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Progress in developing rodent models of age-related macular degeneration (AMD)

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Progress in developing rodent models of age-related macular degeneration (AMD)

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Abstract

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss, typically affecting individuals from mid-life onwards. Its multifactorial aetiology and the lack of any effective treatments has spurred the development of animal models as research and drug discovery tools. Several rodent models have been developed which recapitulate key features of AMD and provide insights into its underlying pathology. These have contributed to making significant progress in understanding the disease and the identification of novel therapeutic targets. However, a major caveat with existing models is that they do not demonstrate the full disease spectrum. In this review, we outline advances in rodent AMD models from the last decade. These models feature various hallmarks associated with AMD, including oxidative stress, hypoxia, immune dysregulation, genetic mutations and environmental risk factors. The review summarises the methods by which each model was created, its pathological characteristics as well as its relation to the disease in humans.

Keywords: Age-related macular degeneration, Animal models, Rodents, Oxidative stress, Hypoxia, Inflammation, Retina.

1. Introduction

Age-related macular degeneration (AMD) is a sight-threatening disease that causes irreversible loss of central vision. AMD typically affects older individuals (>55 years) and is the most common cause of blindness amongst adults in developed countries (Colijn et al., 2017). AMD has a multifactorial aetiology (Nowak, 2006). Apart from age, various factors have been associated with increased risk, including blue light exposure (Taylor et al., 1990), smoking (Cackett et al., 2008; Mitchell et al., 2002), genetic predisposition (Abbas and Azzazy, 2013; Anastasopoulos et al., 2012; Rohrer et al., 2019), and diet (Robman et al., 2007) as well as comorbidities including cardiovascular disease (Chakravarthy et al., 2010). These factors advance the onset of age-related changes in the retina and may contribute to the earlier onset of the disease. The first changes, which can be visualised by fundoscopy, include the presence of yellow spots under the retina, called drusen, as well as hyperpigmentation of retinal pigment epithelium (RPE) in the macula. This phenotype is categorised as early AMD. The disease can progress to either advanced 'dry' AMD, also known as geographic atrophy (GA), or 'wet' AMD, which is referred to as neovascular AMD. Advanced dry AMD is characterised by atrophy of the photoreceptors and RPE in the macular region, while the presence of newly formed leaky/blood vessels typifies wet AMD associated with haemorrhage and ultimately scarring of the retina (Ehrlich et al., 2008; Fine et al., 2000; Hageman et al., 1995). Histopathologically, early AMD is characterised by the presence of soft drusen, loss of photoreceptor outer segments (POS), and RPE abnormalities including hypopigmentation/hyperplasia/hypertrophy and thickening of the Bruch's membrane (BM). Basal laminar deposits (BLamD) also form between the RPE basement membrane and its plasma membrane, whilst basal linear deposits (BLinD) form between the RPE basement membrane and the inner collagenous layer of the BM. Advanced wet AMD is characterised by the presence of drusen, pigment epithelium detachment and sub-RPE or sub-retinal haemorrhage. Terminal stages also feature the absence of POS and RPE in the lesion area, reduced outer nuclear layer (ONL) thickness, reduced choriocapillaris density, and gliosis. The inner nuclear layer (INL) has also been observed in close proximity to the BM and some patients may develop a disciform scar (Bird et al., 2014; Curcio et al., 1998; Green, 1999; Li et al., 2018; Sarks, 1976)

The daily intake of Age-Related Eye Disease Study (AREDS) recommended supplements, including vitamin C, E, lutein, zeaxanthin, zinc and copper which have been shown to delay the progression from early to advanced AMD (Age-Related Eye Disease Study Research, 2001; Hubschman et al., 2009; Moutray and Chakravarthy, 2011; Nowak, 2006). However, there are no disease-modifying treatments for advanced dry AMD. Inhibition of complement and inflammation showed promising effects in phase 2 clinical trials (Li et al., 2017; Zajac-Pytrus et al., 2015), but phase 3 clinical trials of complement inhibition (CHROMA and SPECTRI) failed to demonstrate any beneficial effects (Holz et al., 2018). Currently, antibodies targeting vascular endothelial growth factor (VEGF) are used to treat or manage, but not cure, wet AMD (Hubschman et al., 2009; Moutray and Chakravarthy, 2011; Nowak, 2006). The lack of disease-modifying therapies for early AMD and/or the GA phenotypes of advanced AMD may be partly due to an incomplete understanding of the complex biological processes underlying the disease.

In the past decade, a number of animal models have been developed that recapitulate salient features of AMD. Such models have the potential to enable proof-of-principle studies prior to clinical trials. Efforts to develop animal models which recapitulate the full disease spectrum have thus far failed. A major limitation in widely utilised rodent models is the absence of an anatomical macula, which is defined histologically as two or more layers of ganglion cells (Remington and Goodwin, 2011). Rodents also lack the fovea, a region in the centre of the macula in which only cone cells are present. The macula contains a mixture of rod and cone photoreceptors but is dominated by the former cell-type (Osterberg, 1935). It is increasingly evident that AMD exhibits a multifactorial aetiology, with influences including oxidative stress, hypoxia, inflammation and angiogenesis driven by a combination of genetic and environmental risk factors. Animal models that incorporate multiple factors may therefore be more relevant to studying AMD. To date, models including zebrafish, pigs and non-human primates have been used to study AMD, however rodent models are preferred due to their ease of breeding/handling, rapid onset of disease and relatively low-cost. A review which summarised some of these models was published in 2012 (Pennesi et al., 2012). However, several new models have since been described. In this review, we provide an updated and comprehensive description of recent rodent models developed to investigate AMD and discuss their potential use for translational as well as drug discovery

studies. The described rodent models have been broadly grouped based on disease pathways, associated AMD-like features, the means by which pathology was induced, or a combination thereof.

2. Models of oxidative stress

The POS containing polyunsaturated fatty acids are prone to cellular damage, forming lipid peroxides following exposure to light (Figure 1) (Winkler et al., 1999). The young retina can regulate oxidative stress and prevent damage due to the presence of natural antioxidants (e.g. lutein and zeaxanthin) and antioxidant enzymes such as superoxide dismutase (SOD), catalase and metallothionein (Yildirim et al., 2011). With ageing, these natural protective mechanisms become less effective or are lost, resulting in lipid oxidation, oxidative stress and damage to POS (Beatty et al., 2000). The phagocytic and proteolytic activities of the RPE are also impaired with age, resulting in the accumulation of intracellular lipofuscin and formation of sub-RPE drusen (Figure 1). Moreover, lipoprotein accumulation contributes to oxidative stress in choroidal endothelial cells, leading to elevated reactive oxygen and nitrogen species associated with inflammation and tissue damage (Feeney-Burns et al., 1984; Okubo et al., 1999; Ryan et al., 2012; Sundelin et al., 1998; Zarbin, 2004). These disease features are well-characterised in AMD patients, allowing investigators to develop animal models targeting a single antioxidant system or the nuclear factor responsible for the expression of a number of antioxidants (see table 1).

2.1. Conditional induction of oxidative stress in mice

Superoxide dismutase is an enzyme that acts as an antioxidant to break down reactive oxygen molecules in the cell. Mice deficient in SOD1 ($SOD1^{-/-}$) (Imamura et al., 2006) or SOD2 ($SOD2^{-/-}$) (Justilien et al., 2007) mimic age-related loss of these enzymes. Early $SOD2^{-/-}$ mouse models were generated by the sub-retinal injection of an adenovirus expressing ribozyme. This decreases the enzyme manganese superoxide dismutase (MnSOD), which in turn triggers oxidative stress in RPE cells. $SOD2^{-/-}$ mice show features of early AMD including BLamD, BM thickening, and increased presence of lipofuscin and vacuolisation in RPE cells, which eventually leads to RPE atrophy. A limitation of this model is the variability in the

amount of adenovirus plasmid delivered by sub-retinal injection. The pathology observed in $SOD2^{-/-}$ mice may also be due to inflammation caused by sub-retinal injection-induced retinal detachment. Furthermore, knockout of SOD2 was not sufficient to replicate the progression from the early stages to advanced AMD (Justilien et al., 2007). To refine this model, the RPE monolayer was directly targeted using the cre-lox system (Biswal et al., 2016; Mao et al., 2014). The resulting conditional knockout of *Sod2* was induced by feeding $Sod2^{flox/flox}$VMD2-cre mice with doxycycline (dox). Knockout of *Sod2* was confirmed by evidence of reduced MnSOD expression in the RPE, which was not observed in the absence of dox. After 6 weeks, the RPE showed increased 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunoreactivity. 8-OHdG is the predominant free radical produced by the oxidation of DNA, and its accumulation in the cell acts as a marker for measuring oxidative stress. Hence, it is used to detect oxidative stress in the RPE. Fundus images of 6 and 9 month old $Sod2^{flox/flox}$VMD2-cre mice on dox showed a white reflective area indicating retinal thinning. Spectral domain-optical coherence tomography (SD-OCT) images revealed 15% and 24% reduction in the ONL thickness and increased choroidal porosity at 6 and 9 months respectively, which indicate degeneration in photoreceptor and choroid tissues. The atrophic area observed by fundoscopy showed vascular leakage and retinal blood vessel abnormalities in fluorescein angiography (FA). Functional measurements by ERG showed a significant reduction of scotopic (dark-adapted) alpha (A) and beta (B) waves and photopic (light-adapted) B waves at 6 and 9 months, confirming reduced photoreceptor function in the atrophic retina. In contrast, A and B waves of the $Sod2^{flox/flox}$VMD2-cre mice without dox were similar to wild type (WT) control mice. Clinical symptoms of hypopigmented spots in the fundus, ONL thinning and reduced photoreceptor function is an indication of atrophy. BLam- like deposits were observed between the RPE and the BM by histology. The thickness of the RPE at 9 months was increased by 30% and EM revealed vacuolated RPE cells, and the photoreceptor inner segment (PIS) and POS located above vacuolated RPE were found to be shortened. No such pathogenic changes were observed in mice without dox. Using confocal imaging to measure autofluorescence revealed a 20-fold increase in retinal fluorescence intensity of 4 month old $Sod2^{flox/flox}$VMD2-cre animals compared to WT mice. The investigators did not observe any evidence of choroidal neovascularisation (CNV) in these mice. Thus, this conditional mouse model mimics the dry form of AMD. Nonetheless, it is

worth noting that both OCT and histological analyses do not show true atrophic pathology in the retinal cross-section, which suggests that the hypopigmented area seen in the fundus might not be an atrophic phenotype as seen in patients.

2.2. Nrf2$^{-/-}$ mice

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a negative regulator of oxidative stress. It is a transcription factor that binds to the antioxidant responsive element located upstream of genes encoding detoxifying enzymes [glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1)] or antioxidant enzymes (glutathione and SOD) (Venugopal and Jaiswal, 1998). Hence, deletion of Nrf2 might lead to uncontrolled oxidative stress as seen in early AMD. An Nrf2 knockout (Nrf2$^{-/-}$) model was created by deletion of exon 4 and 5 in *Nfe2l2* gene. Examination of mouse eyes using fundoscopy showed well-defined drusen-like deposits at 8-11 months (Zhao et al., 2011). By 11-18 months of age, these mice develop larger soft drusen-like deposits with ill-defined margins in the mid-peripheral retina, similar to those observed in human fundus images of early AMD patients. In 18% of Nrf2$^{-/-}$ mice, spontaneous CNV develops between 11-17 months, characteristic of wet AMD. Functional measurements of the retina at 12 months show compromised retinal function, which was confirmed by reduced A and B waves compared to age-matched WT controls. Histologically, a continuous basal deposit underneath the RPE was observed in Nrf2$^{-/-}$ mice at 11 months. At 12 months, an increased vacuolisation of the RPE was seen, with increased autofluorescence. The RPE was also hyperpigmented near the vacuolated region and hypopigmented in non-vacuolated areas. Some areas showed RPE atrophy, especially above the BLam-like deposits in 12 month old Nrf2$^{-/-}$ mice. Compromised autophagy as a consequence of dysfunctional lysosomes was found in electron microscopy (EM) images at 12 months. Thickening of the BM, loss of choroidal fenestration, a thickened choriocapillaris endothelium and infiltrating cells in the sub-RPE region are all observed in 12 month old Nrf2$^{-/-}$ mice. CNV was confirmed in 12 month old Nrf2$^{-/-}$ animals by the presence of new choroidal vessels, breaks in the choriocapillaris endothelial processes, and BM abnormalities. Other observed pathologies include the presence of haemorrhages and exudates in the sub-RPE region, RPE hyperplasia and POS atrophy. Evidence of immune dysregulation includes increased levels of complement factor C3d, serum amyloid P,

vitronectin and IgG in the RPE/BM junction (Zhao et al., 2011). This model, therefore, shows features of both dry and wet AMD, phenotypes which are commonly seen in patients (Wang et al., 1998). The limitation of this model is the presence of only a few mid-peripheral drusen-like deposits, which might suggest that they are not true drusen as seen in AMD patients. A high-fat diet can increase the risk of developing AMD (Cho et al., 2001). Interestingly, Nrf2$^{-/-}$ mice fed a high fat diet (HFD) show advanced RPE hyper/hypopigmentation and hyperplasia with a ~2 fold increase in autofluorescence as well as sub-retinal drusen-like deposits at 16 months. CNV was observed in 15% of Nrf2$^{-/-}$ mice with the presence of infiltrating macrophages and an increased number of sub-retinal microglia compared to WT fed a HFD. No pathology was detected in WT fed normal chow (Zhao et al., 2014).

2.3. PPARβ/δ$^{-/-}$ mice

Peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) is one of the nuclear receptor superfamilies, which also acts as a ligand-activated transcription factor. It regulates various signalling pathways related to AMD, including glucose and lipid metabolism, fatty acid oxidation, extracellular matrix (ECM) turnover, inflammation, apoptosis, proliferation, cell differentiation and angiogenesis (Bishop-Bailey, 2008; Bishop-Bailey and Bystrom, 2009; Malek, 2014). PPARβ/δ is expressed and can be activated in the RPE and choroid (Choudhary et al., 2016). The role of this receptor in the retina has been studied in PPARβ/δ$^{-/-}$ mice developed by Choudhary et al., 2016, which show features of an early AMD-like phenotypes. At 18 months, these mice show RPE hypo/hyperpigmentation, damaged basal infolding, increased autofluorescence, a thickened BM, continuous sub-RPE deposits with increased apolipoprotein E (ApoE) expression in BM and sub-RPE deposits, suggesting a protective role of PPARβ/δ in the ageing retina, possibly by regulating lipid metabolism. Interestingly, PPARβ/δ$^{-/-}$ mice exhibit reduced injury following laser-induced CNV, and similar observations were made using the PPARβ/δ antagonist GSK0660. These studies imply that selective targeting of PPARβ/δ may be a suitable strategy for the treatment of different clinical sub-types of AMD, for example using receptor agonists for treating dry AMD and antagonistic treatments for wet AMD. The PPARβ/δ$^{-/-}$ model encompasses some features of early AMD and also acts as a platform to target CNV. However, these animals failed to show

any evidence of retinal abnormalities, or loss of choroidal fenestrations, which are important features of early AMD.

2.4. PGC-1α$^{+/-}$ mice

Peroxisome proliferator-activated receptor gamma coactivator -1 alpha (PGC-1α) is highly expressed in the INL (Guo et al., 2014). This receptor plays a crucial role in the regulation of angiogenesis, oxidative stress, mitochondrial biogenesis, autophagy and retinal and RPE senescence (Iacovelli et al., 2016; Kaarniranta et al., 2018; Saint-Geniez et al., 2013; Satish et al., 2018) and altered function of PGC-1α might contribute to AMD pathology. Indeed, PGC-1α$^{+/-}$ mice develop early AMD-like pathology, when exposed to a HFD (Zhang et al., 2018). Adult PGC-1α$^{+/-}$ mice fed on a HFD for 4 months show PIS/POS thinning in one eye with the contralateral eye unaffected, which is often observed in AMD patients. EM analysis of RPE shows pronounced lipofuscin granule accumulation, collagenous layer thickening, BLamD as well as a loss of choroidal fenestrations, whilst these changes were not observed in WT control mice fed the same HFD diet. PGC-1α$^{+/-}$ mice also possess a thickened or atrophic BM, degenerated RPE with loss of melanosome, and a thinned photoreceptor layer without infiltrating cells. Migration of RPE to the POS layer and presence of carboxymethyl lysine (a component of drusen) in the thickened BM were also noted along with a decrease in autophagy. Increased expression of *Vegfa* was also found in the retina/RPE of PGC-1α$^{+/-}$ mice fed a HFD compared to WT controls on the same diet. Furthermore, PGC-1α$^{+/-}$ mice show increased levels of drusen proteins ApoE, ApoJ, amyloid precursor protein (APP) expression, ROS, a decrease in SOD2 levels, mitochondrial DNA copy number and mitochondrial complex I activity. Thus, the dysregulated lipid metabolism, dysfunctional autophagy and oxidative stress in PGC-1 α$^{+/-}$ mice may be responsible for the AMD-like pathology in this mouse model, although functioning of the photoreceptors has not been evaluated in these mice.

2.5. PGC-1α/Nrf2 double knockout (dko) mice

Mice lacking expression of both Nrf2 and PGC-1α develop early AMD-like pathology at 12 months of age. Functional ERG measurement of Nrf2/PGC-1α double knockout (dko) mice showed a decrease in A and B waves, implying impaired photoreceptor function. Nrf2/PGC-

1α dko mice show more pronounced autophagy and oxidative stress compared to single knockout mice. TEM analysis of RPE in 12 month old dko mice showed vacuolated cells with numerous autolysosomes, damaged mitochondria with lost basal infolding, increased melanosomes and lipofuscin, a thickened BM, focal photoreceptor damage as well as the presence of electron-dense deposits compared to WT control mice. Histologically, 12 month old dko mice showed ONL thinning, altered BM structure and drusen-like deposits, and RPE cells showed evidence of hypertrophy and degeneration. Accumulation of Iba^{+} cells in the RPE flatmount of 12 month old dko mice confirms an inflammatory response to retinal damage (Felszeghy et al., 2019). Mitochondria also accumulate within autophagosomes of RPE cells, and increased autofluorescence and early endosomes further confirm dysregulated mitophagy and autophagy in dko mice, which could explain the AMD-like pathology observed in these animals (Sridevi Gurubaran et al., 2020). Choroidal abnormalities were not evaluated in this model.

2.6. Cryba1 cko mice

Cryba1 is a gene that encodes the crystalline lens proteins βA1 and βA2, but the gene is also expressed in retinal astrocytes and RPE cells, where it plays a role in lysosomal activity (Sinha et al., 2012; Zigler and Sinha, 2015; Zigler et al., 2011). The conditional knockout (cko) of the cryba1 gene was created by Valapala et al., 2014b using the cre-lox system. Fundus images of these cko mice showed late dry AMD pathology with the evidence of hypo/hyperpigmentation and sub-retinal lesions at the posterior pole at 4 months, and by 12 months these lesions had progressed. A decrease in A and B waves in ERG traces indicates a loss of photoreceptor function in 7 month old Cryba1 cko mice. RPE flatmounts from 7 month old Cryba1 cko mice show a disturbed cellular architecture with a loss of tight junctions. TEM analysis of 2 month old cko mice shows evidence of vacuolated RPE, membranous organelles with undigested POS and loss of basal infolding. With age (9 months), Cryba1 cko mice display large vacuoles, partially degraded POS with increased melanosomes, autophagosomes and decreased type 1 lysosomes compared to $Cryba1^{fl/fl}$ mice, indicating impaired lysosomal function and degeneration of the RPE. Histologically, lipid accumulation was predominantly observed in the RPE of 12 month old animals. Cryba1 cko mice also show increased inflammation with increased expression of glial fibrillary

acidic protein (GFAP), increased numbers of Iba1^{+} cells in retinal flatmounts and increased CCL2 mRNA and protein expression in RPE (Valapala et al., 2014a; Valapala et al., 2014b). However, this model does not show evidence of other AMD features, including BM thickening, ONL thinning or choroidal pathology.

2.7. Mice with a high glycaemic (HG) diet

A high glycaemic (HG) diet is known to induce oxidative stress in the retina, which increases the risk of developing AMD (Cho et al., 2001; Mares-Perlman et al., 1995). Indeed, 12 month old C57BL/6 mice fed a HG diet for 12 months show loss of photoreceptors with progressive thinning of the ONL. The retina shows additional features of degeneration, including inner retinal thinning, photoreceptor disorganisation, IS swelling and loss of synapses in the outer plexiform layer (OPL). The HG diet also induces changes to RPE cells, including vacuolisation, hypopigmentation, disorganisation, thinning and increased sub-retinal deposits. The RPE cells adjacent to degenerated photoreceptors were multi-layered and atrophic, with increased phagosomes, lipofuscin granules and autofluorescence. These changes are not observed in mice on a control diet. Advanced glycation end products, as a biomarker of AMD, were highly expressed in the plasma and retina of mice fed a HG diet, and accumulation of oxidative and lipid peroxidised products was evident in the retina and RPE of HG fed mice, but not in the retina of mice on a control diet. HG diet also changed the gut microbiota, which was confirmed by metabolomics analysis. A HG diet promotes an unclassified gut microbiota profile, whereas a low glycaemic (LG) diet enriches for *Bacteroidales* and *Erysipelotrichi*, which may confer protection against AMD (Rowan et al., 2017). A HG diet appears to promote an early AMD phenotype, but this detrimental effect can be reversed by switching to a LG diet. Further analyses including functional measurements of photoreceptor activity, fundoscopy and OCT scans remain to be carried out.

3. Models of retinal hypoxia

Hypoxia plays a key role in AMD (Arjamaa et al., 2009). In the healthy eye, both the inner and outer retina receive oxygen from retinal endothelial cells and choroidal endothelial

cells, respectively (Wangsa-Wirawan and Linsenmeier, 2003). The age-dependent thickening of the BM, increased distance to the retina due to drusen, choroidal thinning and low choroidal blood flow, impede the transport of nutrients and oxygen from the choroid to the outer retina (Figure 1) (Caprara and Grimm, 2012; Linsenmeier and Padnick-Silver, 2000). With increasing age, the decreased oxygen content in the outer retina might lead to ischemia. On the other hand, elevated retinal hypoxia stimulates the production of VEGF, which promotes the formation of new blood vessels (Stefansson et al., 2011). Hypoxia is mediated through transcription factors known as hypoxia inducible factors (HIFs). Among the HIFs, hypoxia-inducible factor 1α (HIF-1α) plays a crucial role in hypoxic conditions of most cell types. Under normal conditions, HIF-1α undergoes oxidation via hydrolase enzyme (prolyl hydroxylase). However, under hypoxic conditions, this process is bypassed leading to the accumulation of HIF-1α linked with increased VEGF synthesis and CNV (Arjamaa et al., 2009). The importance of HIF in neovascularisation was confirmed in AMD donor eyes which showed HIF-1 α and HIF-2α positivity associated with the CNV membrane (Sheridan et al., 2009).

3.1. P4h-tm$^{-/-}$ mice

Transmembrane Prolyl 4-hydroxylase (P4h-tm) is the regulator of HIF-1α (Koivunen et al., 2007; Oehme et al., 2002). P4h-tm is highly expressed in the brain and eye, and most notably in RPE, as demonstrated by real-time PCR (Leinonen et al., 2016). Investigators manipulated HIF-1α signalling by knocking out its regulator P4h-tm to create an AMD phenotype in mice. Functionally, P4h-tm$^{-/-}$ mice showed normal rod and reduced cone function at 5-7 months. However, by 12-13 months, animals developed compromised rod function but with no functional defects in cones (Leinonen et al., 2016). Histologically, at 10 months the RPE of P4h-tm$^{-/-}$ mice showed evidence of disorganisation, broadened intercellular spaces, basal infolding, BLamD as well as drusen-like sub-RPE debris. At 14.5 months, the RPE underwent significant thinning compared to age-matched WT mice. Furthermore, a change in the ONL thickness and a reduction in the length of the PIS and POS were detected. Both the ONL and POS displayed infolds, suggesting defects in the outer limiting membrane (Stuck et al., 2012). Retinal pathology progressed into old age. For instance, by 29 months, mice showed larger vacuoles as a consequence of enlarged

intercellular spaces between RPE cells as well as continuous BLam- drusen-like deposits in the sub-RPE space. EM micrographs displayed RPE with larger phagosomes in the apical region, indicating compromised phagocytosis. Thus, P4h-tm$^{-/-}$ mice represent features of early AMD but do not show BM thickening or choroidal pathology, suggesting that hypoxia alone is not sufficient to promote AMD-like pathology.

4. Models of retinal inflammation

Studies indicate a significant association of inflammation with the onset and progression of AMD. Complement activation is one of the well-known inflammatory mechanisms linked to AMD (Patel and Chan, 2008; Whitcup et al., 2013). Single nucleotide polymorphisms (SNPs) in complement factors of the alternative complement pathway show the strongest association with AMD. The SNP Y402H (rs1061170) of complement factor H (CFH) was the first significant variant associated with AMD (Haines et al., 2005), resulting in exaggerated activation and deposition of complement in the retina (Edwards et al., 2005; Hageman et al., 2005; Klein et al., 1998; Klein et al., 2005). Other complement regulators include CD46 and CD59, the expression of which are decreased in monocytes of wet AMD patients (Singh et al., 2012). Diminished CD46 and CD59 expressions were also seen in the RPE during early AMD, and further decreased in atrophic AMD, implying that complement deposition may be due to the lack of negative regulation (Ebrahimi et al., 2013). Analysis of drusen proteomics demonstrates the presence of C-reactive protein, C3a, C5a, C5b-9, amyloid beta protein, vitronectin and alpha-1 antitrypsin (Donoso et al., 2006; Mullins et al., 2000; Nozaki et al., 2006; Wang et al., 2010), which confirms the link between inflammation and AMD. In AMD patients, retinal microglia also become reactive in response to RPE degeneration (Gupta et al., 2003). Various cytokines and chemokines, such as CCL2, CCL5, CCR2 and CX3CR1, which are secreted by RPE cells and macrophages, are also involved in the activation and recruitment of microglia to the sub-retinal space (Chen et al., 2008; Ma et al., 2012; Xu et al., 2009), that in turn may trigger CNV (Ma et al., 2012). Similarly, macrophages from the choroid might enter the sub-retinal space following degeneration of the RPE/BM (Figure 1) (Chen and Xu, 2015). Several rodent knock-in and knock-out models have been developed that recapitulate features of immune dysregulation including components of the alternative

complement pathway. Examples include, $CFH^{-/-}$ mice (Coffey et al., 2007), CFH Y402H transgenic mice (Ufret-Vincenty et al., 2010), C3 overexpressing mice and C3a and C5a receptor knockout mice (Nozaki et al., 2006). Targeting regulators of the complement pathway, such as CD46, and/or inflammatory factors, such as cytokines/chemokines or microglia/macrophages may create more accurate models of AMD, some of which are summarised below (see table 1). Additionally, combining genetic models of AMD with environmental risk factors that influence the immune system, such as infection and/or diet, may lead to optimized models that mimic human AMD phenotypes more precisely.

4.1. Complement factor H (CFH) transgenic mice

CFH is a complement regulatory protein involved in the alternative pathway, which is strongly associated with AMD (Haines et al., 2005; Klein et al., 2005). The Y402H SNP affects binding of CFH to C-reactive protein and heparan sulfate, leading to chronic inflammation and increased lipoprotein accumulation in BM and sub-RPE deposits (Clark et al., 2010; Laine et al., 2007). This inflammatory response may be enhanced by age and/or environmental factors that induce lipid peroxidation in the retina, such as a high fat diet. To explore the combined effect of diet and AMD associated polymorphisms in CFH, Landowski et al., 2019 developed a transgenic mouse model by inserting the full length of the normal human CFH Y402 gene (CFH-Y/O), or the AMD associated CFH H402 variant gene, into $CFH^{-/-}$ mice. Homozygous (CFH-H/H) mice were generated by inbreeding, and all mice were given a high fat, cholesterol diet (HFC) or control diet for 8 weeks. The levels of CFH, factor B (FB), and C3 in plasma and in the posterior of the eye were similar in all mice, irrespective of the type of diet. Aged CFH-H/H mice (90 week old) showed impaired photoreceptor function, as evidenced by a decrease in A and B waves in ERG measurements, which was dominated by rods (Bmax1) and not observed in age-matched mice on a normal diet. In addition, multinucleated RPE with larger BLamD could be observed in aged CFH-H/H mice fed a HFC diet, suggesting early pathological signs, reminiscent of human AMD. The pathological changes to the retina were not observed in younger (36-40 weeks) CFH-H/H mice or CFH-H/H mice fed a control diet, suggesting an interaction between age, diet and CFH polymorphisms. Only the eyes from aged mice carrying the AMD variant of CFH (Y402H/H) showed an increase in ApoB48 and ApoA1 levels following a HFC, suggesting that the

dysregulation of lipid metabolism and increased lipid accumulation may be responsible for the observed effects (Landowski et al., 2019). There was no evidence of BM thickening, retinal or choroidal changes reported in this model.

4.2. $CFH^{-/-}$ with a glycaemic diet and $CFH^{+/-}$ mice with a high fat, cholesterol (HFC) diet

Previously developed $CFH^{-/-}$ mice by Coffey et al., 2007 only demonstrated few features of AMD including autofluorescence, disorganised POS, compromised photoreceptor function and BM thinning (Coffey et al., 2007). To refine this model, the deletion of CFH was combined with a HG diet. Histologically, no anatomical changes were evident in the retina of 44 week old $CFH^{-/-}$ or C57BL/6J WT mice when placed on either HG and LG diet. Interestingly, $CFH^{-/-}$ mice given a LG diet showed an increase in the number and distribution of vacuoles in the RPE layer. This was confirmed by EM analysis which showed that RPE cells were highly vacuolated and disorganised with disrupted basal infolding. These changes were accompanied by increased lipofuscin, accumulation of BLamD and infiltrating cells. Furthermore, autophagosomes in the basal infolding of RPE cells were recorded. A thickened BM was only observed in 50 week old mice. Overall, the low glycaemic diet seems to induce an early AMD-like pathology in $CFH^{-/-}$ (Rowan et al., 2014). OCT and retinal function measurements were not studied in these mice. There is also no report of any choroidal pathology.

Another study compared heterozygous $CFH^{+/-}$ mice and homozygous $CFH^{-/-}$ mice and the combined effect of a high fat cholesterol diet (HFC). ERG measurement of $CFH^{+/-}$ mice fed on a HFC diet shows reduced function of photoreceptors. $CFH^{+/-}$ mice fed a HFC also show a 40% and 22% reduction in rod dominant (Bmax1) and cone dominant (Bmax2) response, respectively. Though the ultrastructure of RPE showed larger BLamD in $CFH^{+/-}$ and $CFH^{-/-}$ mice fed a HFC diet compared to WT control mice, RPE flatmounts showed increased numbers of multinucleated cells only in the $CFH^{+/-}$ mice fed a HFC diet. Histological analysis showed a reduced ONL and thinned RPE only in $CFH^{+/-}$ mice fed a HFC diet. Accelerated complement activation and corresponding chronic inflammation was confirmed by increased levels of C3a and C5a in the plasma of $CFH^{+/-}$ mice fed a HFC diet and linked to increased immunoreactivity of $CD11b^{+}$ in RPE/choroid. Flow cytometry analysis of RPE/choroid showed increased $CD64^{+}$ cells with 12.8% of classical vs. 9.8% non-classical

monocyte populations in the $CFH^{+/-}$ mice fed a HFC diet (Toomey et al., 2015). Altogether, $CFH^{+/-}$ mice fed a HFC diet show features of early AMD but fail to demonstrate BM thickening and choroidal pathology.

4.3. $CD46^{-/-}$ mice

CD46 inhibits the alternative complement pathway by acting as a cofactor for factor I to degrade C3b and C4b (Liszewski et al., 2005; Liszewski et al., 2000). CD46 is ubiquitously expressed in humans, but its expression is restricted to the retina and testis in mice (Holers et al., 1992). The presence of CD46 in the mouse retina, RPE and choroid suggests that manipulation of this gene might have the same effect as deleting CFH (Lyzogubov et al., 2014a), as both are involved in the degradation of C3b. At 12 months, $CD46^{-/-}$ mice display increased levels of C5b-9 in the RPE/choroid compared to WT controls, suggesting an accelerated alternative pathway in the retina (Lyzogubov et al., 2016). A phenotype resembling dry AMD was observed in $CD46^{-/-}$ mice at 12 months of age. Histological evaluation demonstrated hypertrophic, multinucleated and vacuolated RPE, implying degeneration of this monolayer. The presence of increased autophagosomes in 12 month old $CD46^{-/-}$ mice indicated the activation of autophagy as a result of RPE degeneration. Fluorescent excitation of the RPE using 405nm, 488nm and 561nm wavelengths displayed increased autofluorescence. This data was supported by electron micrographs which revealed the accumulation of lipofuscin in the RPE cytoplasm as well as the deposition of electron-dense material between the BM and the RPE, coinciding with a thickened BM. Choroidal thinning, with reduced choriocapillary lumen and fenestrations and an increased presence of macrophage-like cells in the sub-retinal space, were also evident in 12 month old $CD46^{-/-}$ mice. Apoptosis of photoreceptors was identified by the reduced density of the nucleus, but with unaltered ONL thickness. Consequently, the diminished number of nuclei in the ONL is likely due to rod photoreceptor shortening or cell death, which was confirmed by probing for rhodopsin protein expression, found to be reduced in 12 month old $CD46^{-/-}$ mice. Low levels of VEGF isoforms (43 and 18KD) in the retinal/RPE/choroid of transgenic mice of a similar age confirmed that there was no evidence of neovascularisation (Lyzogubov et al., 2016). This model therefore demonstrates features of early, dry AMD at

retinal, RPE, BM and choroidal levels. However, anatomical changes and functional measurements such as fundoscopy, OCT and ERG were not assessed in this model.

4.4. Inflammasome mice

Studies in GA eyes revealed reduced expression of Dicer1 (microRNA processing RNAse), whose knockdown in human RPE cells causes the accumulation of Alu RNA and RPE degeneration (Kaneko et al., 2011). The exact role of Alu RNA on GA is unclear. However, canonical inflammasome proteins including NRLP3, pro-Casp1, Pycard, and p-IRAK1, as well as non-canonical proteins such as caspase-4, gasdermin D, IFN-β and cGAS, are increased in donor GA eyes compared to healthy age-matched controls. This suggests the role of inflammasome activation in AMD. To prove this mechanism, a study (Tarallo et al., 2012, Kaneko et al., 2011)) investigated TLR3, TLR7, MyD88 and inflammasome-related gene knockout mice. Sub-retinal delivery of Alu RNA into the TLR3, 7 and 9, and Dicer 1 deficient mice led to RPE degeneration. The fundus of these animals showed GA-like lesions, with a disturbed cobblestone morphology of RPE cells in flatmounts. However, Alu RNA receiving MyD88, NLRP3, Casp1/Casp11, Pycard, Cybb, Gsdmd, Infrar, Irf3, stat2, Tmem, as well as ppif knockout mice failed to show any evidence of RPE degeneration. These findings suggest that Alu RNA is activated through both canonical and non-canonical inflammasome pathways to induce RPE degeneration. The pathogenic effects of Alu RNA were rescued by Casp1 and MyD88 inhibition, as well as IL-1β and IFN β overexpression in WT mice, suggesting these may be targets for future GA treatments.

4.5. P2X7 null mice

The P2X7 receptor belongs to the purinogenic receptor family and is a ligand-gated ion channel, which is activated by its native ligand, ATP. In some cases, P2X7 also acts as a scavenger receptor, the activation of which promotes phagocytosis of apoptotic cells and debris. This receptor acquires its scavenger function only in the absence of ATP (Wiley and Gu, 2012), and is expressed on microglial cells and macrophages (Monif et al., 2010). A failure in the function of this receptor might have a role in the accumulation of unprocessed material observed as drusen in AMD patients. The role of the P2X7 receptor has been studied in Caucasian populations with advanced AMD. The rare haplotype of P2X4 Tyr315cys, along with P2X7 150Arg, represented 4-fold increase in AMD patients compared

to healthy, age-matched controls (Gu et al., 2013). Furthermore, immunohistochemistry studies in macaque eyes confirmed P2X4 and P2X7-positive microglial cells in the inner plexiform layer, as well as P2X4 and P2X7-positive infiltrated macrophages from the choroid in the vicinity of drusen. This suggests an association between P2X7 and AMD (Gu et al., 2013). Vessey et al. 2017 developed a P2X7 null mouse model which demonstrates an early, dry AMD phenotype from 12 to 18 months. Examination of 18 month old P2X7 null mice by fundoscopy showed elevated whitish lesions compared to WT littermates. Functional measurements by ERGs revealed a reduced scotopic rod photoreceptor response amplitude as well as a subtle reduction in the cone post photoreceptor timing in 18 month old P2X7 null mice, confirming impaired photoreceptor function. Analysis of RPE flatmounts from 18 month old P2X7 null mice showed hypertrophic, vacuolated and multinucleated RPE in the peripheral and central retina compared to WT controls. Ultrastructural studies revealed a pronounced thickening of the BM, BLam and BLin-like deposits, as well as electron-lucent deposits, in 12 month old P2X7 null mice, with BM thickening persisting up to 18 months. Histological studies at 12 months reported increased autofluorescence in RPE, but retinal thickness was unchanged at 12 and 18 months despite increased gliosis. Histopathology thus confirmed early signs of disease, including BLam-like and drusen-like deposits, with BM thickening and RPE degeneration similar to that seen in AMD pathology. Furthermore, flow cytometry showed that blood-borne macrophages in 4 and 12 month old P2x7 null mice exhibited a progressive reduction in phagocytosis compared to age-matched WT controls. Reduced phagocytosis was also observed in retinal microglia of 4, 12 and 18 month old P2X7 null mice compared to WT controls, alongside increased macrophages in POS, with C3 and C5 deposits in the sub-RPE region (Vessey et al., 2017). This model did not identify any changes in the retinal structure (ONL and photoreceptor) or the choroidal vasculature.

4.6. Polyethylene glycol (PEG)-induced retinal degeneration mouse model

PEG, in the form of phospholipid methoxy PEG, has been shown to modulate both the classical and alternative complement pathways (Moein Moghimi et al., 2006). Lyzogubov V. V. et al. (2014b) created a modified PEG-induced model of early dry AMD, in which a sub-retinal injection of 0.5 mg PEG promoted RPE atrophy (Lyzogubov et al., 2014b). This differed from their previous PEG model, which had injected 1mg PEG to induce CNV in the

mice after 5 days (Lyzogubov et al., 2011). In the modified model, histological evaluation revealed RPE, BM and photoreceptor abnormalities. At 5 days post-injection, apoptosis occurs in the ONL. The pathology observed included thinning of the ONL with a reduced number of nuclei and condensed chromatin, apoptotic bodies in ONL, and shortened PIS/POS suggesting photoreceptor degeneration. The RPE also showed evidence of thinning and was hypopigmented with increased expression of Atg12, indicating increased autophagy. PEG injected mice showed hyperproliferative RPE as well as an increase in the RPE density as a result of undigested POS accumulation and drusen-like deposits was also observed in the sub-RPE space. Thus, this animal shows a phenotype similar to GA. Apart from cellular changes, sub-retinal PEG injection also altered the expression of several risk genes including *C3*, *Mmp9* and *Htra1* in the RPE/choroid, and *C3*, *Cfi*, *serping1* and *lpl* in the retina. However, this model may be less suited to studying BM or choroidal pathology. Studies of retinal function were not carried out in this model.

4.7. CCL2$^{-/-}$ /CX3CR1 $^{GFP/GFP}$ mice

CCl2$^{-/-}$, CCR2$^{-/-}$, *CX3CR1* $^{-/-}$ single knockout mice and *CCL2$^{-/-}$ /CX3CR1* $^{-/-}$ dko mice developed to study the role of retinal microglia and macrophages showed features of AMD from a young age (Ambati et al., 2003; Chan et al., 2008; Combadiere et al., 2007; Luhmann et al., 2013; Tuo et al., 2007). However, the presence of lesions caused by Crb1 and rd8 mutation has cast some doubts on the usefulness of these models (Vessey et al., 2012). *CCL2$^{-/-}$ /CX3CR1*$^{GFP/GFP}$ mice back-crossed onto a pure C57BL/6J background developed GA-like lesions at 12 months of age, and enhanced pathology was observed following exposure to 800 lux light (Chen et al., 2013). Examination of 12-18 month old CCL2$^{-/-}$/CX3CR1$^{GFP/GFP}$ mice revealed progressive yellow/white lesions in the temporal area. However, there was no evidence of CNV in 18 month old transgenic mice with or without chronic light exposure (Chen et al., 2013), suggesting that this model represents a more atrophic phenotype. The atrophic lesions were also pronounced in size and number, following exposure to chronic intense light. RPE flatmounts of 12-18 month old CCL2$^{-/-}$/CX3CR1 $^{GFP/GFP}$ mice probed with F actin revealed the progressive loss of hexagonal morphology in lesion areas with altered cell junctions. Histological and EM studies showed the RPE in 18 month old CCL2$^{-/-}$/CX3CR1$^{GFP/GFP}$ mice to be hypopigmented and vacuolated with an irregular organisation, alongside a

thickened BM. Photoreceptor degeneration as a consequence of RPE dysfunction was observed in 18 month old transgenic mice with reduced cone arrestin and disordered rhodopsin, as well as GABA (marker for amacrine cells) expression. Synaptophysin expression in the ONL also indicated evidence of photoreceptor degeneration. Furthermore, activated Muller glial cells and GFP+ microglial cells were observed in 18 month old $CCL2^{-/-}$ /$CX3CR1^{GFP/GFP}$ mice, which were activated further following exposure to increased light (Chen et al., 2013). Despite some indicators of atrophic AMD, this model failed to develop key features of the disease seen in humans, including wedge-shaped lesion margins and ONL thinning.

4.8. HTRA1 overexpressing mouse model

The Age-Related Maculopathy Susceptibility 2 (*ARMS2)/* Human High Temperature Requirement Serine Protease A1 *(HTRA1)* locus is highly associated with AMD (Abbas and Azzazy, 2013; Ayub et al., 2019; Mohamad et al., 2019). Both fluorescein and indocyanine green angiography in 12 month old HTRA1 transgenic mice show hyperfluorescent lesions with abnormal branching in the fundus. OCT and histology confirm radial branching of choroidal blood vessels, which enter the sub-RPE and retinal space via the BM, indicative of CNV. There is ~ 18% chance of producing a CNV phenotype in these mice. The likelihood of developing CNV was increased further by exposing animals to cigarette smoke (Iejima et al., 2015). Two in ten HTRA1 transgenic mice developed CNV, while one animal developed sub-retinal deposits with vacuolated and hypopigmented RPE (Nakayama et al., 2014). Unlike HTRA1 transgenic mice, ARMS2 transgenic mice do not display any evidence of AMD (Iejima et al., 2015). These findings further support the association of HTRA1 with neovascular AMD. Since only a proportion of HTRA1 transgenic mice develop CNV, the model supports the likelihood that factors such as age and environmental risks also contribute to AMD.

5. Additional models

The following models have distinct mechanisms of action which do not fit into the categories listed above.

5.1. Acute injury GA model

5.1.1. Induced by sodium iodate

An acute injury model of GA was created by sub-retinal delivery of 5mg/ml of sodium iodate (NaIO3) in Norway rats (Bhutto et al., 2018). Fundoscopy and OCT images taken one-day post-injection revealed acute retinal oedema as well as increased retinal thickness. This initial swelling was followed by a reduction in retinal thickness with the outer retinal layer thinning from day 3 to 7, after which it remained stable until day 28. Furthermore, localised/progressive loss of RPE was observed from 3 days post-injection and a complete loss of RPE was seen by day 7, which persisted up to 28 days post- treatment. Histologically, $NaIO_3$-injected rats showed well-demarcated RPE atrophy in the injected area as well as a migration of RPE to the sub-retinal space. Reduced thickness of ONL and loss of its photoreceptors was observed on day 3 and continued progressing to day 28. At day 7, further photoreceptor loss was observed, which progressed to a complete loss of the RPE layer, ONL and the external limiting membrane (ELM) by day 28. The INL was in close juxtaposition to the BM in the degenerated area. The choriocapillaris showed evidence of atrophy at day 14 and continued to deteriorate until 28 days post-injection. A glial membrane-like structure was formed by overlapping activated Muller cell processes with vimentin-positive processes in the degenerating photoreceptor area at 7 to 28 days post-injection. The authors reported no evidence of CNV and no functional measurements were carried out. This model could be used to study some features of advanced dry AMD.

5.1.2. Induced by laser

Recently, our group developed a mouse model of GA (Ibbett et al., 2019; Ratnayaka and Lotery, 2020), which is referred to as the Southampton AMD model. Lesions were made using an 810nm laser at a medium power of 32mW to create atrophy of the RPE and the ONL without disrupting the BM. This model avoids the CNV phenotype seen in many other laser-induced models (Lambert et al., 2013). Fundoscopy carried out 1 week post-laser showed a coalescence of atrophic/lasered regions which progresses to form a single well-defined lesion after 2, 4 and 8 weeks. OCT images showed the collapse of ONL and INL, which brought the INL and IPL in close proximity to the BM and RPE/choroid. There was also a decrease in overall retinal thickness at the lesion site, which was due to focal photoreceptor atrophy. ERGs showed a ~23% reduction in A and B waves, indicating a

functional defect of the photoreceptors and reduced signalling to Müller/ON-bipolar cells. Ultrastructurally, the lasered eye showed complete photoreceptor loss with an intact RPE/BM at 12 weeks post laser treatment. The RPE showed both hypo and hyperpigmentation as well as evidence of disorganisation and diminished RPE microvilli. These changes were most prominent within the lesion area. Other changes included evidence of increased astrocyte and microglial activation. The extent of pathology diminished away from the lesion site in a graded manner, consistent with histopathological changes reported by Sarks and colleagues in donor GA tissues (Sarks et al., 1988; Sarks, 1976). The mRNA expression further confirmed elevated inflammatory responses in lasered eyes at 4 weeks post-treatment, with significantly increased levels of C3, GFAP, FcγRI and inflammasome related genes (casp1, cas-8, IL-1β, IL-18). Absence of leakage using FA, as well as the absence of VEGF mRNA and protein expression, revealed salient features of GA with no evidence of choroidal involvement. Therefore, our model may be suitable to investigate GA features, neuroinflammation and genetic susceptibility (Ratnayaka and Lotery, 2020). This model could also be useful for evaluating various immunotherapy treatments for GA.

5.2. Aryl hydrocarbon receptor deficiency mouse model

Aryl hydrocarbon (AhR) is a nuclear receptor, and its activation regulates cellular responses to ultraviolet and blue light as well as the elimination of cellular metabolic waste, especially in counteracting age-related damage (Ma, 2011). Haplotype analysis in AMD patients vs age-matched healthy controls suggests that AhR has a minor association with AMD (Esfandiary et al., 2005). High levels of AhR protein were reported in healthy human donor eyes, which decreased with age (Dwyer et al., 2011). AhR is expressed in the retina and RPE. To better understand the role of AhR in the retina, $AhR^{-/-}$ mice were created by deletion of exon 2 of the AhR gene in C57BL/6J mice (Hu et al., 2013). Fundus and OCT images showed choroidal thinning or atrophy. Functional evaluation of 11 month old $AhR^{-/-}$ mice showed a 50% decrease in B waves with no noticeable change in A waves compare to WT littermates. Histologically, thinning of the INL and ONL was observed, with no changes in PIS or POS at 11 and 16 months. Compared to WT controls, RPE cells in 11 month old $AhR^{-/-}$ mice showed an increase in lipofuscin with a 70% elevation in autofluorescence. These findings indicate a

key role for this receptor in controlling metabolic waste in tissues of the outer retina. The RPE cells also displayed multi-nucleation, hyper/hypopigmentation with vacuolisation and sub-RPE deposits by 11 months. This progressed to pronounced RPE vacuolisation and atrophy at 16 months. BlinD in the BM as well as a thickened BM was also observed, whilst the choroid showed evidence of thinning and atrophy without neovascularisation in 11 month old $AhR^{-/-}$ mice. Thus, this model represents an early AMD phenotype. A second $AhR^{-/-}$ model was created by deletion of exon 1 on the C57BL/6N background, which also displayed an atrophic phenotype. Fundus images of these animals showed the development of whitish spots at 3 months, which progressed to patches of RPE atrophy by 12 months. OCT images in 12 month old $AhR^{-/-}$ mice showed evidence of PIS/POS defects, including the presence of hyper-reflective areas in the photoreceptor-RPE interface. These mice also displayed increased autofluorescence and vacuolisation in RPE, with sub-retinal accumulation of microglia above degenerated RPE, as well as choroidal thinning. Activation of the complement pathway was indicated by the presence of CFH deposits in the sub-retinal space of atrophic regions (Kim et al., 2014).

5.3. 5xFAD mice

The 5xFAD model was developed to study Alzheimer's disease (AD) with a focus on amyloid beta pathology in the brain. The 5xFAD mice express 5 mutations in two genes (APP and Presenilin-1) related to AD (Oakley et al., 2006). AD has been reportedly linked with visual impairment, which suggests that this model might be useful in the investigation of AMD pathology (Criscuolo et al., 2018; Ratnayaka, 2016). The 5xFAD mice revealed a phenotype recapitulating salient features of early AMD. Impairment of the blood-retinal barrier was confirmed, with reduced and irregular ZO1 expression in RPE cells of 12 month old 5xFAD mice compared to WT littermates. Increased amyloid-beta deposits were found in the basal layer of the RPE, similar to those reported in donor human AMD tissues (Dentchev et al., 2003; Lynn et al., 2017; Ratnayaka et al., 2015). Ultrastructurally, RPE displayed loss of apical microvilli as well as increased lipofuscin in the cytoplasm. Age-dependent thickening of the BM with BLamD and BlinD was reported, but no distinct drusen-like deposits were observed by 12 months in 5xFAD mice. The choroidal vasculature demonstrated reduced

fenestration (Park et al., 2017). The limitations of this model include the absence of any focal geographic atrophy or pathology in the POS/PIS and ONL layers.

5.4. Col18a1$^{-/-}$ mice

Col18a1 is a heparan sulfate proteoglycan which is present in the basement membranes of most epithelial and endothelial cells. Col18a1/endostatin is reported to be present in the basal lamina of RPE, the basement membrane of BM and in the larger vessels of the choroid in aged and AMD donor eyes (Bhutto et al., 2004). Col18a1/endostatin is involved in regulating angiogenesis (Marneros and Olsen, 2005). ERG measurements of Col18a1$^{-/-}$mice at 2 and 16 months showed reduced and progressive A and B waves confirming impaired photoreceptor function, which worsens over time. Altered vitamin A metabolism reportedly led to a reduction in retinal esters and rhodopsin in 16 month old Col18a1$^{-/-}$ mice compared to WT control, which may explain the observed photoreceptor pathology. EM studies of the RPE in 16 month old Col18a1$^{-/-}$ mice showed minor abnormalities such as disorganised POS and a decrease in interactions with the apical RPE microvilli. The RPE contained electron-dense material with membranous debris as well as sub-RPE deposits. Age-dependent enlargement in sub-retinal deposits was observed in 22 month old Col18a1$^{-/-}$ mice compared to age matched WT controls. The RPE is damaged above these pathogenic deposits, which was confirmed by expression of low RPE65 protein levels in 16 month old mice retinal lysates. Col18a1$^{-/-}$ mice also show increased ubiquitin expression in the basal side of RPE cells. RPE degeneration and age-dependent progressive sub-RPE deposition with RPE cell death were evident in 18 month old Col18a1$^{-/-}$ mice (Marneros et al., 2004). P62/sqstm1 and beclin 1 were also increased in the knockout mice (Kivinen et al., 2016), suggesting impaired autophagy. In 16 month old Col18a1$^{-/-}$ mice, increased GFAP and F4/80 immunoreactivity in the ganglion cell layer and in the retinal/vitreous junction, respectively, confirmed the recruitment of immune cells to the damaged area. Despite these features, this model fails to demonstrate any BM thickening or choroidal abnormalities.

6. Conclusion

As the prevalence of AMD increases there is a growing need for disease-modifying treatments, which require a detailed understanding of complex disease mechanisms.

Although *in vitro* assays have been developed to study retinal, RPE/BM and choroidal function, it is challenging to model the complex multi-layered retina, and its associated tissues, in a dish and translate these discoveries to humans. Rodents share their overall retinal structure and close genetic make-up with humans. Hence, numerous rodent models of AMD have been developed, which recapitulate features of the disease that represent an attractive tool for mechanistic studies as well as drug discovery. Unfortunately, most AMD-like models recapitulate only limited features of the disease. The development of some pathogenic features, which are inconsistent with human AMD pathology, have hampered the full use of rodent models. Recent advances in genetic manipulation and in developmental biology have resulted in the creation of new knock-in or knock out models. The use of non-invasive retinal assessments including fundoscopy, OCT, scanning laser ophthalmoscopy and functional studies with ERG, has added significant value allowing in-depth longitudinal studies using fewer animals. This has also made a positive impact on the 3Rs of replacement, reduction and refinement of animals in experimentation. The use of rodent models allows the manipulation of known AMD risk factors and mechanisms such as complement activation, chemokine/cytokine signalling, oxidative stress and hypoxia amongst others. The development of pharmacological or toxic intervention models using refined doses (PEG mouse model for instance) or chemicals ($NaIO_3$ model) also recapitulate important features of the disease. Of the recent models reviewed, the $Nrf2^{-/-}$ (Zhao et al., 2011) and $CD46^{-/-}$ models (Lyzogubov et al., 2016) are suitable to study early AMD phenotypes (Figure 2). Both models display important features of early retinal degeneration seen in humans, such as reduced photoreceptor function, shortened photoreceptor IS as well as increased autofluorescence, activation of autophagy, vacuolisation, hypo/hyperpigmentation in the RPE and electron-dense sub-RPE deposits including thickening of the BM and the loss of choroidal fenestrations. However, to study focal atrophic lesions, the intermediate-advanced GA model (Ibbett et al., 2019) (Ratnayaka and Lotery, 2020) and the $NaIO_3$ (Bhutto et al., 2018) AMD-like model may act as useful tools. Most of the models reviewed here include age as a risk factor in the development of AMD, but increasing evidence suggests a key role for environmental risk factors in driving disease onset/ or progression. For example, older Nrf2-/-, $PGC1\alpha^{+/-}$ mice combined with high glycaemic diet results in late AMD features, while aged human CFH H402 mice on a high fat,

cholesterol-rich diet is be a better model to investigate the early AMD phenotypes. Thus, whilst combining genetic and environmental risk factors are likely to generate more realistic and nuanced models recapitulating specific disease stages, the optimal rodent model differs depending on which stage or type of AMD disease one wants to model. No single rodent model of AMD displays the entirety of AMD phenotypic characteristics.

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Figure 1. A schematic diagram featuring the stages of age-related macular degeneration (AMD) with its associated risk factors. The figure illustrates the underlying pathological mechanisms, which include: 1) oxidative stress by lipid peroxidation and phagocytosis by the RPE; 2) inflammation via drusenoid deposits; 3) hypoxia by a limited nutrient supply from the choroid due to a thickened BM and neovascularization. The presence of drusen in early AMD recruits activated microglial cells which have migrated from retinal endothelial cells. The drusen serve as the substratum for inflammation. Geographic atrophy (GA) develops when the RPE and photoreceptor cells have degenerated. In neovascular AMD, pathological new blood vessels from the choroid breakthrough the BM and enter the sub-RPE space. This is associated with subretinal fluid accumulation, the recruitment of macrophages from the choriocapillaris as well as activation of microglial cells.

Figure 2: Schematic illustration showing AMD features corresponding to the appropriate rodent model. Rodents lacking a specific disease phenotype(s) are indicated in red text.

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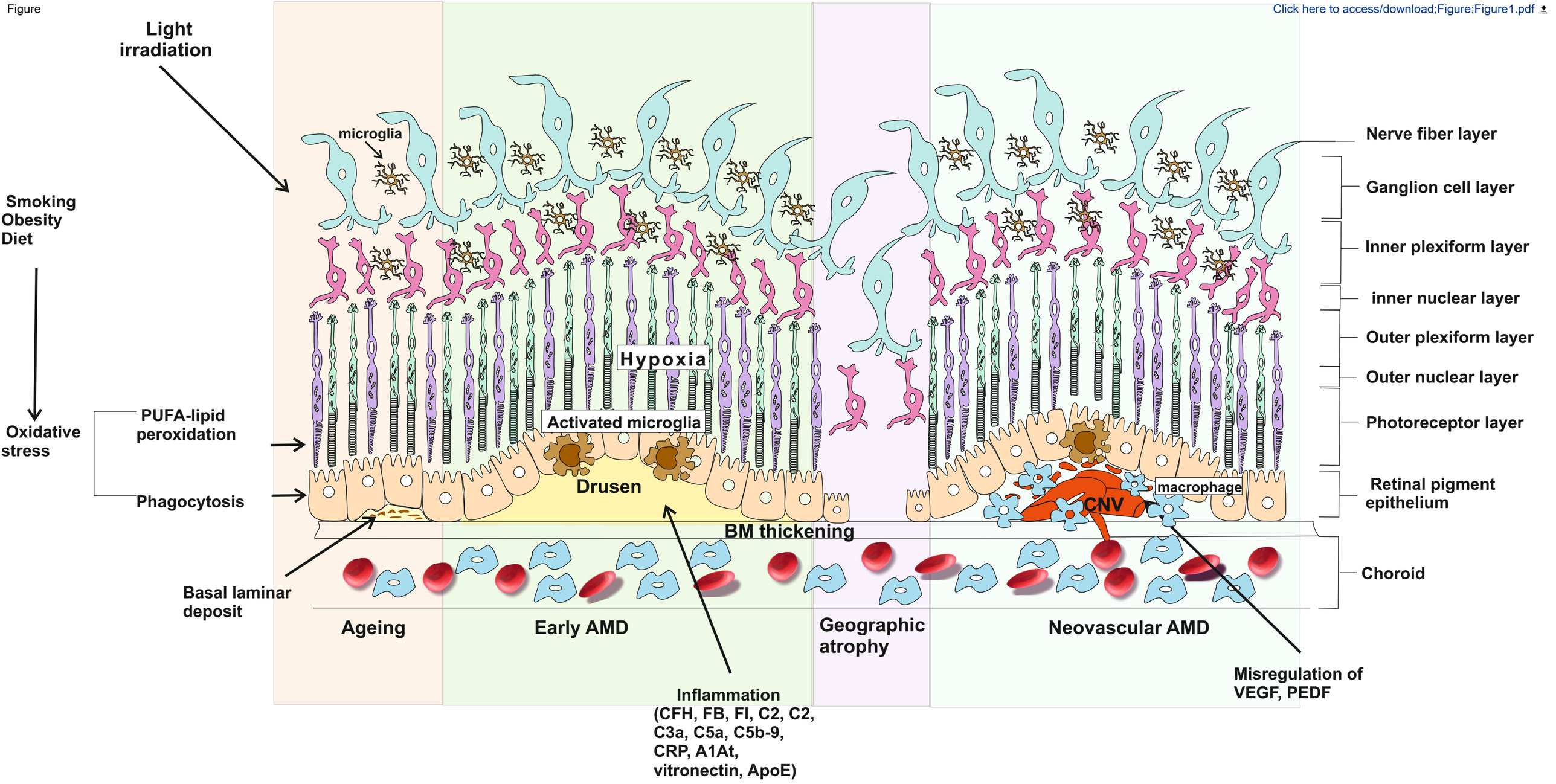
Figure



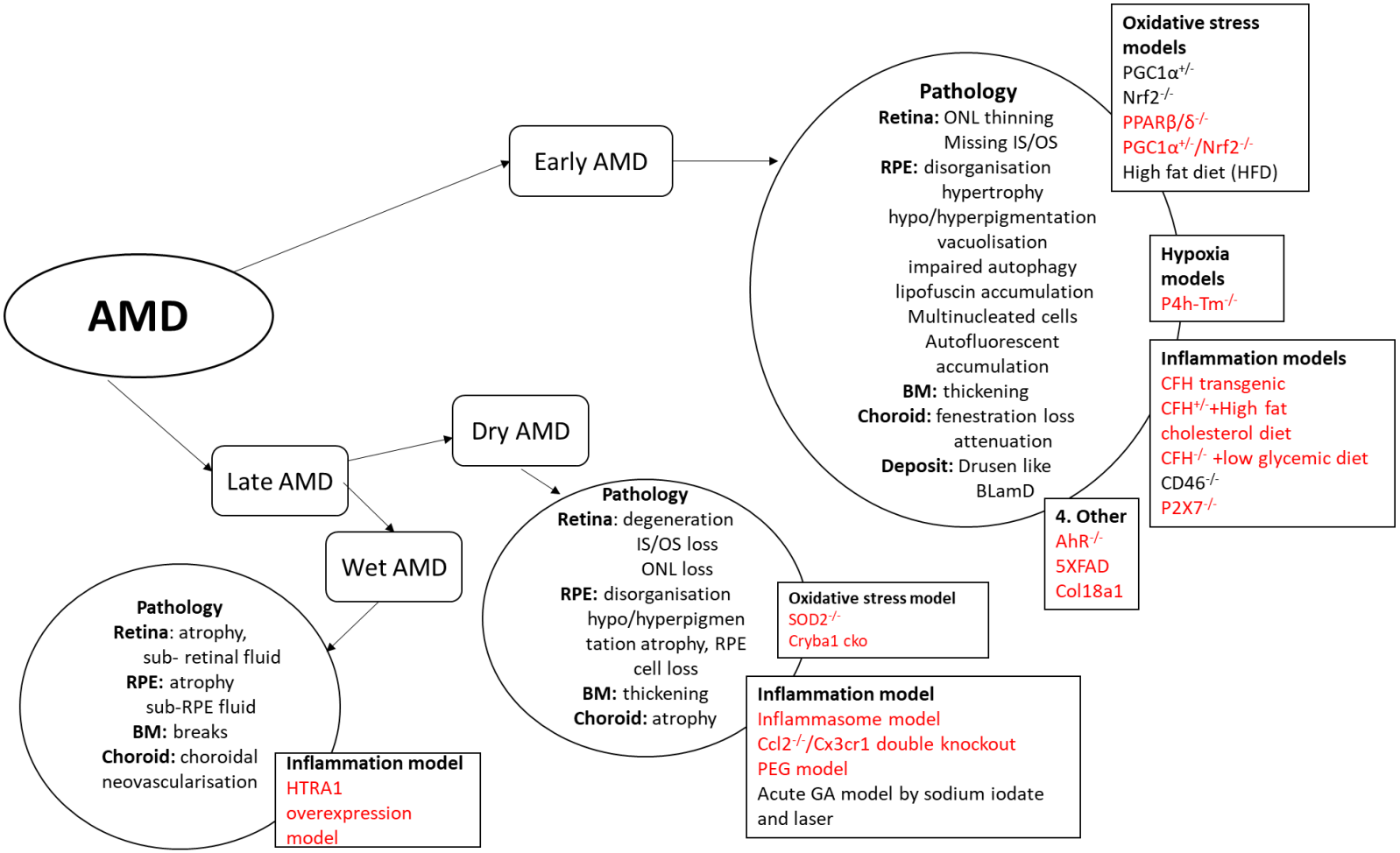


Figure 2: Schematic illustration showing AMD features corresponding to the appropriate rodent model. Rodents lacking a specific disease phenotype(s) are indicated in red text.

Table 1: List of AMD animal model pathology and limitation

	Name	Time	Clinical changes	Functional changes	Histological / Ultrastructural changes	AMD	Limitations	Ref
Oxidative stress models	**SOD2$^{-/-}$ Conditional KO mice**	6 to 9 months	**Fundus**- White reflective area **OCT**- ONL thinning, increased choroidal porosity **FA**- retinal blood vessel abnormality	**ERG**- reduced photoreceptor function	**Retina**- shortened POS and PIS **RPE**-thickened, vacuolated, increased AF **Deposit**-BLam like **Choroid**-no CNV	Late dry	white area in fundoscopy not replicative of atrophy in human eye, unclear pathology of atrophy in histology and OCT	Biswal et al., 2016; Mao et al., 2014
	Nrf2$^{-/-}$ mice	11-18 months	**Fundus**-yellowish soft drusen like deposit	**ERG**- reduced photoreceptor function	**Retina-**POS atrophy **RPE**-Vacuolated, hyper/hypopigmented, hyperplasia, increased AF, compromised autophagy, infiltrated cells in sub-RPE **BM**- Thickened **Deposit**-Continuous basal deposit	Early	few peripheral drusen	Zhao et al., 2011

			Choroid- Lost fenestration, thickened CC endothelium CNV **(18%)**			
PPARβ/δ$^{-/-}$	18 months	**ERG-**reduced B wave	**Retina-** no change **RPE-** hypo/hyperpigmentation, damaged basal infolding, increased autofluorescence **BM**- thickening **Deposit-** patchy/continuous sub-RPE **Choroid-** no change	Early	No change in retina and choroid	Choudhary , 2016
PGC1α+/- mice	3 months +4 months with HFD		**Retina-** thinned IS/OS **RPE-** lipofuscin accumulation, fissured, lost melanosomes, **BM-**thickening **Choroid-** lost fenestration **Deposit**-BLamD	Early	No functional measurement	Zhang et al., 2018
PGC1α/Nrf2 dko	12 months	**ERG-**reduced photorecepto r cunction	**Retina-** focal PR damage, ONL thinning **RPE-**vacuolated, hypertrophic, damaged autolysosomes, damaged	Early	No information of choroidal abnormalities	Felszeghy et al., 2019

			mitochondria, lost basal folding, increased lipofuscin and melanosomes, increased autofluorescence **BM**-thickening **Deposit**- drusen like			Sridevi Gurubaran et al., 2020
Cryba1 cko mice	7-12 months	**ERG- reduced photoreceptor function**	**RPE-** lost tight junction and apical villi, lipid accumulation, vacuolated, more melanosomes, reduced lysosome and autophagaosomes	Late dry	Absence of BM thickening, ONL thinning and choroidal abnormalities	Valapala et al., 2014b
HFD	12 months +12 month HFD		**Retina-** thinned, disorganised PR, swelled IS, lost synapsis **RPE-**vacuolated, hypopigmented, thinned, disorganised, multi-layered, atrophic and increased autofluorescence, lost basal infolding, lncreased lipofuscin, phagosomes. **Deposit-B**LamD, subretinal deposit	Early	No functional or clinical examination	Rowan et al., 2017

Hypoxia targeted model	**P4h-Tm$^{-/-}$ mice**	10 and 14.5 months	**ERG**- reduced cone function (5-7 months), reduced rod function (12-13 months)	***10 months:*** **RPE**-disorder, broadened intercellular space, basal infolding **Deposit**- BLam like and drusen like ***14.5 months:*** **Retina**- ONL thinned, shortened PIS/POS, infolding ONL and POS **RPE**- Thinning, compromised phagocytosis	Early	no clinical examination and unexplained choroidal pathology	Leinonen et al., 2016
Inflammation targeted models	**CFH transgenic mice**	90 week	**ERG-** impaired photoreceptor function	**RPE-**multinucleated **Deposit-** BLamD	Early	unexplained retinal, BM and choroidal abnormalities	Landowski et al., 2019
	CFH$^{+/-}$ mice with High fat cholesterol (HFC) diet	2 year old	**ERG-** impaired photoreceptor function	**Retina-**ONL thinning **RPE-**multinucleated and hypertrophic, thinned RPE **Deposit-**larger BLamD	Early	unexplained BM and choroidal abnormalities	
	CFH-/- with low glycemic diet	44 week		**RPE-**thinned, vacuolated, disorganised, basal folding damage, increased lipofuscin, autophagosomes	Early	No functional measurement and unexplained	Rowan et al., 2014

				BM-thickening **Deposit**-BLamD		choroidal abnormalities	
CD46$^{-/-}$ mice	12 months			**Retina**- Photoreceptor apoptosis, reduced nuclear density in ONL, macrophage like cell in subretinal space **RPE** -Hypertrophic, multinucleated, vacuolated, increased AF, activated autophagy **BM**- Thickened **Deposit**-Electron dense material **Choroid**- Thinning, reduced fenestration	Early	no clinical and functional measures	Lyzogubov et al., 2016
Inflammasome mice			**Fundus**-GA like	**RPE-**disturbed cobble stone structure, degenerated	Late dry		Tarallo et al., 2012
P2X7 $^{-/-}$ mice	12-18 months	**Fundus**- whitish lesion spot	**ERG-**reduced rod photoreceptor function	**Retina-**Gliosis, reduced retinal microglial phagocytosis **RPE**-Hypertrophic, vacuolated, multinucleated, increased lipofuscin and AF **BM**- Thickened **Deposit**-BLam like	Early	ONL, PIS/POS and choroidal changes was not described	Vessey et al., 2017

				BLin like, electron lucent deposit			
	cc12/cx3cr1 double knockout mice	12 -18 months	**Fundus**-yellowish/whitish lesion at periphery No CNV	**Retina**-Photoreceptor degeneration **RPE**-Hypopigmented vacuolated, irregular organisation **BM**- Thickened	Late dry	lack of wedge shaped morphology and ONL thinning	Chen et al., 2013
	HTRA1 over expressing mice	12 months	**FA and indocyanine green angiography**-hyperfluorescent lesion with abnormal branching	**Choroid**-Radial branching of choroidal blood vessel, subretinal haemorrhage	Late wet	low percent of animals developed disease	Iejima et al. 2015
Other models	**PEG induced mice**	5 days		**Retina-**thinned ONL, shortened PIS/POS **RPE**- Thinning, hypopigmentation, hyperproliferative, increased RPE density, increased autophagy **Deposit**-Drusen like	dry	no clinical and functional measures, no description about choroid and BM	Lyzogubov V. V. et al., 2014b
	Acute Geographic atrophy rat	7 days	**Fundus-**retinal thinning **OCT**-RPE loss	**Retina**-Complete loss of photoreceptor, RPE, ONL, glial membrane, wedge shaped atrophy **RPE**-Well demarcated atrophy	Late dry	No functional measurement	Bhutto et al., 2018

	Laser induced acute geographic atrophy like mice	2-8 wk	**Fundus-** whitish lesion spot (retinal thining) **OCT**-wedge shaped atrophy **FA**-absence of choroidal leakage	**ERG-**reduced photoreceptor function	**Retina**-Complete photoreceptor loss, ONL absence **RPE -**Disorganised, hypo/hyperpigmented, reduced apical microvilli	Late dry		Ibbett et al., 2019
	AhR$^{-/-}$ mice	11 months	**Fundus and OCT**-choroidal thinning/atrophy	**ERG**- reduced B wave	**Retina**-INL and ONL thinning **RPE**-Increased lipofuscin and AF, multinucleated, hyper/hypopigmented, vacuolated **Deposit**-BLinD **BM** -thickened **Choroid**-Thinning and atrophy	Early	PIS/POS shortening wasn't shown,	Hu et al., 2013
	AhR$^{-/-}$ mice (C57BL/6N)	12 months	**Fundus-** RPE atrophy **OCT-** Hyperreflective area		**RPE-**Increased AF, vacuolated, subretinal microglial accumulation **Choroid-** Thinned	Late dry	no geographic atrophy like phenotype seen, unexplained POS/PIS and ONL organisation	Kim et al., 2014
	5X FAD mice	12 months			**RPE**-Loss of apical microvilli, increased lipofuscin	Early	No clinical or functional measurement,	Park et al., 2017

			BM- Thickened **Deposit**- BLamD, BLinD **Choroid**- Reduced fenestration		unexplained retinal pathology	
Col18a1-/- mice	16 -18 months	**ERG-** reduced photoreceptor function	**Retina-** reduced rhodopsin, perfused and non-atrophic **RPE-**lost apical villi, damaged RPE layer, reduced RPE65, cell death and increased autophagy **Deposit-** sub-RPE	Early	No BM thickening and choroidal abnormalities	Marneros et al., 2004 Kivinen et al., 2016

OCT- optical coherence tomography, **FA**-fluorescein angiography, **ERG**-Electroretinography, **RPE**-retinal pigment epithelium, **BM**-bruch's membrane, **PIS**-photoreceptor inner segment, **POS**-photoreceptor outer segment, **ONL**-outer nuclear layer, **AF**-autofluorescence, **BLamD**-basal laminar deposit, **BLinD**-basal linear deposit, **CC**-choriocapillary, **CNV**- choroidal neovascularisation, **HFD**-high fat diet