Chapter 1

Effects of Hypercholesterolaemia in the Retina

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1. Introduction

A cholesterol-rich diet causes postprandial hyperlipaemia with an accumulation of chylomicrons. This accumulation leads to a redistribution of the very-low-density lipoproteins (VLDL), thereby determining the elimination of the coarsest particles, the residual chylomicrons, which promote the onset of atherogenesis [1].

For some years, cholesterol-rich food has been associated with the subsequent development of complications such as the formation of atheromatous plaque and lipid deposits at the ocular level. These findings have been reproduced in an experimental rabbit model [2,3], this animal being particularly sensitive to the induction of atheromatous lesions, which faithfully reproduce those caused in human atherosclerosis [4-6].

One of the main barriers of the eye is Bruch's membrane, which, for its strategic situation between the choroidal vascular membrane and the outer retina, constitutes a semi-permeable filtration zone, through which the nutrients pass from the choriocapillaris towards the photoreceptors, while the cell-degradation products of the retina pass in the opposite direction. The accumulation of these waste products thickens Bruch's membrane and the basal layer of the retinal pigment epithelium (RPE) [7]. These changes in the outer retina may be the consequence of metabolic stress associated with the metabolism of fatty acids or of the changes in choroidal perfusion due to atherosclerosis [8]. In any case, the lipids that accumulate in a structurally altered Bruch's membrane cause a hydrophobic barrier that can hamper the free metabolic exchange between the choriocapillaris and the RPE, on interfering with the passage of nutrients and oxygen to the retina. This situation could contribute to the loss of retinal sensitivity and play a pathogenic role in the development of age-related macular degeneration (AMD) [9], the leading cause of blindness among people over 65 years in developed countries. On the other hand, the deposits that accumulate underneath the RPE, which contains unsaturated fatty acids, are oxidized by the light, strengthening lipid peroxidation [10,11] and negatively influencing retinal function.

The changes in the RPE-Bruch's membrane complex contribute to the death of multiple retinal neurons, this translating as a thinning and disorganization of its layers.

Cholesterol is essential for cell functioning. The main cholesterol source for the photoreceptors and the RPE comes from extracellular lipid metabolism, as has been demonstrated on detecting native low-density lipoprotein (LDL) receptors at the RPE level [12], which could be involved in the local production of apolipoprotein E (apoE). The retina also locally produces lipoprotein particles that contain apoE. These particles are secreted fundamentally by the Müller glia to the extracellular retinal compartment and to the vitreous, from which they are transported to the optic nerve [13]. Also, the retinal astrocytes associated with the axons of the ganglion cells participate in the secretion of apoE. This cholesterol transport is essential to supply the retinal neurons the lipids needed for the maintenance and remodelling of their cell membrane.

Studies in apoE-deficient mice have demonstrated the presence of alterations in Müller glia and in amachrine cells, these generating aberrations in the retinal circuit as a consequence of the local disruption of cholesterol homeostasis [14]. In a hypercholesterolaemic rabbit model, cell loss in the inner nuclear layer and in the ganglion-cell layer of the retina has been demonstrated [15,16]. This cell loss probably results from the deprivation of the neurotrophic support [17] and of the CNTF (ciliary neurotrophic factor) and glial fibrillary acidic protein (GFAP) upregulation secondary to the reactivation of the Müller cells [18,19]. In hypercholesterolaemic rabbits, added to the situation of ischaemia at the level of the outer retina induced by the alterations in Bruch's membrane and in the choriocapillaris, is the thickening of the basal membranes of the retinal vessels, which by hampering the passage of oxygen and nutrients towards the inner retina would generate a prolonged situation of ischaemia [15,20]. This chronic ischaemia could increase the concentration of extracellular glutamate, conditioning oxidative damage by a neuronal cytotoxic mechanism [21,22]. This situation can be counteracted so long as the astrocytes maintain their capacity to eliminate cytotoxic neurotransmitters and to supply growth factors and cytokines [23].

In summary, in the present chapter, the structural and ultrastructural changes in the retina of an experimental model of hypercholesterolaemia are described, specifically changes in Bruch's membrane, RPE, and retinal layers as well as the vascular changes responsible for chronic ischaemia. Further on, the effects of the diet-induced normalization of the plasma-cholesterol levels in the retinal structures are discussed. The comparison between the two scenarios suggests that hypercholesterolaemia is a risk factor for the development of chronic ischaemia in the retina and therefore for neuronal survival.

2. Anatomy and physiology of the Bruch's membrane-retinal complex

Bruch's membrane, the innermost layer of the choroid, fuses with RPE as a 5-layered structure consisting of (from outer to inner): a basement membrane of the choriocapillaris, an outer collagenous layer, an elastic layer, an inner collagenous layer, and a basement membrane of the RPE [7,24] (Figure 1, 3A, 4A). Fine filaments from the basement membrane of the RPE merge with the fibrils of the inner collagenous zone, contributing to the tight adhesion between

choroid and the RPE. The basement membrane of the choriocapillaris is discontinuous and is absent in the intercapillary spaces [25]. The collagenous layers surround the elastic layer [7]. Some collagen fibres are arranged parallel to the tissue plane, especially at the inner collagenous zone; others cross from one side of the elastic fibre layer to another, interconnecting the two collagenous layers [7]. Collagen fibres pass through the disruption of the basement membrane to join the collagen fibres of the intercapillary septae. This arrangement may help Bruch's membrane to attach to the choriocapillaris. Vesicles, linear structures, and dense bodies occur in the collagenous and elastic zones but predominantly in the inner collagenous layer [26]. The elastic layer is made up of inter-woven bands of elastic fibres with irregular spaces between them, through which the collagen fibres pass [7,26] (Figure 3A, 4A). The exchange of substances between the choroid and retina (both directions) must traverse Bruch's membrane [7]. The importance of this process is evident in situations in which this membrane is disrupted. During aging, Bruch's membrane gradually thickens [27]. The collagenous layers thicken from the accumulation of membranous lipidic debris [28], abnormal extracellular matrix components (collagen fibres "cross-linking") and the advanced glycation end-product [29]. This decreases the porosity of Bruch's membrane, presumably heightening resistance to the movement of water through it [30]. Also, it has been found that this thickening of Bruch's membrane is accompanied by lower membrane permeability [31]. Although this thickening with aging is relatively minor, greater increases can appear in specific regions. The accumulation of material in the inner collagenous layer bulging toward the retina, is what is known by the term "drusen" [32]. These drusen will deprive the photoreceptors of their nutrition from the choriocapillaris.

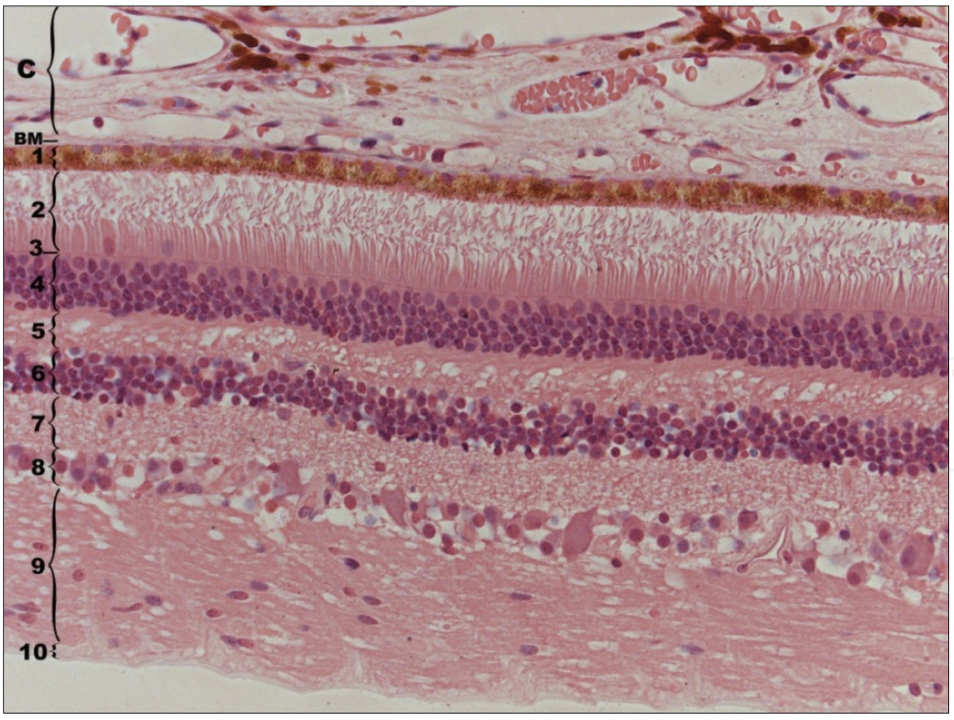

Figure 1. Histological section of the human retina. Retinal layers. Hematoxylin/eosin. 1: retinal pigment epithelium; 2: photoreceptor layer; 3: outer limiting membrane; 4: outer nuclear layer; 5: outer plexiform layer; 6: inner nuclear layer; 7: inner plexiform layer; 8: ganglion-cell layer; 9: nerve-fibre layer; 10: inner limiting membrane. [Bruch's membrane (BM); choroidal vascular layers (C)].

The elastic layer also suffers a disruption with aging, namely, an increase in density and calcification [33]. These aged-related changes could cause cracks and holes in Bruch's membrane. Major breaks in Bruch's membrane are associated with oedema, leading to the accumulation of fluid between the RPE and photoreceptors, and hence to a retinal detachment. This association between the discontinuity of Bruch's membrane and retinal oedema suggests that, under normal conditions, Bruch's membrane could play a role in limiting fluid movement to and from the retina [25].

2.1. Anatomy of the retina

The primary function of the retina is to convert light into nerve impulses which are transferred to the brain via the optic nerve. The retina comprises the retinal pigment epithelium and the neurosensory retina, the latter containing neurons, glial cells and components of the vascular system. Various types of neurons are present, such as: photoreceptors, bipolar cells, ganglion cells, amacrine cells and horizontal cells [34]. The coding function of the retina depends not only on photoreceptors but also on neurons, glial cells and RPE, which amplify the signal [35]. The photoreceptors are the cells that capture light and are situated at the most external side of the neurosensory retina, in the vicinity of the RPE. These cells are of two types: rods (for scotopic vision) and cones (for photopic vision) [34]. The ability of photoreceptors to convert light photons into an electrical signal is due to the presence of a photopigment in their outer segments. These segments consist of a stack of disk membranes that are synthesised in the proximal portion of the outer segment and shed at its apical size [35]. Photoreceptors form contacts with horizontal and bipolar cells in the outer plexiform layer (OPL). Coupling between neighbouring rods and cones in OPL allows the first stage of visual processing. The inner nuclear layer (INL) contains cell bodies of Müller glial, bipolar, amacrine, and horizontal cells. The inner plexiform layer (IPL) consists of a synaptic connection between the axons of bipolar cells and dendrites of ganglion and amacrine cells. The ganglion-cell layer (GCL) contains the cell bodies of retinal ganglion cells, certain displaced amacrine cells, and astrocytes. Inside the eye, ganglion-cell axons run along the retinal surface toward the optic-nerve head forming nerve-fibre layer (NFL) [34,35] (Figure 1).

The neural retina also contains two types of macroglial cells: Müller cells and astrocytes (Figure 2).

Müller cells are long, radially oriented cells which span the width of the neural retina from the outer limiting membrane (OLM), where their apical ends are located, to the inner limiting membrane (INL), where their basal endfeet terminate (Figure 2A). In the nuclear layers, the lamellar processes of the Müller cells can be seen to form basket-like structures which envelope the cell bodies of photoreceptors and neural cells. In plexiform layers, fine processes of these cells are interwoven between the synaptic processes of neural cells. In both the plexiform and nuclear layers, Müller cell processes cover most but not all neural surfaces [36].

Astrocytes are located mainly in the NFL and GCL in most mammals (human, rabbit, rats and mouse, among others) [37-39] (Figure 2B). Astrocyte morphology differs between

species. In humans, two types of astrocytes can be distinguished: elongated (located in the NFL) and star-shaped (located in GCL) astrocytes. In mice and rats the astrocytes are stellate (Figure 2B). The greatest variety of retinal astroglial cell morphologies is found in the rabbit, which possesses two large astrocyte groups: astrocytes associated with the nerve-fibre bundles (AANFB) which are aligned parallel to the axonal bundles in the NFL (Figure 10G), and perivascular astrocytes (PVA), associated with the retinal and vitreous blood vessels (Figure 10A,D). PVA can be further subdivided into: i) type I PVA, which have numerous sprouting, hair-like processes, associated with medium-sized epiretinal vessels, and with capillaries located over the inner limiting membrane (ILM) (Figure 10A), and ii) type II star-shaped PVA, which are located on and between larger and medium-sized epiretinal vessels [15,38,40-42] (Figure 10D). The morphology of retinal astrocytes in different animal species is determined by the way their processes adapt to the surrounding structures [43].

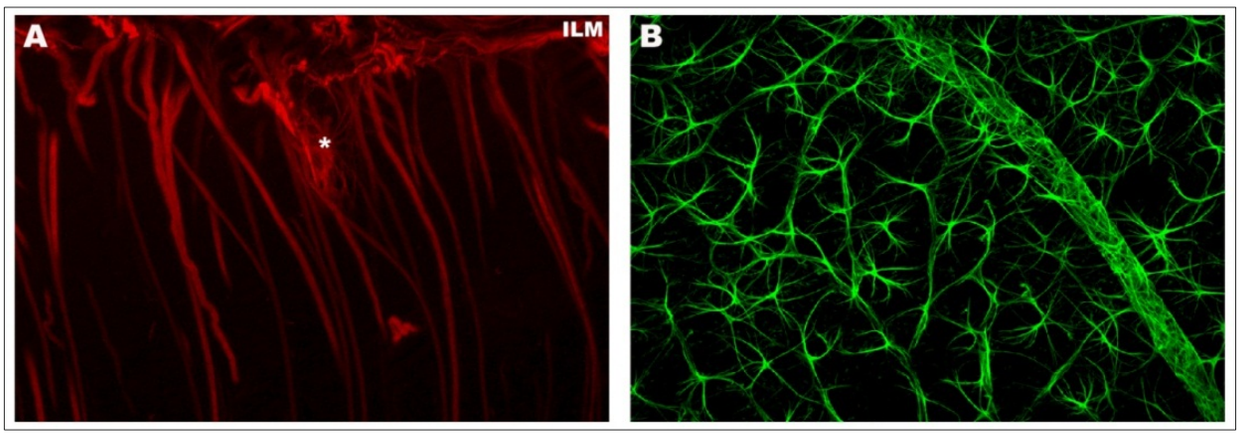

Figure 2. Immunohistochemistry anti-GFAP in mouse retinal whole-mount. A: GFAP+ Müller cells after 15 days of laser-induced ocular hypertension. The pressure exerted by the cover glass on the retinal whole-mount, produced a retinal-like section effect in some retinal borders. Müller cells exhibit a radial morphology that creates a columnar matrix that maintains the laminar structure of the retina [Astrocyte (*); inner limiting membrane (ILM)]. B: Confocal microscopy of normal retinal astrocytes. These cells form a homogeneous plexus on the nerve-fibre-RCG layer constituted by stellate cells. (Modified from Gallego et al [39]).

Macroglial cells perform a variety of essential roles for the normal physiology of the retina, maintaining a close and permanent relationship with the neurons [43]. Thus every aspect of the development, homeostasis, and function of the visual system involves a neuron-glia partnership. Glial cells insulate neurons, provide physical support, and supplement them with several metabolites and growth factors. These cells also play important roles in axon guidance and control of synaptogenesis [44]. Under normal conditions, astrocytes and Müller cells maintain the homeostasis of extracellular ions, glucose, and other metabolites, water, pH and neurotransmitters such as glutamate and GABA [45]. These cells also produce a great quantity of growth factors and cytokines, which may contribute both to neurotoxic as well as neuroprotective effects. It has also been demonstrated that macroglial cells are more resistant to oxidative damage than are the neurons, this trait protecting them against such damage. This potential is due to the fact that these cells contain high

concentrations of antioxidants such as reduced glutathione and vitamin C. Consequently, a depression of these cellular activities could lead to neuronal dysfunction [46]. Macroglial cells induce the properties of barrier in the endothelial cells of retinal capillaries (the blood-retinal barrier), securing immune privilege to protect neurons from potentially damaging effects of an inflammatory immune response. Finally, glial cells can play fundamental roles in local immune responses and immunosurveillance [44].

Macroglial cells also play a part in pathological processes in central nervous system (CNS). Glial cells in the CNS have been cited as participants in the pathological course of neuronal damage after mechanical, ischaemic, and various other insults. Glial cell activation is a hallmark of CNS injury, characterized by an increase in size and number of glial cells and upregulation of GFAP, with additional cellular changes that may cause or relieve neuronal impairment. These reactive cells also have higher metabolic activity. After injury, reactive glial cells participate in the formation of a glial scar, in which there is an accumulation of enlarged astrocyte bodies and a thick network of processes with increased expression of GFAP and vimentin. Macroglial cells become reactive in response to a wide variety of stimuli, including inflammation and oxidative and mechanical stress [47].

Other components of the retina are the blood vessels. Photoreceptors receive nutrients via the choriocapillaris. The inner retinal layers have their own blood supply coming from the blood vessels entering the retina at the optic-nerve head. For its protection, the retina is physiologically and immunologically segregated from the rest of the body by tight junctions between vascular endothelial cells (inner blood-retinal barrier) and RPE cells (outer blood-retinal barrier). This fact is responsible for intraocular tissue to be an immune privileged site, thus protecting the eye from the innocent-bystander effect of inflammation [34]. In addition, only small molecules can cross these barriers, making it difficult for many drugs to reach ocular tissue.

The outermost retinal layer is the RPE (Figure 1), which is formed by a single layer of pigmented hexagonal cells. These cells provide the supportive role necessary to sustain the high metabolic demands of photoreceptors. RPE cells supply nutrients and oxygen, regenerate phototransduction products, and digest debris shed by the photoreceptors. The basal aspect of RPE cells contains numerous infoldings and is adjacent to Bruch's membrane. The apical surface is adjacent the neural retina. The RPE cells contain numerous pigment granules (melanosomes), lipofuscin granules, and degradation products of phagocytosis, which grow in number with age (Figure 4A) [7]. The RPE had several intercellular junctions: zonula occludens, zonula adherents, desmosomes, and gap junctions. The latter allow the cell electrical coupling and provide a low-resistance pathway for the passage of ions and metabolites [48]. The RPE fosters the health of the neural retina and choriocapillaris in several ways: the zonula occludens joining the RPE cells are part of the blood-retinal barrier and selectively control movement of nutrients and metabolites from choriocapillaris into the retina and removal of waste products from the retina into the choriocapillaris [49]. RPE cells phagocytose fragments of the photoreceptor outer segment discs, metabolise and store vitamin A, and produce growth factors, helping to maintain choriocapillaris and retinal function. Other, less well-characterized functions of the RPE are

the absorption of stray light and the scavenging of free radicals by the melanin pigment in the epithelium and the drug detoxification by the smooth endoplasmic reticulum cytochrome p-450 system [50]. From the several functions displayed by RPE, it can be easily concluded that dysfunction of RPE cells has serious consequences on the health of photoreceptors [34].

2.2. The metabolism of lipids in the retina

Recent studies have demonstrated that fatty acids are fundamental for normal visual function [51]. Humans are unable to synthesise essential fatty acids (EFAs) and must acquire them through the food intake. Dietary EFAs are transformed into the endoplasmic reticulum of hepatic and retinal cells [52] into long-chain polyunsaturated fatty acids (LCPUFAs). LCPUFAs perform various functions, e.g. serving as ligands for gene-transcription factors for cell growth and differentiation, to participate in the metabolism of lipids, carbohydrates, and proteins, and to intervene in the inter- and intracellular signal cascades that influence vascular, neural, and immune functions [51].

In the neural retina, the richest LCPUFA-containing lipids are the phospholipids of the cell membranes [53], and the most abundant LCPUFAs in the retina are docosahexaenoic acid (DHA) and arachidonic acid (AA). DHA is a long-chain polyunsaturated fatty acid from the omega 3 series. It is present at high levels in the neurosensory retina [54]. DHA improves the kinetics of the photocycle by creating specific intermolecular associations with rhodopsin [35]. Brain astrocytes [55] and retinal tissue [34] can produce DHA, but in a limited way [56], given that the synthesis process is slow [57] and restricted to the RPE and the endothelial cells of the retinal vessels [58]. Consequently, retinal requirements of LCPUFAs depend on input from the liver (the main site of LCPUFA biosynthesis) [59] and hence on transportation of LCPUFAs from the choriocapillaris to the outer segments of the RPE-photoreceptor.

Cell-membrane permeability is thought to depend on the balance between LCPUFAs and cholesterol [60,61]. Ocular DHA levels are lower in high-cholesterol diets, a fact that could influence the development of ocular disease [62]. Recently, it has been reported the relationship between lipid intake and AMD in patients with low intake of linoleic acid (a LCPUFA) [63].

Cholesterol is present exclusively as the free form in the neurosensory retina, and distributed in all cell layers [54,64]. Cholesterol in the neuroretina originates from *in situ* synthesis and extra-retinal sources. RPE, Müller cells and rods express 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway [65]. RPE cells express various lipoprotein and scavenger receptors which can promote the recognition of cholesterol-rich lipoprotein and enhance the entry of cholesterol in the neurosensory retina [65]. Indeed, cholesterol bound to LDL can reach the RPE and enter the neurosensory retina [66]. Neurosensory retina and RPE cells express proteins which participate to cholesterol export in tissues other than the retina, such as ABCA1, apoE, ApoA1 or SR-BI [65]. RPE cells have the capacity to synthesise lipoprotein-like particles

which may also play a role in these mechanisms of efflux and influx of cholesterol in the retina [67].

Similar to the brain [68,69], the neurosensory retina expresses cholesterol-24S-hydroxylase (CYP46A1) [70]. CYP46A1 is a microsomal cytochrome P450 enzyme which catalyses the hydroxylation of cholesterol at position C24. It has been suggested that CYP46A1 represents a mechanism of cholesterol removal from neurons [71] and strongly induces oxidative stress as well the inflammatory response in RPE cells. RGC specifically express CYP46A1 [70], a hydroxylase that might promote apoptosis of RGC in glaucoma. Cholesterol-27-hydroxylase (CYP27A1) shows a property the similar to that of CYP46A1, converting cholesterol into a more polar metabolite [72]).

7-ketocholesterol is a non-enzymatic-oxidation product of cholesterol. The formation of 7-ketocholesterol in the retina has been thoroughly studied in the retina, in connection with oxidative stress, aging and AMD [73].

With age, the diffusion characteristics of the choriocapillaris-Bruch's membrane-RPE-photoreceptor complex [74,75] change, RPE density decreases [76], and the cytoarchitecture of RPE cells transforms [77]. Such morphological and functional changes lead to AMD in some patients. Additionally, there may be age-related changes in the specific activities of the lysosomal enzymes of the RPE and it has been reported that animals fed a fish-oil-enriched diet presented higher activity of lysosomal acid lipase [78,79]. This could augment the hydrolysis of the intralysosomal lipids of the RPE, thus reducing lipofuscin deposits and oxidative damage of the RPE, this in turn preventing the development of AMD.

Recent studies have demonstrated the relationships between dietary fat and the promotion of vascular disease [51]. Lipoprotein metabolism has also been associated with neurodegenerative disorders in rats [14] but preliminary results showed no marked changes in apo-E knockout mice [80]. Eukaryotic cells require sterols to achieve normal structure and function of their plasma membranes, and deviations from normal sterol composition can perturb these features and compromise cell and organism viability [81]. Given that cholesterol is required by neurons, an intimate relationship could exist between cholesterol homeostasis and the development, maintenance, and repair of these cells [14].

The particular spatial arrangement of retinal macroglial cells (astrocytes and Müller cells) that are intercalated between vasculature and neurons points to their importance in the uptake of nutrients from the circulation, metabolism, and transfer of energy to neurons [37,40,82]. Moreover, apoE lipoprotein, which plays a central role in serum-cholesterol homeostasis through its ability to bind cholesterol with other lipids and to mediate their transport into cells, is produced by glial cells [83]. Müller cells express HMGcoA reductase. Glia is also known to support neurons in the formation and maintenance of synapses in which cholesterol is crucial [84]. Therefore, all together, these data suggest that glial Müller cells may also help deliver cholesterol to neurons [35].

As mentioned above, associations between 24S-hydroxycholesterol in glaucoma and other neurodegenerative diseases are suspected. Glial expression of CYP46A1 has also been

reported in the brain of Alzheimer's patients [85,86]. Glia may compensate for the loss of neurons while expressing CYP46A1. Meanwhile, Müller cells play a key role in the maintenance of RGC bodies in the retina, besides participating in lipid metabolism, including fatty acid oxidation [86].

Reactive gliosis, a general response to injury and inflammation in the adult brain [87,88], is characterized by up-regulation of various kinds of molecules, the best known being GFAP [89]. The *de novo* expression of GFAP by retinal Müller cells is indicative of retinal impairment, whether induced by glaucoma [39,90,91] (Figure 2A), retinal detachment [88,92-94], diabetic retinopathy [88,94], or AMD ([74]. By contrast, retinal astrocytes may not only acquire gliotic features but may also diminish in number when there is either vessel damage with greater permeability of the blood-retinal barrier [95] or a massive loss of neurons [96].

Given the intricate metabolic interdependence between vessels, macroglial cells, and neurons, high cholesterol levels could deregulate a number of cell functions in both macroglial and neuronal cells.

3. Hypercholesterolaemia as a risk factor for retinal ischaemia

Most of the information available on vascular diseases is based mainly on studies of ischaemic heart disease [97] and cerebrovascular diseases [98]. In both, the underlying phenomenon is artherosclerosis, a general term referring to any vascular degeneration causing the thickening and loss of arterial-wall elasticity and that encompasses atherosclerotic and non-atherosclerotic conditions. Atherosclerosis involves a hardening of the arterial intima due to a lipid build-up in artery, a condition that appears in humans at an early age and develops progressively over the aging process [99].

Schematically, we can point to various types of long-recognized vascular risk factors: i) non-reversible factors, such as age, male gender or family history of early atherosclerosis; ii) reversible factors such as smoking, hypertension, obesity or hypercholesterolaemia; iii) partially reversible factors such as hypertriglyceridaemia and other forms of hyperlipidaemia, hyperglycaemia, and diabetes mellitus; and iv) potential risk factors such as physical inactivity or emotional stress. Some new factors can be added to the aforementioned vascular risk factors, including lipoprotein A, homocysteine, coagulation factors and C-reactive protein [99,100].

It bears noting that the importance of hypercholesterolaemia as a cardiovascular risk factor lies not only in its direct effect on the pathogenesis of coronary or cerebrovascular disease, but also in the influence exerted on the course of other pathologies. For ocular diseases, epidemiological studies have demonstrated that hypercholesterolaemia is a risk factor for several pathologies despite not being considered the primary cause of the process.

In the case of retinal lesions, classical risk factors for atherosclerosis seem to lose influence. The Atherosclerosis Risk in Communities Study (ARIC) has suggested that changes in the retinal vessels (arteriolar narrowing, arteriovenous index, and abnormalities where the arterioles cross or arteriovenous nicking) are closely linked to

hypertension but not to other factors [101], although the presence of retinal lesions is associated with a higher prevalence of ischaemic heart disease, myocardial infarction, stroke, or carotid plaques in patients over 65 years [102,103]. It has been suggested that the retinal lesions could reflect the persistence of small-vessel damage due to hypertension and possibly inflammation and endothelial dysfunction, although they have little relation to large-vessel damage [103].

Another work of the ARIC study found that retinal arteriolar narrowing intensifies the risk of ischaemic heart disease in women but not men after adjusting the population for other known risk factors such as blood pressure, diabetes, smoking, and lipids. The authors speculated that the difference between sexes may be due to the fact that microvascular lesions may have a greater role in women than in men. Hormones protect women from macrovascular injury but it is not clear whether small vessels receive the same protection [104].

The examination of the retinal vasculature offers a unique opportunity to investigate cerebral microcirculation [105], which can be of outstanding importance to clarify the role of microcirculation in stroke [106]. The presence of retinal microvascular abnormalities is linked to the incidence of any stroke and also to the presence of high blood pressure, not only at the time of diagnosis, but also beforehand. Furthermore, stroke has been associated with markers of inflammation and endothelial dysfunction, suggesting the possibility of a significant microvascular component in stroke that a retinal examination might reveal [107]. Notably, although the importance of the association between brain and retinal microvascular lesions is still unknown, the prediction of a stroke provided by the white-matter lesions multiply in the presence of retinal lesions [108].

In conclusion, epidemiological studies have shown an association between vascular changes in the retina and elsewhere. This association appears to be related to common factors of microvasculature damage, the role of which, both in ischaemic heart disease and stroke, may be greater than suspected.

4. Animal models of hypercholesterolaemia

Animal models provide a controlled environment in which to study disease mechanisms and to devise technologies for diagnosis and therapeutic intervention for human atherosclerosis. Different species have been used for experimental purposes (cat, pig, dog, rabbit, rat, mouse, zebra fish). The larger animal models more closely resemble human situations of atherosclerosis and transplant atherosclerosis and can also be easily used in (molecular) imaging studies of cardiovascular disease, in which disease development and efficacy of (novel) therapies can be monitored objectively and non-invasively. Imaging might also enable early disease diagnosis or prognosis [109]. On the other hand, the benefits of genetically modified inbred mice remain useful, especially in quantitative trait locus (QTL)-analysis studies (a genetic approach to examine correlations between genotypes and phenotypes and to identify (new) genes underlaying polygenic traits [109].

4.1. Mice

Wild-type mice are quite resistant to atherosclerosis as a result of high levels of anti-atherosclerotic HDL and low levels of pro-atherogenic LDL and very-low-density-lipoproteins (VLDL). All of the current mouse models of atherosclerosis are therefore based on perturbations of lipoprotein metabolism through dietary or genetic manipulations [110].

ApoE-knockout mice

In apoliprotein-deficient mice (apoE-/-) the homozygous delection of the apoE gene results in a pronounced rise in the plasma levels of LDL and VLDL attributable to the failure of LDL-receptor (LDLr-) and LDL-related proteins (LRP-) mediated clearance of these lipoproteins. As a consequence, apoE-/- mice develop spontaneous atherosclerosis. Of the genetically engineered models, the apoE-deficient model is the only one that develops extensive atherosclerotic lesions on a low-fat cholesterol-free chow diet (<40g/kg). The development of atherosclerosis lesion can be strongly accelerated by a high-fat, high-cholesterol (HFC) diet [111].

ApoE-knockout mice have played a pivotal role in understanding the inflammatory background of atherosclerosis, a disease previously thought to be mainly degenerative. The apoE-deficient mouse model of atherosclerosis can be used to: i) identify atherosclerosis-susceptibility-modifying genes; ii) define the role of various cell types in atherogenesis; iii) characterize environmental factors affecting atherogenesis; and iv) to assess therapies [112].

Because of the rapid development of atherosclerosis and the resemblance of lesion to human counterparts, the apoE-/- model have been widely used. However, some drawbacks are associated with the complete absence of apoE proteins: i) the model is dominated by high levels of plasma cholesterol; ii) most plasma levels are confined to VLDL and not to LDL particles, as in humans; and iii) apoE protein has additional antiatherogenic properties besides regulating the clearance of lipoproteins such as antioxidant, antiproliferative (smooth-muscle cells, lymphocytes), anti-inflammatory, antiplatelet, and also has NO-generating properties or immunomodulatory effects [113-115]. The study of the above processes and the effects of drugs thereupon is restricted in this model.

LDLreceptor-deficient mice (LDLr-/- mice)

In humans, mutations in the gen for the LDLr cause familial hypercholesterolaemia. Mice lacking the gene for LDL receptor (LDLr-/- mice), develops atherosclerosis, especially when fed a lipid-rich diet [116]. The morphology of the lesions in LDLr-/- mice is comparable to that in apoE-/-, while the main plasma lipoprotein in LDLr-/- mice are LDL and high-density-lipoprotein (HDL) [117].

*ApoE*3Leiden (E3L) transgenic mouse*

ApoE*3Leiden (E3L) transgenic mice are being generated by introducing a human ApoE*3-Leiden construct into C57B1/6 mice. E3L mice develop atherosclerosis on being fed

cholesterol. Because they are highly responsive to diets containing fat, sugar, and cholesterol, plasma lipid levels can easily be adjusted to a desired concentration by titrating the amount of cholesterol and sugar in the diet. E3L mice have a hyperlipidaemic phenotype with a prominent increase in VLDL- and LDL-sized lipoproteins fractions [118] and are more sensitive to lipid-lowering drugs than are apoE-/- and LDLr-/- mice [110].

4.2. Minipigs

Because of their well-known physiological and anatomical similarities to humans, swine are considered to be increasingly attractive toxicological and pharmacological models. Pigs develop plasma cholesterol levels and atherosclerotic lesions similar to those of humans, but their maintenance is more difficult and expensive than that of smaller animals [109]. The minipig, smaller than the domestic swine, has served as a model of hypercholesterolaemia for more than two decades now. In 1986, the ref. [119] reported that the Göttingen strain had more susceptibility to alimentary hypercholesterolaemia and experimental atherosclerosis than did domestic swine of the Swedish Landrace. Clawn, Yucatan, Sinclair, and Handford are among other general minipigs used for experimental use [120-122].

Down-sized Rapacz pigs are minipigs with familial hypercholesterolaemia caused by a mutation in the low-density lipoprotein receptor. It is a model of advanced atherosclerosis with human like vulnerable plaque morphology that has been used to test an imaging modality aimed at vulnerable plaque detection [123].

The Microminipig (MMP) is the smallest of the minipigs used for experimental atherosclerosis [124]. One of its advantages is that in 3 months an atherosclerosis very similar in location, pathophysiology and pathology to that in humans can be induced [125]. The easy handling and mild character of the MMP make it possible to draw blood and conduct CT scanning under non-anaesthesized conditions.

4.3. Zebra fish

Cholesterol-fed zebra fish represent a novel animal model in which to study the early events involved in vascular lipid accumulation and lipoprotein oxidation [126,127]. Feeding zebra fish a high-cholesterol diet results in hypercholesterolaemia, vascular lipid accumulation, myeloid cell recruitment, and other pathological processes characteristic of early atherogenesis in mammals [128]. The advantages of the zebra-fish model include the optical transparency of the larvae, which enables imaging studies.

4.4. Rabbits

Investigation has continued on hypercholesterolaemic rabbits since 1913, when Anitschkow demonstrated that, in rabbits fed a hypercholesterolaemic diet underwent atherosclerotic changes at the level of the arterial intima similar to those in atherosclerotic humans. The atheromatose lesions in this animal are similar to those in humans also in sequence, as

confirmed in aortic atherosclerosis [3], making this animal a universal model for studying the anti-atherogenic activity of many drugs [129-132].

For the characteristics detailed below, the New Zealand rabbit is an excellent model to reproduce human atheromatosis because: i) it is possible to induce hypercholesterolaemia in a few days after administration of a high-cholesterol diet [2]; ii) it is sensitive to the induction of atheromatose lesions [3]; iii) hypercholesterolaemia results from excess LDL [133]; iv) excess cholesterol is eliminated from the tissues to be incorporated in HDL [134]; vi) it is capable of forming cholesterol-HDL complexes associated with apoE which are transported by the blood to the liver [134]; vii) the lipoprotein profile is similar in size to that of humans in the highest range, with HDL being practically the same [135]; viii) it presents postprandial hyperlipaemia for the existence of chilomicron remnants [136]; ix) the hyperlipaemic diet increases apoE [4]; and x) the sustained alteration of lipids after feeding with a cholesterol-rich diet is reversible when the diet [130] is replaced by a normal one [2].

Studies on hypercholesterolaemic rabbits have improved our knowledge of human atherosclerosis by delving into different aspects of the disease such as lipoproteins, mitogenes, growth factors, adhesion molecules, endothelial function, and different types of receptors. At the vascular level, the importance of endothelial integrity and cell adhesion has been investigated [137]. It has been demonstrated that the high levels of lysosomal iron start the oxidation of the LDL, spurring the formation of lesions [138]. In addition, the expression of VCAM-1 preceding the infiltration of the subendothelial space by macrophages has been studied [139], as have the proteins, including MCP-1. In hypercholesterolaemic rabbits, this protein is over-expressed when the serum-cholesterol levels rise in macrophages and smooth-muscle cells, contributing to the development of fatty streaks [140].

In hypercholesterolaemic rabbits, the expression of Fas-L in cells of the arterial wall help us to understand the progression of the atherosclerotic lesion, as this expression indicates an increase in cell injury, as well as a greater accumulation in the intima of smooth-muscle cells [141]. Also, a hyperlipaemic diet causes a selective alteration of the functioning of certain regulatory proteins that are involved in gene expression, as occurs with the nuclear B factor, which stimulates the proliferation of macrophages and smooth-muscle cells [142].

In this model, a study was also made of the pre-thrombosis state triggered by the platelet aggregation in an altered endothelium and the possibilities of its inhibition [143], as well as the interactions of the LDL with the extracellular matrix to form aggregates that accumulate in the intima of the artery wall [144].

The consequences of hypercholesterolaemia in ischaemic cardiopathy and cerebrovascular pathology are well known. The same does not occur with the functional repercussions of the hypercholesterolaemia at the ocular level, partly because the underlying structural changes are not well known.

The hypercholesterolaemic rabbit constitutes a useful model to explore the repercussions of excess lipids at the ocular level. This is because rabbits are susceptible to both systemic as

well as ocular alterations. One of the broadest contributions made to the implications of experimental hypercholesterolaemia at the ocular level was that of ref. [145]. These authors, apart from analysing the changes in the liver, spleen, adrenaline glands, heart, aorta, and supraaortic trunk, described the most significant ocular findings, such as the accumulation of lipids in the choroid, retinal disorganization, and lipid keratopathy. With respect to the retinal macroglia, the synthesis of the apoE by the Müller cells, its subsequent secretion in vitro, and its being taken up by the axons and transported by the optic nerve enabled the detection of apoE in the latter geniculate body and in the superior colliculus [13].

Studies with electron microscopy on hypercholesterolaemic rabbits have revealed hypercellularity and optically empty spaces in the corneal stroma. These optically empty spaces, with an elongated or needle shape, were previously occupied by crystals of cholesterol monohydrate or crystals of cholesterol esters [146]. In other studies, the analysis in the form adopted for the crystallizations of the different types of lipids revealed that the needles corresponded to esterified cholesterol, and the short, thin ones to triglycerides [134]. Both crystallizations appear to be associated with other components such as collagen.

It had been recently reported that hypercholesterolaemic rabbis had a build-up of lipids (foam cells and cholesterol clefts) mainly at the suprachoroidea and to a lesser extent at the choroidal vascular layers. This lipids compressed the choroidal vessels and causes hypertrophy of the vascular endothelial- and vascular smooth-muscle cells. The ultrastructural analysis of these vascular structures demonstrated numerous sings of necrosis and a severe damage of the cytoplasmic organelles and caveolar system [16,147].

Recently, it has been reported that in comparison with normal control animals, hypercholesterolaemic rabbits had a reduction of the amplitudes of the first negative peak of the visually evoked potentials, the density of the RGCs, and the thickness of the INL and photoreceptor-cell layer. Additionally, the immunoreactivity to eNOS was reduced and increased to iNOSs. Enhanced activity of iNOS in hypercholesterolaemic rabbits might be involved in impaired visual function and retinal histology. Downregulation of eNOS activity might be one of the causes for impairment of the autoregulation [148].

The formation of foam cells is a consequence of phagocytes from the macrophage-oxidized LDL [16], with the retention of cholesterol in the vascular wall and the activation of ACAT (acetyl-cholesterol-acyl-transferase) [149], this point being key to the role of macrophages in the progression or regression of the lesions [134].

Watanabe

The Watanabe heritable hyperlipidaemic (WHHL) rabbit is an animal model for hypercholesterolaemia due to genetic defects in LDL receptors [150] and a lipoprotein metabolism very similar to that of humans [150,151]. These features make WHHL rabbits a true model of human familial hypercholesterolaemia. The first paper on the WHHL rabbit was published in 1980 [152]. The original WHHL rabbits had a very low incidence of coronary atherosclerosis and did not develop myocardial infarction. Several years of

selective breeding led to the development of coronary atherosclerosis-prone WHHL rabbits, which showed metabolic syndrome-like features, and myocardial infarction-prone WHHLMI rabbits. WHHL rabbits have been used in studies of several compounds with hypocholesterolaemic and/or anti-atherosclerotic effects with special relevance for statins [151]. Recently, WHHLMI rabbits have been used in studies of the imaging of atherosclerotic lesions by MRI [153], PET [154] and intravascular ultrasound [155].

5. Hypercholesterolemia induced ultrastructural changes in the Bruch's membrane-retinal complex

Few experimental studies examine the effects of hypercholesterolaemia on the posterior segment of the eye [14,15,145,156-158]. Hypercholesterolaemic rabbits constitute a useful model to delve into the repercussions of excess lipids at the ocular level. Rabbits fed a 0.5% cholesterol-enriched diet for 8 months showed a statistical increase in total serum cholesterol [15,16,147,158,159]. In these animals, the hypercholesterolaemia caused numerous changes in the Bruch's membrane-retinal complex. Bruch's membrane was thicker than in normal animals (Figure 3A,B) due to the build-up of electrodense and electrolucent particles (Figure 3B) in the inner and outer collagenous layers [15]. As in hypercholesterolaemic animals, thickening and lipid accumulation in Bruch's membrane has been described in human AMD [160,161]. These deposits of lipids or lipid-rich material could add resistance to the flow of solutes and water through the Bruch's membrane-RPE complex, as demonstrated by the studies that have measured the hydraulic conductivity of isolated Bruch's membranes [162,163]. The local metabolism and transport of cholesterol, impaired in hypercholesterolaemic rabbits as a result of a thickened Bruch's membrane with changes in its collagenous layers, could play an important role in the contribution of lipids required for retinal neurons to maintain and remodel their membranes.

The cholesterol source for RPE and photoreceptors are the plasma lipids. Given that there is no direct contact between the photoreceptors and the choroidal circulation, adjacent cell types (RPE cells and Müller cells) must facilitate the transfer of lipids to the photoreceptors. In fact, the expression of native receptors for LDL on RPE cells has been reported [12,164]; this could be related to local production of apoE by RPE cells. An abnormal metabolism of lipids secondary to a cholesterol-enriched diet and/or apoE deficiencies could upset the cholesterol balance in RPE and photoreceptors. This could be the situation in hypercholesterolaemic rabbits in which ERP changes have been reported [15]. In this experimental model, RPE showed numerous hypertrophic cells and some nuclei were absent. The cytoplasm of these cells showed numerous dense bodies, debris from cell membranes, and numerous clumps of lipids (Figure 4B) filling the cytoplasm and replacing the nucleus and organelles that could be contributing to the hypertrophy and degeneration of the RPE [15]. Additionally, the basal zone of some RPE cells revealed autophagic vesicles, vacuoles, electrodense deposits, and debris from cell membranes [15] that could correspond to the laminar deposits described by [165] (Figure 4B). As in human AMD, changes of RPE could contribute to the degeneration of the photoreceptors [164] whose metabolism depends on normal RPE function and integrity [15,166].

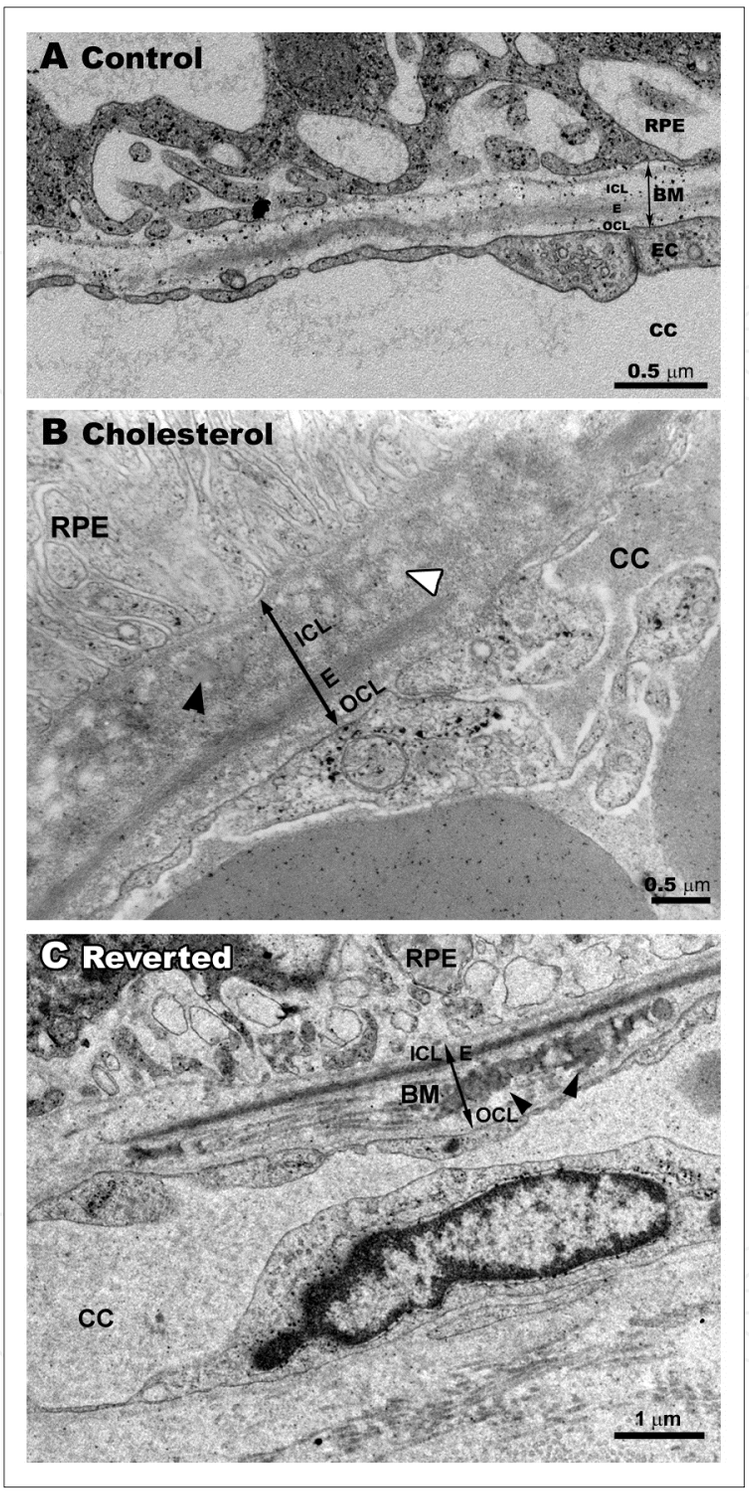

Figure 3. Transmission electron microscopy of Bruch's membrane and choriocapillaris. A: Control rabbit. B: Hypercholesterolaemic rabbit. Electrodense (black arrowhead) and electroluminescent (white arrowhead) particles at the inner collagenous layer Modified from Triviño et al. [15]). C: Reverted rabbit. Bruch's membrane with electrodense particles (black arrowheads) at the outer collagenous layer. [Bruch's membrane (BM); choriocapillaris (CC); retinal pigment epithelium (RPE); inner collagenous layer (ICL); elastic layer (E); outer collagenous layer (OCL); endothelial cell (EC)]. (Modified from Ramírez et al. [158])

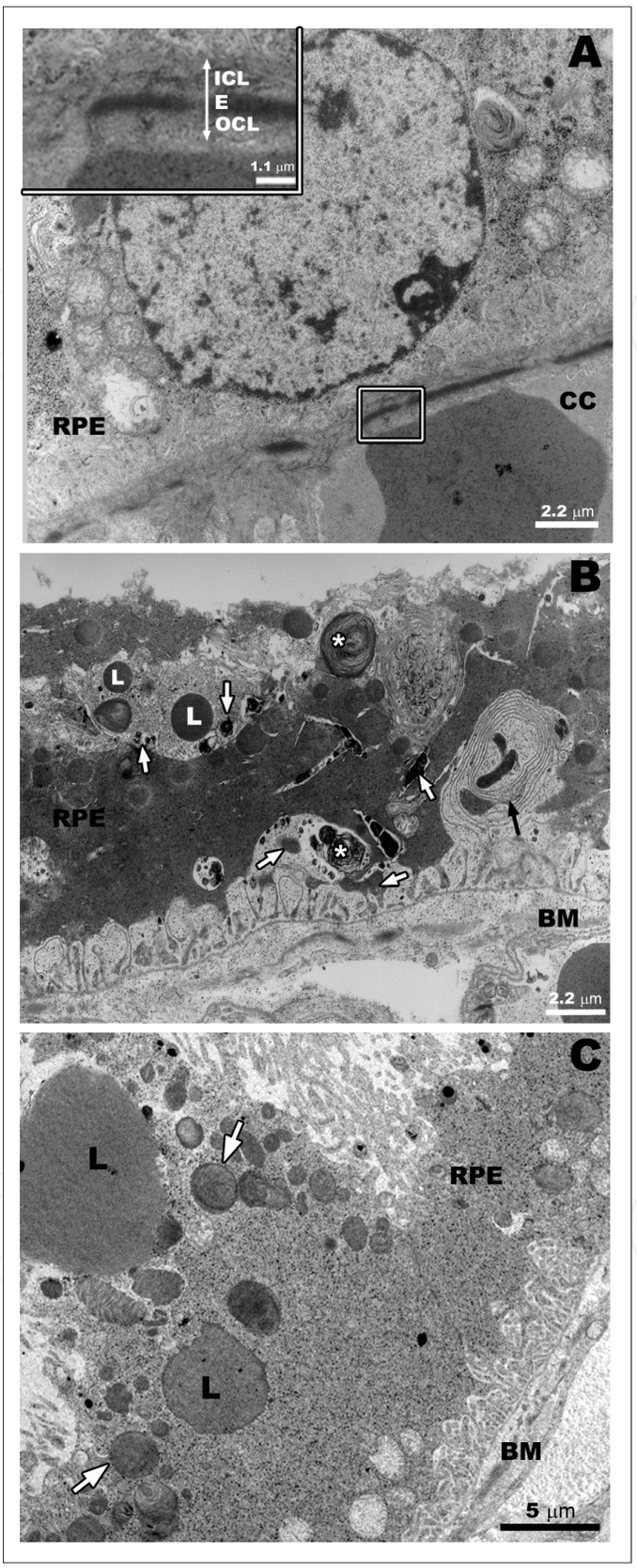

Figure 4. Transmission electron microscopy of Bruch's membrane and retinal pigment epithelium cells (RPE). A: Choriocapillaris - Bruch's membrane - RPE complex from control rabbit. Detail of Bruch's

membrane (insert) showing the outer collagenous layer, elastic layer and inner collagenous layer. B: The cytoplasm of RPE cell in hypercholesterolaemic rabbit shows dense bodies (white arrows), debris from cell membranes (*) and droplets of lipids. The apical microvilli have disappeared and the basal infolding forms lamellar structures (black arrow). C: RPE cells in reverted rabbit. Few lipids, dense bodies (white arrows) and some lamellar structures are visible in the cytoplasm. [Choriocapillaris (CC); retinal pigment epithelium (RPE); Bruch's membrane (BM); inner collagenous layer (ICL); elastic layer (E); outer collagenous layer (OCL); lipids (L)]. (Modified from Ramírez et al. [158] and Triviño et al. [15]).

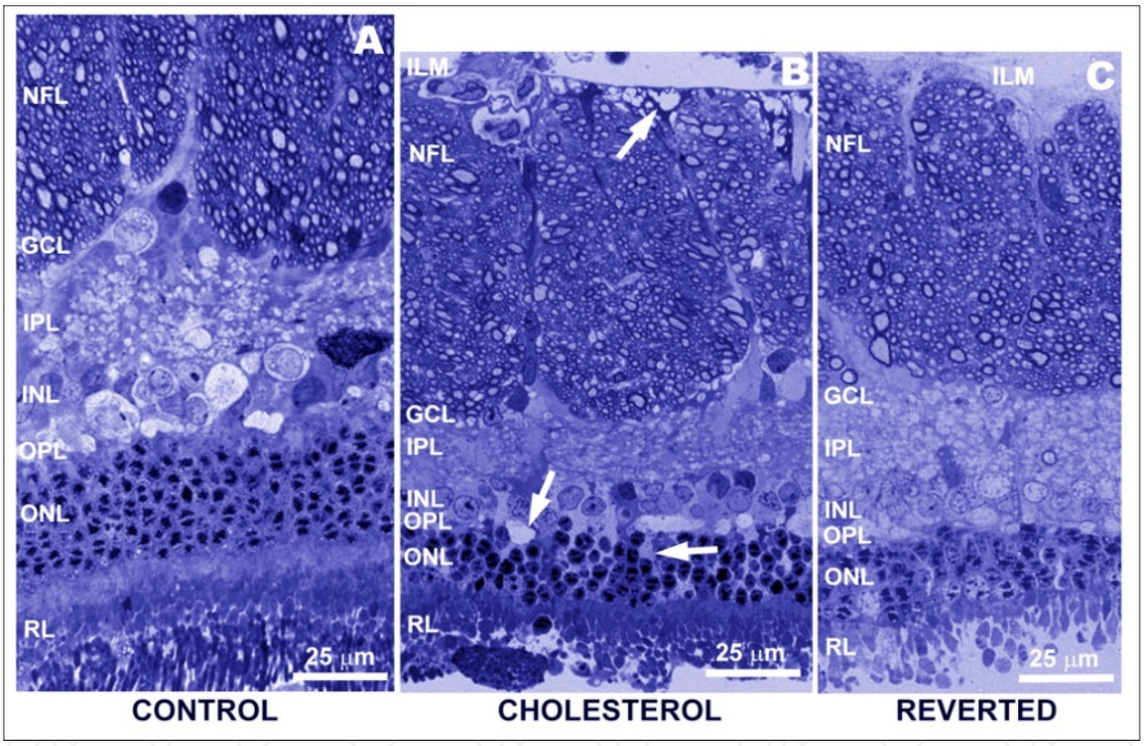

Figure 5. Retinal semi-thin sections (light microscopy). Retinal-layer changes. A: Control rabbit. B: Hypercholesterolaemic rabbit. C: Reverted rabbit. The figure illustrates the overall thinning of the retinal layers in hypercholesterolaemic and reverted animals with respect to control. The empty spaces (arrows) secondary to cell loss and degeneration observed in hypercholesterolaemic (B) are less evident in reverted rabbit (C). [Ganglion-cell layer (GCL); inner nuclear layer (INL); inner plexiform layer (IPL); inner limiting membrane (ILM); nerve-fibre layer (NFL); outer nuclear layer (ONL); outer plexiform layer (OPL); photoreceptor layer (RL)]. (Modified from Ramírez et al. [158]).

The nutrition of the outer retina depends on the integrity of the choriocapillaris vessels and on the diffusion of plasma through the Bruch's membrane-RPE complex. The alterations in

the endothelium of the choriocapillaris and the build-up of lipids (hydrophobic barrier) detected in the Bruch's membrane-RPE complex of hypercholesterolaemic rabbits [15] could interfere with oxygen and nutrient transportation, leading to an ischaemic state [30].

The conditions of hypoxia-ischaemia lead to higher glutamate levels in the extracellular fluid, and thereby could cause oxidative damage by excitotoxic mechanisms in the neurons [21,22]. In hypercholesterolaemic rabbits, neurosensory retinal changes were detected (Figure 5A,B) [15].

These changes were not uniformly distributed throughout the retina, being more intense in the retinal areas overlying the most altered RPE cells. In these areas, the photoreceptor discs were mostly absent. The thickness of the retinal layers (ONL, OPL, INL, IPL, GCL and NFL) were reduced (Figure 5B) and empty spaces were visible at different retinal levels that consisted of different stages of cell degeneration due to necrosis and apoptosis (Figure 6A,7A,B). In necrotic cells, the nucleoplasm, cytoplasm, and cytoplasmic organelles underwent progressive hydropic degeneration (swelling, vacuolization, and disappearance of specific ultrastructural features) (Figure 6A). The nuclear and cytoplasm membranes ruptured and released their contents into the intercellular space (Figure 6A). The remains were taken up and absorbed by neighbouring cells –essentially Müller cells (Figure 6A,7A) and astrocytes -, the latter only in the NFL. The apoptotic cells showed progressive condensation and shrinkage of the nucleoplasm and cytoplasm (Figure 7A,B). Cells in more advanced stages of apoptosis shed part of their substance, which was observed as dense inclusion bodies in neighbouring cells (Figure 6A,7A). The compact bodies appeared surrounded by or engulfed in Müller cells and astrocytes [15,158].

Changes found in the nuclear layers of the retina of hypercholesterolaemic rabbits resemble those described in human AMD [74]. As in human AMD, hypercholesterolaemic rabbits exhibited a loss of ganglion cells and had cell features of apoptosis and necrosis as well as electrodense inclusions (probably lipofuscin) in the cytoplasm of this cell type (Figure 7B). This ganglion-cell loss could be caused, at least partly, by a local disruption of cholesterol homeostasis [14]. A reduced population of ganglion cells could secondarily impair the neurotrophic support of the retinal neurons as a consequence of reduced secretion of brain-derived neurotrophic factor (BDNF) by ganglion cells. This scenario is feasible, given that amacrine cells express the TrkB receptor for BDNF [17] and that BDNF improves the survival of bipolar cells upon activation of the p75 receptor, which then induces the secretion of fibroblast growth factor b (bFGF) [167]. The situations described could contribute to the axon loss observed in hypercholesterolaemic rabbits [158]; this loss parallels human AMD, in which a considerable axonal degeneration has been reported [74].

In hypercholesterolaemic rabbits, the capillaries in the NFL and in the vitreous humour had a thickening of the basal membrane, dense bodies, and cytoplasm vacuoles (Figure 8A,B). These alterations have also been reported in hypercholesterolaemic rats [156].

In summary, the thickening of the basal membrane together with the alterations of the endothelial cells of the intraretinal and epiretinal capillaries, combined with the changes in Bruch's membrane and the build-up of lipids in the outer retina, could contribute to a situation of chronic ischaemia observed in the retina of hypercholesterolaemic rabbits.

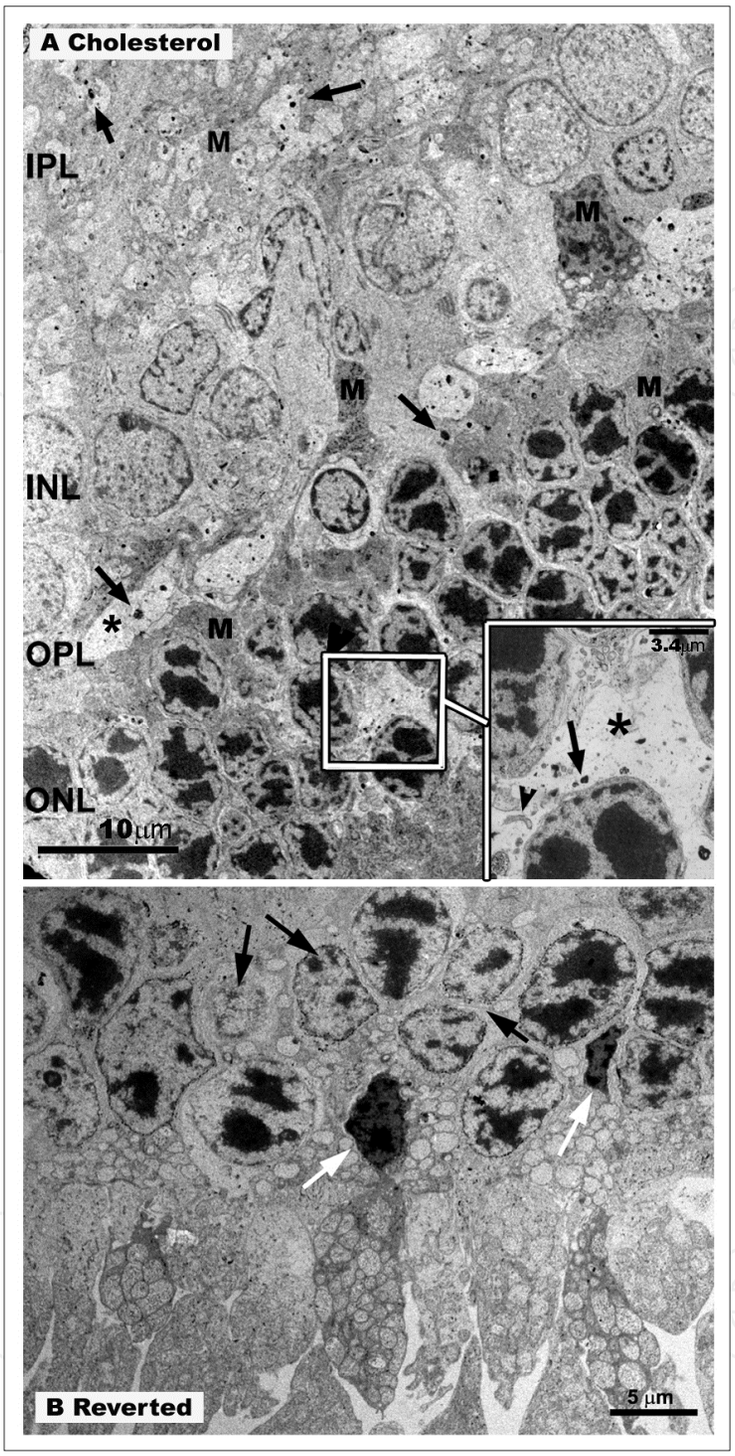

Figure 6. Ultrastructural retinal changes in outer nuclear layer and outer plexiform layer. A: Hypercholesterolaemic rabbit. Numerous dense bodies (black arrows) and empty spaces (*) are visible in these layers. The processes of Müller cells fill the empty spaces left by degenerated cells. Insert: at greater magnification the empty spaces consist of degenerated cytoplasm with numerous dense bodies (black arrow) and cell debris (black arrowhead). B: Reverted rabbit. Apoptosis (white arrows) and necrosis (black arrows) of photoreceptors are visible in the ONL. [Müller cells (M); inner nuclear layer (INL); inner plexiform layer (IPL); outer nuclear layer (ONL); outer plexiform layer (OPL)]. (Modified from Ramírez et al. [158] and Triviño et al. [15]).

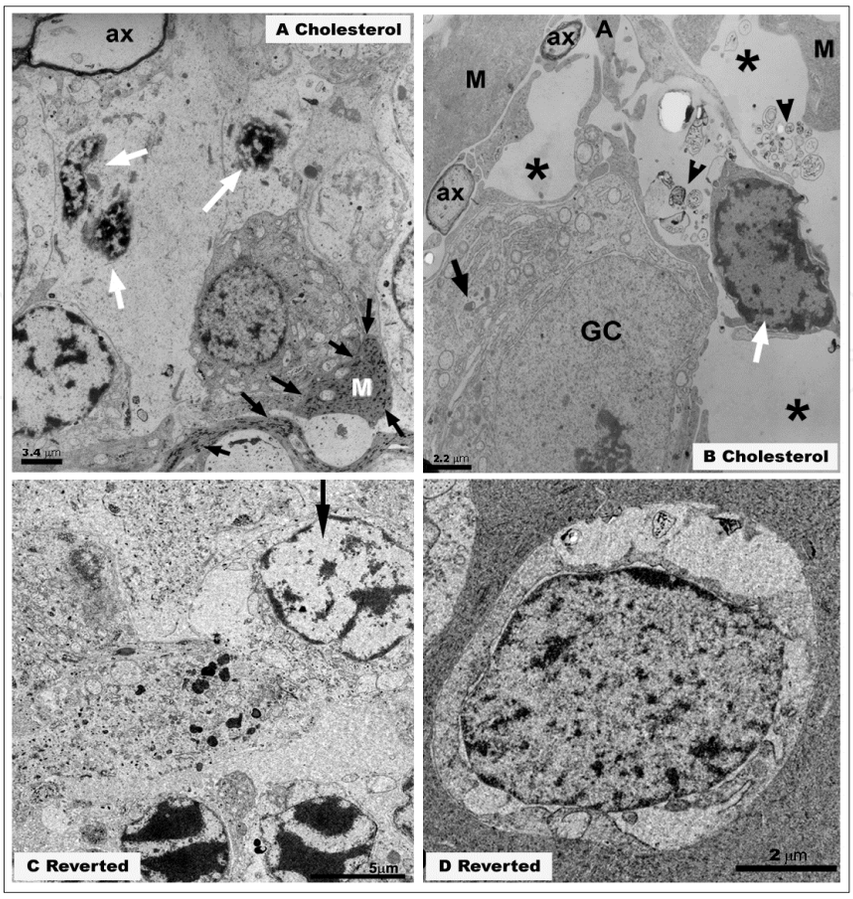

Figure 7. Ultrastructural retinal changes in inner nuclear layer and ganglion-cell layer. A-B: Hypercholesterolaemic rabbit. A: Cells in apoptosis (white arrows) in the inner nuclear layer. Dense bodies (black arrows) inside the Müller cell processes. B: Apoptosis (white arrow) in the ganglion-cell layer. Cell debris (black arrowheads) and dense bodies (black arrow). [Müller cell (M); axon (ax); ganglion cell (GC)]. C-D: Reverted rabbit. C: Cell necrosis (black arrow) in the inner nuclear layer. D: Ganglion cell in advanced stage of necrosis. (Modified from Ramírez et al. [158] and Triviño et al. [15])

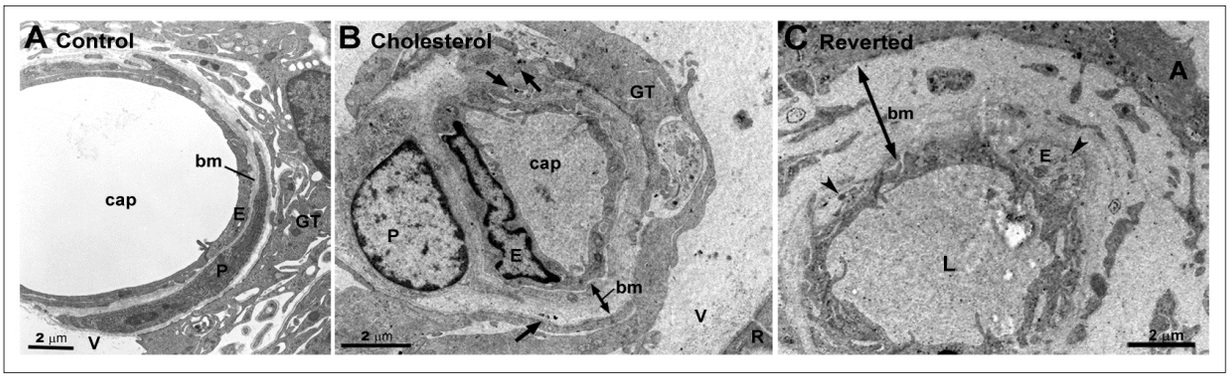

Figure 8. Transmission electron microscopy of capillaries in the vitreous humour. A: Control rabbit. B: Hypercholesterolaemic rabbit. The basal membrane is thickened with respect the control. C: Reverted rabbit. The basal membrane is thicker than control and cholesterol animals. Necrotic features (arrowhead) are visible in some endothelial cells. [Basal membrane (bm); capillary (cap); endothelial cell (E); glial tuft (GT); pericyte (P); vitreous humour (V); dense bodies (black arrows); retina (R); vascular lumen (L); astrocyte (A)]. (Modified from Ramírez et al. [158] and Triviño et al. [15])

6. Hypercholesterolaemia-induced changes in the retinal macroglia

An abnormal metabolism of lipids secondary to a cholesterol-enriched diet and/or apoE deficiencies could upset the cholesterol balance in the retinal layers, as mentioned above. However, it appears that other retinal components can produce heterogeneous particles locally containing apoE [13]. These particles are synthesised mainly by Müller cells, although astrocytes associated with ganglion cells axons could be involved in their production [13]. Müller cells are radially oriented cells that along their course, extend branches that interdigitate with every type of retinal neuron, with other types of glia (Figure 2A), and with the blood vessels of vascularized retinas [168]. Its participation in the cholesterol metabolism (supplying heterogeneous lipoprotein particles and apoE) and transport (due to its anatomical position in the retina) determines its importance as a source of the lipids needed by neurons for maintaining and restructuring their cell membranes [13,168].

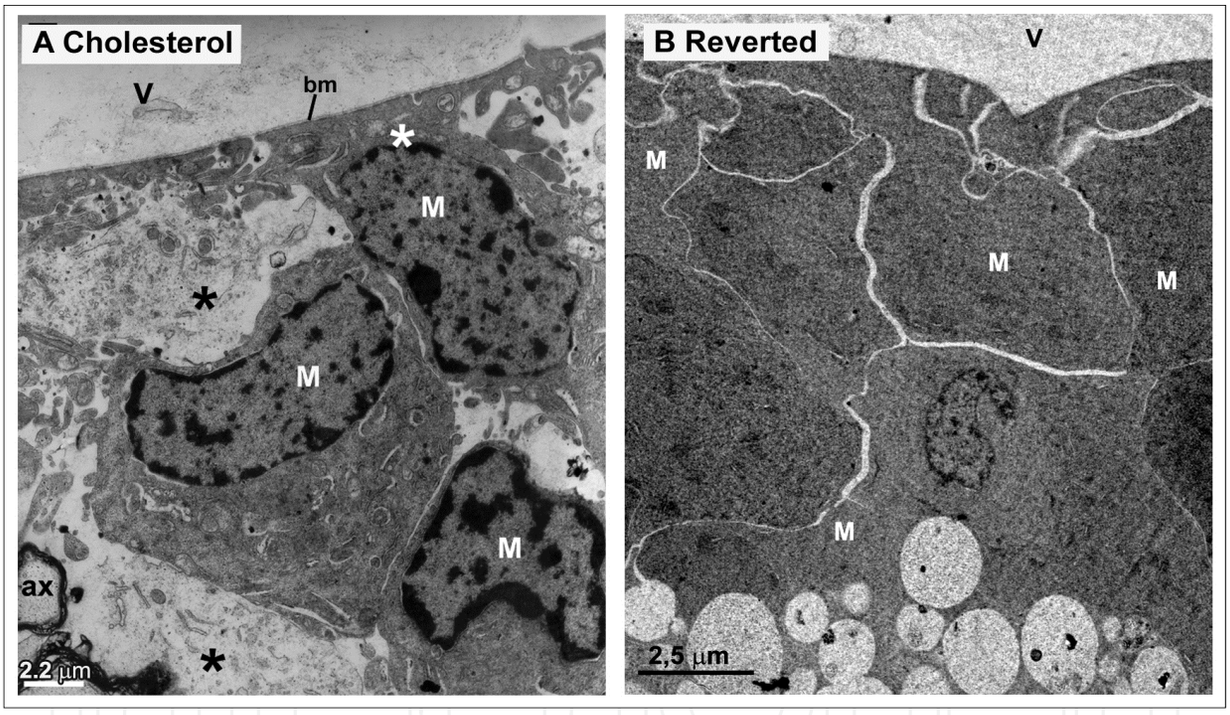

Figure 9. Transmission electron microscopy of retinal astrocytes and Müller cells.
A: Hypercholesterolaemic rabbit. Three nuclei of Müller cells displaced to the nerve-fibre layer. One of the Müller cells participates in the formation of the inner limiting membrane (white asterisk). Astrocytes in advanced stage of necrosis (black asterisk). B: Reverted rabbit. The empty spaces left by degenerated axons in the medullated nerve-fibre region are occupied only by the Müller cells in the retinal periphery. [Axon (ax); basal membrane of the ILM (bm); Müller cell (M); vitreous humour (V)]. (Modified from Ramírez et al. [158] and Triviño et al. [15]).

In situations of sustained hypercholesterolaemia, alterations of lipid metabolism could take place, potentially influencing the glial response. In fact, in hypercholesterolaemic rabbits Müller cells were reactive, exhibiting large amounts of rough endoplasmic reticulum and abundant glial filaments in their cytoplasm (Figure 9A), manifested by a more intense

immunoreaction to GFAP (Figure 10H) [158]. Normally, GFAP is expressed at a low level or is not detectable in mammalian Müller cells (Figure 10G). In pathological situations, the major intermediate filament expressed by reactive Müller cells appears to be GFAP. The loss of retinal integrity as a result of mechanical injury, detachment, photoreceptor degeneration or glaucoma (Figure 2A) provokes intense GFAP immunoreactivity in Müller cells and increases the GFAP content of the retina [39,91,169-171]. This over-expression of GFAP is due to the activation of the transcriptional gene for GFAP in Müller cells [168]. Additionally, Müller cell reactivity transduces an increase in cell metabolism [168].

Another consequence of the reactivity of Müller cells is their capacity to form glial scars, most probably in an attempt to restore the blood-retinal barrier [172]. These scars, formed by hypertrophic cells in which the nuclei were displaced to the NFL, were detected in hypercholesterolaemic rabbits (Figure 9A). In addition, hypertrophic Müller cells occupied some of the empty spaces left by degenerated neurons in the INL, ONL, IPL, and NFL (Figure 6A) [15,158,173]. This type of cell response, which has also been described in human AMD [74] resembles that following photoceptor degeneration, which induces the processes of Müller cells to extend into and fill the empty spaces [168]. Another similarity between human AMD and experimental hypercholesterolaemia are the ultrastructural changes affecting the outer and inner retina. In both instances, the bodies of Müller cells are displaced from the INL to the vitreous in the case of human AMD [74] and to the NFL and ILM in hypercholesterolaemic rabbits [15,158]. It is possible that in both situations Müller cells migrate in an attempt to reach the metabolic reserve in the vitreous. This could be an adaptive system for transporting nutrients and energy substrates to those areas of the retina exposed to the chronic ischaemic insult.

Like Müller cells, astrocytes are related to apoE secretion [174,175], making these cells susceptible to alteration in long-term hypercholesterolaemia. Müller cells and astrocytes are intermediate between neurons and vessels; they are located on the basal membrane of capillaries separating them from neurons [37,82,95,168]. The thickening of the basal membrane and the presence of dense bodies and vacuoles in the endothelial cytoplasm of the retinal blood vessel in hypercholesterolaemic rabbits (Figure 8A) [15] could indicate impaired transport of oxygen and nutrients to the retinal tissue as well as the removal of cellular debris, thus contributing to a situation of chronic ischaemia [20] in the inner retina. It is known that astrocytes protect neurons from ischaemia by different mechanisms: they remove excitotoxic neurotransmitters and ions from the perineural space, doing so partly by glutamine synthetase, which also provides glutamine to neurons ([176,177]. In addition, astrocytes store glycogen, have the potential to provide lactate, and produce growth factors as well as cytokines [23]. Moreover, it has been shown that astrocytes are more resistant to oxidative damage because they possess antioxidant mechanisms such as high concentrations of reduced glutathione and vitamin C [21]. Therefore, a reduction in the protective function of astrocytes could contribute to neural dysfunction.

Differences between rabbit and human retinas and astrocytes must be taken into account when comparing the two species [38,41,42,82]. The rabbit retina has epiretinal vascularization and possesses perivascular astrocytes which are absent in humans. However, in both species,

astrocytes are located at the NFL and GCL. The rabbit retina had two main groups of astrocytes: astrocytes associated with the nerve-fibre bundles (Figure 10A) and perivascular astrocytes (type I and type II) (Figure 10A,D), associated with the vitreous blood vessels [40].

As mentioned above, astrocytes are essential for the maintenance of neural homeostasis, and their susceptibility to alteration in long-term hypercholesterolaemia has been reported [15]. Thus, in hypercholesterolaemic rabbits, all retinal types of astrocytes were reactive, having large amounts of rough endoplasmic reticulum and upregulation of GFAP immunoreactivity (Figure 10B,E,H). The altered lipid homeostasis, in conjunction with increased astrocyte activity, could explain the build-up of electrodense particles, probably lipofuscin and lipids, found in their cytoplasm. The exposure of these electrodense particles to light and high oxygen concentrations provide ideal conditions for the formation of reactive oxygen species that damage cellular proteins and lipid membranes [178], a situation that could impair the mechanism of protection from ischaemia. If we add to this the higher concentrations of extracellular toxic substances (e.g. glutamate) which could damage the neurons by cytotoxic mechanisms [21,22], the possibilities of keeping the cellular machinery intact against ischaemia diminish in favour of neuronal death. All the above-mentioned conditions could contribute to macroglial swelling and subsequent breakdown of intermediate filaments (loss of GFAP staining) and ultimately macroglial death [23]. In fact, hypercholesterolaemic rabbits showed apoptosis and necrosis affecting Müller cells and astrocytes (Figure 7B,9A), resulting in a statistically significant loss of all types of astrocytes in comparison with control animals (Figure 10A,B, 11) [15].

In summary, long-term hypercholesterolaemia lowers the astrocyte number and their antioxidant activity as well as the capability to remove glutamate from the extracellular space; it may also contribute to neuronal dysfunction [15,158]. The reactivation and migration of retinal Müller cells may be reflecting an adaptive system to supply nutrients to those areas of the retina exposed to the chronic ischaemia generated by the hyperlipidaemia.

7. Changes in Bruch's membrane retinal complex after the normalization of hypercholesterol levels

It has been established that the atherosclerotic lesions can undergo regression in experimental animals such as rabbits, dogs, and non-human primates [179]; and the lack of progression or even regression can occur in humans, especially with the introduction of new therapeutic options [180].

Animal models are useful for studying lesion regression after the normalization of cholesterol serum values. When high levels of cholesterol are withdrawn from the diet, rabbits recover some of the biochemical and histological parameters altered in cholesterol-fed animals [16,181]. Serum concentration of total cholesterol, triglycerides, phospholipids, VLDL, HDL, LDL, and intermediate-density lipoprotein (IDL) have reported to increase in rabbits fed with a 0.5% cholesterol-enriched diet for eight months. When the same animals are then fed a standard diet for another 6 months, (reverted rabbits), lipid values returned to normal [158]. Notably, the normalization of serum values was not followed by a complete recovery of the thoracic aorta, choroid [16], or histology of the retina (Figure 5C) [158]. Specifically, in reverted rabbits, Bruch's

membrane (Figure 3C) and RPE alterations (Figure 4C) were still present although to a lesser extent than in hypercholesterolaemic animals (Figure 3B, 4B). Bruch's membrane was thicker in some areas due to collagenous and electrodense material in the outer collagenous layer (Figure 3C). This contrasted with the observations in hypercholesterolaemic rabbits in which the thicker Bruch's membrane resulted from the build-up of electrodense and electrolucent particles, mainly at the inner collagenous layer (Figure 3B) [15]. The cytoplasm of RPE cells contained a considerably lower quantity of lipids in reverted animals (Figure 4C), although in some instances the lamellar structures (the plasma membrane of basal infolding back on itself) described in hypercholesterolaemic rabbits were also seen. This partial structural recovery could improve the diffusion of nutrients from the choriocapillaris and removal of cell debris from RPE, thus exerting a possible effect on the retina. However, reverted rabbits retained features observed in hypercholesterolaemic animals, such as an apparent decrease in retinal thickening (Figure 5C), intense cell degeneration due to necrosis and apoptosis in the ONL, INL, and GCL and axonal degeneration at the NFL (Figure 6B, 7CD). The empty spaces following neuronal death observed in hypercholesterolaemic animals were occupied by Müller cells (in OPL, IPL, NFL) and by astrocytes (in NFL) in reverted rabbits (Figure 6A) [158].

It bears mentioning that the retinal vessel in reverted rabbits showed greater damage than in hypercholesterolaemic animals such as: thickening of the basal membrane with numerous dense bodies, necrosis of endothelial cells, hypertrophy of the muscle layer, and increase in the collagen tissue of the adventitia (Figure 8C) [158]. The maintenance of retinal damage observed in reverted animals could be at least partly due to the greater alterations of retinal vessels and the persistence of the choriocapillaris alterations [16]. The vascular retinal alterations, which extended from the endothelium to the adventitia, could contribute to sustain an ischaemic situation despite the diet-induced normalization of lipid levels. Another factor that could contribute to the maintenance of retinal damage would be the role of Müller cells in neuronal swelling and apoptosis. During ischaemia, over-excitation of ionotropic glutamate receptors not only leads to neuron depolarization, which causes excess Ca^{2+} influx into the cells, but also activates the apoptosis machinery. The ion fluxes in the retinal neurons, associated with water movements that are mediated by aquaporin-4 water channels expressed by Müller cells, can result in neuronal swelling [182]. Thus, during ischaemic episodes in the rabbit retina, the plexiform layers and the cytoplasm of neurons become oedematous.

In summary, normalization of the lipid level is not followed by a complete normalization of the retinal histology. The remaining changes in the retina are due mainly to the sustained chronic ischaemia caused by the alterations in the retinal vessel, Bruch's membrane, and RPE. Such ischaemic situations exert a detrimental impact on the neurons of the different layers of the retina.

8. Changes in the retinal macroglia after normalization of hypercholesterol levels

As described for the Bruch's membrane-retinal complex, the normalization of the blood-lipid levels by the substitution of 8 months of a hypercholesterolaemic diet by 6 months of a

standard one, do not reverse the changes in the retinal macroglial population of hypercholesterolaemic rabbits [158].

In reverted animals, Müller cells were hypertrophic and filled up the empty spaces left by degenerated neurons and axons (Figure 9B). This hypertrophy could be due to the osmotic swelling of Müller cells. A significant correlation between Müller cell hypertrophy and the extent of osmotic Müller cell swelling has been reported in rat retina during retinal inflammation, suggesting that the alterations of swelling properties is characteristic of Müller cell gliosis [183]. It has also been proposed that Müller cell swelling in the post-ischaemic retina is caused by inflammatory mediators, due to the activation of phospholipase A2 by osmotic stress [182]. In both hypercholesterolaemic and reverted rabbits, the hyperlipaemic diet could have caused an imbalance in long-chain polyunsaturated fatty acids (in the neural retina, these are present mainly in the phospholipids of the cell membranes [53]) which could prompt an increase in inflammatory elements such as reactive oxygen species from macrophages, TNF-α, IL-1β, IL-6, Natural Killer, cytotoxic T lymphocyte activation, and lymphocyte proliferation [51]. Therefore, ischaemic and inflammatory processes could trigger Müller cell hypereactivity in hypercholesterolaemic animals and reverted rabbits and provoke the hypertrophy and swelling of this cell type.

The astrocytes of reverted rabbits displayed changes with respect to hypercholesterolaemic animals. The area occupied by the astrocytes associated with the nerve-fibre bundles was significantly lower than in the hypercholesterolaemic group (Figure 10H,I,11). With respect PVA (perivascular astrocytes), a striking feature was the absence of type I PVA, thus the intense GFAP immunoreactivity found in the retinal blood vessels was due mainly to type II PVA (Figure 10C,F). The processes of these cells formed a network similar to that exhibited by the type I PVA of the normal rabbits [158]. The maintenance of the area occupied by the PVA in reverted animals (Figure 11) could be due to the hyperplasia of type II PVA as an attempt to compensate for the loss of type I PVA (Figure 10C,F). This cell proliferation is presumably a response to the sustained retinal ischaemia undergone by reverted rabbits despite of normalization of cholesterol levels. Type II PVA of reverted animals were reactive, hypertrophic, and had an enlargement of their cell bodies and processes (Figure 10F) [158]. These features plus the above-mentioned hyperplasia are typical changes of glial cells in response to nerve damage [184].

The specific function of reactive gliosis is unknown. It has been reported that glial cells undergoing reactive gliosis up-regulate the production of cytokines and neurotrophic factors which may be crucial for the viability of injured neurons [168]. Additionally, it is presumed that reactive gliosis is involved in phagocytosis of debris and in restoring breaches in the blood-brain barrier by scar formation [185]. Müller cells and astrocytes from hypercholesterolaemic and reverted rabbits had cell debris in their cytoplasm [158]. It has been reported that astrocytes [186] as well as Müller cells [187] can exert phagocytic functions and that the microglia (the main phagocytic cell of the nervous system) intervene only when the build-up of debris in the nervous tissue is abundant [188]. Phagocytosis of exogenous particles, cell debris, and hemorrhagic products may be an important scavenging

function of Müller cells [168]. It has been suggested that the phagocytic process of these cells is similar to that associated with macrophages and that in addition they can function as antigen-presenting cells [39,168].

From the above, it can be concluded that the substitution of a hyperlipaemic diet by a standard one in an experimental rabbit model normalizes the blood-lipid levels. However, the progressive and irreversible chronic retinal ischaemia secondary to cholesterol-induced changes in the choroid [16,147] as well as the retinal blood vessels trigger a sustained reactive gliosis that could be exerting neurotrophic, phagocytic or immune-related functions among others.

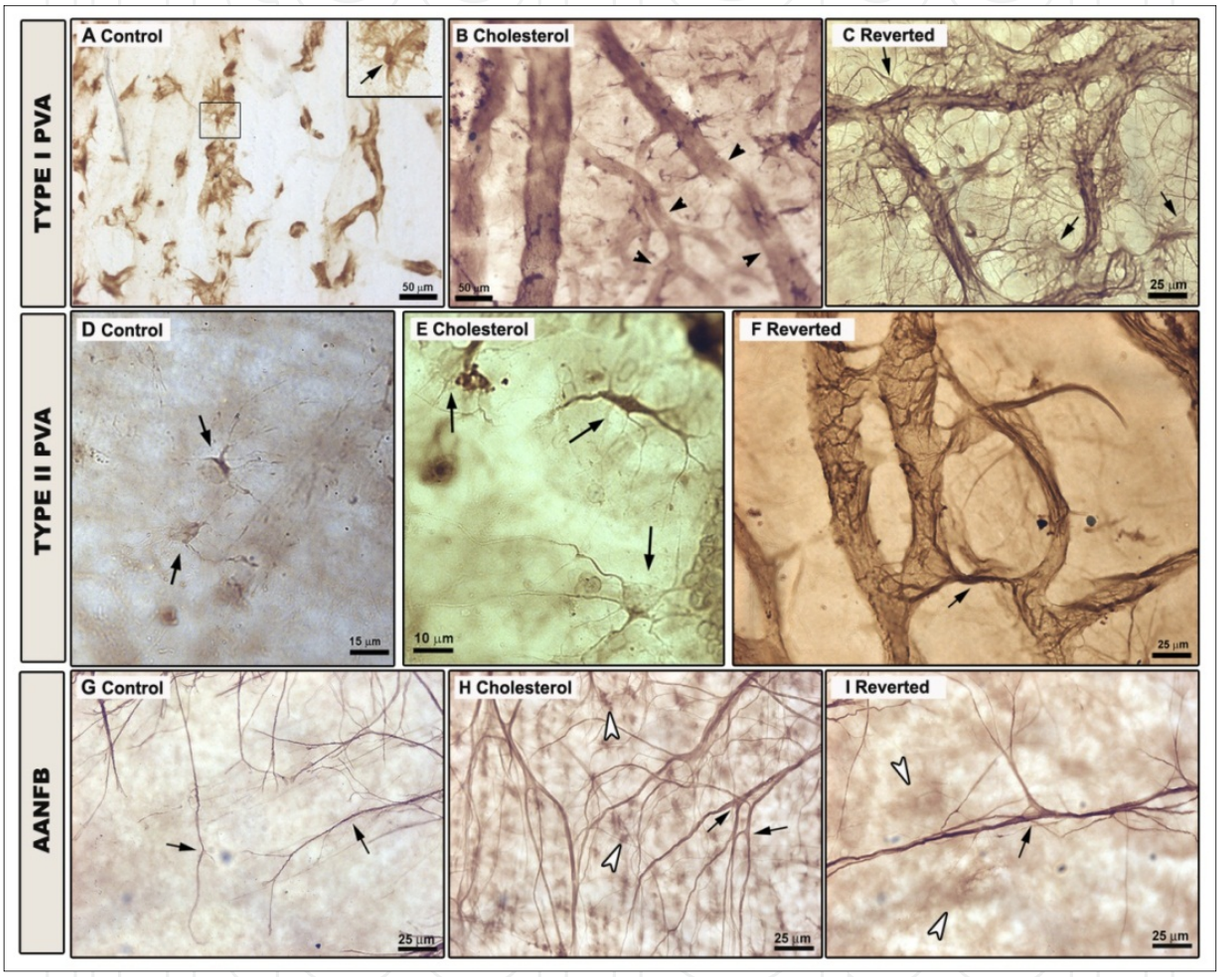

Figure 10. Immunohistochemistry anti-GFAP in rabbit retinal whole-mount. A-C: Type I perivascular astrocytes (PVA). D-F: Type II PVA. G-I: Astrocytes associated with the nerve-fibre bundles (AANFB). A, D, G: Control rabbits. B, E, H: Hypercholesterolaemic rabbits. C, F, I: Reverted rabbits. A-C: In hypercholesterolaemic animals Type I PVA have a higher GFAP+ immunoreactivity than in control animals; these cells are absent from many retinal vessels. In reverted animals a striking feature is the absence of type I PVA. D-F: In hypercholesterolaemic animals Type II PVA have higher GFAP immunoreactivity, robust cell bodies and thicker processes than in control. In reverted animals the intense GFAP+ cells are morphologically similar to the reactive type II PVA of hypercholesterolaemic animals. G-I: In hypercholesterolaemic and reverted animals the AANFB show high GFAP+ immunoreactivity, robust cell bodies, and thick processes. [Astrocytes cell bodies (arrow); vessel free of type I PVA (arrowhead); GFAP immunorectivity of Müller cells (empty arrow)]. (Modified from Ramírez et al. [158]).

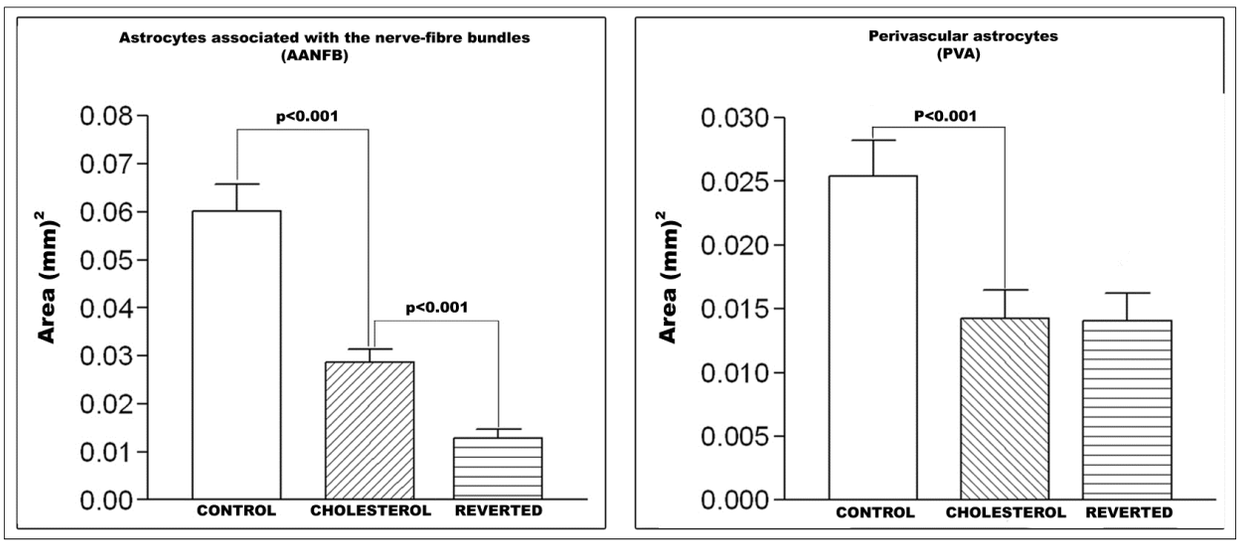

Figure 11. Area occupied by astrocytes per zone measured (0.1899mm2) in Control, hypercholesterolaemic, and reverted animals. (Modified from Ramírez et al. [158]).

9. Conclusions and perspectives

Hypercholesterolaemia is a risk factor for the development of chronic ischaemia in the retina and therefore for neuronal survival [15,158]. It is now recognized that lipids play a key role as structural and signalling molecules. Given that lipid intake is most dependent on food composition, the dietary regimen could contribute to induction or prevention of retinal diseases. In relation to this, a pertinent question would be whether or not the normalization of the plasma-cholesterol levels could restore the retinal changes that take place during hypercholesterolaemia and reverse the chronic ischaemia process generated by this situation. The answer to this question seems to be no, since, although it is true that the lipid accumulations in the choroid and Bruchs' membrane are reduced with the normalization of the blood-lipid level, some structural changes do not reverse [16,158], implying an irreversibly chronic situation and very probably progressive ischaemia in retina.

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