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Simona Gargiulo, Paola Gamba, Giuseppe Poli and Gabriella Leonarduzzi
Department of Clinical and Biological Sciences, University of Turin, Orbassano (Turin), Italy

*Address correspondence to this author at the Department of Clinical and Biological Sciences, Faculty of Medicine San Luigi Gonzaga, University of Turin, Regione Gonzole 10, 10043, Orbassano (Turin), Italy. Tel. +39 011 6705422; Fax: +39 011 6705424;
E-mail: giuseppe.poli@unito.it (G. Poli).

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Abstract: Degradation of the extracellular matrix is an important feature of embryonic development, morphogenesis, angiogenesis, tissue repair and remodeling. It is precisely regulated under physiological conditions, but when dysregulated it becomes a cause of many diseases, including atherosclerosis, osteoarthritis, diabetic vascular complications, and neurodegeneration. Various types of proteinases are implicated in extracellular matrix degradation, but the major enzymes are considered to be metalloproteinases such as matrix metalloproteinases (MMPs) and disintegrin and metalloproteinase domain (ADAMs) that include ADAMs with a thrombospondin domain (ADAMTS). This review discusses involvement of the major metalloproteinases in some age-related chronic diseases, and examines what is currently known about the beneficial effects of their inhibitors, used as new therapeutic strategies for treating or preventing the development and progression of these diseases.

Keywords: metalloproteinases, metalloproteinase inhibitors, atherosclerosis, diabetes mellitus, neurodegenerative diseases, osteoarthritis

1. INTRODUCTION

Metalloproteinases are a large family of important endopeptidases, which include matrixins (matrix metalloproteinases, or MMPs) and adamalysins (a disintegrin and metalloproteinase domain, or ADAMs) [1, 2].

The subunits of ADAMs, which are transmembrane proteases, comprise a catalytic domain at the end of the extracellular extension, which comprises three domains: a disintegrin, a cysteine-rich domain, and a number of epidermal growth factor repeats. The cytoplasmic tail attached to the epidermal growth factor domain protrudes through the membrane, and signals cell-surface events to the cytoplasm [3]. The catalytic domain contains a typical zinc-binding consensus motif; for example, in the case of ADAM-10, HEXGHXXGXXHD.

As the name "ADAM" indicates, the disintegrin or integrin-binding domain binds these molecules to the membrane integrins, and the metalloproteinase domain provides the protease function [4]. Thus ADAMs are important in events at the cell surface, because they attach to integrins on the cell surface and carry out metalloproteinase functions. Their primary functions are cleavage of extracellular matrix (ECM) molecules and of the extracellular domains of many cell-surface membrane proteins, a process known as "ectodomain shedding" [4-6]. Shedding of the tumor necrosis factor-α (TNF-α) receptor, and of interleukin-6 (IL-6), L-selectin, and syndecans, has also been shown to be a function of ADAMs [7]. They are also important in intracellular signaling and cell adhesion [8]. ADAMs are thus implicated in cell proliferation, migration, differentiation, and survival [9]. Of the more than 30 members of this family, only ADAM-10, -15, and -17 have been characterized in vascular cells [10]. ADAM-10 has also been identified in distinct areas of the human brain [11, 12] and peripheral structures [13, 14] (Table 1). ADAMs with a thrombospondin domain (ADAMTS) form another group of metalloproteinases. ADAMTS-4 and ADAMTS-5 have been shown to degrade aggrecan, a proteoglycan of joint cartilage ECM, and to be involved in spinal cord injury [15, 16]. These enzymes are regulated at multiple levels, through control of gene expression, mRNA splicing, and protein processing, as well as through regulation of the expression of various naturally-occurring inhibitors.

With regard to MMPs, there are at least 25 mammalian MMPs, 14 of which have been characterized in vascular cells. Although mammalian MMPs have overlapping specificities for structural ECM components, they are classified into five groups, by differences in their primary structure and substrate specificity. These groups are: interstitial collagenases, gelatinases, stromelysins/matrilysins, membrane-type MMPs (MT-MMPs), and others MMPs. Further, they are assigned MMP numbers, and some members also have trivial names (Table 2). MMPs are extracellular proteins, but recent studies have reported that MMP-1 [17], MMP-2 [18] and MMP-11 [19] are intracellular, and may act on intracellular proteins. Moreover, most MMPs are expressed as inactive, latent proforms, although MMP-11, -21, -23, and -27, and the MT-MMPs, have a furin recognition sequence before the catalytic domain, and are therefore likely to be activated intracellularly and secreted as active enzymes [20, 21].

The protein structure of MMPs follows a basic pattern. A typical MMP consists of a propeptide of about 80 amino acids at the N-terminal, which is attached to a signal peptide, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable length (also called the "hinge region") and a hemopexin domain of about 200 amino acids, which may be attached to a transmembrane domain. Exceptions to this are MMP-7, MMP-26 and MMP-23; these MMPs lack the linker peptide and the hemopexin domain, and MMP-23 has a unique cysteine-rich domain and an immunoglobulin-like domain after the metalloproteinase domain. The two gelatinases, MMP-2 and MMP-9, have three repeats of a fibronectin type II motif in the metalloproteinase domain. Further, MMPs have a zinc binding motif - HEXXHXXGXXH - in the catalytic domain, and a "cysteine switch" motif - PRCGXPD - in the propeptide: the three histidines in the zinc binding motif, and the cysteine in the propeptide, coordinate with the catalytic zinc ion. This Cys-Zn^{2+} coordination keeps proMMPs inactive, preventing catalysis of the zinc atom by water-molecule binding. The

catalytic domain also contains a conserved methionine, forming a "Met-turn" situated eight residues after the zinc binding motif, whose role is to support the structure around the catalytic zinc. The zinc binding motif and the "Met-turn" are also conserved in ADAM family members [20, 21].

Traditionally, MMPs are thought to play a central role in degrading and remodeling ECM components, including fibronectin, laminin, proteoglycans and collagen [21-23]. In recent years, however, findings from several research groups have established that MMPs cleave a wide range of extracellular and bioactive ECM and non-ECM substrates, and that they regulate the activity of these proteins [24, 25]. The established functions of MMPs include releasing cytokines and growth factors from the cell membrane or ECM, cleaving growth factor receptors from the cell surface, activating proforms of cytokines (*e.g.* TNF-α and IL-1β), activating death receptors at cell membranes, shedding cell adhesion molecules, and activating other MMPs and MMP inhibitors, as well as other signaling molecules [24-28]. They are also involved in regeneration, myelin formation, angiogenesis, and axonal growth [29]. The ability of MMPs to modify the structural integrity of tissues is thus essential for certain physiological and pathological processes: MMPs are potent controllers of physiological processes (cell migration, proliferation, differentiation, growth and development) and of pathological processes (tissue remodeling in response to injury, inflammatory processes, neovascularization, cancer progression, apoptosis, etc.) [21, 30]. In this context, and in brief, MMPs are important in normal development, remodeling, wound healing, and also in a wide variety of pathological processes, including the spread of metastatic cancer cells, arthritic destruction of joints, cardiovascular and neurodegenerative diseases, diabetes mellitus, and lung diseases. In these latter pathological processes, which are characterized by an inflammatory response, the abnormal expression and activation of these proteases lead to ECM breakdown.

As cited above, MMPs are secreted as latent enzymes and require activation, which is tightly regulated so as to prevent tissue damage. The activities of most MMPs are very weak or negligible in normal steady-state tissues, but rapidly increase in response to inflammatory and oxidative stimuli. Their activity can be regulated at four levels: induction of MMP genes, vesicle trafficking and secretion, activation of latent proforms, and complexing with specific endogenous tissue inhibitors of metalloproteinases (TIMPs). Activation of MMPs is, thus, an important regulatory step in MMP activity.

MMPs are synthesized as pre-proenzymes, but during translation the signal peptide is removed, generating proMMPs; thus most MMPs are secreted from the cell in the form of proMMPs. MMPs may be stored intracellularly in vesicles, or secreted directly into the intracellular space. The presence of a proteinase-susceptible "bait" region in the propeptide allows tissue and plasma proteinases or opportunistic bacterial proteinases to activate proMMPs. A small number of MMPs, including the membrane-bound MT-MMPs, are proteolytically activated inside the cell by furin proteases, but most MMPs are activated in the extracellular space. Besides proteolytic cleavage, a change in configuration of the propeptide region can activate the enzymes. The proMMPs thus possess a furin-like proprotein convertase recognition sequence, RX[K/R]R, at the end of the propeptide, and are likely to be activated intracellularly and then secreted or cell-surface-bound as active enzymes. ProMMPs can also be activated by oxidants, such as reactive oxygen species (ROS) and nitric oxide (NO) [31] by reacting with the cysteine of the "cysteine switch" in the propeptide, and this activation process takes place under inflammatory and oxidative conditions [32]. Moreover, they can be activated by lysosomal proteases [20], by urokinase type plasminogen activators [33], by angiotensin [34] or by hyperglycemia [35]. In addition, control of the inducible MMP genes can occur at the promoter region, which contains binding sites for transcription factors such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and polyoma enhancer A binding protein-3 (PEA-3); in turn, these are responsive to free radicals, protein kinases, and cytokines,

suggesting that these genes may be induced during inflammation [36, 37]. Activated MMPs can also activate other MMPs, in a stepwise activation cascade [20, 30, 38, 39].

Thus MMP activities are regulated by two major types of endogenous inhibitors: α_2-macroglobulin and TIMPs.

Human α_2-macroglobulin is a plasma glycoprotein of 725 kDa that inhibits most proteinases by entrapping the proteinase within the macroglobulin, after proteolysis of the bait region of the inhibitor; the complex is rapidly cleared by low density lipoprotein (LDL) receptor-related protein-1-mediated endocytosis. MMP activities in the fluid phase are primarily regulated by α_2-macroglobulin. MMP-1 reacts with α_2-macroglobulin more readily than with TIMPs [40].

TIMPs are potent and selective tissue inhibitors of MMPs, consisting of 184-194 amino acids [21, 41]. They are subdivided into an N-terminal and a C-terminal subdomain. Each domain contains three conserved disulfide bonds, and the N-terminal domain folds as an independent unit with MMP inhibitory activity. The N-terminal cysteine is particularly important for inhibition, since its free α-amino group and carbonyl function displace the catalytic water molecule from the essential Zn^{2+} ion at the MMP active site. There are at least four members of the TIMP family (TIMP-1, -2, -3, and -4) that are often secreted by the same cells that secrete MMPs; their expression is closely regulated during embryonic development and tissue remodeling [30, 42]. The four members of the TIMP family have many similarities and overlapping specificities, but their biochemical properties and local expression patterns are distinctive [41]. TIMP-1, -2, and -4 are secreted in soluble form, while TIMP-3 is associated with the ECM. Moreover, their activity is stimulated by platelet-derived growth factor (PDGF) and tumor growth factor β (TGFβ) and is regulated by several cytokines. TIMPs form tight inhibitory 1:1 complexes with MMPs [42]. These interactions are generally rather non-selective, meaning that TIMPs inhibit all MMPs, at least to some extent; however, certain TIMPs have weaker or stronger inhibitory effects on specific proteinases [42]. TIMP-1 mainly inhibits MMP-9 as well as MMP-3, while it only weakly inhibits MMP-14 (MT1-MMP), MMP-16 (MT3-MMP), MMP-18 (MT5-MMP) and MMP-19; TIMP-2 inhibits MMP-2 and, paradoxically, at low concentrations contributes to activating proMMP-2. TIMP-3 is the only TIMP bound to the ECM and cell surface. TIMP-3 inhibits several membrane-bound molecules with sheddase functions, such as MMP-3, MMP-7, and MMP-14. ADAMs also contain an MMP-like catalytic domain, which in some cases remains catalytically active. In general, TIMPs do not bind or inhibit the catalytic site of ADAMs, although TIMP-3 inhibits ADAM-10 and ADAM-17, and TIMP-1 inhibits ADAM-10 [43]. TIMP-4 is chiefly localized in vascular tissue [21].

Several other proteins have been reported to inhibit selected members of the MMP family, although the inhibition mechanism of these protein is still unclear: the secreted form of amyloid β precursor protein (APP) inhibits MMP-2 [44]; a C-terminal fragment of procollagen C-proteinase enhancer protein inhibits MMP-2 [45], and RECK (reversion-inducing cysteine-rich protein with kazal motifs), a GPI-anchored glycoprotein that suppresses angiogenesis, inhibits MMP-2, MMP-9 and MMP-14 [41, 46]. Moreover, MMP expression is determined at a transcriptional level by various cytokines and growth factors [47]. In a number of tissue types, some cytokines and growth factors, including IL-1, TNF-α and PDGF, stimulate MMP expression, while others are inhibitory, *e.g.* TGFβ.

The balance between production, activation, and inhibition of metalloproteinases is critical in maintaining ECM integrity. When proteolytic activity is greater than inhibition caused by TIMPs or other inhibitors, ECM breakdown occurs. Conversely, if inhibitors are too strongly expressed and proteolysis is restricted, there is a build up of ECM proteins, with fibrosis.

2. METALLOPROTEINASES IN AGE-RELATED DISEASES

2. 1. Metalloproteinases in Atherosclerosis

Uncontrolled ECM remodeling of the myocardium and vasculature, by MMPs and other proteolytic enzymes, are features of cardiovascular disorders such as atherosclerosis, stroke, stenosis, left ventricular hypertrophy, heart failure, and aneurysm [20, 48-51].

A number of MMP gene polymorphisms have also been shown to contribute to inter-individual susceptibility and outcome of these cardiovascular disorders. Genetic polymorphisms may, for example, affect MMP expression levels by conferring protection or propensity to vulnerable plaques [52-54]. Moreover, studies using MMP gene knockout mice have indicated that MMP-2 and MMP-9 play key roles in cardiac rupture after myocardial infarction [55, 56]. A critical role of MMP-2 and MMP-9 has also been demonstrated in the development of abdominal aortic aneurysm, using MMP gene deletion mice [57]. MMPs, in particular MMP-1 and MMP-2, might also be involved in triggering acute coronary syndrome, via their ability to promote platelet activation and aggregation [58, 59]. Finally, *Timp-3* deficiency in mice disrupts matrix homeostasis, and causes spontaneous left ventricular dilation, cardiomyocyte hypertrophy, and contractile dysfunction [60].

With regard to the multifactorial disease atherosclerosis, it is characterized by the development of atherosclerotic plaques in susceptible sites of the arterial wall. It is initiated by cholesterol-rich lipid retention and accumulation, oxidation, and modification, which combine to provoke chronic inflammation. Atherosclerotic plaque growth, due to lipid accumulation, smooth muscle cells (SMCs) proliferation, and matrix synthesis, may in turn narrow the arterial lumen and ultimately causing stenosis or thrombosis [61]. The final clinical outcome depends on whether a plaque becomes unstable, leading to acute disruption of the surface and exposure of the thrombogenic core to luminal blood flow [62]. In this context, our understanding of the pathogenesis of atherosclerotic lesions has improved dramatically, helping to clarify the mechanisms of plaque formation and the differences between stable and unstable plaque morphology [63-66]. In particular, it has now been established that inflammation is a key feature in all stages of the disease, especially in plaque destabilization, which leads to plaque rupture [67]. Alongside the inflammatory response, which triggers activation of macrophage-derived MMPs, also apoptosis of vascular cells, especially that of macrophages and SMCs, contributes to plaque destabilization [68, 69].

A variety of intrinsic and extrinsic factors predispones an atherosclerotic plaque to instability and acute disruption. Intrinsic factors characterizing a plaque as vulnerable are: a large lipid core, increased inflammatory-cell infiltration (particularly by monocytes/macrophages), content of foam cells and of T lymphocytes, and reduced collagen and vascular SMCs content associated with increased matrix proteolysis. Extrinsic features include increased blood pressure, hemodynamic shear stress, and vasospam [62, 70-74]. Plaque rupture tends to occur at the shoulder region, which is associated with cap thinning and macrophage infiltration [75]; the shoulder region is also the area of the plaque exposed to the greatest shear stress [74]. Macrophages control many of the inflammatory processes within the plaque, and are the principal cells responsible for the production of MMPs [49]. MMPs are the predominant proteolytic enzymes, thought to participate in weakening the connective tissue matrix in the intima, leading to plaque rupture and acute thrombosis [76, 77]. Of note, human peripheral blood monocytes express MMP-8, -9, -14 and -19, TIMP-1 and TIMP-2 constitutively, but activated macrophages and foam cells express high levels of several MMPs, in response to adhesion and pro-inflammatory mediators, either directly, through mitogen-activated protein kinase (MAPK) activation and the NF-κB pathway, or indirectly, with prostaglandin E_2 (PGE_2) involvement [78, 79]. The upregulation of MMP secretion by differentiated macrophages can be stimulated by contact with endothelial cells (ECs) [80] and by adhesion to matrix components, such as collagen [81]. Contact-mediated upregulation of MMPs seems therefore to be the first stage of MMP induction in activated macrophages. Intracellular accumulation of lipids, typical of foam cells, thus increases MMP expression in macrophages [82, 83] and, in turn, foam cells produce ROS that trigger the transformation

of pro-MMP into active MMP. Several pro-atherogenic cytokines and growth factors, including IL-1β, TNF-α, macrophage colony-stimulating factor (M-CSF) and PDGF respectively, as well as other inflammatory mediators increase macrophage MMP expression [78, 84]. Moreover, immune cells might upregulate MMPs in activated macrophages, including MMP-1, -3, -8 and -11, and MMPs co-localize with CD40 expression in atherosclerotic plaques [85-88]. Moreover, it has been demonstrated that MMP secretion can be induced by both innate immune system, through action on Toll-like receptors, and the acquired immune system, through actions of interferon γ and a variety of interleukins (mainly IL-1, -4, -10, and -13) [78]. IL-8 can for example release a local imbalance between MMPs and TIMPs by inhibition of TIMP-1 expression in macrophages [89].

Besides macrophages, which release mainly MMP-1, -3, -8, -9, -11, -12, and -14, as well as TIMPs, other vascular cells produce and secrete various MMPs [20, 49]. The principal MMPs produced by vascular SMCs are MMP-1, -2, -3, -8, -9, and -14. MMP-2, rather than MMP-9, is constitutively expressed in vascular SMCs, but both MMP-2 activation and MMP-9 induction are rapidly triggered by vascular injury [90, 91]. Both inflammatory and immune activation of MMPs are also reported for SMCs. MMP-1, -3, and -9 are inducible by inflammatory cytokines and growth factors [92-94] as well as by immune cells [88, 95]. Atherosclerotic plaque resident T lynphocytes may also be a direct source of MMP-1, -2, -3, -9 [96, 97]. Mast cells, such as SMCs and ECs, constitutively secrete MMP-2 and upregulate MMP-9 secretion, in response to inflammatory stimuli. MMP-9 induction occurs both through cell-contact-dependent mechanisms with activated T cells, and through autocrine TNF-α secretion [98]. Moreover, inflammatory cytokines and growth factors produced by activated macrophages induce expression of MMPs in ECs, including MMP-1, -3, -2, -3, -7, -8, -9, -10, -11, -13, -14, -15, and -16 [99-101]. Ligation of CD40, expressed by ECs, thus upregulates MMP-1, -3, and -9 and increases activation of MMP-2 [102]. Oxidized LDLs also upregulate MMP-1 in human vascular ECs and human coronary artery ECs, whereas they downregulate TIMP-1 [103]. In addition, ECs express co-activators of MMPs, including urokinase plasminogen activator receptor, tissue plasminogen activator, CD44, and RECK, as well as TIMPs [101]. Furthermore, adventitial fibroblasts produce MMP-1, -3, and -9, while several MMPs have been identified in platelets, including MMP-1, -2, -3, -9, and -14, as well as TIMP-1, -2 and -4, which can modulate platelet activation and aggregation [104]. It has been reported that thrombin, which is produced in large quantities during plaque rupture, leads to MMP-2 activation and that, in turn, MMP-2 may increase platelet activation, leading to further activation of thrombin and to secondary MMP-2 activation, in a feedback mechanism [104, 105]. It has also been shown that oxidized LDLs stimulate MMP-9 and MMP-14 release by macrophages and SMCs [106, 107] and increase MMP-1 expression in ECs [108]. In this context, the mechanism responsible for expression and activation of vascular-cell-derived MMPs seems to involve both inflammatory and immune upregulation, as well as oxidative stress.

The implications of MMP overactivity on atherosclerotic plaque destabilization have been confirmed in *apolipoprotein E* (*ApoE*) knockout mice, by overexpressing individual MMP genes or by deleting TIMPs [77, 109, 110]. In the same type of mice, macrophage overexpression of active MMP-9 was found to induce acute plaque disruption, without significantly affecting lesion size or macrophage content [111]. Another study showed that overexpression of MMP-9 had no effect on the size of early carotid lesions, but disrupted advanced lesions, especially those caused by hypercholesterolemia [112]. It thus appears that MMP-1 overexpression in macrophages reduces atherosclerotic progression in *ApoE* deficient mice, by reducing the amount of collagenous matrix accumulation [113]. Studies in *ApoE* knockout mice with deleted MMP genes have illustrated that MMPs play a dual role in fibrous cup formation and plaque destabilization. Deletion of MMP-13 collagen deposition and greater plaque stability [114, 115]. Conversely, MMP-2 deletion lowered plaque stability in the aortic root, probably due to MMP-2's ability to aid the

migration of vascular SMCs and to build the fibrous cup [116]. *Mmp-3* deficiency produces more stable atherosclerotic plaques and increases plaque size [117]; in the aorta of *Mmp-9* deficient mice, though, plaque size was reduced, as were macrophage content and elastin degradation [118]. Moreover, increased plaque size, macrophage content and buried fibrous layers were observed in the brachiocephalic artery of *ApoE/Mmp-3, ApoE/Mmp-7,* and *ApoE/Mmp-9* double knockout mice [119]. Recruitment of vascular SMCs was also reduced in plaques of both *Mmp-3* and *Mmp-9* deficient mice, which is consistent with impaired intimal thickening in these mice [120]. Conversely, MMP-7 deletion increased SMC proliferation in the plaques of *ApoE* knockout mice [119], consistent with the involvement of MMP-7 in vascular SMC apoptosis [121]. With regard to MMP-12, its deletion caused more stable lesions in the brachiocephalic artery [119] and reduced elastin degradation in the aortic arch [118]. MMP-12 deletion also decreased foam cells apoptosis and reduced calcification into plaques [122]. Conversely, MMP-12 overexpression has also been shown to increase plaque size and inflammation in rabbits [123]. In addition, deletion of MMP-8 reduced inflammation and aortic atherosclerosis [124] while deletion of MMP-13 or MMP-14 had little effect on vascular SMC or macrophage content but, contributed to plaque stability by increasing collagen content [115, 125].

Degradation of ECM by MMPs exerts an influence at various stages of atherosclerotic plaque development [126]. In the initial stages of atherosclerosis after vascular injury, matrix degradation, presumably induced by MMP dysregulation (mainly MMP-2 and MMP-9), is thought to be combined with changes in permeability, macrophages activation and increased SMC migration [20, 49, 127]. For example, monocytes and T-lymphocytes adhering to ECs respectively enhance production of MMP-9 and that of MMP-2 [128, 129]. This enhancement is associated with EC basement membrane degradation, and penetration of the intima by monocytes and T-lymphocytes, leading to enhanced EC permeability [80, 100]. Moreover, MMP-2 and MMP-9 initially, and MMP-14 subsequently, promote the migration and proliferation of vascular SMCs, which could increase fibrous cup thickness and promote stability [127, 130, 131]. MMP-3 has also recently been implicated in this process, via its ability to stimulate MMP-9 activation [120]. Induction of MMPs in vascular SMCs, by a combination of inflammatory cytokines and growth factors, may be the key to matrix remodeling [94]. The regulatory mechanisms by which matrix remodeling can control the SMC cycle, involving growth factors and kinases, and by which it can also regulate plaque stabilization, has been studied in detail [132]. Conversely, MMP-7 mediated cadherin cleavage leads apoptosis of vascular SMCs, with possible decreased intima formation [121]. Large amounts of ECM are deposited in the fibrous cup during atherosclerotic plaque development, which provides its structural integrity and stability, while MMP secretion by vascular cells promotes macrophage invasion, thereby increasing plaque inflammation [133, 134]. During atherosclerotic plaque progression, ECs, vascular SMCs, and foam-cell macrophages secrete a variety of cytokines that perpetuate the recruitment and activation of inflammatory cells, which in turn attract modified vascular SMCs to the neointima [135]. In later atherosclerosis stages, an excess of MMPs versus inhibitors significantly contributes to ECM degradation, rendering the plaque more prone to rupture. Numbers of MMP-1, -2, -3, -7, -9, -12 and -14 positive macrophages, and of SMCs in the plaque increase in parallel with plaque progression [82, 126, 136]. The various different proteolytic enzymes have mainly been observed at the shoulder region of the plaque, where types I and III collagen are degraded; the cells playing the central role in this process are macrophages. Many MMPs (MMP-1, -2, -3, -7, -8, -9, -11, -12, -13, -14, and -16) are thus detectable in the macrophage-rich shoulder region of highly inflamed atheromatous plaques. MMP-7 and MMP-12, however, are detected in a greater concentration in macrophages close to the necrotic core [20, 96, 137, 138]. Co-localization of cleaved collagen with collagenases MMP-1, -8, and -13 suggests these enzymes are active [88, 139]. Interestingly, it has been observed that cells isolated from human plaques overexpress MMP-1 and MMP-3, caused by activation of Toll-like receptor 2 [140]. Moreover, isolated foam cells from cholesterol-fed rabbits induce expression of MMP-1, -3, -

12 and -14 [83, 141, 142]. However, a study carried out in endarterectomy biopsies showed that increased MMP-2 was mainly associated with SMCs and plaque stability, while MMP-8 and MMP-9 were associated with the presence of macrophages, and unstable plaques prone to rupture [138]. Increased MMP expression has also been observed after coronary angioplasty, suggesting that MMP expression may be involved in the formation of restenotic lesions [143]. It has also been reported that increased TIMP-1 levels are consistently present in human atherosclerotic plaques, where they are mainly found in areas of calcification [144] and that increased circulating levels of TIMP-1 are related to stable coronary, carotid and peripheral artery atherosclerosis [145, 146]. Of note, it has been shown that only pro-MMP-2, MMP-14, TIMP-1 and TIMP-2 are constitutively found in normal arteries; no activity was detected by *in situ* zymography [20].

Angiogenesis in the adventitia underlying plaques, and within the plaque itself, is another feature of the pathogenesis of atherosclerosis; it is associated with plaque progression and destabilization. The angiogenetic fibroblast growth factors (FGFs) promote EC migration and upregulation of MMP-1, -2, -3, -7, -9, -10, -11, and -13 [147, 148] whereas vascular endothelial growth factor (VEGF) induce MMP-2 expression [149]. Thrombin also displays pro-angiogenetic effects, by up-regulating MMP-1 and MMP-3 [150] and by enhancing MMP-2 activation via MMP-14 [151].

Endothelial erosion is another process associated with plaque rupture that occurs in highly stenotic and fibrotic plaques. It has been suggested that overproduction of MMPs (principally of MMP-2 and MMP-9) from inflamed or otherwise dysfunctional ECs weakens their interaction with their underlying basement membrane, thereby causing erosion. Moreover, low average shear stress and disturbed flow are also associated with endothelial dysfunction and erosion [101].

Of the various MMPs, MMP-9 has recently been the focus of growing interest in connection with human illnesses, including cardiovascular disorders associated to atherosclerosis [152]. It has then been found that polymorphisms of the MMP-9 gene are linked to atherosclerosis and to complicated coronary lesions [153-155]. Moreover, deficiency of *Mmp-9* reduces the formation of atherosclerotic lesions in *ApoE*-deficient mice [118]: MMP-9 is the enzyme most specifically associated with atherosclerotic plaque instability and rupture. However, MMP-9 is not only important for ECM degradation; it also plays a role in ECM organization [129, 156].

Upregulation of intraplaque MMP-9 leads to increased plaque hemorrhage and rupture in mouse models [111, 112]. MMP-9 has also been found to be highly expressed in unstable human plaques [157, 158], whereas TIMP-1 and TIMP-2 levels remained unchanged, and there was a significant increase in the MMP-9/TIMP-1 ratio [157]. In humans, analysis of coronary atherectomy specimens found higher levels of active intraplaque MMP-9 in patients with unstable angina (i.e. unstable coronary plaques) compared to patients with stable angina (i.e. stable coronary plaques) [159]: MMP-9 has been detected in the vulnerable shoulder region, and in areas of foam-cell formation in atherosclerotic plaques [78]. Moreover, raised levels of MMP-9 and MMP-2 in the coronary arteries carrying the culprit lesion, add further evidence to show the role of these enzymes in plaque rupture, and in the precipitation of an acute vascular event [160]. A significant increase in MMP-9 immunopositivity has also been demonstrated in atherosclerotic lesions of the aorta and carotid artery of rabbits fed a high-cholesterol diet [161]. Increased plasma levels of circulating MMP-9, as well as MMP-2, also correlate with the clinical symptoms of plaque instability and rupture in the coronary and cerebral circulation [162, 163] as well as being found in patients affected by myocardial infarction [164, 165], suggesting that MMP-9 may be useful as a biomarker for acute coronary syndrome [166, 167].

As several studies have also reported, oxidized LDL have been demonstrated to induce expression and activity of MMP-9, and to decrease its endogenous inhibitor, TIMP-1, in human macrophages and ECs [106, 168-170]. A causal

association between oxidized LDL autoantibodies and serum MMP-9 levels *in vivo* has also been demonstrated [171]. Oxysterols, which are cholesterol oxidation products, are present in considerable amounts in oxidized LDL, and appear to be implicated in the pathogenesis of atherosclerosis [172-175]. In this context, several studies have shown that, in cells of the macrophage lineage, oxysterols can initiate specific signal transduction pathways, which are relevant to the development of atherosclerosis [176-179]. A recent study has demonstrated that an oxysterol mixture, of composition similar to that found in advanced human carotid plaques [173], can significantly contribute to destabilizing the fibrotic plaque, by increasing expression and activity of MMP-9, without interfering with expression and synthesis of TIMP-1 or TIMP-2. The consequent net imbalance of the MMP-9/TIMP-1,-2 ratio would in this case then trigger an excessive proteolytic ECM degradation within the advanced lesion, and contribute to plaque instability and likely rupture [180].

2.1.1. Inhibition of Metalloproteinases as Therapeutic Strategies for Atherosclerosis

Although MMP involvement in atherosclerotic pathology and in other vascular diseases goes beyond simple excessive matrix degradation, MMP inhibition may be of therapeutic benefit [101, 181]. Several physiological mediators are present in the vasculature, where they suppress MMP secretion in normal tissues, and in conditions of injury and inflammation. The athero-protective agent NO, produced by ECs, might inhibit MMP-9 production from vascular SMCs [182]. The anti-inflammatory cytokine TGFβ inhibits induction of MMP-1, -3, -7, although paradoxically it upregulates MMP-13 [183], and it may upregulate TIMP-3 [93]. Moreover, interferon-α and/or -γ inhibit the induction of MMP-1, -3, -9, and -13, thereby presumably reducing the contribution of immune mechanisms to MMP induction in SMCs and in macrophages [85, 95].

Furthermore, recent data have shown that treatment with a low-molecular-weight heparin decreases the levels of MMP-9 in patients with abdominal aortic aneurysm [184]. Again, angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (AIIRBs) reduce the raised circulating MMP-9 levels in patients with stable coronary artery disease [185], and the levels of this enzyme are also reduced by ACEI captopril in patients with acute myocardial infarction [186]. Further, since MMP activity may be induced by ROS, antioxidant therapy can be useful to modulate MMPs, including MMP-9, as has been demonstrate in a hypercholesterolemic rabbit model [187].

Targeting specific cytokines or signaling pathways that are involved in mediating MMP upregulation could thus reduce MMP activity in atherosclerotic plaques. For example, inhibition of the CD40 ligand stabilizes plaques in mice, possibly due in part to effects on MMPs [188]. The MMP pathway can also be interrupted by targeting the inflammatory response. One specific target is PGE_2: reducing PGE_2 synthesis, for example with indomethacin or other anti-inflammatory drugs, can contribute to reducing MMP synthesis [189, 190]. Because PGE_2 production is involved in MMP transcription, antagonists at the PGE_2 receptor could also be useful in stabilizing plaques [191, 192]. Aspirin and other cyclooxygenase inhibitors also inhibit MMP production by monocytes [78], although cyclooxygenase independent pathways of MMP production may reduce the beneficial impact of these compounds in man [79]. In addition, MMP-1, -3 and -14 are expressed via NF-κB, therefore inhibition of this transcription factor might reduce expression of these MMPs [83].

Taken together, these findings show that there is a clear potential for the application of TIMPs as endogenous inhibitors [193] through gene therapy [41]. It has been demonstrated that adenovirus-mediated gene transfer aimed at overexpressing TIMPs can reduce MMP activity, intimal thickening, and plaque destabilization in various models. For example, overexpression of TIMP-1 in a mouse model of atherosclerosis caused a reduction in the lesion and macrophage content at the aortic root [194]. Another study examined whether short-term overexpression of TIMP-1 or TIMP-2 would attenuate atherosclerotic plaque development and instability, in *ApoE* knockout mice fed a high-fat diet.

Analysis of brachiocephalic artery plaques showed that overexpression of TIMP-2, but not TIMP-1 infection, resulted in marked reduction in lesion area compared with control animals. TIMP-2 significantly reduced migration and apoptosis of macrophages and foam cells, inhibiting atherosclerotic plaque development and destabilization, whereas TIMP-1 failed to exert similar effects [133]. Moreover, a recombinant TIMP-1 has been shown to reduce the activity of MMP-1, -2, -9, and -3 in the shoulder and core regions of the plaque [101]. Finally, overexpression of TIMP-3 markedly reduced neointima formation both *in vitro* and *in vivo*, by promoting cell apoptosis [195, 196].

Furthermore, several observations suggest that statins, which are potent lipid-lowering drugs, may exert their beneficial effects on the arterial wall in part by means of their effects on the inflammatory response and on MMP and TIMP production. In particular, statins contribute to increasing plaque stability by inhibiting MMP secretion [197]. Statins have been reported to prevent atherosclerosis progression and coronary events, by inhibiting expression and secretion of MMP-1, -2, -3, and -9, in macrophages and SMCs *in vitro*, and in rabbit and human atherosclerotic lesions [198-201]. Cerivastatin also suppresses macrophage growth and reduces MMP-1, -3, and -9 expression in rabbits, while conserving the collagen [202]. Treatment with pravastatin before carotid endarterectomy reduced plaque lipid content, inflammation, MMP activity and cell death, also increasing the collagen content [200]. Statins are also known to exert anti-inflammatory and cardioprotective effects in *ApoE* knockout mice [203]. In addition, statin treatment has been shown to cause a significant reduction of C reactive protein (CRP) circulating levels, which are correlated to the severity of atherosclerosis [204, 205].

Another strategy considers antibody-based inhibitors of MMPs. MMP-2 neutralizing antibodies have shown protective activity in hearts exposed to pro-inflammatory cytokines or ischemia/reperfusion injury. MMP-14 blocking antibodies have also been proposed as targets [206]. Alternatively, synthetic MMP inhibitors have been developed and tested. Synthetic MMP inhibitors are potent Zn^{2+}-chelating mimickers of collagen, and the majority of them are broad-spectrum inhibitors that suppress the activity of a large number of different MMPs [207]. More than 50 synthetic MMP inhibitors have to date been considered for possible clinical development [101]. Among synthetic MMP inhibitors are the hydroxamic acid derived inhibitors, such as BB-94 (batimastat), BB1101, BB-2293, BB-2516 (marimastat), and CPD-845 (CT-1746) [208]. Batimastat and marimastat are competitive MMP-inhibitors but, despite the promising results obtained with both inhibitors, their lack of oral bioavailability has precluded their long-term use; marimastat had better oral bioavailability [209] than batimastat [210]. Moreover, non-selective MMP inhibitors [211] or more selective MMP inhibitors, including pyrimidine-2,4,6-trione derivative Ro-28-2653, inhibit MMP-2, -9, and -14, reducing BBB breakdown in rat models of stroke [212]. Likewise, Ro-28-3555 (trocade) is a selective inhibitor of MMP-1 [213] and IW449 is a selective inhibitor of MMP-2 [208]. Other synthetic inhibitors include PD166793, OPB-3206 [214], BAY12-9566, AG-3340, KBR-7785, KBR-8301, GM-6001 (ilomastat or gelardin) metastat and AE-941 (neovastat) [215]. Moreover, 1,10-phenanthroline is a small organic coumpound inhibiting a broad range of MMPs. The selective MMP-12 inhibitor (RXP470.1) was used to treat established plaques in *ApoE* knockout mice; it blocked plaque enlargement, decreased lipid core formation, improved the ratio of SMCs to macrophages, and also reduced macrophage apoptosis, calcification and medial elastin breaks [122]. Further, a highly-selective inhibitor of MMP-13 also inhibited collagenolysis, thus preserving the collagen content in plaques [216] to a very similar degree as has been observed to occur in both mice transgenic for a collagenase-resistant mutant of mouse collagen I [114] and *Mmp-13* knockout mice [115].

The tetracycline antibiotic doxycycline is also a broad-spectrum MMP inhibitor, reducing both synthesis and activity of MMPs [217, 218]. Its main mode of action is by binding to the active zinc site, resulting in conformational change in the enzyme structure with loss of activity. Clinical trials using doxycycline showed a significant reduction in

MMP-1 levels in carotid endarterectomy samples [219] and of plasma MMP-9 levels in patients with coronary artery disease [220]. Again doxycycline, appeared to be well-tolerated in another clinical trial for the treatment of abdominal aortic aneurysms [221, 222]. However, in all these clinical trials, the clinical benefit was low or remains to be demonstrated. Doxycycline increased SMC adhesion and reduced SMC migration; moreover, it limited the reorganization of fibrillar collagen matrices [217]. Doxycycline and the MMP inhibitors Ro-31-4724 and Ro-31-7467 have also been shown to reduce SMC proliferation *in vivo* [223] and to promote SMC apoptosis [224].

Because angiogenesis within plaques is associated with plaque progression and vulnerability [225] and because MMPs participate in this feature of atherosclerosis, they might be another target for therapy. It has been reported that marimastat inhibits angiogenesis in both collagen and fibrin matrices, by inhibiting MMP expression [149].

2.2. Metalloproteinases and Diabetic Vascular Complications

Type 2 diabetes mellitus is a frequent vascular risk in cardiovascular events, including atherosclerosis, and also in microvascular complications [226]. Considerable evidence points to a role for MMPs and TIMPs in the atherosclerotic process; however, the relationship between MMPs/TIMPs and diabetic angiopathy is less well defined. It is probable that atherosclerosis and diabetes mellitus share common pathways of MMP synthesis and plaque destabilization, including inflammatory pathways and molecules. However, recent *in vitro* and *in vivo* studies have demonstrated that hyperglycemia, either directly, or indirectly via oxidative stress or advanced glycation end-products (AGEs) regulates MMP's expression and activity. Disruption of the ECM may enhance monocyte and vascular SMC migration, which may aggravate atherosclerosis in diabetes mellitus.

The exposure of ECs, macrophages, and SMCs to high concentrations of glucose induces dysregulation of the MMP/TIMP balance. High glucose concentrations have been demonstrated to induce expression of MMP-2 in arterial vasculature *in vivo* [227] and in cultured vascular SMCs [228]. In addition, hyperglycemia induces expression of MMP-1 and MMP-2 in ECs, and expression of MMP-9 in macrophages, decreasing expression of MMP-3 but having no effect on TIMP-1 expression [35]. Another study has found MMP-2 and MMP-9 expression not to be affected, but in a high-glucose environment their activity increased in ECs from umbilical cords [229]. Interestingly, it has also been shown that the effects of hyperglycemia on MMP-2 activity were further enhanced in vascular SMCs that were exposed to intermittent, rather than constant, high-glucose concentrations, more closely resembling a pathophysiological condition [230]. Moreover, increased synthesis of active and latent forms of MMP-2 and MMP-9 was observed in aortic specimens and blood samples from diabetic rats, as well as in cultured ECs, but not in vascular SMCs or macrophages [231]. It was subsequently shown that incubation with plasma LDL from patients with type 2 diabetes significantly increases expression of MMP-9 in monocytes [232]. It has also been suggested that high glucose levels might upregulate transcriptional factors, such as AP-1, or growth factors, such as TGFβ, which in turn enhance MMP gene transcription [233,234].

MMP gene transcription may also be regulated by oxidative stress, through several mechanisms such as alteration of NO synthase activity, and AGE formation [235], as well as by genetic polymorphism [236]. There is increasing evidence to suggest that AGEs, and the interrelationship with their receptors (RAGEs), influence several signaling pathways, which are involved in vascular dysfunction [237]. AGEs are usually localized on the surface of macrophages and bind to the ECM. It has been demonstrated that matrix-glycation products increase monocyte infiltration, especially in the shoulder regions of plaques, and that they stimulate macrophages to release cytokines, thus accelerating the inflammatory response [238, 239]. In the shoulder region, there is thus an accumulation of macrophages, which contain the majority of RAGEs; the overexpression of RAGEs, which sustains inflammation,

triggers vascular SMCs and macrophages to produce MMP-2 and MMP-9 [238]. The preponderance of macrophages coincides with reduced collagen content and with MMP-2 and MMP-9 overexpression in human diabetic plaques [238].

The MMP/TIMP system may interfere with the subcellular insulin signaling pathways. Imbalance of the insulin signaling cascades may be involved in the atherosclerosis-promoting effect of insulin resistance, possibly by promoting MMP-2 and MMP-9 overexpression and compromising the expression of TIMP-3 [240]. Conversely, it has been shown that, in healthy subjects, insulin infusion acutely suppresses plasma levels of MMP-9 and VEGF levels. VEGF is known to contribute to proliferative retinopathy, as well as to plaque evolution and rupture [241]. Thus, insulin might act to prevent atherosclerosis and diabetic complications through its anti-proteolytic and anti-angiogenetic properties. High glucose levels, acting via the AGE/RAGE interaction and altered insulin signaling, thus provide a stimulus to inflammation and ECM degradation within atherosclerotic plaques.

High circulating levels of MMP-2, -8 and -9 have been found in patients with diabetes [242-244] and peripheral arterial disease [245] or acute coronary syndrome [246], while TIMP-1 levels are reported to be lower in diabetic patients than in non-diabetic subjects [247], although other studies have found normal levels of MMP-1, -3, -9 and TIMP-1 in diabetic patients [248, 249].

Apart from the role of MMPs in diabetic macrovascular complications, such as atherosclerosis, MMPs seem to play a key role in the development of the diabetic microvascular complications known as microangiopathies, which include diabetic retinopathy, diabetic nephropathy, and diabetic peripheral neuropathy.

Although normal retinas express MMP-1 and TIMP-2, retinas from diabetic patients show above-normal concentrations of activated MMP-1, -2, -3 and -9, as well as of TIMP-1, -2, and -3 [250, 251]. These processes may contribute to retinal neovascularization. High concentrations of MMP-8, -9, and -14 have also been found in the urine of patients with diabetic nephropathy [252], leading to investigation of the expression of MMP in diabetic kidneys and mesangial cells. Accumulation of ECM within the glomerulus contributes to diabetic renal dysfunction, and the amount and composition of mesangial matrix in diabetic nephropathy reflects the imbalance between synthesis and degradation of the ECM [253]. Additionally, hyperglycemia reduces MMP expression whereas it accelerates TIMP expression, consequently suppressing ECM degradation, leading to the accumulation of matrix components in the glomerular mesangium [254]. Moreover, it has been observed that the increased AGE formation within glomeruli renders the ECM less susceptible to degradation, by reducing MMP activity, and thus playing a role in the pathogenesis of diabetic nephropathy [255]. Interestingly, alongside their direct role in ECM turnover, MMPs have been demonstrated to release or activate various growth factors that have been associated with renal hypertrophy, tubular cell proliferation, and renal fibrosis, and which contribute to development of the renal abnormalities characteristic of diabetic nephropathy [256].

2.2.1. Inhibition of Metalloproteinases as Therapeutic Strategies for Diabetic Vascular Complications

There is increasing interest in the influence of anti-diabetic drugs on the MMPs/TIMPs balance. Rosiglitazone, a thiazolidinedione, reduces circulating levels of MMP-9, IL-6, white blood cells and other inflammatory markers in type 2 diabetes [257, 258], subsequently reducing neointimal hyperplasia [259]. Moreover, gliclazide reduces oxidized LDL-mediated MMP-9 expression in human aortic ECs *in vitro* [168] and pioglitazone, compared with placebo, significantly decreases plasma MMP-9 levels in diabetic patients with coronary artery disease [260]. Thiazolidinediones may also influence MMP-1 expression in vascular ECs [261].

It has also been reported that both ACEIs and AIIRBs exert beneficial effects by preventing or slowing the progression of diabetic nephropathy, favoring, for example, MMP-2 activity [262]. In addition, it has been shown that diabetic patients benefit from statin treatment [263-265]. In this context, it is speculated that statins may exert their beneficial

effects in diabetic patients through a similar mechanism to that occuring in atherosclerosis: they appear to protect from diabetic complications by decreasing lipid levels, lipid oxidation, inflammation, MMP expression, and cell death, and by increasing the TIMP and collagen content of human atherosclerotic plaques, thus contributing to their stability. Statins may also act by reducing vascular SMC migration and proliferation [266], and exerting an inhibitory action on MMP-1, -3, and -9 secretion from the same cells [267]. Statin treatment also causes significant suppression of MMP activity and significant activation of TIMPs (MMP-2 and TIMP-2, respectively) preventing, for example, glomerular ECM accumulation [268, 269]. Statins are also reported to possess anti-inflammatory action: in particular they reduce the serum concentrations of CRP, which is considered to be a marker of vascular risk [270-272]. These drugs can also improve renal function and, of note, they lower serum urate concentrations, which can influence MMP activity [273-275].

Peroxisome proliferator activator receptors (PPARs) are nuclear receptors that regulate fatty acid oxidation, adipocyte differentiation, and insulin sensitivity, as well as atherosclerosis pathogenesis; PPAR ligands are thus another compound that is used in the treatment of diabetes mellitus. The thiazolidinediones, which are insulin-sensitizing drugs, are PPARγ activators, while fibric acid derivatives (fibrates) have lipid-lowering properties, and activate PPARα [276]. There is evidence suggesting that both a PPARα activator (fenofibrate) and a PPARγ activator (rosiglitazone) inhibit MMP-9 expression in vascular SMCs and in monocytes/macrophages [277, 278]. Fibrates may also decrease plasma levels of inflammatory markers (*e.g.* CRP) that, in turn, influence MMP expression [279, 280]. Based on these findings, it has been suggested that the fibrate-plus-statin combination could be a promising therapeutic strategy for diabetic atherosclerosis [281].

2.3. Metalloproteinases and Neurodegenerative Diseases

An increasing body of evidence points to the critical role of inflammation in the neurodegenerative process. During neuroinflammation, molecular cascades occur whose goal is to remove damaged cells and prepare the brain for repair, but the overactivated and/or chronically activated state of the microglia contributes to neuronal death and dysfunction. Microglia can be activated by MMPs as well as by amyloid β (Aβ) and α-synuclein [282, 283].

MMPs and ADAMs are important in acute and chronic neuroinflammation, and recent studies have linked their actions to neurodegenerative disorders that are often associated with vascular cognitive impairment, such as Alzheimer's and Parkinson's diseases [23, 284-286].

Several key MMPs and ADAMs have been implicated in neuroinflammation: MMP-2, MMP-3, MMP-9, MMP-14, ADAM-9, ADAM-10, and ADAM-17. However, with regard to ADAM functions in the brain, they are also implicated in cell survival, proliferation, differentiation, and migration, as well as in axonal growth and myelination.

Brain cells express both constitutive and inducible MMPs in response to cellular stress [7]. For example, MMP-2 is a constitutively expressed molecule that is normally present in brain tissue and in the cerebrospinal fluid (CSF). MMP-9 is normally expressed in brain tissue at low levels, but is markedly upregulated by various inflammatory stimuli (*e.g.* cytokines and growth factors) in many brain disorders; conversely, MMP-9 is not present in the CSF [7,23].

The initial phases of neuroinflammation are characterized by activation of constitutive proteases that begin the process of disassembling the ECM, opening the blood-brain barrier (BBB), preventing normal cell signaling, and initiating cell death by apoptosis [287].

In the active form, MMPs play a number of important roles in normal development, but they are highly destructive in case of inflammation of the central nervous system (CNS). These enzymes, indeed, increase the

permeability of the BBB by attacking the ECM, basal lamina, and tight junctions proteins in ECs; this increased permeability is a feature of the acute neuroinflammatory response, and allows cells to enter the CNS, contributing to white-matter damage. When matrix proteins around the neurons are degraded, there is loss of contact and cell death by anoikis [32]. Proteolysis of the matrix protein of blood vessels and brain cells by MMPs, in particular by the constitutive MMP-2, and subsequently by the inducible MMP-1, -3, and -9, increases the risk of cerebral edema, hemorrhage, and cell death [288]: it has been shown that MMP-2, -3, and -9 increase permeability of the BBB [287, 289-292] leading to the infiltration of inflammatory cells, such as neutrophils, which play an important role in neuroinflammation [293]. Loss of oxygen and energy substrates releases glutamate into the extracellular space, initiating molecular events in the injured cells that might result in loss of membrane integrity and necrosis. Inhibitors of MMPs can reduce damage to the BBB, and thus reduce cerebral edema and hemorrhage [290]. *Mmp-9* or *Mmp-3* knockout mice were also found to have reduced infarct size and significantly less BBB damage and neutrophil infiltration [289, 293, 294]. Direct injection of TNF-α into the rat brain results in a dramatic increase in the expression and activation of MMP-9 and MMP-3, which is associated with a significant opening of the BBB. Microglia and neurons surrounding the injection site are the major cellular sources of MMP-3 and MMP-9, through cycloxygenase-derived product involvement, following intracerebral TNF-α administration [295, 296]. Moreover, in the brain, microglia and astrocytes are major sources of many pro-inflammatory cytokines, and of other mediators that stimulate increased MMP production [297, 298]. Secondly, MMPs are involved in tissue repair, driving angiogenetic and neurogenetic processes [299, 300] and in the end ECM remodeling occurs, with impenetrable scar tissue formation that blocks re-growth and re-projection of axons.

However, much has been learned about the function of the MMPs in the brain, by using cell cultures such as neurons, ECs, astrocytes and microglia. The two major inducible MMPs that have been identified in the neuroinflammatory response are MMP-3 and MMP-9. Rat brain ECs stimulated with lipopolysaccharide (LPS) showed induction of MMP-9 [301]; cultured rat astrocytes stimulated with LPS, IL-1β, TNF-α, or bradykinin secreted normal MMP-2, while expression and activity of MMP-9 was upregulated [302-305]. Other studies have further demonstrated that several external stimuli can upregulate MMP-9 expression, via the MAPK/AP-1 pathway, in different cell types [306-308]. Expression of MMP-9 can also be induced by oxidized LDL in rat brain astrocytes, through the MAPK-extracellular signal-regulated kinase kinase (MEK)1/2, and the phosphatidyl inositide 3 (PI3)/Akt-c-Jun N-terminal kinase (JNK)1/2 signaling pathways, leading to AP-1 activation [309]. This data implies that oxidized LDL might play a crucial role in the development of brain injuries and CNS diseases. Oxidized LDL, indeed, has been reported to exhibit a wide range of biological activities, including alteration of neuronal apoptosis, capillary homeostasis, and modulation of inflammatory protein activity in various brain cells [310-312]. Moreover, astrocytes also release MMP-1 when stimulated with IL-1β, and MMP-1 has been shown to be toxic to human neurons in culture [313]. In addition, incubation of this cell type with the 1-40 fragment of Aβ induced MMP-9 and MMP-3 production, suggesting that MMPs may be involved in amyloid processing in AD [314]. Stimulated astrocytes might also be induced to produce the inactive form of MMP-9, thus failing to produce its active form; however, cultures of microglia and astrocytes stimulated with LPS have been shown to produce the active form of MMP-9 and MMP-3 [303, 315, 316]. This evidence stresses that the microglia are necessary for activation of the proMMP-9, possibly through MMP-3 or free radical production [315]. It has also been suggested that the effects of LPS on the MMPs expression are due in part to the formation of pro-inflammatory cytokines. TNF-α and IL-1β produce a significant increase in the production of MMP-3 and MMP-9 in cultured astrocytes and microglia [302, 317]. In LPS-stimulated astrocytes, smaller increases of MMP-10, -12, and -13 were also seen [318].

Of note, a number of immunohistochemistry studies, conducted in human autopsy brain tissue from patients with various pathologic conditions, or in animal models, have identified the cell types expressing the MMPs in the brain. In patients with multiple sclerosis and cerebral infarction, antibodies to MMP-9 were localized in blood vessels and neutrophils, but in more chronic lesions, MMP-2 and MMP-7 were the prominent enzymes found in the inflammatory cells. Astrocytes around the infarctions immunostained positive for MMP-2 [319]. Furthermore, in the progressive form of dementia, due to atherosclerosis of the blood vessels with demyelination of the white matter, tissue macrophages stained for MMP-3 in regions of damaged white matter [320]. There is some evidence to indicate a role for MMP-3 in neurodegeneration, because it plays a critical role as an intercellular signaling molecule that modulates neuroinflammatory responses [321-323].

In neuronal cells, MMP-3 expression is increased in response to cell stress, and the active MMP-3 participates in apoptotic signaling [324] and triggers microglia activation and production of pro-inflammatory cytokines [321] triggering the formation of ROS [322, 323]. Active MMP-3 may also induce microglial activation near the site where apoptosis occurs, to promote clearance of apoptotic cells [321]. Despite these data, the exact molecular mechanisms through which active MMP-3 activates microglia cells are still not clear. In this connection, it has been shown that MMP-3 deficient mice display a significant reduction in microglia activation following the *in vivo* administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [322]. This would appear to indicate that, in the extracellular space, MMP-3 triggers the microglia to produce pro-inflammatory and cytotoxic molecules, which in turn contribute to neuronal damage and to the removal of damaged neurons by phagocytosis. In addition, MMP-3 produced by the activated microglia can be released into the extracellular matrix, further exacerbating the neuroinflammatory process [324]. In contrast, overexpression of TIMP-1 results in the attenuation of apoptosis in neuronal cells, along with the suppression of MMP-3 activity [325]. TIMP-1 is also neuroprotective, both against excitotoxic neuronal death [326] and against traumatic and ischemic brain injury in mice [327]. In an animal model of temporary ischemia-reperfusion, MMP-3 was mainly seen in the neurons and microglia, while MMP-9 was present in the neurons. After 3 weeks of reperfusion, the principal MMP present was MMP-2, in astrocytes [315].

In this connection, evidence is emerging that MMPs and ADAMs play roles in neurodegenerative diseases, including in the damage to white matter in patients with vascular cognitive impairment, in degradation of amyloid peptides in AD, and in apoptosis of dopaminergic neurons in PD.

a) Vascular cognitive impairment

In vascular cognitive impairment, MMPs are induced by hypoxic hypoperfusion in the white matter [328]. The brain tissues of patients with vascular cognitive impairment express hypoxia-inducible factor 1α (HIF1α). HIF1α increases during hypoxia or ischemia and, because hypoxia seems to play a crucial part in vascular cognitive impairment, an understanding of the role of HIF1α is important.

The increase in HIF1α leads to the expression of many genes implicated in injury and repair [329]. HIF1α can activate furin, an intracellular convertase that activates several enzymes involved in injury, including the MMPs; these increase permeability of the BBB and demyelination in the white matter, as well as elevating vasoconstriction, with subsequent marked hypoxia [330, 331]. Conversely, HIF1α can stimulate the expression of genes involved in repair, such as VEGF and TGFβ [332, 333]. It has also been observed that patients with vascular cognitive impairment have increased expression of MMPs in the white matter, particularly around blood vessels in regions with loss of myelin. Gliotic regions have reactive astrocytes that overexpress MMP-2; macrophages around damaged blood vessels are immunopositive for MMP-3 [320]. The MMPs might damage blood vessels, disrupting the BBB, and thus activating

microglia and recruiting macrophages that contribute to white-matter injury. Demyelination of white matter might also occur, through MMP-mediated mechanisms, mainly by MMP-2, MMP-3 and MMP-9 [320, 330, 334]. Moreover, patients with vascular cognitive impairment have high concentrations of MMP-9 in the CSF [335]. Patients with vascular cognitive impairment also have increased levels of endothelin-1 in the white matter, where it is highly expressed by MMP-2 [336, 337]; endothelin-1 is a strong vasoconstrictor that may compromise blood flow to the deep white matter [338].

b) Parkinson's Disease

Parkinson's disease (PD), a progressive neurodegenerative disorder, is characterized by the selective loss of dopaminergic neurons in the substantia nigra, and degeneration of projecting nerve fibers in the striatum, leading to extrapyramidal motor dysfunction, which is associated with microglia activation [339]. MMPs have been implicated in the death of dopaminergic neurons; in particular, various experimental models have linked increased MMP-3 levels with PD. An animal model of PD showed an increase in MMP-3 immunoreactivity in the substantia nigra region [340, 341]. It has also been shown that, *in vitro*, apoptotic dopaminergic neurons release MMP-3, which acts as a microglia-activating molecule. This evidence suggest that, in addition to ECM degradation, MMP-3 is a signaling molecule in the neuronal apoptotic process, mediating the interaction between apoptotic neurons and microglia, and consequently causing neuroinflammation [321]. The activated microglia release pro-inflammatory cytokines, including TNF-α, which directly induce neuronal death, contributing to neuronal degeneration. It has also been suggested that, in addition to the extracellularly-triggered apoptotic mechanisms, MMP-3 might also act intracellularly in apoptotic signaling in the dopaminergic neurons [342]. Furthermore, MMP-3 may play an important role in the pathophysiology of PD, by contributing to the generation of the toxic α-synuclein aggregates [340, 343].

Finally, MMP-3 mediates BBB disruption, which would allow infiltration of blood immune cells such as neutrophils to the damaged region [293]. Indeed, BBB leakage has been observed in PD patients [344] as well as in animal models of PD [345, 346]. Interestingly, the substantia nigra region is reportedly more prone to BBB disruption and neutrophil infiltration than is the cortical region [347].

c) Alzheimer's Disease

Alzheimer's disease (AD), the most common form of dementia, is characterized by the progressive loss of neurons and synapses, and by extracellular deposits of Aβ in the form of senile plaques, Aβ deposits in the cerebral blood vessels, and intracellular inclusions of hyperphosphorylated tau in the form of neurofibrillary tangles (NFT) [348, 349]. Several mechanisms contribute to AD development and progression, and deposition of improperly processed amyloid is thought to be a major factor in its pathophysiology. Amyloid precursor protein (APP) comprises a transmembrane and an extracellular component, and is degraded into various fragments by the secretases. The physiological pathway results in the cleavage of APP by α-secretase, which produce a soluble fragment (sAPPα) that can be broken down for clearance, and a membrane-bound COOH terminal fragment (CTF). Both β-secretase and γ-secretase act together to produce an ectodomain derivative (sAPPβ) and Aβ peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$) that can aggregate to form insoluble dimmers, oligomers, and subsequently fibrils, which are deposited extracellularly and intracellularly, forming senile plaques [350, 351]. Both MMPs and ADAMs have been implicated in APP shedding [352].

Three members of the ADAM family have been shown to act as an α-secretase: ADAM-9, ADAM-10, and ADAM-17; these enzymes cleave APP into the soluble sAPPα for clearance [353, 354]. ADAM-17 also activates TNF-α, which induces cell death, contributing to neurodegeneration [355].

Overexpression of ADAM-9 has been reported to increase sAPPα release, although mice lacking ADAM-9 revealed no difference in the production of the α-secretase cleavage product of APP [356]. The impact of ADAM-9 on sporadic AD [357] might therefore rely on a more indirect mechanism: ADAM-9 has been shown to proteolytically process ADAM-10 [358-360]. Unlike ADAM-9, ADAM-10 was found to have constitutive and regulated α-secretase activity [354, 361].

With regard to ADAM-10, it has also been observed, in an AD mouse model in which the animals were crossbred with ADAM-10 transgenic mice, that plaque pathology was greatly attenuated, production of α-secretase was enhanced, and subsequently also that of sAPPα [361]. Furthermore, these mice had increased learning and memory potential [361, 362], which might correlate with the observed enhanced cholinergic and glutamatergic synaptogenesis [363]. Conversely, mice with a dominant negative mutant of ADAM-10 had lowered sAPPα levels, accompanied by an enhanced number of plaques [361] and learning deficiencies [364]. In addition, axonal guidance is conveyed by ADAM-10, as has been shown for retinal and peripheral axons [365, 366], and this enzyme regulates axon withdrawal by ephrin cleavage [367, 368]. However, it remains a matter of controversy whether there is a substantial decline of neuronal ADAM-10 in ageing or in the pathological context.

Experiments performed with ADAM-17-deficient cells indicated a participation of ADAM-17 in the regulated [369, 370] and the constitutive α-secretase pathways [371, 372]. Although there is no evidence that ADAM-17 acts as an *in vivo* APP-sheddase in transgenic mice, ADAM-17-positive neurons are found to co-localize with amyloid plaques in AD brains, supporting its role as an α-secretase [373].

In the CNS, MMPs are produced by astrocytes, microglia, neurons, ECs, oligodendrocytes and leukocytes [301, 374, 375]. Elevated levels of MMPs have been reported in the cortex and hippocampus of AD patients compare with controls [376]. Several studies have suggested that MMPs, like ADAMs, participate in the formation and clearance of Aβ [377, 378]. It has been shown that MMPs, such as MMP-2, -3, and -9, are induced endogenously by the toxic amyloid fragments ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) in astrocytes, microglia, mixed hippocampal neurons, and blood vessels [379-381]. Moreover, it has been demonstrated that the upregulation of MMP-9, expressed by SK-N-SH cells in the presence of $A\beta_{1\text{-}40,}$ is mediated by $\alpha_3\beta_1$ and $\alpha_2\beta_1$ integrin receptors, and that MMP-9 can directly process APP, by interacting on the cell surface with α-secretase-like activity, increasing sAPPα release and substantially reducing levels of secreted Aβ peptides [382]. These findings indicate that MMP-9 might act as a neuroprotective mechanism whereby the shedding of APP to soluble fragments precludes the formation of aggregating Aβ peptides. In addition, metal-mediated activation of MAPKs resulted in upregulated MMP-2 and MMP-3 activity, in turn leading to enhanced cleavage of extracellular Aβ, and preventing its accumulation [383-385].

In the area around the amyloid plaques, the microglia are activated and contribute to neuronal death by releasing inflammatory molecules [386]; elevated production of MMPs in the brain tissue of AD patients is part of the inflammatory response. It has been shown that expression of MMP-2, -3 and -9 is increased in astrocytes around amyloid plaques compared with areas without Aβ deposition [377]. MMP-3, for example, has been detected around senile plaques, in the grey matter and in the interstitium between myelinated axons and astrocytes in the white matter of AD patients [387]. MMP-3 is also significantly elevated in the plasma and CSF of AD patients [388]. More recently, the CSF of cognitively-healthy individuals with risk markers for future AD has been found to have higher MMP-3

levels and a higher MMP-3/TIMP-1 ratio than healthy individuals without risk markers [389]. Furthermore, in *postmortem* AD brain tissue, MMP-9 has been found expressed in hippocampal neurons around the amyloid plaques, and in the NFT and vascular wall [289, 390]; it has been suggested that MMPs are synthesized in response to Aβ, and that their activation degrades the peptide *in vivo*, reducing its aggregation [390]. Moreover, plasma concentrations of MMP-9 are increased in AD patients, and it has been suggested that this may be due to ECs releasing the enzyme in response to circulating Aβ [391]. Conversely, MMP-9 concentrations are not increased in the CSF [335]. Active MMP-9 has been reported to degrade synthetic $A\beta_{1-40}$ *in vitro*, acting directly at the α-secretase cleaving site [390], and also to cleave extracellular and fibrillar Aβ through BBB [377, 380]. In this connection, *Mmp-2* and *Mmp-9* knockout mice had higher levels of Aβ in the brain tissue than wild-type mice, and treatment with the MMP inhibitor GM-6001 increased Aβ in the transgenic mice, overexpressing the Swedish variant of APP (mutations at positions 670 and 671) [377]. Besides MMP-9, increased expression of MMP-3 has also been detected in hippocampal neurons, around amyloid plaques in the cortex, and in the interstitium of white matter [387]. It has also been suggested that MMP-14, -16, and -18 might play important roles in regulating APP function, inducing cleavage and shedding of the APP ectodomain when co-expressed with APP adaptor protein Fe65 [352].

2.3.1. Therapeutic Strategies for Inhibiting Metalloproteinases in Neurodegenerative Diseases

Currently, no therapy is available clinically that delays the neurodegenerative process itself, and therefore the development of selective inhibitors and/or other therapeutic strategies against the proteolytic enzyme actions, together with a knowledge of their side-effects, will be of great interest to contrast the progression of neurodegenerative diseases.

In vascular cognitive impairment, treatment with a drug that blocks MMPs, such as minocycline, might be useful to decrease white-matter damage. The possible use of MMP inhibitors in treating neurodegenerative diseases such as AD and PD is even more speculative. Regarding AD, the role of MMPs and ADAMs is complex because of their dual function, in breaking-down amyloid to form Aβ, and in clearance of the peptide from the brain. MMP inhibitors could thus interfere with Aβ clearance. With regard to PD, the use of MMP inhibitors might be promising, since death of dopaminergic neurons appears to be stimulated when activated microglia release MMP.

The major synthetic MMP inhibitors are based on a hydroxamate structure [392]. These compounds interfere with the action of the zinc catalytic domain in the MMP molecule; however, the use of these inhibitors in clinical trials gave controversial results. More encouraging results have been obtained with other inhibitors, in terms of reducing BBB damage, infarct size, and cell death, in animal models of CNS diseases [23]. For example, TIMP-2 blocked the MMP-2-induced BBB opening in MMP-induced brain injury [393]. Damage to the BBB was blocked with hydroxamate treatment [394-396]. BB-94 blocked BBB opening after intracerebral injection of TNF-α, and BB-1101 was effective in LPS-induced BBB injury [295, 397] as well as in BBB opening after stroke, in the rat brain [398]. In addition, GM-6001 reduced BBB injury and improved the outcome [394, 399]. Moreover, a highly-specific thiirane gelatinase inhibitor of MMP-9, namely SB-3CT, rescues laminin from proteolysis, and protects neurons from apoptosis, in cerebral ischemia [400]. MMP inhibitors can also protect the brain from hemorrhagic complications of alteplase, a recombinant tissue plasminogen activator, by reducing BBB permeability and preventing alteplase from entering the brain and activating MMPs [401, 402]. Although there are MMP inhibitors that are selective for MMP-2 and MMP-9 [403], most available MMP inhibitors are broad-spectrum drugs [207]. Indeed, although MMP-3 is thought to contribute to neurodegeneration via multiple mechanisms, development of selective inhibitors for this proteolytic enzyme has been difficult, because the MMP family members share many structural characteristics. Pyrone-based

inhibitors with potency against MMP-3 and selectivity for MMP-1, -2 and -3 have been studied [404]; because MMP-3 prefers a more acidic environment than other MMPs, more acidic inhibitors, such as the carboxylates, have also been shown specific for MMP-3 [405].

However, the problem with these inhibitors, especially if used for long-term therapy, is that as side-effects they might block important functions, for example remodeling the ECM, leading to excessive fibrosis, and in particular causing joint stiffness [406]. Short-term use of MMP inhibitors may be less controversial in neurological disorders, particularly for treating cerebrovascular diseases. In addition, most non-peptidic MMP inhibitors reported thus far contain hydroxamic acid, carboxylic acids, phosphonates or thiols, and may not easily cross the BBB. Moreover, the most commonly used MMP inhibitors have poor solubility, and further studies are needed to improve delivery systems; the development of selective MMP inhibitors, with high BBB penetration rates and good solubility, would be of great benefit.

Alternatively, because MMP-3 gene expression is induced to a dramatic extent in response to cellular stress, while it is very low in basal conditions, it should be possible to suppress its upregulation without altering basal levels. In this connection, modification of MMP-3 gene expression could be a promising novel approach to providing neuroprotection. Tetracycline derivatives that penetrate the BBB, such as doxycycline and minocycline, downregulate the MMP-3 expression induced by cell stress, cytokines and other stimuli, with subsequent attenuation of neuroinflammation and apoptosis of dopaminergic neurons, both *in vitro* and *in vivo* [407-411]. Other compounds, such as ghrelin (an endogenous ligand for growth hormone secretagogue receptor 1a), exendin-4 (analog of glucagon-like peptide-1) or glycitein (a bacterial metabolite of the isoflavone glycitin) have also been found to attenuate MMP-3 expression, as well as loss of nigrostriatal dopaminergic neurons and hippocampal neurons [412-415].

Furthermore, because of its involvement in the non-amyloidogenic processing of APP, ADAM-10 overexpression or activation in the brain might be beneficial for the treatment of neurodegenerative diseases, in particular AD. In ADAM-10 overexpressing mice it has been shown that cortical synaptogenesis is enhanced [363]; in AD model mice, long-term potentiation deficiency is rescued [361], and learning, as well as memory, is positively influenced by ADAM-10 [362]. ADAM-10 activity can be enhanced, for example, by cholesterol depletion [416, 417] or by statin application [416]. Various results obtained from *Adam-10* transgenic mice have suggested that increasing ADAM-10 activity might be a valuable alternative to other strategies for treating AD, such as inhibiting β- or γ-secretase, or immunization. However, α-secretase activation must be moderate in extent, and needs to be closely monitored, since overexpression of ADAM-10 in the brain might affect homeostasis of the entire organism [354]. Again, also in this case, the crucial question is whether there are any side-effects connected with enhanced ADAM-10 activity in the brain or in peripheral tissues.

2.4. Metalloproteinases and Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disease that causes disability in the elderly, in the form of pain, stiffness and loss of function in articulating joints. OA is characterized by changes in the anatomy of load-bearing joints, which lead to degradation of the articular cartilage by proteolytic enzymes, inflammation of the synovium (synovitis), changes to subchondral bone, and growth of new bone and cartilage (osteophytes) at the joint edge [418-420]. The primary causes of OA are mechanical factors such as joint injury and obesity, with other risk factors, including age (associated intraarticular crystal deposition, muscle weakness and peripheral neuropathy), gender, and genetics, contributing to disease development and progression [418, 421, 422].

In OA, degradation of ECM molecules, which are synthetized and catabolized by chondrocytes, exceeds their synthesis, resulting in a net decrease in the amount of cartilage matrix, and eventually leading to total or partial erosion of the cartilage. Among the articular cartilage components, aggrecan and collagen are the main molecules that are slowly degraded. Aggrecan is a large proteoglycan, containing numerous chondroitin sulphate and keratin sulphate glycosaminoglycan moieties, which are important for the molecular function since they draw water into the cartilage matrix, giving it the ability to withstand compressive deformation during joint articulation. Degradation of aggrecan is an important manifestation of OA [15]. The depletion of aggrecan from articular cartilage is confirmed by the release of aggrecan catabolites into the synovial fluid [423]. Along with aggrecan breakdown, degradation of collagen, in particular of type II collagen, which provides strength to the tissue, is also a central feature of OA [424, 425]. Aggregan is lost in the initial phases of the disease, while collagen is lost at the later stages.

Molecules mediating matrix degradation, including MMPs and ADAMTSs, are upregulated in OA chondrocytes [426, 427]. Studies on transgenic mice have confirmed the central role of ADAMTS-4 and ADAMTS-5 in aggrecan degradation, and that of the collagenolytic MMP-13 in collagen degradation [16].

Although ADAMTS-1, -8, -9, -15, -16 and -18 can degrade aggrecan *in vitro* [428-431] ADAMTS-5 is the most active "aggrecanase" *in vitro*, followed by ADAMTS-4 [432]. ADAMTS-4 and ADAMTS-5 are thus considered to be the major enzymes responsible for pathological cleavage of aggrecan at the Glu^{373}-Ala^{374} bound in the interglobular domain [433-435]. Of note, studies in synovial cells suggest that ADAMTS-5 is constitutively expressed, whereas ADAMTS-4 is only induced by pro-inflammatory cytokines [436, 437].

The pathological importance of ADAMTS-5 in the development of OA was demonstrated by the finding that *Adamts-5* knockout mice develop less severe cartilage damage, both in a murine surgical model of OA, and in an antigen-induced arthritis model [438, 439]. In comparison, *Adamts-4* knockout mice did not show any significant suppression of aggrecanase activity in an arthritic model [440], indicating that ADAMTS-5 is the primary aggrecanase, at least in mice. Conversely, the role of ADAMTS-4 as aggrecanase appears more evident in humans [441, 442]. It has then been observed that ADAMTS-4 mRNA is induced in chondrocytes by IL-1 [443], while *Adamts-4/Adamts-5* double knockout mice are protected from cartilage degradation by IL-1, but not by retinoic acid, suggesting that other aggrecans apart from ADAMTS-4 and -5 are capable of retinoic acid-induced cartilage breakdown, at least in animal models [444]. In addition, suppressing ADAMTS-4 and/or ADAMTS-5 in human cartilage explants, via transfection of these ADAMTSs by siRNAs, significantly decreased aggrecan release and catabolism induced by a combination of IL-1β, TNF-α and oncostatin M [441].

MMPs normally present in articular cartilage and bone, such as MMP-1, -2, -3, -7, -8, -9 and -13, are also thought to be capable of degrading cartilage components, primarily type II collagen. For example, fibrillar collagens, which are highly stable molecules, can be degraded by the collagenolytic MMP-1, -8, -13 and -14 and by cathepsin K. However, MMP-13 is thought to be the primary collagenase in OA, with its expression increased in OA cartilage [424, 445, 446], whereas *Mmp-13* knockout mice were protected by MMP-13 collagen degradation in a surgical OA model [447]. MMP-13 is also up-regulated during chondrocyte terminal differentiation, and deficiency of *Mmp-13* results in impaired endochondral ossification [448, 449].

However, in addition to ADAMTS-4 and -5 and MMP-13, expression of various other proteolytic enzymes (*i.e.* MMPs, ADAMs and ADAMTSs) has been reported to increase in OA. ADAM-8, for example, has been suggested to contribute to OA pathogenesis, by cleaving fibronectin, generating fragments that stimulate further cartilage catabolism [450]. MMP-3 is, then, the most abundantly secreted enzyme in OA cartilage, and it is known to induce activation of other MMPs, such as MMP-1 and MMP-13, raising the possibility that it may contribute to OA by

activating latent collagenases [436]. It has also been suggested that MMP-3 promotes collagenase activation, and either direct or indirect MMP-mediated aggrecan cleavage [451]. Higher levels of MMP-3 have, indeed, been found in the synovial fluid and serum of OA patients, compared with normal individuals [452, 453]. Expression of MMP-1 and MMP-8 as well as MMP-13 was also detected by immunostaining in the superficial zone of OA cartilage [454].

Fragmentation of aggrecan, type II collagen, fibronectin and hyaluronan reveals cryptic epitopes, which also stimulate proteolytic enzymes including MMPs and ADAMTSs. Proteolytic fragments also stimulate the release of NO, chemokines and cytokines [455]. Of note, it has been observed that human chondrocytes cultured with intact monomeric type II collagen induced expression of MMP-1, -3, -13, -14, as well as that of IL-1β, IL-6, and IL-8 [456]. Similarly, collagen fragments generated during arthritis may influence matrix turnover. A fragment of type II collagen has been reported to upregulate levels of MMP-2, -3, -9 and -13 in bovine chondrocyte explants [457] and in human chondrocytes [458]. These results may suggest that type II collagen causes sequential activation of MMPs and cytokines during cartilage damage.

It is increasingly accepted that inflammation plays a role in OA pathogenesis [459-463]. Inflammatory cells, cytokines and growth factors that are not normally present in the cartilage matrix are found in OA patients [437, 464]. Inflammatory cytokines, for example, can increase chondrocyte expression of ADAMTS-4 and ADAMTS-5, leading to cartilage damage [465]. The cytokine IL-1 is suggested to be a principal mediator of joint damage in OA. Chondrocytes from OA cartilage display high levels of IL-1α and IL-1β, but are also more sensitive to IL-1. Moreover, IL-1 has the ability to stimulate chondrocytes to degrade both aggrecan and collagen. In cultured chondrocytes or cartilage explants, ADAMTS-4 has been shown to be induced following stimulation with IL-1, TNF-α or TGFβ [441, 443, 466, 467] whereas ADAMTS-5 was upregulated by IL-1α in an immortalized human chondrocyte cell line [468]. Using a model of cultured human synovial cells from digested OA synovium, it was observed that ADAMTS-4 gene expression is dependent on TNF-α, and that IL-1 is produced by the synovial macrophages, whereas the level of ADAMTS-5 is not significantly change by either of the two pro-inflammatory cytokines [437]. In contrast, monocytes from wild-type mice, but not those from *Il-1* deficient mice, upregulated ADAMTS-5 mRNA in chondrocytes, without affecting ADAMTS-4. This data suggest that murine ADAMTS-4 is unresponsive to IL-1 [469]. The role of NF-κB in regulating ADAMTS gene expression has recently been clarified: ADAMTS-4, but not ADAMTS-5, has several NF-κB binding sites. Of note, it has also been reported that IL-1 and TNF-α increase ADAMTS-4 expression in a NF-κB dependent manner [470].

Moreover, IL-1β is known to stimulate expression of MMPs, for example MMP-1 and -13, in OA cartilage [436,471]. The role of IL-1, together with TNF-α, in matrix degradation has been clarified [471]. Of note, when IL-1 is combined with TNF-α and the two are injected simultaneously, there is enhanced cartilage destruction, which exceeds the effects observed with either cytokine alone [472]. The combination of IL-1 and oncostatin M also upregulates matrix-degrading proteinases in cartilage [473]. In addition to inducing the synthesis of MMPs and other proteinases by chondrocytes, IL-1 and TNF-α increase the synthesis of PGE_2, by stimulating expression or activity of COX-2, microsomal PGE synthase-1, and soluble phospholipase A2 (PLA2); they also upregulate NO production via inducible NO synthetase (iNOS), contributing to the inflammatory response [474]. *Il-1β* knockout mice showed protection against OA induced by destabilization of the medial meniscus [475]. Further, IL-17 and -18 are also thought to play a role in cartilage catabolism [476]. Osteoarthritic chondrocytes also overexpress TGFβ, which has dual effects: it can be protective as well as deleterious for articular cartilage. This dual effect can be explained by the fact that TGFβ can signal via different receptors and related the Smad signaling routes [477]. Importantly, signaling via anaplastic

lymphoma kinase (ALK)1, but not that via ALK5, stimulates MMP-13 expression by chondrocytes. In cartilage of aging mice, and in experimental OA models, it has been found that the ALK1/ALK5 ratio is significantly increased, favoring TGFβ signaling via the Smad1/5/8 (ALK1) route, and augmenting changes in chondrocyte differentiation and MMP-13 expression [478]. Moreover, in human OA cartilage there is a significant correlation between ALK1 and MMP-13 expression [477]. Chemokines have also been reported to play a role in OA [479, 480]; expression of chemokine receptors has been demonstrated in OA chondrocytes [481] and synovial cells [482].

MMPs are strongly inhibited by all four of the mammalian TIMPs [483] but, conversely, ADAMTS-4 and ADAMTS-5 are effectively inhibited only by TIMP-3 [484-486]. As TIMP-3 can inhibit MMPs and ADAMTSs, it is considered to be a central inhibitor of cartilage degradation. The addition of TIMP-3 blocks cartilage degradation in explant cultures [487]. The key role played by TIMP-3 in cartilage protection has been confirmed by the finding that *Timp-3* knockout mice develop increased cartilage degradation upon aging [488] and display increased cartilage damage in an antigen-induced arthritis model [489]. In addition, TIMP-3 protein levels are reduced in human OA cartilage [490]. With regard to the other TIMPs, unlike their action on MMPs, TIMP-1 has been shown to partially inhibit glycosaminoglycan release from human cartilage, whereas TIMP-2 has no effect on cartilage components [465, 484]; the level of TIMP-4 is decreased in OA cartilage [446].

2.4.1. Therapeutic Strategies for Inhibiting Metalloproteinases in Osteoarthritis

OA remains a disease with insufficient disease-modifying treatments. With an increasing number of people suffering from the disease, the identification of novel therapeutic targets is a priority.

The routine therapies to moderate OA clinically are intraarticular injection of hyaluronic acid, which has the ability to aggregate aggrecan, or of steroids, or oral administration of anti-inflammatory molecules. These conventional OA therapeutics, however, do not inhibit the underlying tissue catabolism, and thus they allow the disease to progress into irreversible ECM loss and chronic disability.

Since the central role of aggrecanases (*e.g.* ADAMTS-4 and -5) and collagenases (*e.g.* MMP-13) in cartilage degradation has now been verified by studies on transgenic mice, identification of the key role played by these proteinases is now of importance for the successful development of specific inhibitors to be used as therapeutic agents in OA. It has been reported that blocking aggrecanase cleavage in the interglobular domain of aggrecan diminishes aggrecan loss and cartilage erosion in knockin mice, in surgically-induced OA and also in a model of inflammatory arthritis, and the procedure also appeared to stimulate cartilage repair following acute inflammation [435].

The design of small molecule inhibitors or protein antagonist inhibitors, to block the increase catabolism of matrix components in OA, is thus an area of considerable interest for the pharmaceutical industry. New inhibition strategies using small molecules inhibitors and TIMPs, engineered to increase their inhibitory specificity, or using new reagents such as ribozymes and siRNAs, which repress expression of specific enzymes, are thus now under consideration [491]. However, disappointing results from clinical trials with small molecule inhibitors have highlighted the critical importance of inhibitor specificity, and the need to better identify the individual enzymes responsible for joint destruction. The potential side-effects of inhibiting these enzymes, which are expressed in a number of tissue, are also still unclear. However, among agents targeting aggrecanase inhibitors, α-amino hydroxamate has been discovered to be a potent and selective aggrecanase inhibitor [492]; in subsequent attempts to optimize the potency and pharmacokinetic profile of this inhibitor, anti-succinate hydroxamates containing cyclic P1 substituents were identified [493]. Other potential selective synthetic inhibitors of ADAMTS-5, as well as of ADAMST-4, have been investigated, *e.g.* a series of 5-((1H-pyrazol-4-yl)methylene)-2-thioxothiazolidin-4-one [494, 495].

Studies on mice with specific gene ablations have also identified a network of factors and cellular signals that regulate MMP-13 and ADAMTS expression in chondrocytes [16]. Given that many pathways can stimulate an increase in proteinase expression, the development of inhibitors targeting the effector proteinases, and their use in combination, may block cartilage damage more effectively than therapies targeting a single activating factor. For example, it has been reported that the OA process is driven by loss of the Smad2/3 block on differentiation in articular chondrocytes, leading to progression of chondrocyte differentiation and an autolytic phenotype. In the early stages of OA, some chondrocytes will have progressed in their differentiation to an OA chondrocytes phenotype, triggered by a loss of the Smad2/3 block; others will still be in a quiescent, healthy state of differentiation [477]. The latter cells could be targets for therapy, to block further progression of the OA process: blocking the progression of chondrocyte differentiation will block further expansion of the OA process in the remaining healthy cartilage; compounds specifically stimulating the Smad2/3 route could be developed as a therapeutic strategy [496, 497]. An alternative therapy could be stimulation of one of the other Smad2/3 routes in chondrocytes. In this connection, signaling via the activin ALK4 and ALK7 receptors leads to activation of the Smad2/3 pathway [498]. These receptors could be potential targets to enhance Smad2/3 signaling in chondrocytes in OA. Alternatively, ALK1, which is involved in vessel formation, or the Smad1/5/8 pathway, which is associated with the activity of ALK1, ALK2, ALK3 and ALK6, could be blocked in chondrocytes (for example with kinase inhibitors) with subsequent blocking of aberrant chondrocyte differentiation [477]. These possible therapeutic strategies, however, might trigger side-effects in other tissues: stimulation of Smad2/3 pathway using TGFβ mimetics, or of the ALK4/7 pathway, might result in excessive induction of fibrosis; blocking ALK1 might reduce blood vessel formation; general inhibition of the Smad1/5/8 pathway might interfere with bone metabolism [477].

Furthermore, ADAMTS-4 is induced by IL-1 and TNF-α, and depends on the NF-κB [470, 499, 500], and it has been reported that treatment of bovine cartilage explants with small-molecule IκB kinase inhibitor leads to the prevention of IL-1-induced aggrecan degradation. These data suggest that aggrecan degradation occurs in a NF-κB dependent manner [501]. Conversely, ADAMTS-5 has been found to be NF-κB independent and to lack κB elements on its promoter [500]. The differential regulation of ADAMTS-4 and ADAMTS-5 could thus have important implications for the development of disease-modifying OA drugs [502].

In addition, a more complete knowledge of the pathways and receptors used by endogenous matrix molecules in concert with cytokines and chemokines [455] may improve future possibilities for developing new therapeutic strategies for OA.

3. CONCLUSIONS

Metalloproteinases are a large family of enzymes that have been implicated in the pathophysiology of several chronic diseases related to aging, including atherosclerosis, type 2 diabetes mellitus, neurodegenerative diseases, and osteoarthritis (Figure 1). They thus represent potential therapeutic targets. In order to develop therapeutic strategies against the action of these proteolytic enzymes, it is fundamental to understand the regulation of and roles played by MMPs, ADAMs and ADAMTSs, and their endogenous inhibitors, especially of TIMPs, in all phases of the pathogenesis of these age-related diseases. More research is thus needed to clarify the effects of MMPs, not only on ECM degradation, but also on cell types involved in the pathogenesis of these diseases.

The design of agents that may treat or prevent the excessive degradation of ECM components, induced by MMP dysregulation, or the effects of MMPs on cells is now an area of considerable interest for the pharmaceutical industry. However, the use of broad spectrum synthetic inhibitors of MMPs, or of other exogenous therapeutic agents,

has not always replicated the effects of TIMPs in preclinical models and in clinical trials, possibly due to their side-effects, but also because these proteolytic enzymes possess reparative as well as pathogenetic properties. In this connection, the emphasis is therefore shifting to the development of MMP-modulating agents of restricted specificity. Despite this new impetus for developing more beneficial therapeutic strategies, more scientific studies are essential to improve our knowledge of protease function, thus helping to identify targets for targeted pharmacology therapy.

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Figure legend

Figure 1.. Several stimuli induce specific activated cells to release metalloproteinases which are involved in the pathogenesis of age-related chronic diseases.

Figure 1.

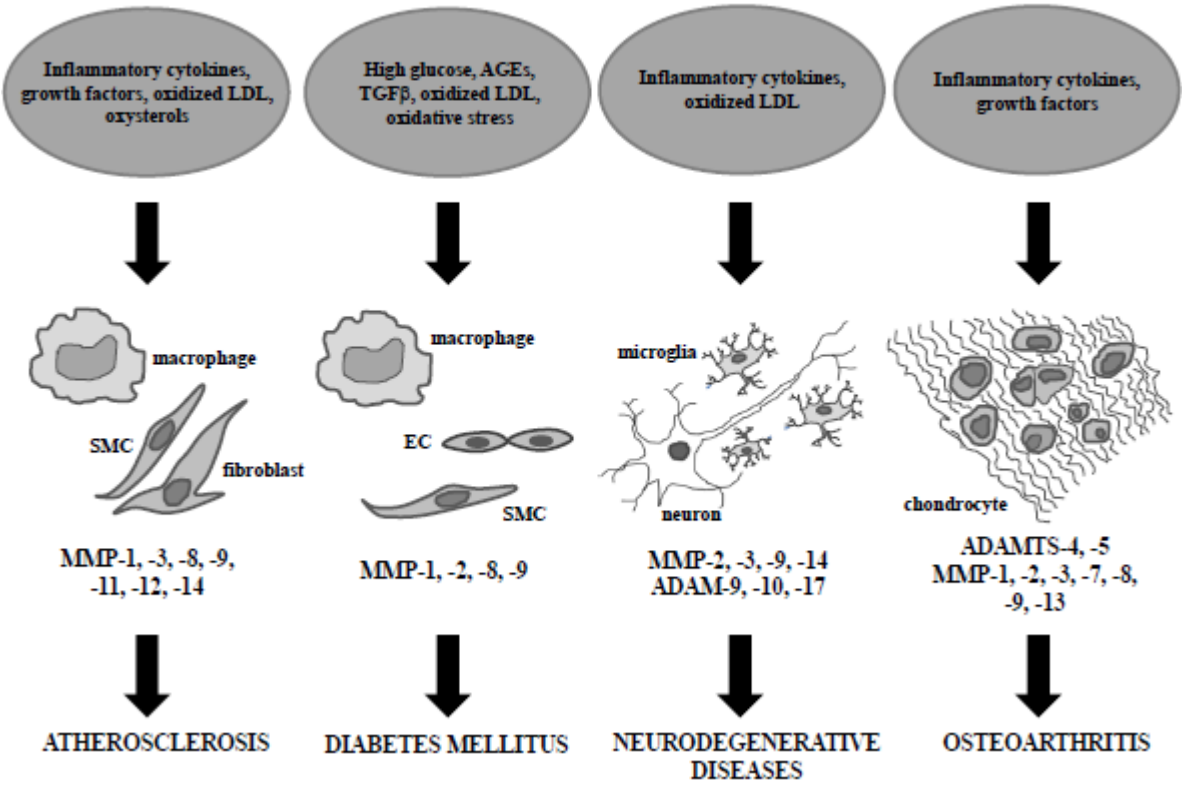
Inflammatory cytokines, growth factors, oxidized LDL, oxysterols
High glucose, AGEs, TGFβ, oxidized LDL, oxidative stress
Inflammatory cytokines, oxidized LDL
Inflammatory cytokines, growth factors
macrophage
SMC
fibroblast
macrophage
EC
SMC
microglia
neuron
chondrocyte
MMP-1, -3, -8, -9, -11, -12, -14
MMP-1, -2, -8, -9
MMP-2, -3, -9, -14 ADAM-9, -10, -17
ADAMTS-4, -5 MMP-1, -2, -3, -7, -8, -9, -13
ATHEROSCLEROSIS
DIABETES MELLITUS
NEURODEGENERATIVE DISEASES
OSTEOARTHRITIS

Table 1. Human ADAM members and suggested functions.

ADAM	Proteolytic activity	Suggested functions
ADAM-1	No	Possibly involved in sperm-egg fusion
ADAM-2	No	Possibly involved in sperm-egg fusion
ADAM-3A	No	Possibly involved in sperm-egg fusion
ADAM-5P	No	Unknown
ADAM-6	No	Unknown
ADAM-7	No	Possibly involved in sperm-egg fusion
ADAM-8	CD23, TNFRI, IL-1RII	Involved in cell adhesion during neurodegeneration; a target for allergic respiratory diseases, including asthma
ADAM-9	APP	Involved in induced ectodomain shedding of membrane-anchored heparin binding-epidermal growth factor (HB-EGF)-like growth factor; suggested to cleave APP at the α-secretase site
ADAM-10	APP, CX3CL1, collagen IV	Involved in shedding of various transmembrane proteins, including cadherins; α-secretase for APP; control wound healing, neurogenesis, skin homeostasis
ADAM-11	No	Candidate tumor suppressor gene for human breast cancer; pain transmission, synaptic modulation
ADAM-12	HB-EGF sheddase	Involved in myogenesis and skeletal muscle regeneration; upregulated in tumor progression; involved in osteoclast formation from mononuclear precursors
ADAM-15	collagen IV, gelatin	Involved in cell adhesion through integrin binding; wound healing, mediates heterotypic T-cell interactions; cleaves E-cadherin; glomerular cell migration and pathological neovascularization; cartilage remodeling
ADAM-17	APP, TNF-α, TNFRI and RII, IL-1RII	Proteolytic release of TNF-α, p75TNF receptor, IL-1R type II, p55 TNFR, TGF-α, L-selectin and APP; activation of Notch pathway
ADAM-18	No	Possibly involved in sperm-egg fusion
ADAM-19	TRANCE	Involved in neurogenesis and synaptogenesis; osteoblast differentiation and/or activity in bone
ADAM-20	Yes	Possibly involved in sperm-egg fusion
ADAM-21	Yes	Possibly involved in sperm-egg fusion and in epithelia functions
ADAM-22	No	Ligand for integrin in the brain; involved in regulation of cell adhesion, inhibition of cell proliferation, neuronal receptor for leucin-rich-glioma inactivated1 (LGI1)
ADAM-23	No	Involved in cell-cell and cell-matrix interactions in the brain
ADAM-28	CD40, FasL	Role during lymphocyte migration; shedding of lymphocyte surface target protein; sperm maturation
ADAM-29, -32	No	Involved in spermatogenesis and fertilization
ADAM-30	Yes	Involved in spermatogenesis and fertilization
ADAM-33	Yes	Involved in asthma and bronchial hyperresponsiveness

Table 2. Human MMP members and principal biological effects.

Group name	MMP	Substrates	Biological effects
I. Collagenase			
Collagenase-1	MMP-1	Collagens I, II, III, VII, X, gelatin, proteoglycan, link protein, entactin, tenascin	Keratinocyte migration and reepithelialization, platelet aggregation, release of bFGF, cell proliferation, pro- and anti-inflammatory, osteoclast activation, cell migration, enhanced collagen affinity, apoptosis , increased bioavailability of TGFβ
Collagenase-2	MMP-8	Collagens I, II, III, gelatin, proteoglycan, link protein	
Collagenase-3	MMP-13	Collagens I, II, III, IV, IX, X, XIV, proteoglycan, fibronectin, tenascin	
II. Gelatinase			
Gelatinase A	MMP-2	Gelatin, collagens IV, V, VII, XI, laminin, fibronectin, elastin, proteoglycan, link protein	Neurite outgrowth, generation of angiostatin-like fragment, enhanced collagen affinity, epithelial cell migration, tumor cell resistance, pro-inflammatory, increased bioavailability of TGFβ, generation of vasoconstrictor, recruitment of osteoclasts
Gelatinase B	MMP-9	Gelatin, collagens III, IV, V, elastin, entactin, link protein	
III. Stromelysin			
Stromelysin-1	MMP-3	Proteoglycan, collagens III, IV, IX, X, laminin, fibronectin, gelatin, tenascin, link protein, elastin	Mammary epithelial cell apoptosis and alveolar formation, generation of angiostatin-like fragment, release of bFGF, increased bioavailability of IGF1 and cell proliferation , anti-inflammatory, increased bioavailability of TGFβ, increased cell invasion
Stromelysin-2	MMP-10	Collagens III, IV, V, fibronectin, laminin, proteoglycan, link protein, elastin	
Stromelysin-3	MMP-11	Fibronectin, laminin, proteoglycan, gelatin	
IV. Membrane-type			
MT1-MMP	MMP-14	Collagens I, II, III, gelatin, proteoglycan, fibronectin, laminin	Cell migration, kidney tubulogenesis, epithelial cell migration, reduced cell adhesion and spreading, embryo attachment to uterine epithelia
MT2-MMP	MMP-15	Fibronectin, tenascin, entactin, aggrecan, perlecan, laminin	
MT3-MMP	MMP-16	Collagen III, gelatin, fibronectin	
MT4-MMP	MMP-17	?	
MT5-MMP	MMP-24	Proteoglycan	
MP6-MMP	MMP-25	Gelatin	
V. Others			
Matrilysin	MMP-7	Proteoglycan, gelatin, fibronectin, tenascin, elastin, collagen IV, laminin, link protein	Adipocyte differentiation, generation of angiostatin-like fragment, increased bioavailability of IGF1 and cell proliferation, epithelial cell migration, increased bioavailability of TGFβ, disrupted cell aggregation and increased cell invasion, Fas-receptor mediated apoptosis, pro-inflammatory, osteocleast activation, vasocontriction and cell growth
Metalloelastase	MMP-12	Elastin	
Collagenase 4	MMP-18	Collagen I	
RAS I-1	MMP-19	Tenascin, gelatin, aggrecan	
Enamelysin	MMP-20	Enamel, gelatin	
XMMP	MMP-21	?	
No common name	MMP-23	?	
Matrilysin 2	MMP-26	Collagen IV, fibronectin, gelatin	
No common name	MMP-27	?	
Epilysin	MMP-28	Casein	