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Abstract: Atherosclerosis underlies most cardiovascular diseases, and is accepted as a primary cause of mortality worldwide. Proteases have been implicated in the development and progression of atherosclerosis, due to their ability to provoke focal destruction of the vascular extracellular matrix. Members of the metalloproteinase family, especially matrix metalloproteinases (MMPs), and their endogenous tissue inhibitors (TIMPs) have been suggested to perform complex dual roles during late-stage progression and rupture of atherosclerotic plaques. Proposed favourable actions of metalloproteinases include the promotion of vascular smooth muscle growth and survival which stabilises plaques, while conversely extracellular matrix destruction alongside interminable monocyte/macrophage accumulation can encourage plaque rupture. This review provides a summary of the cogent evidence connecting the contribution of individual metalloproteinases to atherosclerotic plaque development, progression, and instability. Topics discussed include structural, functional and cell-specific diversity of MMP members; evidence from animal models of atherosclerosis and comparisons with findings in humans; the dual role of MMPs and the requirement to selectively target individual MMPs; and the need for efficient surrogate markers of MMP inhibition. Accordingly, as our knowledge of the complex roles individual MMPs play especially during the progression and rupture of atherosclerotic plaques expands, new impetus is required for clinical trials evaluating the therapeutic potential of selective MMP inhibition, which could limit cardiovascular morbidity and mortality worldwide.

Metalloproteinases in atherosclerosis

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Abstract/Summary

Atherosclerosis underlies most cardiovascular diseases, and is accepted as a primary cause of mortality worldwide. Proteases have been implicated in the development and progression of atherosclerosis, due to their ability to provoke focal destruction of the vascular extracellular matrix. Members of the metalloproteinase family, especially matrix metalloproteinases (MMPs), and their endogenous tissue inhibitors (TIMPs) have been suggested to perform complex dual roles during late-stage progression and rupture of atherosclerotic plaques. Proposed favourable actions of metalloproteinases include the promotion of vascular smooth muscle growth and survival which stabilises plaques, while conversely extracellular matrix destruction alongside interminable monocyte/macrophage accumulation can encourage plaque rupture. This review provides a summary of the cogent evidence connecting the contribution of individual metalloproteinases to atherosclerotic plaque development, progression, and instability. Topics discussed include structural, functional and cell-specific diversity of MMP members; evidence from animal models of atherosclerosis and comparisons with findings in humans; the dual role of MMPs and the requirement to selectively target individual MMPs; and the need for efficient surrogate markers of MMP inhibition. Accordingly, as our knowledge of the complex roles individual MMPs play especially during the progression and rupture of atherosclerotic plaques expands, new impetus is required for clinical trials evaluating the therapeutic potential of selective MMP inhibition, which could limit cardiovascular morbidity and mortality worldwide.

Index words

Atherosclerosis; proteases; metalloproteinase; MMP; TIMP; ADAM; ADAMTS cell

1 Introduction

The destruction and loss of collagen, elastin, and other extracellular matrix proteins from the fibrous cap and adjacent shoulder regions overlying the necrotic core of atherosclerotic plaques, is considered to precipitate plaque rupture and consequent clinical sequelae such as myocardial infarction and stroke. Accordingly, researchers have examined the production of extracellular proteases from vascular cells resident within the blood vessel wall and atherosclerotic plaque, especially foam cell macrophages as they are an abundant source of proteases and accumulate in advanced unstable plaques (Johnson and Newby 2009). Cysteine proteases (including cathepsins), serine proteases (for example the plasminogen activator-plasmin system, and mast cell related

proteases including chymase and tryptase), and metalloproteinases (including the MMP family) all exist within human plaques. These differing but complimentary protease families use different modes of substrate hydrolysis (for example, cysteine, serine, metalloproteases) and harbour the potential to moderate essentially all aspects of atherosclerosis, from lesion formation, development and subsequent progression to plaque instability and thrombosis, predominantly through secondary effects on extracellular matrix proteolysis alongside modulating vascular cell proliferation, migration, and apoptosis. Indeed, a host of biochemical and histological studies have defined a prominent role for proteases in atherosclerotic plaque progression and vulnerability, alongside delineating the cellular sources of potential culprit proteases. However, to determine causality over guilt by association, researchers have turned to animal models to elucidate the contribution of select proteases in atherogenesis, the formation of advanced plaques, and the progression to unstable and rupture-prone lesions. Most of the published studies have focussed on members of the metalloproteinase family, and as such, this group of proteases will form the focus of this review.

2 Metalloproteinases

The metalloproteinase family of proteases (also termed metzincins) consist of ADAMs (a disintegrin and metalloproteinase family), ADAMTSs (ADAM with thrombospondin motifs), and MMPs (matrix metalloproteinase), as they all contain a zinc atom and a conserved methionine in the catalytic domain (Murphy 2010). The roles of these three related families will be discussed separately, with particular focus on MMPs, as they have been extensively researched in the atherosclerosis arena.

2.1 ADAMs

ADAM proteases cleave numerous cell-surface molecules including receptors for cytokines, chemokines, and growth factors, alongside a range of adhesion molecules, and as such their role in cardiovascular diseases is now gaining interest (van der Vorst et al. 2012). ADAM9, ADAM10, ADAM15, ADAM17, and ADAM33 have all been shown to be present within human atherosclerotic plaques and at increased levels compared to healthy vessels (Satoh et al. 2008, Oksala et al. 2009, Donners et al. 2010, Holloway et al. 2010). While ADAM9, 15 and 17 expression is localised to macrophage-rich regions (Oksala et al. 2009), and ADAM10 within neovascular endothelial cells and macrophages (Donners et al. 2010), ADAM33 was preferentially present within plaque smooth muscle cells (Holloway et al. 2010). Genetic studies have also provided associative evidence for ADAM8 (Levula et al. 2009), ADAM17 (Holdt et al. 2008) and ADAM33 (Holloway et al. 2010) expression in atherosclerosis. Animal studies have been recently undertaken in an attempt to

demonstrate causality over association alone (see Table 1). Myeloid-specific deletion of ADAM10 in Ldlr KO mice did not affect plaque size, but favourably altered composition through increased collagen content and decreased macrophage accumulation in advanced atherosclerotic lesions (van der Vorst et al. 2015), supporting a pro-atherosclerotic role for ADAM10. Genetic ablation of ADAM15 in Apoe KO mice limited plaque development, attributable to ADAM15 promoting endothelial cell dysfunction and monocyte transmigration (Sun et al. 2012). In direct contrast gain- and loss-of-function studies in athero-susceptible rabbits demonstrated that ADAM15 activity exerts protective effects on plaque progression, potentially through ADAM15-mediated cleavage of endothelial adhesion molecules which act to facilitate monocyte recruitment (Bültmann et al. 2011). These discordant findings may highlight the importance of determining the temporal and spatial expression of ADAM15 during atherogenesis. Plaque size was increased and composition adversely altered in ADAM17 hypomorphic mice (used as ADAM17 global KO mice are not viable) ascribed to over-activation of TNFR2 signalling, and therefore implying an atheroprotective role of ADAM17 (Nicolaou et al. 2017). The aforementioned ADAMs have been shown to have many substrates, a number of which may underlie their effects on atherosclerosis. In particular, ADAMs can influence the recruitment and differentiation of inflammatory cells through the release of chemokines (CX3CL1 and CXCL16), adhesion molecule modulation (ICAM-1 and VCAM-1), and generation of active pro-inflammatory mediators (TNFα) (Dreymueller et al. 2012, van der Vorst et al. 2012). Evidently further research is required to clarify the contribution of individual ADAMs to atherosclerotic plaque progression and instability, particularly if these proteases are to be pursued as therapeutic targets.

2.2 ADAMTSs

ADAMTS proteases have been implicated in cardiovascular diseases, especially atherosclerosis due to their ability to cleave proteoglycans (Salter et al. 2010), which populate pre-atherosclerotic adaptive intimal thickenings and early lesions, and contribute to the retention of lipids alongside adhesion and recruitment of monocyte/macrophages (Nakashima et al. 2007, Otsuka et al. 2015). Within human atherosclerotic lesions, ADAMTS1 is expressed by smooth muscle cells (Jönsson-Rylander et al. 2005), and can stimulate migration of vascular smooth muscle cells in culture (Jönsson-Rylander et al. 2005), and therefore ADAMTS1 may favour plaque stability. Conversely, ADAMTS4 and ADAMTS8 are prominent in the macrophage-rich regions and their expression increases during lesion progression (Wågsäter et al. 2008). Moreover, elevated circulating levels of ADAMTS4 have been reported in patients with coronary artery disease and acute coronary syndromes (Zha et al. 2010). Concurring, loss of ADAMTS4 reduced atherosclerosis and enhanced

plaque stability in Apoe KO mice (Kumar et al. 2016). A protective role in atherosclerosis has been ascribed to ADAMTS5 as its expression is depleted in atherosclerotic aortas, leading to the accumulation of proteoglycans such as versican and biglycan, which may favour lipid retention and macrophage accumulation (Didangelos et al. 2012).

Genome-wide association studies have identified single-nucleotide polymorphisms within the genomic region of ADAMTS7 which associate with coronary artery disease (Reilly et al. 2011) (Patel and Ye 2013). However, there is controversy over the contributory role of ADAMTS7 to atherogenesis and disease progression. Histological studies have demonstrated vascular smooth muscle cells (VSMCs) express abundant ADAMTS7 within carotid and coronary atherosclerotic plaques (Reilly et al. 2011, Pu et al. 2013). In association, ADAMTS7 has been shown to play a functional role in promoting VSMC migration and neointima formation (Wang et al. 2009, Pu et al. 2013, Bauer et al. 2015), in part through cleavage of its primary substrate cartilage oligomeric protein (COMP), which normally exerts an inhibitory effect on VSMC motility (Hanby and Zheng 2013, Pu et al. 2013). Further mechanistic studies highlighted that VSMCs with an ADAMTS7 SNP leading to a Ser-to-Pro substitution in its pro-domain (rs3825807), displayed reduced ADAMTS7 maturation, COMP cleavage, and VSMC migration, and the variant associated with protection from atherosclerosis (Pu et al. 2013). Proof-of concept studies in Ldlr or Apoe KO mice also deficient for ADAMTS7, further supported a role for this protease in VSMC growth and atherogenesis, demonstrating that aortic plaque size was reduced in ADAMTS7 KO atherosclerotic mice, which was associated with diminished VCSMC content (Bauer et al. 2015). While ADAMTS7 may foster atherogenesis by promoting VSMC migration and intimal growth, its role in facilitating VSMC motility may act beneficially in advanced plaques through maintaining fibrous cap stability. However, recent evidence suggested elevated ADAMTS7 expression is associated with macrophage number and concomitant with an unstable plaque phenotype. In human carotid lesions, high levels of ADAMTS7 positively correlated with macrophage and lipid content, but with low VSMC and collagen content, which are considered characteristics of a vulnerable plaque phenotype (Bengtsson et al. 2017). Moreover, above median ADAMTS7 levels were associated with increased risk for future cardiovascular events (Bengtsson et al. 2017).

The expression of ADAMTS2, 3, 13, and 14 has also been documented within culprit coronary plaques of patients with acute myocardial infarction or stable angina (Lee et al. 2012). Relatedly, diminished ADAMTS13 activity correlates with increased risk of stroke (Sonneveld et al. 2015). In accordance, genetic deletion of ADAMTS13 accelerates early atherosclerotic plaque formation in Apoe KO mice, presumed as a consequence of augmented von Willebrand factor cleavage, platelet

deposition and inflammatory cell recruitment (Jin et al. 2012). As such, strategies to promote ADAMTS13 expression may prove advantageous to dampen atherosclerosis.

Collectively ADAMTS proteases share many common substrates, such as the aforementioned COMP, versican, biglycan and aggrecan, all ECM proteins associated with vascular pathologies (Kelwick et al. 2015). While ADAMTS-mediated degradation of these ECM molecules may limit the retention of atherogenic lipids and therefore be protective during atherogenesis, their cleavage can also promote atherosclerosis through the stimulation of VSMC migration (Didangelos et al. 2012, Pu et al. 2013).

2.3 Matrix metalloproteinases

The MMP family consists of 23 human zinc-dependent endopeptidases. MMPs are a large family of proteolytic enzymes and have been shown to play fundamental roles during morphogenesis, wound healing, tissue repair and remodelling. They also participate in the pathophysiology of numerous major diseases including cancer progression and atherosclerosis, and are therefore considered prominent therapeutic targets for preventative medicine. With regards to the vasculature, collectively MMPs harbour the potential to degrade all the major components of the blood vessel wall. Accordingly, their activity is tightly regulated by a family of endogenous inhibitors termed the tissue inhibitors of metalloproteinases (TIMPs) that, alongside MMPs, maintain a vital balance over ECM homeostasis during physiological and pathophysiological conditions. In addition to their proteolysis of matrix proteins, numerous non-ECM molecules have also been highlighted as potential MMP substrates (such as cell-surface and soluble mediators), bestowing new roles for this broad family of proteases and their inhibitors in processes pertinent to atherosclerosis such as proliferation, migration/invasion, and apoptosis of resident blood-vessel cells, such as vascular smooth-muscle cells (Johnson 2007), endothelial cells (van Hinsbergh and Koolwijk 2008), and several inflammatory cell types including monocytes (Khokha et al. 2013).

The MMPs are multi-domain proteases that share similar structure to ADAMs and ADAMTSs, while also displaying a high degree of homology between themselves (Fig.1), and are regulated by a medley of inflammatory cytokines, growth factors, hormones, and physical cell–cell and cell–matrix interactions (Nagase et al. 2006). Specifically within the MMP family, members are further divided into sub-families based on subtle variances in their structure, function, activation, and cellular location, which confers substrate specificity (Fig.1) (Nagase et al. 2006). Consequently, MMPs are grouped into collagenases (MMP-1, -8, and -13) that preferentially cleave fibrillar collagens, gelatinases (MMP-2 and -9) that efficiently cleave denatured collagen (ie, gelatin), and stromelysins (MMP-3, -10, and -11) that have broad specificity but do not effectively cleave intact fibrillar collagens (Visse and Nagase 2003, Nagase et al. 2006). Similarly, Matrilysin (MMP-7) does not

efficiently degrade fibrillar collagens, but can cleave many extracellular components including fibronectin, collagen type IV, laminin and elastin (Burke 2004). Macrophage metalloelastase (MMP-12), as it name suggests, primarily cleaves elastin (Visse and Nagase 2003, Nagase et al. 2006). With the exception of MMP-11 which is activated intracellularly by furin before secretion (Pei and Weiss 1995), the aforementioned MMPs are secreted in a latent form and require activation. Conversely, membrane-type MMPs (MT-MMPs) are membrane-bound in an active form (including MMP-14 to -17, -25, and -26) (Fig.1) (Visse and Nagase 2003, Nagase et al. 2006), and thought to direct peri-cellular proteolysis of a wide-range of substrates including cell-surface molecules (Seiki 2002). Accordingly, MT-MMPs have been referred to as 'sheddases' due to their membrane-bound location, and can effectively modulate cell behaviour through the proteolysis of membrane proteins including cell-cell and cell-matrix contacts, integrins, cytokine and growth factor receptors (Seiki 2002, Murphy 2010).

Activation of the biologically inactive MMP (pro-form) is a multi-step process also known as 'stepwise activation'. Firstly, a 'proteinase susceptible bait region' is cleaved through the action of plasmin or bacterial proteinases, which weakens the cysteine-Zn^{2+} negative interaction within the pro-domain, inducing the creation of a MMP intermediate form. In order achieve full activation, the *in-trans* activity of other intermediaries or other active-MMPs is required to fully dislocate the inhibitory pro-domain (Nagase et al. 2006). Within the plaque environment, there are numerous specific pathways of MMP activation which include members of the plasmin system such as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), both of which can generate plasmin from plasminogen, and therefore indirectly activate multiple MMPs including MMP-1, MMP-3, MMP-7, MMP-9 and MMP-12 (Lijnen 2001). Further supporting a link between thrombosis and ECM degradation, thrombin can promote activation of certain MMPs, including MMP-2 (Galis et al. 1997). Mast cells are present in abundant numbers within advanced atherosclerotic plaques (Kaartinen et al. 1998, Willems et al. 2013) and release specific proteases (chymase and tryptase) which are capable of activating select MMPs; for example, MMP-1 and MMP-3 (Lees et al. 1994, Johnson et al. 1998). Many cathepsins are present in atherosclerotic lesions, such as cathepsin G and K (Sukhova et al. 1998, Legedz et al. 2004), which have been identified to harbour the ability to activate MMPs, notably MMP-1, MMP-3 and MMP-9 (Okada and Nakanishi 1989, Saunders et al. 2005, Wilson et al. 2009, Christensen and Shastri 2015). Circulating levels of the serine protease tissue kallikrein are related to carotid atherosclerosis severity (Porcu et al. 2004), and has been shown to activate MMP-9 (Desriviéres et al. 1993). Finally, MMPs themselves can promote proteolytic amplification through the activation of other family members, such as the well-characterised activation of MMP-2 by MMP-14 (Strongin et al. 1995).

MMP activity is tightly regulated and counterbalanced by endogenous inhibitors (such as TIMPs and α2-macroglobulin), proteolysis, or internalization and recycling (Nagase et al. 2006). Further proteins have been identified which also display the ability to inhibit MMPs, such as; the reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tissue factor pathway inhibitor-2 (TFPI-2), and the pro-collagen C-terminal proteinase enhancer (PCPE). Still, TIMPs are the most potent endogenous inhibitors of MMPs and therefore considered key regulators of MMP activity under physiological and pathophysiological conditions. Four TIMPs have been identified (TIMP-1, -2, -3 and -4), which exhibit varied inhibitory actions towards different MMP family members (Baker et al. 2002). For example, TIMP-1 displays a poor inhibitory effect on MMP-9, -14, -15, -16, and -24, while TIMP-2 possesses a higher affinity for pro-MMP-2 (Strongin et al. 1993). TIMPs also harbour the ability to inhibit members of both the ADAM and ADAMTS family of proteinases, particularly TIMP-3 (Baker et al. 2002). Like MMPs, TIMPs are finely regulated during development and remodelling, and their expression is tissue. The inhibitory capacity of TIMPs has mainly been ascribed to the N-terminal domain since it is able to form, when isolated, a stable native molecule with an inhibitory effect on MMPs (Brew and Nagase 2010). TIMPs are secreted soluble proteins with the exception of TIMP-3 which is insoluble and has a high affinity for ECM proteins, permitting its accumulation within the extracellular milieu and prolonging its half-life (Baker et al. 2002). However through interactions with MT-MMPs, several TIMPs can localise to the cell membrane (Strongin et al. 1993, Hernandez-Barrantes et al. 2001).

Therefore, and as previously inferred, maintaining equilibrium between MMPs and TIMPs is essential in homeostasis. As such alterations in this balance can trigger patho-physiological conditions associated with aberrant ECM turnover and/or dysregulation of processes involved in wound healing, remodelling and inflammation. Arthritis, cancer and cardiovascular diseases represent pathologies where an imbalance between MMPs and TIMPs is evident (Brew and Nagase 2010). Consequently, over the last thirty years there has been great efforts into defining the expression and potential contribution of MMPs in the pathogenesis of atherosclerosis. The cellular sources of MMPs within human plaques are shown in Fig.2 and their potential roles in animal models summarised in Table 2 and discussed below.

2.3.1 Collagenases (MMP-1, MMP-8 and MMP-13)

The thrombogenic core of advanced atherosclerotic plaques is composed of extracellular lipids, cell debris, and lipid-laden macrophages (termed foam cell macrophages). In stable plaques, this core is characteristically protected from the circulating blood by a fibrous cap, which is rich in type I, II, and III fibrillar collagens. It has been proposed that proteolysis of fibrillar collagens within the protective

fibrous plaque precipitates plaque destabilisation (Libby 2013). Accordingly, several studies have documented elevated expression and activity of MMP-1 (Nikkari et al. 1995, Johnson et al. 1998, Sukhova et al. 1999), MMP-8 (Herman et al. 2001, Molloy et al. 2004) and MMP-13 (Sukhova et al. 1999) in human atherosclerotic plaques, associated with ECs, vascular smooth muscle cells, and macrophages. Moreover, polymorphisms within the promoters of the MMP-1 (Ghilardi et al. 2002), MMP-8 (Djurić et al. 2011), and MMP-13 (Yoon et al. 2002, Vašků et al. 2012) genes are associated with aortic (Yoon et al. 2002), carotid (Ghilardi et al. 2002, Djurić et al. 2011), and coronary (Vašků et al. 2012) atherosclerosis. In addition, enhanced collagenolytic activity attributed to MMP-1, -8, and -13 has been detected in plaques characterised as unstable (Sukhova et al. 1999, Molloy et al. 2004). Furthermore, elevated plasma levels of MMP-1 (Cavusoglu et al. 2015) and MMP-8 (Djurić et al. 2010, Momiyama et al. 2010) have been detected in patients with atherosclerosis, while no association was detected for plasma levels of MMP-13 (Momiyama et al. 2010). Finally, elevated intra-plaque and plasma levels of MMP-8 are predictive for systemic cardiovascular events (Peeters et al. 2011) and subsequent all-cause mortality (Cavusoglu et al. 2015), respectively.

Unlike humans, mice do not actively express MMP-1. Nonetheless a study where human MMP-1 was over-expressed selectively in macrophages of apolipoprotein E knockout (Apoe KO) mice, showed unexpectedly a reduction in plaques size and collagen content, an indication of less advanced atherosclerosis and intimating that MMP-1 is beneficial in delaying the progression of atherosclerosis (Lemaître et al. 2001). MMP-8-deficiency in the Apoe KO mouse model reduced aortic atherosclerotic plaque coverage and the lesions contained fewer macrophages and smooth muscle cells (Laxton et al. 2009) implying reduced atherogenesis, although it has also been demonstrated that MMP-8 is necessary for vascular smooth muscle growth (Xiao et al. 2014), suggesting a role in maintaining fibrous cap integrity. It must be noted that the effects of MMP-8 on atherogenesis maybe secondary as it has been shown that MMP-8 cleaves angiotensin (Ang) I to generate Ang II and modulate blood pressure in the Apoe KO mouse (Laxton et al. 2009), and affect stem/progenitor cell migration and subsequent recruitment to atherosclerotic lesions (Xiao et al. 2013). A study using Apoe KO mice that were also deficient for Mmp13 had negligible effects on aortic plaque size, or plaque macrophage and vascular smooth muscle cell content, although fibrillar collagen regulation and organisation was hampered, indicating a role for MMP-13 in regulating plaque collagen accumulation and structure (Deguchi et al. 2005). A further study using MMP-8 and MMP-13 double deficient and single deficient mice within the Apoe KO mouse model, also revealed negligible effects of these collagenases on plaque size (at both the aortic root and brachiocephalic artery), or macrophage and smooth muscle cell density within the plaques, either singularly or in combination (Quillard et al. 2014). However, MMP-13 did predominate over MMP-8 with regards to

collagenolytic activity and associated plaque collagen content (Quillard et al. 2014). Encouraged by the above findings, pharmacological MMP-13 inhibition in the Apoe KO mouse was assessed on nascent and developed plaques within the aortic root (Quillard et al. 2011). In agreement with the above gene deletion studies, MMP-13 inhibition (ascertained through reduced intra-plaque collagenolytic activity) did not affect plaque size, or macrophage and smooth muscle cell plaque content, but favourably increased collagen accumulation in plaques (Quillard et al. 2011).

2.3.2 Stromelysins (MMP-3, MMP-10 and MMP-11)

Stromelysins display a broad range of substrates underlying their ability to modulate numerous biological activities, including the activation of numerous other proteases such as proMMPs (Nagase et al. 2006). Of the stromelysins, MMP-3 has been given the most attention in atherosclerosis-related research. In situ mRNA hybridisation revealed MMP-3 expression within human rupture-prone atherosclerotic plaques (Henney et al. 1991), which has been further corroborated at the protein level in smooth muscle cells and macrophages, and associated with increased intra-plaque proteolytic activity (Galis et al. 1994, Galis et al. 1994, Johnson et al. 1998). These findings suggest a role for MMP-3 in advanced atherosclerosis, but genetic studies demonstrated that the progression of coronary atherosclerosis and acute myocardial infarction was associated with a polymorphism in the human MMP-3 promoter which results in reduced gene expression (Ye et al. 1995, Ye et al. 1996, Terashima et al. 1999). However elevated plasma levels of MMP-3 have been associated with the presence of carotid atherosclerosis (Beaudeux et al. 2003) and prediction of recurrent acute myocardial infarction (Cavusoglu et al. 2016), although this may represent the healing of existing plaque disruptions as MMP-3 expression and activity regulates smooth muscle cell growth (Johnson et al. 2011). In agreement, MMP-3 deletion in Apoe KO mice resulted in larger aortic and brachiocephalic artery plaques (Silence et al. 2001, Johnson et al. 2005), associated with reduced smooth muscle cell content and a concomitant increase in buried fibrous layers (Johnson et al. 2005) (a surrogate marker of previous plaque disruption (Johnson et al. 2005)), indicating MMP-3 affords greater stability.

MMP-10 has been localised to macrophage-rich regions and endothelial cells within advanced human atherosclerotic plaques (Montero et al. 2006). Elevated plasma levels of MMP-10 were detected in patients with carotid plaques compared to controls, and associated with the systemic pro-inflammatory marker high-sensitivity C-reactive protein (hs-CRP) (Orbe et al. 2007). Raised circulating plasma levels of MMP-10 in patients with chronic kidney disease or peripheral arterial disease correlated with atherosclerosis severity (Coll et al. 2010) and predicted all-cause and cardiovascular disease-related mortality (Martinez-Aguilar et al. 2015), respectively. Recent studies have proposed that MMP-10 regulates macrophage migration and invasion (Murray et al. 2013), and

through induction by thrombin may exert a fibrinolytic action (Orbe et al. 2009). Consequently, it is suggested that MMP-10 is pro-atherogenic, however the direct role of MMP-10 in atherosclerosis has not yet been assessed.

MMP-11 expression has been co-localised with endothelial cells, smooth muscle cells, and macrophages within carotid and aortic plaques (Schönbeck et al. 1999), and is up-regulated in these cell types by the pro-inflammatory molecule CD40 ligand in vitro and in vivo (Schönbeck et al. 1999). Interestingly, studies in knockout mice demonstrated that MMP-11 impairs smooth muscle growth and neointima formation (Lijnen et al. 1999), implying that in atherosclerotic plaques MMP-11 may exert deleterious effects on fibrous cap integrity. As such the direct examination of the contribution of MMP-11 to atherosclerosis in an in vivo model warrants further investigation.

2.3.3 Gelatinases (MMP-2 and MMP-9)

The gelatinases, MMP-2 and MMP-9, have been extensively studied in several cardiovascular diseases, due in part to their fundamental role in regulating vascular smooth muscle cell migration and proliferation (Johnson 2007). Within non-atherosclerotic and atherosclerotic arteries, MMP-2 is expressed at similar levels in intimal and medial smooth muscle cells and luminal endothelial cells, while plaque macrophages and microvascular endothelial cells also express notable levels (Galis et al. 1994, Galis et al. 1994). Increased MMP-2 expression and activity has been correlated with the development of human aortic plaques (Li et al. 1996), and elevated plasma MMP-2 levels are detected in patients which present clinically with coronary (Kai et al. 1998) or carotid (Alvarez et al.) atherosclerotic plaque complications. However, other studies have shown no relationship between intra-coronary plaque MMP-2 expression and clinical cardiovascular outcomes (Fiotti et al. 2008) or indeed demonstrated MMP-2 expression is associated with less advanced and stable carotid artery atherosclerosis (Choudhary et al. 2006, Sluijter et al. 2006). Pertinent studies in animal models of atherosclerosis have also yielded discordant findings. Whilst studies in hypercholesterolaemic rabbits intimated that elevated MMP-2 levels in aortic plaques drives plaque progression towards and unstable phenotype (Zaltsman and Newby 1997, Kuge et al. 2007), deletion of MMP-2 in atherosclerosis-susceptible mice results in decreased vascular smooth muscle cell accumulation within aortic plaques, although plaque areas are smaller than those in MMP-2 wild-type animals (Kuzuya et al. 2006, Sasaki et al. 2013), and are less calcified (Sasaki et al. 2013). As such, MMP-2 may contribute to plaque development, particularly through the formation of adaptive intimal thickenings (Kuzuya et al. 2003), but conversely protect from plaque progression and instability by preserving vascular smooth muscle cell accumulation.

In opposition to the ubiquitous expression of MMP-2, MMP-9 expression is increased in plaque smooth muscle cells and endothelial cells, alongside high expression in macrophages and

microvascular endothelial cells, resulting in heightened proteolytic activity when compared to non-atherosclerotic arteries (Galis et al. 1994). It has been suggested that increased MMP-9 expression and activity is in response to a preponderance of pro-inflammatory molecules within atherosclerotic plaques, such as IL-1β, TNFα and CD40L (Galis et al. 1994, Schönbeck et al. 1997). Human carotid plaque MMP-9 expression and activity has been shown to associate with an unstable plaque phenotype (Loftus et al. 2000, Peeters et al. 2011), but failed to function as predictive for the occurrence of systemic cardiovascular outcomes on follow-up (Peeters et al. 2011). However, numerous studies have shown elevated circulating levels of MMP-9 are associated with cardiovascular disease, including patients presenting with acute coronary syndrome (Kai et al. 1998, Inokubo et al. 2001), and patients with documented coronary artery atherosclerosis (Blankenberg et al. 2003). These findings have been interpreted as evidence that MMP-9 is causally involved in the process of acute plaque rupture (Silvello et al. 2014). However it is plausible that MMP-9-dependent processes are activated in response to acute plaque rupture, triggering a healing response that involves the recruitment of smooth muscle cells and the elaboration of matrix, a well-described role for MMP-9 in the vasculature (Johnson 2007). As such, after an acute coronary event, an increase in circulating MMP-9 concentration may be a consequence of smooth muscle cell growth during plaque healing. Indeed, a recent study demonstrated that carotid atherosclerotic plaques stabilise after stroke, as evidence by an increase in smooth muscle cell content which was preceded by elevated MMP-9 levels (Peeters et al. 2009).

Further support for this supposition comes from mouse studies. Relevant to the pathogenesis of atherosclerosis, vascular injury rodent models have demonstrated a fundamental role for MMP-9 in facilitating vascular smooth muscle cell migration and subsequent intimal thickening (Cho and Reidy 2002, Johnson and Galis 2004, Johnson et al. 2011) or geometric remodelling (Galis et al. 2002). Indeed, brachiocephalic plaques from Apoe KO mice also deficient for MMP-9 contained larger plaques with increased macrophage number and concomitant decreased vascular smooth muscle cell content (Johnson et al. 2005). These findings mirror those observed in MMP-3 knockout atherosclerotic mice (Johnson et al. 2005), which may be explained by the recent elucidation that MMP-3 can activate MMP-9 and facilitate vascular smooth muscle cell migration (Johnson et al. 2011). Conversely, plaque burden within the aorta of Apoe and MMP-9 double KO mice was decreased compared to control animals, although this observation was associated with reduced collagen content and synthesis by vascular smooth muscle cells (Luttun et al. 2004). Transgenic experiments deploying macrophage-specific over-expression of MMP-9 did not influence atherosclerotic lesion stability within the brachiocephalic artery (Gough et al. 2006). However, over-expression in macrophages of a mutated form of MMP-9, which is fully auto-activated, induced

multiple features associated with plaque instability within the brachiocephalic artery and aorta such as signs of previous haemorrhage, although no changes in lesion area or composition were reported (Gough et al. 2006). Similarly, lesional over-expression of MMP-9, which induced increased proteolytic activity, did not affect plaque size or composition in a perivascular collar model, although the incidence of intra-plaque haemorrhage was markedly increased in advanced lesions (de Nooijer et al. 2006). Bone-marrow transplantation studies have also provided conflicting results, implying that MMP-9 produced from vascular smooth muscle cells regulates atherosclerosis in a carotid-ligation model (Choi et al. 2005), while myeloid cells were considered the principal source of MMP-9 during the progression of atherosclerosis in hypercholesterolaemic Apoe KO mice (Luttun et al. 2004).

Evidently MMP-2 and MMP-9 are required for the proliferation and migration of vascular smooth muscle cells and probably play an important role in the development and maintenance of the fibrous cap. However, increased levels of MMP-9 in advanced plaques, particularly in an active form and in association with macrophage accumulation, may favour plaque disruption through promoting intra-plaque haemorrhage.

2.3.4 Matrilysin (MMP-7)

Matrilysin, now commonly known as MMP-7, has been shown to be expressed by human macrophages and exert potent elastolysis, after activation by uPA (Filippov et al. 2000). Several studies have identified MMP-7 mRNA and protein expression within advanced human atherosclerotic plaques, while detectable levels are negligible in healthy arteries (Halpert et al. 1996, Abbas et al. 2014). Elevated plasma levels of MMP-7 have been associated with symptomatic carotid (Abbas et al. 2014) and coronary (Nilsson et al. 2006) atherosclerosis and independently allied to adverse outcomes in patients including all-cause mortality (Abbas et al. 2014), suggesting MMP-7 may serve as a biomarker for disease progression. Within advanced human plaques, MMP-7 expression is restricted to foam cell macrophages between the fibrous cap and the lipid-rich necrotic core (Halpert et al. 1996), suggesting MMP-7 may contribute to core expansion and/or fibrous cap rarefaction. Indeed, MMP-7 has been shown to promote human vascular smooth muscle cell apoptosis and associates with apoptotic vascular smooth muscle cell in human and mouse plaques (Williams et al. 2010). Supportingly, brachiocephalic plaques from Apoe KO mice also deficient for MMP-7 displayed an increased vascular smooth muscle cell content, without affecting plaque size (Johnson et al. 2005), consistent with a pro-apoptotic role of MMP-7 on vascular smooth muscle

cells (Williams et al. 2010). These findings imply a deleterious role for MMP-7 in atherosclerotic plaque progression, although more work is needed to clarify its importance to plaque instability.

2.3.5 Metalloelastase (MMP-12)

Macrophage metalloelastase, more commonly referred to as MMP-12, as its name suggests preferentially degrades elastin although it can also cleave numerous other non-matrix substrates including angiostatin (Morrison et al. 2009) and the uPA receptor (u-PAR) (Koolwijk et al. 2001), and may therefore modulate neovascularisation, a characteristic associated with plaque progression (Michel et al. 2011). Mouse and human macrophages express abundant MMP-12, and MMP-12 expressing macrophages are prominent in advanced mouse (Johnson et al. 2011) and human plaques (Halpert et al. 1996, Scholtes et al. 2012). Like MMP-7, macrophage MMP-12 expression is confined to a sub-population of foam cell macrophages that reside at the periphery of the lipid-rich necrotic core within carotid plaques (Halpert et al. 1996, Scholtes et al. 2012), and similar localisation is observed in rabbit aortic plaques (Thomas et al. 2007). Related, foam cell macrophages undergoing apoptosis commonly also express MMP-12 (Scholtes et al. 2012), implying MMP-12 may potentiate foam cell macrophage death and contribute to necrotic core expansion. Indeed, MMP-12 has been shown to promote macrophage apoptosis potentially through cleavage of the pro-survival protein N-cadherin (Johnson et al. 2011). Of note, some carotid plaques have little or no MMP-12 expression while lesions deemed unstable and therefore vulnerable to rupture display the greatest proportion of MMP-12 positive foam cell macrophages (Scholtes et al. 2012). Moreover, endarterectomy patients whose plaques contained the highest number of MMP-12 positive foam cell macrophages were identified at increased risk of recurrent stroke and major adverse cardiovascular event (Scholtes et al. 2012). These findings tally with genetic studies demonstrating coronary plaque progression in diabetic patients carrying a functional gain-of-function polymorphism in the MMP-12 gene promoter (Jormsjo et al. 2000).

Studies in animal models of atherosclerosis have supported a deleterious role for MMP-12 in plaque advancement. Macrophage over-expression of an active form of MMP-12 in athero-susceptible rabbits accelerates the development (Yamada et al. 2008) and progression (Liang et al. 2006) of aortic plaques suggesting that MMP-12 activity promotes atherosclerosis progression. In agreement, Apoe KO mice also deficient for MMMP-12 harbour smaller plaques which have characteristics of increased stability (Johnson et al. 2005), while also limiting elastin fragmentation (Luttun et al. 2004). Concurring, MMP-12 inhibition in Apoe KO blocked plaque progression and improved stability through reduction of lipid core expansion and macrophage apoptosis, increased vascular smooth

muscle cell to macrophage ratio, decreased plaque calcification and attenuated elastinolysis (Johnson et al. 2011). All these effects, together with a reduction of buried fibrous layers in plaques mirrored those observed on MMP-12/Apoe double KO mice (Johnson et al. 2005). Underlying an adverse role for MMP-12 in plaque progression, both studies suggested a prominent role for MMP-12 in facilitating monocyte/macrophage invasion, confirming previous findings (Shipley et al. 1996). Collectively, MMP-12 appears to be a prime candidate in therapeutic clinical trials for the prevention of atherosclerosis. Indeed, a MMP-12 inhibitor (which also targets MMP-9) has already been used in a phase II clinical trial for chronic obstructive pulmonary disease (Dahl et al. 2012). However identification of a robust surrogate marker of MMP-12 activity is required to validate inhibitor outcome trials, perhaps based on proteomics identification of circulating shed proteins (Stegemann et al. 2013) or molecular imaging (Lenglet et al. 2012).

2.3.6 Membrane-type MMPs

Of the MT-MMP family members, MMP-14 (MT1-MMP) has received the most attention, with histological studies demonstrating that this membrane-active protease is up-regulated during human atherosclerotic plaque progression and in symptomatic carotid plaques (Rajavashisth et al. 1999, Johnson et al. 2014). While medial cells of healthy and diseased coronary arteries were shown to express MMP-14 (Rajavashisth et al. 1999), within advanced plaques the cellular source was most prominent in foam cell macrophages (Rajavashisth et al. 1999, Johnson et al. 2014). Moreover, MMP-14 protein expression is limited to a subpopulation of foam cell macrophages that preferentially populate human carotid and coronary plaques characterised as unstable (Di Gregoli et al. 2014, Johnson et al. 2014), and bestow macrophages with augmented invasive capacity (Johnson et al. 2008, Di Gregoli et al. 2014, Di Gregoli et al. 2016) as has also been shown in monocytes (Di Gregoli et al. 2014, Di Gregoli et al. 2016). Furthermore, the MMP-14 positive foam cell macrophage subpopulation also exhibit increased proliferation and susceptibility to apoptosis (Johnson et al. 2008), which alongside their heightened invasive ability would render these cells with attributes to promote plaque progression and instability.

In vivo studies from rabbits and mice support a pro-atherosclerotic role for MMP-14. The progression of aortic plaques in rabbits has been associated with increased MMP-14 expression, again predominantly by foam cell macrophages (Kuge et al. 2007, Johnson et al. 2008). In Apoe KO mice, although bone-marrow restricted deletion of MMP-14 had negligible effects on plaque macrophage and vascular smooth muscle cell composition within aortic root plaques, increased fibrillar collagen content was observed in advanced lesions, indicating a role in plaque instability and

also highlighting MMP-14 as a prominent collagenase in atherosclerosis (Schneider et al. 2008). Recent evidence has demonstrated that macrophage MMP-14 protein expression is tightly regulated by the microRNA (miR)-24 (Di Gregoli et al. 2014, Johnson et al. 2014). Thus, miR-24 inhibition with an anti-miR increased macrophage MMP-14 protein levels and promoted their invasion using a mouse subcutaneous granuloma model (Di Gregoli et al. 2014). Administration of the anti-miR-24 to Apoe KO mice with existing atherosclerosis, promoted plaque progression through increased plaque size and histological surrogates of plaque instability (Di Gregoli et al. 2014). Consistent with these observations in mice, MMP-14 positive foam cell macrophages in human atherosclerotic plaques lack miR-24 and associate with unstable lesions (Di Gregoli et al. 2014). Consequently, these above findings strongly suggest that MMP-14 promotes plaque progression and instability. Accordingly, strategies to inhibit MMP-14 expression or activity may be favourable to prevent plaque rupture, although MMP-14 has also been shown to foster vascular smooth muscle cell migration and neointima formation (Filippov et al. 2005), which would favour fibrous cap preservation.

Although the other MT-MMP family members have not been examined in animal models of atherosclerosis, human pathological studies suggest other membrane-type MMPs may play a role in atherosclerosis. MMP-15 (MT2-MMP) (Horozoglu et al. 2014), MMP-16 (MT3-MMP) (Uzui et al. 2002) and MMP-24 (MT5-MMP) (Horozoglu et al. 2014) have all been shown to be expressed in atherosclerotic coronary arteries. While the mRNA expression of MMP-15 and MMP-24 did not differ between healthy and diseased coronary arteries (Horozoglu et al. 2014), MMP-16 expression is elevated during coronary plaque progression (Uzui et al. 2002). Like MMP-14, MMP-16 is expressed by vascular smooth muscle cells within the media of healthy arteries and by plaque vascular smooth muscle cells, although in advanced plaques abundant MMP-16 expression is co-localised with foam cell macrophages, and associates with their increased accumulation (Uzui et al. 2002). MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP) expression has not been determined in atherosclerotic plaques. However, a protective role for MMP-17 in maintaining vascular smooth muscle cell phenotype and consequent protection from aortic aneurysm formation in mice has been recently shown (Martín-Alonso et al. 2015), suggesting that MMP-17 may be protective in atherosclerosis. Conversely, MMP-25 is induced in human (Huang et al. 2012) and mouse (Hayes et al. 2014) macrophages upon stimulation with pro-inflammatory molecules such as IFNγ and LPS, effects which may be expected to translate to increased atherosclerosis. Accordingly, the roles of both MMPs in atherosclerosis warrant further investigation.

2.3.7 TIMPs

The tissue inhibitors of MMPs (TIMPs) play a key homeostatic role in regulating the activity of MMPs (and ADAMS), and as such they are commonly increased where MMP activity is prevalent. However, while stimulation of vascular smooth muscle cells or macrophages with pro-inflammatory molecules induces the expression of multiple MMPs, a concomitant increase in TIMP-1 and TIMP-2 expression is not observed (Galis et al. 1994, Huang et al. 2012). Accordingly, TIMP-1 and TIMP-2 levels remain constant in human atherosclerotic plaques while the expression of several MMPs is elevated, accounting for the abundant proteolytic activity detected in advanced lesions (Galis et al. 1994). In agreement, systemic adenovirus-mediated over-expression of TIMP-1 (Rouis et al. 1999) or TIMP-2 (Johnson et al. 2006) retarded plaque initiation and progression of early lesions. Extended over-expression of TIMP-2, but not that of TIMP-1, suppressed plaque progression and stability of existing lesions in Apoe KO mice (Johnson et al. 2006), in part through limiting macrophage invasion and their susceptibility to apoptosis. Transgenic over-expression of human TIMP-1 in Apoe KO mice also failed to protect from plaque development (Cuaz-Perolin et al. 2006). Complimentary studies performed in TIMP-1 or TIMP-2 KO mice, revealed modest or no effects of TIMP-1 deletion (Silence et al. 2002, Lemaître et al. 2003, Di Gregoli et al. 2016), whereas TIMP-2 deficiency led to plaques with increased markers associated with plaque vulnerability (Di Gregoli et al. 2016). These above findings provide support for pro- and anti-atherosclerotic effects of MMPs, as TIMP-1 and TIMP-2 display prominent differences in their inhibitory efficiencies toward specific MMPs, particularly MT-MMPs (Baker et al. 2002).

TIMP-3 is also expressed by vascular smooth muscle cells and macrophages in vitro, and by medial vascular smooth muscle cells and plaque macrophages within advanced lesions, most likely to counteract MMP activity and hence influence plaque stability (Fabunmi et al. 1998). Further studies have revealed that TIMP-3 protein expression is restricted to a subpopulation of foam cell macrophages in vitro (Johnson et al. 2008, Johnson et al. 2014) and within atherosclerotic plaques (Fabunmi et al. 1998, Johnson et al. 2008, Johnson et al. 2014, Di Gregoli et al. 2017). TIMP-3 positive foam cell macrophages are most prevalent within the fibrous cap of human plaques and inversely correlate with and fail to co-localise with MMP-14 positive foam cell macrophages (Johnson et al. 2014). Accordingly, TIMP-3 positive foam cell macrophages mainly occur in stable plaques rich in vascular smooth muscle cells and with small necrotic cores, while MMP-14 foam cell macrophages are dominant in unstable plaques (Johnson et al. 2008, Di Gregoli et al. 2014, Johnson et al. 2014, Di Gregoli et al. 2017). Animal studies have provided further supporting evidence for a protective role of TIMP-3 in atherosclerosis. Macrophage restricted over-expression of TIMP-3 decreased plaque size and increased features of stability such as increased collagen content and

decreased necrotic core size (Casagrande et al. 2012). Concordantly, two studies have shown TIMP-3 deficiency in Apoe KO mice exacerbates atherosclerosis development and progression (Stöhr et al. 2014, Di Gregoli et al. 2017). Finally, the regulation of TIMP-3 protein expression by select microRNA has been exploited to elevate TIMP-3 expression. Inhibition of miR-181b, which is over-expressed in symptomatic human plaques (Di Gregoli et al. 2017), was shown to increase foam cell macrophage TIMP-3 protein levels, reduce their invasion and apoptosis, and retarded both the development and progression of existing plaques in Apoe KO mice (Di Gregoli et al. 2017). Delivery of anti-miR-712 to Apoe KO mice reduced disturbed flow induced atherogenesis, through up-regulation of endothelial TIMP-3 expression and suggesting inhibition of ADAMs as well as MMP activity may be implicated (Son et al. 2013).

TIMP-4 has received less attention but its expression has been reported in vascular smooth muscle cells, particularly after vascular injury and neointima formation in rats, where it is proposed to limit vascular smooth muscle cell migration (Dollery et al. 1999). TIMP-4 is also expressed by many inflammatory cell types including lymphocytes, monocyte/macrophages and mast cells (Koskivirta et al. 2006). TIMP-4 is also expressed in human atherosclerotic plaques, especially macrophages around the necrotic core (Koskivirta et al. 2006). However, the direct role of TIMP-4 in atherosclerosis is yet to be examined but the above observations indicate further scrutiny is required.

2.3.8 Synthetic MMP inhibitors

The above studies lend compelling evidence for MMP inhibition as a therapeutic approach to prevent the clinical events associated with atherosclerotic plaque instability, such as myocardial infarction and stroke. Accordingly, there have been substantial efforts to develop and test synthetic inhibitors of MMPs. However, the results obtained from studies utilising inhibitors containing zinc-chelating groups (such as thiol or hydroxamate groups, or tetracycline derivatives) have been far from encouraging. Non-selective hydroxamic acid-based MMPs inhibitor provided no beneficial effects on plaque development or progression in Lldr KO mice (Prescott et al. 1999) or Apoe KO animals (Johnson et al. 2006). Similar unfavourable effects were observed with the widely used antibiotic doxycycline, which also displays broad spectrum MMP inhibitory properties, in Apoe KO mice (Manning et al. 2003). Similar disappointing results have been recapitulated in human studies where treatment with sub-antimicrobial doses of doxycycline in two independent, prospective placebo controlled pilot clinical trials in patients with symptomatic coronary and carotid artery disease, failed to exert any positive effects on plaque phenotype or clinical outcome (Axisa et al. 2002, Brown et al. 2004). The paucity of any distinct benefits against atherosclerosis in either animal

or clinical studies may support the rationale that MMPs play divergent roles during disease progression.

2.3.9 MMP expression and macrophage heterogeneity

Macrophages accumulate within atherosclerotic plaques and express a wide array of proteases including MMPs (see Fig.2), which as outlined above can exert divergent effects on disease progression (Newby 2008). Multiple lines of evidence have revealed that macrophages display marked diversity and can (co)-exist in a number of different phenotypes within human atherosclerotic plaques (Chinetti-Gbaguidi et al. 2015). Accordingly, there have been recent efforts to characterise the MMP and TIMP profile of the varying macrophage phenotypes with regards to atherosclerosis. Phenotypically, at one end of the spectrum macrophages are classified as classically activated through co-stimulation with toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and pro-inflammatory mediators, including interferonγ (IFNγ), and are commonly termed M1 macrophages (Martinez et al. 2006). In contrast, macrophage exposure to interleukin (IL)-4 or IL-10 gives rise to alternatively activated phenotypes, referred to as M2a and M2c macrophages, respectively (Martinez et al. 2006). In vitro studies conducted on human peripheral blood-derived monocyte/macrophages consistently demonstrate that mRNA expression of MMP-1 and MMP-14 are up-regulated in classically activated M1 macrophages, while MMP-11 and TIMP-3 are increased in M2 macrophages (Huang et al. 2012, Jager et al. 2016). Interestingly, while MMP-9 mRNA expression is unaffected under any of the polarisation conditions (Huang et al. 2012, Jager et al. 2016), MMP-9 protein levels are increased in M2 macrophages (Jager et al. 2016), suggesting the involvement of a post-transcriptional regulatory mechanism. The colony stimulating factors (CSF), granulocyte/macrophage-CSF (GM-CSF) and macrophage-CSF (M-CSF), are also utilised to generate in vitro pro- and anti-inflammatory macrophage phenotypes, respectively (Di Gregoli and Johnson 2012). Moreover, it has been proposed that these two CSFs may give rise to the macrophage heterogeneity observed within human atherosclerotic plaques (Waldo et al. 2008). With regards to MMPs, GM-CSF polarised macrophages exhibit elevated MMP-12 mRNA and protein expression (Wu et al. 2000, Waldo et al. 2008). GM-CSF polarisation has also been proposed to underlie the TIMP-3 low/MMP-14 high macrophage subpopulation (Johnson et al. 2008) which dominates in advanced human carotid and coronary plaques (Di Gregoli et al. 2014, Johnson et al. 2014, Di Gregoli et al. 2017), through microRNA-dependent post-transcriptional mechanisms, which appear independent of M1 or M2 polarisation (Di Gregoli et al. 2014). These findings demonstrate that only a small number of MMPs display specificity towards select macrophage phenotypes when assessed robustly and validated in human atherosclerotic lesions.

3 Conclusion

As the accumulating evidence discussed within this review suggests, MMPs appear to play dual beneficial and detrimental roles in atherosclerosis. Collectively the findings indicate that some MMPs (MMP-2, MMP-3 and MMP-9) facilitate vascular smooth muscle cell growth and therefore promote fibrous cap integrity, while other MMPs (MMP-7, MMP-12, MMP-13 and MMP-14) precipitate the loss of vascular smooth muscle cells and matrix proteins within the fibrous cap and contribute to the perpetual accumulation of monocyte/macrophages (summarised in Fig.3). Considering the poor outcomes achieved with broad-spectrum MMP inhibition cognate with the successes in animal models attained through selective intervention (Johnson et al. 2011, Quillard et al. 2011), supports the continued effort of developing therapies for atherosclerosis that focus on inhibiting individual MMPs. However, due to the large number of ADAMS, ADAMTS, and MMPs, and their wide-range of substrates which display broad overlap, surrogate endpoints will be required to ascertain specificity and efficacy, which may involve shed circulating molecules identified by proteomics (Stegemann et al. 2013) or non-invasive imaging (Lenglet et al. 2012).

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Figure Legends

Figure 1: Metalloproteinase subfamily domain structures

Generalised domain structures for the major classes of the metalloproteinase families, ADAMs, ADAMTS, MMPs, and MT-MMPs (a subclass of MMPs). Common major domains include the signal peptide (SP), pro-domain (Pro), catalytic domain containing the active site zinc and sometimes referred to as the metalloprotease domain, the hinge domain (HG), the hemopexin-like domain, and in some cases either a transmembrane domain (TM) or GPI-anchor domain (GPI) within the cytoplasmic tail (CT). ADAMs contain an epidermal growth factor-like (EGFL) domain, whilst ADAMTS family members have variable numbers of thrombospondin-like (TSL) motifs. The MMP shown is of the gelatinase class and contains fibronectin-like type II repeats (FN), whilst other subclasses of MMPs lack this domain and/or hemopexin-like sequences. A furin cleavage site (F) between the pro-domain and the catalytic domain is found in MT-MMPs. are also present.

Figure 2: Cellular sources of MMPs in human atherosclerotic plaques

This figure depicts the cellular sources of MMPs within human atherosclerotic plaques, including endothelial cells, vascular smooth muscle cells (VSMC), and macrophages.

Figure 3: Opposing roles of MMPs in atherosclerotic plaque progression and stability

Hypothetical model of the potential beneficial and deleterious roles of MMPs and TIMPs during atherosclerotic plaque progression and instability. Matrix metalloproteinase (MMP)-2, -3 and -9 can facilitate vascular smooth muscle cell (VSMC) migration from the media into the developing atherosclerotic plaque where they participate in fibrous cap formation and maintenance, thus promoting plaque stability. In opposition, MMP-1, MMP-8, MMP-12, MMP-13 and MMP-14 can degrade extracellular matrix proteins present in the fibrous cap whilst also encouraging the recruitment and accumulation of monocytes and macrophages, and their subsequent susceptibility to apoptosis as foam cells – which collectively enhance lipid core expansion, thrombogenicity of the plaque, and thinning of the fibrous cap. Similarly, MMP-7 can induce VSMC apoptosis and therefore compromise plaque stability, while excess macrophage-derived MMP-9 can trigger intra-plaque haemorrhage (IPH), possibly through destabilising the neovascularisation, and subsequently contribute to plaque expansion and instability. Consequent to the expression of these deleterious MMPs, the stability of the plaque is compromised and vulnerable to plaque rupture and ensuing thrombus formation. More recently, microRNA (miR) have been identified which can regulate MMP and TIMP expression/activity, exerting direct effects on plaque progression.

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Table 1

Table 1. Results of in vivo animal studies evaluating the effects of modulating ADAMs and ADAMTS on atherosclerotic plaque size and cellular composition

Abbreviations: VSMCs, vascular smooth-muscle cells; Mø, macrophages; BCA, brachiocephalic artery; ND, not determined; ↓, decreased; ↑, increased; ↔, no change.

In separate studies where different vascular sites have been assessed and discrepancies in effects are observed, red, green and blue text colour are used to define the different sites.

ADAM/ADAMTS	Modulation	Site	Effect on plaque size and composition	Reference
ADAM				
ADAM10	Knockout	Aorta	Size (↔), VSMCs (↔), Mø (↔)	Van der Vorst et al, 2012
ADAM15	Knockout	Aorta	Size (↓), VSMCs (↓), Mø (↓)	Sun et al, 2012
	Over-expression	Aorta/carotid	Size (↔/↓), VSMCs (ND/ND), Mø (ND/↓)	Bültmann et al, 2011
ADAM17	Knockdown	Carotid	Size (↑), VSMCs (↑), Mø (↑)	Nicolaou et al, 2016
ADAMTS				
ADAMTS7	Knockout	Aorta/BCA	Size (↓/ND), VSMCs (↔/↔), Mø (↔/↔)	Bauer et al, 2015
ADAMTS13	Knockout	Aorta	Size (↑), VSMCs (ND), Mø (↑)	Jin et al, 2012

Table 2

Table 2. Results of in vivo animal studies evaluating the effects of modulating matrix metalloproteinases (MMP) or tissue inhibitors of metalloproteinases (TIMPs) on atherosclerotic plaque size and cellular composition

Abbreviations: VSMCs, vascular smooth-muscle cells; Mø, macrophages; BCA, brachiocephalic artery; ND, not determined; ↓, decreased; ↑, increased; ↔, no change.

In separate studies where different vascular sites have been assessed and discrepancies in effects are observed, red and green text colour are used to define the different sites.

MMP/TIMP	Modulation	Site	Effect on plaque size and composition	Reference
Collagenases				
MMP-1	Over-expression	Aorta	Size (↓), VSMCs (↔), Mø (↔)	Lemaître et al, 2001
MMP-8	Knockout	Aorta	Size (↓), VSMCs (↔), Mø (↓)	Laxton et al, 2009
MMP-13	Knockout	Aorta	Size (↔), VSMCs (↔), Mø (↔)	Deguchi et al, 2005
	Inhibitor	Carotid	Size (↔), VSMCs (↔), Mø (↔)	Quillard et al, 2014
Gelatinases				
MMP-2	Knockout	Aorta	Size (↓), VSMCs (↓), Mø (↔)	Kuzuya et al, 2006
MMP-9	Knockout	Aorta/BCA	Size (↓/↑), VSMCs (ND/↓), Mø (↓/↑)	Luttun et al, 2004 Johnson et al, 2005
	Over-expression	Arch/carotid	Size (↔), VSMCs (↔), Mø (↔)	Gough et al, 2006 de Nooijer et al, 2006
Stromelysins				
MMP-3	Knockout	Aorta/BCA	Size (↑/↑), VSMCs (ND/↓), Mø (↔/↑)	Silence et al, 2001 Johnson et al, 2005
MMP-10	Not assessed			
MMP-11	Not assessed			
Others				
MMP-7	Knockout	BCA	Size (↔), VSMCs (↑), Mø (↔)	Johnson et al, 2005
MMP-12	Knockout	Aorta/BCA	Size (↔/↓), VSMCs (↔/↑), Mø (↔/↓)	Luttun et al, 2004 Johnson et al, 2005
	Over-expression	Aorta	Size (↑), VSMCs (↑), Mø (↑)	Yamada et al, 2008 Liang et al, 2006
	Inhibitor	Aorta/BCA	Size (↓), VSMCs (↑), Mø (↓)	Johnson et al, 2011
MT-MMPs				
MMP-14	Knockout	Root	Size (↔), VSMCs (↔), Mø (↔)	Schneider et al, 2008
TIMPs				
TIMP-1	Knockout	Aorta/BCA	Size (↔), VSMCs (↔/↓), Mø (↔)	Silence et al, 2002 Lemaître et al, 2003 Di Gregoli et al, 2016
	Over-expression	Aorta/BCA	Size (↓/↔), VSMCs (ND/↔), Mø (↓/↔)	Rouis et al, 1999 Johnson et al, 2006
TIMP-2	Knockout	Aorta/BCA	Size (↔), VSMCs (↓), Mø (↑)	Di Gregoli et al, 2016
	Over-expression	Aorta/BCA	Size (↓), VSMCs (↑), Mø (↓)	Johnson et al, 2006
TIMP-3	Knockout	BCA	Size (↑), VSMCs (↓), Mø (↑)	Di Gregoli et al, 2017
TIMP-4	Not assessed			

Figure 1

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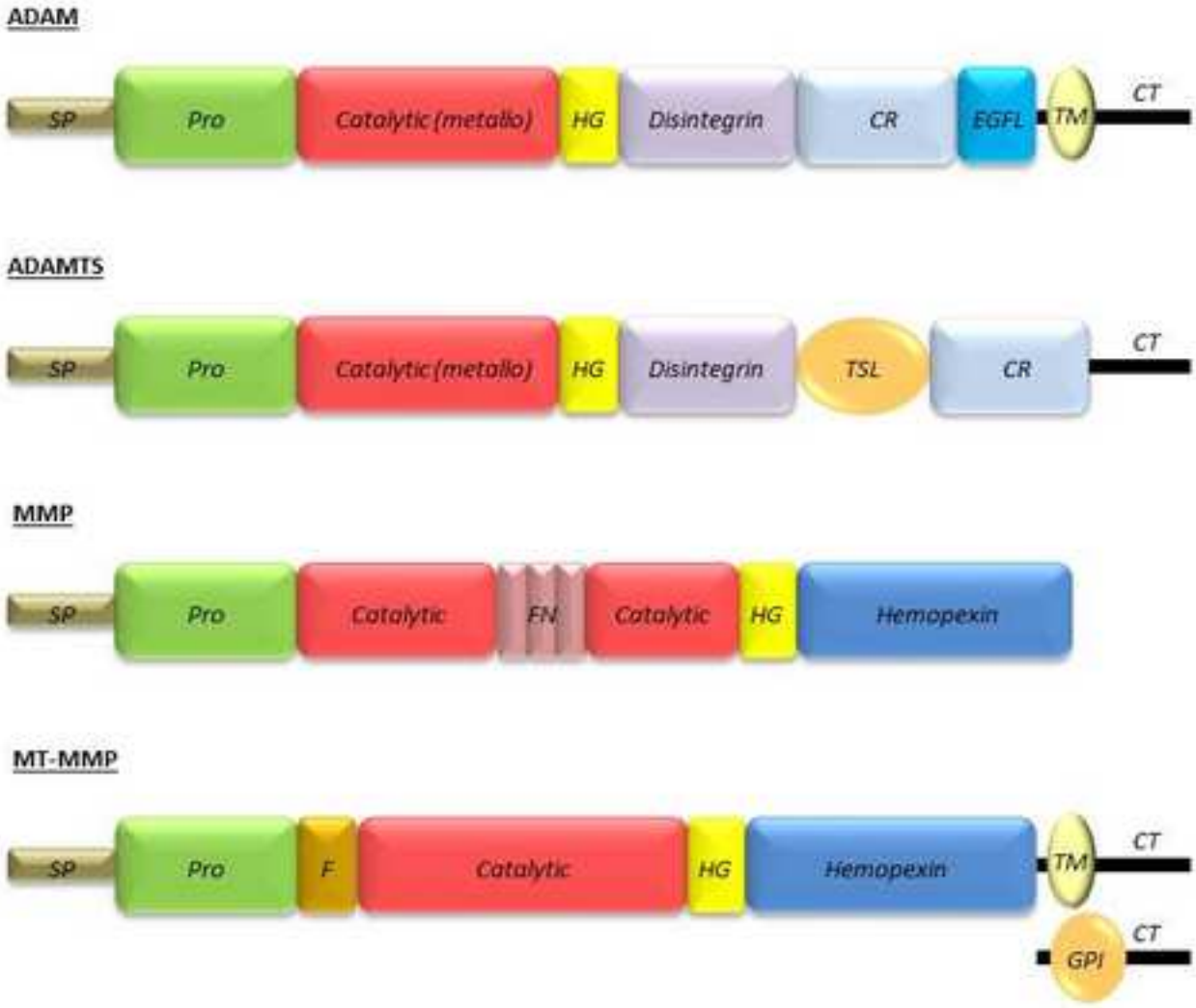
Figure 1: Metalloproteinase subfamily domain structures



Generalised domain structures for the major classes of the metalloproteinase families, ADAMs, ADAMTS, MMPs, and MT-MMPs (a subclass of MMPs). Common major domains include the signal peptide (SP), pro-domain (Pro), catalytic domain containing the active site zinc an sometimes referred to as the metalloprotease domain, the hinge domain (HG), the hemopexin-like domain, and in some cases either a transmembrane domain (TM) or GPI-anchor domain (GPI) within the cytoplasmic tail (CT). ADAMs contain an epidermal growth factor-like (EGFL) domain, whilst ADAMTS family members have variable numbers of thrombospondin-like (TSL) motifs. The MMP shown is of the gelatinase class and contains fibronectin-like type II repeats (FN), whilst other subclasses of MMPs lack this domain and/or hemopexin-like sequences. A furin cleavage site (F) between the pro-domain and the catalytic domain is found in MT-MMPs, are also present.

Figure 2

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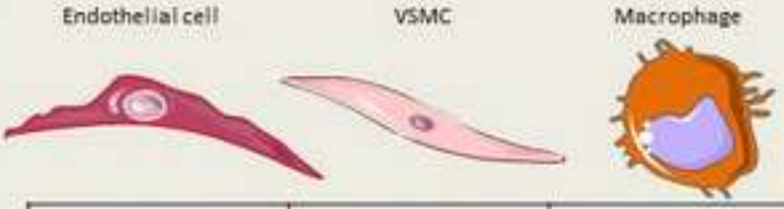
Figure 2: Cellular sources of MMPs in human atherosclerotic plaques



Endothelial cell	VSMC	Macrophage
MMP-1	MMP-1	MMP-1
	MMP-2	MMP-2
MMP-3	MMP-3	MMP-3
		MMP-7
MMP-8	MMP-8	MMP-8
MMP-9	MMP-9	MMP-9
MMP-10		MMP-10
MMP-11	MMP-11	MMP-11
		MMP-12
		MMP-13
	MMP-14	MMP-14
	MMP-16	MMP-16

Figure 3

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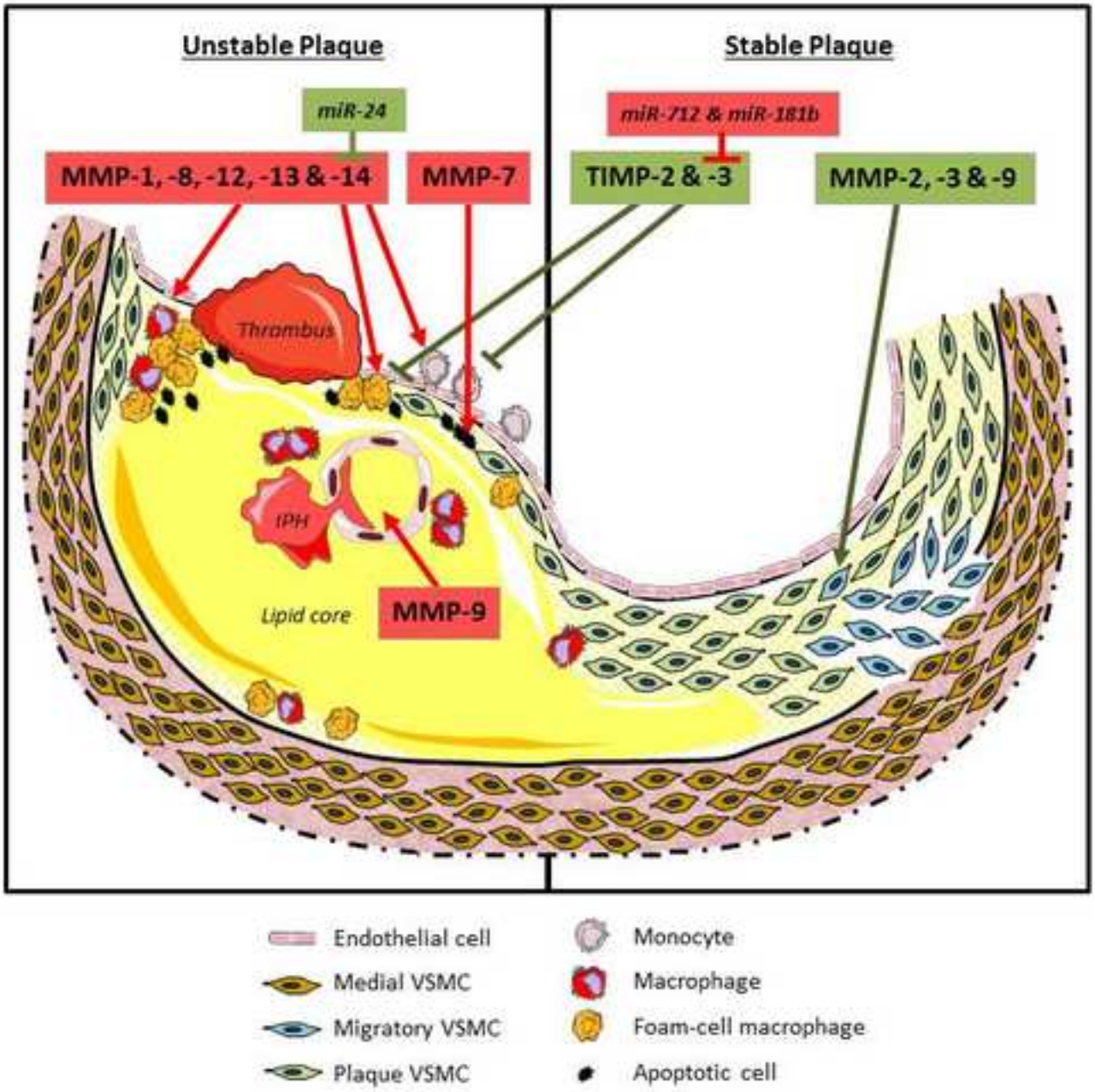

Figure 3: Opposing roles of MMPs in atherosclerotic plaque progression and stability

Hypothetical model of the potential beneficial and deleterious roles of MMPs and TIMPs during atherosclerotic plaque progression and instability. Matrix metalloproteinase (MMP)-2, -3 and -9 can facilitate vascular smooth muscle cell (VSMC) migration from the media into the developing atherosclerotic plaque where they participate in fibrous cap formation and maintenance, thus promoting plaque stability. In opposition, MMP-1, MMP-8, MMP-12, MMP-13 and MMP-14 can degrade extracellular matrix proteins present in the fibrous cap whilst also encouraging the recruitment and accumulation of monocytes and macrophages, and their subsequent susceptibility to apoptosis as foam cells – which collectively enhance lipid core expansion, thrombogenicity of the plaque, and thinning of the fibrous cap. Similarly MMP-7 can induce VSMC apoptosis and therefore compromise plaque stability, while excess macrophage-derived MMP-9 can trigger intra-plaque haemorrhage (IPH), possibly through destabilising the neovascularisation, and subsequently contribute to plaque expansion and instability. Consequent to the expression of these deleterious MMPs, the stability of the plaque is compromised and vulnerable to plaque rupture and ensuing thrombus formation. More recently, microRNA (miR) have been identified which can regulate MMP and TIMP expression/activity, exerting direct effects on plaque progression.