Age-related macular degeneration: current knowledge of zinc metalloproteinases involvement

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Abstract:

Background: Advanced age-related macular degeneration (AMD) is the leading cause of blindness in the elderly with limited therapeutic options. The disease is characterized by photoreceptor loss in the macula and reduced retinal pigment epithelium (RPE) function, associated with matrix degradation, cell proliferation, neovascularization and inflammation. Matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) play a critical role in the physiology of extracellular membrane (ECM) turnover and, in turn, in ECM pathologies, such as AMD. A balance between the activities of MMPs and Tissue inhibitors of metalloproteinase (TIMPs) is crucial for the integrity of the ECM components; indeed, a dysregulation in the ratio of these factors produces profound changes in the ECM, including thickening and deposit formation, which eventually might lead to AMD development.

Objective: This article reviews the relevance and impact of zinc metalloproteinases on the development of AMD and their roles as biomarkers and/or therapeutic targets. We illustrate some studies on several inhibitors of MMPs currently used to dissect physiological properties of MMPs. Moreover, all molecules or technologies used to control MMP and ADAM activity in AMD are analyzed.

Conclusions: This study underlines the changes in the activity of MMPs expressed by RPE cells, highlights the functions of already used MMPs inhibitors and consequently suggests their application as therapeutic agents for the treatment of AMD.

Keywords: AMD, RPE, MMPs, ADAMs, ADAMTSs, MMP Inhibitors

1. Introduction

Age-related macular degeneration (AMD) is a multifactorial disorder influenced by interaction between genetic and environmental risk factors. AMD is characterized by a pathological extracellular matrix (ECM) remodeling and by deposition of lipids or lipoproteins within the Bruch's membrane (BrM) in the retinal area [1]. Bruch's membrane is a penta laminated extracellular matrix responsible for the diffusion between the retinal pigment epithelium (RPE) and the choroidal vasculature. The aging process causes a progressive thickening of this membrane due to the deposition of matrix components and the consequence is a decreased efficiency of the hydraulic conductivity of BrM [2].

A large number of proteases are responsible for the proteolytic processes in the BrM. The main enzymatic group involved in the degradation of ECM is the superfamily of the zinc metalloproteinases, which includes the matrix metalloproteinases (MMPs), also known as matrixins, the A Disintegrin and Metalloproteinase Domain (ADAMs) and the A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTSs) [3].

1.1 What is AMD?

AMD is the most common cause of irreversible blindness in people aged 50 and over in the developed world [4, 5]. It occurs when the small central portion of the retina, known as the macula, deteriorates causing a progressive visual impairment due to the neurodegeneration at the level of photoreceptors/retinal pigment epithelial complex. In particular, the pathological area involved in AMD is represented by photoreceptors, the retinal pigment epithelium (RPE), the Bruch's membrane (BrM), and the choriocapillaris. So far, there is no treatment for this pathology, and for this reason, the identification of risk factors is a significant point to provide cause and possible intervention strategies. Unfortunately, nowadays the understanding of biological nature, risk factors and disease therapies remains limited [6].

Epidemiological studies have shown that genetic predisposition, and other several risk factors (modifiable and non-modifiable) such as smoking, obesity, age, gender, hypertension, hyperlipidemia, cardiovascular disease and nutritional deficiencies are associated with the development of AMD (**Table 1**).

Table 1. Risk Factors for Age-Related Macular Degeneration.

Risk Factors	First Author	Publication year	References
Genetic factors	Fritsche L.G.	2016	[6]
Hypertension	Vingerling J.R.	1995	[7]
Low dietary intake of antioxidants	Seddon J.M.	1994	[8]
Age > 65 years	Friedman D.S.	2004	[9]
White race	Chang M.A.	2008	[10]
A history of smoking within the past 20 years	Nano M.E. McCarty C.A.	2013 2001	[11], [12]
Obesity (BMI > 30 Kg/m ²)	Rowan S.	2017	[13]
Gender (female)	Armstrong R.A.	2015	[14]

Age is the most important risk factor identified to date. In fact, AMD is rare in people younger than 50 years, and its frequency of development increases considerably after 75 years of age [15].

Moreover, a family history of AMD confers an established risk factor, which is typically multifactorial in nature. Different studies [16] have identified polymorphism in genes that encode complement factors, important immunosystem elements, as a positive or negative influencer for AMD risk. In fact, the development of AMD includes mutations in the complement gene H (CFH) [17] and, in contrast, other studies showed protective effects of genetic polymorphism in complement component 2 (C2) and complement factor B (CFB) [18]. In particular, CFH acts as a complement inhibitor and it is believed that a polymorphism of this gene reduces its ability to down-regulate complement activity. This allows continuous inflammation and pathogenic degeneration of the macula [19]. Therefore, the genetic defects of proteins may play a fundamental role in development of AMD by disrupting the homeostatic balance between pro and anti-inflammatory molecules generated by RPE cells after exposure to

inflammatory stimuli [20], either locally-generated or transmissible by blood. In addition to the role played by genetic polymorphisms, environmental factors also contribute to the development of AMD.

1.2 Pathophysiology and Classification: Wet and Dry AMD

In general, the most relevant pathogenic mechanisms responsible for AMD development are: the accumulation of a cellular debris, hypoxia, local inflammation, and neovascularization. Among them, the accumulation of extracellular matrix and polymorphous material, “drusen”, in the macula is the hallmark of AMD.

Human retinal pigment epithelium (RPE) is the structure in the retina responsible for the maintenance of photoreceptor cells, and the death of photoreceptor seems to be strictly connected with a RPE degeneration due to drusen accumulation. Typically, drusen are spread out between RPE basement and the inner collagenous area of Bruch’s membrane (**Figure 1**) [21]. An inefficient disposal of the accumulated ECM molecules by RPE or a dysregulation of inflammatory mediators may be the hypothesized mechanisms for drusen accumulation [22].

Figure 1. Classification of AMD, according to Age-Related Eye Disease Study. GA= Geographic atrophy. [Copyright: **Figure 1.1** This image was originally published in the Retina Image Bank® website. H. Michael Lambert, MD. Normal Retina. Retina Image Bank. 2014; Image Number 16035. © the American Society of Retina Specialists. **Figure 1.2** This image was originally published in the Retina Image Bank® website. H. Michael Lambert, MD. Dry AMD. Retina Image Bank. 2013; Image Number 8688. © the American Society of Retina Specialists. **Figure 1.3:** This image was originally published in the Retina Image Bank® website. Jerald A. Bovino, MD. Dry Macular Degeneration. Retina Image Bank. 2013; Image Number 6379. © the American Society of Retina Specialists. **Figure 1.4:** This image was originally published in the Retina Image Bank® website. Young Hee Yoon, MD, PhD. Geographic Atrophy. Retina Image Bank. 2012; Image Number 530. © the American Society of Retina Specialists.]

AMD may progress from early signs (early AMD), to its intermediate and advanced forms [23]. While early stages of the disease are mostly asymptomatic, the late stages cause severe vision loss due to disruption of the retinal anatomy and ultimately photoreceptor degeneration. AMD is classified as intermediate AMD, the so called “Dry form” (geographic atrophy, GA, when angiogenesis is absent), and advanced AMD, the so called “Wet form” (choroidal neovascularization, CNV, accompanied by angiogenesis) [24]. AMD classification was made by the Age-related Eye Disease Study (AREDS) and it is based on drusen, atrophy and neovascularization (**Figure 1**): a situation of “no AMD” is characterized by none or a few small drusen ($<63\ \mu\text{m}$), Intermediate AMD (Dry) is defined by extensive intermediated-size drusen ($63\text{--}124\ \mu\text{m}$) or GA not involving foveal center, Advanced AMD (Wet) is determined by GA and CNV [25].

Dry AMD is the most frequent and less dangerous form of disease. It represents 85 to 90 % of cases and its course is usually slow and degenerative. In the dry type of AMD, the deterioration of the retina is associated with the development of drusen, microcalcifications and irregular dystrophy with distorted and blurred vision at first, followed by a loss of central vision (**Figure 1**) [26]. This phenomenon leads to a thinning and drying out of the macula, causing loss of macula functionality. Dry AMD is also called non-neovascular AMD and non-exudative AMD because it does not involve the leakage of fluids from blood vessels. Advanced cases – “late dry” AMD – are called geographic atrophy (GA) because large sections of the retina that are well demarcated (geographies), stop functioning. GA is characterized by the gradual expansion of atrophic macular changes, which results in a degeneration of vision abilities [27].

The wet/neovascular type affects approximately 10–15% of individuals with AMD, but accounts for approximately 90% of all cases of severe vision loss from the disease. Wet AMD is a condition characterized by choroidal neovascularization or CNV [28] where new pathogenic blood vessels grow in the choroid layer breaching the BrM and going across the sub-retinal space. Since these new blood vessels are abnormal, they tend to break, bleed, and leak fluid, damaging the macula that lifts up and pulls away from its base. The leaking gets into the layers of the retina – including the layers of the macula – and can cause scar tissue to form and retinal cells to stop functioning.

1.3 Bruch's membrane and RPE involvement

Bruch's membrane (BrM) is a thin (2–4 μm), acellular, extracellular matrix located between the retina and choroid and consists of five layers: the basement membrane of the RPE, the inner collagenous layer, a central band of the elastic fiber, the outer collagenous zone, and the choriocapillaris endothelium basement membrane (**Figure 2**).

Figure 2. The composition of human extracellular matrix in retinal area. RPE BM= Retinal Pigment Epithelium Basal Membrane, composed by Coll IV, V, laminins, E/N -1,-2, PGs (mainly HSPGs), fibulins; ICL= Inner Collagene Layer, composed by Coll I, III, V, fibronectin, HSPGs, fibulins; EL= Elastin layer, composed by elastin, coll VI, bronectin, HSPGs, fibulins; OCL= Outer Collagene Layer, composed by Coll I, III, V, fibronectin, HSPGs, fibulins; Choriocapillaris BM= Choriocapillaris Basal Membrane, composed by Coll IV, V, VI (XV, XVIII*), laminins, E/N -1,-2, PGs (mainly HSPGs), fibulins; E/N= entactin/nidogen; PGs= proteoglycans; HSPGs= heparin sulphate proteoglycans.

In physiological conditions, BrM is a physical barrier permeable to fluids and small molecules, it serves as a selective conduit for nutrients transported from the choroidal vasculature to the retina and for metabolic wastes transported from the retina to the circulation [29]. Moreover, it gives a structural support against

neovascularization i.e. the invasion of new vessels into the retina. In fact, containing the anti-angiogenic molecules in the elastin layer it prevents the growth of neovascular capillaries from the choroid, separating the vascular choroid from the avascular outer retina.

Several recent findings support the involvement of BrM in AMD development [30]. Studies of hydraulic conductivity have invariably shown that the BrM permeability decreases with ageing [31]. Over the course of time, BrM tends to accumulate debris in the elastin layer (EL) and also drusen between the inner collagen layer (ICL) and RPE basal lamina (RPE-BL). This debris accumulation causes a reduction of the permeability and a thickening of BrM elastic layer [32]. The resulting porous BrM elastic layer becomes a feeble structure and may trigger the development of neovascularization [33].

RPE is constituted by a thin layer of cuboidal cells close to photoreceptor cells. The relationship between photoreceptors and RPE cells is fundamental for the correct function of sight. In fact, the dysregulation of RPE cells is responsible for photoreceptors death and for a consequent degeneration of the visual ability causing retinal pathologies such as AMD [34]. In particular, RPE plays a central role in the development and maintenance of adjacent photoreceptors in the vertebrate retina as well as in retinal physiology by forming the outer blood-retinal barrier. RPE cells regulate the production of various matrix proteins (collagen type I, collagen type IV, and laminin) but also BrM structural elements thus suggesting to be the site of the primary lesion in AMD [35].

Several authors have shown that human RPE in cell culture releases several members of the family of matrix metalloproteinases (MMPs), and the tissue inhibitor of metalloproteinases (TIMPs) (see *next paragraph*) [36,37]. Modulation of ECM turnover by changing RPE secretion of these matrix metalloproteinases and their TIMPs may play a central role in the normal functions and in the pathologies of the retina. The secretions of RPE could be altered in the retinal interphotoreceptor matrix (IPM), as well as in the BrM. The fraction associated with RPE-IPM may manifest interferences with the metabolism of retina as a whole, leading indirectly to stimulation of RPE, and causing the development of AMD. Nowadays, the regulation of RPE cellular functions is not completely understood and it is still under investigation.

1.4 Emerging drug treatments for Dry and Wet AMD

Although in the last years many research efforts have been directed toward understanding the pathophysiology of the disease in order to develop new therapies, none of the current treatments can cure the disease or reverse its course. In general, Dry and Wet AMD require different treatments due to the different main pathological manifestations, GA and CNV.

Dry AMD: The most promising therapeutic agent for treating Dry AMD is the human monoclonal antibody Lampalizumab, discovered by the 18-month Phase II MAHALO study. The intravitreal administration of this molecule, which is a selective complement factor D inhibitor, was associated with a significant decrease (44%) in the GA progression. Lampalizumab reached Phase III in the Chroma and Spectri clinical trials, so far the largest

studies on GA, but results published at the end of 2018 demonstrated that it could not reduce GA enlargement during 48 weeks of treatment. [38,39].

Another clinical trial (TOGA) demonstrated the efficacy of tetracyclines. In particular, a low-dose administration of Doxycycline, a broad-spectrum antibiotic, could slow the progression of GA in patients with AMD [40].

A topical ophthalmological drug under development is MTP-131 (Ocuvia), a mitochondrial protective compound really effective in several experimental models and currently under clinical trial in order to evaluate the tolerability in AMD patients [41,42].

Wet AMD: the treatment of wet AMD focused on the development of CNV that is mainly due to VEGF activation [43]. Pegatinib is the first anti-VEGF approved drug and it is a specific RNA aptamer able to bind the VEGF-165 isoform. Its efficacy was demonstrated in the clinical trial VISION based on intravitreal administration and it was the starting point to develop more effective anti-VEGF treatments. Successively, the recombinant humanized IgG1 monoclonal antibodies were largely studied and analyzed, reporting a strong anti-VEGF-A activity: Ranibizumab, Bevacizumab [44] and the most recent Aflibercept [45], which is a VEGF-A receptor decoy. Recently, these anti-VEGF agents were shown to be highly effective by intravitreal administration but they are not suitable for a long lasting therapy. An innovative drug is Pegpleranib which is a platelet-derived growth factor inhibitor, promoting the pericyte density in the neovascular membrane, making it more receptive to anti-VEGF agents. The Phase III program of Pegpleranib consists of evaluating its efficacy and tolerability in combination with the anti-VEGF agents (Ranizumab, Bevacizumab and Aflibercept)[46,47]. Unfortunately, at the end of 2016 Ophthotech Corporation announced that the co-administration of Pegpleranib with Ranizumab did not result in a major benefit compared to Ranizumab monotherapy for AMD treatment as measured by the mean change in visual activity at the 12 month time point.

2. Matrix Metalloproteinases (MMPs), A Disintegrin and Metalloproteinase Domain (ADAMs) and A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTSs): a role in AMD

The extracellular matrix is involved in the physiological processes of tissue architecture characterized by a natural balance between synthesis and degradation of ECM constituents. The continuous rebuilding of connective tissue in pathological and physiological processes is due to specific endopeptidases, the zinc metalloproteinases, which include the superfamily of the metzincins zinc-dependent endopeptidases. Metzincins are able to cleave proteins of the extracellular matrix participating both to unspecific protein degradation and to specific cleavage events, such as enzyme activation. It is evident that the deregulation of their degrading potential can lead to various serious pathologies. Based on sequence and structural similarities, metzincins are classified in four distinct subfamilies: the astacins, the matrixins (matrix metalloproteinases, MMPs), the serralyins (large bacterial proteinases) and the adamalysins (ADAMs and ADAMTSs) (**Figure 3**).

Figure 3. Schematic representation of Metzincins classification.

In this chapter the role of Metzincins in AMD disease is analyzed, considering that MMPs, ADAMs and ADAMTSs play an important role in extracellular matrix (ECM) turnover. MMPs are essential for maintaining ocular physiology and their inordinate activities have been linked to several neurodegenerative disorders of the retina, including age-related macular degeneration. Expression of most MMPs in ocular tissues, under normal conditions, is low and it is induced when remodeling of ECM is required.

2.1 MMPs

Matrix Metalloproteinases (MMPs), or Matrixins, constitute a family of more than twenty zinc-dependent endopeptidases homogeneous for structure, function and localization. The widely used classification of MMPs is based on substrate specificity, sequence similarity, domain organization. They are divided in five main groups:

- Collagenases (MMP-1, MMP-8, MMP-13 and MMP-18) that cleave interstitial collagens I, II, III and a various number of ECM and no-ECM molecules.
- Gelatinases (MMP-2 Gelatinase A and MMP-9 Gelatinase B) that digest type IV collagen and gelatin, and are discriminated by the presence of gelatin-binding region inside the catalytic domain.
- Stromelysins (MMP-3 and MMP-10) that display a broad ability to digest ECM proteins but are unable to cleave the triple helical fibrillar collage.
- Matrylisins (MMP-7 and MMP-26) that are characterized by the lack of the hemopexin domain.
- Membrane-Type MMPs (MT-MMPs) that are divided in type I (MMP-14, MMP-15, MMP-16 and MMP-24) and the glycosylphosphatidylinositol (GPI)-anchored proteins MT5-, and MT6-MMP (MMP17 and MMP-25). All MT-MMPs have a furin cleavage site in the propeptide and they are all capable of activating

other proteins. In particular MT1-MMP has a collagenolytic activity on type I, II, and III collagen and other components of ECM

- Other MMPs (MMP-11, MMP-12, MMP-19, MMP-20, MMP-22, MMP-23, and MMP-28) that are not classified in the above categories.

MMPs activity is strictly regulated by various factors but in particular by their tissue inhibitors, TIMPs (Tissue inhibitors of Metalloproteinases). The complexes MMPs-TIMPs are responsible for the balance in the regulation of numerous cascade of enzymatic reactions involved in many physiological processes.

The turnover of matrix in the retina bordering the apical interphotoreceptor matrix (IPM) and the basolateral aspect of RPE cells (Bruch's membrane) is thought to be mediated by metalloproteinases and their related inhibitors (TIMPs).

From a structural point of view the family of MMPs mostly shares a common three domain-based structure that consists of:

- a signal sequence responsible for the detection of the molecules and the consequent MMP secretion
- a pro-peptide domain at the amino terminal, which is removed for MMP activation.
- a catalytic domain that contains a zinc ion essential for the proteolytic activity.
- a hemopexin domain at the carboxylic-terminal, that has a functional role in substrate binding and interactions.

2.1.1 MMPs in the eye

Recent studies using eye tissues or cell culture systems suggest that MMPs and their inhibitors play a key role in the homeostasis of extracellular matrix in the eye. A balance between the activities of MMPs is significant to the remodeling of the ECM components, including collagen, vitronectin, fibronectin, laminin, elastin and proteoglycans. A small dysregulation in the ratio of these factors can produce profound changes in the retinal ECM, including thickening and deposit formation [48]. Nowadays, the exact sources of MMPs in the retinal area are still unknown even if they are mainly released by BrM and RPE cells [49].

In general, MMPs level increases in the BrM proportionally with the BrM thickness with age. After the release from plasma or from RPE or choroidal cells, MMPs can diffuse across BrM following a molecular weight exclusion limit. In fact, only those MMPs with a small molecular weight, such as MMP-1, MMP-2 and MMP-3, can diffuse. Nevertheless, analyzing another potential pathway MMPs may be passively incorporated into the ECM of BrM if their release is coincident with the synthesis of BrM components. In this case, even MMPs with high molecular weight such as MMP-9 could be incorporated [50].

The presence of some MMPs and TIMPs in RPE cells is well known [51, 21]. MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-9 (gelatinase B) and MT1-MMP but also TIMPs 1 and 3 are present in human IPM and vitreous and they are the most reported. Eichler et al. [52] and Li et al. [49] demonstrated that MMP-1, -2, -3, and -9 were expressed in cultured RPE cells. Moreover, Platner et al. [53],

showed a highly significant increase in the level of gelatinase A in RPE-associated Interphotoreceptor matrix (IPM) in AMD compared to normal donors, therefore suggesting a role for gelatinase A in the pathology of AMD.

Not only MMPs are responsible for the accumulation or the degradation of ECM components in BrM but also TIMPs have a role. In particular, the age-related increased level of TIMP-3 is connected with a decreased level of ECM components in BrM [54]. In fact, the TIMP-3 growth is somehow counterbalanced by an age dependent production of proMMP-2 and -9 [55], that remain in the deactivate form.

A dysfunction of RPE cells activity causes alterations in MMPs/TIMPs balance and it is responsible for an mutated activity of MMPs [35]. Alteration in the expression and activity of MMPs expressed by RPE, has been associated with pathologies involving matrix degradation, cell proliferation, neovascularization and inflammation and cell adhesion in vitreo-retinal disorders, such as Choroidal neovascularization (CNV) and Proliferative vitreoretinopathy (PVR)[56,57].

The MMPs role in the retinal pathological processes, principally in inflammatory and in the oxidative stress, is reported in **Table 2**.

Table 2. MMPs, ADAMs and ADAMTSs expressed by RPE Cells and their involvement in different biological processes (↑↑ = relevant upregulation; ↑ = upregulation; - = no regulation; ↓ = downregulation; ↑↓ = both upregulation and downregulation observed)

MMP-1, MMP-2, MMP-3, MMP-9 are expressed in RPE cells			
Process	MMP and TIMP involved	Notes	References
Neo-angiogenesis (VEGF and COCL2)	↑ MMP-9, MMP-2 (after VEGF and chemical hypoxia)	<ul style="list-style-type: none"> • Feedback positive between MMP-9 and VEGF • Feedback negative between MMP-2 and VEGF • Probably MMP-9 is not constitutively expressed but secreted only in inflammatory cases • MMP-9 reduce VEGF expression through a direct stimulation on cells facilitating neovascularization. 	[52] [55] [58] [59] [60] [61] [62] [63]
Oxidative stress	↓ MMP-2, -14 ↓ TIMP2	<ul style="list-style-type: none"> • Both MMP-14 and TIMP-2 are essentials to reduce the loss of MMP-2 activation • MMP-14 is a possible therapeutic target 	[52] [64] [65] [66]
ADAM9, ADAM10, ADAM17 are expressed in RPE cells			
Inflammation	↑ ADAM10, 17	<ul style="list-style-type: none"> • Shedding of proinflammatory mediator 	[67]
Migration	↑ ADAM10	<ul style="list-style-type: none"> • ADAM10/E-cadherin interaction regulates inflammatory diseases 	[68]
Development of the retina	↑ADAM9, 10, 17		[69]
Ectodomain shedding	↑ADAM 9, 10, 17	The activation of ADAM17 by VEGF suggests a role in the neovascularization of the retina	[70] [71] [72]
Epidermal integrity	ADAM10, 17	<ul style="list-style-type: none"> • ADAM10 main sheddase for notch receptors. • ADAM10 and 17 involved in epidermal integrity 	[73] [74] [75]

ADAMTS-1, ADAMTS-3, ADAMTS-5, ADAMTS-7, ADAMTS-9 are expressed in RPE cells			
Inflammatory and Neo-angiogenesis	↑ ADAMTS-1, ADAMTS-6, ADAMTS-9	• Compromising the retina structure	[76] [77] [78]

2.1.2. MMPs in Choroidal Neovascularization: controversial in MMP-2 and -9 role and VEGF mechanism

In the CNV (Choroidal Neovascularization) newly formed vessels originate from the choroid and release fluids in the subretinal space, causing reversible visual loss that in this conditions could evolve in permanent loss of central vision [79]. Unaltered production of MMPs from RPE cells can help to modify their proteolytic activity in the subretinal space and participate, together with the endothelial cells, to regulation of choroidal neovascularization [80,81]. Choroidal neovascular membranes surgically removed from patients suffering from AMD also show a strong expression of some MMPs, indicating that they could cooperate in the progression of choroidal angiogenesis [57].

In the eye, high levels of MMP-9 and MMP-2 have been found in human diabetic vitreous fluids, and in the areas of new vessel formation [82,83], suggesting that these enzymes can contribute to the pathogenesis of retinal neovascular diseases [84].

Currently, knowledge about MMP-2 and MMP-9 involvement in AMD pathogenesis and also in CNV development, is rather controversial. In particular, MMP-2 and -9 are the metalloproteinases most studied for AMD until now for their specificity for gelatin and collagen.

Concerning MMP-2, a decreased level in BrM, i.e. a positive association between MMP-2 and CNV was reported by some studies [55, 85] but, on the contrary, a protective role of MMP-2 has been shown by other studies [86].

The research on the involvement of MMP-9 in AMD is vague as well. In fact during macular degeneration, some studies showed a decreased level of MMP-9 in choroidal neovascular membranes [59] and in serum [86], meanwhile a MMP-9 growth was found in plasma [87], in choroidal neovascular membranes [88], and in the aqueous humor [89,90]. Furthermore, MMP-9 polymorphism was studied in order to better understand the CNV process. Fiotti et al. [62] found a relationship between polymorphisms in MMP-9 and neovascularization in AMD. In addition to this hypothesis, Liukeviciene et al. [63] reported the significant role of MMP-9 Rs3918242 (C→T) single nucleotide polymorphism in the development of AMD, and the effect was more pronounced in people aged less than 65. To arrest the pathological angiogenesis of CNV, typical of wet AMD, is considered as an important strategy to limit the development of AMD. In particular, the main angiogenic factor in the retina able to promote neovascularization is the vascular endothelial growth factor (VEGF) [91,92]. VEGF is a growth factor able to upregulate the expression of MMPs in RPE cells [52, 60].

In cancer vascularization, the role of MMPs in regulating VEGF factor release is well known [93]. However, how MMPs modulate the secretion of VEGF from RPE cells is still under investigation. Holborn et al. [59], evaluated the involvement of MMP-9 and MMP-2 altered gene expression in the secretion of VEGF in RPE cells. In hypoxic conditions, RPE cells upregulate the expression of MMP-2 and MMP-9 and while MMP-9 has a positive feedback on VEGF production and secretion, MMP-2 slightly reduces them. The hypothesis was that MMP-9 could work as a proteolytic promoter on basement membrane and ECM components but also could stimulate the VEGF expression through a direct stimulation on cells thus facilitating neovascularization.

2.1.3 MMP-14 and TIMP-2: involvement in proMMP-2 activation.

MMP-14 is one of the most studied enzymes among the Membrane-type MMPs. It is a proteolytic enzyme that has a broad substrate specificity, especially against ECM components: fibrillar collagens (type I, II, III), laminin-1 and -5, fibronectin, vitronectin, fibrin and aggrecan [94]. MT1-MMP degrades ECM components directly, and indirectly by activating proMMP-2 forming a trimolecular complex with TIMP-2. In fact, MMP-2 activation process requires two MT1-MMP molecules bound to forming a dimeric interaction through their hemopexin and transmembrane domain. Dimeric MT1-MMP is bound to its endogenous inhibitor, TIMP-2 (Tissue Inhibitors Metalloproteinases), resulting in a homodimeric complex (MT1-MMP-TIMP2-MT1-MMP) that allows MT1-MMP to cleave the propeptide of proMMP-2 [95]. For this mechanism in the retina, RPE cells express high levels of proMMP-2 and active MMP-2, but also of TIMP-2 and MMP-14 [52, 64, 65].

As reported by Elliot et al. [66] MMP-14 and TIMP-2 are crucial to reduce the loss of MMP-2 activation induced by oxidative stress in RPE cells. The prevention of the dysregulation of the components of the trimolecular complex is therefore fundamental, suggesting that these enzymes can be possible therapeutic targets.

2.2 ADAMs in RPE cells

ADAMs (a disintegrin and metalloproteinase) belong to the metzincin superfamily and together with snake venom metalloproteinases and ADAMTSs constitute the Adamalysin subfamily of zinc metalloproteinases (**Table 2**).

ADAMs is a family of transmembrane and secreted proteins with functions in cell adhesion and proteolytic processing of ectodomain of cell surface receptors and signal molecules. ADAMs structure is constituted by a Metalloprotease domain and a unique Disintegrin receptor binding integrin domain, which inspires their name. Similarly to MMPs, their domain structure consists of a pro-domain, a metalloprotease domain, a disintegrin domain, a cysteine rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic tail.

ADAMs are involved in multiple biological events including proteolysis, cell adhesion, cell fusion, cell proliferation and cell migration and therefore play a pivotal role in developmental processes. Proteolysis is the best established ADAMs function, and the cleavage of transmembrane proteins, known as “ectodomain shedding” has emerged as a principal biological event. This process mainly affects type I and type II transmembrane proteins including growth factors, cytokines, chemokines and adhesion molecules [96].

ADAMs have numerous functions in the retina involved in the cell-cell and cell-matrix interactions, in the proteolytic shedding of other membranes proteins and in the intracellular signal transduction [97,98].

At the retina level, ADAMs are involved in different processes and the most significant are summarized in **Table 2**:

- **Development of the retina:** ADAMs are involved in the development of the retina, but there is insufficient knowledge of the expression of ADAMs during retinal development. As reported by Yan et al. [99], ADAMs are expressed in different layers of the developing retina, but with a partial overlapping. The authors demonstrated that the mRNAs of ADAM9, ADAM10 and ADAM17 are expressed in the RPE. In particular, ADAMs may play a role in the development of RPE, probably by remodeling the extracellular matrix between RPE and external segments of the photoreceptors. Other studies confirmed that both ADAM10 and ADAM17 are widely expressed also in human retina during the embryonic period, showing an important role in maintaining epidermal integrity and resulting essential for the determination of cell fate, angiogenesis, cell migration and wound healing [69].

- **Ectodomain shedding:** ADAMs are the most important proteinases that mediate the ectodomain shedding through an activity similar to that of α -secretase. It is known that ADAM9, ADAM10 and ADAM17 are the principal proteases involved in the activation of TNF- α and EGF receptor pathways by shedding of pro-TNF- α and EGF receptor ligands [70,71]. ADAM10 and ADAM17 ectodomain shedding activity has a large amount of substrates including cytokine receptors, TNF receptor, EGF receptor, adhesion molecules, transformant-growth factor, and VEGF receptors [96,100]. The involvement of ADAM17 by VEGF-A activation in vascular endothelial cells suggested a possible role in regulating neovascularization of the retina [72]. In fact, after the stimulation by VEGF-A, ADAM17 is able to shed VEGF2 promoting the cascade signaling.

- **Migration and invasion of RPE cells:** As reported by Park et al., ADAMs might be involved in the migration and invasion of RPE cells through the control of E-cadherin expression and secretion of epithelial-mesenchymal transition (EMT) cytokines as TGF- β [68]. ADAM10/E-cadherin interaction represents a mechanism that regulates many inflammatory epidermal diseases, which are characterized by loss of E-cadherin expression and loss of epithelial integrity [101]. Loss of this interaction generally affects the adhesion of epithelial cells and cell migration. There are no specific data on the modulation of EMT processes mediated by ADAM10 in the eye diseases.

- **Inflammatory processes:** recent studies support a role for ADAMs in mediating inflammation by the shedding of pro-inflammatory mediators as TNF- α [67]. The authors explored the expression of ADAM10 and 17 in post-mortem eyes and in experimental model of AMD in which β -amyloid (A β), a drusen component, promotes pro-inflammatory events. It was shown that older postmortem eyes expressed higher levels of ADAM10 and 17, mostly in RPE and drusenoid deposits.

- **Epidermal integrity:** both ADAM10 and ADAM17 have an important role in maintaining epidermal integrity through Notch-mediated regulation signaling [73-74]. Studies using ADAM10 complete knockout mice have suggested a role of this protease in the Notch1 cleavage (an independent splitting within the extracellular-juxtamembrane region of Notch) during mouse embryogenesis but also in adults. The results indicate that ADAM10 functions as the main sheddase for all three Notch receptors. In addition, loss of ADAM9 function causes a disorganization of RPE cells, resulting in the interruption of photoreceptor cell functions [75].

2.3 ADAMTSs in RPE cells

Considerable attention has been reserved to the ADAMTSs (A Disintegrin and Metalloproteinase with Thrombospondin motifs) enzymes that are multi-domain zinc metalloendopeptidases with important roles in tissue morphogenesis and patho-physiological remodeling, in inflammation and in vascular biology. Structurally the ADAMTSs are similarly organized as the other metalloproteinases, MMPs and ADAMs. Basically, ADAMTSs present two main structures: a proteinase domain and an ancillary domain. More specific is the ancillary domain which is composed by one or more thrombospondin type 1 sequence repeats and it gives variability between ADAMTS members [102].

The family of the ADAMTS proteins, as the others zinc metalloproteinases, is involved in different processes at the retina level. First of all, the discovery of ADAMTS expression in RPE cells extends the number of enzymes potentially involved in the turnover of the retinal extracellular matrix both in normal and pathological conditions. From the literature, the transcription of several ADAMTS genes into the ARPE19 ocular cell line was first described by Bevitt et al. [78]. In particular, the authors showed that the mRNAs of ADAMTS-1, ADAMTS-3, ADAMTS-5, ADAMTS-7 and ADAMTS-9 are expressed in RPE cells, while the mRNA for ADAMTS-2, ADAMTS-4, ADAMTS-8 and ADAMTS-6 were not detected.

Moreover, the secretion of proteoglycans from RPE cells in the matrix is fundamental for the turnover of some proteoglycan molecules in IPM and Bruch Membrane and, as evidenced by Hollyfield et al. [103], requires the action of particular ADAMTSs.

ADAMTS-2 and ADAMTS-3 are procollagen N-pro-peptidases (PCNP1 and PCNP2, respectively) able to process the type I, II and III of collagens in Bruch's membrane [104,105]. For other ADAMTSs there are no data on their activity at the retina level; it is therefore difficult to state what roles these enzymes can have at the level of the retinal extracellular matrix. They have similar structural domains to the other members of the ADAMTS family and therefore could show similar targets for ECM proteins.

2.3.1 ADAMTSs involved in inflammatory processes and in retinal neovascularization

In the human retina hypoxia and migration of RPE cells are characteristic of senile macular degeneration (ARMD) and other retinal disorders such as proliferative vitreoretinopathy (PVR) and diabetic vitreoretinopathy (DVR). These processes are known to be mediated by upregulation of some MMPs, but Bevit et al. [78], suggested the possibility that ADAMTS proteins may also be involved. The authors observed a potent upregulation of some ADAMTSs by human RPE cells, in response to tumor necrosis factor α (TNF- α). TNF α is known to play a role in the development of neovascularization in the murine retina in response to hypoxia [76]. In particular, the expression of various ADAMTSs were observed after stimulation with TNF α : ADAMTS-1, -6 and -9 transcripts, expressed in ARPE-19 cells, showed a potent upregulation [77]. ADAMTSs are involved principally in inflammatory conditions of the retina. In fact, especially in ARMD they are able to compromise the structure of the retinal matrix. In conclusion, further studies are needed to deeply dissect the role of ADAMTSs in pathological neo-angiogenesis.

3. Modulation of MMPs and ADAMs activity in AMD.

For the treatment of AMD, the modulation of metalloproteinase activity could be a therapeutic tool to exploit considering metalloproteinase role in the progression of the pathology. Herein, we analyze the molecules and the technologies recently described as modulators of MMP activity that could have an important role as therapeutic agents in this pathology. Further studies are needed to clarify the role of these molecules with a monotherapy or in combination with other drugs or technologies (**Table 3**).

Table 3. Modulation of MMPs and ADAMs activity in AMD

(↑ = upregulation; - = no regulation; ↓ = downregulation; ↓↓ = relevant downregulation)

Molecules able to modulate MMPs activity			
Name	Effect	Notes	References
Triamcinolone acetoneide	↓MMP-2, -9 expression at BM level ↓MMP-9 secretion at BM level ↓VEGF expression and secretion at BM level ↓ MMP-2 activation	<ul style="list-style-type: none"> • Downregulation of feedback between MMP-9 and VEGF so the inhibition of MMP-9 capable methods in the efficiency of anti VEGF therapies 	[106] [107] [108]
Doxycycline	↓MMP-2, -9 activity at the BM level	<ul style="list-style-type: none"> • Simultaneous presence of a MMP-dependent mechanism with a MMP-independent mechanism concerning the modulation of the PI3K/Akt-eNOS pathway. 	[109]
Vandetanib + ADAM10 (G12 54023X) and ADAM17 (Marimastat) inhibitors	↓↓ADAM10 ↓↓ADAM17	<ul style="list-style-type: none"> • Combination of Vandetanib and ADAM10 and ADAM17 inhibitors may be a therapeutic measure for retina pathologies. 	[110]
MMP and ADAM Inhibitors tested in AMD			
Prinomastat	↓MMP-2, -3, -9, MT-MMP-1	<ul style="list-style-type: none"> • Positive effect on Oxygen-induced CNV 	[111] [112]

BPHA	↓MMP-2, -9, -14	<ul style="list-style-type: none"> • ↓ MMP degradation of gelatin and thickness of CNV lesion 	[113]
Batimastat	↓MMP-1, -2, -3, -7, -9	<ul style="list-style-type: none"> • Suppress neovascularization 	[114]

3.1 MMP and ADAM Inhibitors tested in AMD

In recent years, ADAMs and MMPs have been studied as potential therapeutic targets. Several synthetic inhibitors of these metalloproteinases have been developed and some of them have been proposed as investigative or therapeutic tools for retinal diseases. Unfortunately, due to the high degree of homology between the members of the MMPs and ADAMs family as well as the complexity of their physiological functions, to date the efforts spent in the development of synthetic inhibitors have not led to the desired results.

Figure 4 Chemical structure of the principal MMP inhibitors tested in AMD

Prinomastat: AG3340, also called Prinomastat (Figure 4), is the first synthetic matrix metalloproteinase inhibitor with selective inhibitory activity on MMP-2, MMP-9, MMP-3, and MT-MMP1. It was developed by Agouron Pharmaceuticals Inc [110] until Phase II/III study, demonstrating a good pharmacokinetic profile in reducing neovascularization in new born mice and in a hypoxia-induced model of retinopathy. In 2004 the positive effect of AG3340 in the early stages of laser induced rat CNV model was reported. Hypothetically, it could be used in combination with photodynamic therapies to inhibit recurrence from temporarily closed new vessels [112]. Further

investigations were performed by Garcia et al. [115], demonstrating the positive effect of Prinomastat on oxygen-induced CNV using systemic administration.

BPHA: *N*-Biphenyl sulfonyl-phenylalanine hydroxamic acid (BPHA, Figure 4) is a selective MMP inhibitors for MMP-2, MMP-9 and MMP-14, showing antiangiogenic properties. BPHA effect on CNV was studied by Kohri et al. [113] with good results on reducing the development of the pathology. In particular on laser-induced CNV, BPHA reduced the MMP degradation of gelatin in a dose-dependent fashion. Moreover, BPHA caused a reduction in the thickness of CNV lesion in patients.

Batimastat (BB-94): Batimastat (Figure 4) is a potent broad-spectrum MMPs inhibitor for MMP-1, MMP-2, MMP-9, MMP-7 and MMP-3 and also ADAM17. In a murine model of retinal neovascularization, BB-94 has been shown to suppress neovascularization due to a disruption of potential matrix interactions necessary for the maintenance of survival and motility of endothelial cells [114]. BB-94 can be used only at low doses (1 mg / kg), while at higher doses it has been described as toxic in *in vivo* models.

Triamcinolone acetonide

Doxycycline

GI254023X

Marimastat

Verteporfin

Vandetanib

Figure 5. Chemical structures of Triamcinolone acetate, Doxycycline, GI254023X, Marimastat, Verteporfin and Vandetanib.

Triamcinolone acetate: Triamcinolone acetate (TA, Figure 5) is a corticosteroid and is one of the first drugs evaluated for the treatment of choroidal neovascularization (CNV) secondary to AMD [108].

Triamcinolone is able to reduce the expression of MMP-2 and MMP-9 in choroidal endothelial cells and prevents endothelial cell migration by inhibiting MMP-2 activation [106]. Similarly Webb et al. [107], showed that TA decreases the gene expression of MMP-2 and MMP-9 and inhibits the secretion of MMP-9 by RPE cells induced by chemical hypoxia or VEGF (see *CNV paragraphs*). Basically, TA inhibits the positive feedback regulation between MMP-9 and VEGF under hypoxic conditions through inhibition of the gene expression of MMP-9.

Doxycycline: Doxycycline (Figure 5) is a semisynthetic tetracycline antibiotic with strong antimicrobial properties, used principally for acne treatment, infective diarrhea, anthrax, Lyme disease [116]. For ages, it has been used as a safe drug in clinical setting as antibiotics but it also possesses versatile non-antibiotic properties deeply explored in ocular angiogenesis. In fact, many studies reported the effective inhibitory effect of doxycycline on ocular angiogenesis with oral or topical administration [109, 117, 118]. Moreover, the co-administration of doxycycline and bevacizumab improves the inhibitory effects of the monotherapy on CNV in animal model [119]. So far, the Doxycycline mechanism is not completely understood but some evidences suggested that doxycycline antiangiogenic effect may be a consequence of MMPs inhibition.

In 2013 Su et al. [120] reported a potential Doxycycline mechanism demonstrating the simultaneous presence of a MMP-dependent mechanism with an MMP-independent mechanism concerning the modulation the PI3K/Akt-eNOS path. MMP-2 and MMP-9 are involved in Doxycycline-mediated vascular epithelial cell growth on CNV *in vivo*.

Mass spectrometry studies indicated Doxycycline as a non-competitive MMP inhibitor, interacting with the zinc or calcium atoms within the structural center of these proteins necessary for stability [121]. Other studies showed that Doxycycline effectively reduces the progression of CNV, inhibiting the activity of MMP-2 and MMP-9 gelatinases [109]. These results are in agreement with previous studies indicating the contribution of MMP-2 and MMP-9 in CNV progression. These results suggest that Doxycycline, when orally administered, can reach the choroid and inhibit proteolytic enzymes that remodel the membrane and may therefore reduce neovascularization.

Vandetanib and ADAM inhibitors co-administration: Vandetanib (Figure 5), an oral bioavailable kinase inhibitor, is considered a potential post-operative adjuvant chemotherapy drug to inhibit proliferation, progression, migration and the survival of tumor cells, as it plays an important role in regulating the VEGFR and EGFR signaling pathways [122].

A recent research studied the effect of Vandetanib on the expression of ADAMs, epithelium marker and mesenchymal markers, to evaluate their application in the clinical setting, as new targets or drugs to prevent

progression of AMD [110]. The researchers used EBV-infected RPE cells (ARPE19 / EBV), as an *in vitro* model of CNV or PVR, which expressed mesenchymal phenotypes and which secreted VEGF.

Regarding ADAM10 and ADAM17, the treatment of ARPE19/EBV cells with Vandetanib led to the downregulation of their expression in a dose-dependent manner. Moreover, the activity of these ADAMs has been effectively blocked after the co-treatment with ADAM10 (GI254023X) and ADAM17 (Marimastat) inhibitors and low-dose of Vandetanib. The combination of Vandetanib with an ADAM inhibitor influenced the signaling pathway induced by VEGF. Moreover, the combination of low-dose Vandetanib with an ADAM10 or ADAM17 inhibitor attenuated the migratory capacity of EBV-infected ARPE19 cells in an *in vitro* model of CNV compared to treatment with a single drug (Vandetanib) previously tested. The results of the present study suggested that the inhibition of VEGF-mediated signal transduction cascade (at the basis of pathological migration of RPE cells) through co-treatment of Vandetanib and an ADAM inhibitor, may be a suitable therapeutic measure to treat neovascular AMD or other retinal pathological conditions.

Photodynamic therapy and MMPs: The photodynamic therapy (PDT) of Verteporfin (Figure 5) (Visudyne, Ciba Vision Corp., Duluth, GA, USA) was found useful in clinical studies in retinal diseases [123,124]. However, the use of PDT showed a very high recurrence percentage that exceeds the benefits of its use. Tatar et al. [125], verified that PDT induced firstly a temporary decrease in MMP-9 expression level in the retina but in a second time, MMP-9 expression increased again due to the dual role of MMP in angiogenic process. In fact MMP inhibitors play an inhibitory activity on microvessel formation at the begin of angiogenesis process but in the angiogenesis growth phase they prevent vascular regression [126]. However, a similar dual role of MMPs should be expected in the vascularisation and involution stages of CNV. In fact, MMPs inhibitors can be used as PDT adjuvants: in longer intervals after PDT may additionally inhibit CNV rather than the PDT used as the only therapy for neovascular AMD.

Nanosecond laser: MMPs role in AMD is controversial (see *paragraph Metalloproteinases*) and it is demonstrated that the administration of MMP-2 and MMP-9 in activate form could improve the BrM permeability [66]. Along this line, an alternative technique, the nanosecond laser, was used to induce RPE-mediated release of MMPs [127]. One year of clinical trial demonstrated that a single laser application can improve the functionality of the surface of the macula.

Conclusion

Age-related macular degeneration (AMD) is a multifactorial disorder characterized by an ECM remodeling that is generally pathological. The main enzymatic group involved in the degradation of ECM is the superfamily of Metzincins, that includes Matrix metalloproteinases (MMPs), ADAMs and ADAMTSs. Many recent findings highlight the importance of metzincins in the development of AMD. Alteration of these enzymes plays a very important role in AMD pathogenesis, especially in the early phases of choroidal neovascularization.

We have separately analyzed the different groups of zinc metalloproteinases: MMPs, ADAMs and ADAMTSs. Many metzincins are expressed in the human retina: MMP-2, MMP-9, MMP-14, ADAM9, ADAM10, ADAM17, ADAMTS-1, ADAMTS-3, ADAMTS-5, ADAMTS-7 and ADAMTS-9 and may provide clues to the role of the matrix-degrading proteases in the pathogenesis of the complex phenotype of AMD.

In particular, the localization of MMPs in the areas of new vessel formation and in the enveloping Bruch's-like membrane suggests that MMPs may be cooperatively involved in the progressive growth of choroidal neovascular membranes in AMD. However, knowledge on MMPs action in AMD pathogenesis is still controversial, because different studies demonstrates the protective effect of these enzymes.

Over the last decade, numerous therapeutic options have become available for neovascular AMD, most notably anti-VEGF medications. In order to be effective, anti-VEGF drugs must be used relatively early in the disease onset, before scar formation has occurred. These drugs have the potential to increase the risk of thromboembolic events in an already susceptible population.

The use of molecules or technologies able to modulate these enzymes with a potential therapeutic benefit in the treatment of many proliferative retinopathy conditions was analyzed, considering metzincins deeply involved in the process of neovascularization. So far, different promising molecules were proposed as MMP, ADAM and ADAMTS modulator useful in AMD treatment, or even in association with other promising treatment.

In conclusion, the complexity of the research field examined points out that AMD therapy has changed dramatically over the last decade. While our knowledge of the molecular mechanisms and the role of metalloproteinases involved in AMD is increasing, new treatments are emerging and innovative therapeutic approaches to AMD can be provided.

LIST OF ABBREVIATIONS

ADAM	= A disintegrin and metalloproteinase
ADAMTS	= A disintegrin and metalloproteinase with thrombospondin motifs
AKt	= Protein kinase B
AMD	= Age-related macular degeneration
ARMS2 / HTRA1	= Age-related maculopathy 2/HtrA peptidase serine 1
BPHA	= <i>N</i> -Biphenyl sulfonyl-phenylalanine hydroxamic acid
BrM	= Bruch's Membrane
C2	= Complement component 2
C3	= Complement component 3
CFB	= Complement B

CFH	= Complement gene H
CNV	= Choroidal neovascularization
DVR	= Diabetic vitreoretinopathy
ECM	= Extracellular matrix
EL	= Elastin layer
EMT	= Epithelial-to-mesenchymal transition
E/N	= Entactin/nidogen
FN-439	= Tetrapeptidyl hydroxamic acid
GA	= Geographic atrophy
GPI	= Glycosylphosphatidylinositol
HSPGs	= Heparin sulphate proteoglycans
ICL	= Inner collagen layer
IL-1	= Interleukin-1 family
IPM	= Interphotoreceptor matrix
MAPK	= Mitogen-activated kinase protein
MMPs	= Matrix metalloproteinases
Notch	= Notch-mediated regulation signaling
OCL	= Outer Collagene Layer
PCNP	= Procollagen Npropeptidases
PDT	= Photodynamic therapy
PGs	= Proteoglycans
PI3K	= Phosphatidylinositide 3-kinases
PVR	= Proliferative Vitreoretinopathy
RPE	= Retinal pigment epithelium
RPE-BL	= RPE basal lamina
RPECs	= Retinal Pigment Epithelium cells
SRF	= Sorafenib
TA	= Triamcinolone acetonide
TGF-2	= Transforming growth factor alfa

TIMP	= Tissue inhibitor of metalloproteinase
TNF- α	= Tumor necrosis factor- α
VEGF	= Vascular endothelial growth factor
VEGFR	= Vascular endothelial growth factor receptor

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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