plaque accumulation in the aorta, but in the brachiocephalic artery there was increased VSMC infiltration, decreased macrophage penetration, and decreased elastin degradation, suggesting properties of a more stable plaque phenotype in the knockout mouse and supporting a destructive role for MMP-12 in plaque progression [50, 53].

## Tissue Inhibitors of Metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are expressed during development and tissue remodeling and play a major role in regulating MMP activity under healthy as well as pathologic conditions [92]. Considering the four TIMPs identified in humans, all MMPs may be inhibited but individual affinity does vary, such as a lack of activity between TIMP-1 and MT-MMPs [93]. TIMP-1 and TIMP-2 are constitutively expressed by VSMCs, but expression cannot be augmented by inflammatory cytokines that contribute to enhanced MMP production by VSMCs in atherosclerotic disease, providing one avenue by which the balance may favor proteolytic activity in these regions [94]. Interestingly, TIMP-1 production by VSMCs has been enhanced by PDGF, a frequent contributor to matrix remodeling [95]. Shared utilization of binding sites for the AP-1 transcription factor may indicate how TIMP-1 may be coexpressed with collagenases such as MMP-1 and MMP-13 to modulate matrix degeneration [96]. Experimental manipulation of TIMP-1 production has suggested that this protein plays a significant role in limiting plaque progression, however, since larger lesions grew in TIMP-1/ApoE knockout mice [97]. Localization of TIMP-1 to macrophage-rich regions of plaque led to further experimentation regarding local expression and the observation that ox-LDL decreased TIMP-1 expression in macrophages, while the production of several MMPs was not affected [98]. Oxidation of LDL molecules trapped in the intima has been described as an early trigger for EC expression of adhesion molecules and chemokines that initiate fatty streak deposition [8]; therefore, the decreased TIMP-1 production by these early infiltrating macrophages may provide an early mechanism for MMP/TIMP imbalance and ECM degradation.

High levels of TIMP-2 have been noted in macrophages overlying the lipid core as well as along the intima-media border, potentially stabilizing locations confirmed by the decreased activity observed by in situ gelatin zymography in these regions [95, 99]. TIMP-3 has minimal secretion from healthy VSMCs, is tightly sequestered in the ECM, and has demonstrated augmented expression by VSMCs in response to PDGF and TGF- $\beta$  but no change for inflammatory cytokines associated with atherosclerosis, potentiating a state of dysregulated MMP activity during atherogenesis [94, 100]. Interestingly, TIMP-3 colocalized with apoptotic markers in the necrotic lipid core of mature atherosclerotic plaques, implying that the described role of nonphysiologic TIMP-3 overexpression in VSMC apoptosis in vitro may contribute to plaque physiology in vivo [93, 101]. TIMP-3 expression by activated macrophages was decreased following exposure to ox-LDL or inflammatory cytokines, known stimulators of MMP production in these cells [79]. TIMP-4 has a restricted expression profile limited to cardiovascular tissues [102], and production of TIMP-4 has been reported in the media and adventitia of healthy vessels, but localization to macrophages bordering the necrotic lipid core has also associated this protein with active inflammatory processes [101, 103].

## Regulation by microRNA

Short, single-stranded segments of RNA, known as microRNA (miRNA), are recognized regulators of gene expression implicated in numerous physiologic and pathophysiologic processes, including the initiation and progression of vascular diseases [104]. These



molecules can downregulate target genes through mRNA degradation or by repressing translation, and each miRNA can influence several genes, demonstrating how these interactions may influence entire signaling pathways [105]. Particular to atherosclerotic disease, multiple miRNAs have amplified expression in plaque specimens from peripheral arteries, including miR-21, miR-34, miR-146a, miR-146b-5p, and miR-210, with corresponding attenuation of expected target gene levels. The inflammatory response known to contribute to atheroma growth has likewise been associated with particular miRNAs [106, 107], but those regulating MMP and TIMP production will be selectively further discussed.

The association of miR-21 with adverse cardiovascular events due to the promotion of neointimal lesions was strengthened with the identification of RECK as a target gene [108]. Overexpression of miR-21 in macrophages induced increased secretion of pro-MMP-9 as well as active MMP-9 by inhibiting RECK expression [108]. Additional epigenetic manipulation of MMP-9 and MMP-2 production has been attributed to miR-29b targeting of DNA methylation genes that are upstream of gelatinase transcription [109], and a single-site polymorphism has been demonstrated to effect a significant amplification of MMP-9 production due to the loss of activity of miR-491, ultimately leading to an increased risk of ischemic stroke [110]. This relationship between miRNAs and atherosclerotic plaque stability has been greatly expanded with the identification of a group of miRNAs that influence plaque homeostasis. Through comparative analysis of miRNA profiles in symptomatic versus asymptomatic carotid plaques, miR-100, miR-127, miR-133a, miR-133b, and miR-145 were found to be jointly upregulated in symptomatic – and presumed unstable – plaques [111]. Bioinformatic analysis identified MMP-9 as a potential target of miR-133a and miR-133b, and this association was subsequently proven by decreased MMP-9 production in the presence of miR-133a mimic, and additional reports implicate this group of miRNAs in regulating MMP-13 as well, thereby providing a means of impacting plaque remodeling and potential stability through two powerful proteases [111]. Stabilizing effects have also been demonstrated with miRNA-24 through the downregulation of MT1-MMP and decreased invasion of macrophages [112]. Factors regulating miRNA production continue to be explored and utilizing this avenue to modulate MMP production may be a viable asset in the future.

## Biomarkers

Having recognized several MMPs and TIMPs as key players in atherosclerotic plaque rupture with consequent clinical cardiovascular manifestations such as myocardial infarction or stroke, interest in measuring the plasma levels of these proteins to help identify high-risk patients has continued to rise. Early studies following patients with moderate carotid atherosclerotic disease found elevated MMP-9 levels to be associated with an increased incidence of stroke or cardiovascular death over a 10-year follow-up timeframe [113]. More specifically, elevated MMP-9 correlated to ultrasonographic as well as postsurgical histologic signs of plaque instability, including increased intima-media thickness (IMT), a thinner fibrous cap, and the presence of intraplaque hemorrhage [114, 115]. Examining a broader scope of the proteolytic environment in patients presenting for carotid endarterectomy, those with unstable plaques had higher plasma levels of MMP-1, 2, 3, and 9 coinciding with lower levels of TIMP-1 and 2 when compared to those with stable plaques

as well as to healthy age-matched volunteers [116, 117]. Continuing to monitor these proteins along with the surveillance carotid artery duplex ultrasound imaging in postendarterectomy patients may prove efficacious given that those with late symptomatic recurrence of carotid stenosis have shown increased MMP-2 and 9 and decreased TIMP-1 and 2 plasma levels compared not only to healthy volunteers, but also to those with asymptomatic restenosis, suggesting that these proteins may mark patients with more aggressive, unstable disease [118]. Of the participants in the Atherosclerosis Risk in Communities Carotid MRI Study, those with high IMT also demonstrated elevated MMP-1, 3, and 7 levels, while the normalized wall index, an MRI-derived value comparing the wall area to total vessel area, positively correlated with MMP-3, 7, and TIMP-1 [119]. Because this study associated serum levels with high-resolution cross-sectional imaging, it was also able to make a positive association between TIMP-1 and fibrous cap thickness, likely indicating plaque stability [119].

Alternatively, the ultrasonographic evidence of hypoechogenic plaque has been associated with elevated MMP-8 serum levels in asymptomatic patients undergoing carotid endarterectomy, and those with high levels of MMP-8 within the endarterectomized plaque had a greater than 4-fold increased risk of a cardiovascular event in the ensuing 3 years [120, 121]. Likewise, patients with plaques possessing elevated MMP-12 have a greater than 3-fold risk of stroke over the same timeframe [89]. Those with preoperative symptomatic carotid lesions have demonstrated elevated plasma levels of MMP-7 and associated increased all-cause mortality during an 8-year follow-up period, with this correlation being stronger than that between either age or increased CRP and mortality in this population [71]. Tracking the inflammatory biomarker CRP has repeatedly proven to be prognostic for stroke, however, and colocalization of MMP-10 with CRP in rupture-prone regions of carotid atherosclerotic plaques has led to the investigation of MMP-10 as a clinically relevant biomarker, particularly in patients with elevated CRP [67, 68]. Patients lacking clinical evidence of cardiovascular disease but maintaining elevated CRP values have demonstrated concurrent elevation of MMP-10 as well as increased carotid IMT, suggesting that this enzyme may serve as an effective screening tool for subclinical atherosclerotic disease [68, 122]. When studied in smokers with cardiovascular risk factors but no clinical symptoms, MMP-10 was significantly elevated while inflammatory markers, other MMPs, and TIMP-1 remained stable [123]. Integration of biomarker and biochemical data may facilitate the identification of which MMP isotypes would be most efficacious to target pharmaceutically.

## Plaque Neovascularization and Vulnerability

Advanced atherosclerotic lesions may become infiltrated by vessels of the vasa vasorum in response to hypoxia and inflammatory mediators [124]. This centripetal angiogenesis is frequently localized to the plaque shoulders, suspected to begin early in lesion progression beyond fatty streaks, and regarded as the likely source of intraplaque hemorrhage, providing another mechanism for plaque instability [3, 125-127]. In fact, a greater density and tortuosity of infiltrating vessels has been associated with symptomatic carotid plaques [128]. Furthermore, carotid plaque neovascular density and the presence of intraplaque hemorrhages have been identified as independent predictors of cardiovascular events [129].

Therefore, promoting plaque stability may require quenching matrix proteolysis along with deterring EC invasion. MMPs are the point of intersection between these vascular remodeling processes.

Potent angiogenic growth factors such as FGFs, VEGF, and TGF- $\beta$  may be released from ECM sequestration through the local activity of MMPs induced during plaque progression, and these proteases are also instrumental in degrading pericyte and EC attachments [17]. For instance, MMP-1 and 7 can promote signaling through the VEGF receptor to upregulate EC proliferation, migration, and tube formation [130, 131]. The gelatinases and MT1-MMP have been heavily studied in developmental and pathologic angiogenesis and are of particular interest given that FGF as well as VEGF can stimulate ECs to secrete vesicles containing MMP-2, MMP-9, and MT1-MMP, thereby increasing and potentially initiating local proteolysis [132, 133]. MMP-9 can release ECM-bound growth factors, expose proangiogenic collagen-binding sites, and contribute to basement membrane degradation, while selective inhibition of MMP-9 in vitro results in decreased EC organization [40, 134]. MMP-2 is constitutively expressed by ECs and plays an essential role in proliferation, differentiation, and migration, but variations in the MT1-MMP activation complex may also cause MMP-2 to promote apoptosis, an additional mechanism for angiogenesis regulation that may be exploited during plaque progression [135]. In its location on the EC surface, MT1-MMP plays a significant role in pericellular proteolysis for migration and invasion; in fact MT1-MMP collagenase activity is required for EC tube construction in 3D collagen matrices as a precursor for vessel formation [136, 137].

Animal models exploring the functional overlap of MMPs involved in atherogenesis and angiogenesis have provided interesting results. Utilizing ApoE/MMP-8 double knockout mice to establish a role for this enzyme specifically in plaque neovascularization, investigators discovered that the atherosclerotic burden was decreased, aortic specimens had less EC sprouting, and cultured ECs from double knockout mice had impaired proliferation and migration [138]. Viral transfection to inhibit MMP-8 translation in human ECs in vitro confirmed decreased migration and identified downregulation of the angiotensin II signaling pathway as a key contributor to the altered gene expression and EC phenotype in the absence of MMP-8 [138]. Alternatively, in a hypercholesteremic rabbit model of aortic atherosclerotic plaque deposition, in situ levels of MMP-1, 2, 3, 9, MT1-MMP, and VEGF were quantitated at 2-week intervals and the plaques underwent histologic examination to document IMT, the vulnerability index (VI), and microvessel density (MVD) [139]. With progressive plaque growth, the expression of MMP-1, 2, 3, 9, and VEGF increased along with the VI, a value describing the macrophage and lipid components divided by the VSMC and collagen components [139]. Microvessels began to incorporate into plaques at 8 weeks and the MVD increased until 12 weeks at the conclusion of the study [139]. Surprisingly, MT1-MMP expression decreased throughout the study and was negatively correlated with VI as well as MVD [139], suggesting that EC liberation from the basement membrane may be achieved through different MMPs in diseased adventitia versus healthy tissues. Mediation of neovascularization through the VEGF signaling pathway appears to be preserved in atheromas, however, providing another avenue for potential therapeutic intervention. Despite significant progress, the definition of the key growth factors, proteases, and timeframe for

neovascularization will be instrumental in further attempts to stabilize atherosclerotic plaques through the inhibition of angiogenesis.

## Targets to Promote Plaque Stabilization

Medical management of atherosclerotic disease has significantly improved over the past 2 decades, reducing the incidence of major cardiovascular events in Westernized countries and supporting the paradigm that pharmaceutical plaque stabilization is a clinically relevant treatment goal [1]. Given the mounting pathophysiologic evidence that MMPs play a major role in plaque vulnerability, significant efficacy may be expected by targeting these remodeling proteases. Mouse models describing significant plaque alterations with selective MMP knockout or TIMP overexpression certainly support this line of pharmaceutical development, but the congenital abnormalities identified in these strains also forewarn of complex side effect profiles [140]. Further proof of concept has been derived from clinical reports of decreased rates of major cardiovascular events together with in vitro studies reporting modulation of MMP activity by HMG CoA reductase inhibitors (statins) as well as peroxisome proliferator activator receptor (PPAR- $\alpha$  and  $\gamma$ ) agonists, two medication classes frequently administered to patients with peripheral arterial disease [141-147]. Unfortunately, plaque rupture events do still occur, however, and directly targeting MMPs may provide additional preventive measures to decrease the associated morbidity and mortality.

Interest in engineering molecules to target MMPs has progressed since their role in tumor metastasis was identified and has not only endured despite several failures, but also expanded to consider therapies for arthritis, periodontal disease, and several cardiovascular diseases. Basic inhibition involves chelation of the active site zinc ion with the addition of alternative affinity sites to provide specificity and attempt to minimize side effects, most notably the musculoskeletal syndrome [148, 149]. Several synthetic nonselective MMP inhibitors have been constructed but studies of systemic as well as local administration have failed to demonstrate a meaningful impact on vascular remodeling, though it ought to be noted that very few of these investigations focused on peripheral arteries [150-153]. Doxycycline, a downregulator of general MMP activity, is currently approved in a low-dose form to treat periodontal disease, but its efficacy in attenuating atherosclerotic disease has been questionable [154]. This pharmaceutical has failed to minimize cardiovascular events in a randomized study of patients with stable coronary artery disease, and although carotid endarterectomy specimens have demonstrated good doxycycline penetration and reduced MMP-1 expression in the plaque after oral therapy for 2–8 weeks, no changes in plaque morphology or clinical outcome were noted, suggesting that the inhibition of alternative or additional MMPs may be necessary [155, 156].

Targeting a select MMP within an atherosclerotic plaque poses a significant challenge and these complex molecules have not yet reached clinical trials. Advanced biochemical methods have produced selective inhibitors of MMP-1, 2, 3, 8, 9, 12, and 13, but few have been tested in animal models thus far [157-171]. Increased collagen was noted in the plaques of ApoE knockout mice treated with an MMP-13 inhibitor, a marker of plaque stability, but no change in plaque burden could be appreciated [170]. The most promising results have been observed when these proatherogenic mice were treated with a selective MMP-12

inhibitor, where a 50% decrease in plaque burden, increased VSMC:macrophage ratio, smaller necrotic cores, and increased fibrous cap thickness were documented, supporting MMP-12 as a pertinent target for therapeutic strategies [172]. An alternative approach has involved the generation of humanized monoclonal antibodies and successful inhibition of MT1-MMP activity has been reported in tumor cells, with a concurrent decrease in the activation of pro-MMP-2 [173, 174]. An animal model of inflammatory bowel disease has been utilized to demonstrate the efficacy of an antibody for MMP-2 and 9, but applications to vascular remodeling have not been pursued [175]. An MMP-9-specific antibody has also been developed and proved to effectively inhibit hematopoietic cell mobilization in nonhuman primates, but further investigation into human trials has not occurred [176]. These encouraging results in animal models support the continued development of small-molecule MMP inhibitors to maximize bioavailability and minimize side effects so that efficacy in atherosclerotic disease may be assessed.

Interestingly, despite the significant morbidity of the musculoskeletal syndrome with general MMP inhibitors, overexpression of TIMPs has not produced this debilitating tendonitis in murine models. TIMP-1 overexpression in ApoE knockout mice has been shown to attenuate plaque progression [177]. TIMP-2 overexpression in ApoE knockout mice also reduced plaque size and increased histologic markers of plaque stability through inhibition of macrophage migration and apoptosis [178]. Targeted overexpression of TIMP-3 in macrophages effectively reduced plaque size and inflammatory infiltrate while promoting plaque stabilization through increased intact collagen, supporting TIMP-3 as a key mediator of proteolytic activity [179]. Adenovirus-mediated overexpression of TIMP-4 in carotid and aortic VSMCs effectively inhibited MMP-2 activation and VSMC migration while inducing apoptosis in these cells [180], but appropriate application of this data to atherosclerotic plaque stability requires further investigation. The safe and effective delivery of these adenoviruses to atherosclerotic plaques poses a significant challenge, but investigation into utilization of genetic variants has been initiated [181].

Another approach to stabilizing existing plaques may be through the inhibition of angiogenesis. For instance, treating ApoE knockout mice with angiostatin, endo-statin, or TNP-470, all known angiogenesis inhibitors that prevent EC proliferation and migration, successfully decreased atherosclerotic plaque growth and neovascularization when the drug was administered at late stages of plaque progression [182, 183]. Initiation of treatment at a younger age and continuation during the administration of a hypercholesterolemic diet, however, did not influence fatty streak and early fibrous lesion development in these mice [183], supporting the contribution of angiogenesis to late plaque growth and instability, and suggesting that an alternative method of targeting vessel growth may be more beneficial. As discussed earlier, several proangiogenic growth factors may be liberated from the local ECM through MMP activity, but these peptides also stimulate MMP expression. FGF-1 can impact on the early stages of angiogenesis by upregulating MMP-1 expression, FGF-2 can induce synthesis of MMP-2, 3, 7, 9, 10, 11, and 13, PDGF promotes MMP-13 transcription, and VEGF can stimulate the production of MMP-2 [184-187], all major contributors to EC migration and atherosclerotic plaque vulnerability, therefore modulation of growth factor binding may decrease MMP secretion and prevent EC migration to combine two potent mechanisms of plaque stabilization. Unfortunately, clinical trials of monoclonal antibodies

for some proangiogenic growth factors/receptors, especially those targeting VEGF, have demonstrated a prohibitively high rate of cardiovascular complications [188]. Exploration of this paradox with the ApoE knockout mouse has yielded conflicting results. Adenovirus-mediated overexpression of VEGF had no effect on aortic atherosclerotic plaque deposition or neovascularization [189], but systemic therapy with VEGF inhibitor led to accelerated atherogenesis [190]. Newer oncologic antiangiogenesis agents targeting combinations of the FGF, PDGF, and/or VEGF receptors have not demonstrated similar toxicities but evaluation in the treatment of peripheral arterial disease has not been pursued [191-193]. The long-term therapy required for a chronic disease such as peripheral artery disease may be another limiting factor in the utilization of these pharmaceuticals; therefore, research pursuing the more specific alternative of a selective MMP inhibitor to mitigate plaque neovascularization may be a more productive avenue.

## Conclusion

Despite advances in pharmacologic therapy, atherosclerosis with vulnerable plaque morphology continues to contribute to morbidity and mortality in Westernized countries. Knowledge of the intraplaque pathophysiology resulting in instability continues to accumulate, but viable therapies to quench proteolysis or angiogenesis are increasingly complex due to the recognized necessity for selective, targeted agents so that patient tolerance may be maximized. Focusing on the generation and activity of MMPs in both atherosclerosis and angiogenesis provides a unified pathway toward plaque stability and continued research into the delivery and bioavailability of selective MMP inhibitors is warranted.

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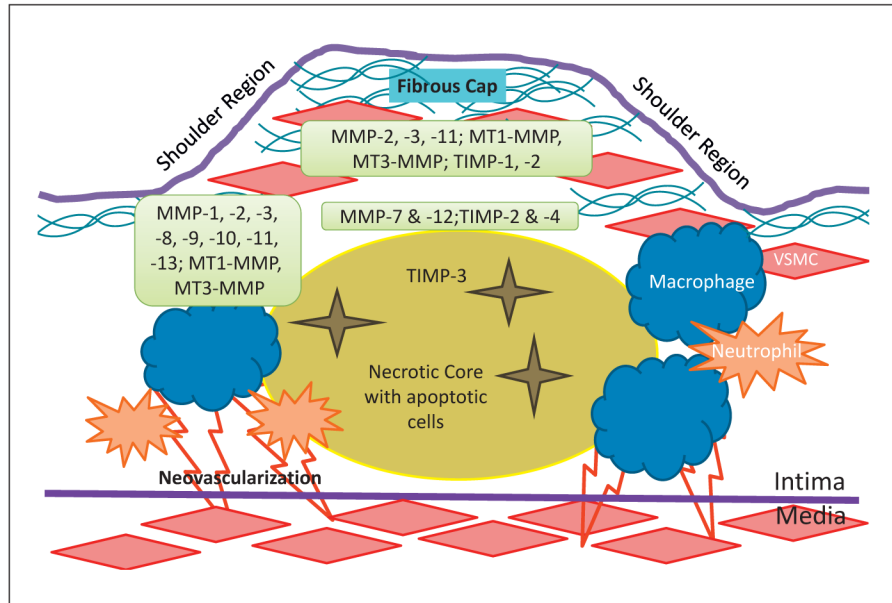


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**Fig. 1.** Illustration of the regional distribution of MMPs in atherosclerotic plaque.

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**Table 1.**

Summary of MMP and TIMP contributions to plaque vulnerability and angiogenesis as well as current data on selective MMP inhibitors

<b>MMP/TIMP</b>	<b>Effect on plaque vulnerability</b>	<b>Contribution to angiogenesis</b>	<b>Inhibitors tested</b>	<b>References</b>
MMP-1	Increased at shoulders and intraplaque rupture	Promotes VEGF signaling	Doxycycline, expression reduced in carotid plaques	24-30, 118, 143, 144
MMP-2	Potentially stabilizing through VSMC migration	Promotes EC migration	Antibodies, efficacy in inflammatory bowel	32, 48, 49, 119, 120, 123
MMP-3	Potentially stabilizing			50, 61-64
MMP-7	Proteoglycan degradation and VSMC apoptosis under fibrous cap	Promotes VEGF signaling		50, 68-71, 118
MMP-8	Increased at shoulders and areas of neovascularization	EC migration		32-37, 126
MMP-9	Potentially regional, colocalizes to vulnerable regions but also evidence of promoting collagen organization	Releases proangiogenic growth factors from the ECM, promotes EC organization	Antibodies, efficacies in inflammatory bowel and hematopoietic cell migration	32, 40, 50-56, 120, 121, 163, 164
MMP-10	Localized to rupture-prone regions			66
MMP-11	Localized to inflammatory mediators			67
MMP-12	Proteolysis between lipid core and fibrous cap		Synthetic, increased plaque stability and 50% decrease in burden	29, 50, 53, 68, 88, 160
MMP-13	Contributes to disorganized collagen		Synthetic, increased plaque stability but no change in burden	31, 158
MT1-MMP	Proteolysis in rupture-prone areas	Pericellular proteolysis for EC migration	Antibodies, reduced activity in tumor cells	73, 74, 77-81, 124, 125, 161, 162
MT3-MMP	Proteolysis in rupture-prone areas			82
TIMP-1	Stabilizing		Overexpression attenuates plaque	92, 165
TIMP-2	Stabilizing		Overexpression reduces plaque size	91, 94, 166
TIMP-3	Unclear, may promote VSMC apoptosis		Overexpression reduces plaque size	90, 96, 167
TIMP-4	Unclear, localizes to the border of the lipid core, potentially inhibits VSMC migration		Overexpression inhibits VSMC migration	96-98, 169