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Matrix Metalloproteinases in Aortic Aneurysm – Executors or Executioners?

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1. Introduction

Despite numerous studies focusing on the aortic aneurysm pathogenesis, the mechanism of aneurysm formation, especially – initiation of this process, remains unclear. The research concerning both, structural and molecular studies, is based on two main data sources. The first source of information are patients with already formed aneurysm, and with well defined biochemical and morphological changes in aortic wall architecture. The other source of data are experimental studies based on laboratory animals with artificially induced aneurysms. This approach enables verification of various hypotheses concerning pathogenesis of aortic aneurysm. Regrettably, animal aneurysm models, although similar, are not exactly the same, as human pathology. Thus, since the link between both mentioned data sources is still lacking, the knowledge achieved to date, even being highly profound, is not sufficient to fully understand this disease. Besides well defined factors, predisposing to formation of aortic aneurysm (patient's age, cigarette smoking, arterial hypertension, atherosclerosis, as well as the Marfan's and the Ehlers-Danlos's syndrome-associated mutations), increasing popularity is currently being gained by the hypothesis concerning the pivotal role of proteolytic enzymes - matrix metalloproteinases (MMPs) in aortic wall destruction. The involvement of MMPs in extracellular matrix damage in aortic aneurysm is doubtless. However, it needs to be elucidated, what the sequence of events is and what the exact role of MMPs is in these events. MMPs could play a role of "executioners", that are produced and activated in the aortic wall as constituents of inflammatory reaction, in response to some yet poorly defined triggers. On the other hand, it is plausible, that aortic wall destruction, followed by inflammatory response to tissue degradation products, results from primary local overproduction and/or activation of proteases. It could be due to some mutations or polymorphisms of MMP genes, or some impairment in their controlling mechanisms. In that circumstance MMPs could rather be considered as "executors", with causative role in aortic aneurysm pathogenesis. Although the majority of studies suggest the first scenario as being more possible, there is some evidence, that could support the second alternative, too.

2. Chronic inflammation – where the chaos begins…

The histopathological assessment of aneurismal aortic wall specimens reveals widespread chronic inflammatory reaction. This reaction is associated with extensive destruction of

elastic fibers in the tunica media layer, and infiltration of both, media and adventitia, by macrophages and lymphocytes, mainly the Th2 subset. Moreover, as has been found recently, outer media and adventitia of human aortic aneurysm samples contain numerous mast cells (Miyake & Morishita, 2009; Michel et al., 2011). In addition to the previously mentioned lymphocytes and macrophages, mast cells are currently recognized as a third considerable source of pro-inflammatory cytokines, including tumor necrosis factor (TNF), various chemokines, and interleukins. Furthermore, in cooperation with macrophages, mast cells produce and release large quantities of various proteases and, thus, they are also actively engaged in aortic wall destruction (Tsuruda et al., 2008). However, a trigger of inflammatory reaction still remains to be a missing component of this scenario.

2.1 Chronic proteolytic atherothrombosis – a new concept

Recently, it has been proposed, that the aneurysm pathogenesis could be explained, at least to some extent, by the model of chronic proteolytic atherothrombosis (Michel et al., 2011). This model is based on the observation that the development of aortic aneurysms is accompanied by the formation of chronic intraluminal thrombus inside the aneurysmal sac. It has been shown that the presence of intraluminal thrombus is associated with widespread degradation of elastic fibers, increased apoptosis and loss of vascular smooth muscle cells (VSMC) in tunica media, and with extensive inflammatory reaction in adventitia. It may suggest, that the thrombus rather, than the aortic wall, could be the primary source of various pro-inflammatory factors, including proteolytic enzymes (Michel et al., 2011). On the other hand, one can argue that formation of thrombus on the inner, luminal surface of the aortic wall could be secondary to already existing aortic wall inflammation, due to the damage of endothelium and tunica intima. Nevertheless, it is plausible, that independently of the sequence of events, the thrombus-aortic wall interface may be "the place, where the chaos begins"…

The pathophysiological role of intraluminal thrombus may be described by various activities of its components. The first activity could be a generation of free radicals and induction of oxidative stress reaction, mainly due to a degradation of red blood cells and release of the potent pro-oxidant mediator – iron-rich hemoglobin. The oxidative stress leads to the production of reactive oxygen and reactive nitrogen species, which are both components of a self-augmenting mechanism (Miyake & Morishita, 2009). Reactive oxygen and nitric oxide increase expression of pro-inflammatory cytokines, followed by further up-regulation of reactive oxygen species production, peroxidation of membrane phospholipids and generation of eicosanoids and pro-apoptotic ceramides. Finally, reactive oxygen species induce activation of nuclear factor kappa-B (NF-κB), that leads to additional increase in MMPs expression and initiates the apoptosis of VSMC in the aortic wall. In addition to erythrocytes, intraluminal thrombus consists of an approximately 12-fold higher number of neutrophils, as compared to circulating blood (Michel et al., 2011). These cells produce a large number of proteinases, including elastase, cathepsin, MMP-8 and -9. Moreover, strong proteolytic activity between the thrombus and the adjacent aneurysm wall is revealed by the plasmin. This activity, although originally aimed at the thrombus fibrin network, may also contribute to aortic wall destruction. It may occur mainly through degradation of fibronectin, thus resulting in mesenchymal cells detachment and apoptosis, as well as direct activation of pro-MMPs.

2.2 *Chlamydia pneumoniae* and MMPs in aortic aneurysm

According to "infection hypothesis", the chronic inflammatory reaction, which takes place in the aortic wall, may be initiated by some pathogens. However, studies focusing on the presumed importance of various *Chlamydia* species, *Helicobacter pylori*, *Borrelia burgdorferi*, *Cytomegalovirus*, *Herpes simplex virus*, and most recently, some comensal, or weak pathogenic bacteria from the oral cavity, including *Porphyromonas gingivalis* and *Streptococcus mutans*, have failed to reveal a direct relationship between the presence of pathogen and aneurysm formation. Nevertheless, there is still no consensus in the debate concerning the significance of intracellular bacteria *Chlamydia (Chlamydophila) pneumoniae* in that event. It has been shown that almost half of aortic aneurysm specimens contained this pathogen. Moreover, a high prevalence of *C. pneumoniae* seropositivity and the presence of *C. pneumoniae*-reactive T lymphocytes in aortic aneurysm-suffering individuals, seemed to further support this hypothesis. Strong evidence was also provided by the results of experimental studies indicating, that in animal models *C. pneumoniae* antigens stimulated an elastin degradation followed by dilatation of aorta (Petersen et al., 2002). It is plausible that *C. pneumoniae* infection may reveal such destructive influence on the aortic wall due to activation of inflammatory reaction, mainly by stimulation of tissue macrophages with *C. pneumoniae* heat shock protein 60. This stimulation could lead to the release of a variety of pro-inflammatory molecules, eicosanoids, cytokines and several MMPs. Thus, although the exact role of *C. pneumoniae* in pathogenesis of aortic aneurysm remains to be clarified, the results of several prospective clinical trials could provide some contribution to this matter. It has been proven, that antibiotics effective against *C. pneumoniae* – tetracyclines (doxycyclin) and macrolides (roxithromycin, but not azithromycin), may reduce the progression of small aortic aneurysm (Høgh et al., 2009, see also chapter 3.5.2.2). Based on mentioned observations one can expect a direct correlation between the presence of *C. pneumoniae* and tissue levels of MMPs in aneurismal aortic wall specimens. This hypothesis was tested by Petersen and coauthors (Petersen et al., 2002). Surprisingly, the authors found, that mean levels of MMP-2 and MMP-9 in *C. pneumoniae*-positive aortic wall specimens were lower, than in *C. pneumoniae*-negative samples. This astonishing result was explained by the authors as a consequence of possible irregular distribution of bacteria in the aortic wall. Since the *C. pneumoniae* detection and determination of MMPs activity were done using specimens from different locations, some of them could possibly display false negative results. It is noteworthy, that problems with detection of *C. pneumoniae* DNA in tissue specimens of *Chlamydia*-seropositive patients with abdominal aortic aneurysms have also been reported by other authors (Falkensammer et al., 2007). An additional cause behind such results in Petersen's study could be the relatively small patient groups (28 individuals, divided into 4 groups, 7 patients in each). Moreover, the results of gelatin zymography could be affected by components of the extraction buffer used for analysis of MMP activity, especially EDTA and potent proteinase inhibitor – phenyl methylsulphonylfluoride (PMSF). Finally, authors suggested that *C. pneumoniae* infection may result in activation of some other, different from MMP-2 or MMP-9, proteolytic enzymes, e.g. neutrophil- or mast cells-derived proteinases, like cathepsins G, or chymase. These enzymes can also reveal elastolytic activity, and therefore may directly contribute to the aortic wall destruction (Miyake & Morishita, 2009; Michel et al., 2011). Furthermore, it has been demonstrated, that mast cell-derived chymase could activate pro-enzyme forms of MMP-2 and -9 in aneurysm tissue. However, although these observations could confirm the association of *C. pneumoniae* with MMPs activation in pathogenesis of aortic aneurysm, this issue still requires further studies.

3. Matrix metalloproteinases – the dark side of the Force…

Matrix metalloproteinases (MMPs), also known as matrix metallopeptidases, or matrixins, belong to the large and still expanding family of zinc endoproteinases. The members of this evolutionarily ancient group were found in various organisms, from bacteria and plants, through hydra and worms, to humans. So far, at least 25 distinct MMPs have been identified in vertebrates. In humans a presence of 23 proteins, encoded for 24 distinct genes, has been confirmed. This discrepancy is due to the fact that human MMP-23 was found to be encoded by two identical genes located on chromosome 1. Together with the astacins, the adamalysins, and large bacterial proteinases – serralysins, MMPs constitute a huge superfamily of enzymes, called metzincins, which are characterized by the presence of the zinc-binding motif, with a conserved methionine nearby.

MMPs play a crucial role in extracellular matrix (ECM) turnover. They are able to cleave main ECM components, including collagens, elastin, fibronectin, gelatin and aggrecan, as well as a variety of non-ECM molecules – transforming growth factor (TGF)-β, pro-IL-1β, pro-IL-8, Fas ligand, and pro-TNF. Moreover, MMPs are responsible for the release of cryptic fragments and neo-epitopes from extracellular matrix and non-ECM macromolecules, which may reveal bioactivities different from those of the parent molecules. Furthermore, MMPs may liberate numerous growth factors (e.g. vascular endothelial growth factor – VEGF and TGF-β) and cytokines, which are embedded in extracellular matrix and require proteolytic release from binding proteins for their activation. Finally, MMPs may modify cells' attachment to the ECM by processing of syndecans, dystroglycan and other adhesion molecules (Endo et al., 2003; Yamada et al., 2001; Mott & Werb, 2004). These properties of MMPs make them key players in the majority of physiological conditions (e.g. pregnancy, embryogenesis, wound healing), but also in various pathologies, including cancer progression with metastases, liver fibrosis, periodontal disease, multiple sclerosis and vascular diseases, especially atherosclerosis and aortic aneurysm (Hadler-Olsen et al., 2011).

As mentioned previously, MMPs should not only be recognized as typical effector/"executioner" molecules, but also, at least in some circumstances, they may be considered as real causative factors/"executors". This status may be supported by results of studies concerning the genetic polymorphisms of MMP genes. The polymorphisms are natural differences in DNA sequence that occur in more than 1% of the entire population. The vast majority of them concern variability of single nucleotides and are known as single nucleotide polymorphisms (SNPs). The effect of particular SNP is determined by its position in a gene structure. Most SNPs are functionally neutral. However, some of them may lead to an amino acid substitution, thus influencing the structure and properties of encoded protein. Furthermore, some SNPs located in a promoter region may alter the level of gene transcription. There is the reason, for which functional SNPs may contribute to the individual susceptibility to common diseases, including aortic aneurysms. In this chapter authors will shortly review several polymorphisms of selected MMP genes, which have been suspected of being involved in aortic aneurysm development.

3.1 MMPs structure

The overall scheme of a protein structure is common among all MMPs, with more or less significant differences between particular groups (Fig. 1).

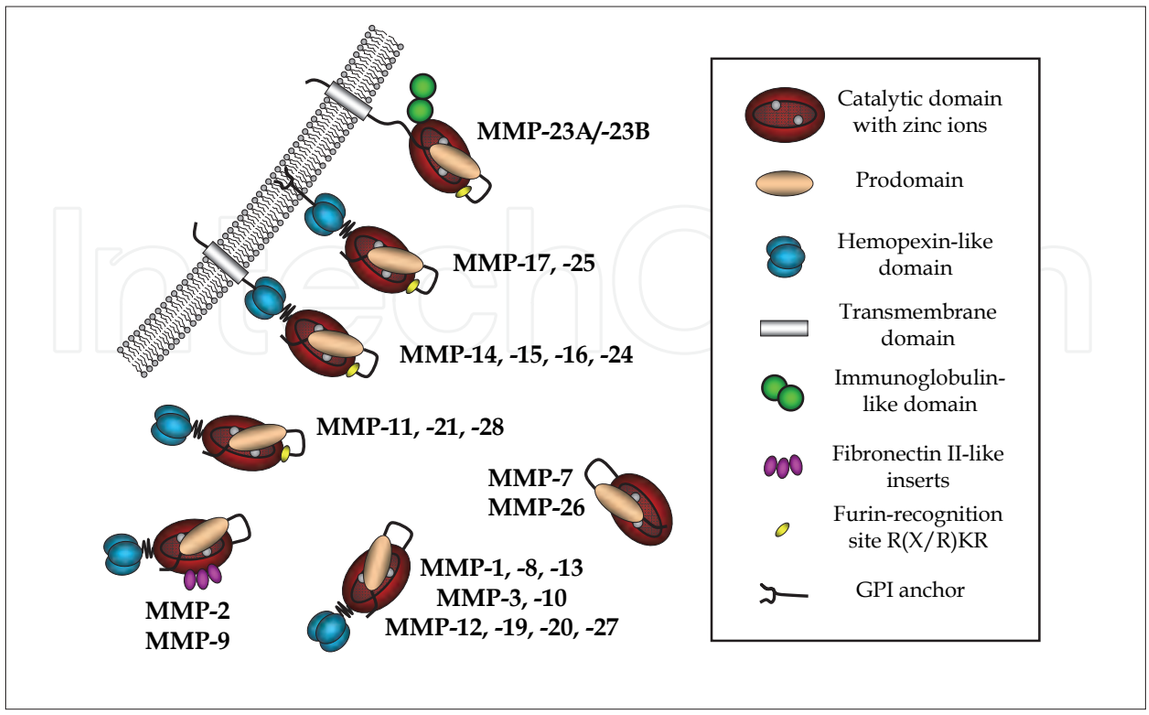

Fig. 1. The schematic structure of MMPs family

In general, on its N-terminus the MMP molecule contains a signal sequence, which directs the protein to the secretory pathway, and is removed during insertion of the protein into an endoplasmic reticulum. The signal sequence is followed by the propeptide composed of approximately 80 amino acid residues, that contains a characteristic conserved PRCGXPD motif, known as "cysteine-switch". The role of this sequence is to block a catalytic zinc and thus maintain the latent form of an enzyme. The next, the catalytic domain, has a sphere-like shape with an active site containing two atoms of zinc inside a large, shallow cleft. The catalytic domain is composed of approximately 160-170 amino acids, with a unique HEXXHXXGXXH sequence, that binds zinc ions. The last, approximately 200 amino acid residues-long C-terminal domain, called the hemopexin-like domain is found in all MMPs except for MMP-7, -23 and -26. In most MMPs the hemopexin-like domain is linked to a catalytic domain through a short, approximately 10-30 amino acid residues-containing hinge region. Exceptionally, the hinge region of MMP-9 is 64 amino acids-long, and is strongly O-glycosylated. Furthermore, six representatives of the membrane type (MT) MMPs subgroup hold either a type I transmembrane domain with a short intracellular segment (MT1, -2, -3 and -5-MMP) or a cell membrane-anchoring glycosylphosphatidylinositol (GPI) moiety (MT4- and -6-MMP).

Unlike other MMPs, in the MMP-23 molecule, a cystein-rich segment with an immunoglobulin-like domain is present, instead of the hemopexin-like domain on C-terminus, whereas the N-terminal signal peptide has been replaced by an N-terminal type II transmembrane domain. In addition to the previously mentioned common components, other elements, attached to the catalytic domain are fibronectin II-like inserts, which are found in MMP-2 and -9 molecules exclusively. Furthermore, three of the secreted MMPs (MMP-11, -21 and -28), as well as all the membrane-anchored MMPs, have a unique

sequence R(X/R)KR between the prodomain and the catalytic domain. This motif is recognized and cleaved by a serine proteinase – furin, that results in removal of prodomain from the active site of the catalytic domain, followed by intracellular activation of mentioned MMPs (Fanjul-Fernandez et al., 2010).

3.2 Classification of MMPs

Traditionally, MMPs were classified into 6 main groups – collagenases, gellatinases, stromelysins, matrilysins, membrane type MMPs and others, unclassified to former groups. However, increasing knowledge, concerning the molecular structure, substrate specificity and mechanism of MMPs activation contributed to an arrangement of their new classification. According to this classification, MMPs are divided into four groups: archetypal MMPs, matrilysins, gelatinases and furin-activated MMPs (Fanjul-Fernandez et al., 2010, Hadler-Olsen et al., 2011)

3.2.1 Archetypal MMPs

Archetypal MMPs have the structure typical for all MMPs. They are further divided into three subgroups: collagenases, stromelysins and other archetypal MMPs.

3.2.1.1 Collagenases

This subgroup of archetypal MMPs is represented by three enzymes: collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13). Their main common feature is the ability to cleave native collagens into characteristic N-terminal ¾ and C-terminal ¼ fragments (Fanjul-Fernandez et al., 2010). Since the triple helix conformation of native collagens is highly resistant to cleavage mediated by other proteinases, collagenases are crucial enzymes for initiation of collagen degradation. After the cleavage mediated by collagenases, the native collagens rapidly denature to gelatin and thus they become susceptible to degradation by other MMPs.

Besides the native fibrillar collagens (types I, II, III, V, and XI), the other targets for collagenases are numerous extracellular matrix components, as well as non-ECM molecules, including IL-8, pro-TNF, protease-activated receptor-1, several insulin-like growth factor-binding proteins (IGFBPs), etc… (Gearing et al., 1994; Boire et al., 2005; Amalinei et al., 2007).

The activation of MMP-1 requires a presence of active MMP-3 or plasminogen activator/plasmin system. The main sources of collagenases are stimulated fibroblasts (MMP-1), neutrophils (MMP-8) and VSMC (MMP-1 and MMP-13). Increased levels of mRNA and proteins for collagenases were found in aortic aneurysm tissue (Kadoglou & Liapis, 2004). Moreover, they are supposed to be involved in aneurysm rupture.

The studies focused on a presumable connection between aortic aneurysm formation and known SNPs in genes encoding for collagenases, including potentially clinically relevant SNP in MMP-1 promoter region (-1607 G/GG), did not reveal any significant correlation (Ogata et al., 2004; Sandford et al., 2007; Saratzis et al., 2011).

3.2.1.2 Stromelysins

The members of this group are stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10). Both stromelysins have a structure analogous to that of collagenases, however, in contrast to those enzymes, stromelysins are not able to cleave native collagen. Their substrates include processed collagen types III, IV, V, IX and X, laminin, gelatin, fibronectin,

proteoglycans and several other molecules, e.g. plasminogen, fibrinogen and IL-1β (Amalinei et al., 2007). Although both stromelysins display similar substrate specificity, MMP-3 reveals a grater proteolytic activity, as compared to MMP-10. Furthermore, MMP-3 is known to activate various pro-MMPs (collagenases and gelatinases) by removal of their pro-domain, and therefore it is crucial for their activation (Suzuki et al., 1990; Visse & Nagase, 2003). Stromelysins may be produced by fibroblasts and epithelial cells, however, in aortic aneurysm tissue their main source seem to be macrophages (Fanjul-Fernandez et al., 2010).

The possible clinical relevance of nucleotide polymorphism in promoter region of genes encoding for both, MMP-3 and MMP-10, has been studied by several authors (Sandford et al., 2007; Saratzis et al., 2011). It has been found, that in the MMP-3 promoter, at the position -1171, corresponding to the transcriptional start site, two variants – one of them, containing 5 adenosines (5A) and the other one with 6 adenosines (6A), may be present. *In vitro* tests have shown, that the 5A allele has nearly two fold higher activity, than the 6A variant. Presumably, this could be due to the higher affinity of the transcriptional repressor p50/p50 to 6A, than to the 5A allele. Epidemiological studies have shown, that the mentioned 5A/6A polymorphism of MMP-3 promoter may be associated with various cardiovascular diseases. It was revealed that 5A/5A homozygotic individuals are significantly more susceptible to hypertension, myocardial infarction and coronary artery aneurysms.

Interestingly, the 6A/6A variant carriers displayed a higher growth rate of atherosclerotic plaque, which, on the other hand, was more stable, than in patients with the 5A allele. Yoon and coauthors have observed a trend ($p=0.06$) for a higher 5A allele frequency in a small cohort of 47 Finnish patients with abdominal aortic aneurysm (Yoon et al., 1999). The higher 5A allele frequency among aortic aneurysm-suffering patients compared to a control group was also confirmed in a British population (Ye, 2006).

In contrast to MMP-3, studies concerning the presumable role of MMP-10 gene polymorphisms, including -180 A/G SNP, have failed to reveal any significant correlation with a prevalence of aortic aneurysm (Ogata et al., 2005).

3.2.1.3 Other archetypal MMPs

This subgroup of archetypal MMPs includes four enzymes: MMP-12, MMP-19, MMP-20 and MMP-27. The representative of this subgroup – MMP-12, also known as macrophage metalloelastase, is mainly expressed and secreted by activated macrophages and is necessary for its migration (Visse & Nagase, 2003; Kadoglou & Liapis, 2004). The main substrate for MMP-12 is elastin, but the enzyme may cleave some other ECM molecules, including aggrecan, fibronectin, laminin, and type IV collagen.

The increased expression of MMP-12 was found exclusively in aortic aneurysm wall, but not in the control, or atherosclerotic aorta specimens. Immunohistochemical studies have localized MMP-12 within the media of aortic aneurysms, predominantly in zones adjacent to non-dilated aorta. However, although MMP-12 is recognized as a key player in aneurysm formation, its prominent role has been neglected in the elastase-induced aneurysm animal model. Interestingly, it has been found that MMP-12-deficient mice revealed aortic dilatation similar to that of wild type animals, whereas mice lacking MMP-9 were resistant to aneurysm induction. Therefore, it was suggested that MMP-12 role is restricted rather to supporting other MMPs in aneurysm formation (Kadoglou & Liapis, 2004).

The analysis of polymorphic sites in the MMP-12 gene and *in vitro* studies suggested the possible connection between A to G substitution in -82 position of the promoter and the

aortic aneurysm expansion. However, none of the clinical trials have confirmed the expected correlation to date (Sandford et al., 2007; Saratzis et al., 2011).

Group	Subgroup	MMP	Common name	Substrates	
				ECM	Non-ECM
Archetypal MMPs	Collagenases	MMP-1	Collagenase-1	Collagens (I, II, III, VII, VIII and X), gelatin, proteoglycan link protein, aggrecan, veriscan, tenacin, entactin	α1-PI, ILb-1, pro-TNF, IGFBP-3, MMP-2, MMP-9
		MMP-8	Collagenase-2	Collagens (I, II, III, V, VII, VIII and X), gelatin, aggrecan	α1-PI, α2-antiplasmin, fibronectin
		MMP-13	Collagenase-3	Collagens (I, II, III, IV, IX, X, XIV), gelatin, aggrecan, perlecan, large tenascin-C, fibronectin, osteonectin	MMP-9, plasminogen activator inhibitor-2
	Stromelysins	MMP-3	Stromelysin-1	Collagens (III, IV, V and IX), gelatin, aggrecan versican, hyaluronidase-treated versican, perlecan, decorin, proteoglycan link protein, large tenascin-C, fibronectin, laminin, entactin, osteonectin	α1-PI, antithrombin-III, ovosstatin, substance P, IL-1β, serum amyloid A, IGFBP-3, fibrinogen and cross- linked fibrin, plasminogen, MMP-2/TIMP-2 complex MMP-1,-7,-8,-9,-13
		MMP-10	Stromelysin-2	Collagens (III, IV and V), gelatin, casein, aggrecan, elastin, proteoglycan link protein	MMP-1,-8
	Other Archetypal	MMP-12	Metalloelastase	Collagen IV, gelatin, elastin, casein, laminin, proteoglycan monomer, fibronectin, vitronectin, enactin	α1-PI, fibrinogen, fibrin, fibrin, plasminogen, myelin basic protein
		MMP-19	RASI	Gelatin	ND
		MMP-20	Enamelysin	Amelogenin	ND
		MMP-27	-	ND	
Matrilysins		MMP-7	Matrilysin	Collagens IV and X, gelatin, aggrecan, decorin, proteoglycan link protein, fibronectin, laminin, insoluble fibronectin fibrils, entactin, large and small tenascin-C, osteonectin, β4 integrin, elastin, casein, transferrin	MMP-1,-2,-9 α1-PI, MMP-9/TIMP-1 complex, plasminogen
		MMP-26	Matrilysin-2	Collagen IV, gelatin, fibronectin	ProMMP-9, fibrinogen, α1-PI

ND-not determined

3.2.2 Matrilysins

Matrilysins are represented by matrilysin-1 (MMP-7) and matrilysin-2 (MMP-26, or endometase). The most prominent feature of matrilysins group members is the lack of the hemopexin domain. MMP-26 is the smallest known MMP; it is composed of only 261 amino acids and may activate itself by autocatalysis. Matrilysins play an important role in degradation of ECM molecules, including type IV collagen, laminin, entactin, as well as several cell surface molecules, e.g. Fas ligand, E-cadherin and syndecan-1 (Fanjul-Fernandez et al., 2010). Due to a shedding of membrane-bound Fas ligand and generation of its soluble form, matrilysins may stimulate apoptosis. Furthermore, while cleaving plasminogen to produce an angiostatin fragments, matrilysins are involved in inhibition of angiogenesis. Interestingly, MMP-26 has an ability to activate pro-MMP-9 in a specific site, that results in better stability of MMP-9 (Zhaoet al., 2003; Amalinei et al., 2007). Moreover, intracellular MMP-26 was found to process the estrogen receptor β, therefore in patients with breast cancer an increased MMP-26 level was found to correlate with longer survival (Hadler-Olsen et al., 2011)

3.2.3 Gelatinases

The group of gelatinases consists of two members: gelatinase A (MMP-2) and gelatinase B (MMP-9). Both of them are constitutively expressed by many cells, including fibroblasts, keratinocytes, endothelial cells, polymorphonuclear leukocytes, monocytes, alveolar macrophages and osteoclasts. When compared to other MMPs, the most significant difference in the structure of gelatinases is the presence of three type II fibronectin-like repeats within the catalytic domain. They are responsible for recognition and binding of denatured collagen and gelatin molecules (Visse & Nagase, 2003). Gelatinases may cleave various extracellular matrix molecules, e.g. collagen types I, IV, V, VII, IX, X, elastin, fibronectin, aggrecan, vitronectin, laminin, as well as numerous non-ECM molecules, including pro-TNF, TGF-β, pro-IL-1β and pro-IL-8 (Fanjul-Fernandez et al., 2010). Moreover, they are involved in generation of several pro- and anti-angiogenic factors, which may originate from both, ECM and non-ECM substrates (Mott & Werb, 2004).

Gelatinases are considered the most important members of the MMPs family, involved in pathogenesis of aortic aneurysm. The amounts of mRNA and specific proteins in the aneurysm wall, as well, as plasma levels of both, MMP-2 and MMP-9, were statistically significantly higher in patients with aortic aneurysms, as compared to healthy controls. Moreover, it has been found that plasma levels of gelatinases noticeably correlate with aneurysm expansion rate and therefore they were suggested as presumable predictors of the aneurysm rupture risk. In an animal experimental study it was proven, that infusion of gelatinases resulted in development of aortic aneurysms. Interestingly, MMP-2- and/or MMP-9-deficient mice were resistant to aneurysm formation in this model (Longo et al., 2002; Baxter, 2004; Kadoglou & Liapis, 2004).

Recently, it has been postulated, that, in addition to elastin degradation, gelatinases may also be engaged in the pathogenesis of aortic aneurysm in a proteolysis-independent fashion. This hypothesis is based on the observation, that both, MMP-2 and MMP-9, may display some inhibitory influence on the calcium-dependent contraction of VSMC isolated from the aortic wall (Raffetto & Khalil, 2008). In addition to maintenance of aortic wall integrity, the contraction of those cells is considered as a counterbalance of hemodynamic forces, that protects the aorta against dilatation during each cardiac cycle. Thus, the MMP-

mediated reversible inhibition of vascular myocytes contraction could promote aneurysm progression. However, this issue requires further elucidation.

3.2.3.1 MMP-2 (Gelatinase A)

MMP-2, or gellatinase A, is constitutively expressed by vascular myocytes, however, it may be produced in small amounts by macrophages and fibroblasts, too. As described in chapter 3.4, the activation of MMP-2 occurs mainly by its interaction with MT1-MMP/TIMP-2. Due to its elastolytic activity, MMP-2 is believed to play a pivotal role in aortic aneurysm development. It was found, that VSMC isolated from aortic aneurysm tissue produced higher amounts of MMP-2, as compared to those from atherosclerotic, or normal aortic wall. Surprisingly, the activity of MMP-2 negatively correlated with aneurysms diameter. This observation could support the opinion, that MMP-2 may be essential in the early stages of aneurysm development. Besides weakness of the aortic wall, MMP-2-mediated degradation of elastic fibers may lead to production of elastin-derived peptides, which have a great chemotactic potential. These peptides may promote recruitment of inflammatory cells and enhance further proteolysis. Moreover, MMP-2 liberates TGF-β from an inactive extracellular complex consisting of TGF-β, latent TGF-β binding protein and TGF-β-latency associated protein, which may stimulate various MMPs expression and results in further progression of the disease (Rizas et al., 2009).

Among 18 polymorphisms found in the MMP-2 gene, the SNP located in a promoter (-1306 C/T) was initially considered as clinically relevant. The studies focusing on the influence of this particular SNP on promoter function have shown, that the presence of C allele was associated with a higher promoter activity (Price et al., 2001). However, none of clinical studies, addressed at verifying a possible correlation between -1306 C/T SNP and aortic aneurysm prevalence, has revealed such a connection (Eriksson et al., 2005; Ogata et al., 2005; Sandford et al., 2007; Saratzis et al., 2011).

3.2.3.2 MMP-9 (Gelatinase B)

MMP-9, or gellatinase B, is mainly produced by macrophages and neutrophils. Subsequently to proteolytic activation by a variety of factors, including plasminogen activators and other MMPs (MMP-2, -3, -12), MMP-9 displays elastolytic, collagenolytic and gelatinolytic activity. The leader position of MMP-9 in the pathogenesis of aortic aneurysm may be confirmed by several observations. Animal studies have shown, that MMP-9-deficient mice were resistant to experimentally induced aortic dilation and they did not reveal elastin degradation despite the presence of inflammatory cell in the aortic wall (Kadoglou & Liapis, 2004; Baxter, 2004). On the other hand, among all metalloproteinases, MMP-9 is the most abundantly expressed enzyme in aortic aneurysm wall, in contrast to normal aortic tissue, where it was not present. Also, huge amounts of MMP-9 are released by neutrophils trapped in the intraluminal thrombus (see also chapter 2.1). Furthermore, high MMP-9 concentrations were detected in ruptured aneurysms. However, although patients with an aortic aneurysm have significantly higher levels of MMP-9, than control individuals, there is no consensus regarding a direct correlation between MMP-9 plasma levels and aneurysm progression and rupture risk to date (Takagi et al., 2009; Eugster et al., 2005). Therefore, this issue still requires further elucidation.

Several polymorphisms have been found in the MMP-9 gene. Most of the studies have focused on -1562 C/T polymorphism in a promoter region. It has been proven that a common T to C substitution in -1562 position of the MMP-9 promoter is associated with

approximately 50% increase of the promoter activity. A clinical study by Medley and coauthors has shown that mRNA level, protein concentration, as well as enzymatic activity of MMP-9 in aortic aneurysm tissues of -1562 T allele carriers were significantly higher, as compared to those with the -1562 C variant. Also, plasma levels of MMP-9 were much higher in -1562 T carriers, than in patients with -1562 C allele (Medley et al., 2004). Furthermore, Jones and coauthors have also reported a higher frequency of the -1562 T variant in a group of patients with aortic aneurysm (n=414), in comparison to individuals with atherosclerotic peripheral vascular disease (n=172, adjusted odds ratio 2.94), or healthy control subjects (n=203, adjusted odds ratio 2.41) (Jones et al., 2003).
Interestingly, subsequent studies, independently conducted by Ogata and Eriksson on large groups of aortic aneurysm-suffering individuals (n=387 and n=455, respectively) have failed to confirm this association (Ogata et al., 2005; Eriksson et al., 2005). In a large study by Smallwood and coauthors, carried out on 678 patients with aortic aneurysms and 659 healthy controls, no statistically significant association between occurrence of the -1562 T allele and development of aortic aneurysm was found (Sandford et al., 2007; Smallwood et al., 2008; Saratzis et al., 2011).
It is noteworthy, that although recent studies have negated the direct involvement of -1562 C/T polymorphism in pathogenesis of aortic aneurysm, the potential importance of other functional polymorphisms in the MMP-9 gene cannot be excluded. Presumably, two additional functional SNPs – the first located in the exon encoding for catalytic domain (Q279R), and the next relating to the sequence encoding for hemopexin domain (P574R), could be considered as attractive candidates for further studies.

3.2.4 Furin-activated MMPs

The members of this group are characterized by the presence of an unique RXKR or RRKR sequence, inserted between the prodomain and the catalytic domain. This site is recognized and cleaved by pro-protein convertases or serine proteinases – furins, thus resulting in an activation of enzyme. The furin-activated MMPs are further divided into small subgroups: secreted MMPs, membrane-type I and type II MMPs and GPI-anchored MMPs (Fu et al., 2008; Fanjul-Fernandez et al., 2010).
The furin-activated secreted MMPs include MMP-11, MMP-21 and MMP-28. In contrast to other secreted MMPs, members of this subgroup undergo intracellular processing by furin, or furin-like proteases and therefore they are secreted already in active form (Fanjul-Fernandez et al., 2010).

3.2.4.1 Membrane type MMPs

All the membrane type MMPs (MT-MMPs) contain a hydrophobic component that enables their insertion in the cell membrane. They control the close neighborhood of both, normal and pathological cells, thus being involved in promoting of cell migration, invasion, experimental metastasis and angiogenesis (Hernandez-Barrantes et al., 2002).
Type I transmembrane MMPs include: MT1-, MT2-, MT3-, and MT5-MMP (MMP-14, -15, -16, and -24, respectively). They are characterized by a long hydrophobic transmembrane sequence followed by a short cytoplasmic tail, which could participate in several signaling pathways. The main representative of this subgroup, MT1-MMP (MMP-14) may be expressed on the cell surface of various cells, however, activated macrophages and VSMC are considered as its most important producers. It may cleave native type I collagen into ¾ – ¼ fragments in a collagenase-specific fashion. Apart from collagen I, MT1-MMP is able

to process various components of extracellular matrix, including collagens type II and III, gelatin, fibronectin, as well, as non-ECM molecules, e.g. hyaluronan receptor (CD44), myelin-inhibitory protein and α-2 macroglobulin (Fanjul-Fernandez et al., 2010). Furthermore, as previously mentioned, MT1-MMP plays a key role in the proteolytic activation of pro-MMP-2 (see chapter 3.4).

Group	Subgroup	MMP	Common name	Substrates	
				Extracellular matrix (ECM)	Non-ECM
Gelatinases		MMP-2	Gelatinase A	Collagens (I, IV, V, VII, X, XI and XIV), gelatin, elastin, fibronectin, laminin-1, laminin-5, galectin-3, aggrecan, decorin, hyaluronidase-treated versican, proteoglycan link protein, osteonectin	IL-1b, α1-PI, prolysyl oxidase fusion protein, MMP-1, MMP-9, MMP-13
		MMP-9	Gelatinase B	Collagens (IV, V, VII, X and XIV), gelatin, elastin, galectin-3, aggrecan, fibronectin, hyaluronidase-treated versican, proteoglycan link protein, entactin, osteonectin	α1-PI, IL-1β, plasminogen
Furin-activated MMPs	Secreted	MMP-11	Stromelysin-3	Casein, laminin, fibronectin, gelatin, collagen IV and carboxymethylated transferrin	α1-PI, casein, IGFBP-1
		MMP-21	XMMP	ND	
		MMP-28	Epilysin	ND	
	Type I transmembrane	MMP-14	MT1-MMP	Collagens (I, II and III), casein, elastin, fibronectin, gelatin, laminin, vitronectin, large tenascin-C, entactin, proteoglycans	α1-PI, MMP-2,-13
		MMP-15	MT2-MMP	Large tenascin-C, fibronectin, laminin, entactin, aggrecan, perlecan	MMP-2
		MMP-16	MT3-MMP	Collagen-III, gelatin, casein, fibronectin	MMP-2
		MMP-24	MT5-MMP	ND	
	GPI-anchored	MMP-17	MT4-MMP	ND	
		MMP-25	MT6-MMP	ND	
	Type II transmenbrane	MMP-23A	-	ND	
		MMP-23B	-	ND	

ND – not determined

Recently, it was found, that mRNA and protein levels of MT-MMP-1 were significantly higher among patients with an aortic aneurysm, than in healthy controls or atherosclerosis-suffering subjects. Therefore, it is suggested, that MT-MMP-1 may also be important in pathogenesis of aortic aneurysm, especially due to its multidirectional action. Besides direct destruction of extracellular matrix components, MT1-MMP activates MMP-2 and facilitates migration of macrophages, thus promoting inflammatory infiltration of the aortic wall (Kadoglou & Liapis, 2004).

Type II transmembrane MMPs are represented by enzymes: MMP-23A and MMP-23B, which, despite being encoded by two different genes, actually have an identical amino acid sequence. Their structure is significantly distinct from other MMPs, since they lack the signal peptide, the cysteine-switch motif and the hemopexin domain. Moreover, opposite to type I MT-MMPs, they have a transmembrane domain located in their N-terminal tail, whereas C-terminus contains a cysteine array and immunoglobulin-like domains (Fanjul-Fernandez et al., 2010).

The last subgroup of MT-MMPs includes MT4-MMP and MT6-MMP (MMP-17 and -25, respectively). Both of them have the glycosylphosphatidylinositol (GPI) anchor instead of a transmembrane domain on their C-terminus, that enables binding of the MMP molecule to the cell membrane. To date, little is known about the presumable role of other MT-MMPs in pathogenesis of aortic aneurysm, with the exception of MT1-MMP, therefore, this subject still requires further studies.

3.3 Regulation of expression

The critical role of MMPs in physiology, as well as their involvement in various pathological conditions decipher the tight control of production and activation of these enzymes. Among all MMPs, only MMP-2 and MMP-9 were found to be expressed constitutively, whereas the expression of the remaining members of the MMP family has to be induced, e.g. in response to a tissue remodeling, or inflammatory reaction. Numerous factors, including pro-inflammatory cytokines (IL-1β, IL-6, TNF, etc.), growth factors (platelet-derived growth factor – PDGF; epidermal growth factor – EGF; TGF-β; etc.) and corticosteroids are involved in the control of MMPs gene expression Kadoglou & Liapis, 2004; Diehm et al., 2007). The common feature of the majority of inducible genes, including these encoding for MMPs, is the presence of the binding sites for transcription factors AP-1 and/or NF-κB in their promoter region. AP-1 transcription factors are heterodimer complexes, composed of two proto-oncogene family proteins – Jun and Fos. Together with NF-κB, a p50/p65 heterodimer, which controls the expression of numerous immune response- and inflammation-engaged molecules, AP-1 complexes interconnect a number of growth factor- and cytokine-mediated pathways. Another group of transcription factors involved in regulation of MMPs expression are members of Ets family. They recognize and bind to the conserved polyomavirus enhancer activator protein-3 (Pea3) binding site, which is found in MMP promoters. Since the Pea3 binding site is located contiguously to at least one AP-1 element, the interaction between both transcription factors will presumably modulate promoter response to various stimuli. Most recently, it was suggested that, in addition to the formerly discussed, the modification of chromatin structure through its acetylation-deacetylation could be another mechanism, involved in the regulation of MMP genes expression. Interestingly, it has been found that inhibition of histone deacetylase (HDAC) results in enhanced MMP-3, but decreased MMP-1 and MMP-9 expression in response to

stimulation with IL-1β or TNF (Clark et al., 2007; Clark et al., 2008). Therefore, this aspect of MMPs expression control remains unclear and requires further studies. The next issue to be clarified is the role of mechanotransduction and the involvement of putative mechanoreceptors or mechano-responsive elements in MMPs expression control. This pathway seems to be of great importance especially in tissues permanently exposed to dynamic stress, like joint cartilage chondrocytes or VSMC in blood vessels (Blain, 2007). Finally, it is noteworthy, that besides transcriptional level, MMPs expression in response to various stimuli, including cytokines and growth factors, may also be controlled by the modulation of specific mRNA stability (Chakraborti et al., 2003).
Most recently, it has been found that MMP-2 activity may also be modulated by protein kinase C–mediated phosphorylation at the post-translational level (Sariahmetoglu et al., 2007). This modification may involve at least 5 amino acid residues from active site of catalytic domain and may result in modulation of its activity. Possibly, 3′-5′ cyclic adenosine monophosphate (cAMP) pathway may be involved in this step, too.

3.4 Activation of MMPs

As mentioned previously, all MMPs are produced and secreted in an inactive zymogen form. This status is maintained due to the presence of the "cysteine-switch", the particular interaction between the thiol group of the prodomain cysteine and the zinc ion from the catalytic domain. The disruption of this interaction is essential for enzyme activation and may occur in two different manners (Fig. 2).
The first mode is an alteration of the cysteine thiol group by some physiological factors, including disulfides, oxidants and electrophiles, as well as non-physiological compounds, among them denaturing surfactants with sodium dodecyl sulphate (SDS), alkylating agents, organomercurials with 4-aminophenylmercuric acetate (APMA), and heavy metal ions. This alteration results in some allosteric changes in MMP structure, followed by an exposure of the catalytic site of the enzyme. It may lead to the auto-cleavage and removal of prodomain, and thus is associated with reduction of enzyme molecular size. On the other hand, the prodomain may stay attached to the active enzyme and thus the molecular weight of activated MMP remains unchanged (Fu et al., 2008; Hadler-Olsen et al., 2011) (Fig. 2A).
The second mechanism of MMPs activation is direct cleavage of their prodomain by another proteolytic enzyme. It has been shown that proteolytic activation of MMPs may be conducted by other MMPs, as well as a broad spectrum of extracellular serine-, cysteine- or aspartate-proteinases. Moreover, this mechanism is also utilized during an intracellular activation of MMPs by furin, the subtilisin-like serine proteinase from the trans-Golgi network (Fig. 2A).
An interesting combination of both, allosteric and proteolytic mode of action, represents activation of MMP-2 by the membrane type 1-MMP (MT1-MMP, or MMP-14) in the presence of the endogenous MMP inhibitor – the tissue inhibitor of MMPs (TIMP)-2. The TIMP-2 molecule serves as a link between MMP-2 and MMP-14, where the N-terminal part of TIMP-2 inactivates MMP-14 and its C-terminal part binds to the hemopexin domain of pro-MMP-2 (Fig. 2B). The prodomain of immobilized MMP-2 is then cleaved by another recruited MMP-14 molecule, thus resulting in proteolytic activation of MMP-2. Remarkably, other membrane type MMPs: MT2-MMP and MT3-MMP are able to activate pro-MMP-2 without involvement of TIMP (Hadler-Olsen et al., 2011, Klein & Bischoff, 2010).

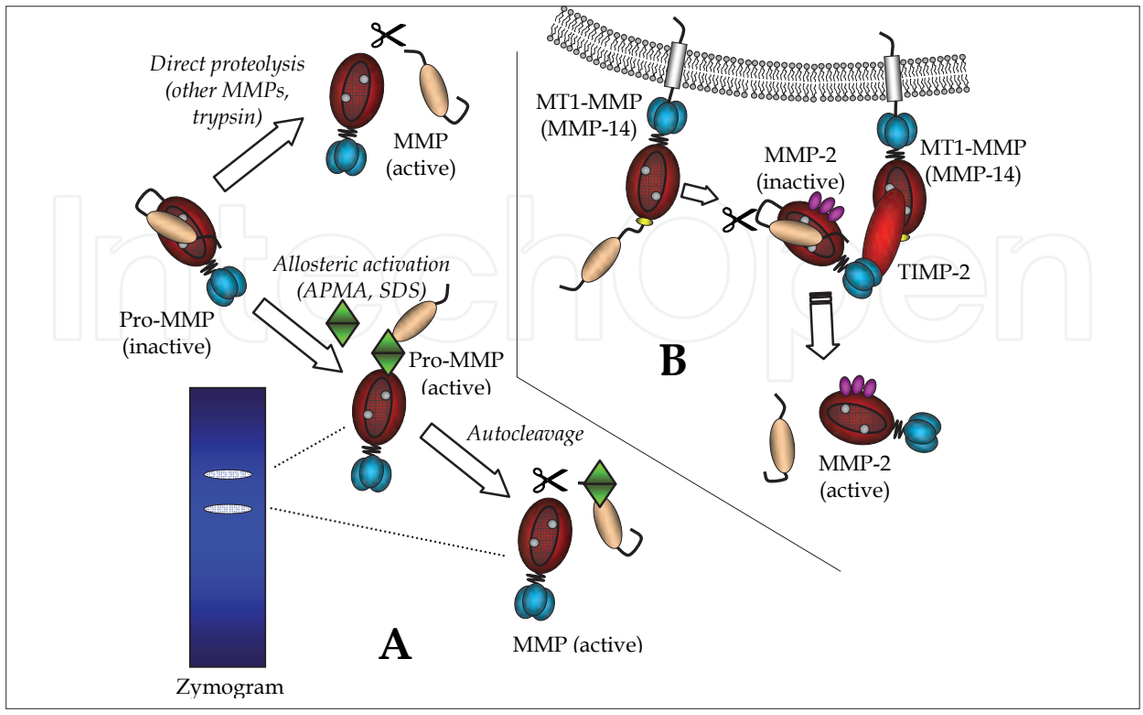

Fig. 2. The MMP activation pathways (a detailed description in text)

3.5 Inhibition of MMPs

When firmly supervised, matrix metalloproteinases control various physiological reactions very precisely. However, if this supervision appears incompetent, MMPs reveal their "dark side of the Force" and become highly dangerous molecules, which are engaged in many pathologies, including the development of an aortic aneurysm. Therefore, mechanisms responsible for this regulation and factors participating in these mechanisms are potentially useful in some therapeutic approaches.

Standard mechanisms of MMPs silencing include interaction with the specific tissue inhibitors of metalloproteinases and other endogenous inhibitors, such as α2-macroglobulin, reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and tissue-factor-pathway-inhibitor 2 (TFPI2) (Maskos & Bode, 2003).

Surprisingly, although reactive oxygen species are known to activate autolytic cleavage of MMPs due to disruption of their "cysteine switch", it has been suggested that oxidants may also inactivate MMPs by modification of some amino acids critical for their catalytic activity (Fu et al., 2008).

3.5.1 TIMPs

Tissue inhibitors of metalloproteinases are secreted by different types of cells, including macrophages, VSMC and platelets. The basically defined function of TIMPs is a suppression of the MMPs activity by biding to their catalytic domain and blocking enzymatic activity. Based on *in vitro* studies, TIMPs are recognized as highly efficient MMPs inhibitors (with K_i at the level of 10^{-11} M for TIMP-1/MMP-1 interaction). The affinity of various TIMPs to specific MMPs differs among them, e.g. TIMP-1 preferably binds to MT-MMPs, whereas

other TIMPs are generally less selective. However, a direct interaction between TIMPs and active MMPs may be difficult to observe *in vivo*, mainly due to a presence of α2-macroglobulin, a common MMP-neutralizing agent (Fu et al., 2008). On the other hand, as clearly shown for TIMP-2, as well as for other TIMPs, their binding to the hemopexin domain of pro-MMP-2 is actually necessary for the activation of this enzyme by MT1-MMP (Hadler-Olsen et al., 2011).

It is plausible, that functional polymorphisms in genes encoding for TIMPs could influence the activity of MMPs in the aneurysm wall. Thus, several SNPs supposed to affect the level of TIMP transcription, were assessed in patients with aortic aneurysms. It is noteworthy, that the genotyping results were analyzed for male and female patients separately due to the fact, that TIMP-1 gene is located on the X chromosome. To date, the association with aortic aneurysm has been suggested for two polymorphisms in gene for TIMP-1 (434 C/T and rs2070584 T/C) (Ogata et al., 2005) and two in TIMP-2 (a promoter SNP -479 C/T, and 573 G/A, but in male patients group only) (Wang et al., 1999; Hinterseher et al., 2007). Nevertheless, since the mentioned analyses concerned relatively small groups, this issue still requires additional investigation (Sandford et al., 2007; Saratzis et al., 2011).

3.5.2 Pharmacological modulation of MMPs

Since the modulation of MMPs activity by endogenous inhibitors in pathology seems to be inefficient, several strategies with exogenous MMP modulators have been developed. It is noteworthy, that some of these modulators are already used in clinical practice, although the primary indication for their application did not concern inhibition of MMPs.

An interesting approach may be the use of MMP-specific antibody-based inhibitors. The experimental model with neutralizing antibodies directed against MMP-2 has shown their protective influence on a heart exposed to ischemia/reperfusion injury (Cheung et al., 2000). However, the verification, whether this procedure would be useful in management of aortic aneurysms, needs further studies. Apart from endogenous inhibitors, various pharmacological agents may reveal inhibitory activity against MMPs. The small hydroxamate-based zinc-chelating synthetic agents, such as batimastat, marimastat or ilomastat (galardin), were first used in oncology, to suppress MMPs-dependent metastasis and tumor invasion. However, due to non-selective, generalized inhibition of MMPs activity with numerous adverse events, accompanied by relatively poor effectiveness, their clinical career was ended very soon (Baxter, 2004).

3.5.2.1 Tetracyclines

Tetracyclines are natural antibiotics derived from *Streptomyces*. They are known for many non-antibiotic properties, including inhibition of proteolysis, or anti-apoptotic and anti-inflammatory activity. The most intensively tested representative of tetracyclines – doxycycline, has been shown to prevent formation of aortic aneurysm in several animal models. Also, the results of three small clinical trials in humans have shown that doxycycline in a well tolerated dose 150-200 mg/day significantly reduced aneurysm expansion rate. It has been suggested, that doxycycline inhibits MMPs directly, by binding to their catalytic site. Furthermore, it may reduce the MMP expression attenuating the inflammatory cascade. However, the results of the studies on putative effects of tetracyclines on MMP-9 are not fully consistent. The first published study by Cruci and coauthors has shown that doxycycline treatment in a dose 200 mg/day resulted in a 2.5-fold reduction of MMP-9 protein levels and 82% reduction of MMP-9 mRNA expression (Cruci et al., 1998).

Surprisingly, in a subsequent placebo-controlled study Ding and colleagues did not observe any effect of 1 month 100 mg/day doxycycline therapy on MMP-9 mRNA, or protein expression (Ding et al., 2005). Finally, Lindeman and coauthors have shown that 2-weeks doxycycline treatment decreased MMP-9 protein levels ($p<0.0026$), but did not affect mRNA expression level ($p=0.206$). The authors have concluded, that the beneficial effect of doxycycline on aneurysm progression might be due to the reduction of aneurysm wall infiltration by neutrophils and T CD8-positive lymphocytes rather, than the decrease of MMP-9 gene expression (Lindeman et al., 2009).

3.5.2.2 Macrolides

The beneficial influence of tetracyclines on aneurysm expansion rate may be multidirectional, including their antimicrobial activity against *Chlamydia pneumoniae*, and thus, indirect MMPs modulation (also discussed in chapter 2.2). This concept led to the introduction of macrolides, other antibiotics with potent *Chlamydia*-killing activity, to the arsenal of anti-aneurysm agents. In a randomized trial by Vammen and coauthors, the aortic aneurysm expansion rate in patients treated with roxithromycin was 1.56 mm/year, in comparison to 2.75 mm/year in the placebo-receiving control group. Interestingly, no significant correlation between *C. pneumoniae* titers and roxithromycin ability to suppress the aneurysm expansion was observed (Vammen et al., 2001) .

The mechanism of anti-aneurysm protective action of macrolides remains unclear. Recently, another macrolide derivative, rapamycin, was tested in animal aortic aneurysm model. Due to its strong anti-inflammatory and immunosuppressive properties, rapamycin is commonly used to prevent transplanted organ rejection. It has been found, that rapamycin administration in experimentally induced aortic aneurysms in rat significantly inhibits activation of NF-κB and, subsequently, MMP-9 expression thus resulting in 40% decrease of aneurysm expansion rate, as compared to control animals (Lawrence et al., 2004).

3.5.2.3 Statins

The inhibitors of hydroxymetylglutaryl-coenzyme A reductase, better known as statins, are widely used in a treatment of patients with cardiovascular diseases. Apart from their main activity, which decreases atherogenic lipoproteins, statins reveal various pleiotropic effects, including an augmentation of pro-apoptotic properties of some anticancer drugs, or an inhibition of inflammatory cell activity. Possibly, these anti-inflammatory properties of statins result from suppression of NF-κB, that is also involved in regulation of MMPs expression. Indeed, *in vitro* studies have demonstrated, that simvastatin and cerivastatin may decrease the production of MMPs, especially MMP-9, in VSMC, macrophages and neutrophils. A subsequent small clinical study by Nagashima, and a randomized prospective trial by Evans group independently showed, that statins were potent inhibitors of inflammatory cells *in vivo*, and they effectively suppressed the MMP-9 production in the aortic aneurysm wall (Nagashima et al., 2002; Evans et al., 2007). Also, Wilson and his colleagues observed, that simvastatin, pravastatin and atorvastatin decreased local concentrations of MMP-3 and MMP-9 in the anterior wall of aortic aneurysms in patients undergoing open heart surgery. Furthermore, as shown by Schouten and coauthors, statin use resulted in lower aneurysm expansion rate with 2.0 mm/year in statin-treated versus 3.6 mm/year in the placebo control group (Schouten et al., 2006). Interestingly, in contrast to reports mentioned previously, Hurks and coauthors did not observe any statistically

significant differences in MMP-9 level between patients treated with statins and a control group. Moreover, they have found, that a level of active MMP-9 in patients treated with pravastatin was higher, than in control subjects (Hurks et al., 2010). This may suggest, that a pleiotropic actions profile of these drugs may differ among the various statins.
Unfortunately, the issue concerning statins influence on aortic aneurysm progression seems unlikely to be resolved in the near future, because of two reasons at least. Firstly, the prevalence of statin use in aortic aneurysm-suffering patients is very high, mainly due to other concomitant cardiovascular diseases. Therefore, it may be difficult to assemble a control group for a large randomized study. Secondly, according to current recommendations, statins should be used as perioperative protection during open surgical, as well as endovascular aneurysm repair (Diehm et al. 2007). Thus, such a study, additionally to being difficult to design, appears to be ethically controversial.

3.5.2.4 Low molecular weight heparin

As was discussed previously (chapter 2.1), the aortic aneurysm is usually accompanied by an intraluminal thrombus, that is always associated with more or less clinically overt coagulation abnormalities. It has been shown, that these coagulopathies may be successfully treated with small doses of low molecular weight heparin (LMWH) (Jelenska et al., 2004). Moreover, our subsequent studies revealed, that in addition to the attenuation of coagulation abnormalities, a LMWH treatment resulted in statistically significant decrease of circulating MMP-9 plasma activity. Interestingly, the *in vitro* studies did not show any direct influence of LMWH on MMP-9 production by activated leucocytes, or enzyme activity, either. Therefore, the most plausible explanation seems to be an indirect mechanism of LMWH action. According to our concept, the stabilized thrombus does not capture any new leukocytes. In such circumstances the number of MMP-9-producing cells, which are already entrapped in fibrin clot, decreases slowly. Finally, theses conditions lead to the decrease of MMP-9 amounts, released from intraluminal thrombus to the circulating blood. However, the verification of this hypothesis, as well as further clinical studies, concerning LMWH influence on aneurysm progression, are necessary.

3.5.2.5 JNK inhibitor

Based on results of studies focused on molecular aspects of aortic aneurysm pathophysiology, the c-Jun N-terminal kinase (JNK) has been selected as a new target for therapy. Using pharmacological JNK inhibitor Yoshimura and coauthors prevented the development, or induced the regression of already formed aneurysm in calcium-, or angiotensin II-induced aortic aneurysm mouse models. This effect was associated with decreased MMP-9 activity, but also with restoration of aortic tissue architecture, mainly due to the upregulation of various extracellular matrix proteins synthesis. Obviously, the results of further studies are expected highly impatiently (Yoshimura et al., 2005; Miyake & Morishita, 2009).

3.5.2.6 Renin-angiotensin system modulators

Recently, it has been suggested, that the renin-angiotensin system and its main effector molecule – angiotensin II, may also be involved in the initiation of inflammatory reaction in the aortic wall and, thus, it significantly contributes to the development of aneurysm. Therefore, antagonists of the angiotensin II receptor were tested for their presumable protective effect on aneurysm progression. Indeed, it was shown, that losartan and

valsartan, two representatives of the group, were effective in preventing aneurysm formation. Interestingly, although they do not appear to influence MMPs activity directly, their beneficial influence was associated with decrease of MMPs expression in aneurismal tissue, possibly due to the suppression of NF-κB-controlled inflammatory reaction (Fujiwara et al., 2008; Miyake & Morishita, 2009). However, since the career of angiotensin II receptor antagonists in a management of cardiovascular diseases is rather short, their value in aortic aneurysm treatment still remains to be verified.

The next group of pharmaceuticals, addressed to interfere with the renin-angiotensin system are angiotensin converting enzyme (ACE) inhibitors. In contrast to the previously described angiotensin II receptor antagonists, they bind to the active site of MMPs and directly block their enzymatic activity in a dose dependent manner. Using animal models, it has been shown, that ACE inhibitors, including captopril, enalapril, lisinopril and perindopril, reduced the aortic wall degeneration and aneurysm progression. Surprisingly, data provided by several clinical trials are highly inconsistent. Reports by Lederle and Taylor, or Hackam group suggested independently, that patients with aortic aneurysms, which received ACE inhibitors, displayed lower aneurysm expansion rate and were better protected from aneurysm rupture, as compared to individuals treated without convertase inhibitors (Hackam et al., 2006; Lederle & Taylor, 2006). On the other hand, Schouten and colleagues did not find any correlation between aneurysm dilatation rate and ACE inhibitors (Schouten et al., 2006; Baxter et al., 2008; Miyake & Morishita, 2009). Moreover, the abdominal aortic aneurysm screening program in UK revealed an increasing aneurysm growth rate, that was apparently associated with ACE inhibitors intake (Sweeting et al., 2010). Therefore, this approach urgently needs further elucidation in multicenter prospective randomized trials, although, due to the common use of ACE inhibitors in cardiovascular diseases, the study design will possibly meet similar obstacles, as in statins case.

3.5.2.7 Decoy oligodeoxynucleotides

Recently, the novel strategy based on targeting transcription factors, which are known to be involved in regulation of inflammatory reaction and MMPs expression, appears to be highly promising approach in the management of aortic aneurysm. A principle of this strategy is the use of synthetic oligodeoxynucleotides (ODNs), referred as "decoy" ODNs, which specifically recognize and bind to respective transcription factors and thus competitively block their binding to promoter of gene of interest.

This decoy strategy was used to inhibit two transcription factors – NF-κB and ets-1, which are suggested to control gene expression of several MMPs, including MMP-1, -2, -3 and -9. Using elastase-induced aneurysm rat and rabbit models, and chimeric anti-NF-κB/ets-1 decoy ODNs, Miyake and colleagues have analyzed the effects of the simultaneous suppression of both, NF-κB and ets-1. They found, that this approach resulted in marked decrease of aortic aneurysm progression, that was accompanied by significant decrease of MMP-1 and MMP-9 expression in the aneurysm wall. Moreover, in the rabbit model the application of the mentioned decoy system onto already formed aneurysms led to their significant regression, which in addition to MMPs inhibition, was possibly due to induction of collagen and elastin synthesis, that is negatively controlled by ets-1. Hopefully, this strategy will be effective also in human, since the anti-NF-κB/ets-1 ODNs decreased MMPs expression in *ex vivo* experiments with cultured human aortic tissue (Miyake & Morishita, 2009). It is noteworthy, that the decoy ODNs-based therapy has some limitations, too. The main problem is the rapid degradation and relatively short half-life of therapeutic ODNs,

that require their local application. This in turn raises the problem with an effective delivery system. In experiments by Miyake and coauthors the cellulose-based ODNs delivery system was applied intraoperatively on the outer surface of dilated aorta. In clinical practice this could be done using low-invasive procedures, such as laparoscopy, or more likely by endovascular access. However, this issue remains to be resolved.

3.6 Methods of MMP detection and analysis

The increasing evidence concerning the involvement of MMPs in various pathologies, including aortic aneurysm, results in the necessity to develop methods of analysis that would be sufficiently sensitive and specific enough to detect minute amounts of MMPs.

3.6.1 ELISA

The above mentioned main requirements are met by an enzyme-linked immunosorbent assay (ELISA). Despite its high sensitivity (picograms per ml) and availability for all known MMPs, the ELISA method provides data reflecting only the specific protein amount, without any information about its enzymatic activity. This obstacle is partially resolved by a new generation of ELISA kits, that can discriminate between full length pro-enzyme and proteolytically activated MMP (Fig. 3A). Obviously, similar data could be obtained using the western-blot method with respective anti-MMP antibodies. Nevertheless, the previously mentioned non-proteolytic activation of full-length molecules may result in false negative, or at least, underestimated results of MMP measurement. Thus, the studies focusing on MMPs require another method that would enable convenient analysis of their enzymatic activity.

3.6.2 Substrate-specific zymography

Currently, the standard method used for the analysis of MMPs activity is a substrate-specific zymography with sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The method is based on a molecular size-dependent electrophoretic separation of tested samples in a polyacrylamide gel, containing substrate specific for MMP being analyzed, e.g. gelatin for MMP-2 and MMP-9, casein for MMP-1, -3, -7, -10, -12 and -13 or collagen for MMP-1 and -13 (Kupai et al., 2010). Then, the gel is incubated in zinc- and calcium-rich reaction buffer to re-activate enzyme particles separated in the gel. The high local concentration of enzyme molecules results in a substrate digestion, that is restricted to the area close to the position of respective MMP in the gel. The visualization of enzymatic activity of assayed samples occurs after incubation of the whole gel with the staining solution, e.g. Coomassie blue. The presence of an unstained area in a gel corresponds to a substrate cleavage, due to enzymatic activity of respective MMP, whereas the size of this area directly correlates with amount and/or activity of the enzyme. Thus, the standard SDS-PAGE zymography allows a semi-quantitative comparison of respective MMP amount, or more precisely – activity between various samples. When used with known amounts of recombinant MMP, as a reference, the method permits the quantitative assessment of respective MMP. Furthermore, due to the gel electrophoresis of analyzed samples, this method enables the molecular size-based identification and individual measurement of both, full length pro-MMP and its short processed form, too (Fig. 3D, also Fig.2). It has been proven that sensitivity of standard SDS-PAGE zymography is sufficient to detect similar amounts of active MMP, as those using ELISA method (Table 1).

Substrate	Enzyme	Detection limit
gelatin	MMP-2	10 pg
casein	MMP-7	1 ng
collagen	MMP-1	0.1 pg

Table 1. Examples of SDS-PAGE zymography detection limits (Kupai et al., 2010)

It is noteworthy, that the standard SDS-PAGE zymography is not free of some problems, either. The important issue is the unspecific, SDS-mediated activation of pro-MMP, that may occur during electrophoresis. Obviously, this event affects the results, and is usually associated with an overestimation of MMP activity. Additional difficulty with the use of this method is the long, time-consuming procedure. Therefore, although the SDS-PAGE zymography is sensitive enough and relatively inexpensive, in some circumstances the better choice for a fast MMP assessment could be the fluorescent zymography.

3.6.3 Fluorescent zymography

The fluorescent zymography is based on a technology known as the fluorescence resonance energy transfer (FRET). Briefly, the tested sample is incubated in small volume (10-20 µl) of reaction mixture containing the substrate, that is labeled with fluorochrome and quencher tags. When a substrate remains intact, the energy emitted by fluorochrome is entirely absorbed by the quencher, therefore no fluorescence is detected. The substrate degradation abolishes the suppressive effect of the quencher on fluorochrome, thus resulting in increase of fluorescence in the reaction mixture. The intensity of fluorescence corresponds to the amount of digested substrate, that, in turn, reflects the amount of active enzyme in a sample (Fig. 3B).

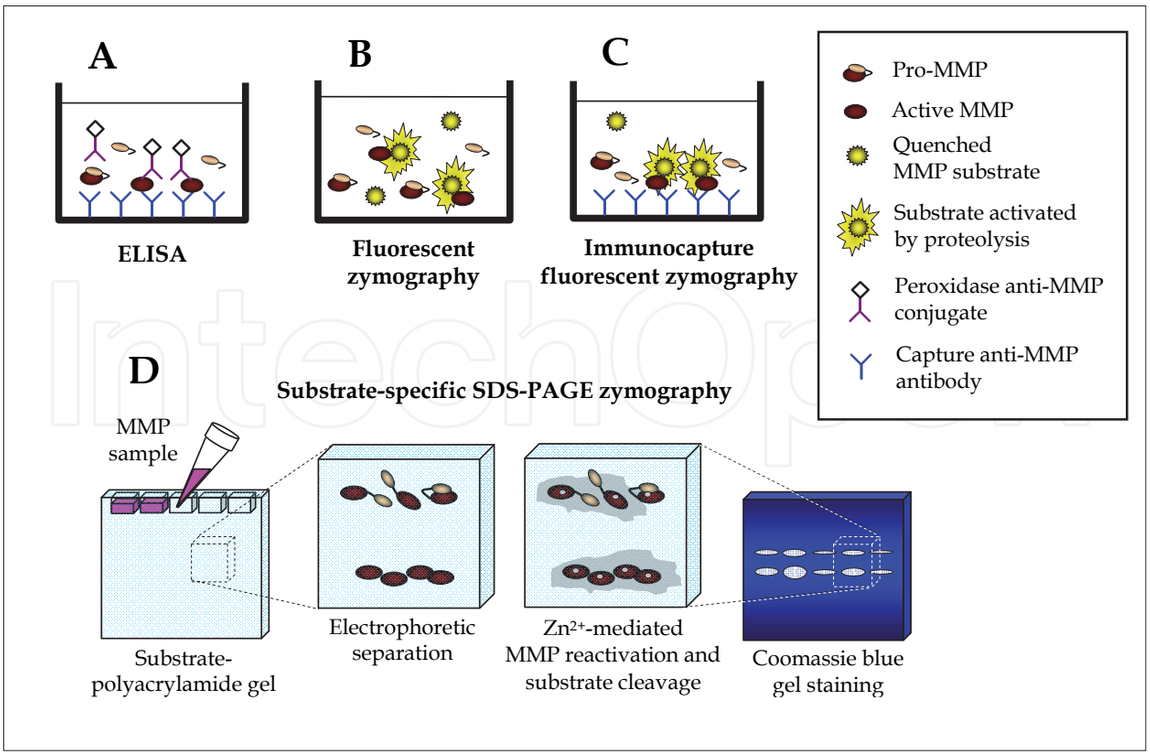

Fig. 3. Schematic representation of main methods of MMPs detection (description in text).

Oppositely to standard SDS-PAGE zymography, this method is very fast and enables the measurement of proteolytic activity in analyzed samples within several minutes. In contrast to the previously mentioned ELISA and SDS-PAGE zymography, the fluorescent zymography allows an assessment of the direct influence of various factors on MMP activity. However, the outcome of the analysis is highly sensitive to sample preparation and the reaction conditions. The samples should be prepared without addition of EDTA or other metal-ion chelators, and without addition of protease inhibitors. This step may be of great importance for final results of analysis, especially since the extraction procedures themselves may induce MMPs activation or lead to release of natural MMP inhibitors. After tissue disruption during sample preparation, these inhibitors, although initially localized in different compartments, can interact with active enzymes, and thus, may affect the results of assay. Another important issue may be the concentration of non-ionic detergents in the reaction mixture, which may "neutralize" small molecules that potentially could interfere with MMP activity (Kupai et al., 2010).

The most significant disadvantage of the fluorescent zymography is, that it does not determine MMP, that directly contributes to the observed substrate degradation. This obstacle may be omitted by the use of respective, MMP-specific, monoclonal antibodies, which block the proteolytic activity of the selected enzyme. With a panel of blocking antibodies, this modification allows the quantitative assessment of each constituent of MMP-mediated proteolytic activity in the analyzed sample.

Another solution to this problem may be the method that combines the idea of both, ELISA and fluorescent zymography. Briefly, the plate is coated with specific anti-MMP antibodies, which capture molecules of the respective enzyme from the sample being assessed. Then, non-captured, unspecific proteins are washed out and the fluorochrome/quencher-labeled substrate is added to the plate followed by incubation. Finally, since the intensity of MMP-mediated fluorescence corresponds to the activity of specific MMP, it may be measured using the fluorescence plate reader. This solid-phase, or immunocapture fluorescent zymography represents the key advantages of both, ELISA and "normal" fluorescent zymography (Fig. 3C). It enables a measurement of enzymatic activity of individual MMPs, is specific, sensitive, rapid and lacks the previously mentioned main deficiencies of both methods.

3.6.4 *In situ* zymography

In studies concerning distribution of MMPs in affected tissues, the use of *in situ* zymography may be very advantageous. This test allows the detection of MMPs activity in histological specimens, directly on the place of their production or activation. Similarly to previously mentioned fluorescent zymography, this method also exploits FRET technology. The fluorochrome/quencher-labeled substrate is applied on the surface of a specimen, and is incubated. Then, the specimen can be assessed with a fluorescent microscope, or using a confocal laser scanning microscope.

The main disadvantage of this method is that it does not allow precise measurement of the amount of active MMP. Moreover, similarly to "normal" fluorescent zymography, it is necessary to use neutralizing antibodies, to determine MMPs that contribute to substrate cleavage.

3.6.5 Reverse zymography - detection of MMP inhibition

The studies concerning the role of MMP in pathogenesis of aortic aneurysm include also a method that enables assessment of the natural tissue inhibitors of MMP (TIMPs). This

method, known as reverse zymography, utilizes polyacrylamide gel with specific substrate, that is additionally supplemented with respective MMP. After electrophoresis of tested samples the gel is subjected to the same processing as in routine zymography. Then, the MMP, which is equally distributed in the whole gel, cleaves the substrate with the exception of those places, where TIMPs inhibit its proteolytic activity. Those places are visible as Coomassie blue-stained bands, whereas the other parts of the gel remain unstained.

In addition to reverse zymography, the expected interaction between MMPs and their tissue inhibitors can also be analyzed using a modified western blotting (SDS-PAGE and immunoblotting) method. The formation of higher molecular weight complex by interacting molecules results in its slower migration during polyacrylamide gel electrophoresis, and thus reveals a band shift of specific MMP or its inhibitor, depending on the antibody used for detection.

3.6.6 Possible applications

Currently, the methods of MMPs analysis in aortic aneurysm clinics described above are predominantly used for research purpose. However, due to an increasing interest in the monitoring of MMPs level and/or activity in aneurysm-suffering patients, it is plausible that some of these methods will become the standard diagnostic tools in the near future.

The first postulated indication for clinical use could be the screening of plasma MMPs activity in patients with newly recognized aortic aneurysm. Although there is no consent, whether low plasma levels of MMPs may be considered as a low risk indicator of aneurysm progression and/or rupture, it is nevertheless widely accepted that the higher concentration, or more precisely, their higher activity should at least encourage more frequent monitoring of aneurysm diameter. The second, important clinical application for MMP assessment seems to be the periodic monitoring of MMPs activity in patients with small aneurysms and subjected to pharmacological treatment. Obviously, the method selected for monitoring should depend on the mechanism of action of controlled MMP modulator. Thus, the monitoring of pharmacological treatment of aortic aneurysm using compounds displaying indirect influence on MMP amount and/or activity, including statins, heparins or ODNs, may engage a broad spectrum of methods mentioned above. However, in regimens using potent direct MMP inhibitors, e.g. tetracycline-derivatives, or angiotensin converting enzyme-inhibitors, the fluorescent zymography rather, than other methods, including substrate-specific SDS-PAGE zymography, should be used preferentially.

It has been found that open aneurysm repair leads to transient (usually within first 3 moths) MMP-9 increase, followed by its later significant reduction. Nevertheless, in some patients high levels of MMPs may still persist even a very long time after surgery (Kadoglou & Liapis, 2004). An interesting example of this condition may by the case of a 50-year old men with an abdominal aortic aneurysm, subjected to successful open aneurysm repair. Surprisingly, after 4 years the patient was qualified again for surgical treatment due to the significant enlargement of the thoracic aorta, remaining suprarenal part of abdominal aorta and common iliac arteries. The plasma level of MMP-2 was similar to those observed in other aortic aneurysm-suffering individuals. However, his MMP-9 plasma activity was extraordinarily (almost 10-fold) higher, than mean MMP-9 level, observed in other patients bearing an aortic aneurysm.

In contrast to open repair, patients subjected to endovascular aneurysm exclusion usually reveal a gradual decrease of circulating MMP-3 and -9, whereas the persisting high concentration and/or activity of circulating MMP-9 actually correlates with some postoperative complications. They include graft migration, or presence of endo-leak, where both conditions are accompanied by the contact of circulating blood with the thrombus still existing in the aneurysm lumen (Kadoglou & Liapis, 2004). Therefore, the temporary monitoring of MMP activity may be an important diagnostic procedure also in patients after both, open surgical, but also endovascular intervention.

4. Conclusion

Despite numerous studies, the role of MMPs in pathogenesis of aortic aneurysm remains unclear. Also, the question, whether they are "executors", or "executioners", is still unanswered. Since MMPs are obviously engaged in all stages of the aneurysm development, from the beginning, till the fatal aneurysm rupture, they are very attractive targets in approaches, concerning pharmacological treatment of this pathology. However, due to some discrepancies between results of experimental, as well as clinical studies conducted so far, this issue still requires intensive research. Nevertheless, it is plausible that in the near future the majority of patients with aortic aneurysms might simply require regular drug intake, without the necessity for surgical intervention, which would be reserved for particular cases only. Since it is very similar to the history of management of gastric ulcers, this vision seems to be quite realistic in the near future…

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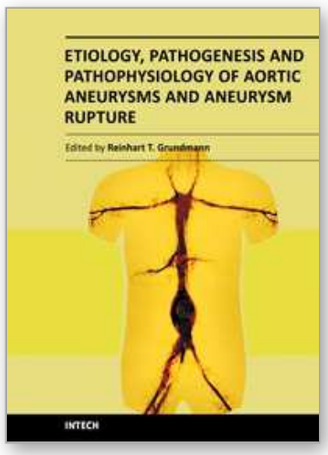

Etiology, Pathogenesis and Pathophysiology of Aortic Aneurysms and Aneurysm Rupture

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This book considers mainly etiology, pathogenesis, and pathophysiology of aortic aneurysms (AA) and aneurysm rupture and addresses anyone engaged in treatment and prevention of AA. Multiple factors are implicated in AA pathogenesis, and are outlined here in detail by a team of specialist researchers. Initial pathological events in AA involve recruitment and infiltration of leukocytes into the aortic adventitia and media, which are associated with the production of inflammatory cytokines, chemokine, and reactive oxygen species. AA development is characterized by elastin fragmentation. As the aorta dilates due to loss of elastin and attenuation of the media, the arterial wall thickens as a result of remodeling. Collagen synthesis increases during the early stages of aneurysm formation, suggesting a repair process, but resulting in a less distensible vessel. Proteases identified in excess in AA and other aortic diseases include matrix metalloproteinases (MMPs), cathepsins, chymase and others. The elucidation of these issues will identify new targets for prophylactic and therapeutic intervention.

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