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Saponin-Mediated Rejuvenation of Bruch's Membrane: A New Strategy for Intervention in Dry Age-Related Macular Degeneration (AMD)

Yunhee Lee, Eun Jung Ahn and Ali Hussain

Abstract

At present, there is no treatment modality for the vast majority of patients with dry AMD. The pathophysiology of AMD is complex but current evidence suggests that abnormal ageing of Bruch's membrane imparts a metabolic insult to the retinal pigment epithelium (RPE) and photoreceptor cells that leads eventually to the inflammatory-mediated death of these cells. Underlying mechanisms contributing to the pathology of Bruch's membrane include the accumulation of 'debris' and malfunction of the matrix metalloproteinase (MMP) system resulting in diminished metabolic support of the retina and inefficient removal of toxic pro-inflammatory mediators. Saponins are amphipathic molecules that have a hydrophobic tri-terpenoid lipid region and hydrophilic glycosidic chains that allow for the dispersion of these deposits in Bruch's and re-activation of the MMP system leading to a 2-fold improvement in the transport properties of the membrane. Such an intervention is expected to improve the bi-directional exchange of nutrients and waste products, thereby slowing the progression of dry AMD. This will be the first drug-based interventionist possibility to address dry AMD.

Keywords: macular degeneration, Bruch's membrane, extracellular matrix, ageing, saponins

1. Introduction

Blindness due to AMD is of great concern to the ageing elderly population since the prevalence of the disease in Europe, in those aged 60 years and over has been estimated to be 27.7% with a projected increase in numbers from 67 to 77 million by 2050 [1]. Clinically, AMD is broadly divided into early, intermediate and late (or advanced) forms. Early AMD is characterised by the presence of large drusen and pigmentary abnormalities such as hypo- or hyperpigmentation of the fundus. Progression to the late form results in geographic atrophy of the RPE followed by photoreceptor degeneration, known as 'dry' AMD. The late phase is also associated with secondary complications of neovascular episodes (comprising 10-20% patients), these being designated as 'wet' AMD.

The wet form of the disease can lead to rapid visual loss and considerable efforts at intervention have resulted in anti-vascular endothelial growth factor (anti-VEGF) intra-vitreous injections with a considerable degree of success in managing the neovascularisation, but the underlying progression of the disease is not altered. Thus, the vast majority of AMD patients (falling in the 'dry' AMD category) still await the development of a suitable treatment modality that can either slow or arrest the progression of the disease [2, 3].

The pathophysiology of AMD is highly complex due to the diverse genetic associations and considerable gene-environmental interactions exacerbated by the additional association of dietary and cardiovascular risk factors [4–6]. Furthermore, all these factors are superimposed on the normal ageing changes in the visual unit making it very difficult to nominate specific targets for intervention. Since age is the highest risk factor for the development of AMD, an understanding of the inherent stresses in the visual system would allow us to predict the likely effect of additional risk factors providing a more targeted approach towards therapy.

Briefly, in the visual unit, the photoreceptor is the primary site of sustained damage producing highly toxic compounds that can trigger an inflammatory response. However, this damage is rapidly transferred to the RPE by the daily shedding of outer segment discs and phagocytosis. Since the RPE also operates in the same oxidative environment as the photoreceptor cell, the engulfed discs undergo further damage resulting in compromised lysosomal degradation. Non-degradable material, comprising mainly lipofuscin-related products is either packaged and stored in the RPE or extruded as membranous debris onto Bruch's membrane. With age, this debris accumulates in Bruch's compromising its ability to transport nutrients, anti-oxidants, and vitamins essential for RPE and photoreceptor function. The toxic metabolites in Bruch's are pro-inflammatory mediators and in the normal elderly, lead to a low-grade inflammatory response [7]. In advanced ageing associated with AMD, a chronic inflammatory response is precipitated leading to the death of RPE and photoreceptors.

For therapeutic intervention to be effective, the functional aspects of the RPE and Bruch's membrane need to be restored. We will examine the compositional and functional alterations of ageing RPE and Bruch's membrane, nominate suitable targets for intervention, and assess the potential for amphipathic saponin molecules to reverse these ageing changes as a potential therapy for dry AMD.

2. Underlying stresses in the visual unit leading to ageing and pathophysiology

Photoreceptor physiology is dependent on the supportive roles provided by the RPE and Bruch's membrane. Inherent stresses in these compartments lead to morphological and functional deterioration manifesting as 'normal' ageing changes but in the advanced ageing scenario of AMD culminate in the transition to pathology. The stresses within each compartment of the visual unit will be identified, providing therapeutic targets for intervention.

2.1 Stresses generated in photoreceptor cells

The photoreceptor is a highly specialised neuronal cell capable of detecting a single photon of light. Absorption of light by rhodopsin (R) present in the outer segment disc membranes leads to isomerization of the 11-cis retinal chromophore to its all-trans form (AT-RL), producing activated rhodopsin (R*). Amplification of the light signal begins by rapid lateral diffusion of R* over the disc membrane

and interaction with many transducin molecules. Such high mobility of R^* requires a very fluid membrane conferred by the high level of unsaturated docosahexanoic acid (DHA) in its membrane phospholipids. Further enzymatic amplification of the light signal by the guanylate cyclase-phosphodiesterase system leads to closure of sodium channels in the outer segment membrane resulting in hyperpolarisation of the cell and concomitant modulation of transmitter release. To meet the energy demands of these processes, the photoreceptor maintains the highest rates of oxidative metabolism of any cell in the body. Associated with this activity is the release of damaging oxygen radicals by the mitochondrial electron transport chain.

The presence of toxic retinoids, highly unsaturated fatty acids, high oxygen tension, high oxidative metabolism, and light is an explosive mixture for the generation of free-radical mediated damage. Since released AT-RL is highly toxic, it is rapidly reduced to all-trans retinol. However, AT-RL can react with phosphatidylethanolamine to form retinylidene-phosphatidylethanolamine (NRPE) [8]. NRPE can react with a second molecule of AT-RL to form a bis-retinoid. Further modifications produce a variety of all-trans retinal dimers including A2E, the auto-fluorescent fluorophore of lipofuscin [9]. These bis-retinoids can undergo photo-oxidation to form oxo-aldehydes which then react with proteins to form advanced glycation end-products (AGEs) that are triggers of inflammatory processes [10].

Peroxidation of polyunsaturated fatty acids such as DHA results in fragmentation of the molecule leading to a mixture of compounds that bind to proteins [11, 12]. Oxidation of DHA produces carboxy-ethyl-pyrrole (CEP)-protein adducts. Thus, oxidation of PUFAs results in lipid aggregates, lipid-protein complexes, protein cross-link formation and CEP-adducts. These CEP-adducts have been localised to the RPE and drusen and being strongly immunogenic, activate the immune system [13].

Some protection from oxidative damage is afforded by the impressive anti-oxidant machinery (vitamins C&E, macular pigments, and enzymes such as catalase, peroxidase, and superoxide dismutase) [14, 15]. However, this protection in photoreceptors is dependent on an adequate supply of anti-oxidants and essential metals for the enzymic system by the RPE and Bruch's membrane. Despite these protective mechanisms, considerable damage is sustained by photoreceptors. Fortunately for the photoreceptor, this damage is confined to the outer segment discs and transferred to the RPE.

Therapeutic intervention to combat this damage has been considered resulting in the Age-Related Eye Disease Study (AREDS) vitamin and anti-oxidant supplements and their effectiveness will be discussed later.

2.2 Oxidative damage in the RPE

The RPE operates in the same oxidative environment as the photoreceptor cell and therefore, the toxic reactions initiated in the outer segments will continue in the phagolysosome. Lysosomal enzymes hydrolyse the normal, undamaged protein and lipid components, recycling the base metabolites back to the photoreceptor cell. Damaged proteins, lipid-derived adducts, protein cross-links due to lipid-carbonyl attack, and aggregated lipid complexes that are no longer susceptible to lysosomal enzymes remain in the phago-lysosomal sac [16]. The lysosomal hydrolysis of bis-retinoids results in the formation of the primary age pigment, A2E. A2E and other bis-retinoids undergo further oxidation to produce a variety of toxic products that not only damage lysosomal enzymes but also damage the lysosomal membrane inhibiting the proton pumps with the subsequent increase in pH that will further diminish lysosomal enzyme activity [17].

Un-hydrolysed lipoprotein and aggregated protein complexes together with bis-retinoids are packaged and stored as the auto-fluorescent pigment lipofuscin in membrane enclosed sacs. Lipofuscin content of the RPE increases with age and can amount to nearly 20% of cytoplasmic volume in the elderly [18]. Increased oxidative stress is inferred from the accumulation of AGEs in both ageing RPE and Bruch's membrane [19]. The RPE has a battery of anti-oxidants and a robust enzymic machinery to neutralise the oxidative stress and again, the components of the protective machinery are supplied by transport across Bruch's membrane. However, the age-related accumulation of bis-retinoids and damaged proteins suggests that the anti-oxidant system is not effective in tackling this threat.

The primary functions of the RPE are (a) phagocytosis of shed outer segment discs and their degradation, (b) vectorial transport of nutrients, lipids, metals, vitamins and anti-oxidants, and the removal of waste products generated in the photoreceptor cell, and (c) fluid transport from the sub-retinal space to the choroid. The effect of the age on the various functional parameters of the RPE are poorly understood. One report has suggested that phagocytic activity is halved between the ages of 30 and 80 years [20]. Another important function of the RPE is the delivery of nutrients, anti-oxidants, vitamins, etc supplied by the choroidal circulation to the photoreceptor cell. Since the RPE is the site of the outer blood-retinal barrier, all metabolites must cross the interior of the cell to gain access to photoreceptors. Therefore, transport across the RPE is mediated by passive diffusion or facilitated by active and passive carriers in the membrane. Most active carriers utilise the sodium electro-chemical gradient generated primarily by mitochondrial respiration [21]. However, A2E generated in the RPE binds to cytochrome C of the electron transport chain impairing mitochondrial respiration and this is expected to impact on the effectiveness of active carrier transport [22, 23].

There is little information of the effect of age on the activity of ligand carriers of the RPE due largely to interference from the adjacent Bruch's membrane. This is best illustrated with the transport of retinol (vitamin A). In elderly subjects and patients with early AMD, the recovery in dark-adaptation following a strong bleach is delayed [24, 25]. This delay is thought to be due to low levels of retinoids in the RPE and therefore slower transfer of 11-cis retinal to photoreceptors for regeneration of rhodopsin. Lowered levels of retinoids in the RPE could be due to lowered uptake by the RPE itself or diminished transport of retinol across Bruch's membrane. The fact that there is improvement in dark-adaptation following vitamin A supplementation would suggest inefficient delivery across Bruch's, rather than reduced uptake by the RPE as the contributory factor [26].

Fluid transport is another important function carried out by the RPE. Retinal fluids (originating from retinal capillary beds and retinal metabolism) are transported out by the RPE predominantly by an active process [27, 28]. The daily output of fluid from the RPE has been determined to be about $0.13 \pm 0.11 \mu\text{l}/\text{hour}/\text{mm}^2$ and metabolic insufficiency in the RPE would lead to fluids accumulating on top of the RPE resulting in macular oedema and/or retinal detachment [29, 30].

Therapeutic intervention in support of the RPE would require effective delivery of anti-oxidants and strengthening of its metabolic capability so as to reduce the generation of toxic products and assist in their rapid removal.

2.3 Compositional changes in ageing Bruch's membrane

Bruch's membrane mediates the exchange of nutrients and waste products between the choroidal blood supply and the RPE. An age-related compromise in these functions will reduce the capacity to supply essential nutrients to the RPE and photoreceptor cells increasing the risk of damage in these compartments.

The most obvious morphological change in Bruch's with age is increased thickness from about 1.5 μm in the young to 5.5 μm in the elderly [31]. This is due primarily to the deposition of normal and abnormal extracellular matrix (ECM) material. In the elderly, cross-linked and denatured (damaged) collagen accounts for nearly 50% of total collagen in Bruch's membrane [32]. There is also an increase in oxidative and non-enzymic glycosylation of proteins and lipids leading to the accumulation of toxic advanced glycation end-products, AGEs [33]. The membrane also shows an exponential increase in the level of lipid-rich debris [34]. Most of this debris arises from inefficient phagocytic processing of damaged outer segment discs in the RPE that is then extruded onto Bruch's membrane. This material then undergoes further oxidative modification with both the inherent matrix proteins and with passer-by constituents leading to further damage and deposition. Finally, the lipid components undergo free-energy driven aggregation leading to the accumulation of 100 nm diameter lipid-rich particles observed in the inner collagenous layer of Bruch's membrane [35].

Thus, in addition to the toxic metabolites mentioned above, deposits in Bruch's contain phospholipids, triglycerides, cholesterol, cholesterol esters, peroxidised lipids and apolipoproteins, immunoglobulins, amyloid, complement, and proteins specific to RPE function [36]. Heavy metal deposition has also been demonstrated that stabilises the debris in Bruch's [37].

The above changes result in gross morphological alteration of ageing Bruch's membrane that are expected to be detrimental to its transport functions (**Figure 1**).

Mechanisms exist to counteract the deleterious changes described above for Bruch's membrane. This involves the continuous synthesis and degradation of the extracellular matrix, the latter process being mediated by the matrix metalloproteinase (MMP) system [38]. Although this system performs well in the young, it deteriorates rapidly with age and more so in AMD [39].

2.4 Functional deterioration of ageing Bruch's membrane

Since Bruch's membrane is crucial for the exchange of nutrients and waste products, a deficiency in its transport functions will increase the risk of damage in the RPE and photoreceptor compartments for the reasons outlined earlier. The extent to which the compositional alterations of ageing Bruch's impact on its ability to remove

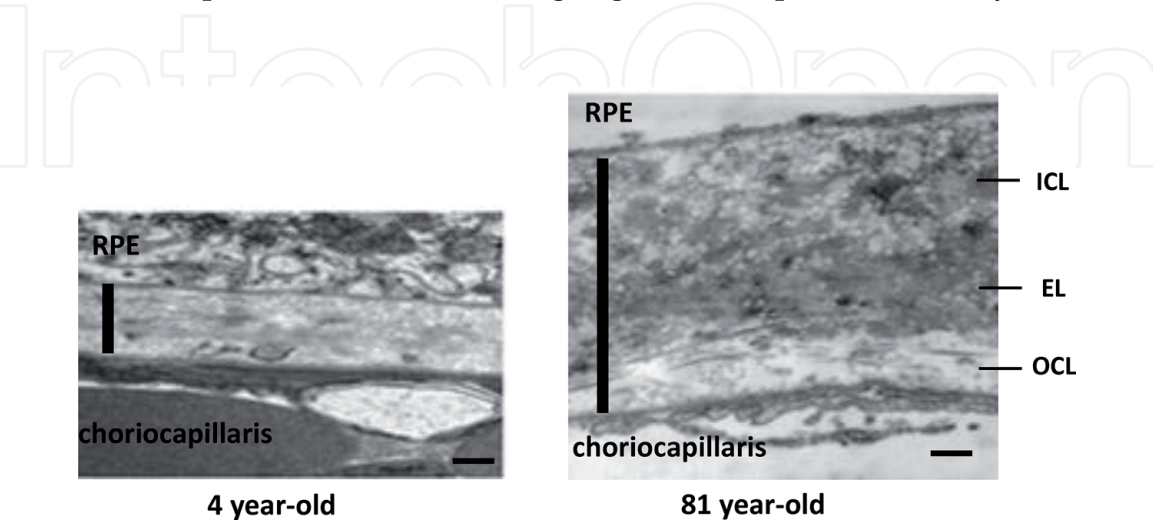


Figure 1. Morphology of ageing Bruch's membrane. With age, Bruch's becomes thicker and contains a lot of debris rich in lipids, and abnormal matrix and non-matrix material. The increase in thickness alone will reduce the diffusional gradients for the transport of nutrients and waste products. Vertical bar denotes the thickness of Bruch's membrane. ICL, inner collagenous layer; EL, elastin layer; OC, outer collagenous layer. Bar marker: 1 μm .

fluids into the choroidal circulation, to supply adequate levels of essential nutrients, antioxidants, and vitamins to the RPE and photoreceptors, to maintain the rejuvenation potential of its membrane, and to modulate the occurrence of inflammatory responses will now be examined in both normal ageing and in the advanced ageing scenario of AMD.

2.4.1 Diminished fluid transport

The capacity for fluid transport across a membrane is designated by its hydraulic conductivity. As previously indicated, the daily output of fluid from the RPE and onto Bruch's membrane is about $0.13 \pm 0.11 \mu\text{l}/\text{hour}/\text{mm}^2$. To effectively transport this amount of fluid, Bruch's needs to have a minimum hydraulic conductivity of $0.65 \times 10^{-10} \text{ m/s/Pa}$, and this level is referred to as the failure threshold [40, 41]. If hydraulic conductivity falls below this level, then fluid will accumulate on top of the membrane leading to a RPE detachment. Hydraulic conductivity of human Bruch's has been determined in 56 donors spanning the age range 1-91 years (**Figure 2**, modified from reference [40]). Conductivity was shown to decline exponentially with age and in the semi-log plot, the transformation is shown as a straight line. The half-life of the decay process was 16 years, i.e., conductivity was halved for every 16 years of life. Excess capacity is present in the younger population but with age, there is a drift towards the failure threshold. Extrapolating the straight line shows that the shelf-life of human Bruch's is about 123 years, but in the data of **Figure 2**, two of the normal donors have already reached the failure threshold. Bruch's from AMD donors showed a faster rate of decline in hydraulic conductivity [40] and as such, complications of RPE detachment are observed in about 12-20% of AMD patients [42].

For an effective therapeutic intervention in AMD, the exponential decay line in **Figure 2** needs to be elevated so as to avoid the failure threshold within the life-time of an individual.

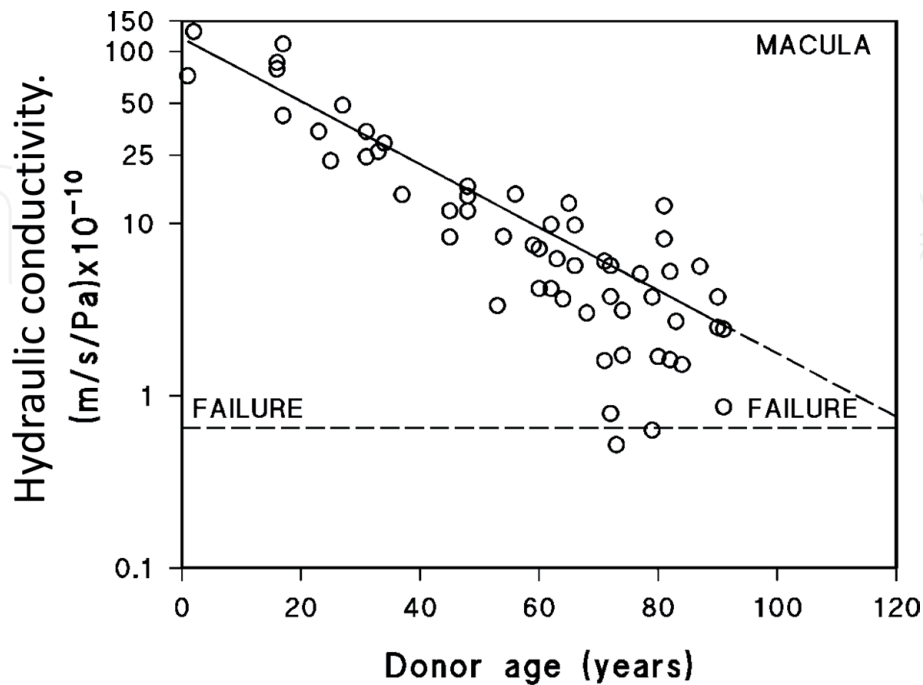


Figure 2.
Semi-logarithmic plot to show the exponential decay in the hydraulic conductivity of human Bruch's with age. (Modified from reference [40]).

2.4.2 Diminished metabolite and waste transport

Metabolites ranging from the simple sugars and amino acids to the much larger lipo-protein complexes are released from the fenestrated endothelium of the choriocapillaris vessels and traverse Bruch's by passive diffusion. Most of the essential metabolites such as heavy metals, vitamins (including vitamin A), and lipids are transported bound to carrier proteins that generally have a hydrodynamic radius of about 3-12 nm.

To assess the effect of age on the diffusional status of human Bruch's membrane, a FITC-albumin test probe was utilised that has a hydrodynamic radius of about 3.5 nm, similar to most carrier proteins. Diffusional experiments were conducted in standard Using chambers utilising isolated Bruch's membrane preparations from 33 donors, age range 12-92 years. The diffusional status of Bruch's membrane was observed to decrease exponentially with age, with a half-life of 18 years (**Figure 3**) [43]. Thus, over a human life-span, diffusional status was reduced by about 10-fold. We do not know the value of the failure threshold for diffusion across Bruch's membrane. However, since most elderly subjects show delayed dark-adaptation due to inefficient transport of vitamin A, the albumin diffusion values of subjects aged 77-87 years (0.024 nmol/6 mm/hour) were taken as the failure threshold.

Other in-vitro studies utilising serum proteins (MW 40-200 kDa) or FITC-dextran molecules (MW 21 kDa, radius 3.3 nm) have also shown a >10-fold reduction in diffusion capability over a human life-span [44, 45].

In AMD, the reduction in diffusional transport across Bruch's membrane was much more severe compared to age-matched controls [45]. This reduction in transport is expected to impact on the nutritional and anti-oxidant support of both RPE and photoreceptor cells, increasing oxidative stress. Similarly, transport in the opposite direction i.e., removal of toxic waste products from Bruch's membrane will also be diminished leading to greater oxidative modifications and generation of further toxic products.

As with hydraulic conductivity, for effective therapeutic intervention, the diffusional decay curves should be elevated away from the failure threshold.

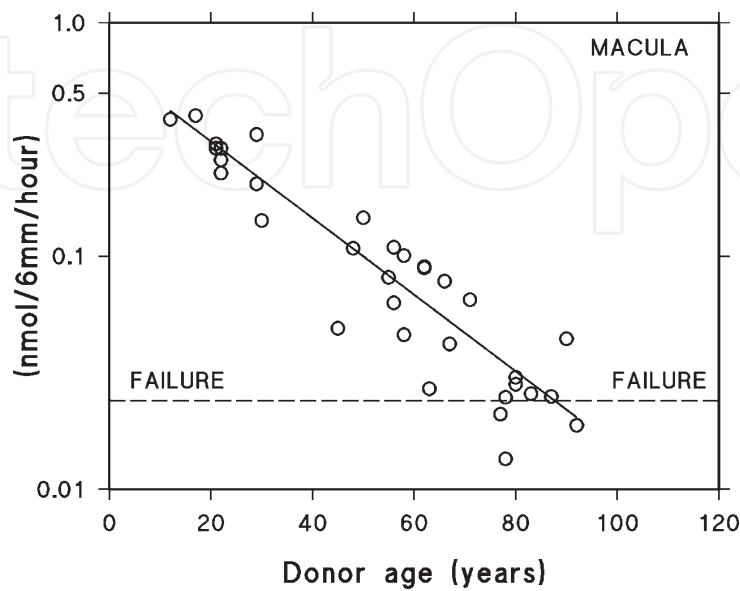


Figure 3.
The effect of age on the diffusion of albumin across human Bruch's. Semi-logarithmic plot showing the decay half-life to be 18 years.

Reduced diffusion within Bruch's membrane will also affect the protective mechanisms that depend on rapid mobility such as the interactions of complement factor H (CFH) with its many ligands and activation of pro-MMP2 in the regeneration of Bruch's. These aspects are described below.

2.4.3 Impaired regeneration in ageing Bruch's membrane

The ECM of Bruch's is continuously regenerated by coupled processes of synthesis and degradation. This ensures that damaged material is removed and replaced by new ECM components synthesised by the RPE, thereby maintaining the transport integrity of Bruch's membrane. Since abnormal collagen accumulates in ageing Bruch's (amounting to 50% of total collagen in the elderly), the regeneration process appears to be dysfunctional [32]. Little is known about the synthetic rate of ECM by the ageing RPE but the accumulation of damaged ECM components suggests problems with the degradation machinery.

Matrix degradation is mediated by a family of proteolytic enzymes called the matrix metalloproteinases (MMPs). These are synthesised in the RPE and released into Bruch's membrane as latent pro-enzymes (pro-MMPs) that on activation, following the removal of a small inhibitory peptide, can degrade almost all components of the ECM [38, 46]. In Bruch's, the major MMP species are pro-MMP2 and pro-MMP9, the former being the homeostatic enzyme in the system and the latter being the inducible form. Activation of pro-MMP2 occurs on the basolateral surface of the RPE by the initial formation of a binary complex between the membrane bound MMP-14 and the tissue inhibitor of MMPs, TIMP2. This then binds pro-MMP2 to form a tertiary complex that then results in the hydrolysis of the inhibitory peptide on pro-MMP2 by a second molecule of MMP-14, to release activated MMP-2 [47].

Thus, optimal pro-MMP2 activation requires adequate levels of pro-MMP2 and TIMP2 and good mobility of these two components within the matrix of Bruch's to interact with the MMP-14 enzyme on the RPE basal membrane. The age-related reduction in diffusion within Bruch's is expected to compromise this activation potential (**Figure 3**). Furthermore, pro-MMP2 covalently binds pro-MMP9 to form the high molecular weight complex termed HMW2, reducing its level for the activation process [48]. A polymorphism in the microsatellite region of the MMP9 gene (present in most AMD patients) results in elevated levels of pro-MMP9 in both plasma and Bruch's membrane, increasing the potential for further sequestration of pro-MMP2 from the activation step [39, 49–51].

The gross alterations of ageing Bruch's membrane together with the reduction in diffusional competence are expected to hamper the mobility of pro-MMP2 and TIMP2, diminishing the activation of this MMP. Thus, levels of activated MMP2 decrease with age, and in AMD, the level was reduced by 50% compared to age-matched controls [39]. It should also be noted that activated MMP2 may not be able to diffuse adequately to interact with its substrate and in the gross morphology of **Figure 1**, may be trapped within the membrane. The decreased turnover of Bruch's leads to the deleterious morphological and functional changes described earlier culminating in diminished support of RPE and photoreceptors.

2.4.4 Ageing and increased susceptibility to inflammatory intervention

Many of the toxic products produced in the RPE and present in Bruch's (as outlined earlier), including A2E, bis-retinoids, malonaldehyde, carbonyl lipids, C-reactive protein, etc., are capable of activating the complement system [52]. Thus, in elderly subjects, the exponential increase in A2E in Bruch's may be associated with a low-grade complement activation [53, 54]. A low-grade inflammatory

response may be beneficial for eliminating toxic metabolites present in Bruch's or in drusen and may serve to prevent the transition from normal ageing to pathology. Thus, the presence of the membrane attack complex and other complement factors in drusen and inter-capillary columns may allow their removal by macrophages [55].

However, indiscriminate activation of the complement system can lead to a chronic inflammatory response damaging RPE and photoreceptor cells. CFH (a 155 kDa glycoprotein) plays important roles in modulating the activation of the complement cascade. Firstly, it can bind to the toxic entities to prevent complement activation and secondly, by binding to the C3b complement component, block the progression of the cascade [56–58].

Levels of CFH in Bruch's are maintained by synthesis in the RPE and binding to glycosaminoglycans in the membrane, and delivery of plasma-derived CFH. With age, and under oxidative stress, the production of CFH by the RPE is reduced [59, 60]. Similarly, the nearly 10-fold decrease in diffusion across elderly Bruch's is expected to compromise delivery from the blood. Furthermore, in the presence of inflammatory activity in Bruch's, CFH is nitrated [61]. This nitrated CFH does not bind lipid peroxidation products nor C3b, diminishing its protective ability. Also, plasma levels of nitrated CFH are elevated in AMD patients and this may contribute to AMD progression [61].

A polymorphism in the CFH gene (Tyr402His) has been detected in about 50% of AMD patients [62, 63]. This mutated CFH shows diminished binding to toxic ligands such as malondialdehyde and C-reactive protein, and thus becomes ineffective in modulating the inflammatory response [64–66]. Mutated CFH also shows poor binding to heparin sulphate in Bruch's and hence its enhanced presence in Bruch's is compromised [67].

Ageing changes in Bruch's and the RPE therefore compromise the protective effects of CFH and in the aged AMD patient may exacerbate the inflammatory response leading to the death of RPE and photoreceptors.

3. Requirements for effective therapeutic intervention in dry AMD

Oxidative damage in the RPE and Bruch's membrane is the primary driver of ageing changes in the normal elderly and more so in patients with AMD. These ageing changes diminish the supply of key anti-oxidants and vitamins required to combat oxidative stress and therefore a vicious cycle is set-up that leads to the degenerative changes in AMD.

Anti-oxidant and vitamin supplement regimes have been devised as a possible interventionist measure to reduce oxidative stress and hopefully slow the progression of the disease. Thus, the AREDS dietary supplementation cocktail was devised (vitamin C (500 mg), vitamin E (400 IU), beta-carotene (15 mg), zinc oxide (80 mg), and cupric oxide (2 mg)) and initial results showed it to be effective in reducing the risk of visual loss [68, 69]. The supplement was further modified (as the AREDS 2 formulation) by removing beta-carotene and adding lutein (10 mg) and zeaxanthine (2 mg) but did not confer any additional benefits [70].

Despite the wide use of AREDS supplements for over 10 years, controversy remains as to its usefulness since it does not prevent legal blindness in advanced AMD [71]. It has been pointed out that the earlier reported decrease in progression was related to the occurrence of neovascularisation rather than slowing the progression of dry AMD [72].

Given the fact that the diffusion of metabolites across Bruch's membrane is reduced by nearly 10-fold in the elderly, and perhaps more so in AMD, one must

question the likely effectiveness of such dietary supplementation. The major problem with supplementation therapies is that they do not address transport in the opposite direction across Bruch's membrane i.e., the removal of toxic metabolites that are the likely triggers of neovascular and inflammatory episodes.

For effective therapeutic intervention, it would be ideal to improve the bi-directional transport pathways across Bruch's membrane, to improve nutritional and anti-oxidant delivery and to remove toxic waste products. This would require the destabilisation and dispersal of the lipid-rich debris and the removal of normal and damaged proteinaceous deposits. Such a strategy would also release trapped activated MMP enzymes that could participate in hydrolysing the altered collagenous components. The expected improvement in intra-membrane mobility would favour greater activation of pro-MMP2, kick-starting the normal rejuvenation machinery in Bruch's membrane. Therapeutic success would be realised if the transport decay curves shown in **Figures 2 and 3** could be elevated so that they no longer crossed the failure threshold within the lifetime of an individual. The potential implementation of such a strategy using saponin molecules is discussed next.

4. Saponin characteristics enabling therapeutic intervention in dry AMD

Saponins are amphipathic molecules that have hydrophobic and hydrophilic domains that can partition into lipoidal deposits and assist dispersal [73–76]. Saponins extracted from the ginseng plant (*Panax ginseng* CA Meyer) have a 4-membered triterpenoid ring and are often referred to as ginsenosides or steroidal glycosides because of the structural similarity to the cholesterol molecule (**Figure 4A**). The type and number of sugar units attached at sites R1, R2, and R3

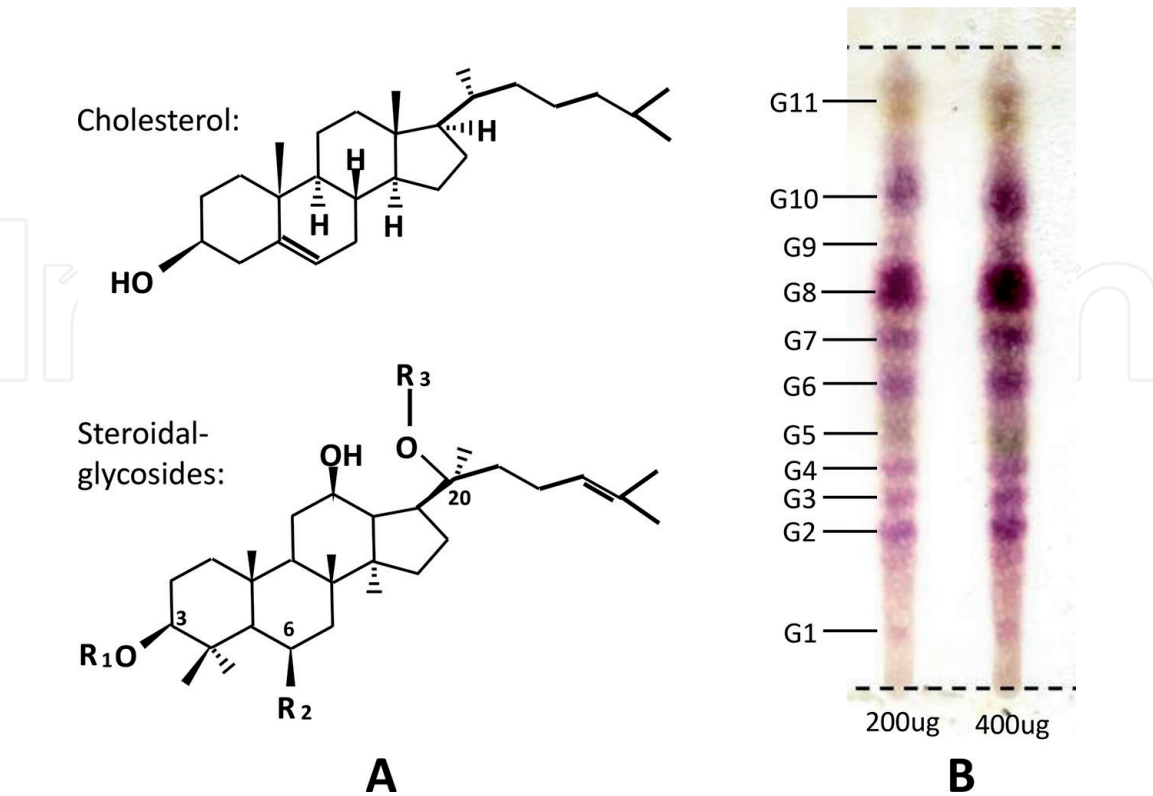


Figure 4. Saponins extracted from the ginseng plant. (A) Structural similarity of saponins to the cholesterol molecule. Sugar attachment sites R1, R2, and R3 lead to the diversity of saponin species. (B) TLC of extracted saponins. G1 to G11 denote the major spots, each spot comprising several species.

gives rise to a myriad of species and over 30 have been structurally characterised. Saponins extracted from the roots of the ginseng plant were separated on Silica Gel thin-layer-chromatography (TLC) plates using a solvent mixture comprising chloroform: methanol: acetic acid: water (50:30:8:3 v/v) and colour developed by spraying with 20% sulphuric acid in methanol and heating to 100 °C for 5 minutes (**Figure 4B**). Since the separation was dependent on the degree of hydrophilic/hydrophobic properties, each spot on the chromatogram represents a collection of several species.

These saponin molecules not only bind to various lipid classes, they also display transition metal chelating properties [77, 78]. Thus, saponins can chelate the heavy metal deposits in Bruch's membrane and therefore assist in destabilising the lipid aggregates.

4.1 Saponin mediated dispersal of lipid deposits

The potential for saponin mediated dispersal of lipids was assessed in both isolated deposits and intact human Bruch's membrane. Bruch's from four donor eyes (ages 50 and 82 years) was homogenised in Tris-buffer and spun to obtain pellets containing the deposits. Pellets were resuspended in Tris buffer containing ginseng-derived saponins in the range 0-1.2 mg/ml and incubated for 12 hours at 37°C. Samples were then spun and any lipids released into the supernatants extracted with chloroform: methanol (2:1 v/v). Lipids were then separated on Silica Gel thin-layer chromatography (TLC) plates using solvent system #1, chloroform: methanol: acetic acid: water (50:30:8:3 v/v) and solvent system #2, heptane: diethyl ether: acetic acid (70:30:2 v/v). Lipid spots were visualised by staining with amido-black 10B stain and following densitometry, levels quantified with reference to standard curves. Saponins were observed to rapidly release various lipid classes from the deposits in a dose dependent manner (**Figure 5**).

In addition, 14 Bruch's preparations were obtained from 4 human donors (age range 64-75 years) and mounted in Ussing chambers. All chambers were perfused with Tris buffer to remove loosely attached debris and then half the chambers were incubated with Tris buffer and the other half with Tris buffer containing 4.6 mg/ml saponins for 12 hours at 37°C. Chambers were rinsed in Tris buffer and the content of the various lipid classes present in Bruch's membrane quantified as detailed above. The content of lipids in the samples incubated with saponins was significantly reduced compared to controls (**Figure 6**). Thus, saponin molecules are able to solubilise and disperse the lipid deposits in Bruch's membrane.

4.2 Saponin-mediated release of deposited proteins

Soluble proteins that are either damaged due to chemical modification or denatured tend to unfold exposing the hydrophobic regions to the aqueous environment. This leads to aggregation and deposition within Bruch's membrane. The possibility that saponins could interact with these 'amphipathic' structures to de-segregate and solubilise them was also assessed. Bruch's membrane from four human donors (age range 49-77 years) was mounted in 8 Ussing chambers and perfused with Tris buffer. The perfusate was collected every 5 hours and the protein content determined. As indicated in **Figure 7**, perfusion with Tris buffer resulted in slow release of loosely adherent proteins up to the fourth perfusion period (20 hours). In four chambers, the perfusion fluid was then switched to one containing 167 µg/ml of saponin Rb1. This resulted in the rapid and copious release of further, presumably trapped proteins from the membrane (**Figure 7**). The possibility that trapped MMP enzymes may also have been released is assessed below.

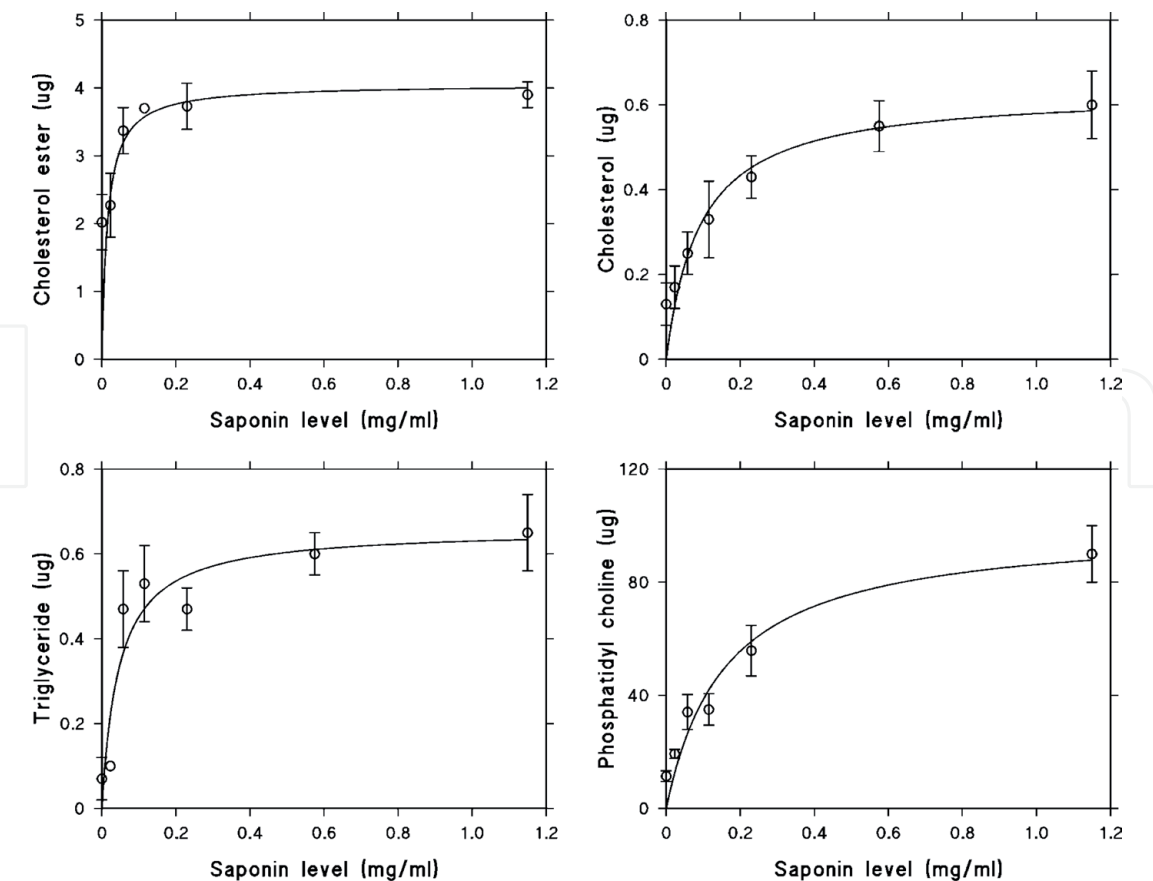


Figure 5. Saponin-mediated release of lipids from deposits extracted from Bruch's membrane. Extracted deposits were incubated with saponins in the range 0-1.2 mg/ml for 12 hours, spun, and lipids present in the supernatant quantified. Saponins released cholesterol, cholesterol esters, phospholipids, and triglycerides in a dose-dependent manner. Data is given as Mean \pm SD.

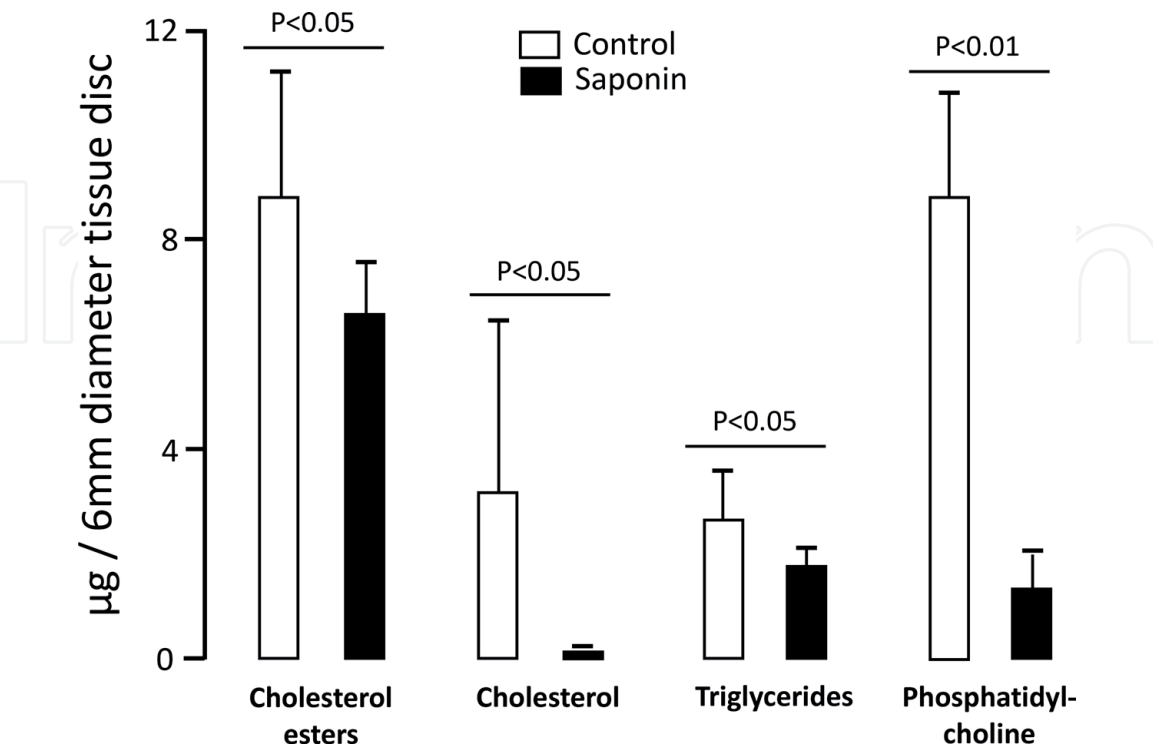


Figure 6. Saponin-mediated release of lipids from intact human Bruch's membrane. The level of the major lipid classes was reduced following saponin treatment. Data is given as Mean \pm SD.

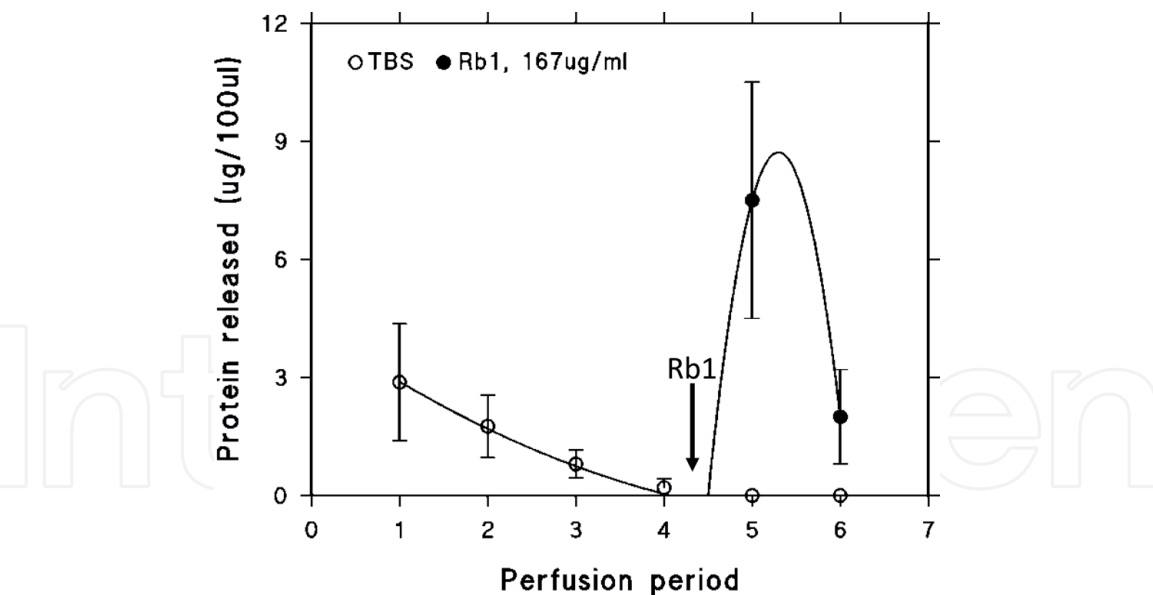


Figure 7.
Solubilisation and release of trapped proteins from Bruch's membrane. On perfusion with saline, loosely adherent proteins are released slowly. When there was no further release (after period 4), the perfusion medium was switched to one containing Rb1, resulting in the copious release of trapped proteins. TBS-Tris buffered saline; Rb1-Ginsenoside Rb1. Data as Mean \pm SD.

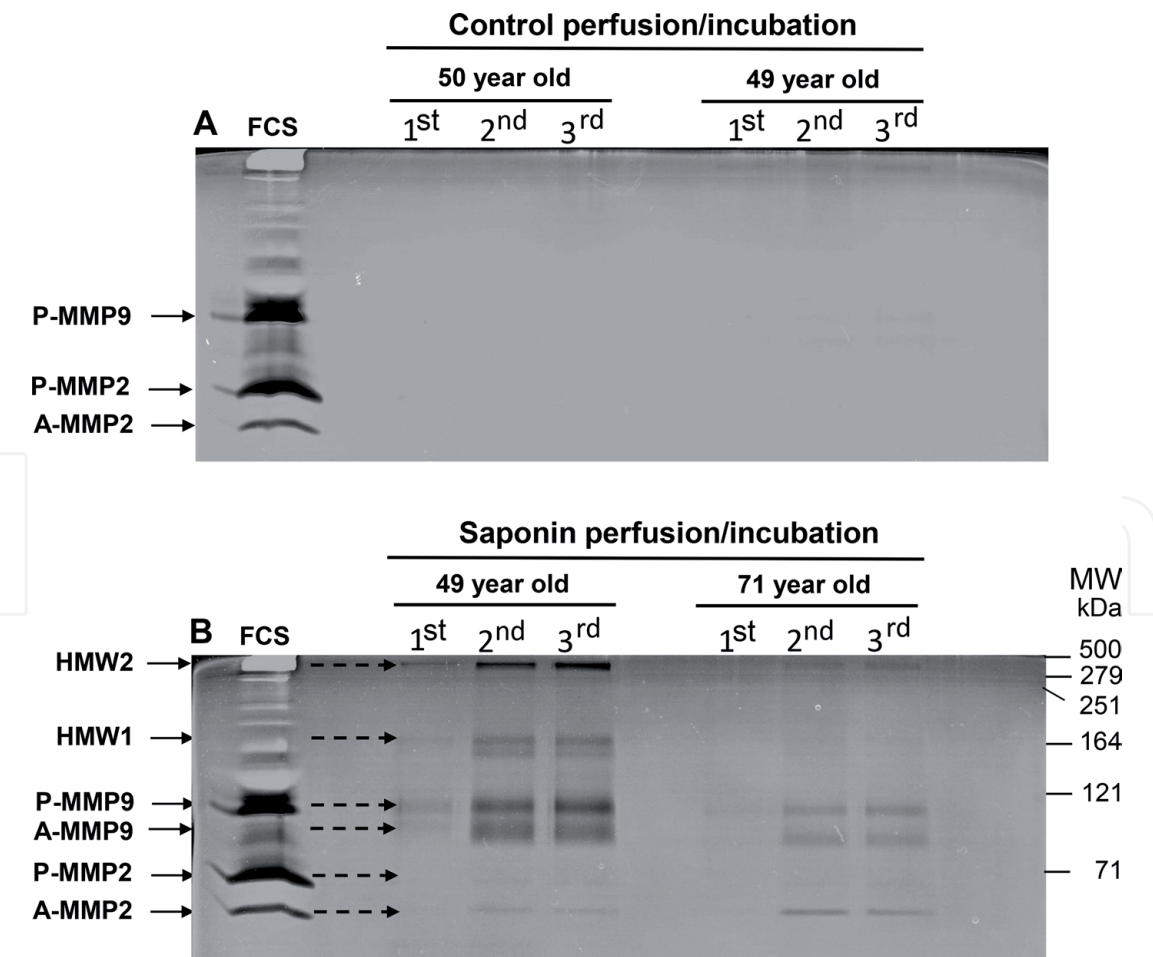


Figure 8.
Saponin-mediated release of trapped MMP enzymes. (A) Perfusion with Tris buffer did not release any MMP species from Bruch's membrane. (B) Saponin perfusion released trapped MMP species and in particular activated forms of MMPs 2&9. FCS-foetal calf serum standard. P- pro-MMPs; A-activated MMPs. From reference [43].

4.3 Saponin-mediated release of trapped MMP enzymes

MMPs are detected by the technique of gelatine zymography. This is standard electrophoresis but with the gel containing 1% gelatine, a substrate for MMPs. Samples are electrophoresed with the MMPs migrating according to molecular weight, the smaller ones running fastest. Following electrophoresis, the gel is incubated in Tris-buffer (containing calcium) for a period of 18-24 hours to allow the MMP enzymes to digest the gelatine in their locality. The gel is then stained with Coomassie Blue and after de-staining, regions containing gelatine stain blue but where the gelatine has been hydrolysed (by MMP enzymes), the region is colourless. Reversing the grey-scale of the gel image shows the MMP regions as dark bands and the identity of the MMP is confirmed from its molecular weight.

To assess the likely effect of saponins on release of trapped MMP enzymes, Bruch's membrane from donors aged 49-71 years was mounted in Ussing chambers and perfused for a period of 12 hours to remove plasma-derived and loosely bound MMP species. Half the chambers were then perfused with Tris buffer for three periods of 3 hours each and the remaining half with saponins at a level of 4.6 mg/ml. After each period, the perfusate was collected and examined by zymography. Incubation with Tris did not show the release of any MMP species (**Figure 8A**). However, perfusion with saponins showed a trace release of MMPs in the first period followed by greater release in the two subsequent periods (**Figure 8B**). The release profiles showed the presence of activated MMP9 and MMP2. If this release occurred in vivo, it would kick start the MMP degradation machinery, rejuvenating the membrane.

5. Functional improvement of Bruch's membrane

Saponins have been shown to disperse and release lipid deposits, protein aggregates and trapped active MMP enzymes as described earlier. This is akin to reversing the ageing process of Bruch's membrane. The likely impact of these changes on the functional properties of Bruch's has also been assessed by monitoring hydraulic conductivity and diffusional status.

5.1 Hydraulic conductivity of saponin treated human Bruch's

Hydraulic conductivity measurements were undertaken in 23 preparations of Bruch's membrane from donors over the age range 13-90 years. After determining basal hydraulic conductivity, samples were incubated with saponins (4.6 mg/ml) for 24 hours at 37°C. After a thorough rinse in Tris buffer, the hydraulic conductivity was re-assessed. Treatment with saponins improved the hydraulic conductivity about 2-fold ($p < 0.0001$) elevating the exponential decay curves upwards, away from the failure threshold (**Figure 9**).

The curves were shifted by 19 years, reversing the ageing decline in fluid transport. Such a shift means that the curves will meet the failure threshold outside the normal human life span. In AMD, this improvement will reduce the risk of pigment epithelial detachments that normally affects 12-20% of these patients. These results were obtained after a single exposure to the saponins. A second subsequent exposure to the saponins (1.15 mg/ml) further increased the improvement in hydraulic conductivity indicative of potential to further elevate the decay curves (**Figure 10**). This is the likely result of continued removal of lipid and protein debris and release of MMP enzymes.

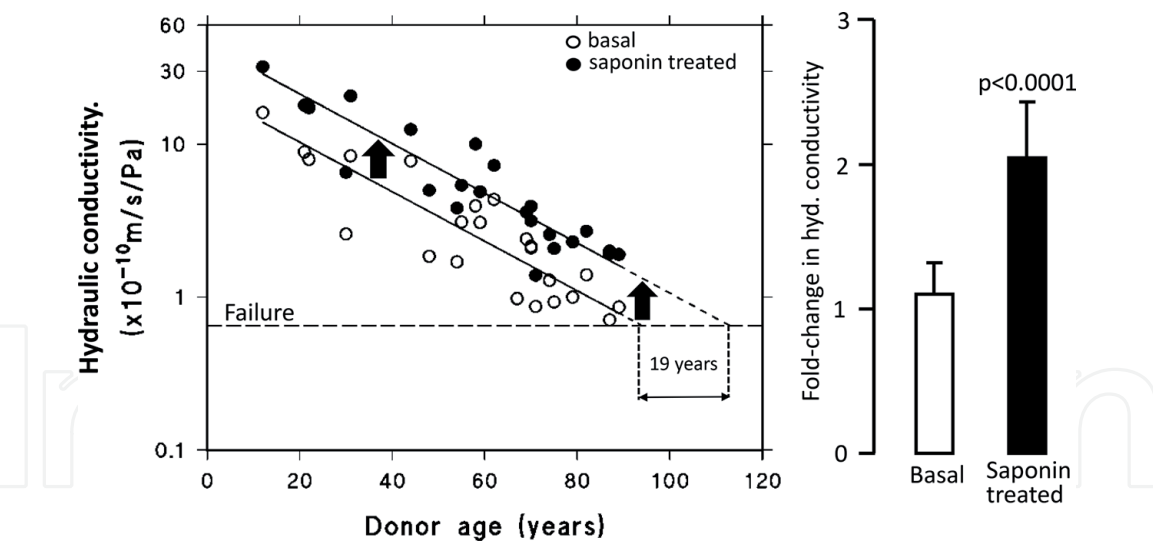


Figure 9.
Effect of saponins on the hydraulic conductivity of human Bruch's membrane. Saponins improved the hydraulic conductivity by 2-fold across the age range examined ($p<0.0001$). Decay curves were elevated so that failure threshold was met 19 years later. Modified from reference [43].

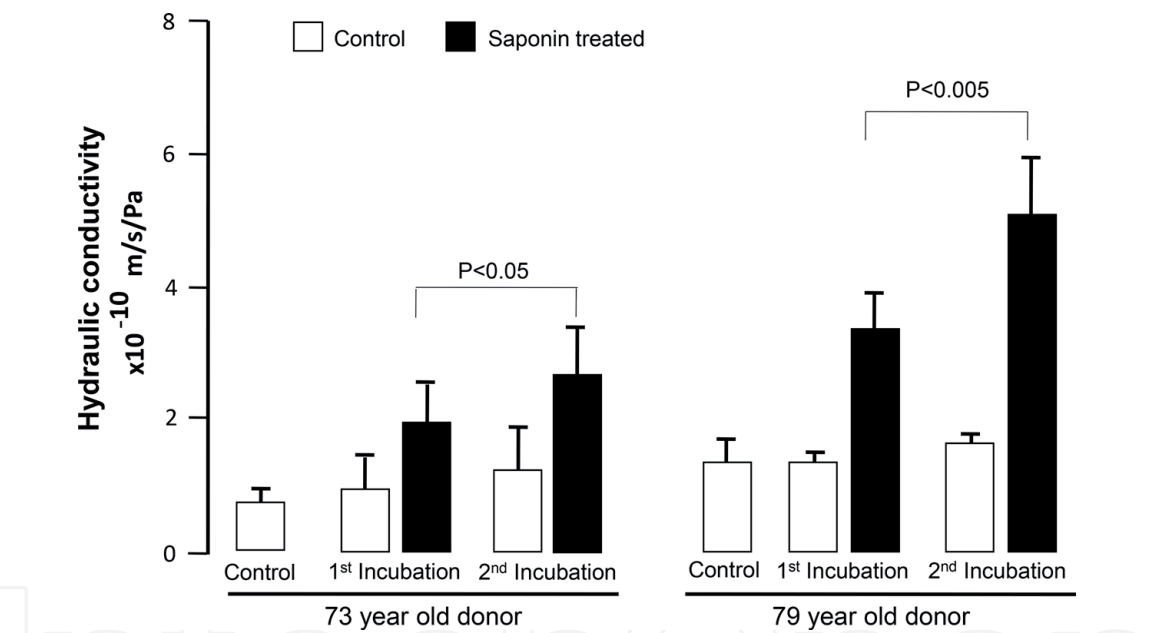


Figure 10.
Effect of repeated saponin exposure on the hydraulic conductivity of Bruch's membrane. Initial exposure to saponins improved hydraulic conductivity by over 2-fold. This was further increased by a second exposure.

5.2 Diffusional status of saponin treated human Bruch's

Diffusional status of Bruch's was assessed by following the transport of an FITC-labelled albumin test molecule (MW 65 kDa, hydrodynamic radius 3.5 nm) across the membrane, at a concentration gradient of 0.1 mM. Bruch's was obtained from 21 human donors (age range 12-92 years) and the basal diffusion rate was first determined. After a thorough wash in Tris buffer for 3 hours to remove all traces of albumin, the samples were incubated with saponin solution (4.6 mg/ml) for 24 hours at 37°C. Following a rinse with Tris buffer, the diffusional status was re-assessed. Exposure to saponins improved the diffusional status of Bruch's membrane by 2-fold ($p<0.0001$) shifting the ageing decay lines upwards, effectively reversing the ageing process by ~15 years (**Figure 11**).

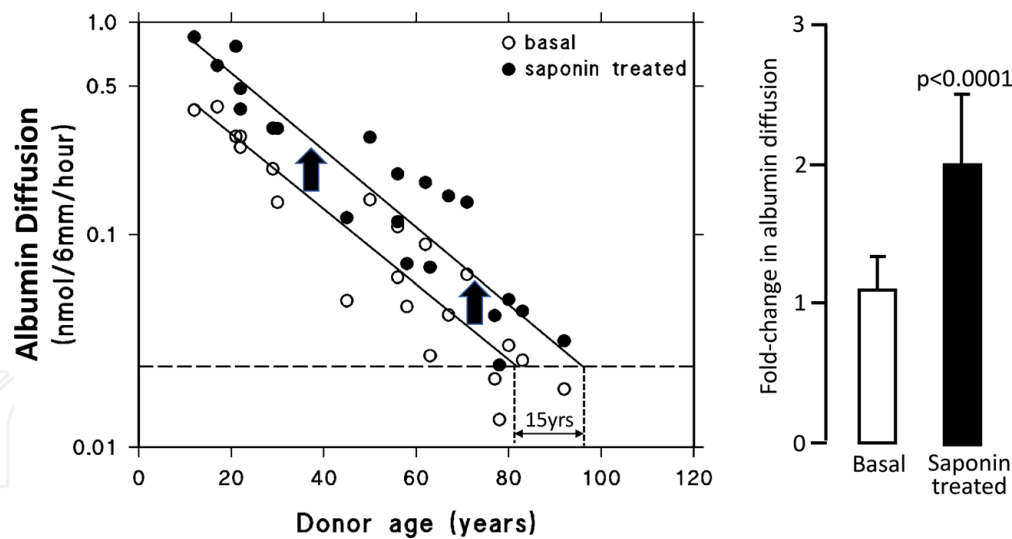


Figure 11.

Effect of saponins on the diffusional status of Bruch's membrane. Saponin incubation improved diffusion ($p < 0.0001$) elevating the decay curves upwards so that they reached failure thresholds 15 years later. Modified from reference [43].

Saponin mediated improvement in diffusion within Bruch's membrane would allow greater delivery of nutrients, vitamins and anti-oxidants to both the RPE and photoreceptor cells. Reduction in oxidative stress would result in reduced production of A2E and other bis-retinoids minimising the generation of pro-inflammatory mediators. Similarly, the removal of toxic waste products from Bruch's would be facilitated. This would also improve the mobility of TIMP2 and proMMP2 allowing greater activation of the MMP species and thus improved turnover of Bruch's membrane. Furthermore, improved mobility of CFH would minimise the risk of inflammatory involvement. In AMD, these changes are expected to slow the degenerative phase of the disease.

6. Conclusions: potential for saponin-mediated therapy in dry AMD

In-vitro work, using donor human Bruch's preparations has demonstrated the potential for saponin molecules to disperse and remove lipoidal and proteinaceous debris, releasing trapped activated MMP enzymes that can then mediate the normal degradation processes essential for rejuvenation of the membrane. Associated with these changes was a significant improvement in the bi-directional transport properties of Bruch's membrane. In vivo, improved transport would considerably augment the delivery of protective anti-oxidants and related nutrients to the RPE and photoreceptor cells and more importantly stimulate the removal of toxic products that underlie the progression of the disease.

Saponins constitute a large mixture of amphipathic molecules, the diversity being due to the varied nature of sugar residues at the several attachment sites on the aglycone ring structure. Individual species, such as ginsenoside Rb1 or Compound K have been shown to improve the functional status of Bruch's membrane preparations [43]. These compounds could be administered by intra-ocular injection as a potential therapy. But being very difficult to synthesise, reliance would have to be on isolation and purification from natural sources and therefore not likely to be cost-effective.

The alternative would be to utilise a varied mixture of saponins and this has many advantages. Firstly, they can be administered orally and in the Far East have been used as nutritional supplements for centuries. Secondly, and most

importantly, a mixture can target a broad spectrum of substrates that are normally encountered in the deposits in Bruch's membrane. From the pharmacokinetic data currently available for saponins, and our in-vitro dose response curves, calculations suggest that a 200 mg dose of an appropriate saponin mixture, taken twice daily, should significantly improve the transport characteristics of Bruch's membrane over a period of 4-6 months. Work is in progress to develop a clinical protocol for assessing the usefulness of saponin-mediated therapy for dry AMD.

Unlike other therapeutic interventions in AMD where outcome has been judged by following the progression of the disease over a period of 2-5 years, the saponin intervention can be assessed at 4-6 months using dark-adaptation kinetics. Since the saponin intervention aims at improving the transport systems in Bruch's membrane, the increased delivery of vitamin A would supplement the retinoid stores in the RPE allowing faster delivery of 11-cis retinal to the photoreceptor, and hence faster dark adaptation would be expected as proof of principle of saponin intervention.

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