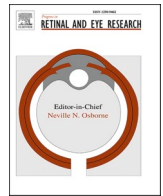




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## 10q26 – The enigma in age-related macular degeneration

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## ABSTRACT

Despite comprehensive research efforts over the last decades, the pathomechanisms of age-related macular degeneration (AMD) remain far from being understood. Large-scale genome wide association studies (GWAS) were able to provide a defined set of genetic aberrations which contribute to disease risk, with the strongest contributors mapping to distinct regions on chromosome 1 and 10. While the chromosome 1 locus comprises factors of the complement system with well-known functions, the role of the 10q26-locus in AMD-pathophysiology remains enigmatic. 10q26 harbors a cluster of three functional genes, namely *PLEKHA1*, *ARMS2* and *HTRA1*, with most of the AMD-associated genetic variants mapping to the latter two genes. High linkage disequilibrium between *ARMS2* and *HTRA1* has kept association studies from reliably defining the risk-causing gene for long and only very recently the genetic risk region has been narrowed to *ARMS2*, suggesting that this is the true AMD gene at this locus. However, genetic associations alone do not suffice to prove causality and one or more of the 14 SNPs on this haplotype may be involved in long-range control of gene expression, leaving *HTRA1* and *PLEKHA1* still suspects in the pathogenic pathway. Both, *ARMS2* and *HTRA1* have been linked to extracellular matrix homeostasis, yet their exact molecular function as well as their role in AMD pathogenesis remains to be uncovered. The transcriptional regulation of the 10q26 locus adds an additional level of complexity, given, that gene-regulatory as well as epigenetic alterations may influence expression levels from 10q26 in diseased individuals. Here, we provide a comprehensive overview on the 10q26 locus and its three gene products on various levels of biological complexity and discuss current and future research strategies to shed light on one of the remaining enigmatic spots in the AMD landscape.

## 1. Introduction

### 1.1. Age-related macular degeneration: an increasing concern in the aging population

Age-related macular degeneration (AMD) is a complex,

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multifactorial disease that threatens the sight of millions. It is listed as the fourth most common cause of blindness worldwide (Jonas et al.,

that would allow us to develop and employ highly effective treatment strategies to ease the disease burden on both the individual and the

### Abbreviations

10q26	chromosomal region 10q26	LYTAC	Lysosome-targeting chimaeras
AD	Alzheimer's disease	MAF	minor allele frequency
AMD	age-related macular degeneration	MMP	matrix metalloproteinases
APP	amyloid precursor protein	MNV	macular neovascularization
ARMS2	Age-Related Maculopathy Susceptibility 2	MZ	myoid zone
A $\beta$	amyloid beta	nAMD	neovascular AMD (=wet AMD)
BrM	Bruch's membrane	ndtGA	non-diffuse-trickling GA
CARASIL	cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy	neo-CC	neo-choriocapillaris
CC	choriocapillaris	OA	osteoarthritis
CLU	clusterin	OCL	outer collagenous layer
COMP	cartilage oligomeric matrix protein	ONL	outer nuclear layer
CSVD	cerebral small vessel disease	OPL	outer plexiform layer
dAMD	dry AMD	OR	odds ratio
DKK3	Dickkopf-related protein 3	OS	photoreceptor outer segments
dtGA	diffuse-trickling GA	p.A69S	missense mutation in ARMS2 that leads to an exchange of the alanine at position 69 with a serine
ECM	extracellular matrix	PDZ	postsynaptic density protein (PSD-95)/Drosophila disc large tumour suppressor (dlg)/tight junction protein (ZO1) domain
EL	elastin layer	PH	pleckstrin homology domain
ELM	external limiting membrane	PI3K	phosphatidylinositol 3-kinase
ELN	Elastin	pLDDT	per-residue confidence score
EMILIN	elastin microfibril interface-located protein	PLEKHA1	Pleckstrin Homology Domain Containing A1
EOAD	early-onset Alzheimer's disease	PR	photoreceptor
EZ	ellipsoid zone	PROTAC	Proteolysis Targeting Chimera
FBLN3	Fibulin-3	PSG	pregnancy-specific $\beta$ -1 glycoprotein
FBLN6	Fibulin-6	PTPL1	protein-tyrosine-phosphatase-like protein-1
FN1	fibronectin 1	R38X	nonsense mutation in ARMS2 that leads to a premature stop-codon at position 38
GA	geographic atrophy	RA	rheumatoid arthritis
GCL	ganglion cell layer	RBP3	retinol-binding protein 3
GWAS	genome-wide association study	RCA	regulators of complement activation
HL	Haller's layer	RNFL	retinal nerve fiber layer
HTRA1	High-Temperature Requirement A Serine Peptidase 1	RPE	retinal pigment epithelium
IAMDGC	International AMD Genetics Consortium	RPE/BrM	retinal pigment epithelium/Bruch's membrane complex
ICL	inner collagenous layer	SL	Sattlers's layer
INL	inner nuclear layer	SNP	single nucleotide polymorphism
IPL	inner plexiform layer	SRF	subretinal fluid
iPSC	induced pluripotent stem-cells	TAILS	terminal amine isotopic labeling of substrates
IRF	intraretinal fluid	TIMP	tissue inhibitors of metalloproteinases
IZ	interdigitation zone	WBSR	Williams Beuren syndrome chromosome region 27
LD	linkage disequilibrium		
LTBP-1	latent TGF- $\beta$ binding protein 1		

2017) and is the leading cause of blindness among the elderly in industrialized countries (Bourne et al., 2018). Due to its close association with age accompanied with demographic changes and extended life-expectancies, AMD has an ever-increasing prevalence with estimates projecting a rise in global cases from 196 million in 2020 to 288 million in 2040 (Wong et al., 2014). It is important to note that, while not a life-threatening condition, impaired vision is reported to decrease the subjective quality of life more than other chronic conditions like for example type II diabetes or coronary disease (Langelaan et al., 2007), and recent patient surveys reveal a devastating emotional burden and impact on a patient's independence and mental well-being (Caballe-Fontanet et al., 2022). Additionally, the clinical management of AMD causes substantial costs for healthcare systems worldwide (Schmier et al., 2012). Despite tremendous research efforts over recent decades, that were able to partially elucidate the pathophysiology of AMD, we are still lacking a comprehensive understanding of the disease

economic level.

### 1.2. The clinical course of AMD

AMD manifests as a progressive degeneration of structures in and around the macula that ultimately lead to photoreceptor (PR) death and consequent vision impairment. From a clinical point of view, drusen are usually the first observable sign of AMD. Drusen are small, yellowish deposits comprising a wide range of lipids and proteins that form between retinal pigment epithelium (RPE) and Bruch's membrane (BrM) (L. G. Wang et al., 2010). While small drusen (<63  $\mu$ m in diameter; hard drusen, drupelets) without pigmentary abnormalities in the RPE may be considered signs of normal retinal aging, medium-sized drusen (>63  $\mu$ m in diameter; soft drusen) are indicative of early stage AMD. Disease progression may lead to intermediate AMD, characterized by large drusen (>125  $\mu$ m in diameter) and large drusen area, with or without

pigmentary abnormalities in the RPE. Further progression in intermediate AMD can ultimately lead to the development of late-stage AMD. Late stage AMD is commonly divided into neovascular AMD (nAMD; wet AMD) and atrophic AMD (dAMD, dry AMD) (Bird et al., 1995; Ferris et al., 2013). While wet AMD is characterized by the presence of macular neovascularization (MNV) (Spaide et al., 2020), dry AMD lacks MNV and solely presents with drusen and pigmentary abnormalities in early and intermediate stages. Late stage dry AMD presents with patchy, map-like areas of RPE atrophy and is therefore commonly referred to as geographic atrophy (GA) (Fig. 1). It is however important to note that not every early or intermediate stage AMD will progress to GA. The MNV vessels formed in wet AMD have suboptimal barrier functions that often lead to hemorrhages or fluid leakage and formation of subretinal (SRF) or intraretinal fluid (IRF) accumulations. Development of such fluid accumulations frequently cause a rapid and pronounced decline in vision, which can at least be partially counteracted when treated timely and consistently with intravitreal injections of anti-VEGF agents (Fig. 2). In contrast to wet AMD, there is currently no approved treatment for intermediate or dry AMD, and interventions are limited to life-style modulatory measures and oral supplementation of vitamins and antioxidants (Chew et al., 2009). Importantly, there is no sharp pathogenic border between wet and dry AMD as both entities may converge into one another. A detailed review covering the clinical aspects of AMD including risk factors, general pathophysiology and clinical management has recently been published by Fleckenstein et al. (2021).

## 2. 10q26 genetics in AMD

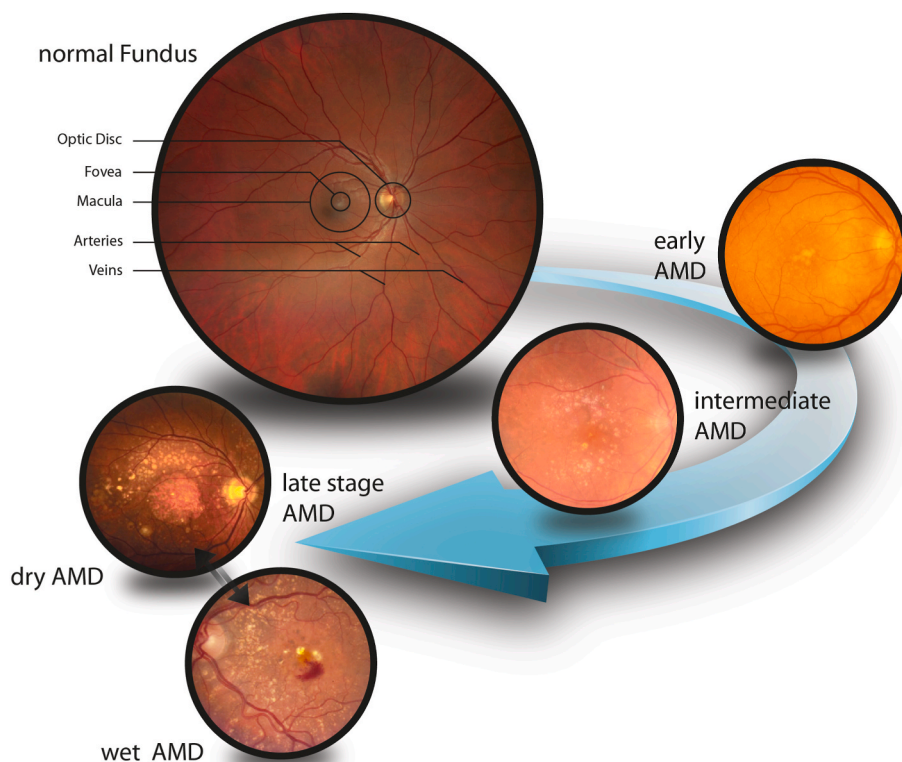
### 2.1. The multifactorial nature of AMD and the prominent role of chromosomal region 10q26

An individual's risk of developing AMD can be defined by a number of different factors. Besides age, genetic as well as environmental and life-style related factors like smoking and low dietary intake of antioxidants have been associated with increased risk for AMD development

and progression (Fleckenstein et al., 2021; Mitchell et al., 2018). Since publication of the first genome-wide association studies (GWAS) of AMD in 2005 (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005) many more have followed and detected a plethora of genetic variants associated with AMD. The largest GWAS performed to date defined a set of 52 common variants mapping to 34 loci (Fritsche et al., 2016). In addition, various studies have identified high impact rare variants in complement genes as well as other genes involved in innate immunity, energy homeostasis and neuronal signaling, making AMD a complex genetic disorder (de Breuk et al., 2021; Geerlings et al., 2017; Ratnapriya et al., 2020; Saksens et al., 2016; Seddon and Ferrara, 2017). Pathway analyses based on the GWAS revealed that the complement system, lipid metabolism and extracellular matrix regulation are among the highest ranked candidate pathways implicated in AMD pathogenesis (Fritsche et al., 2016). Over all, exceedingly high genetic risk for AMD is associated with variants mapping to chromosomal region 10q26 (Fig. 3). Within this region, three genes lie in close proximity, namely *PLEKHA1*, *ARMS2* and *HTRA1* (Fig. 4) (Wang, 2014). Importantly, AMD-associated *ARMS2* and *HTRA1* variants are in almost complete linkage disequilibrium (LD) with each other, making it exceedingly hard to reliably define the causative variant. As a result, there is an ongoing controversy as to which of these variants confer the risk for developing AMD.

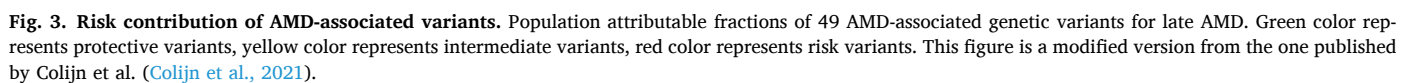
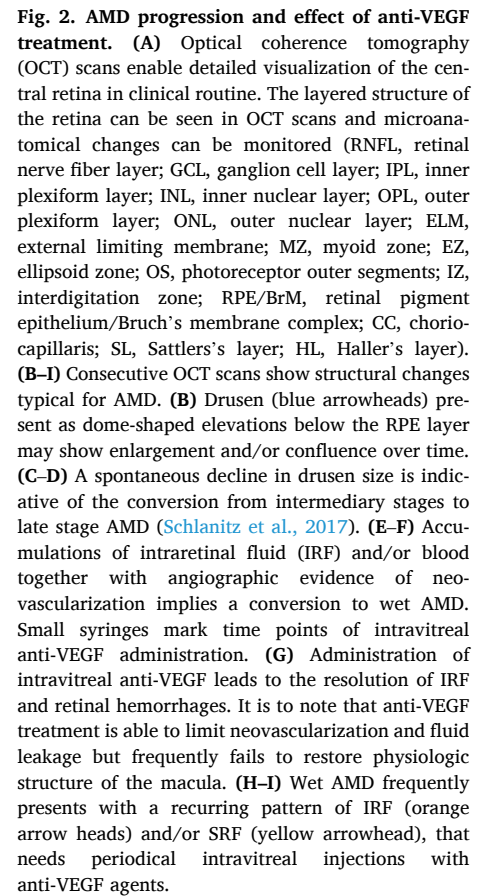
### 2.2. Polymorphisms, associations and controversies

Shortly after publication of the first ever GWAS (Ozaki et al., 2002), a report on the association of chromosomal region 10q26 (from now on referred to as 10q26) and AMD was published (Majewski et al., 2003). This was followed by several studies replicating this result (Kenealy et al., 2004; Seddon et al., 2003; Weeks et al., 2004) and a Meta-analysis (Fisher et al., 2005) confirming the reported association. 10q26 contains three adjacent genes: *PLEKHA1*, *ARMS2* (*LOC387715*, from now on only referred to as *ARMS2*) and *HTRA1* (Fig. 4). In-depth analysis of 93 single nucleotide polymorphisms (SNPs) in this region reported the strongest association with a missense SNP in *ARMS2* (rs10490924) that leads to an

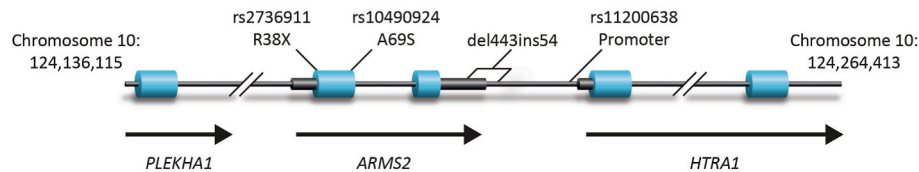


**Fig. 1. Fundus anatomy, the macula and the clinical course of AMD.** The human retina is a neurosensory tissue that enables vision by converting electromagnetic stimuli into electric impulses and transmitting those to specialized areas of the brain. The term macula refers to a specific retinal area located at the center of the eye's posterior pole. The fovea, the most central part of the macula, principally confers central vision and enables high-resolution vision due to densely packed cone photoreceptors. The optic disc is the entry site of the optic nerve into the eye, which transmits signals to the brain and comprises the axons of the retinal ganglion cells. The retinal arteries and veins enter and leave the eye through the optic disc via the optic nerve, and provide blood supply to the innermost layers of the retina, while the outer retinal layers are supported by the vessels of the choroid/choriocapillaris. In AMD, the macula undergoes gradual pathologic changes that lead to degenerative processes that ultimately can cause loss of central vision. Drusen are yellowish, protein- and lipid-rich deposits beneath the RPE and can be seen as the clinical hallmark of AMD. In early AMD only small drusen and RPE pigmentary abnormalities can be seen. These drusen can enlarge over time and the disease can progress to intermediate AMD, which can then develop into late stage AMD. Late stage AMD is commonly divided into two forms: dry AMD and wet AMD. While wet AMD is defined by the presence of MNV, the late stage of dry AMD presents with patchy areas of RPE and outer retinal atrophy that is commonly known as GA.





the *HTRA1* promoter region (rs11200638) to be the causative variant (Dewan et al., 2006; Yang et al., 2006). In this manner, the controversy continued as to which of the three 10q26 genes is actually conferring the risk for AMD. While several follow-up studies reported the association of *PLEKHA1* variants with AMD (Cameron et al., 2008; Leveziel et al.,



**Fig. 4.** The genetic structure of chromosomal region 10q26 and important AMD-associated variants. Blue tubes represent exons, black tubes represent 3'- and 5'-untranslated regions (UTRs). For *PLEKHA1* only the last exon and for *HTRA1* only the first two exons are depicted.

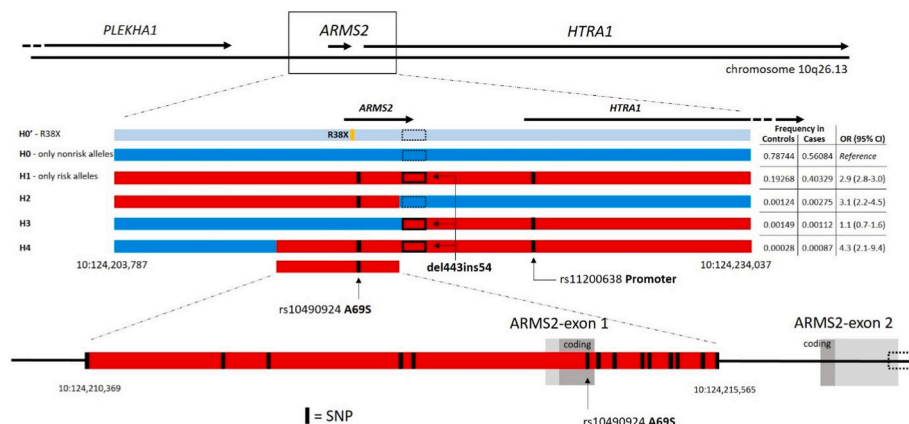
2007), this finding was not consistently reproducible in all tested populations (Conley et al., 2006). In contrast, the p.A69S variant in *ARMS2* (rs10490924) was consistently associated with increased AMD risk in numerous studies and, together with rs11200638 in *HTRA1*, gradually became a variant of prime interest (Francis et al., 2007; Ross et al., 2007; Seddon et al., 2007; Tanimoto et al., 2007). In parallel, numerous studies also confirmed the reported association of the variant in the *HTRA1* promoter region (rs11200638) with AMD (Cameron et al., 2007; Chen et al., 2009; Mori et al., 2007; Weger et al., 2007). Due to a near complete linkage disequilibrium (LD) between *ARMS2* and *HTRA1* (Hughes et al., 2007; Leveziel et al., 2007), the early genetic studies were unable to definitively detect the risk causing SNP and several subsequent studies argued for either *ARMS2* (Kanda et al., 2007) or *HTRA1* as the causal gene (Cameron et al., 2008; Gibbs et al., 2008).

A few years later, the largest GWAS on AMD was published by Fritsche et al., in 2016, that narrowed the genetic risk in 10q26 down to 30,250 base pairs (LD  $R^2 > 0.8$  around the GWAS tag SNP rs3750846 in the *ARMS2* region (OR 2.7) (Fritsche et al., 2016). These 30,250 base pairs harbour 25 risk variants; 14 in *ARMS2* including the p.A69S variant, and 11 in *HTRA1* (Fig. 5, haplotype H1 and Supplementary Table 1; source Haploreg v3 (Ward and Kellis, 2012)). No additional independent signals in 10q26 were found in the GWAS, ruling out an association of the stop-codon variant p.R38X within *ARMS2* with AMD (Fig. 5, haplotype H0'). Subsequently, the International AMD Genetics Consortium (IAMDGC) analyzed recombination events in the *ARMS2* region in their cohort to further narrow down the associated region. They identified several rare haplotypes which made it possible to further narrow down the AMD risk associated region to 5196 base pairs. The most important haplotypes are shown in Fig. 5 (modified from (Grassmann et al., 2017)). A rare recombinant haplotype was identified that had the variants in *HTRA1* and none of the variants in *ARMS2* - except for a deletion insertion in *ARMS2*. This particular haplotype was not associated with AMD (OR 1.1, 95%CI 0.7–1.6, Fig. 5, haplotype H3), indicating that neither variants in the *HTRA1* promoter or gene, nor the deletion-insertion in *ARMS2* determined the risk at locus 10q26.

Conversely, a rare recombinant haplotype that did not carry all inter-genetic risk alleles between *PLEKHA1* and *ARMS2*, was associated with an AMD risk that was similar, or even higher than that of the entire region (OR 4.3 vs OR 3.1, although with overlapping confidence intervals) (Fig. 5, haplotypes H2 and H4), narrowing down the risk region to a haplotype of 14 SNPs with complete LD and very high  $R^2$  located in front of *ARMS2* gene and around exon 1.

As mentioned, most epidemiologic studies investigated the risk *ARMS2/HTRA1* haplotype by analysis of only one variant (rs10490924 (p.A69S) or rs3750846, which is in LD with p.A69S). Given this limitation at the genetic side, they did study epidemiologic associations in great detail. The Rotterdam Study and EYE-RISK consortium found that the risk haplotype was associated with higher risks of large sized drusen  $>125 \mu\text{m}$  (OR 2.1), large drusen area (OR 6.6), reticular pseudodrusen (OR 7.2), wet AMD (OR 11.2), GA (OR 8.6) and mixed wet AMD and GA (ORs 12.2) (Thee et al., 2022). It did not increase the risk of hard or small drusen, suggesting that the risk haplotype did not initiate AMD but rather enhance it after disease onset. In comparison to chromosome 1, 10q26 had higher risks of wet AMD (OR, 4.1; 95% CI, 3.2–5.4), but similar risks of large drusen and GA. Approximately 65% of patients with late AMD were carriers of the *ARMS2* risk haplotype (Thee et al., 2022), 21% of patients even carried two copies thereof. A prospective analysis revealed that by the age of 85 years, 26.8% of homozygous carriers had developed late AMD, and 15.9% became bilaterally severely visually impaired. This makes the risk haplotype at 10q26 the one with the highest disease burden.

Gene-environmental interactions studies focusing on *ARMS/HTRA1* as a single genetic risk factor suggested a strong interaction with smoking (Lee et al., 2010; Schmidt et al., 2006), although this interaction was not supported by all (Francis et al., 2007). Interestingly, the risk haplotype particularly appeared to induce a 5 year earlier onset of late AMD (Lechanteur et al., 2015). Other remarkable associations that involved the risk haplotype were an increased risk of polypoidal choroidal vasculopathy (Woo et al., 2015) and a more therapy-resistant course with poor visual outcome for homozygotes suffering from central



**Fig. 5.** Haplotype analyses in the *ARMS2* locus associated with AMD. The upper section shows chromosomal region 10q26 and the localization of *PLEKHA1*, *ARMS2*, and *HTRA1*. The middle section shows five recombinant haplotypes (H0 to H4) defined by 25 variants in LD ( $R^2 > 0.8$ , including the tag SNP of the IAMDGC GWAS (Fritsche et al., 2016). The frequency of the haplotypes in AMD cases and controls is shown on the right. The H0' haplotype is defined by the p.R38X variant (minor allele frequency (MAF) ranging from 0.21 in Japanese to 0.05 in African populations). The lower section provides detailed information on the narrowed down region in and around *ARMS2* which maintained a high risk of AMD. The region includes 14 SNPs. Additional information on the variants can be found in Supplementary Table 1. Grey boxes = exons; dark grey boxes = coding regions within exons; black rectangles = SNPs. Genome positions indicated in the figure are from the genome build GRCh37/hg19. This figure

is a modified version from that published by Grassmann et al. (Grassmann et al., 2017).

serous choroidopathy (Hayashida et al., 2020). Apart from the risk, the impact of 10q26 is also determined by the relatively common frequency, with minor allele frequencies (MAFs) between 19 and 21% in European ethnicities, and 24% in Africans (1000Genome; dbSNP database). A GWAS study performed on Asians (Cheung et al., 2017), including Koreans (Woo et al., 2015) and Japanese (Akagi-Kurashige et al., 2015), confirmed the ARMS2 variant rs10490924 (p.A69S;  $P = 1.20 \times 10^{-103}$ ) as one of the strongest risk alleles (OR 2.4) in this ethnicity (Cheng et al., 2015). Strikingly, the frequency of this variant is the highest in Asian populations, ranging from 34% for South Asians to 40% in East Asians (1000G; dbSNP database). The high population frequency raises the question whether there may be an evolutionary advantage linked to the risk haplotype, but this remains as of yet unresolved.

### 2.3. GWAS in complex diseases: chances and limitations

GWAS are of great value in defining genetic risks within the genetic make-up of an individual or a group. They may further lead to the identification of pathomechanisms and define pathways and factors that could be targeted for rational therapy development. It is, however, important to keep in mind that stronger associations in genetic studies do not readily translate to a higher contribution to disease causality and that consequently all variants in high LD have to be considered. Accordingly, identification of genetic variants alone does not suffice to generate mechanistic insights that allow for translational developments and experimental laboratory data are obligatorily needed to complement GWAS data. Strikingly, Strunz et al. showed that since the publication of the largest GWAS on AMD in 2016 (Fritsche et al., 2016), only around 19% of the published studies included new experimental results (Strunz et al., 2020). This lack of generated experimental insight inevitably hampers the development of efficient new treatments. Over the last decade, clinical trials on AMD almost exclusively focused on the complement system. Several of these trials failed and so far not a single candidate gained EMA or FDA approval (Armento et al., 2021). Although, APL-2, a complement inhibitor targeting C3, recently showed efficacy during phase three trials with an approximate 25% reduction in the speed of GA growth. This drug is expected to receive FDA approval in the final quarter of 2022 ("Apellis Announces FDA Acceptance and Priority Review of the New Drug Application for Pegcetacoplan for the Treatment of Geographic Atrophy (GA)," 2022), which will make it the first approved therapeutic intervention for GA, albeit with a suboptimal efficacy. This illustrates the need for a broader understanding of disease mechanisms and other potential targets. Interestingly, several studies reported increased levels of HTRA1 protein in AMD (Chan et al., 2007; Iejima et al., 2015), and an anti-HTRA1 antibodies has entered clinical trials (Khanani et al., 2021). By contrast, another study failed to reproduce these results (Kanda et al., 2007), and a recent study even reported downregulation of HTRA1 in AMD patient's RPE (Williams et al., 2021). If the latter holds true, treatment with anti-HTRA1 agents may potentially have negative effects on the clinical course of AMD.

### 2.4. The best of both worlds: linking genetic and phenotypical data

Since the discovery of AMD-associated genetic risk variants, several studies trying to provide a link between genetics and the clinical course of AMD have been published (Ratnapriya and Chew, 2013). Besides some intriguing insights, the results were often inconsistent or irreproducible and the similarity between the clinical course of disease associated with complement mediated risks and 10q26 associated risks gives rise to speculations on a possible common pathomechanism. At this point, it is reasonable to argue that the genotype alone is insufficient to explain the varying time of disease onset, the different rates of progression and the multifaceted types of clinical manifestation that we observe among patients. However, we strongly believe that AMD should be considered a disease spectrum, in which several subtypes exist, that in turn may benefit from different drugs or interventions. So far, we are not

able to properly define those subgroups, which in consequence hampers detailed molecular analyses and the development of specific treatment strategies. With the emergence of artificial-intelligence based methods and tools, we are now able to perform high-throughput analyses and detect patterns that no human observer could reliably make. This was impressively illustrated when Poplin et al. reported the ability of a deep learning model to reliably predict subject age, gender and cardiovascular risk-factors solely based on fundus photographs (Poplin et al., 2018). When provided with large, multimodal, high quality image data-sets in conjunction with genetic data, these analyses have the potential to reliably define disease-subtypes. To this end, generation of large and well-curated datasets is of utmost importance (Wagner et al., 2022). Once a sub-classification is achieved, patient-derived induced pluripotent stem-cells (iPSCs)-based experimental models can be used to obtain mechanistic insights that in turn may fuel translational approaches (Achberger et al., 2019a).

In line with this, detailed phenotypical analyses identified a distinct subtype of geographic atrophy (GA) that presented with a diffuse-trickling appearance in fundus autofluorescence photographs. In comparison to non-diffuse-trickling GA (ndtGA), diffuse-trickling GA (dtGA) was associated with a more rapid progression and therefore with a worse visual prognosis (Fleckenstein et al., 2011; Holz et al., 2007). Intriguingly, a follow-up study was able to identify distinct genetic associations with either subtype and showed a significantly higher frequency of the ARMS2 variant rs10490924 among dtGA patients (Fleckenstein et al., 2016).

Additionally, Corvi et al. recently reported presence of a functional, so-called neo-choriocapillaris (neo-CC) in a subset of AMD patients with type 1 MNV that may protect from developing atrophy after anti-VEGF therapy (Corvi et al., 2022). However, no information on the genotype was provided. The formation of a functional vascular tissue, like the neo-CC, is among other factors obligatorily reliant on a tightly regulated and intact extracellular matrix (ECM). Although being highly speculative at this point, the fact that HTRA1 and ARMS2 may play an important role in ECM homeostasis should prompt us to perform further analyses on the potential link between an observable phenotype and the respective genotype. If such an association can be made, we are one step closer in finding a potentially druggable molecular target for a defined subgroup of AMD patients.

## 3. 10q26: the current stage of knowledge

### 3.1. ARMS2: more than just a pseudogene

As mentioned before, several studies have argued against a relevant role of ARMS2 in AMD pathogenesis (Dewan et al., 2006; Yang et al., 2006). However, others attributed the 10q26 mediated risk predominantly, or even exclusively, to ARMS2 (Fritsche et al., 2008; Kanda et al., 2007). One frequently raised argument against a relevant role of ARMS2 was the doubt about its actual expression in the retina and adjacent ocular tissues. The fact that ARMS2 is highly expressed in the placenta (Yang et al., 2010) shows that the ARMS2 gene is capable of producing functional mRNAs that are potentially translated to create protein. Likewise, several studies have shown the presence of ARMS2 mRNA in human retina via reverse-transcriptase (RT)-PCR (Fritsche et al., 2008; Kanda et al., 2007; Rivera et al., 2005; G. L. Wang et al., 2010) or RT-qPCR (Kanda et al., 2010). Another study found ARMS2 to be expressed in the neuroretina and the RPE of rhesus monkeys as well as in human RPE, but yielded inconsistent findings in human neuroretina (Francis et al., 2008). Additionally, higher levels of ARMS2 expression were reported in central nervous tissues such as the hippocampus, the cortex, the cerebellum (Gatta et al., 2008) as well as in the testes (Liao et al., 2017). Although some of these studies only reported low expression, transcriptomic studies have shown that there is no universal cutoff for mRNA abundance to have functional relevance (Lee and Young, 2013). Nevertheless, evidence for the existence of ARMS2 on the protein



level is scarce as only a few studies, including our own, reported the presence of the ARMS2 protein in human retinal samples (Fritsche et al., 2008; Kortvely et al., 2010). Additionally, a study by Micklisch et al. (2017) showed ARMS2 protein expression in human genotyped retinal sections and in purified monocytes derived from AMD patients not carrying ARMS2 risk variants. It is important to note that these studies, as well as our own, did not use commercial ARMS2 antibodies and that the availability of high-quality antibodies with proven specificity may be a limiting factor for the conduction of studies alike.

Taken together, the available data on ARMS2 expression is limited but does not justify ruling out a potential role of the ARMS2 protein in the pathogenesis of AMD. Therefore, further research on ARMS2 and HTRA1 in the context of AMD should be encouraged as it may yield critical information for the discovery of novel treatment strategies.

### 3.2. Transcriptional interplay at 10q26

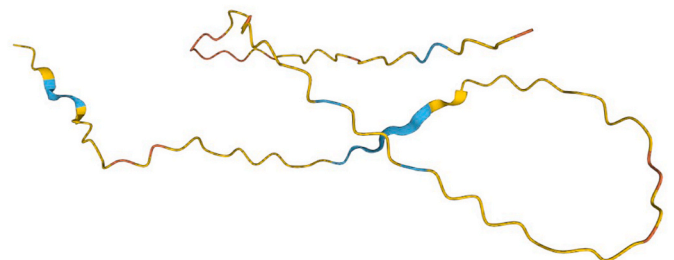
To date, the role of ARMS2 gene variants remains largely enigmatic. While one report claimed that AMD-associated ARMS2 variants would contribute to AMD development and progression by increasing HTRA1 expression (Yang et al., 2010), another study found that HTRA1 mRNA is reduced in RPE but not in neural retina or choroid tissues derived from human donors with homozygous risk at the 10q26 locus (Williams et al., 2021). Other studies found these observations to be either inconsistent or not reproducible (Friedrich et al., 2011; Kanda et al., 2010; Wang et al., 2013). In this light, a potential role of ARMS2 variants beyond modulation of HTRA1 expression is at least debatable given that the ARMS2 gene contains all relevant structural elements to produce functional mRNA as well as protein: It holds a well-defined transcriptional start site (Fritsche et al., 2008), two exons interspaced with an intron holding a GT-AG splicing site, and a canonical polyadenylation signal (Kortvely and Ueffing, 2016). Intriguingly, a functional alternative splice acceptor site was found within exon 2, that gives rise to a second mRNA isoform in the healthy human retina (Wang et al., 2012). This second isoform is reported to be highly expressed in the RPE/choroid, theoretically matching our observed protein localization within the choriocapillaris intercapillary septa (Kortvely et al., 2010). In a follow-up study, we analyzed the exon-intron structure of transcripts originating from the 10q26-locus and found chimeric transcripts containing elements from PLEKHA1 and ARMS2. Importantly, no chimeric transcripts including elements from ARMS2 and HTRA1 were detected (Kortvely and Ueffing, 2016). This may seem surprising since the intergenic distance between ARMS2 and PLEKHA1 is significantly larger than the distance between ARMS2 and HTRA1. However, transcriptomic analyses found chimeric transcripts originating not only from neighboring genes but also from distant DNA regions and even from genes located on different chromosomes (Gingeras, 2009). In general, the existence of chimeric transcripts is a common phenomenon (Sun and Li, 2022). The separation of ARMS2 and HTRA1 transcripts may therefore imply a spatial and/or functional separation of both mRNAs or proteins. Despite being hypothetical at this point, this observation is well in line with the fact that both ARMS2 and HTRA1 proteins use different routes of secretion (Kortvely et al., 2016), which will be discussed in the following chapters.

### 3.3. ARMS2 on the protein level

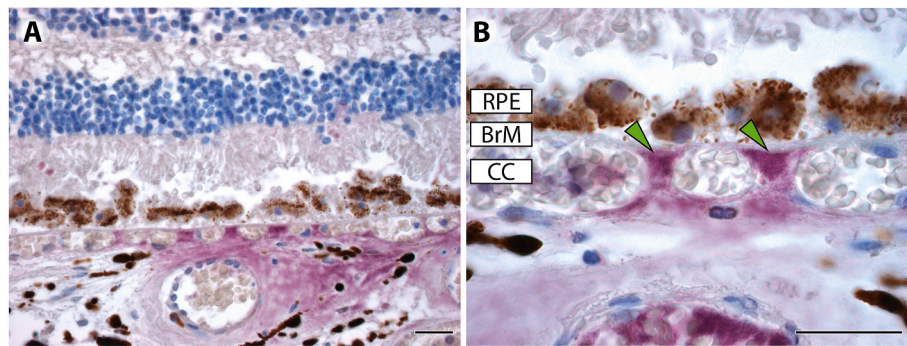
The ARMS2 gene codes for a small (11-kDa) protein of unknown function with a total length of 107 amino acids. The gene itself is composed of two exons and is flanked by PLEKHA1 and HTRA1 (Fig. 4). Intriguingly, the ARMS2 protein does not share any similarity with known protein motifs. Homology-modelling-based approaches failed to provide reliable results (unpublished data), whereas an *in silico* study provided a structure model and tried to assess the functional consequences of the p.A69S substitution (Jahanfar and Hamishehkar, 2018). Given that ARMS2 lacks any significant homology to known proteins,

domains or motifs, and was proposed to be a product of de novo gene birth, any such model remains rather speculative. Up to now, no experimentally derived three-dimensional protein-structure of ARMS2 has been published. Our own previous attempts to use X-ray crystallography have failed due to insufficient crystal formation (unpublished data). Along with the fact that AlphaFold does not predict any extensive secondary structures (Fig. 6) (“Age-related maculopathy susceptibility protein 2 - AlphaFold structure prediction,” 2022), this may be explained by a highly flexible or even intrinsically disordered structure of ARMS2. Elucidating the structural properties in the future may help to characterize potential modes of interaction and inform future experiments. In order to provide reliable structural data, we argue to use methods better capable of capturing dynamic protein structures, such as NMR-spectroscopy. If it holds true that ARMS2 belongs to the group of intrinsically disordered proteins (IDPs) or at least harbors disordered regions, its role may be even more complex than anticipated. IDPs depict a relatively new group of proteins that were found to show complex molecular behavior and to be involved in complex interactions and diverse biological and biophysical processes (Uversky, 2019).

An initial study reported that the ARMS2 protein localizes to the mitochondrial outer membrane (Kanda et al., 2007). However, the study used overexpression of a tagged protein and did not provide information on the endogenous protein. Fritsche et al. then used a commercially generated polyclonal ARMS2 antibody on human retinal sections and found ARMS2 localization to the mitochondria-rich ellipsoid region of the photoreceptor inner segments (Fritsche et al., 2008). However, we and others were unable to replicate these findings (Kortvely et al., 2010; Wang et al., 2009). In our experiments, peptide epitope based ARMS2 antibodies were generated to evaluate the localization of endogenous ARMS2 protein in formalin-fixed, paraffin-embedded sections from human retina (Kortvely et al., 2010). Besides rather weak expression in the RPE and retinal cells, we found ARMS2 to be enriched within the intercapillary pillars of the choriocapillaris with a gradient towards Bruch’s membrane (Fig. 7). The observed signal was completely lost in negative controls that excluded the primary ARMS2 antibody or when the primary ARMS2 antibody was pre-blocked with the corresponding peptide. Additionally, we did not observe any relevant staining in sections from species that by nature do not possess the ARMS2 gene (Kortvely et al., 2010). Therefore, as evidenced by these results and western blot experiments within the same publication, we conclude that ARMS2 is a secreted protein. Subcellular localization in ARPE-19 cells revealed that it co-localizes with endoplasmic reticulum (ER) but not Golgi markers. Importantly, we observed mitochondrial staining only in cases where the signals for ER and mitochondria overlapped and the staining was not lost upon biochemical disruption of mitochondrial integrity (Kortvely et al., 2010).



**Fig. 6. ARMS2 protein structure as predicted by AlphaFold.** The predicted structure is devoid of any extensive secondary structure. The colors indicate per-residue confidence scores (pLDDT), with blue colors representing high pLDDTs and yellow/orange colors representing low pLDDTs. Low pLDDT scores have been shown to be associated with structural disorder (Tunyasuvunakool et al., 2021). It should be noted that no experimentally derived three-dimensional structure of ARMS2 has been published to date. Protein structure taken from (“Age-related maculopathy susceptibility protein 2 - AlphaFold structure prediction,” 2022).



**Fig. 7. Localization of the ARMS2 protein in human eyes.** Immunostainings of ARMS2 using custom antibodies in human donor eyes. (A) ARMS2 enrichment was found in the extracellular space of the CC/choroid. (B) In higher magnification, ARMS2 was found to localize to the intercapillary pillars of the CC (green arrowheads) with an observable gradient towards BrM. The pictures were previously published in (Kortvely et al., 2010). Scale bars correspond to 20  $\mu$ m.

We observed that C-terminal tagging of ARMS2 results in loss of ER localization, while N-terminal tagging did not impact on subcellular localization (Kortvely et al., 2010). As conventional protein secretion is dependent on an N-terminal secretion signal, this rather atypical finding prompted us to investigate the exact mode of ARMS2 secretion. We generated mutants in the C-terminal region of ARMS2 and found that elimination or substitution of the di-isoleucine present in the ARMS2 C-terminus (-SIIHTAAR) lead to disruption of subcellular localization and prevented secretion (Kortvely et al., 2016). It is known that di-hydrophobic motifs like ARMS2's di-isoleucine may serve as ER-export signals (Wendeler et al., 2007). Additionally, we found that ARMS2 interacts with cytosolic lectin chaperones and co-localizes with calnexin (CANX)-positive and protein disulfide isomerase (PDI)-negative vesicle-like structures. The observed ARMS2 co-localization with GRASP65 points towards involvement of autophagic factors in the export process. However, we found that Interleukin-18 (IL-18), the best studied candidate undergoing secretory autophagy, did not co-localize with ARMS2 (Kortvely et al., 2016). This implies that ARMS2 is secreted via an unconventional, Golgi-bypassing route. This unique way of secretion may hold functional relevance as it may provide ARMS2 with a specific biochemical environment allowing for proper maturation or preventing premature interaction with other proteins undergoing conventional secretion.

On the tissue level, ARMS2 was detected in the intercapillary septa of the choriocapillaris CC adjacent to BrM (Fig. 7) (Kortvely et al., 2010). Interestingly, autofluorescent drusen exhibit a non-random distribution and localize to the same area (Lengyel et al., 2004). Mullins et al. postulated that the deposition of complement pathway complexes in the CC acts as an activating event for the CC degeneration and drusen formation observed in early AMD (Mullins et al., 2011). Therefore, ARMS2 staining patterns imply a localization to both, the principal sites of drusen formation and complement depositions. Interestingly, amyloid aggregates of amyloid beta ( $A\beta$ ) have been found in drusen associated with products of complement activation (Anderson et al., 2004) and intrinsically disordered proteins have a proven ability to impact on the aggregation kinetics of amyloid proteins (Falsone et al., 2012; Ikeda et al., 2020; Merle et al., 2019). Considering that ARMS2 localizes to the site of drusen formation and complement activation along with the high likelihood of being at least partially intrinsically disordered, one could speculate that ARMS2 may have an impact on protein aggregation during drusen formation and that the missense variant (p.A69S) could have an influence on these properties.

### 3.4. HTRA1 on the protein level

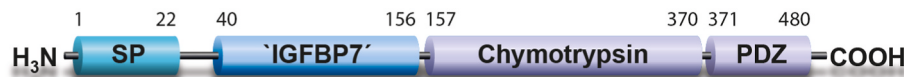
Despite a multitude of research efforts trying to pinpoint the role of HTRA1 in AMD pathogenesis, fundamental shortcomings in our understanding remain. The reported, but rather inconsistent, upregulation of

HTRA1 in AMD (Chan et al., 2007) was recently fundamentally challenged when Williams et al. showed reduced HTRA1 levels in the RPE of post mortem AMD donor eyes (Williams et al., 2021). At this point we need to critically reevaluate how to address this challenging scientific question. Based on the complex and largely context-dependent regulation of HTRA1, focusing on simple up- or downregulation may be an oversimplification, while the true role of HTRA1 may be more subtle and variable than previously thought. We therefore want to take a step back and provide insights into the basic physiology of HTRA1 that are not commonly covered in AMD-related studies or reviews. A comprehensive review on the possible role of HTRA1 in AMD has recently been published by May et al. (2021).

HTRA1 is highly conserved across species and belongs to the high temperature requirement A family of serine proteases. The first *htrA* gene was discovered in *E. coli* when a mutant was isolated that stabilized a hybrid protein (Strauch and Beckwith, 1988). The gene name resulted from the observation that *htrA* (*degP*) null mutants failed to grow at elevated temperatures (Lipinska et al., 1988; Strauch et al., 1989). Human HTRA1 is synthesized as a 51 kDa precursor that harbors a N-terminal signal sequence, followed by a partial insulin-like growth factor binding 7 (IGFBP7) domain of unknown function, a highly conserved chymotrypsin-like S1 serine protease domain and one C-terminal PDZ domain (Fig. 8) (Clausen et al., 2002). It is a peculiarity of HTRA1 that following its secretion, about 15% of the HTRA1 pool re-enters the cell, a process that can be mimicked when adding purified HTRA1 to the medium in *in vitro* experiments (Grau et al., 2005; Poepsel et al., 2015).

HtrAs are homooligomeric proteins with trimers representing the functional unit. While classic serine proteases are synthesized as enzymatically inactive precursors, termed zymogens that require proteolytic processing for irreversible activation (Hedstrom, 2002), HtrA proteases are reversibly activated by peptidic ligands (Clausen et al., 2011). Here, the degree of activation is determined by the affinity and sequence of the ligands. In line with reports on *E. coli* HTRAs, the PDZ-domain of human HTRA1 serves as an allosteric site. The binding of 3–4 C-terminal residues of a wide variety of proteins or protein fragments triggers activation of its proteolytic activity (Rey et al., 2022). Another function of the PDZ domain is to locate HTRA1 to specific subcellular sites such as cytoplasmic microtubules (Chien et al., 2009) or fibrillar collagens of the ECM (Murwantoko et al., 2004). As the peptide binding site of the PDZ domain is rather hydrophobic, it allows HTRA1 to detect hydrophobic C-termini in misfolded proteins (Runyon et al., 2007). Interestingly, HTRA1 remains functional even in the absence of its PDZ domain, because ligand binding to the catalytic domain is sufficient for activation (Truebestein et al., 2011). Together, ligand-induced activation and targeted cellular location mediate the temporally and spatially fine-tuned regulation of proteolytic activity that is based on the classic biochemical principles of allostery and cooperativity (Merdanovic et al.,





**Fig. 8. HTRA1 protein domain structure.** The HTRA1 protein consists of a N-terminal cleavable signal peptide (SP), an N- and C-terminally truncated fragment of insulin growth factor binding protein (IGFBP) 7 (truncations indicated by ‘), a central S1 serine protease domain resembling chymotrypsin and a C-terminal PDZ domain (Clausen et al., 2011).

2020). As there are 6 ligand binding sites in an HTRA1 trimer (one in each PDZ and one in each protease domain, respectively), an up to 6-fold positive cooperativity can be reached with suitable high affinity ligands. This regulatory complexity represents an interesting feature in light of the hypothesis that modulation of HTRA1 activity may have pathological effects in the eye.

The fact that the N-terminal partial IGFBP7-domain has no effect on the catalytic activity of purified HTRA1 does not mean it has no function in tissue whatsoever. The crystal structure of this domain revealed the presence of eight disulfide bonds (Eigenbrot et al., 2012) that are expected to contribute to protein stability and therefore increase its half-life in the extracellular space. Several reports describe autocatalytic cleavage between the N-terminal and protease domains, for example in urothelial carcinoma (Lorenzi et al., 2013) and pre-eclampsia (Lorenzi et al., 2009). However, these studies did not distinguish between extra- and intracellular HTRA1. As the intracellular milieu is reducing, we would expect that processing occurs mainly in the cytoplasm, where the disulfide bonds are probably reduced following protein uptake. Indeed, it was shown with purified protein that reduced HTRA1 is autocatalytically cleaved (Risør et al., 2014). Whether the disulfide bonds are implicated in redox-sensing in the extracellular matrix and the regulation of HTRA1's activity remains to be experimentally addressed in AMD in particular because AMD is strongly associated with oxidative stress.

Another critical question concerns the specific roles of HTRA1 in the biology of the relevant tissues of the eye that could be deduced from the identification of its substrates. This is a greatly interesting but also hugely challenging issue as, for example, the human eye proteome project has identified at least 237 proteases (Supplementary Table 2) and 70 natural protease inhibitors (Supplementary Table 3) (Ahmad et al., 2018) of potentially overlapping functions and interactions. Clearly, HTRA1 is expected to have hundreds of substrates as well as numerous natural activating and inhibiting ligands, only a few of which have been identified to date (Rey et al., 2022; Schillinger et al., 2018; Wright et al., 2020). This knowledge is likely of critical importance for a mechanistic understanding of the dysregulation of HTRA1, its consequences for the onset of pathological features as well as for devising therapeutic strategies that work without causing additional imbalances of the already disturbed biology of the eye.

### 3.5. Interfering with HTRA1 function

One straightforward strategy to test the hypothesis that the deregulation of HTRA1 is implicated in various pathological aspects of AMD and whether its inhibition is a sensible therapeutic strategy is to develop an array of tools for inhibiting or activating HTRA1 in cell- and animal-based studies. We feel that currently, it is useful to explore various avenues as too little is known about the effects of most HTRA1 inhibitors on the healthy and diseased eye. In this light, gene editing or gene silencing approaches in animal models are typically complex and time-consuming procedures. They are often hampered by long-term pleiotropic effects and unpredictable cellular adaptations. Conversely, small molecules act reversibly on a much more rapid timescale, allow a facile spatiotemporal control of their action and enable a ‘fine-tuning’ of modulation by applying the chemical tools at appropriate concentrations. As a consequence, small molecule HTRA1 inhibitors seem ideally suited to test the hypothesis that the hyperactivity of HTRA1 represents one critical cause for the development of AMD.

So far, most developed HTRA1 inhibitors use classic mechanism of

action (Powers et al., 2002), acting either by covalent and thus often irreversible modification of HTRA1 or as non-covalent inhibitors for reversible inhibition. Many of them have only been disclosed in patents and are not accessible to interested researchers. In contrast, the boronic acid-based inhibitor NVP-LEB748 (originally developed at Novartis) has emerged as the most frequently applied HTRA1 inhibitor in academic research, including AMD studies in the eye (Vierkotten et al., 2011). NVP-LEB748 inhibits HTRA1 with an  $IC_{50}$  of around 0.21  $\mu$ M (Grau et al., 2006). Unfortunately, NVP-LEB748 is currently also no longer available, but an alternative boronic acid-based HTRA1 inhibitor, termed DPMFKL-BoroV, that binds to the active site of HTRA1 with a  $K_d$  of 0.8  $\mu$ M (Merdanovic et al., 2020; Truebestein et al., 2011) has been recently used in an AMD mouse model (Kumar et al., 2017). A drawback of both inhibitors is that their full cellular target repertoire is largely unknown and we cannot exclude effects on other proteases and proteins. Irreversible covalent binding phosphonate-based HTRA1 inhibitors, and activity-based probes, have also been recently reported (Nam et al., 2020). These compounds produced a promising HTRA1 inhibition selectivity profile in HeLa cell cultures as detected by activity-based protein-profiling (ABPP): a chemical biology technique for monitoring the activity state of enzymes under various physiological conditions. Moreover, fluorophore-tagged variants of these compounds have also been used successfully in imaging studies to probe HTRA1 activity, thereby offering new opportunities for functional studies. Nevertheless, for some chemical biology and potential chemotherapeutic applications, non-covalent small molecule inhibitors are more desirable due to their often superior pharmacological properties. Recently, such a non-covalent, reversible HTRA1 inhibitor has also been generated (Köcher et al., 2017). This compound is a synthetic derivative from the Ahp-cyclodepsipeptide natural product family (Köcher et al., 2020).

Besides these classic small molecule modulators, alternative chemical modalities to probe HTRA1 function are highly desirable. Along these lines, the development of an inhibitory monoclonal antibody FHTR2163 (also known as RO7171009 or Fab15H6.v4.D221) for the treatment of geographic atrophy has recently been reported (Khanani et al., 2021; Tom et al., 2020) and is currently being evaluated in two concurrent phase II clinical trials (NCT03972709 and NCT04607148). In addition macrophage migration inhibitor factor (MIF) was discovered to be the first known endogenous inhibitor of HTRA1 (Fex Svenningsen et al., 2017), indicating that biologic HTRA1 inhibitors could also be developed from native protein inhibitors. An alternative route to HTRA1 inhibition employs targeted protein degradation approaches such as the emerging Proteolysis Targeting Chimeras (PROTAC) (Békés et al., 2022), or, due to the mainly extracellular localization of HTRA1, Lysosome-targeting chimaeras (LYTAC) (Banik et al., 2020) technologies. Despite their merits, clinical development of these approaches have not yet been reported.

Finally, a complementary targeted enzyme activation of HTRA1 may provide additional insights into the protein's role in AMD. In biochemical assays, HTRA1 activation upon binding of activating peptides (Rey et al., 2022) as well as by application of substoichiometric amounts of HTRA1 inhibitors (Merdanovic et al., 2020) has been observed. These promising results have however so far not been developed further into chemical tools for customized HTRA1 activation. It must be stressed that any potential therapeutic modification of HTRA1 in any indication requires a detailed elucidation of HTRA1's function as HTRA1 dysfunction results in serious conditions like for example cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy

(CARASIL), a severe vascular disease (199).

### 3.6. *PLEKHA1* on the protein level

First identified in 2000, *PLEKHA1* (Pleckstrin homology domain containing A1) is widely expressed across tissues, with highest expression in skeletal muscle, thymus, pancreas, placenta and lung, and lower levels in many tissues including retina and brain (Dowler et al., 2000; Marshall et al., 2002). The strong association of genetic variants in *ARMS2* and *HTRA1* and the absence of risk polymorphisms linked to *PLEKHA1* has resulted in a strong bias towards those genes, leaving *PLEKHA1* largely ignored in experimental settings. Based on our current knowledge, we are, however, by no means able to exclude it from having an important role in AMD pathogenesis since genetic associations alone do not suffice to unravel the complex functional interplay within chromosomal region 10q26.

In line with our data on *PLEKHA1/ARMS2* chimeric transcripts, alternative splicing gives rise to multiple *PLEKHA1* transcripts. The canonical transcript encodes a 404 amino acid containing two pleckstrin homology (PH) domains. This intracellular protein is recruited to the plasma membrane when it binds the second messenger phospholipid phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P(2)), through a binding motif in the second PH domain (Dowler et al., 2000; Kimber et al., 2002). In addition, *PLEKHA1* interacts with proteins containing PDZ (postsynaptic density protein (PSD-95)/Drosophila disc large tumour suppressor (dlg)/tight junction protein (ZO1)) domains, including the protein-tyrosine-phosphatase-like protein-1 (PTPL1), to recruit them to the plasma membrane (Kimber et al., 2002, 2003). This has been suggested to contribute to phosphatidylinositol 3-kinase (PI3K) signal transduction following insulin- and growth-factor-stimulation. In both *in vitro* and *in vivo* studies, *PLEKHA1* appears to promote cytoskeletal rearrangements in stimulated B cells (Marshall et al., 2002), and has an important role in B cell activation and autoimmunity (Jaya-chandran et al., 2017; Landego et al., 2012). However, the role of *PLEKHA1* in other tissues including the eye, remains largely unexplored and leaves a lot of potential for future experimental studies.

## 4. 10q26: ideas and concepts

### 4.1. From structure to function: 10q26 in the light of evolution

*HTRA1* is member of a highly conserved group of serine proteases, whereas *ARMS2* is very young when looked at from an evolutionary perspective. Orthologues to human *HTRA1* are even found in bacteria (Chien et al., 2009). In contrast, besides being present in humans, *ARMS2* is only present in old world monkeys and apes (Francis et al., 2008). Here, however, the gene locus displays high conservation among those species (Kortvely and Ueffing, 2016). Intriguingly, the emergence of *ARMS2* as a potential functional gene coincides with the development of the typical macular architecture including the predominance of cones, central displacement of other retinal cell types as well as formation of a foveal pit and a foveal avascular zone (Pennesi et al., 2012). Although being associative at this point, it is imaginable that the emergence of *ARMS2* along with the macula has functional implications. The macula enables prime visual acuity due to increased density of photoreceptors and almost 1:1 representation in afferent neurons. However, the high level of cellular activity and the absence of intraretinal vasculature results in increased metabolic demand from the choriocapillaris and renders this region susceptible to nutritional shortages and other molecular stressors. It seems reasonable that an age-related disease like AMD develops in such a region as aging processes are known to impact on AMD-relevant processes like inflammation, cellular metabolism and cellular responses towards oxidative stress (Armento et al., 2021). Accordingly, given the likely possibility, that *ARMS2* function is protective, the coevolution of *ARMS2* and the macula points towards a putative functional interrelationship and a potential role of *ARMS2* in

macular homeostasis.

On a molecular level, the evolutionary conserved organization of the 10q26 locus and the tight clustering of its genes is also unlikely to be a product of pure chance as recent research clearly rejects the hypothesis of random arrangements of genes in the genome (Gherman et al., 2009) and argues for clustering of functionally related genes (Foflonker and Blaby-Haas, 2021). Furthermore, neighboring genes have been reported to impact on each other's expression (Arnone et al., 2012; Ghanbarian and Hurst, 2015) and genes with similar expression profiles have a tendency to cluster (Ghanbarian and Hurst, 2015). This can likely be explained by chromatin-state dependent regulation of gene expression, another aspect that is experimentally underrepresented in 10q26-related AMD research. Going a step further, *ARMS2* and *HTRA1* may jointly form an operon-like structure. While operons have for a long time been viewed as exclusively present in bacteria, there is evidence that similar mechanisms exist in eukaryotes (Ben-Shahar et al., 2007). Additionally, co-expressed genes have been reported to physically interact (Ge et al., 2001; Wuchty et al., 2006). Therefore, an interaction between *ARMS2* and *HTRA1* is a distinct possibility, notably not only on the protein level, but also on the RNA or even DNA level.

### 4.2. Hypothetical roles of *ARMS2* mRNA

RNA has the ability to form complex three-dimensional structures and has more diverse functions as initially anticipated. Accordingly, mRNA is not limited to its function as a template for translation (Cech and Steitz, 2014; Chooniedass-Kothari et al., 2004), but can simultaneously act as a non-coding RNA (Kumari and Sampath, 2015). Non-coding RNAs (ncRNAs) is an umbrella term for a functionally versatile group of RNA molecule with roles beyond transcription and translation (mRNA, tRNA). Among others, many ncRNAs have important roles in gene expression regulation and associated processes. A detailed review on the different types of ncRNA has been published elsewhere (Cech and Steitz, 2014). Although there is published evidence for the existence of *ARMS2* on the protein level, it was speculated that *ARMS2* predominantly exists as a non-coding mRNA (Kortvely and Ueffing, 2016) and may directly impact on the transcription of other genes. *In silico* analysis using the intaRNA 2.0 tool (Mann et al., 2017) predicted an interaction between the *ARMS2* and *HTRA1* mRNAs ( $\Delta G$  of  $-21.83$  kcal/mol). Interestingly, the predicted interaction site lies within the 5'-UTR of the *HTRA1* mRNA in close proximity to the functional start-codon (see sup. Fig. 1B). The 5'-UTR of eukaryotic mRNA is known to impact on translational regulation (Leppek et al., 2018) and nuclear export (Cenik et al., 2011). Hence, any changes in *ARMS2* mRNA sequence (e.g. rs10490924) or expression can have a potential impact on *HTRA1* protein levels. In this manner, the strongly AMD-associated *ARMS2* deletion-insertion variant, resulting in destabilization of *ARMS2*-mRNA (Rivera et al., 2005), might exert its effect *via* modulation of *HTRA1* expression. Furthermore, mRNA is able to specifically interact with proteins (Sanchez de Groot et al., 2019). *In silico* analysis of the *ARMS2*-mRNA using the Vienna RNA websuite's RNAfold tool (Gruber et al., 2008) predicts extensive secondary structure formation in *ARMS2* mRNA and *in silico* analysis *via* RPIseq (Muppirala et al., 2011) predicted a high likelihood of interaction between *ARMS2* mRNA and *HTRA1* protein (random-forest (RF) and support vector machine (SVM) classifiers of 0.9 and 0.71, respectively; 0.5 positivity threshold for actual RNA-protein interaction). Although these findings remain highly speculative, the highly enigmatic nature of 10q26 should prompt us take non-conventional hypotheses into account when planning our future experiments. Additionally, we suggest to extend the general idea of non-coding mRNAs being involved in 10q26 regulation and to consider transcripts of all three 10q26 genes as potential actors.

#### 4.3. Coding vs non-coding variants: lessons learnt from chromosome 1

Genetic variants that map to coding regions of the genome and lead to missense or nonsense mutations have traditionally been the focus of biomedical research as they directly affect a protein's primary structure and potentially its function. In this light, the ease of establishing experimental setups with coding mutants, for example via PCR-based site directed mutagenesis in *in vitro* settings, is more intuitive and far less complicated than studying non-coding variants in advanced experimental settings. This general idea is reflected by the strong bias towards data on coding variants in published literature. While monogenic diseases, are in majority the result of a single coding mutation, this is not the case for complex diseases, where the vast majority of variants discovered in GWAS map to non-coding regions (Farh et al., 2015; Maurano et al., 2012; Zhang and Lupski, 2015). Importantly, recent research highlighted the importance of non-coding variants in pathologic processes (<https://pubmed.ncbi.nlm.nih.gov/31748530/>).

This concept is illustrated by recent studies on chromosomal region 1q31.3, the locus that harbors the second highest genetic risk for AMD. 1q31.3 contains the genes *CFH*, *CFHR1*, *CFHR2*, *CFHR3*, *CFHR4* and *CFHR5*, coding for complement factor H (FH)/Factor H-like protein 1 (FHL-1) and Complement Factor H Related 1 to 5, respectively. Numerous SNPs in this region are associated with increased risk for AMD. For a long time, the non-synonymous SNP rs1061170 in the coding region of *CFH*, that leads to the exchange of the tyrosine at position 402 with a histidine (Y402H), was the predominant focus of scientific endeavors. While the functional relevance of Y402H was shown on the protein level (Clark et al., 2010, 2014), recent studies largely extended our understanding by analyzing the impact of non-coding SNPs in this region. Surprisingly, AMD-associated, non-coding SNPs around the *CFH* gene were not associated with altered FH protein plasma levels but increased levels of FHR proteins (Cipriani et al., 2020, 2021). Although further research is needed to elucidate the exact mechanism, this example beautifully illustrates how neglected non-coding SNPs can have drastic molecular consequences that are highly relevant to understand disease pathophysiology.

Furthermore, research over the last decades has largely extended our understanding of gene expression regulation and findings like the ones mentioned above are likely to be mediated by epigenetic or higher-level genetic mechanisms. Besides classic epigenetic mechanisms that tune transcriptional activity by altering local chromatin structure and dynamics (Klemm et al., 2019), a plethora of long-range interactions has been discovered. Intriguingly, functional genetic interactions not only take place between neighboring regions or loci on the same chromosome but also between loci residing on different chromosomes (Dekker and Misteli, 2015). Hence, the three-dimensional organization of the genome has important functional implications (Mohanta et al., 2021) and future studies in this direction have the potential to significantly extend our understanding of AMD's complex genetic basis. In line with this, a recent report on the human retinal genome topology by Marchal et al. found evidence for higher-level genetic interactions at 10q26 (Marchal et al., 2022). In addition, recent advances in high-throughput proteomic analyses (protein quantitative trait loci; pQTL) are able to correlate changes in serum protein amounts with individual SNPs (Gudjonsson et al., 2022). Recently, a first pQTL-study assessed the impact of AMD-associated SNPs on serum protein levels. Although this study found no correlation between rs3750846 and altered levels of the analyzed serum proteins, similar analyses of ocular tissues may provide important insights based on the putative tissue-specific and context-dependent regulation of 10q26 (Emilsson et al., 2022).

#### 4.4. Potential links between chromosome 1 and chromosome 10

Among the many variants defining risk for developing AMD, the 10q26 risk locus remains the most highly-associated. The second most risk-conferring locus is found in chromosomal region 1q32, in the

regulators of complement activation (RCA) cluster. This RCA cluster comprises a number of genes associated with the regulation of the complement system, a powerful part of a host's innate immune system (Armento et al., 2021). The major risk-conferring genetic variants occur in and around the genes encoding the factor H (FH) family of proteins: primarily the *CFH* gene itself, but also the five factor H-related genes (*CFHR1-5*) that sit immediately downstream of *CFH* (García-Fernández et al., 2021). The biochemical consequences of these genetic variants and their potential association with AMD pathogenesis have been extensively reviewed elsewhere (Armento et al., 2021).

There is currently very little validated association between *HTRA1*, *ARMS2* or complement on the protein level, although the identification of clusterin and vitronectin, both inhibitors of complement-mediated cell lysis, as substrates for *HTRA1* (An et al., 2010) strongly hints to a potential functional link. Additionally, Micklisch et al. reported a possible link between *ARMS2* and the complement system. According to this study, *ARMS2* binds to human apoptotic and necrotic cells and initiates complement activation by recruiting the complement activator properdin, thereby augmenting C3b surface opsonization for phagocytosis, which in consequence results in clearance of cellular debris (Micklisch et al., 2017). But despite this, the fact that disease risk association from these two risk loci dwarfs all remaining genetic risk, the lack of direct and validated data confirming an interplay between these two risk loci has led to the hypothesis that their risks present independently. This has, in part, put new emphasis on genetically stratifying patients and samples when studying AMD and/or delivering therapeutics. Nevertheless, it is not possible to segregate wet vs dry AMD based on risk loci stratification only. Additionally, large European population-based studies showed that patients carrying homozygous risk solely at either loci have a similar phenotypic course and that patients cannot be separated based on phenotype (Thee et al., 2022). However, there may be a difference in the speed of clinical readouts, such as MNV development and GA lesion growth (Keenan et al., 2018; Thee et al., 2022). In fact, our recent study suggests, that carriers of the risk haplotype at *ARMS2/HTRA1* have a particularly high risk of late AMD at a relatively early age. Our data further suggest that risk variants at *ARMS2/HTRA1* act as a strong catalyst of progression once early signs are present. The phenotypic spectrum resembles that of complement genes, only with higher risks of MNV (Keenan et al., 2018). It has to be considered, that the prevalence of individuals with homozygous risk solely at either genetic loci is relatively low; most AMD patients (>85%) have simultaneous risk in both genetic loci (Colijn et al., 2021). Further studies have pointed out that the combined risk of both genetic trajectories is more detrimental for AMD than the separate effects, suggesting some interplay between these two genetically defined sub-groups of AMD patients (Schmitz-Valckenberg et al., 2022; Thee et al., 2022).

Indeed, a recent study investigating the protein content of BrM, enriched from genotyped human donor eyes, shows a remarkable similarity in donors carrying either genetic risk loci (Mcharg et al., 2022). The study compared BrM enriched from human donor eyes carrying no genetic risk for AMD with donors carrying homozygous risk only at the chromosome 1 loci, or homozygous risk only at the chromosome 10 loci: all without evidence of any macroscopic AMD changes. Although the study showed clear differences in elevated or reduced proteins in the two risk groups, strikingly the top seven biggest protein changes were shared by both genetic-risk groups. Of these, the top four changes were significant elevations of proteases found in mast cells with the top three proteases (*i.e.* chymase, carboxypeptidase A3, and tryptase) derived solely from mast cells: see Table 2 in (Mcharg et al., 2022). Subsequent histological analysis showed increased mast cell accumulation within the inner choroid of both genetic-risk groups and evidence of mast cell degranulation and collagen IV degradation. This result shows that patients carrying genetic risk at either loci both have mast cell protease-mediated ECM remodeling in their outer blood/retinal barrier even before evidence of AMD even begins, and implies that they share a common early biochemical pathogenesis. Given these findings and the



striking similarity in course of disease between 10q26 associated risk and complement risk carrying individuals, these findings further increase the likelihood that not only various complement associated risks but also 10q26 associated risks contribute to a common overlapping pathogenic molecular mechanism of disease. At which stage the biochemistry merges between these two risk groups remains to be elucidated. Although the study did not investigate driving mechanisms behind this observation, it is intriguing to note that a major stimulant of mast cell recruitment and degranulation is the engagement of the anaphylatoxins C3a and C5a with their respective receptors on the mast cell surface (West et al., 2020), implying a role of complement over-activation in both genetic-risk groups.

In addition, a recent pQTL-study on serum proteins of AMD patients found an association between the chromosome 1 SNP rs6677604 and calnexin (CANX) serum levels (Emilsson et al., 2022). Intriguingly, we were able to show that CANX is involved in ARMS2 secretion and that ARMS2 closely co-localizes with CANX (Kortvely ARMS2 ECM), pointing towards another potential level of interaction between chromosome 1 and chromosome 10.

## 5. HTRA1 and ARMS2 in ECM homeostasis

### 5.1. ECM homeostasis

Both, HTRA1 and ARMS2 are found in ECM-rich compartments. The ECM is a network of proteins and other substances that supports and sustains the cellular components of a specific tissue (Hubmacher and Apte, 2013). ECM composition and structure helps controlling nutrient exchange and facilitates the elimination of metabolic products. The ECM is involved in signaling transduction and regulation of cellular processes, like migration and proliferation (Gkretsi and Stylianopoulos, 2018; Klein et al., 2009). Moreover, controlled ECM alterations are important for a correct formation of blood vessels (Neve et al., 2014). Therefore, it is vital that the ECM is maintained in physiological balance. To achieve this, ECM components need to be degraded, redeposited, and remodeled to ensure proper stiffness, structure and function. Changes in ECM can affect cellular functions, and, at the same time, cell damage can impact the cellular production of ECM components or enzymes needed for its regulation, creating a negative feedback loop. Under physiologic conditions, the turnover of ECM components is ensured by enzymes like matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) (García-Onrubia et al., 2020).

In the eye, a multilayered and highly structured ECM boundary called Bruch's membrane (BrM) separates the RPE and the neurosensory retina from the vascular system of the choroid/choriocapillaris. It is commonly proposed that BrM itself is composed of five layers: basement membrane of the RPE, the inner collagenous layers (ICL), the elastin layer (EL), the outer collagenous layer (OCL) and the basement membrane of the CC endothelial cells. By an alternative definition, it has been proposed that BrM itself does include only three layers, excluding the CC and RPE basal lamina. The basement membranes are similar in their compositions and contain, among others, collagens, laminin, fibronectin, heparan and dermatan sulphate. The ICL and OCL present highly organized structures of collagen fibers, while the EL is rich in elastic fibers of diverse shapes and sizes (Nita et al., 2014).

The region of the retina affected in AMD is the interface between the CC/BrM/RPE with changes in BrM appearing in the early stages of the disease. Non-genetic AMD-risk factors, like age, unhealthy diet or smoking, have detrimental effects on ECM homeostasis, affecting both BrM and CC functions. Aged BrM becomes thicker, due to an accumulation and cross-linking of collagen fibers (Curcio and Johnson, 2013). Moreover, with age, changes in the amount of other proteins and substances occurs, such as a reduction in heparan sulphate (Keenan et al., 2014) and Fibrillin 2 (FBN2), a glycoprotein of the elastin-rich extracellular matrix (Ratnapriya et al., 2014). In parallel, accumulation of advanced glycation end products (AGEs), which consist of glycated and

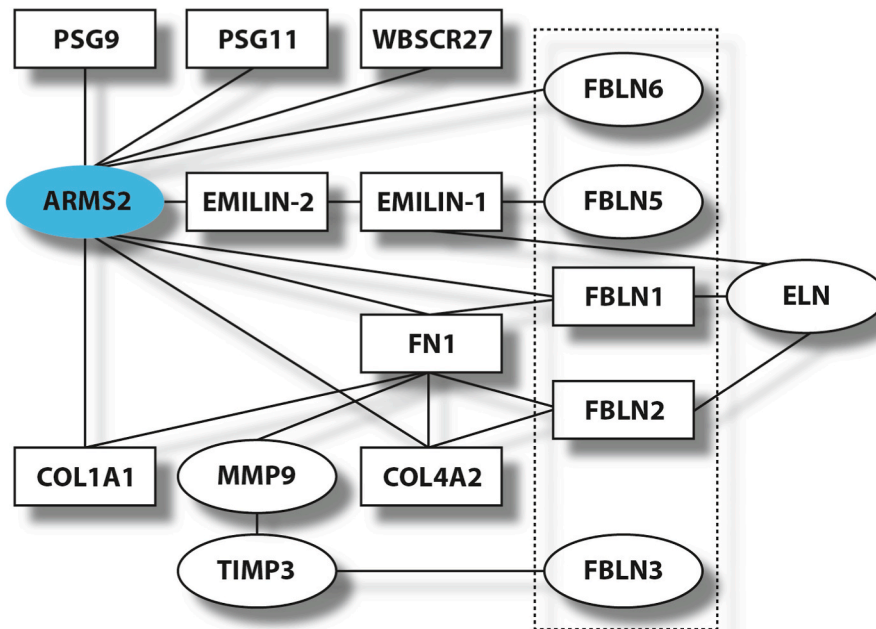
oxidized proteins and lipids and are pro-inflammatory molecules, has been observed (Howes et al., 2004). The choroid is also affected by age, showing reduced plasticity and flexibility (Wakatsuki et al., 2015). Moreover, life-style habits, like smoking or unhealthy diet induce oxidative stress, which can damage the ECM of the BrM and choroid (Woodell and Rohrer, 2014). Those changes impair the permeability of BrM, causing a reduction in transport of nutrient and oxygen through the BrM to the RPE, and a transport of discard material from the RPE to the choroid (Chirco et al., 2017). On one hand, the homeostasis and metabolism of RPE is impaired and on the other hand, discard material accumulates in the BrM, forming drusen, which are the hallmark of AMD (Bird et al., 1995). Of notice, many SNPs associated with AMD have been found in loci that overlap or are close to genes associated with ECM composition and turnover: rs5749482 near *TIMP3*, rs13081855 in *COL8A1*, rs6795735 in *ADAMTS9*, rs3812111 in *COL10A1* (Fritsche et al., 2014) and rs154001 in *FBN2* (Chen et al., 2010; Fritsche et al., 2013, 2014; Ratnapriya et al., 2014). Moreover, many studies suggest that also HTRA1 (Dewan et al., 2006; Vierkotten et al., 2011; Yang et al., 2006) and ARMS2 (Biarnés et al., 2020; Kortvely et al., 2010; Nita et al., 2014) are involved in AMD pathogenesis via modulating the ECM.

### 5.2. ARMS2 in ECM homeostasis

Given that comprehensive analyses of the 10q26 risk haplotype have narrowed down the genetic risk region to ARMS2, suggesting that this is the true AMD gene at this locus, the discovery of ARMS2's function holds great relevance to understand the overall functional implication of 10q26 in AMD. In order generate mechanistic information we performed Yeast two-hybrid screens using ARMS2 as bait. Due to low expression in most tissues but high expression in connective tissue and placenta, we chose to use a placental library for the experiment. In line with the localization experiments, we found ARMS2 to directly interact with several ECM proteins and ECM regulators (Kortvely et al., 2010). Intriguingly, we found ARMS2 to directly or indirectly interact with several factors that have been implicated in macular dystrophies (Fig. 9). As an example, missense mutations in FBLN5 (fibulin-5) have been associated with AMD (den Hollander and de Jong, 2014; Stone et al., 2004) and a point-mutation in FBLN3 (fibulin-3; EFEMP1) causes Malattia Leventinese (Doyle honeycomb retinal dystrophy; Familial Dominant Drusen), a macular dystrophy that shows striking similarities with AMD (Livingstone et al., 2020). FBLN6 (fibulin-6; Hemicentin-1) was reported to be associated with AMD in a large family (Schultz et al., 2003), however, follow-up studies were largely unable to confirm a clear association (Hayashi et al., 2004; Schultz et al., 2005). Furthermore, fibulins have been reported to play important roles in AMD-relevant processes like inhibition of angiogenesis (Xie et al., 2008) and ECM homeostasis (Kobayashi et al., 2007; Timpl et al., 2003). Given the association of fibulins with macular disease, the ARMS2-fibulin interaction likely play an important role in AMD-related ECM homeostasis and warrant further research in the context of 10q26.

ARMS2 was also found to directly interact with Elastin Microfibril Interface-Located Protein 2 (EMILIN-2), which regulates elastogenesis and vascular cell maintenance by stabilizing molecular interactions between elastic fiber components (Zanetti et al., 2004). Dysregulated elastogenesis may lead to loss of BrM elasticity and stimulate the secretion of pro-angiogenic factors, which is thought to be an important contributor to MNV development in wet AMD (McLeod et al., 2009). Furthermore, smoking, a well-known risk factor for AMD, was shown to induce the degradation of elastin fragments (Lee et al., 2007). This may explain, why smokers who are homozygous for the ARMS2 p.A69S allele exhibit a 22-fold higher risk for AMD compared to homozygous non-smokers (Jabbarpoor Bonyadi et al., 2017).

Taken together, the binding partners of ARMS2 form an interlinked and closely ECM-related interactome. Within this protein network, several proteins have been implicated in the development of macular dystrophies including ARMS2, fibulin-3, fibulin-5, fibulin-6, and TIMP3



**Fig. 9. The ARMS2 Interactome.** Using data from a GAL4-based yeast two-hybrid system along with published interaction data, ARMS2 was shown to directly or indirectly with several ECM proteins. Proteins with reported roles in macular degenerations are shown in circles. The dashed box specifically marks the group of Fibulins. ARMS2, age-related macular susceptibility 2; COL, collagen; ELN, elastin; EMILIN, elastin microfibril interface-located protein; FBLN, fibulin; FN1, fibronectin 1; MMP9, matrix metalloproteinase-9; PSG, pregnancy-specific  $\beta$ -1 glycoprotein; TIMP3, tissue inhibitor of metalloproteinase 3; WBSCR, Williams Beuren syndrome chromosome region 27. This figure is a modified version from that published by Kortvely et al. (Kortvely et al., 2010).

(Fisher et al., 2007; Iyengar et al., 2004; Klein et al., 1998; Schultz et al., 2003). Besides, ARMS2 physically interacts with several other ECM-factors including collagen type IVa2 (COL4A2), a component of basement membranes, collagen type I a1 (COL1A1), a component of the choroidal basement membrane, the pro-angiogenic factor fibronectin 1, and elastin (Kortvely et al., 2010). Those interactors support the existence of a network of extracellular proteins connecting ARMS2 and the ECM. Using in-house developed anti-ARMS2 antibodies for ARMS2 and commercially available antibodies for fibulin-1 and -6, we observed overlapping staining patterns in human retinal tissue sections (unpublished data). We therefore hypothesize that ARMS2 has a yet enigmatic role in maintaining the integrity of the RPE/BrM/choriocapillaris interface.

### 5.3. HTRA1 in ECM homeostasis

As a secreted protease, HTRA1 can be directly or indirectly implicated in the processing and degradation of a large variety of ECM proteins such as aggrecan, bone sialoprotein, collagens, decorin, elastin, fibronectin and fibulin 5 (Tiaden and Richards, 2013). However, these substrates are considered exemplary as a recent terminal amine isotopic labeling of substrates (TAILS) mass spectrometry approach identified 321 cleavage sites in 109 intra- and extracellular proteins of healthy articular cartilage (Bhutada et al., 2022). A similar approach using vitreous humor of rabbits and cynomolgus monkeys identified clusterin (CLU), Dickkopf-related protein 3 (DKK3) and retinol-binding protein 3 (RBP3) as substrates of HTRA1, leading to the insight that DKK3 represents a useful biomarker of HTRA1 activity in cell culture and animal studies (Tom et al., 2020). Indirect effects of HTRA1 activity can occur via the activation of other proteases such as MMPs either by processing them from a zymogen into the proteolytically active form, or even when HTRA1 produces protein fragments that trigger increased production of proteases as observed with fibronectin and MMPs (Grau et al., 2005; Tiaden et al., 2012). Both mechanisms are likely to potentiate the effects caused by HTRA1 alone. While imbalances in ECM composition resulting from increased HTRA1 activity are best studied in skeletal disorders such as osteo- and rheumatoid arthritis (Tossetta et al., 2022), effects on BrM of the eye were also reported (Vierkotten et al., 2011). Moreover, a mouse model overexpressing HTRA1 in RPE cells exhibited lesions in BrM and choroidal vessels attributed to the degradation of the elastic

layer (Vierkotten et al., 2011).

HTRA1 not only affects structural elements of tissue but is also implicated in the modulation of signaling pathways (Oka et al., 2022). Perhaps the best studied of which is the TGF- $\beta$  pathway. Here, HTRA1 can cleave TGF- $\beta$ , TGF- $\beta$ -receptors and TGF- $\beta$ -binding proteins (LTBP), the latter being relevant in the context of the monogenetic disease CARASIL that is driven by loss of function mutations in HTRA1 (Beaufort et al., 2014). Other pathways that are affected by HTRA1 include NOTCH, Wnt and insulin-like growth factor signaling (Globus et al., 2017; Hu et al., 1998; Klose et al., 2018). While all these and many other data are interesting and useful, there is no clear picture of how exactly and under which conditions HTRA1 affects ECM composition and cellular signaling, suggesting a need for more hypothesis driven, mechanistic research, ideally also including kinetic aspects. Such research is required to understand the implications of HTRA1 in the formation of drusen, the destabilization of BrM, neoangiogenesis and ultimately the destruction of photoreceptors. In this respect, it might be worth considering that as a protein quality control factor, HTRA1 prefers misfolded or fragmented proteins as substrates while folded proteins are also degraded but much more slowly. It might therefore be that "upstream" events, causing folding defects, such as oxidative or inflammatory stress, are predisposing the ECM for HTRA1-mediated damages.

## 6. ARMS2, HTRA1 and PLEKHA1 beyond AMD

Molecular alterations of a certain gene and/or protein may have different consequences with respect to the affected tissues or cell type. Nevertheless, binding partners, pathways and the regulatory framework around it frequently show significant overlaps between cell, tissues and even conditions. Therefore, it is often possible to draw meaningful conclusions by looking at other conditions that are associated with a certain molecular alteration.

### 6.1. ARMS2

ARMS2 research has so far been almost exclusively focused on AMD. However, its expression in non-ocular tissues, especially in placenta, suggests roles beyond the eye and AMD. In line with this, a study assessing placental transcriptomes of nine pre-eclamptic and nine healthy pregnant women found upregulation of ARMS2 in the pre-

eclamptic group (Kaartokallio et al., 2015). Intriguingly, the placenta has some structural similarities to the retina when it comes to blood supply and vascularization. The placenta serves to provide blood-borne nutrients, oxygen and other essential factors and substrates to the growing fetus while simultaneously eliminating harmful waste-products - a functional profile closely resembling the RPE/BrM/Choriocapillaris interface. Therefore, understanding the role of ARMS2 in placenta may provide insights with relevance for AMD pathogenesis and collaborative cross-disciplinary projects should be encouraged.

## 6.2. HTRA1

Over the last 20 years, it has become apparent that HTRA1 contributes to a number of diseases. These can be broadly grouped into conditions in which HTRA1 expression is altered resulting in dysregulated proteolysis (osteoarthritis (OA), rheumatoid arthritis (RA), Alzheimer disease (AD), cancer, pre-eclampsia) or Mendelian diseases, like for example CARASIL, in which rare mutations lead to loss-of-function of HTRA1.

A role for HTRA1 in growth factor signaling was first reported by Oka et al., who showed that HTRA1 inhibits BMP and TGF- $\beta$  signaling, and regulates availability of IGFs by cleaving IGF-binding proteins (Oka et al., 2004). *In vitro* assays using deletion mutants of HTRA1 showed that protease activity was required for inhibition of TGF- $\beta$  signaling. This was subsequently confirmed by the identification of pathological loss-of-function variants in HTRA1 that result in uncontrolled TGF- $\beta$  signaling, leading to CARASIL (Hara et al., 2009). This rare, autosomal recessive cerebral small-vessel disorder (OMIM #600142), caused by homozygous or compound heterozygous mutations in HTRA1, is characterized by early-onset alopecia, spondylosis, impaired mobility, strokes and dementia. Pathological examinations of cerebral small arteries revealed loss of vascular smooth muscle cells, dense collagen fibers contributing to thickening of the tunica intima, and degeneration of the tunica media in CARASIL (Beaufort et al., 2014; Oide et al., 2008).

The spectrum of variants in HTRA1 that cause CARASIL has expanded since the initial report in 2009 (Hara et al., 2009), and over 50 pathogenic or likely pathogenic missense or nonsense changes in CARASIL or in HTRA1-cerebral small vessel disease (CSVD) have now been identified (as recently reviewed in (Uemura et al., 2020)). Some, but not all, heterozygous carriers of mutations exhibit mild-to-moderate phenotypes inherited in an autosomal dominant manner. Frameshift and premature stop codon variants identified in CARASIL result in nonsense-mediated decay of the HTRA1 mRNA (Hara et al., 2009); haploinsufficiency can lead to CSVD in carriers. Functional studies of recombinant missense mutant HTRA1s has revealed that some mutations directly impair activation of protease activity *in vitro*, while others have a dominant negative impact on trimerisation or protease activity by altering linker domains, interfering with signal transduction between monomers in assays designed to reflect heterozygous state in carriers (Uemura et al., 2019).

Elevated expression of TGF- $\beta$ 1 was detected in the tunica media of CARASIL patients, consistent with increased TGF- $\beta$  signaling in cerebral small vessels. This was further supported by significant upregulation of transcription of TGF- $\beta$ 1 in reporter assays performed in patient-derived fibroblasts, in cell lines over-expressing HTRA1 with CARASIL mutations, and by loss of repression of TGF- $\beta$  family signaling in *in vitro* assays (Beaufort et al., 2014; Hara et al., 2009). The underlying molecular mechanism remains to be elucidated, but the identification the extracellular matrix protein latent TGF- $\beta$  binding protein 1 (LTBP-1) as a substrate for HTRA1-mediated proteolysis may have implications for TGF- $\beta$  bioavailability (Beaufort et al., 2014) and pathological thickening of the ECM in cerebral vessels.

HTRA1 is expressed in a variety of tissues, including retina, brain, liver, adipose tissue, but with strongest expression in placenta during the third trimester of pregnancy, where it may act in placental development and function (De Luca et al., 2004; Zumbunn and Trueb, 1996). This

role is supported by the observation that placentas from Htra1 $^{-/-}$  mice are smaller and vascularization is impaired, giving rise to smaller pups (Hasan et al., 2015). This indicates that the function of the placenta – to support fetal growth – is impaired. During healthy pregnancies, serum HTRA1 levels increase in the third trimester (Teoh et al., 2015), but in pre-eclampsia, a condition where hypertension and proteinuria develops during pregnancy, HTRA1 levels in serum are elevated (Ajayi et al., 2008; Teoh et al., 2015) compared to healthy pregnancies. Increased HTRA1 mRNA expression has been reported compared to placentas obtained following non-complicated pregnancies (Kang et al., 2011; Liu et al., 2018). This was proposed to alter trophoblast cell function by altering differentiation, migration and invasion in response to growth factors or by altering processing of ECM proteins.

HTRA1 expression is also up-regulated at the transcriptomic and proteomic levels in OA, a condition in which progressive loss of articular cartilage causes joint pain and stiffness (Chamberland et al., 2009; Hu et al., 1998; Milner et al., 2010; Rosenthal et al., 2011; Swingler et al., 2009; Wu et al., 2007). There are multiple lines of evidence that HTRA1 has a role in the dysregulated proteolysis of the cartilage extracellular matrix in OA. Single-cell RNA-seq of chondrocytes from patients revealed that HTRA1 expression level correlated with disease progression (Ji et al., 2019), while HTRA1 was the most abundant protease detected in human OA cartilage by mass spectrometry (Bhutada et al., 2022). Consistent with these data, in two post-traumatic models of OA, Htra1 $^{-/-}$  mice showed significantly delayed cartilage destruction leading to the suggestion that HTRA1 is a rate-limiting factor for OA development (Chen et al., 2019). These proteomic datasets identify substrates of HTRA1, with ECM components aggrecan, biglycan, fibromodulin, cartilage oligomeric matrix protein (COMP), fibronectin and decorin robustly identified as substrates in multiple studies (Bhutada et al., 2022; Chamberland et al., 2009; Grau et al., 2006).

RA is an autoimmune disease in which chronic inflammation leads to joint damage. Increased expression and secretion of HTRA1 by synovial fibroblasts appears to contribute to cartilage destruction in RA directly, by cleaving fibronectin, a component of the ECM, and indirectly, when HTRA1-generated fibronectin fragments induce expression of matrix metalloprotease 1 (MMP1) and matrix metalloprotease 3 (MMP3) (Grau et al., 2006). The increased expression of HTRA1 in RA may arise as a downstream consequence of inflammatory signaling pathways, with the interplay between TLR4 activation by lipopolysaccharide (LPS), IFN- $\gamma$  (interferon- $\gamma$ ; a protective cytokine in RA) and HTRA1 regulation highlighted by mouse studies and in human RA tissue to be of particular interest (Hou et al., 2013, 2014).

AD, an age-related, polygenic condition, is the most common form of dementia. AD is characterized pathologically by formation of protein aggregates, called  $\beta$ -amyloid (A $\beta$ ) plaques and Tau neurofibrillary tangles, in the brain. Together, post-mortem proteomic or immunohistochemical assessment of pathological samples, animal models and GWAS case-control studies of increasing size have highlighted the contribution of inflammation and aberrant protein catabolism to AD (Bellenguez et al., 2022; Wightman et al., 2021). For some years, HTRA1 activity has been implicated in AD as it has been shown in *in vitro* and cellular studies to disaggregate and degrade both A $\beta$  (Grau et al., 2005) and aggregated tau (Poepsel et al., 2015; Tennstaedt et al., 2012). These activities have been proposed to be part of the protein quality control pathways that maintain healthy tissues, and to be protective against the accumulation of extracellular amyloid deposits and intracellular tau aggregates in the brain. Consistent with this, tau over-expression induced upregulation of HTRA1 expression and activity in PC12 rat pheochromocytoma cells (Tennstaedt et al., 2012), and expression of HTRA1 is increased in a transgenic mouse model of AD expressing a mutant form of human amyloid precursor protein (APP) (Searcy et al., 2014). In homogenised samples from AD brains, HTRA1 and tau (total and phosphorylated, P396) levels measured by ELISA were inversely correlated (Tennstaedt et al., 2012). Treating astrocytoma U373 cells with the HTRA1 inhibitor NVP-LBG976 resulted in accumulation of A $\beta$



in cell culture media (Grau et al., 2005). This early study also showed co-localization of HTRA1 with amyloid plaques in AD brain by immunohistochemistry. Subsequently, proteomic studies have determined that plaques contain many proteins in addition to A $\beta$ , including apolipoprotein E (ApoE), a known interactor of A $\beta$  (Wisniewski and Frangione, 1992) and a substrate for HTRA1 *in vitro* (Chu et al., 2016; Wisniewski and Frangione, 1992). Quantitative proteomics of the amyloid plaques in human AD brains compared to non-AD aged brains, in early-onset AD (EOAD), and in the APP/PS1 mouse model reveal that HTRA1 is more abundant in AD (Drummond et al., 2022; Xiong et al., 2019). These data imply that HTRA1 plays a key role in protein homeostasis in the brain, but additional mechanistic insights are required to fully understand whether increased HTRA1 is a cause or consequence of AD.

Dysregulated expression of HTRA1 has also been implicated in cancer, with poor prognosis, increased metastasis and resistance to chemotherapy associated with reduced or absent HTRA1 expression in malignancy in multiple tissues (Chien et al., 2006). The mechanisms governing altered gene regulation remain unclear, although promoter methylation (Lehner et al., 2013), the nuclear receptor RXR $\alpha$ , and the epigenetic regulator HDAC (Wang et al., 2020) have been implicated. Functional insights from *in vitro* studies in cancer cell lines show that over-expression of HTRA1 inhibits cell proliferation (Baldi et al., 2002) and induces cell death (Chien et al., 2004). Some chemotherapeutic agents including cisplatin and paclitaxel upregulate HTRA1 expression in ovarian cancer cell lines, and treatment response correlated with HTRA1 expression level in primary ovarian tumors (Chien et al., 2006). This has led to the suggestion that HTRA1 may be a tumor suppressor, and efforts made to elucidate the mechanism of cell death. The protease activity of HTRA1 appears key to this role, required for degradation of X-linked inhibitor of apoptosis protein (XIAP) (He et al., 2012), activating caspases (Chien et al., 2006) and inhibiting epidermal growth factor receptor (EGFR) signaling in ovarian cancer cells (He et al., 2010) to permit anoikis.

### 6.3. PLEKHA1

PLEKHA1 variants have not been implicated in monogenic disorders to date. A rare variant, rs142473166, resulting in PLEKHA1 c.530G > A, p.S177N, was identified by whole-exome sequencing in a family with early onset AMD; this variant was heterozygous in affected individuals who did not carry the ARMS2 p.A69S variant (Shoshany et al., 2019). No unaffected family members were available for testing; the alternate allele for this variant has a frequency of 0.001691 in gnomAD V2.1.1 ("gnomAD," n.d.) and has not been associated with disease in other studies. Conserved motifs and specific amino acids contribute to the binding of phosphatidylinositides by PLEKHA1 (Dowler et al., 2000; Thomas et al., 2001), but no missense or loss-of-function (LOF) variants have been identified so far. Consistent with a role for PLEKHA1 in transducing PI3K signaling in response to growth-factors or insulin, genetic association studies have implicated non-coding, putative regulatory variants in or near to *PLEKHA1* in contributing to morphometric traits, impaired insulin signaling and autoimmune disorders. Intronic variants in *PLEKHA1* have been associated with a variety of quantitative traits, including birth weight (rs2292626;  $p = 1 \times 10^{-8}$ , (Warrington et al., 2019); rs6585827,  $p = 3 \times 10^{-9}$ , (Plotnikov et al., 2020); rs2421016,  $p = 6 \times 10^{-9}$  (Horikoshi et al., 2016),); body weight and height (rs6585827,  $p = 2 \times 10^{-18}$ , rs3850765,  $p = 4 \times 10^{-33}$  (Sakaue et al., 2021),). Eye-related traits showing association with *PLEKHA1* variants include increased central corneal thickness (rs4311997,  $p = 8 \times 10^{-9}$  (Choquet et al., 2020);) and corneal resistance factor (rs10510110,  $p = 2 \times 10^{-8}$  (Jiang et al., 2020),).

Intronic and 3' UTR variants in *PLEKHA1* have also been associated with altered risk of complex diseases. A recent, large, GWAS of AD including 111,326 clinically diagnosed or 'proxy' AD cases and 677,663 controls reported a novel, suggestively significant association between

*PLEKHA1* variants and AD risk (Bellenguez et al., 2022). Although PheWAS analysis of the lead SNP, rs7908662, identified that the same variant was also significantly associated with AMD risk (although not in LD with rs10490924 and rs11200638), gene prioritization informed by brain single-cell expression data sets ranked *PLEKHA1* over *HTRA1* as the likely causal gene at this locus. This highlights that GWAS associated variants may be pleiotropic, and functional follow-up must be performed in cell types implicated in disease processes for an understanding of biological relevance.

Well-replicated associations with the complex metabolic disease Type 2 diabetes in multiple, cross-ancestry, studies have been reported over the past decade (Mahajan et al., 2014, 2018; Sakaue et al., 2021; Spracklen et al., 2020; Suzuki et al., 2019; Vujkovic et al., 2020; Xue et al., 2018; Zhao et al., 2017) along with reported associations with autoimmune disease (Kichaev et al., 2019; Saevarsdottir et al., 2020). Notably, these lead variants from GWAS for different morphometric traits and complex diseases of autoimmunity are in high LD with one another, but are not in high LD with variants on the 10q26 AMD-risk haplotype.

## 7. Conclusions and future directions

Given that all attempts to develop curative therapy for AMD have failed so far, the lack of understanding may be one of the reasons that keep us away from developing and providing patients with effective treatment options. Here, we have discussed a wealth of data on 10q26, and yet the mist of ignorance on this enigmatic locus remains. "I know, that I don't know", would perhaps be a suitable and poignant conclusion to end this article. Nevertheless, we can draw a few conclusions: On one hand, all three genes harbored at 10q26 are in close proximity in regions of strong linkage disequilibrium, a functional interlink seems likely. On the other hand, comprehensive analyses of the 10q26 risk haplotype have narrowed the genetic risk region to ARMS2, suggesting that this is the true AMD gene at this locus. However, genetic associations do not suffice to prove disease causality and we still lack definite evidence. In this light, any of the 14 SNPs in this haplotype may be involved in long-range control of gene expression, leaving HTRA1 and PLEKHA1 still suspects in the pathogenic pathway.

As the role of all three proteins for AMD remains enigmatic, current and future approaches for their therapeutic targeting or use have to be taken with great care. In particular, HTRA1 targeting may be a double-edged sword, as we do not understand its regulatory role in the choriocapillaris - Bruch's membrane - RPE microenvironment. Protective as well risk-associated variants in 10q26 suggest a sensitive balance of expression and function for both proteins and an interdependency of their expression, localization and context specific function. The lack of knowledge calls for new approaches to the clinical phenotyping of AMD involving big data and artificial intelligence (AI) (Topol, 2019). AI based approaches combined with advanced functional proteomic studies may also shed light on a possible functional connection between complement associated risk proteins and 10q26 associated risk proteins that merge to an overlapping pathogenic molecular mechanism of disease. Many recent efforts have focused on creation of much larger clinical datasets, with a focus on advanced retinal imaging (e.g., the INSIGHT Health Data Research Hub in the United Kingdom (Denniston et al., 2022)). This work will be facilitated by increased migration of hospital information technology infrastructure to the Cloud (Keane and Topol, 2020), and the rapid recent advances in privacy protecting technologies such as federated learning (Kaissis et al., 2020). The combination of larger datasets, privacy protecting techniques, and the latest advances in AI such as self-supervised learning (Krishnan et al., 2022), is likely to yield new insights into disease progression and prognosis, particularly if paired with genetic data. The lack of knowledge also calls for new approaches to deconvolution of this unique operon-like gene cluster using both laboratory methods and bioinformatic analyses.

As the organization of the genetic architecture of *HTRA1* and *ARMS2*

is primate specific, protein centric and cell type specific investigations focusing on the retina, RPE, and choriocapillaris within a primate or humanoid context are needed to elucidate the specific functions and regulation of ARMS2 and HTRA1 with relevance for AMD. Human iPSCs based retina on a dish systems (Achberger et al., 2019b; Haderspeck et al., 2019) that contain choriocapillaris like structures (Cipriano et al., 2022) as well as genotyped human post mortem eye tissues are valuable resources to address a multitude of open questions that remain. Answering those questions will be a necessary prerequisite, not only for understanding 10q26, but also towards closing one of the last open gaps in understanding the genetic contribution of AMD.

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## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

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