**Secretory proteostasis of the retinal pigmented epithelium: impairment links to age-related macular degeneration**

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# **Highlights**

* The retinal pigment epithelium (RPE) spends a huge amount of cellular energy for producing its secretome.
* Multiple pathways are employed to allow protein release from RPE cells.
* RPE secretory proteins govern multiple processes (apical/basolateral ECM (re)modelling, angiogenesis, immune response).
* Altered RPE secretory proteostasis contributes to age- and disease-associated loss of retinal integrity.
* Mutated secreted proteins cause intracellular proteotoxicity and ER stress, alongside extracellular proteolytic imbalance.

# **Abstract**

Secretory proteostasis integrates protein synthesis, processing, folding and trafficking pathways that are essential for efficient cellular secretion. For the retinal pigment epithelium (RPE), secretory proteostasis is of vital importance for the maintenance of the structural and functional integrity of apical (photoreceptors) and basal (Bruch’s membrane/choroidal blood supply) sides of the environment it resides in. This integrity is achieved through functions governed by RPE secreted proteins, which include extracellular matrix modelling/remodelling, angiogenesis and immune response modulation. Impaired RPE secretory proteostasis affects not only the extracellular environment, but leads to intracellular protein aggregation and ER-stress with subsequent cell death. Ample recent evidence implicates dysregulated proteostasis as a key factor in the development of age-related macular degeneration (AMD), the leading cause of blindness in the developed world, and research aiming to characterise the roles of various proteins implicated in AMD-associated dysregulated proteostasis unveiled unexpected facets of the mechanisms involved in degenerative pathogenesis. This review analyses cellular processes unveiled by the study of the top 200 transcripts most abundantly expressed by the RPE/choroid in the light of the specialised secretory nature of the RPE. Functional roles of these proteins and the mechanisms of their impaired secretion, due to age and genetic-related causes, are analysed in relation to AMD development. Understanding the importance of RPE secretory proteostasis in relation to maintaining retinal health and how it becomes impaired in disease is of paramount importance for the development and assessment of future therapeutic advancements involving gene and cell therapies.

**Keywords**

Retinal pigment epithelium RPE; secretory proteostasis; age-related macular degeneration AMD; proteases; cathepsins; protease inhibitors; cystatin C; secretion; leader sequence; ECM; degeneration; neurotrophic; angiogenesis; cytokine; amyloid β; apical; basolateral

# **Introduction to RPE and proteostasis**

The RPE is a highly specialised tissue consisting of post-mitotic cuboidal epithelial cells that play a critical role for the protection, survival and function of photoreceptors (Zinn and Marmor, 1979; Strauss, 2005). RPE functions that help maintain retinal homeostasis include establishment of an outer retinal blood barrier (RBB), transport of nutrients between the choroid and photoreceptors, phagocytosis of spent photoreceptor outer segments (POS), regeneration of visual cycle pigments, and absorption of scattered and reflected light (Bok, 1993; Strauss, 2005). Typically, the apical surface of an epithelium faces an open lumen but the RPE differs as its apical surface is in immediate contact with an extracellular matrix (ECM) known as the interphotoreceptor matrix (IPM) (Rohlich, 1970; Hollyfield, 1999). The highly specialised photoreceptors are surrounded by the IPM establishing a close structural interaction with the RPE (Tien et al., 1992). Basolaterally, RPE cells form the RBB with an underlying support matrix called the Bruch’s membrane (BrM), which separates the neuronal tissue from the vascular network (Booij et al., 2010). The RPE maintains the structure and the function of the microenvironments at both apical and basal sides through the secretion of several proteins (Strauss, 2005; Kay et al., 2013). Protein secretion is tightly regulated in order to ensure that correct protein homeostasis, or proteostasis, is achieved in the surrounding environment.

Generally, proteostasis is achieved by the action of many proteins collectively known as the proteostasis network (PN). The PN is directly involved in processes such as protein synthesis, folding, trafficking, aggregation/disaggregation and degradation. This incorporates translational machinery, chaperones as well as proteins involved in the ubiquitin-proteasome system (UPS) and the autophagic pathway. Components that govern import/export machinery, post-translational modification as well as stress responses, such as unfolded protein response (UPR), are considered supplementary but crucial to the PN (Labbadia and Morimoto, 2015). Proteins destined for secretion employ the PN in order to be secreted at the right levels, in the right structure and at the right location for the right amount of time. Proteins destined for secretion are generally folded inside the cell with subsequent targeting for release through various sorting mechanisms. ER plays a primary role in ensuring secretory proteostasis integrity through quality control pathways that select non-native proteins either towards ER folding or degradation. If non-native proteins are unable to obtain a native folded state, they will be removed from the ER and degraded through ER-associated degradation (ERAD) via the proteasome and autophagy, thus preventing the secretion of non-native dysfunctional proteins into the extracellular environment. Imbalance in the ER quality control mechanisms, caused by environmental, age- and/or genetic-based alterations, results in the accumulation of inappropriate non-native proteins in a condition called ER-stress. When ER-stress occurs, the ability of the ER to fold and traffic proteins outside the cell is compromised and thus affects the secretory proteostasis. A direct consequence of altered levels or misfolded proteins reaching the extracellular milieu is impairment of their normal function in the extracellular environment. In an attempt to restore homeostasis, the UPR is activated.

The UPR system is a short term adaptive response that exerts its effects by temporary upregulation of genes involved in ER protein folding (e.g. molecular chaperones), lipid biosynthesis, and degradation of cellular aggregates (Travers et al., 2000) in coordination with a temporary decrease of ER-localised mRNA translation (Hollien and Weissman, 2006; Hollien et al., 2009). This results in lower levels of ER synthesised proteins. UPR activation also causes an upregulation of macroautophagy activity (Kroemer et al., 2010), likely as a tool for disposing of damaged ER or ER-bound protein aggregates. This pathway can be initiated by one of three independent ER-localised membrane proteins: Inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which all react to ER-stress. The UPR system has been reviewed in detail previously (Bravo et al., 2013). The combined effects of the different arms of the UPR system result in alleviation of ER-stress within the cell. Should homeostasis not be achieved, a delayed response associated with activation of apoptosis pathways is induced (Szegezdi et al., 2006; Lin et al., 2007; Tabas and Ron, 2011). Prolonged chronic ER-stress may then cause disease progression. These ER pathways highlight how levels of proteins destined for secretion are tightly regulated. In addition to ER pathways that regulate secretory proteostasis, secreted proteins themselves can exert chaperone-like activity where they inhibit the aggregation of toxic deposits. Examples of secreted proteins with chaperone-like activity expressed by the RPE include αB crystallin, cystatin C and clusterin (Humphreys et al., 1999; Kaeser et al., 2007; Mi et al., 2007; Boelens, 2014). Thus, proteostasis is integral to both intracellular as well as extracellular protein regulation.

Maintenance of proteostasis is of utmost importance for the post-mitotic RPE cells as they have to survive and function a lifetime in an efficient manner to preserve their own integrity as well as that of the surrounding environment. Not surprisingly therefore, increasing amount of evidence implicates a progressive dysregulation of proteostasis, both inside and outside the RPE, as a key contributor to the pathology of age-related macular degeneration (AMD), the leading cause of blindness in the western world (An et al., 2006; Wang et al., 2009; Golestaneh et al., 2017). AMD is classified as either dry (atrophic), characterised by the formation of yellow aggregates (drusen) in the macula between the RPE and BrM (Jager et al., 2008; Bhutto and Lutty, 2012) or wet (exudative), which in addition to the degenerative alterations seen in the dry form (e.g. drusen formation) is characterised by choroidal neovascularisation (CNV) with presence of blood leakage in the subretinal space. RPE dysfunction is a prominent event in the pathogenesis of both forms. A recent review discussed defects in intracellular RPE cell proteolysis regulation and links with AMD, with particular focus on degradative pathways such as autophagy (Ferrington et al., 2016). The scope of the present review is to focus on RPE secretory proteostasis and its role in health and disease. Firstly, the assessment of the top 200 most abundantly expressed transcripts in the RPE/choroid highlights the specialised secretory nature of the RPE: the analysis emphasises the importance of RPE secretome in the regulation of many important processes and functions performed by the RPE, which help maintain its own function as well as that of its surrounding environment. Thereafter, aberrant alterations of RPE secretory proteostasis, due to age-related and genetic changes and how this links to AMD pathogenesis are exemplified.

# **RPE: a specialised secretory epithelial cell layer**

Top 200 most abundantly expressed transcripts in the RPE/choroid (Wistow et al., 2002); National Eye Institute (NEI) Bank database: <https://neibank.nei.nih.gov/cgi-bin/showDataTable.cgi?lib=NbLib0047>) were subjected to analysis using Signal P and Phobius programmes implemented on the Human Protein Atlas website ([http://www.proteinatlas.org](http://www.proteinatlas.org" \t "_blank); Uhlén M et al, 2015), to predict the presence of signal peptides from amino acid sequences and to select predicted secretory proteins. Notably, 54 proteins were thus predicted to be secretory proteins, with experimental evidence of secretion from the RPE existing for 30 of these (Table 1). In addition, two other proteins influence secretion protein function by being membrane receptors. We suggest a gap in research exploring the specific functions of the top RPE transcripts may explain the difference between predicted and actual number of proteins detected as secreted from the RPE. The fact that computer software used to predict secretion is limited in its ability to fully differentiate between signal peptides and N-terminal transmembrane helices (Petersen et al., 2011) may also contribute to this discrepancy. On the other hand, false positives can be predicted and may explain why some plasma membrane proteins, such as low density lipoprotein receptor-related protein 1 (LRP-1) (Hollborn et al., 2004), are predicted to be secreted (Table 1). Nevertheless, the fact that approximately 30 proteins, all from the top 1% of expressed transcripts, are secreted supports the concept that a huge amount of cellular energy is used by the RPE to synthesise proteins destined for secretion, thus underpinning the specialised secretory nature of the RPE.

## **Multiple secretory pathways are engaged to release proteins from RPE**

The specialised secretory nature of the RPE is further demonstrated by the variety of mechanisms it employs to maximise efficient secretion. Proteins secreted from the RPE generally contain an N-terminal signal peptide or leader sequence that directs them co-translationally to the ER (Paraoan et al., 2001). It is here where they are subsequently processed, folded and post-translationally modified before being released via the Golgi apparatus in what is known as the ‘classical secretory pathway’ (Walter et al., 1984; Harter and Wieland, 1996) (Figure 1A). Proteins can also be released from the cells via unconventional protein secretory pathways and these will be discussed further below (Figure 1B).

Focusing on the classical secretory pathway, the importance of the integrity of the leader sequence can be demonstrated via *in vitro* transfection experiments of fusion proteins. We used the secreted protease inhibitor cystatin C as a model for RPE protein secretion and the functional defects associated with its impairment. For example, we demonstrated for the first time experimentally the essential role of the N-terminal leader sequence as the secretory signal for the precursor form of this protein, with the trafficking of the mature form (lacking the signal/leader peptide) missing the processing through the secretory pathway (Paraoan et al., 2003) (Figure 2A). Conversely, while enhanced green fluorescent protein (EGFP) is exclusively retained within the cell, N-terminal fusion with the cystatin C signal/leader peptide (importantly with removal of the EGFP start codon to ensure single transcription initiation) resulted in expression of a fusion protein that is efficiently processed through the secretory pathway and released outside of RPE cells (Figure 2B). Moreover, mutation of the signal/leader sequence, introducing the naturally occurring A25T amino acid substitution in the penultimate position of the sequence (found in the variant B cystatin C associated with increased risk of AMD), led to a decrease in efficiency of EGFP secretion from cells (Figure 2B). The functional role of cystatin C in RPE biology and AMD will be discussed later in this review. Taken together, these studies emphasise the importance of the signal/leader peptide in the correct trafficking and processing of RPE proteins for secretion, so that physiological amounts are released outside the cell.

Although the signal/leader peptide facilitates protein secretion, the presence of such a signal does not necessarily mean that a protein is destined for release into the extracellular environment. Specific transmembrane domains may be present downstream of the N-terminal signal/leader peptide determining retention of these proteins in membranes (Harter and Wieland, 1996). Furthermore, as these proteins move through the ER-Golgi system, they may undergo post-translational modifications that influence their targeting, such as the mannose-6-phosphate modification for proteins destined for the lysosome (Coutinho et al., 2012). These processes further explain why some predicted secretory proteins are not actually secreted owing to the cells various sorting mechanisms.

In addition to the classical secretion pathway, cells can employ unconventional protein secretory pathways to release proteins in the extracellular environment. There are at least two types of proteins that are secreted unconventionally. The first comprise proteins that lack a signal peptide, which are usually excluded from the ER and Golgi system. Other proteins that contain a signal peptide enter the ER but bypass the Golgi on their way out of the cell (Rabouille, 2017). The mechanism by which these proteins are released into the extracellular environment include exosomal secretion, direct translocation across the plasma membrane into the extracellular space, lysosomal secretion and membrane blebbing (Nickel and Seedorf, 2008). The RPE is known to implement unconventional routes to release proteins outside the cell. For example, the RPE can secrete proteins via exosomal release, which is elaborated below and in the next section (Sreekumar et al., 2010; Klingeborn et al., 2017a; Klingeborn et al., 2017b). [[1]](#footnote-1)

Another route that is likely to be implemented by RPE cells is direct translocation across the plasma membrane; the classic example for this is the fibroblast growth factor 2 (FGF2). This protein, abundantly expressed by the RPE, is involved in angiogenesis and cell survival, and is known to be secreted from the RPE (Mousa et al., 1999; Kolomeyer et al., 2011) most likely by direct translocation across the plasma membrane as it lacks a signal peptide (La Venuta et al., 2015).

Although unconventional secretion pathways can occur under normal conditions, cells usually implement these signalling pathways in situations of stress. One such example of stress that highlights how secretion pathways can change is in the situation of damaged mitochondria in RPE, a characteristic feature in ageing and AMD scenarios. In an *in vitro* aged RPE model in which mitochondrial DNA damage was induced, a decrease in cathepsin D levels and lysosomal activity was observed. This decrease in lysosomal activity was shown to be accompanied by increased exosomal release (Wang et al., 2009). Furthermore, in this model an increase in autophagic markers Atg5 and LC3B was also observed, which indicated an increase in autophagy after damage to the mitochondrial DNA. In healthy cells, material such as damaged mitochondria is removed by the autophagy/mitophagy. However, in the model described the decreased lysosomal activity would suggest that overall autophagic flux may be impaired and that exosomal release may be an alternative mechanism by which the cells initiate clearance of intracellular debris when lysosomal activity is compromised. If stress persists, the continuous release of material could contribute to the formation of extracellular deposits such as drusen. This concept is supported by the presence of intracellular proteins and exosome markers within drusen (Wang et al., 2009).

Moreover, recent data points towards an emerging role of autophagy machinery in protein secretion. A number of autophagy effectors (ATG genes) have been implicated in unconventional secretion of proteins lacking a leader sequence. The mechanism by which these effectors play a role is yet to be fully elucidated but different mechanisms have been suggested, which include formation of autophagosome intermediates carrying cargo fusing with endosomal multivesicular bodies (MVBs) and being secreted (Levine and Kroemer, 2019). In addition, an ATG-related lysosomal secretory mechanism has also been implicated in causing release of proteins into the extracellular environment. This mechanism requires the ATG protein conjugation machinery to insert LC3 into the plasma membrane so that lysosomes can fuse and release material outside (DeSelm et al., 2011). The links between autophagy and secretion have been reviewed extensively recently (Levine and Kroemer 2019; Cavalli and Cenci, 2020). Thus, intracellular and extracellular proteolytic events are clearly co-ordinated and the correct secretory function of cells/tissues such as the RPE is therefore of crucial importance for maintaining both the internal and external protein homeostasis.

## **RPE secretion polarity**

Extra complexity of RPE secretion is afforded by the polarised nature of these cells that helps direct proteins into the apical and basal environments. The importance of polarity in RPE cells is demonstrated by overall secretion increasing when polarity is attained (Sonoda et al., 2009), showing that polarity is a crucial element in ensuring that optimal levels of proteins are secreted from RPE cells. Given the two different environments that RPE faces on its apical and basal side in the form of photoreceptors and BrM/choroidal network, respectively, the polarised secretion from the RPE is crucial for supporting its interaction and maintenance of its surrounding environment.

Due to this directionality in secretion towards the apical and basal environment, additional intricate mechanisms are in place to ensure proteins are targeted and secreted from the appropriate site. Potential mechanisms of polarised protein secretion are understood through knowledge of membrane bound protein trafficking. The sorting mechanisms by which membrane proteins move to the apical and basal regions have been previously reviewed (Folsch et al., 2009; Prydz et al., 2012; Kay et al., 2013; Stoops and Caplan, 2014). It is likely that similar mechanisms exist for secreted proteins. Generally, mechanisms that help sort proteins to the apical and basal membranes occur at the level of the trans-Golgi network (TGN). It is here where incorporation of directional sorting signals occurs within proteins, which leads to proteins moving into distinct apical and basal vesicles (Figure 3).

Basal secretion sorting mechanisms likely involve the addition of signals that are tyrosine based (YxxØ; Ø=represents any amino acid with hydrophobic side chains) or double leucine motifs found within the cytoplasmic region of transmembrane proteins (Figure 3A). These sequence-based signals interact with heterotetrameric adaptor protein complexes (APs) at the cell surface or at different intracellular sites (Traub, 2005; Park and Guo, 2014). APs are involved in transport between the TGN and endosomes, which suggests that basolateral bound proteins traverse recycling endosomes before reaching the basal membrane and that trafficking signals are also recognised at these sites en route to their final destination. An example of a secreted protein that uses a typical basolateral signal is transforming growth factor-α (TGF-α). This protein is synthesised as a membrane-anchored precursor (proTGF-α) that is cleaved proteolytically to release the mature form outside the cell. It is within the cytoplasmic domain of proTGF-α where a dominant basolateral signal is found and drives basolateral sorting and secretion (Dempsey et al., 2003).

Apical secretion sorting signals (Figure 3B) are more varied and include post-translational modifications as well as protein sequences. Post-translation modifications include carbohydrates being present on proteins such as N- and O-linked glycans. For instance, the importance of N-glycans in driving apical secretion was demonstrated through the use of N-glycosylation inhibitors that caused missorting of glycoprotein gp80 in Madin-Darby canine kidney (MDCK) epithelial cells (Urban et al., 1987). Additionally, when an N-glycan sequence was added to the sequence of rat growth hormone, this protein was secreted apically from cells (Scheiffele et al., 1995). Moreover, a deletion of the N-glycan sequence from erythropoietin impaired its apical secretion (Kitagawa et al., 1994). The importance of O-linked glycans for apical sorting has also been demonstrated through similar experiments (Yeaman et al., 1997; Alfalah et al., 1999). Glycosylphosphatidylinositol (GPI) anchors also allow sorting of proteins through apical plasma membrane-bound lipid rafts (Fritz and Lowe, 1996). In addition, apical sorting can occur independently from sorting signals where specific cells provide certain cellular conditions to allow secretion in this direction. For example, thyrocyte cells package and secrete human growth hormone apically in a manner that is independent of glycation but dependent upon cellular conditions such as availability of ions (Prabakaran et al., 1999).

In relation to the RPE, an example of an apically secreted protein is pigment epithelium-derived factor (PEDF), a growth factor with neurotrophic function for the photoreceptors (Cayouette et al., 1999; Becerra et al., 2004). On the other hand, cystatin C, which is secreted abundantly from the basal side of RPE cells, is believed to be important in maintenance and turnover of the BrM (Paraoan et al., 2001; Paraoan et al., 2004). RPE cells also release proteins such as αB crystallin through polarised exosomal release in a polarised manner (Sreekumar et al., 2010; Klingeborn et al., 2017b). The polarised exosomal release of proteins further exemplifies the complexity of the secretory nature of RPE cells. The functions of these proteins, as well as others in health and disease, are detailed later in this review. Compromised RPE polarity affects secretory proteostasis as proteins are not secreted appropriately. Mislocalisation of proteins due to disturbances in cell polarity has been implicated in age-related degenerative diseases such as Alzheimer’s disease (AD) (Yang et al., 2011). Furthermore, age-related stress such as exposure to advanced glycation end products (AGEs) impairs the ability of RPE cells to maintain their polarised state, which in turn affects directional secretion of proteins such as PEDF and cystatin C (Kay et al., 2014). Therefore, the maintenance of a polarised secretory state is without doubt essential for maintaining the homeostasis of tissues surrounding the RPE. Any age-related or genetic alterations in protein secretion from the RPE (be it in targeting, location or amount of protein) will ultimately disturb extracellular proteostasis. Furthermore, not only will the outer environment be affected, but an accumulation of aggregated protein as well as chronic ER-stress will also occur inside RPE cells.

**Table 1:** Proteins from the top 200 most abundantly expressed RPE/choroidal transcripts (expression level rankings as per NEI database <https://neibank.nei.nih.gov/cgi-bin/showDataTable.cgi?lib=NbLib0047>; last accessed December 2019) predicted to be secreted according to information from The Human Protein Atlas website.

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| --- | --- | --- | --- |
| **Expressed transcript from RPE/choroid** | **Transcript expression ranking** | **RPE secretion/polarity** | **Function/processes involved** |
| Insulin-like growth factor binding protein 5 (IGFBP5) | 3 | Secreted by RPE cells (Yang and Chaum, 2003). | IGFBP5 – involved in binding to and regulating biological activity of insulin-like growth factors (IGF) such as IGF-I, a secreted growth factor, which acts in an autocrine manner to influence processes such as RPE proliferation, migration and survival. In addition, the IGF/IGFBP system is known to influence the secretion of the angiogenic factor VEGF from RPE cells (Slomiany and Rosenzweig, 2004; Weng et al., 2009; Zheng et al., 2017).  |
| Prostaglandin D2 synthase | 4 | Secreted by RPE cells and found in IPM (Beuckmann et al., 1996; Kolomeyer et al., 2011). | Prostaglandin D2 synthase is responsible for the biosynthesis of prostaglandin D2, a molecule shown to neuroprotective role as well as being a secretory retinoid transporter (Liang et al., 2005; Tanaka et al., 1997).This enzyme is secreted by RPE cells and suggested to be taken up by photoreceptors (Beuckmann et al., 1996) where it possibly performs similar roles listed above. |
| TIMP metallopeptidase inhibitor 3  | 7 | Secreted basolaterally towards BrM (Kamei and Hollyfield, 1999; Qi et al., 2003).  | MMP inhibitor - Present in normal BrM and postulated to regulate/inhibit local MMP to help maintain rate of turnover and limit choroidal growth (Kamei and Hollyfield, 1999). |
| Prosaposin | 10 | Secreted by RPE cells (Kolomeyer et al., 2011; Toyofuku et al., 2012).  | Neurotrophic factor and role in phagocytosis of POS (Van Den Berghe et al., 2004; Toyofuku et al., 2012). |
| Clusterin  | 11 | Secreted by RPE cells (An et al., 2006). | Found in BrM, chaperone-like activity, drusen biogenesis (Sakaguchi et al., 2002; Yerbury et al., 2007).  |
| Serpin family G member 1 (SERPING1) also known as complement 1 inhibitor (C1IHN) | 17 | Secreted by RPE cells (Kolomeyer et al., 2011). | Inhibits complement activation by interfering with the proteolytic activity of C1r/C1s classical pathway and mannose binding protein-associated serine proteases in the lectin pathway (Wagenaar-Bos and Hack, 2006). |
| Ceruloplasmin | 18 | Secreted by RPE cells (Kolomeyer et al., 2011; Harned et al., 2012). | A ferroxidase enzyme that is important in conversion of ferrous iron to ferric iron. Ferric iron binds to transferrin, which then transport iron to sites of storage or utilisation. Iron bound to transferrin is unable to catalyse free radical reaction and thus Ceruloplasmin has an indirect antioxidant role.  |
| Pigment epithelium-derived factor (PEDF) | 20 | Secreted apically by RPE cells (Becerra et al., 2004). | Growth factor that provides neurotropic support to photoreceptors and maintains a non-angiogenic environment (Cayouette et al., 1999; Dawson et al., 1999). |
| Opticin | 24 | Secreted by RPE cells (Ma et al., 2012). | ECM protein known to have anti-angiogenic properties by binding to collagen and regulating ECM adhesive properties (Le Goff et al., 2012a; Le Goff et al., 2012b).  |
| Complement component 4BPrecursor | 30 | Secreted by RPE cells (Kolomeyer et al., 2011). | This is a protein that helps form, along with complement component 2B, the C3 convertase enzyme specific for the classical and mannose-binding lectin pathways. C3 convertase cleaves C3 to form C3b that eventually leads to activation of the membrane attack complex (MAC) that is involved in pathogen and cell death (Sarma and Ward, 2011).  |
| Secreted protein, acidic, cysteine-rich (SPARC a.k.a. osteonectin) | 32 | Secreted basolaterally by RPE cells (Ratnayaka et al., 2007a). | ECM remodelling (Wei et al., 2012).  |
| Laminin subunit beta 2(LAMB2) | 36 | Produced and potentially secreted towards the BrM by RPE cells (Aisenbrey et al., 2006). | Laminin beta 2 is a subunit that helps produce the ECM heterodimeric proteins such as laminin 14 and 15, which are both present in IPM (Libby et al., 2000). RPE secreted laminin subunit may contribute to IPM matrix development and modelling. In addition, RPE cells are known to produce and secrete Laminin beta 2 into the BrM where it helps form laminin 11 (Aisenbrey et al., 2006). The presence of laminins in the BrM is important for RPE adhesion and stability.  |
| Low density lipoprotein receptor-related protein 1 (LRP-1) | 37 | LRP-1 is a receptor expressed on the RPE cell surface. Although not secreted from RPE cells, interaction with secreted proteins makes this an important protein to include in this review (Hollborn et al., 2004).  | Binds to molecules such as RPE-secreted molecule α2-macroglobulin, where it helps regulate levels of cytokines and growth factors (Hollborn et al., 2004).  |
| Complement component 1, r subcomponent (C1r) | 43 | Secreted by RPE cells (An et al., 2006). | C1r associates with C1s and C1q to form the first component of the classical pathway C1. C1r activates C1s that subsequently cleaves C4 and C2 to form the C3 convertase [28]. |
| Insulin-like growth factor binding protein 7 | 47 | Secreted by RPE cells (Kolomeyer et al., 2011). | Function has not been characterised in RPE cells. This protein differs from other IGFBPs in that it lacks a C-terminal and has 100 times lower affinity for IGF-1 (Yamanaka et al., 1997). Nevertheless, it can still bind to and influence the function of IGFs. Therefore, it could be involved in affecting the secretion of VEGF from RPE cells as the IGF-IGFBP system has been shown to be involved in (Slomiany and Rosenzweig, 2004; Weng et al., 2009; Zheng et al., 2017).  |
| Coagulation factor V (proaccelerin, labile factor) | 48 | No experimental evidence of secretion from RPE cells to date. | Coagulation factor V is a protein that is part of the coagulation system where it functions as a procoagulant factor. It is also involved in the physiological anticoagulant pathway and thus has a dual role in the coagulation system (Duga et al., 2004).  |
| α2-macroglobulin (α2M) | 52 | Secreted by RPE cells (Kolomeyer et al., 2011). | A protease inhibitor that is an important regulator of growth factor homeostasis and extracellular proteostasis (Westwood et al., 2001). α2M binds to several growth factors and cytokines and regulates their clearance via LRP-1 receptor mediated endocytosis (Etique et al., 2013). |
| Myocilin, trabecular meshwork inducible glucocorticoid response (MYOC) | 53 | Released from RPE cells via exosomes (Locke et al., 2014).  | Myocilin interacts with the G-protein coupled receptor, GPR143, in a signal transduction manner to regulate exosome release from RPE cells (Locke et al., 2014).  |
| GNAS complex locus | 54 | No experimental evidence of secretion from RPE cells to date. | GNAS (guanine nucleotide-binding alpha subunit) is a protein subunit, which helps form and make up G proteins. G proteins are located within the cell and are a key element in signal transduction pathways that help link receptor-ligand interactions with the activation of intracellular pathways.  |
| Glycoprotein (transmembrane) nmb | 55 | No experimental evidence of secretion from RPE cells to date.  | Important for the development of RPE layer (Bachner et al., 2002; Loftus et al., 2009). |
| Follistan-like 1(FSTL1) | 59 | Secreted by RPE cells (An et al., 2006). | FSTL1 is a secreted protein that has a role in promoting endothelial cell migration and differentiation. In addition this protein has been shown to help revascularisation in mice ischemic tissue (Ouchi et al., 2008). It is possible that FSTL1 is basolaterally secreted from the RPE where it helps maintain the choroidal endothelial network. However, if this secretion is uncontrolled, it could lead to irregular endothelial growth and contribute to CNV. |
| αB crystallin | 65 | Secreted apically by RPE cells exosomal release (Sreekumar et al., 2010; Kolomeyer et al., 2011). | αB crystallin is a molecular chaperone and has cytoprotective roles. In normal retina, αB crystallin is found in interphotoreceptor matrix. Under stress such as oxidative stress, there is increased uptake of αB crystallin that helps protect photoreceptors from apoptosis by inhibiting caspase 3 and activating DNA repair and apoptosis-related Poly (ADP-ribose) Polymerase (PARP) (Sreekumar et al., 2010). |
| DEAD (Asp-Glu-Ala-Asp) box helicase 17 (p72) | 67 | No experimental evidence of secretion from RPE cells to date. | DEAD box proteins are RNA helicases that are implicated in alteration of RNA structure, microRNA processing. P72 has been shown to influence cellular processes such as autophagy, cell invasion and apoptosis (Zhang et al., 2016).  |
| Cystatin C | 77 | Secreted basolaterally (Paraoan et al., 2001).  | Role in RPE is not fully characterised. However, being a potent inhibitor of cysteine proteases such as cathepsin B, L and S, cystatin C is likely to have an important role in proteolytic control at its site of action, for example in ECM modelling. In addition, cystatin C may be involved in the regulation of amyloid formation and complement regulation.Interestingly, a polymorphism in the cystatin C, which leads to an alternate precursor protein, is associated with increased risk of exudative AMD development. The alternate protein product has impaired intracellular trafficking with subsequent reduced secretion.  |
| Endoplasmic reticulum protein 29(Erp29) | 80 | No evidence of secretion from RPE. However it is expressed at mRNA and protein level in RPE cells (Verma et al., 2007; Huang et al., 2015). | Erp29 is an endoplasmic reticulum protein that functions as an escort chaperone and in protein folding. In an AMD mouse model, Erp29 levels were decreased, which may contribute to accumulation of misfolded protein in affected RPE (Verma et al., 2007). In addition, overexpression of Erp29 is known to protect RPE cells from external stresses i.e. cigarette smoke by enhancing cell viability and barrier integrity (Huang et al., 2015). |
| Decorin | 81 | Secreted by RPE cells (An et al., 2006). | A small leucine rich proteoglycan that has been shown to inhibit angiogenic potential of choroid-retinal endothelial cells by regulating expression of VEGF in RPE cells (Du et al., 2013). In addition, involved in preserving RPE barrier integrity under stressful condition (Wang et al., 2015). |
| Aldolase A, fructose-bisphosphate(ALDOA) | 84 | No experimental evidence of secretion from RPE cells to date. | ALDOA is an enzyme involved in the glycolysis pathway where it catalyses the reversible conversion of fructose-1, 6 bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. It has been reported to be attached to cytoskeletal proteins such as actin where it can aid cell migration (Tochio et al., 2010). Interestingly, insulin stimulation of cells leads to a phosphoinositide 3-kinase (PI3K) pathway-dependent release of actin-bound ALDOA protein and increase in glycolysis (Hu et al., 2016).  |
| CD59 molecule, complement regulatory protein | 96 | CD59 is a cell surface glycophospholipid (GPI) - anchored protein expressed on RPE cells (Yang et al., 2009). Exosomal release from RPE cells occurs under stressful conditions. | CD59 prevent complement membrane attack complex (MAC) assembly (Huang et al., 2006).  |
| TIMP metallopeptidase inhibitor 2(TIMP2) | 99 | Secreted by RPE cells -evidence suggests a predominantly apical secretion (Padgett et al., 1997). | TIMP2, in combination with MMP-14, is involved in regulation of MMP-2 (Butler et al., 1998). MMP-2 is a key enzyme involved in IPM and BrM turnover. TIMP2 has also shown to exhibit MMP independent antiangiogenic effect (Seo et al., 2003). Disrupted MMP-2 activity (reduced levels) has been observed in AMD BrM, which is said to be responsible for impaired turnover and dysfunction of BrM (Hussain et al., 2011). In addition, RPE cells exposed to AMD related stresses i.e. cigarette smoking components leads to increased TIMP2 levels (Zhang et al., 2017). As high levels of TIMP2 are known to inhibit MMP2 activity (Butler et al., 1998), an increase may further augment the physiological effects of reduced MMP2 observed in AMD.  |
| Apolipoprotein D(ApoD) | 100 | No experimental evidence of secretion from RPE cells to date.  | ApoD is a secreted glycoprotein that has many functions including lipid transport. This apolipoprotein is expressed in several tissues including the central nervous system (CNS) (Drayna et al., 1986). Known to have neuroprotective roles by exerting anti-oxidant and inflammatory effects (Do Carmo et al., 2008; Ganfornina et al., 2008). Immunolocalisation study showed that ApoD is localised mainly to the inner retina and the outer plexiform layer. ApoD was not found in the RPE (Zheng et al., 2015). Role in retina is not fully characterised. |
| Secreted phosphoprotein 1(SPP1; also known as osteopontin)  | 105 | RPE is known to express SPP1, which is most likely secreted (Bednar et al., 2013).  | SPP1 is a matricellular protein that is implicated in inflammation, cell migration and angiogenesis (Hamada et al., 2003; Lund et al., 2009). In inflammation, it can regulate macrophage migration as well as modulating cytokine production by macrophages. Role in RPE is not fully characterised. Interestingly, SPP1 is present in drusen and basal deposits in human eyes with AMD (Bednar et al., 2013). Furthermore, IPSC-RPE cells exposed to Bis-retinoid N-retinyl-N-retinylidene (A2E), a component of lipofuscin, led to increased expression of SPP1 (Parmar et al., 2018).The presence of this protein in drusen may be a signal for promoting AMD progression, through recruitment and retention of macrophages and inflammation, as well as CNV. |
| Osteosarcoma amplified 9, endoplasmic reticulum lectin(OS9)  | 108 | No experimental evidence of secretion from RPE cells to date. | OS9 is a ubiquitously expressed ER-associated protein that regulates trafficking of proteins at the ER via its ability to recognise folding states of proteins (Alcock and Swanton, 2009). OS9 is upregulated in response to ER stress where it facilitates ubiquitination and transfer of misfolded proteins to ER-associated degradation pathways (Alcock and Swanton, 2009). One example of a protein that is recognised by OS9 at the ER is the transient receptor potential TRPV4, a plasma membrane protein (Wang et al., 2007). Interestingly, TRPV4 is a receptor localised to apical plasma membrane of RPE cells (Zhao et al., 2015). This receptor is a calcium-permeable channel involved in barrier function (Arredondo Zamarripa et al., 2017). The presence of TRPV4 in RPE cells also suggests that OS9 is expressed in RPE cells. |
| Selenoprotein P, plasma, 1(SEPP1)  | 111 | No experimental evidence of secretion from RPE cells to date. | SEPP1 is an abundant extracellular protein that functions as an antioxidant. In the eye, SEPP1 secreted from the lacrimal glands protects ocular corneal cells from oxidative stress (Higuchi et al., 2010).  |
| Prion protein  | 113 | Prion protein is not secreted but expressed on the basolateral membrane of the RPE (Asthana et al., 2017).  | Prion protein is involved in mediating the transport of iron from the choroidal capillaries to the neuroretina (Asthana et al., 2017). |
| Biglycan  | 119 | No experimental evidence of secretion from RPE cells to date. | Biglycan is secreted member of the class I family of small leucine-rich proteoglycans that is known to interact with many components of the ECM including type I collagen and elastin (Reinboth et al., 2002; Schonherr et al., 1995). Through its interaction with these molecules, Biglycan is said to have role in ECM turnover (Embree et al., 2010). Interestingly, these ECM components including Biglycan are found in the BrM (Booij et al., 2010; Keenan et al., 2012). In addition, biglycan is able to modulate growth factors and cytokine functions through interaction with molecules such as transforming growth factor (TGF) β and tumour necrosis factor (TNF) α (Hildebrand et al., 1994; Tufvesson and Westergren-Thorsson, 2002).  |
| Cathepsin D | 120 | Expressed by the RPE where it functions as a major proteolytic lysosomal enzyme. However, in certain situations, it was shown to be secreted (Frost et al., 2013).  | Cathepsin D is an aspartic protease that plays a critical role in the digestion of engulfed POS in RPE (Rakoczy et al., 1997). Taking this role into account, Cathepsin D processing and regulation is very important in ensuring overall retinal health. A key regulator of cathepsin D processing is a protein called melanoregulin (MREG). In a situation where there is a lack of MREG, cathepsin D processing was impaired with the enzyme also being secreted. This in turn leads to impaired POS degradation (Frost et al., 2013).  |
| Protein disulphide isomerase family A, member 6(PDIA6) | 123 | No experimental evidence of secretion from RPE cells to date. | PDIA6 is a disulphide isomerase that resides in the eukaryotic ER (Hatahet and Ruddock, 2009). This protein interacts with protein kinase RNA-like ER kinase (PERK) and inositol requiring enzyme (IRE)-1 and acts as a negative regulator of the unfolded protein response (UPR), a stress response that functions to restore proteostasis in cells (Eletto et al., 2014; Groenendyk et al., 2014). PDIA6 thus prevents excessive activation of UPR signalling, which favours apoptosis.  |
| Latent transforming growth factor beta binding protein 2(LTBP2) | 128 | No experimental evidence of secretion from RPE cells to date.  | The LTBPs are a group of extracellular multi domain proteins. LTBP2 associates with the ECM components such as fibrillin-1, fibulin-5 and proteoglycans such as heparan sulphate and is shown to be involved in elastic fibre assembly (Hirai et al., 2007; Hirani et al., 2007; Parsi et al., 2010). Deficiency in LTBP2 results in loss of proper ECM assembly. LTBP2 also contains a common integrin recognition site and has been shown to be involved in cell adhesion (Vehvilainen et al., 2003). Recently, LTBP2 was shown to be present in the elastic layer of the BrM alongside other molecules such as fibrillin-1 and fibulin 5 (Mori et al., 2019).  |
| Cathepsin B  | 130 | Secreted by RPE cells (Kolomeyer et al., 2011; Sharif et al., 2014). | Cathepsin B is normally targeted and localised to the lysosome where it is likely to function in photoreceptor outer segment (POS) degradation (Katz and Shanker, 1989) and autophagic processes (Tatti et al., 2012) inside RPE cells. Although functions in relation to the RPE are not understood, work on other cellular systems suggests a role for cathepsin B in ECM remodelling (Buck et al., 1992). Interestingly the known molecular inhibitor of cathepsin B, cystatin C, is predominantly secreted from the RPE towards the BrM (Paraoan et al., 2001), which suggests an interplay between inhibitor and cathepsins in modulating BrM matrix turnover.  |
| Microfibrillar-associated protein 4(MFAP4)  | 142 | No experimental evidence of secretion from RPE cells to date.  | MFAP4 is an extracellular glycoprotein that is involved in elastic fibre assembly (Pilecki et al., 2016). MFAP4 was also shown to regulate cell proliferation and migration (Schlosser et al., 2016).  |
| C1q and tumour necrosis factor related protein 5(C1qTNF5) | 143 | Secreted by RPE cells -evidence suggests a predominant apical secretion (Mandal et al., 2006). | Role in RPE is not fully characterised. However is known to colocalise with ZO1, a tight-junction protein, and is secreted into the IPM (Mandal et al., 2006). A mutation (S163R) in C1qTNF5 causes an autosomal dominant late onset retinal degeneration (L-ORD) disorder, which results in loss of peripheral and central vision. In a similar manner to AMD, a characteristic of this disease is the formation of abnormal extracellular deposits between the BrM and RPE cell layer (Hayward et al., 2003). Furthermore, the mutation causes impaired secretion of C1qTNF5 (Dinculescu et al., 2018).  |
| Nuclear factor, erythroid 2-like 1(NFE2L1) | 144 | No experimental evidence of secretion from RPE cells to date.  | NFE2L1 is a transcription factor that plays an important role in the transcriptional regulation of genes involved in oxidative stress response, inflammation, differentiation and proteostasis (Kim et al., 2016).NFE2L1 signalling occurs in the RPE and plays an important role against oxidative stress (Sachdeva et al., 2014; Takayama et al., 2016). Ageing RPE cells demonstrate impaired protective NFE2L1 signalling following oxidative insult (Sachdeva et al., 2014). Thus, appropriate NFE2L1 signalling is protective for RPE cells against oxidative stress.  |
| C3 and PZP-like, alpha-2-macroglobulin domain containing 8(CPAMD8) | 146 | No experimental evidence of secretion from RPE cells to date.  | CPAMD8 gene encodes a protease inhibitor that belongs to the α2M family of proteins that are involved in innate and acquired immunity regulation (Li et al., 2004). Although the function of this protein is not understood, its structure is consistent with other members of the α2M proteins, which may point towards similar functions. These proteins are generally secreted or membrane-associated and have been shown to be involved in protease inhibition that influences ECM remodelling and growth factor homeostasis. It is likely that CPAMD8 may be involved in similar functions.  |
| Collagen, type I, alpha 2(COL1A2) | 148 | Secreted by RPE cells (An et al., 2006). | COL1A2 gene encodes one of the chains for type 1 collagen, a protein that is found within the BrM (Booij et al., 2010).  |
| Proline/arginine-rich end leucine-rich repeat protein(PRELP) | 155 | PRELP is a secreted protein and experimental evidence suggests that it is synthesised in the photoreceptor and RPE cells after which it is secreted (Birke et al., 2014). | PRELP is an ECM protein that functions as a basement membrane anchor through its interaction with ECM components such perlecan, heparin sulphate and collagen (Bengtsson et al., 2000; Bengtsson et al., 2002), all of which are components of the BrM (Booij et al., 2010; Keenan et al., 2012). Interestingly, PRELP has been shown to inhibit angiogenesis and laser induced CNV, a characteristic of wet AMD, in mice (Birke et al., 2014). As a prerequisite of CNV is the breakage of ECM, it is possible that alterations of PRELP may contribute to wet AMD. Additionally, PRELP reduces MAC formation in laser induced CNV in mice, hence showing a role of PRELP in complement regulation (Birke et al., 2014).  |
| Amine oxidase, copper containing 2 (retina-specific)(AOC2) | 156 | No experimental evidence of secretion from RPE cells to date.  | AOC2 is a retina-specific copper amine oxidase, which are enzymes that catalyse the oxidative conversion of amines to aldehydes (Imamura et al., 1998). The physiological functions of these enzymes are not well understood.AOC2 was shown to be expressed in the ganglion cell layer of the retina (Zhang et al., 2003). The presence of AOC2 in the list of abundantly expressed transcripts for the RPE/choroid encourages further investigation.  |
| Daz associated protein 2 (DAZAP2)  | 158 | No experimental evidence of secretion from RPE cells to date. | DAZAP2 was originally identified as an interacting protein for the germ line specific RNA binding protein DAZ (Tsui et al., 2000). DAZAP2 is also able to modulate the transcription signalling pathway Wnt through interaction with an effector protein called TC4 (Lukas et al., 2009).  |
| Transferrin | 160 | Expressed by RPE cells so likely to be secreted from cells where it helps maintain extracellular iron regulation (Yefimova et al., 2000). Transferrin receptors that are bound to by transferrin protein, are located on the basolateral membrane of the RPE (Martin et al., 2006), which suggests potential RPE secretion of transferrin occurring in a basolateral manner.  | Transferrin is a major extracellular iron transport protein. Iron bound to transferrin is taken up by cells via receptor-meditated endocytosis after which it is stored or used inside the cell (Hentze et al., 2004). With age, iron accumulation occurs in the RPE as well as associated changes in iron regulatory proteins. Transferrin protein levels exhibit an age-related decrease in the RPE, which likely impact iron extracellular transport of iron (Chen et al., 2009).  |
| Chondroitin polymerizing factor(CHPF) | 162 | No experimental evidence of secretion from RPE cells to date.  | CHPF is an enzyme that plays a major role in chondroitin sulphate (CS) (Ogawa et al., 2010). CS is a glycosaminoglycan sidechain of proteoglycans found in the ECM and is known to influence processes of tumour metastasis, proliferation and adhesion (Fthenou et al., 2009). Knockdown of CHPF leads to inhibition of cell growth and increases cell apoptosis of tumour cells (Fan et al., 2017).  |
| Fibulin 1  | 171 | Secreted by RPE cells (Kolomeyer et al., 2011). | Its role in relation to RPE is not fully characterised. However, Fibulin-1 is a secreted glycoprotein that is usually incorporated into the ECM where it regulates processes such as adhesion and motility (Twal et al., 2001). Binds to fibronectin, laminin and nidogen, all of which are found in BrM (Booij et al., 2010; Kunze et al., 2010). Fibulin-1 has been shown to suppress angiogenesis (Xie et al., 2008). Therefore, alterations of this protein may contribute to CNV development in AMD.  |
| Complement component 1, s subcomponent (C1s) | 172 | Secreted by RPE cells (An et al., 2006; Kolomeyer et al., 2011).  | C1s associates with C1r and C1q to form the first component of the classical pathway C1. C1r activates C1s, which subsequently cleaves C4 and C2 to form the C3 convertase (Sarma and Ward, 2011).  |
| Von Willebrand factor (VWF) | 175 | No experimental evidence of secretion from RPE cells to date.  | VWF is an adhesive plasma glycoprotein that has a central role in blood homeostasis. It mediates platelet adhesion to damaged vascular and subsequent platelet aggregation (Peyvandi et al., 2011). As the transcript data presented here is from the RPE/choroid database, it is likely that the high expression of this protein is due to expression by choroidal endothelial cells (Lou and Hu, 1987).  |
| Major histocompatibility complex, class I, C(HLA-C) | 177 | Expressed on the surface of RPE cells (Sugita et al., 2016).  | Major histocompatibility complex proteins play a pivotal role in adaptive immune response. In the RPE, these proteins are involved in maintaining the immune privilege of the eye.  |
| Tissue factor pathway inhibitor 2(TFPI2) | 179 | No experimental evidence of secretion from RPE cells to date.  | TFPI2 is a serine protease inhibitor that is synthesised and secreted into ECM where it is thought to inhibit activity of MMPs and prevent ECM hydrolysis (Rao et al., 1998; Herman et al., 2001; Chand et al., 2005). TFPI2 is a growth promoting factor for RPE cells (Tanaka et al., 2004). In addition, TFPI2 was shown to protect RPE cells from apoptosis and rescue photoreceptor loss in retinal degeneration animal models (Obata et al., 2005).  |

# **Key retinal processes regulated by secreted proteins: Impairment of secretory proteostasis contributes to AMD progression**

The analysis of the profile of secreted proteins establishes that the RPE secretome is involved in the governance of many processes that are vital for the overall retinal health. Interestingly, these processes often require the interplay of more than one secreted protein, which adds extra complexity to the RPE secretory proteostasis in relation to the retina. In addition, a single protein may be involved in more than one process, which means that a defect in the secretion of a specific protein may have deleterious consequences on more than one cellular process. The analysis herein concentrates on specific roles of abundantly secreted RPE proteins and the implications of their impaired function on the compromised proteostasis contributing to AMD pathogenesis. Specific processes regulated by the RPE secretome considered are neuroprotection/neurotrophic functions, ECM (re)modelling and angiogenesis, modulation of protein folding/misfolding, iron transport and immune control.

## **5.1 Dysregulation of neurotrophic factors secreted by RPE cells leads to photoreceptor dysfunction/death**

*Pigment epithelium-derived factor (PEDF)*

PEDF is a highly expressed RPE/choroid transcript known to be secreted predominantly from the apical side of RPE cells (Becerra et al., 2004; Maminishkis et al., 2006). PEDF has neurotrophic roles in the retina where it is involved in photoreceptor cell development and protection from cell death (Cayouette et al., 1999; Volpert et al., 2009). PEDF promotes the development of cone photoreceptors in retina from embryonic chicks by increasing cell proliferation, observed as an increase in number of cone receptors (Volpert et al., 2009). With regards to its role in photoreceptor survival, injection of PEDF into eyes of a retinal degeneration mouse model led to a transient delay of photoreceptor loss, thus showing the survival-promoting ability of this protein (Cayouette et al., 1999). Notably, immunostaining experiments of AMD donor eyes displayed decreased PEDF levels in the RPE and BrM/choroidal complex when compared to age-matched normal eyes (Bhutto et al., 2006). Furthermore, polymorphisms within the PEDF gene are linked to increased risk of AMD development (Hao et al., 2018). Although research investigating the functional consequences on a molecular level of these AMD-linked polymorphisms are lacking, it is conceivable that these mutations may affect proteostasis of extracellular PEDF, e.g. folding, trafficking and levels, which in turn leads to compromised neurotrophic support for photoreceptors.

Another important function of PEDF results from its anti-angiogenic properties in the retinal environment (Dawson et al., 1999). Although predominantly secreted in the apical environment, low levels of basal amount of PEDF may help prevent vascularisation in the BrM/choroid region. In order to fully comprehend the role PEDF plays in angiogenesis, it is important to highlight that a delicate balance exists between PEDF and another secreted protein, vascular endothelial growth factor (VEGF). VEGF is a pro-angiogenic growth factor that is secreted towards the basal side of the RPE (Maminishkis et al., 2006). The regulation of VEGF is critical in ensuring that its activity does not pass a certain threshold where it is able to induce CNV. An important player in this regulation is PEDF, which counteracts the angiogenic potential of VEGF (Bhutto et al., 2006). This scenario is supported by the fact that alterations of PEDF and VEGF expression promote vascularisation whilst also causing retinal cell death, as seen in AMD (Bhutto et al., 2006).

*Prosaposin*

Prosaposin is another highly expressed RPE/choroid transcript, which is secreted from RPE cells (Kolomeyer et al., 2011). This multifunctional glycoprotein was initially identified as a precursor for lysosomal activator products saposins (Sphingolipid Activator Proteins) A, B, C and D. Saposins are lysosomal proteins that facilitate the breakdown of glycosphingolipids (Kishimoto et al., 1992). In addition to its lysosomal function, prosaposin can also be released as a secretory protein that has neurotrophic activity, i.e. regulates survival, development and function of neuronal cells (Meyer et al., 2014). In relation to the retina, a study that investigated prosaposin gene expression in normal and dystrophic RCS rat retina at various stages of development showed that mRNA was detected in rat RPE cells and ganglion cells 14, 21, and 45 days after birth (Van Den Berghe et al., 2004). Prosaposin mRNA levels increased between days 14 and 45 in normal retina but not in the RCS retina, suggesting a role for prosaposin in normal retinal development. Indeed, the increase in prosaposin mRNA in normal retina coincided with photoreceptor development and maturation in addition to the beginning of the phagocytosis process. Furthermore, RPE cells from RCS rats were characterised by the deficiency of ingestion of POS, which coincided with deficient prosaposin expression (Van Den Berghe et al., 2004). Hence, this study highlighted a role for prosaposin in photoreceptor development and maintenance as well as in efficient phagocytosis of POS from RPE cells. Considering these roles, it is likely that the secretion of prosaposin from RPE cells occurs apically to exert its influence.

Prosaposin neurotrophic function was also shown through its interaction with another important glycoprotein expressed by RPE cells called semaphorin 4A. In response to oxidative stress, semaphorin 4A switches endosomal sorting of lysosomal destined prosaposin to exosomal release, which helps prevent light-induced photoreceptor apoptosis (Toyofuku et al., 2012). Interestingly, a point mutation (F350C) in semaphorin 4A, known to be associated with retinal degenerative diseases such as retinitis pigmentosa (RP) and cone rod dystrophy (CRD) (Abid et al., 2006), caused its abnormal localisation in RPE cells both in an *in vivo* mouse model and in overexpressing RPE *in vitro* cell systems. This in turn impaired endosomal sorting of molecules such as prosaposin, which was also accompanied by retinal degeneration *in vivo* (Nojima et al., 2013). Moreover, a further study examining semaphorin 4A through overexpression experiments in ARPE-19 cells showed that disease-linked mutations caused altered localisation of semaphorin 4A from the cell membrane to ER retention (Tsuruma et al., 2012). The ARPE-19 cells with increased expression of semaphorin 4A also presented suppressed phagocytosis ability as well as increased susceptibility to oxidative and ER stress. This recent knowledge about the role played by semaphorin 4A in regulating prosaposin trafficking supports further investigations of mutations in semaphorin 4A and their link to AMD.

These studies collectively highlight, on one hand, how single protein mutations can cause detrimental effect on multiple cellular processes dependent on proteostasis both inside and outside the cell, and on the other hand, the functional link between intra- and extracellular proteostasis. In this case, a single mutation not only causes mistrafficking of semaphorin 4A but also of prosaposin. This loss of proper localisation of interacting proteins contributes to photoreceptor degeneration as well as impairment of RPE phagocytic capability whilst also increasing susceptibility of cells to ER stress.

*αB crystallin*

αB crystallin is a small heat shock protein secreted from the apical side of RPE cells and taken up by photoreceptors where it plays a protective role under situations of stress. αB crystallin performs its cytoprotective effects by inhibiting the activity of caspase-3, a crucial mediator of the apoptotic pathway, as well as activating DNA repair mechanisms to provide protection from oxidative stress (Sreekumar et al., 2010). The exposure of RPE cells to heat shock or oxidative insult caused a significant increase in αB crystallin mRNA and protein, although this was accompanied by apoptotic cell death (Alge et al., 2002). However, RPE cells that were stably transfected with an αB crystallin vector that induced overexpression were more resistant to stress-induced cell injury, showing that an increase in αB crystallin in non-transfected control cells was a possible survival mechanism after stress (Alge et al., 2002).

Interestingly, αB crystallin was proposed as a biomarker for AMD, with increased expression linked to the presence of the disease. Maculae from human patients with dry and wet AMD were analysed immunohistochemically for αB crystallin expression and compared to age-matched controls. The data showed that αB crystallin was expressed in dry and wet AMD RPE cells in the macular region and in areas of RPE hypertrophy (De et al., 2007). This clearly shows that the RPE undergoes changes in protein expression and secretion during disease, with functional implications for surrounding tissues. αB crystallin is also found within drusen (Crabb et al., 2002). Under constant and severe oxidative stress, RPE barrier function may be compromised leading to loss of polarity with αB crystallin being found in the basal side instead of the apical side of RPE cells. The fact that αB crystallin is recognised as a molecular chaperone, having the ability to bind and attempt to correct misfolded/unfolded proteins, opens the possibility that, under these circumstances, it acts at the site of drusen biogenesis. The increased basolateral presence of αB crystallin also suggests that there is less of it at the apical aspect of RPE cells, thus not being able to provide a protective role to photoreceptors under stress leading to degeneration.

# **5.2 ECM (re)modelling and angiogenesis alterations in AMD**

*TIMPs*

The interaction of matrix metalloproteinases (MMPs) and TIMPs play an important role in regulating turnover of ECM. MMPs are involved in degradation of ECM in the normal renewal and turnover of matrix (Birkedal-Hansen et al., 1993). In addition, MMPs are also involved in neovascularisation as they degrade components of capillary basement membrane, which is an initial stage before outgrowth of new vessels. TIMPs, through their inhibitory effects on MMPs, are able to suppress/limit excessive ECM degradation and neovascularisation (Moses and Langer, 1991; Woessner, 1991). Both TIMP3 and TIMP2 are abundantly expressed transcripts in RPE (positions 7 and 99, respectively), and both are documented to be secreted from RPE cells (An et al., 2006; Zhang et al., 2017).

TIMP3 is unique compared to other TIMPs in that it can bind directly to components of the ECM. TIMP3 is present in normal BrM and a postulated role here is to regulate/inhibit local MMPs that helps maintain the rate of turnover and limit choroidal growth (Qi et al., 2003). Notably, TIMP3 content in the BrM increases significantly with age and further in AMD eyes, which may contribute to BrM thickening (Kamei and Hollyfield, 1999). Furthermore, in areas where CNV is observed, the RPE is absent, and virtually no TIMP3 is evident in sub-adjacent BrM. This observation may indicate that the lack of TIMP3 in these areas contributed to CNV development. Mutations in the TIMP3 gene are found in families with an early-onset, inherited form of macular degeneration called Sorsby’s fundus dystrophy (Weber et al., 1994). Immunohistochemical analysis from Sorsby’s fundus disease tissue showed extensive TIMP3 accumulation in thickened BrM in a similar manner to what is observed in AMD donor eyes. In addition, TIMP3 mutations associated with Sorsby’s fundus dystrophy were shown to impair the turnover of TIMP3 in the matrix of RPE cells. The mutations in TIMP3 induce multimerisation and render a portion of the protein resistant to degradation. This feature may explain the increased deposition of the protein in the BrM and eyes from these patients (Langton et al., 2005). Due to the similarity in clinical phenotype between Sorsby’s fundus dystrophy and AMD, it is possible that mutations in TIMP3 may also play a role in the development of AMD. Indeed, studies investigating the genetic link between TIMP3 and AMD were performed (Chen et al., 2010; Kaur et al., 2010), and a susceptibility locus near the TIMP3 region was identified in their genome-wide association study.

*SPARC*

Another protein abundantly expressed and secreted by RPE cells is the Secreted Protein Acidic and Rich in Cysteine (SPARC – also known as osteonectin), a matricellular protein with chaperone-like activity involved in basement membrane (BM) assembly and modelling. SPARC protects BM molecules such as collagen against unfolding and aggregating, with loss of SPARC leading to abnormalities in BM structure (Martinek et al., 2007; Yan et al., 2005). SPARC is synthesised and secreted from RPE cells preferentially towards the BrM (Ratnayaka et al., 2007a). This observation indicates a role for SPARC in the BrM environment where it may help maintain ECM integrity. Indeed, SPARC interacts with and modulates many proteins expressed by the RPE and found in BrM such as laminin-1 and collagen IV (Yan et al., 2005; Aisenbrey et al., 2006; Bradshaw, 2009). An age-related decline in SPARC expression in human RPE cells was evidenced by immunohistochemistry, as intensity of SPARC immunostaining was significantly lower in cells from older donors (Howard et al., 2010). In addition, cultured AMD donor macular RPE cells showed a reduction in SPARC protein production compared to age-matched control cells. Given the BM assembly and chaperone roles that SPARC performs, it is conceivable that any alteration of this protein will contribute to the age-related changes of the BrM, linked to AMD development, by causing abnormal BM proteins aggregation.

*Cystatin C*

Cystatin C is one of the most abundantly expressed and secreted proteins from the RPE (Paraoan et al., 2000; Kay et al., 2014). Being one of the most potent protease inhibitors, cystatin C targets enzymes such as cathepsin B (also abundantly expressed by the RPE), L and S, all of which having experimental evidence of intracellular expression and secretion from the RPE (Kolomeyer et al., 2011; Sharif et al., 2014) (Figure 4).Due to its high level of expression and versatility of functions, cystatin C is likely to have an important role in local proteolytic control at its sites of secretion through balanced interaction with these enzymes. Remarkably, a polymorphism (CST3 SNP rs1064039 causing G to A substitution) of the cystatin C gene, which results in the production of a mutant protein called variant B (with A25T amino acid substitution in the leader sequence), was the first to be associated with increased risk of development of both AMD (exudative form) and AD (Zurdel et al., 2002; Hua et al., 2012; Butler et al., 2015). Variant B cystatin C displays impaired intracellular trafficking, resulting in a fraction of likely incorrectly processed forms being retained/delayed intracellularly in association with the mitochondria and reduced secretion from RPE cells (Paraoan et al., 2004). A similar secretion pattern was also observed in RPE cells differentiated from induced pluripotent stem cells (iPSCs) gene-edited bi-allelically by CRISPR/Cas9 to express the AMD-linked cystatin C (Carlsson et al., 2019) and in donor fibroblasts homozygous for the variant B compared to heterozygous and WT donor fibroblasts (Benussi et al., 2003). The impaired processing and trafficking of variant B cystatin C is most likely due to the change in the hydrophobicity of the secretory signal sequence caused by the substitution of alanine with threonine at the penultimate position of the leader sequence (Paraoan et al., 2004; Ratnayaka et al., 2007b).

Given its preferential basolateral secretion (Paraoan et al., 2001), it was hypothesised that cystatin C is a key modulator of ECM in and around BrM. The fact that RPE secretion of wild-type cystatin C decreases with age (Kay et al., 2014), together with the AGE-induced alterations in the expression of cathepsins by RPE (Sharif et al., 2019), support the concept that an imbalance between levels of cystatin C and its targets in and outside the RPE occur with age and in AMD. Imbalances between cathepsin S and cystatin C expression levels were also shown to play a part in the deregulated proteolytic pathways that contributes to the invasive phenotype of uveal melanoma cells (Paraoan et al., 2009). In a similar manner, an imbalance in the interaction between target enzymes and cystatin C, be it due to age and/or presence of the variant, in relation to the RPE could have detrimental effects on proteolytic regulation and contribute to AMD disease characteristics such as impaired matrix turnover in the BrM and angiogenesis. Indeed, most recent data from studying the ECM-related functional changes in RPE cellular behaviour induced by the variant in iPSCs-derived RPE cells evidenced cellular processes directly linked to development of the exudative AMD phenotype. Specific changes include an increased rate of cell migration, significantly higher rate of laminin and fibronectin degradation, lower transepithelial resistance and increased pro-angiogenic potential ensuing from reduced cystatin C expression (Carlsson et al., 2019).

Further to its role in modulating cathepsin activity and subsequent processes, cystatin C displays chaperone-like activity where it influences Aβ aggregation. Wild-type cystatin C is protective against AD development by inhibiting Aβ deposition (Kaeser et al., 2007; Mi et al., 2007). In addition to its protective effects against Aβ deposition, cystatin C also plays a role in complement activation as it interacts with the member of the complement cascade, C4 (Ghiso et al., 1990). Reduced amounts of cystatin C may lead to increased complement activation, a process linked with AMD pathogenesis (Sparrow et al., 2012; Schramm et al., 2014). Aβ, which is found in drusen, is a known activator of the complement system by inhibiting complement factor I (Wang et al., 2008). Complement factor I is an inhibitor of complement activation and therefore when blocked by Aβ, low grade chronic inflammation occurs in sub retinal tissues (Wang et al., 2008). In addition to its interaction with C4, cystatin C may also influence complement regulation through its interaction with Aβ. In relation to the RPE, cystatin C may target amyloid-β and aid in cell protection, i.e. reducing drusen biogenesis by reducing production of Aβ as well as complement activation. Taken together, reduced amounts of cystatin C at its sites of action, as seen with age as well as in the variant B scenario, not only allow uncontrolled cathepsin activity but also affect Aβ deposition, drusen formation and complement activation/inflammation. This is turn can contribute to loss of retinal integrity and subsequent AMD development.

*Fibulin 1*

The fibulin family, comprising seven different proteins (de Vega et al., 2009), are ECM glycoproteins secreted from a wide variety of tissue (Timpl et al., 2003) – with fibulin 1 being one of the family members most abundantly expressed and secreted by the RPE. Although its role in relation to RPE is not fully characterised, fibulin 1 is usually incorporated into ECM where it regulates processes such as adhesion and motility (Twal et al., 2001). Fibulin 1 is known to bind to fibronectin, laminin and nidogen, all of which are found in BrM (Booij et al., 2010; Kunze et al., 2010). Interestingly, fibulin 1 suppresses angiogenesis and alterations of this protein may therefore contribute to CNV in AMD (Xie et al., 2008).

# **5.3 Dysregulated iron transport and links to retinal degeneration**

In the retina, iron is an essential micronutrient necessary for important functions such as visual phototransduction cascade, synthesis of photoreceptor discs and RPE phagocytosis activity. For example, RPE65, the key isomerase responsible for the conversion of all-trans-retinyl esters to 11-cis-retinal in RPE cells, is dependent on iron for its activity (Moiseyev et al., 2006; Song and Dunaief, 2013). Photoreceptor cells shed and synthesise new disc membrane and this process is dependent on iron-containing enzymes such as fatty acid desaturase. Although iron is needed for many essential processes, excess iron can become a source of free radicals that cause damage to lipid membranes, proteins and ultimately tissue.

RPE cells express proteins involved in iron regulation. An example is *ceruloplasmin*, which is abundantly expressed and secreted from the RPE. Ceruloplasmin is a ferroxidase enzyme that converts ferrous iron to ferric iron, the form that binds to transferrin, an iron transport protein. Interestingly, *transferrin* is also a highly expressed transcript from the RPE/choroid database and RPE is a major site of transferrin synthesis in the body (Yefimova et al., 2000). It is likely that transferrin is secreted out of the RPE cells where it helps regulate iron levels. In addition to transferrin, RPE cells express transferrin receptors 1 and 2 on their basolateral membrane (Yefimova et al., 2000). This observation indicates that the RPE plays a pivotal role in regulating the entry of transferrin-bound iron from the choroid into the retina. Interestingly, iron accumulation is observed in the aged RPE/choroid (Chen et al., 2009). This increased iron inside the RPE is accompanied by expression alterations of key iron regulating proteins such as transferrin and ceruloplasmin, and contributes to a decrease in RPE phagocytosis ability and lysosomal activity (Chen et al., 2009). Furthermore, in a mouse model where a deficiency of ceruloplasmin and another ferroxidase called Hephaestin (Heph) was present, an age-related accumulation of iron alongside retinal degeneration, characteristic of AMD, was observed (Hahn et al., 2004; Hadziahmetovic et al., 2008).

In addition to ceruloplasmin and transferrin, another important protein involved in iron regulation is Prion protein (PrPc). PrPc is not a secreted protein but is found expressed on the basolateral membrane RPE cells where it modulates retinal iron homeostasis by transporting iron from the choroid to the neuroretina (Asthana et al., 2017). Interestingly, prion disease associated retinal degeneration is attributed to a misfolded isoform of prion protein called scrapie (PrPs). The accumulation of misfolded PrPs impairs iron uptake by the RPE and also causes inflammation that subsequently contributes to cytotoxicity (Asthana et al., 2017). Taken together, the importance of iron regulation by the RPE is evident in relation to maintenance of retinal integrity as loss of balance in age and/or due to presence of mutated proteins causes phenotypical characteristics of AMD such as retinal degeneration.

# **5.4 Interaction of LRP-1 and α2M axis – regulation of growth factors, ECM remodelling, cytokine and amyloid β clearance/accumulation**

LRP-1 is a highly expressed endocytic receptor that is found on the membranes of neurons, vascular and RPE cells (Herz and Strickland, 2001; Hollborn et al., 2004). Its level of expression in the RPE is indicative of a significant cellular effort required to produce it and a possible major RPE function. Although it is not a secreted protein *per se*, LRP-1 interacts with a variety of secreted proteins that are able, through endocytosis and signal transduction properties, to influence a diverse group of cellular processes such as ECM proteolysis, cell growth and inflammation (Etique et al., 2013).

LRP-1 functions as a large endocytic receptor that has ligands that include α2M and Aβ. α2M, itself a highly expressed protein by the RPE, is a protease inhibitor that binds to all classes of proteases, including the MMPs. As mentioned earlier, MMPs are involved in degradation of ECM in normal renewal and turnover of matrix, and in neovascularisation (Moses and Langer, 1991; Woessner, 1991; Birkedal-Hansen et al., 1993). α2M complexed to proteases bind to LRP-1 receptor, which leads to the endocytic internalisation and elimination of the active proteases from the external environment. LRP-1 is also able to carry out endocytosis independently from α2M to regulate extracellular protease activities (reviewed previously by (Etique et al., 2013). RPE cells are known to secrete MMPs and their inhibitors TIMPs. The role of LRP-1 and α2M in maintaining MMP activity is thus part of the quality control processes that help maintain ECM homeostasis and ultimately extracellular proteostasis.

Another molecule that is a target of LRP-1 mediated clearance is Aβ. In the brain, LRP-1 is thought to be expressed at endothelial cells in the blood brain barrier, where it helps mediate the clearance of Aβ out of the brain (Zlokovic et al., 2000). The accumulation and aggregation of Aβ peptides trigger the development of progressive neurodegeneration and dementia associated with AD. Transgenic mice strains where there is a deletion of Lrp1 gene specifically within the brain endothelial cells showed reduced removal of injected Aβ peptides across the blood brain barrier (Storck et al., 2016). In addition, an increase in soluble brain Aβ was observed in a mouse model of AD, where Lrp1 gene was also specifically deleted in blood endothelial cells, along with impaired spatial learning and memory deficits due to the lack of LRP-1 protein (Storck et al., 2016). In addition to endothelial cells, *in vitro* experiments showed that neurons also aid clearance of Aβ via LRP-1 endocytosis and lysosomal degradation (Kanekiyo et al., 2013).

In relation to AMD development, it is possible that alterations of LRP-1 may influence the accumulation of Aβ aggregation in the RPE/retina environment. Given the high level at which they express LRP-1, it is likely that RPE cells are involved in clearing Aβ via LRP-1 receptor mediated endocytosis in a similar manner to neurons. An age-related increase in sub-RPE accumulation of Aβ in mice was reported (Hoh Kam et al., 2010) while in human tissue accumulation of Aβ was exclusive in drusen containing eyes and AMD eyes with no Aβ detected in age-matched control eyes with no drusen (Luibl et al., 2006). Hence, it appears that the accumulation of Aβ is a disease-associated event. The increase in Aβ deposits with age/AMD may be due to a shift to increased production and/or reduced ability to remove aggregates. Therefore, it is plausible that a decrease in LRP-1 expression in RPE cells contributes to the lack of removal of Aβ from the external environment, which then subsequently creates an environment where free Aβ can be incorporated in drusen formation. Further work investigating the expression of LRP-1 in age-related and AMD RPE cells and choroidal endothelial cells is needed in order to get a better understanding of this protein and the processes it is involved in.

# **5.5 Clusterin – a secreted chaperone that maintains extracellular proteostasis**

One of the most important mechanisms by which extracellular proteostasis is regulated is through secreted chaperone activity (Wyatt et al., 2013). Extracellular chaperones can bind to and keep soluble proteins that have misfolded due to stress and stop them from forming aggregates. An example of an extracellular chaperone that is secreted by the RPE is clusterin (An et al., 2006; Humphreys et al., 1999). Although the role of clusterin in relation to the RPE is not fully understood, the extracellular chaperone is most likely involved in helping maintain proteins in their correct form. Interestingly, clusterin secretion is increased from AMD donor RPE cells (An et al., 2006) and is also found to be a major constituent of drusen, suggesting a role in drusen formation (Sakaguchi et al., 2002). The increased secretion of clusterin from AMD donor RPE cells could be a response mechanism for keeping extracellular proteostasis in check. Clusterin is known to influence amyloid formation and has been shown to have both protective and harmful effects. Binding of clusterin to pre-amyloidogenic proteins (i.e. prefibrillar forms) stops further aggregation (Yerbury et al., 2007). As RPE cells synthesise and secrete amyloid peptides, it is possible that the secretion of clusterin from RPE cells is a safety mechanism to stop their aggregation (Wang et al., 2012). Therefore it is plausible that an increase in clusterin may be a response of the cells to compensate for the accumulation of Aβ that occurs with age/AMD.

Another hypothesis for the elevated clusterin secretion is that it contributes to drusen formation. Clusterin has a very promiscuous nature and thus is able to bind to many molecules (Bailey et al., 2001). It is conceivable that it acts at sites where biogenesis of drusen occurs. This hypothesis is supported by the findings from a clusterin knockout mouse model of AD in which reduced amyloid deposition and cell death were observed hence showing that clusterin promotes adverse events in AD. Elevated clusterin secretion seen from RPE cells of AMD donors may promote amyloid production and therefore be a drusen promoting event. While the precise mechanisms are yet to be determined, its altered secretion from AMD RPE cells, presence in drusen as well as its promiscuous nature suggest that clusterin dysregulation may play a role in AMD development.

# **5.6 Complement regulation by the RPE secretome in AMD**

The complement system, a major component of the innate immune system, is important in helping maintain tissue homeostasis. This system contains over 30 small proteins which are produced by the liver as soluble proteins found in blood circulation or as proteins associated to cell membranes (Sarma and Ward, 2011). The activation of the complement system can occur by three pathways – the classical, mannose-binding lectin and the alternative pathway. For the activation of the full complement system pathways, C3 and C5 cleavage must occur. This subsequently results in the formation of the MAC and death of pathogens and cells. Interestingly, five highly expressed proteins from the top 200 transcripts from the RPE/choroid are involved in regulation of the complement system. These include serpin family G member 1 (SERPING1) also known as complement 1 inhibitor, complement component 4B precursor, complement component 1r and complement 1s - all having evidence of secretion from RPE cells. In addition, other proteins that are involved in the regulation of complement activation are the complement regulatory protein, CD59 as well as PRELP. CD59 is also one of the highly expressed transcripts from the RPE/choroid database and although not secreted, CD59 is found on RPE cell membranes where it prevents MAC formation (Kim and Song, 2006; Yang et al., 2009). Similarly, PRELP, a secreted protein, also plays a role in prevention of MAC formation (Birke et al., 2014). The expression and secretion of these components highlights that the RPE is involved in complement regulation under normal physiological conditions. The control of complement is tightly regulated and uncontrolled activation can contribute to inflammation and cell/tissue damage during disease.

The role of complement in AMD pathogenesis was studied extensively over the last decade. A series of publications showed that activation products, such as C5a and MAC, can regulate the expression and secretion of pro-inflammatory cytokines as well as growth factors, such as VEGF (Fukuoka et al., 2003; Cortright et al., 2009; Kunchithapautham and Rohrer, 2011). Components of complement system (C3a, C5a and CFH) were also detected in drusen and AMD lesions showed to promote CNV (Nozaki et al., 2006). In addition, increased plasma levels of complement components such as C3a, C3b, C3d, C4a and C5a were documented in AMD patients (Scholl et al., 2008; Reynolds et al., 2009; Lechner et al., 2016). Two proteins highly expressed and secreted from RPE cells highlighted by the current analysis, C1r and C1s, were shown to have increased secretion from AMD donor RPE cells (An et al., 2006). C1r and C1s are proteins that associate with C1q to form the first component of the classical complement pathway C1. The classical pathway is activated when immune complexes are formed after IgG or IgM binds to pathogens or other foreign or non-self-material. The immune complexes are then bound to C1, specifically the C1q component. Activation of C1r and C1s occurs when C1q binds to the immune complexes after which C1s subsequently cleaves C4 and C2 to form the C3 convertase (Sarma and Ward, 2011). The increased secretion from AMD-RPE cells observed in these two components of the C1 complex along other components of complements showing plasma-associated increase in AMD suggests increased local and systemic complement activation in this disease (An et al., 2006; Scholl et al., 2008; Reynolds et al., 2009; Lechner et al., 2016).

To further highlight the link of complement with AMD, polymorphisms in a number of complement genes such as CFH and SERPING1 increase the risk of AMD (Gibson et al., 2012). In this review, one of the proteins identified to be abundantly expressed and secreted from RPE cells is complement 1 inhibitor that is encoded by the SERPING1 gene. Complement 1 inhibitor is the sole regulator of classical pathway of the complement system and acts by inactivating C1r and C1s of the C1 complex. It may be hypothesised that increased C1r and C1s secretion from AMD RPE cells leads to increased complement activation and this may in turn be supported by decreased complement 1 inhibitor levels. Indeed, in some diseases such as hereditary angioedema, complement 1 inhibitor deficiency leads to swelling to the airways and face, thus supporting that a decrease in complement 1 inhibitor leads to inflammation (Longhurst et al., 2015). However, plasma levels of complement 1 inhibitor have been shown to be higher in AMD compared to control samples (Gibson et al., 2012). An increase in complement 1 inhibitor may occur to counteract the increases observed in C1r and C1s in AMD. On the other hand, in patients with chronic inflammatory skin disease an increase in complement inhibitor 1 was seen. This shows that increase in complement 1 inhibitor production is associated with chronic inflammation. What role an increase in complement inhibitor 1 has in AMD is not understood. Therefore, dissecting the role of complement inhibitor 1 and how its plasma-associated increase contributes to AMD will allow better understanding of complement system involvement in this disease.

The combined molecular and genetic evidence led to the current knowledge that complement system dysregulation plays a role in AMD development. The RPE produces and secretes complement proteins in particular those associated with the classical pathway; as such, the alterations of the RPE secretome most likely contribute to complement activation in regards to AMD. In addition, it can be established that complement dysregulation observed in AMD samples is affected by how the RPE secretome changes in different patho-physiological conditions. Under age-related stresses, the RPE is known to lose polarity in secretion (Kay et al., 2014), which may also affect secretion of complement proteins. This may be important with regards to complement proteins where apparent levels of secretion may not be altered but where the level of secreted proteins is affected. This may be important in processes such as drusen formation as well as RPE dysfunction and death. Also, it is important to note that although levels of inhibitors of complement such as C1 inhibitor levels increase in AMD samples, where they are secreted and how they interact with molecular targets will also affect complement activation. A scenario could exist where the RPE secretes more of C1 inhibitor in order to counteract the increases in C1r and C1s but due to loss of polarity in RPE secretion, an imbalance exists between these molecules in a way that pushes towards more activation. Further investigation into how the RPE secretome is disturbed with age with particular focus on directional secretion of complement proteins will increase understanding on how complement system is altered in AMD.

Regarding non-secreted complement proteins, CD59 is a membrane-associated protein that is highly expressed by RPE cells and involved in preventing MAC formation (Kim and Song, 2006; Yang et al., 2009). Reduction of CD59 was suggested to play a role in AMD pathogenesis as it was shown that while CD59 staining in control human RPE cells is minimal, strong apical staining is observed in RPE cells during AMD (Ebrahimi et al., 2013). However, the staining was reduced in RPE that were overlying drusen as well as in RPE cells at the edge of atrophic regions (Ebrahimi et al., 2013). The decreased levels of CD59 during the AMD process were in part explained by the exosomal and apoptotic membranous release of CD59 protein. The decrease in CD59 on RPE cells may also be responsible for causing inadequate control of complement leading to RPE damage and development of AMD.

# **5.7 ER stress in RPE – do mutated secretory protein variants play a role?**

ER stress plays a role in the pathogenesis of many neurodegenerative diseases such as AD and Parkinson’s disease, where loss of proteostasis, misfolded proteins and cell death are common features (Mercado et al., 2016; Gerakis and Hetz, 2018). Similarly, AMD is a degenerative disease that is associated with high levels of proteotoxic stress, oxidative stress, inflammation and cell death. An emerging theme in the understanding of AMD development is the cross talk that exists between these insults and ER stress (Salminen et al., 2010; Kheitan et al., 2017). Prolonged ER stress is implicated as a trigger for angiogenesis through upregulation of VEGF (Roybal et al., 2004; Roybal et al., 2005a). This is particularly relevant as increased VEGF expression is a trigger for CNV, a characteristic of the exudative form of AMD. Furthermore, ER stress has a major impact on the secretory proteins as it compromises the ability of the ER to traffic proteins efficiently. ER stress can lead to alternative cellular secretory mechanisms that aim to compensate the impaired ER function such as the use of autophagy linked secretion, a mechanism described earlier in this review (section 4.1). A recent study showed that intestinal Paneth cells infected with bacteria rerouted antimicrobial lysozyme secretion via ER stress-induced autophagy linked secretory (Bel et al., 2017). In the ageing RPE, autophagy linked secretion may be a compensatory mechanism implemented by cells to alleviate the burden on the ER blockage. However, this process is likely to be disrupted with age as key effectors of autophagy are susceptible to age and AMD-related changes (Golestaneh et al., 2017). Ultimately, hindered ER function and altered secretion from cells would affect the downstream environments these proteins are destined for. Therefore, the role of ER quality control processes in the RPE for maintaining secretory proteostasis cannot be underestimated. If ER stress persists, then mechanisms that ultimately lead to impaired proteostasis and apoptosis would have serious detrimental consequences as RPE cells are post-mitotic, non-regenerative and play vital functional roles in relation to the surrounding environment.

The general mechanisms by which the ER quality systems such as the UPR regulate secretory proteostasis have been reviewed recently (Plate and Wiseman, 2017). Being a specialised secretory cell layer, the RPE invests a very substantial amount of energy into expression and regulation of its secretome. This means that the ER is constantly in use processing and trafficking proteins that will leave the cell. It is possible that mutations in abundantly expressed and secreted proteins from the RPE may cause a persistent ER stress that would ultimately compromise cell function. The concept of mutations in proteins destined for secretion impacting ER function is highlighted in a study in which a mutation in a cutinase protein led to impaired secretion in *Saccharomyces cerevisiae* (Sagt et al., 1998; Sagt et al., 2002). This was also accompanied by significant increase in protein aggregation in the ER, oxidative stress as well as UPR and ERAD. More recently, a missense mutation in the laminin β2 gene (LAMB2), the product of which is important for the basement membrane formation, was shown to impair secretion from podocyte cells. This impaired secretion was again accompanied by ER stress, likely to be from protein misfolding (Chen et al., 2013).A growing body of evidence from varied tissues supports the idea that mutations within proteins that impair their secretion cause misfolding in the ER and promote ER stress. Identification of mutated proteins with impaired secretion from RPE and their possible links with ER stress will give a better understanding of how RPE dysfunctions and ultimately AMD progresses.

One such example is provided by cystatin C, which is expressed at very high levels by the RPE (Kay et al., 2014) and is constantly being moved through the ER. As the polymorphism responsible for producing the AMD- and AD-associated variant occurs at the level of the signal peptide and thus outside of the sequence for mature, biochemically active inhibitor, no functional difference of cleaved protein can be expected, although disruption in the signal sequence determines changes in the processing of the precursor. This explains the impaired secretion of variant B cystatin C as well as the partial intracellular retention with the mitochondria (Paraoan et al., 2004). In addition, variant B cystatin C presents a signal/leader sequence that has higher amyloidogenic propensity compared to the wild type signal sequence (Sant'Anna et al., 2016) (Figure 5). In light of this data, it is highly likely that the presence of the aggregation-prone sites of the signal peptide in the partially (un)processed precursor could lead to the formation of aggregated proteins, either within or outside ER. While the presence of such aggregates within ER may contribute to ER stress, formation of aggregates through the ‘sticky’ hydrophobic residues of the signal peptide elsewhere in or outside the cell may recruit other proteins with additional deleterious effects. The latter scenario may offer an additional explanation for the function of cystatin C in amyloidosis.

In addition to cystatin C, other proteins such as fibulins were highlighted as potentially playing a role in ER-stress linked to retinal degenerations. For example, a single point mutation in the fibulin 3 gene, corresponding to a R345W mutation in the amino acid sequence of the mature protein was associated with development of Malattia Leventinese and Doyne honeycomb retinal dystrophy (Stone et al., 1999). Although the mutation was not found in AMD patients, these conditions share some of the hallmarks of AMD, such as accumulation of sub-RPE drusen. Functional studies on this protein in RPE cells showed that the mutation results in poorly secreted and misfolded protein (Marmorstein et al., 2002) that accumulates in the ER and activates the UPR (Roybal et al., 2005). The low secretion of R345W fibulin 3 in HEK cells could be partially offset by activation of the PERK arm of the UPR (Hulleman et al., 2012). Amino acid-altering mutations in five fibulin genes were observed in AMD patients (Stone et al., 2004). Of these, the secretion of fibulin 5 harbouring AMD-linked mutations was significantly reduced compared to WT protein (Lotery et al., 2006). Biophysical characterisations of some of these mutant fibulins revealed that the G267S mutation in fibulin 5 has an impact on the overall protein structure consistent with extensive misfolding (Schneider et al., 2010). The G412E mutation in fibulin 5 has been shown to result in increased monomeric hydrodynamic radius and increased levels of self-association, indicative of a propensity for aggregation (Jones et al., 2010). Although the fibulin proteins mentioned are not found amongst the top 200 expressed transcripts analysed in this review, the fact that such mutations in other secreted proteins are linked to misfolding, aggregation and ER-stress, further supports a plausible mechanism to how misfolding of abundantly expressed transcripts may occur and contribute to RPE dysfunction and pathology.

# **Future directions**

We have herein highlighted and exemplified the specialised secretory nature of the RPE. The importance and sophistication by which it affects its immediate environment through tightly regulated protein secretion is demonstrated by the abundance of secreted proteins and the multitude of biological pathways they participate in. From an evolutionary perspective, the energy exerted to fuel this machinery simply would not have developed unless the functions it governs were crucial for survival. However, like in any fine-tuned mechanism, seemingly minor disruptions can have major consequences. We now know that dysregulated function of certain proteins expressed and secreted by the RPE dramatically increases the risk of RPE cell death and development of AMD. Altered protein secretion that occurs due to age and in AMD not only affects important extracellular functions such as photoreceptor protection and ECM (re)modelling, but also intracellular proteostasis through accumulation of misfolded protein aggregates and damaged organelles (Figure 6). In some of these examples, the effects can be attributed to direct specific functional changes, e.g. point mutations leading to a change in activity; more elusive are the processes through which combination of proteins and the interplay between them can disrupt RPE proteostasis in a progressive age-related manner, where the cumulative long-term effects have pathological potential. This is why further work into investigating the molecular pathways affected by the RPE secretome and how disrupted secretory proteostasis contributes to disease development is necessary. Using novel cellular and molecular biology techniques, the ability to study the functional effects of genetic mutations and secretome interference in a defined environment are improving rapidly. Our group recently applied CRISPR/Cas9 gene editing technique in an innovative way to generate models of RPE cell lines with bi-allelic mutations (for recessive disease variants) and induced pluripotent stem cells expressing the AMD-linked variant of cystatin C (Supharattanasitthi et al., 2019). This model is currently being used to study how the presence of the disease-associated cystatin C protein impacts proteostasis inside and outside the RPE. The use of CRISPR-Cas9 methodology is now being applied widely to other disease-associated variants to create ‘disease in a dish’ models. These models will undoubtedly allow us to gain a better understanding of the molecular processes affected in RPE leading to AMD and potentially provide points of intervention for much needed novel therapeutic directions.

# **Acknowledgements**

The members of the Ocular Molecular Biology and Mechanisms of Disease group in the University of Liverpool gratefully acknowledge the continued generous support of The Humane Research Trust UK and The Macular Society UK.

The authors have no competing interests.

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**Figure legends**

**Fig. 1.** Cellular pathways employed by the RPE for protein secretion. Protein secretion from the RPE is broadly classified as being via the classical secretion pathway (A) or unconventional secretion pathways (B). Classical secretion involves the ribosomal synthesis of proteins harbouring an ER/Golgi-targeting signal sequence. Translation is accompanied by translocation to the ER where some ER resident proteins are retained, while proteins destined for secretion enter transport vesicles (1) that are moved through the Golgi apparatus and released in secretory vesicles (2) that move to the basolateral or apical side of the RPE monolayer, where they are released into the extracellular space. All other forms of secretion are designated unconventional secretion pathways. These include proteins that initially follow the classical secretory pathway, but bypass the Golgi and instead are released directly from the ER in secretory vesicles. Other secreted proteins lack a signal sequence all together and never enter the ER, but are instead released via direct membrane translocation (4). In addition, cytoplasmic proteins can be released in exosomes. Exosome biogenesis begins with the endosomal system where early endosomes mature into late endosomes or multivesicular bodies (MVBs) (5). Cellular machinery then transports MVBs towards the plasma membrane where they fuse leading to extracellular exosome release (6). MVBs may also fuse with lysosome where vesicle degradation occurs (7).

**Fig. 2.** Role of N-terminal leader sequence in driving protein secretion in RPE cells, investigated using the RPE most abundantly expressed and secreted cysteine proteinase inhibitor, cystatin C as a model. The inhibitor is synthesised as a precursor with a 26-amino acid N-terminal leader sequence (pCysC; WT-wild-type); a naturally occurring point mutation in the leader sequence is translated into amino acid change A25T in variant B (VB) precursor cystatin C. (A) Confocal microscopy images showing transiently transfected living ARPE-19 cells expressing (a) EGFP-tagged cystatin C lacking leader sequence (pMcysC-EGFP), (b)EGFP only and (c) EGFP-tagged precursor cystatin C (pCysC-EGFP), 24 hours post-transfection. CellTracker OrangeTM reagent (Molecular Probes, Inc., Eugene, USA), staining defined boundaries of viable cells through visualisation of membranous intracellular structures (d), (e) and (f). Corresponding bright field images (g), (h) and (i) are indicative of cell viability. Precursor cystatin C is processed through the secretory pathway whereas the ‘leaderless’ cystatin C (mature form) is not. (Image adapted from Paraoan et al., 2003). (B)Immunoblotting analysis of cell lysates and conditioned media (cell number/volumes equalised for quantitative analysis) collected 24 hours after ARPE-19 cells were transiently transfected with EGFP only construct, WT leader sequence fused to EGFP (WT-LS-EGFP) or VB leader sequence fused to EGFP (VB-LS-EGFP). Both WT-LS-EGFP and VB-LS-EGFP fusion constructs were created by removing the start codon for EGFP to ensure start of single transcription in order to allow investigation of how cystatin C leader sequence affects EGFP trafficking. The variant B leader fusion resulted in lower amounts of secreted EGFP compared to the wild-type-driven secretion. EGFP without a leader sequence was not detected in the conditioned media, demonstrating the importance of an intact leader sequence for driving secretion, and how mutations in the leader sequence can impair secretion. Figure 2B contains original data that has not been previously published.

**Fig. 3.** Cellular mechanisms for targeted protein secretion to apical and basal plasma membranes of RPE cells via the endoplasmic reticulum (ER)-Golgi secretory pathway. (A) Proteins destined for basolateral plasma membrane (PM) are labelled with signals in the trans-Golgi network (TGN) after which they are packed in vesicles ((1) circular = secretory; (2) rectangle = transmembrane)). Vesicles will then be trafficked to the basolateral PM directly (3) or via endosomes (4, 5). Some proteins may contain a cytoplasmic domain which has to be cleaved for the release of the mature form into the extracellular environment (6). (B)Proteins, labelled with apical signals (glycans, GPI linkages) in the TGN, are packed into vesicles which are then trafficked directly to the apical PM for secretion (1). In addition, proteins destined for apical secretion may be trafficked to endosomes (2) and then released from the PM (3) or sent to the basolateral PM (4) where they are redirected to the apical PM via transcytosis or via endosomes (5).

**Fig. 4.** Cathepsins protein expression and secretion from RPE cells. (A)Immunohistochemical analysis of human donor RPE tissue shows abundant expression of ECM-degrading cysteine proteases cathepsin B (I), L (II) and S (III). These enzymes drive the breakdown of substrates such as collagen, fibronectin and laminin, processes which are tightly regulated by protease inhibitors such as cystatin C. The negative control (no primary antibody) showed no staining (IV). (B) Immunoblotting analysis of media conditioned by ARPE-19 cells expressing wild-type cystatin C and bi-allelically gene edited to express variant B cystatin C shows increased amounts of active forms of cathepsin B, L and possibly S, compared with the unconditioned media (UcM) (volume of samples equalised before loading and densitometry data normalised to cell number in order to allow comparative secretion per cell analysis shown in the lower panel). The data evidences, for the first time, that RPE cells secrete active forms of cathepsin B and L as well as possible cathepsin S and these are likely to be modulated by cystatin C in the extracellular environment. Figure presents original data, not published previously.

**Fig. 5.** Characterisation of aggregation properties of the variant B (VB) cystatin C leader peptide. Aggregation properties of synthesised VB leader peptide were analysed through an *in vitro* peptide aggregation assay; morphology of aggregates visualised using transmission electron microscopy (TEM) (A) and analysed by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FITR) (B) and dynamic light scattering (DLS) (C). Data collectively show an increase in β-sheet structures and in size of structures highlighting possible presence of mature fibrils accumulating over time, demonstrating that the variant leader peptide has a high amyloidogenic propensity. This can thus impart a higher amyloidogenic propensity to the full-length variant precursor in case of impaired processing. (D) Model of some possible effects of the A25T mutation on VB precursor cystatin C cell trafficking and its possible implications for protein aggregation. WT cystatin C is processed in the ER-Golgi network where the leader sequence is cleaved and the mature protein is secreted. In the extracellular environment, cystatin C can inhibit cysteine proteases (cathepsin B, L and S) as well as interact with Aβ and modulate amyloid formation. On the other hand, VB cystatin C is inefficiently processed and can contribute to formation of protein aggregates through the unprocessed ‘sticky’ hydrophobic residues of the signal peptide, either intracellularly (where some of the molecules are delayed/retained) or extracellularly (not shown) if unprocessed forms are exported. (adapted from Sant’Anna et al., 2016).

**Fig. 6.** Model of normal RPE secretion vs aged/AMD RPE secretion.Healthy RPE cells have a polarised state which leads to directional-specific secretion of proteins into their appropriate environment, in the right amount and at the right time. At the apical side, proteins such as PEDF and aβ crystallin contribute to the protection of the neuronal photoreceptors. At the basal side, secretion of proteins such as SPARC and cystatin C contribute to regulation of ECM (re)modelling. In addition, iron transport transferrin receptors, which are located on the basal plasma membrane, are likely to interact with the basally secreted ceruloplasmin and transferrin proteins and regulate iron transport. As RPE cells age and/or AMD develops, polarised secretion becomes compromised leading to diminished or mis-directed secretion. Age- and AMD-associated accumulation of advanced glycation end products (AGE) in and around the RPE affects expression and contributes to significantly altered secreted protein levels. Subsequently altered extracellular proteostasis causes photoreceptor death, BrM damage and contributes to drusen formation. Within the cell, an accumulation of iron, due to loss of proper secretion of iron regulatory proteins, can impair lysosomal function and thus affect processes such as autophagy/mitophagy that are vital for removal of damaged molecules and cellular organelles. It is hypothesised that mutations linked with increased risk of developing AMD may lead to impaired processing and misfolding of proteins which can cause ER stress as well as formation of aggregates within and outside the cell. The (partial) retention/failure of clearance of such altered proteins may also impact the activity of different cellular organelles. Altered secretion patterns affect both intra- and extracellular RPE proteostasis influencing progression of AMD, thus substantiating the importance of secretion from RPE cells.

**List of abbreviations**

AD Alzheimer’s disease

AGE advanced glycation end product

AMD age-related macular degeneration

AP adaptor protein complex

ATF6 activating transcription factor 6

BM basement membrane

BrM Bruch’s membrane

CNV choroidal neovascularisation

CRD cone rod dystrophy

ECM extracellular matrix

EGFP enhanced green fluorescent protein

ER endoplasmic reticulum

ERAD ER-associated degradation

FGF2 fibroblast growth factor 2

GPI Glycosylphosphatidylinositol

IPM interphotoreceptor matrix

iPSC induced pluripotent stem cell

IRE1 Inositol requiring enzyme 1

LRP-1 low density lipoprotein receptor-related protein 1

MDCK Madin-Darby canine kidney

MMP matrix metalloproteinase

NEI National Eye Institute

PEDF pigment epithelium-derived factor

PERK protein kinase (PKR)-like ER kinase

PN proteostasis network

POS photoreceptor outer segments

PrPcPrion protein

PrPs scrapie

RBB retinal blood barrier

RP retinitis pigmentosa

RPE retinal pigment epithelium

SERPING1 serpin family G member 1

SPARC Secreted Protein Acidic and Rich in Cysteine

TGF-α transforming growth factor-α

TGN trans-Golgi network

UPR unfolded protein response

UPS ubiquitin-proteasome system

VEGF vascular endothelial growth factor

1. We note that there has been substantial discussion regarding nomenclature of the vesicles released by cells. Due to the number of papers in the area reviewed and referenced here, which have used the term “exosomes” and to denote vesicles that have originated from the endosomal system, we are using this term throughout this review. However, readers who wish to understand the current views around various choices of nomenclature may refer to the recent excellent summary of this discussion (Witwer and Thery, 2019). [↑](#footnote-ref-1)