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Benefits of genetic and immunohistochemical markers in understanding abnormalities in aging retina

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Abstract

The aim of the study was to better understand the interplay between genetic factors and the aging process in the human retina through mapping complement factor H (CFH) and related proteins. Two human eyes, from 92- and 64-year-old donors, were genotyped for the expression of CFH-related 1 (*CFHR1*) and CFH-related 3 (*CFHR3*) genes. Deoxyribonucleic acid (DNA) was extracted and analyzed for concentration and purity with a spectrophotometer, at 260 nm. The results showed a DNA concentration of 469.17 ng/μL in the aged retina and of 399.20 ng/μL in the younger one. Through polymerase chain reaction (PCR) genotyping, the DNA *CFHR1* and *CFHR3* were visible as bands of 175 bp and 181 bp. Immunohistochemistry by immunofluorescence method was used with a panel of specific antibodies for CFH, CFHR1, CFHR3 and GFAP, a marker for Müller cells. All the samples were examined, and images captured using confocal microscopy. In the younger retina, CFH was localized in the inner plexiform layer and below the outer nuclear layer, while in the aged retina, it was found in the photoreceptors. CFH was also detected in the choriocapillaris and within the end-feet of the Müller cells. Our controls showed autofluorescence of the retinal pigment epithelium shedding light on a false positive CFH immunostaining of this layer. GFAP immunoreactivity highlighted an increased gliosis within the aged retina. CFHR3 signal was found in the microglia, while CFHR1 was detected in the choriocapillaris. In summary, underpinning the expression of these components can show the potential involvement of these modulators in implementing new treatment strategies.

Keywords: retina, AMD, complement system, *CFHR* gene, gliosis, immunofluorescence.

Introduction

Genetic studies have revealed a strong association between degenerative retinal disorders as age-related macular degeneration (AMD) and complement system genes, especially complement factor H (*CFH*), CFH-related 1 (*CFHR1*) and CFH-related 3 (*CFHR3*) [1–6]. A deletion of the *CFH* gene encoding the regulatory protein, CFH, leads to abnormal complement activation and is seen as a major AMD susceptibility gene [7, 8]. The deletion of the two genes, *CFHR1* and *CFHR3* encoding the complement-related proteins (CFHR1 and CFHR3), was found to be protective in AMD, however, their exact function is still unveiled [8, 9].

Current research incriminates different pathways that describe the degenerative process in the aged retina by showing the morphological changes that occur in the eye [10–14]. Medzhitov first introduced the idea of para-inflammation as an adaptive response of the tissues to stress [15–17]. In this chronic inflammatory process, the activated microglia migrate and accumulate in the subretinal space [18–21]. Also, as a response to the state of chronic inflammation, the Müller cells, specialized glial cells found only in the retina, induce a neuronal repair process called gliosis [22–25].

To understand the adaptive response of the retina to stress (para-inflammation), the aging process of the retina and the complement association with human retinal diseases, a deeper insight needs to be provided. Through modern methods of molecular biology or digital morphometry, it is important to determine the expression of the complement system genes in the human retina and mapping the complement system proteins in the aging retina. Also, the importance of different markers for gliosis and para-inflammation expressed in the retinal cells is a vital key to comprehend the degenerative process in the retina [16, 26, 27].

Aim

The aim of our preliminary research was to study the *CFHR1* and *CFHR3* genes, their associated proteins, and the markers for retinal stress in the aging process of the human retina through immunohistochemistry (IHC) by immunofluorescence, with a panel of specific antibodies directed against the most important proteins associated with aging degenerative disorders. This is a first step towards understanding the pathogenesis of AMD and could lead to new treatment strategies (*e.g.*, complement-based immunotherapies).

Materials and Methods

Two human formalin-fixed donor eyes, from 92- and 64-year-old patients, were used to perform the study. Both donor eyes were procured from the Department of Ophthalmology, University of Bern, Inselspital, Bern,

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Switzerland. Tissues were acquired with ethical approval obtained from the local Ethics Committees, in accordance with the principles outlined in the Declaration of Helsinki. The study was realized in the Laboratory of Immunotherapy for Retinal Degeneration, Experimental Ophthalmology, Department of Ophthalmology, University Hospital Regensburg, Germany.

First, the deoxyribonucleic acid (DNA) samples from the two human eyes were extracted using Promega Kit (ReliaPrep™ FFPE gDNA Miniprep System; Promega Corp., Madison, USA) according to the manufacturer's protocol for alternative methods for DNA isolation without deparaffinization. As positive controls, we used DNA samples from tissues like cow retina, mice liver, and spleen (BL6 mice, Department of Ophthalmology, Regensburg, Germany). The quantity and quality of the DNA was measured using spectrophotometry analysis (DeNovix DS-11 spectrophotometer, Wilmington, USA) and the DNA samples were amplified using the following primer sets (Metabion, Planegg, Germany): *CFHR1* specific primer and *CFHR3* specific primer, while a positive control glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) specific primer was included to ensure that all polymerase chain reaction (PCR) reagents were working well and could generate a band. The purified amplified products were then genotyped for the two genes previously shown to be associated with AMD, *CFHR1* and *CFHR3*, while *GAPDH* was used as positive control. The PCR-based genotyping was realized using PCR Thermocycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) and the expression of *CFHR1*, *CFHR3* and *GAPDH* was analyzed using FluorChem FC2 Imaging System (Alpha InnoTec, Kasendorf, Germany). Genotypes were determined using the AlphaView Software (Alpha InnoTec, Kasendorf, Germany). In the end, the products were separated with agarose gel electrophoresis (Gibco BRL H% Horizontal Gel Electrophoresis Apparatus; Life Technologies, Carlsbad, USA) to visualize the analysis. After separation, the resulting DNA fragments were visible as clearly defined bands of 175 bp *CFHR1* and 181 bp *CFHR3*.

The associated proteins of the complement system genes, *CFHR1* and *CFHR3*, were studied through IHC by immunofluorescence. We used antibodies directed against the most representative complement system proteins related to AMD: CFH, CFHR1, CFHR3. To study the gliosis process, we used glial fibrillary acidic protein (GFAP), an immunomarker for retinal stress expressed in the Müller cells and to examine the adaptive response of the retina in the aging process, we used ionized calcium-binding adaptor molecule 1 (Iba1), an immunomarker expressed in the microglia. For the examination of the nuclei of the retinal cells, 4',6'-Diamidino-2-phenylindole (DAPI) staining was used.

The following antibodies were used for both retinae (92- and 64-year-old patients): primary antibodies: anti-mouse CFH C5H5 antibody (kindly provided by Berra & Clivio [28] mouse monoclonal antibody, 1:200 dilution) and anti-rabbit GFAP antibody (Abcam, P7 goat monoclonal anti-rabbit antibody, 1:500 dilution); secondary antibodies: goat anti-mouse CF488A-conjugate antibody (Biotium, Hayward, CA, USA; Cat. #20018-1, 1:1000 dilution), goat anti-rabbit Cy3-conjugate (Thermo Fisher Scientific, Braunschweig, Germany; Cat. #A10520, 1:500 dilution), and DAPI (Thermo Fisher Scientific, Braunschweig, Germany; 1:1000 dilution). A control sample was also used only with the secondary antibodies.

For the younger retina, a few more antibodies were added as this study was part of a bigger project: primary antibodies: anti-mouse monoclonal CFHR1 P163 (R&D Systems Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany), rabbit anti-Iba1 polyclonal antibody P155 (Wako Chemicals, Neuss, Germany; Cat. #019-19741, 1:500 dilution) and an in-house anti-mouse monoclonal antibody FHR-3 [RETC (REgensburg Therapy Complement)-2: 269-5, Department of Ophthalmology, Regensburg Hospital, Germany]; secondary antibodies: goat anti-mouse CF488A-conjugate antibody (Biotium, Hayward, CA, USA; Cat. #20018-1, 1:1000 dilution), Alexa Fluor 546 rabbit anti-goat (Thermo Fisher Scientific, Braunschweig, Germany; Cat. #A21085, 1:1000 dilution), goat anti-rabbit Cy3-conjugate (Thermo Fisher Scientific, Braunschweig, Germany; Cat. #A10520, 1:500 dilution), and DAPI (Thermo Fisher Scientific, Braunschweig, Germany; 1:1000 dilution). The control samples contained only the secondary antibodies.

IHC by immunofluorescence was performed on 25-µm thick cryostat frozen sections. For the immunostaining were used: Roti® liquid barrier marker (red) (Carl Roth GmbH & Co, Karlsruhe, Germany), blocking solution containing 3% Dimethyl sulfoxide (DMSO)/0.3% Triton X-100/5% donkey serum in phosphate-buffered saline (PBS) (Sigma-Aldrich; Cat. #D8537), PBS (Sigma-Aldrich; Cat. #D8537), antibody solution containing 1% bovine serum albumin (BSA) in PBS, fluorescence mounting medium (Dako; Cat. #S302380-2).

The immunostaining was done on cryostat-sectioned human retina and visualized after by electron microscopy (EM). The slides were previously cryo-protected in a 10–30% sucrose gradient (3 d) and embedded in Neg-50 frozen section medium (Thermo Fisher Scientific, Braunschweig, Germany). Before the immunolabeling, the retinal sections were bordered with Roti® liquid barrier marker (red) (Carl Roth GmbH & Co, Karlsruhe, Germany) and the reactive sites were blocked using 150 µL of blocking solution containing 3% DMSO/0.3% Triton X-100/5% donkey serum in PBS. Primary antibody binding was performed overnight, at room temperature. Antibodies were diluted to a specific concentration in their blocking solution. After primary antibody exposure, sections were washed in PBS (Sigma-Aldrich; Cat. #D8537) three times and then incubated with the appropriate secondary antibody diluted in antibody solution containing 1% BSA in PBS for 30 minutes. DAPI (1:1000 dilution) was used for the staining of the cells' nuclei. The slides were then washed again three times and covered in fluorescence mounting medium (Dako, Cat. #S302380-2).

All the samples were examined, and images captured with a laser scanning confocal microscope VisiScope CSU-X1 Confocal System (Visitron Systems, Puchheim, Germany) equipped with a high-resolution scientific complementary metal–oxide–semiconductor (sCMOS) camera (PCO AG, Kelheim, Germany).

Results

The extracted DNA from the two human eyes underwent a spectrophotometry analysis and the measurements were compared to three different types of cow retina tissue, mouse liver, and mouse spleen (Table 1).

Table 1 – ***DNA spectrophotometry analysis***

DNA samples	Dilution in 30 µL	DNA concentration	DNA purity (A260/A280)
DNA 1	0.108 g	469.17 µg/µL	1.82
DNA 2	0.214 g	399.20 µg/µL	1.81
Pig retina	0.772 g	202.65 µg/µL	1.8
Mouse spleen	0.177 g	1001.46 µg/µL	1.82
Mouse liver	0.164 g	179.4 µg/µL	1.91

A260/A280: The ratio of absorbance (A) at 260 nm and 280 nm; DNA: Deoxyribonucleic acid.

The results revealed a higher concentration of DNA in the mouse spleen tissue (1001.46 ng/µL), while compared to aged human retina (469.17 ng/µL) and in descending order the young human retina (399.20 ng/µL), the cow retina (202.65 µg/µL) and the mouse liver sample (179.4 ng/µL).

When referred to the quality of the samples, the purity (A260/A280) of the DNA was higher for the mouse liver tissue (1.92), followed by the same values for the aged human retina and mouse spleen (1.82), when compared to the young human retina (1.81), and the lowest value found was in the cow retina tissue (1.8).

The genotyping of the two eyes performed in our study depicted the expression of two genes *CFHR1* and *CFHR3* previously shown to be associated with AMD. The extracted genomic DNA was pure and sufficient from both eyes. PCR-based genotyping was performed, and the specific DNA fragments were visible as defined bands of 175 bp (*CFHR1*) in the young retina and 181 bp (*CFHR3*) in the older one. DNA samples from the team members were used as controls (Figure 1).

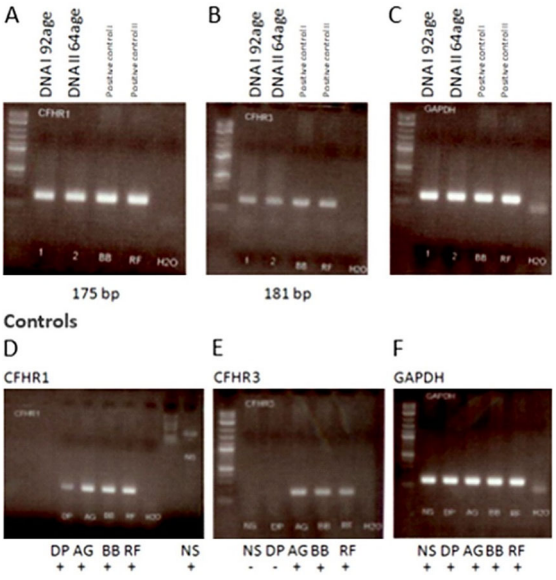

Figure 1 – ***(A–E) PCR-based genotyping. CFHR1: Complement factor H (CFH)-related 1; CFHR3: CFH-related 3; DNA: Deoxyribonucleic acid; GAPDH: Glial fibrillary acidic protein; PCR: Polymerase chain reaction. AG, BB, DP, NS, RF: The initials of the DNA donors used as control samples.***

We investigated the distribution of the complement system proteins in the human retina through confocal microscopy analysis comparing the significantly different pattern of the proteins expression in the aged human retina and the younger eye. Our results were consistent with a potential role of the CFH and related proteins in the diagnosis of age-related retinal disorders.

In the 64-year-old retina, CFH was localized in the inner plexiform layer (IPL) and below the outer nuclear layer (ONL), in contrast to the aged retina where interestingly CFH was clearly expressed predominantly in the photoreceptors (Figure 2).

Our immunostaining controls showed a certain amount of autofluorescence of the retinal pigment epithelium (RPE), which interfered with the evaluation of CFH immunostaining in this layer (Figure 3).

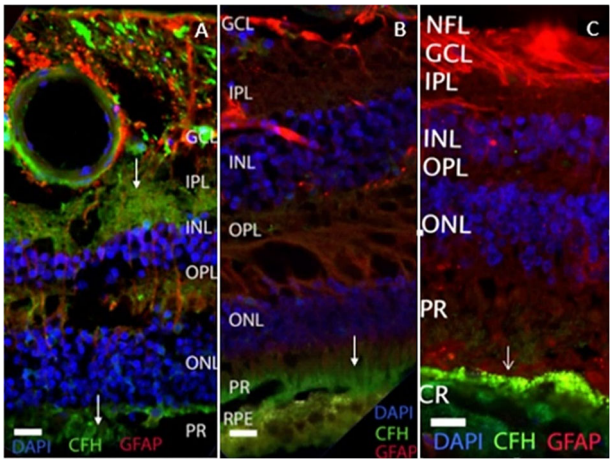

Figure 2 – ***(A–C) Different immunoexpression pattern of CFH in aged and young human retina. CFH: Complement factor H; CR: Choroid; DAPI: 4',6'-Diamidino-2-phenylindole; GCL: Ganglion cell layer; GFAP: Glial fibrillary acidic protein; INL: Inner nuclear layer; IPL: Inner plexiform layer; NFL: Nerve fiber layer; ONL: Outer nuclear layer; OPL: Outer plexiform layer; PR: Prelaminar region; RPE: Retinal pigment epithelium.***

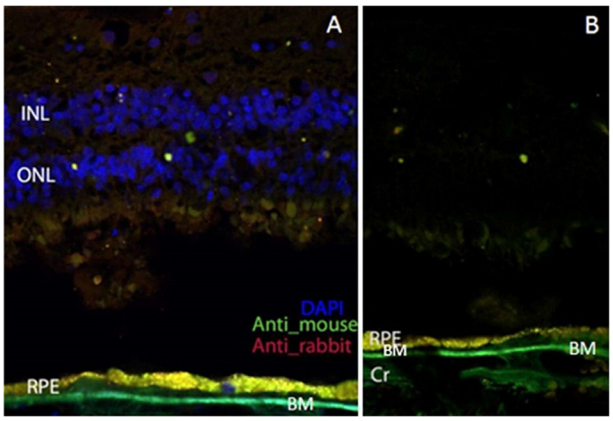

Figure 3 – ***(A and B) Autofluorescence of the RPE and BM. BM: Bruch's membrane; Cr: Choroid; DAPI: 4',6'-Diamidino-2-phenylindole; INL: Inner nuclear layer; ONL: Outer nuclear layer; RPE: Retinal pigment epithelium.***

When we analyzed the CFHR3 signal, it was found only in the microglia, while CFHR1 was detected in the choriocapillaris (Figure 4).

IHC detected CFH in the choriocapillaris, demonstrating the systemic involvement of this protein. CFH was also found to be colocalized with GFAP within the end-feet of the Müller cells (Figure 5).

We also compared the GFAP immunoreactivity, highlighting an increased process of gliosis within the ganglion cell layer (GCL) of the aged retina in comparison to the younger tissue (Figure 6).

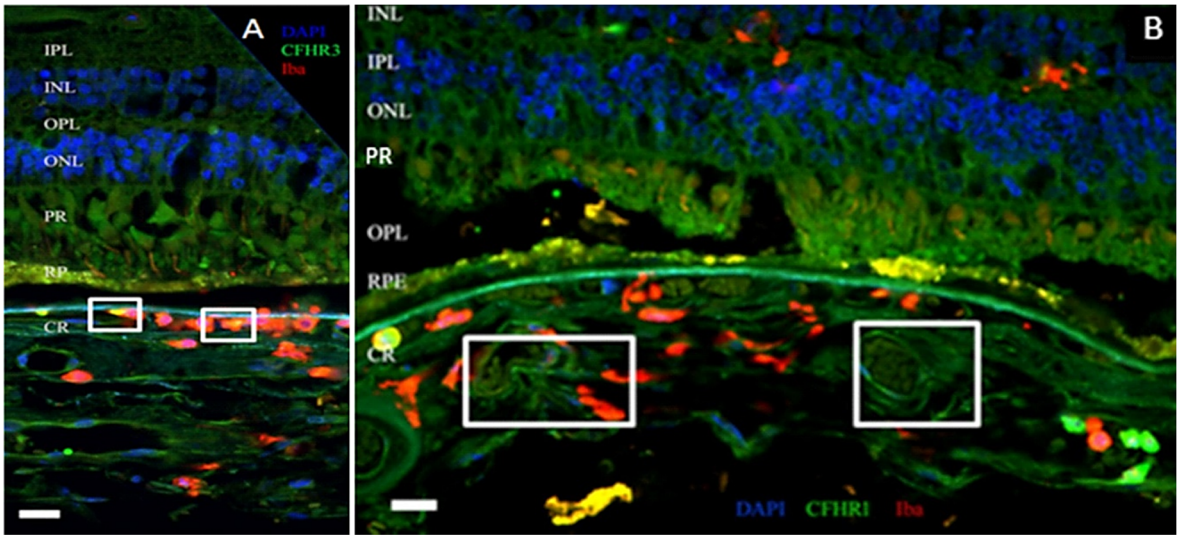

Figure 4 – *(A and B) CFHR3 immunolabeling when compared to CFHR1 deposition. CFHR1: Complement factor H (CFH)-related 1; CFHR3: CFH-related 3; CR: Choroid; DAPI: 4',6'-Diamidino-2-phenylindole; Iba: Ionized calcium-binding adaptor molecule; INL: Inner nuclear layer; IPL: Inner plexiform layer; ONL: Outer nuclear layer; OPL: Outer plexiform layer; PR: Prelaminar region; RPE: Retinal pigment epithelium.*

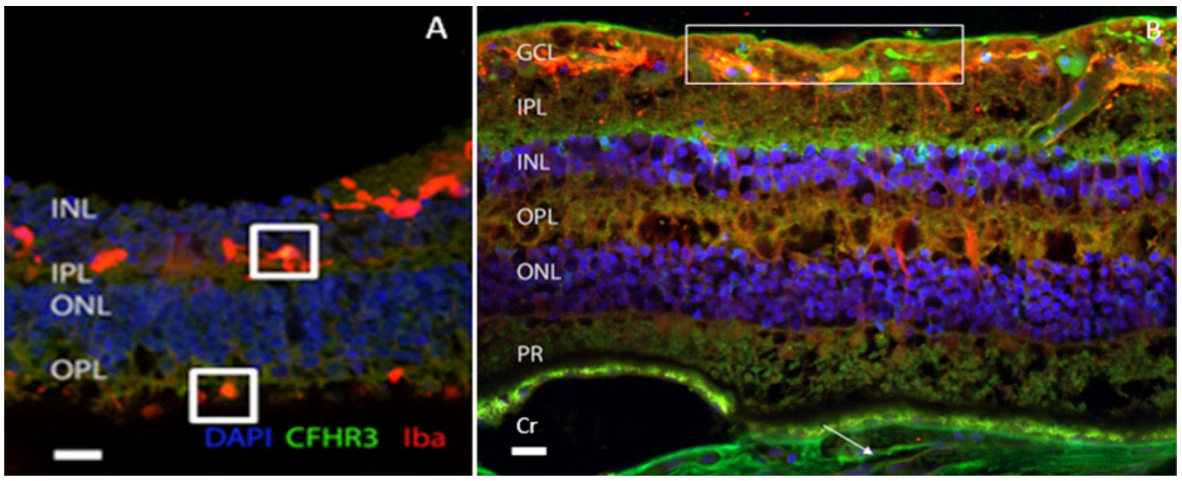

Figure 5 – *(A and B) CFHR3 immunoexpression in comparison to CFH specificity. CFHR3: Complement factor H (CFH)-related 3; Cr: Choroid; DAPI: 4',6'-Diamidino-2-phenylindole; GCL: Ganglion cell layer; Iba: Ionized calcium-binding adaptor molecule; INL: Inner nuclear layer; IPL: Inner plexiform layer; ONL: Outer nuclear layer; OPL: Outer plexiform layer; PR: Prelaminar region.*

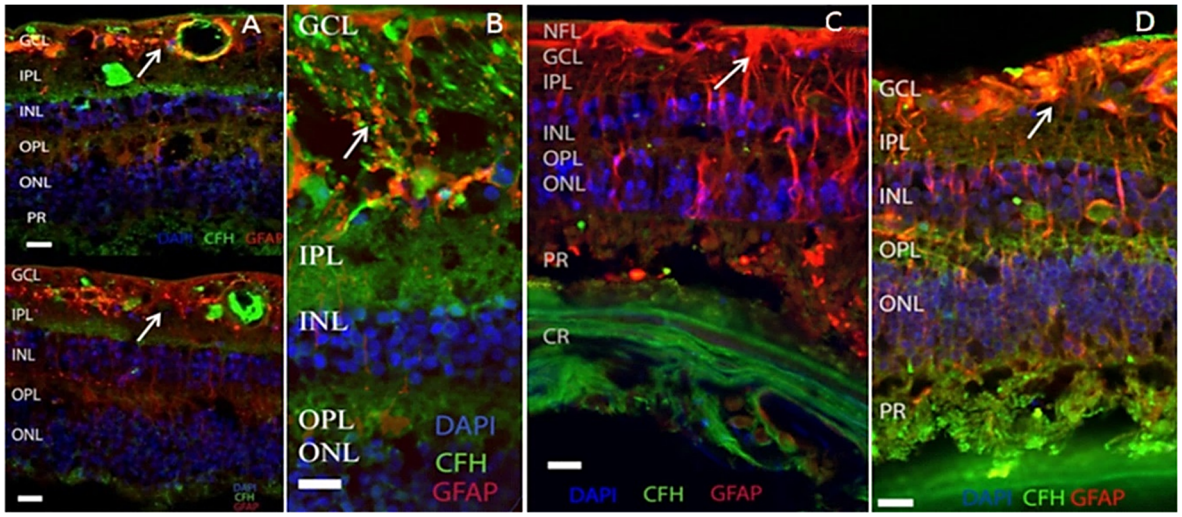

Figure 6 – *(A–D) Gliosis in younger versus aged human retina. CFH: Complement factor H; CR: Choroid; DAPI: 4',6'-Diamidino-2-phenylindole; GCL: Ganglion cell layer; GFAP: Glial fibrillary acidic protein; INL: Inner nuclear layer; IPL: Inner plexiform layer; NFL: Nerve fiber layer; ONL: Outer nuclear layer; OPL: Outer plexiform layer; PR: Prelaminar region.*

Discussions

Genetic and immunohistochemical studies on human retina have been a real challenge for researchers since the fixation of this sensitive tissue requires a lot of precision. Smit-McBride *et al.* observed that the mouse and human tissues are similar but not identical [29]. Experimental comparisons for every level of detail from the structure of the retina to the genes expressed in the eye and the physiology of the entire complement system have not been fully explored [29].

Nevertheless, depicting the expression of the complement system proteins in the human retina offers the most accurate knowledge about the changes that appear in the aging retina.

In our study, we genotyped two eyes and depicted the deposition of two genes, *CFHR1* and *CFHR3*, previously shown to be associated with AMD [30]. The deletion of these genes encoding the complement-related proteins (CFHR1 and CFHR3) was found by several genetic groups like Spencer *et al.* (2008), Fritsche *et al.* (2010) and Hughes *et al.* (2006) to be protective in AMD; however, their exact function is still unveiled [7, 9, 31].

We recently concluded that CFH expression within the choriocapillaris reveals a wider systemic involvement which may advance our understanding of how complement dysfunction contributes to the development of AMD. Various researchers like Fett *et al.* have found strong associations between AMD disorder and variants of several complement pathway-associated genes, *CFH*, being one of many complement-regulatory genes expressed in the retina [32]. A deletion of this gene encoding the regulatory protein, CFH, which leads to abnormal complement activation. Kawa *et al.* defined it as a major AMD susceptibility gene and a dual player in the pathogenesis of AMD [1].

Seth *et al.* suggested that the retinal pigmented epithelium cell represents a local retinal source for complement activation [33]. Furthermore, Kim *et al.* described that the main site of CFH synthesis within the retina appears to be the retinal pigmented epithelium from where it may be secreted directly into the retina to provide localized protection against unwanted complement activation [34]. Despite this, our controls demonstrate that the retinal pigmented epithelium itself exhibits a certain amount of autofluorescence, which needs to be considered when the CFH immunostaining pattern in the human retina is discussed.

In accordance with other previous researches, both on mouse (Smit-McBride *et al.*) and human retina (Fett *et al.*) in our study, CFH and related proteins express modifications in their localization patterns as the human retina ages [29, 32]. We observed that the distribution of the CFH protein, when the two eyes were compared, reveals its specificity, staining the IPL in the young retina while affecting the photoreceptor layer in the aged retina, in accordance with Schäfer *et al.* [35]. This is perhaps another early indicator of the age-induced changes within the human eye.

Another interesting finding of our immunostaining revealed that CFH is colocalized with GFAP within the end-feet of the Müller cells. This distribution of CFH was also described by Schäfer *et al.* [35].

Interestingly, another strong indicator of the changes in retinal homeostasis is the immunopattern of the CFHR1, which appeared to be localized within the choriocapillaris in the 64-year-old retina. Furthermore, it describes an unveiled systemic involvement of this protein.

Our results were consistent with an essential role of the CFH and related proteins in the age-related pathology of the eye.

In our study, we observed a co-expressing pattern of the CFHR3, with the migrating microglia emphasizing its role in the inflammatory process within the aged retina. This was observed with the specific immunomarker for the microglia cells, Iba1.

Our immunohistochemical analysis revealed a significant activation of microglia within the layers of the retina and a migration of these cells towards the subretinal space. Immunostaining the retina sections with the Iba microglial specific marker, we observed this specific migration, and we depicted more cells in the older retina when compared with the younger one. This phenomenon occurring with the microglia cells was compared by Madeira *et al.* in a study showing a different pattern of the cell movement in each of the following eye diseases: AMD, glaucoma, and diabetic retinopathy [18]. Seth *et al.* suggested that microglial cells become activated and migrate toward complement deposits; therefore, their increased frequency in the older retinal tissue is justified [33].

The microanalysis of our immunostaining suggested an increased gliosis within the GCL of the aged retina in comparison to the number of activated ganglion cells in the younger tissue. The result was shown by immunostaining the Müller cells with GFAP, a specific marker expressed in these structures. This process induced by the ganglion cells has been previously studied by Hippert *et al.* and Ganesh & Chintala who emphasized that degenerative eye disorders culminate with photoreceptor loss and this loss induces the process of reactive gliosis through the upregulation of GFAP [22, 36]. Nevertheless, it is considered a strong indicator of the neuronal repair occurring in a state of chronic inflammation as a response to the retinal oxidative stress [22].

Our results suggest that age plays a role in activating the complement cascade in the retina. Aging leads to structural and functional changes in all compartments of the retina including the neuroretina, retinal pigmented epithelium and Bruch's membrane (BM).

Study limitations

One of the limits of this study is the use of post-mortem tissue and the time delay between the death of the donor and fixation of eye tissue. This delay affects the quality of the samples. The difficulty of fixation for the human retina sections in comparison to the mouse samples makes it hard to work with the tissues during the immunostaining protocol that needs to be followed. Due to this fact, to date there are few studies on human samples. As perspectives for the improvement of the study would be to introduce a higher number of eye donors and to include a comparison between healthy aged human retina and the pathological eye to be able to understand where the balance between healthy aged tissue and the pathological aging gets disrupted, so that we can use the histological changes for the development of new treatment strategies.

Conclusions

Our study depicted the expression of two genes, *CFHR1* and *CFHR3*, previously shown to be associated with AMD. While mapping the complement system proteins in the human retina, our results were consistent with a potential role of the CFH and related proteins in the diagnosis of age-related retinal disorders. IHC detected CFH in the choriocapillaris of both retinae demonstrating the systemic involvement of this protein. In the 64-year-old retina, CFH was localized in the IPL and below the ONL, in contrast to the aged retina, where it was expressed in the photoreceptors. Our immunostaining controls showed autofluorescence of the RPE, which needs to be considered for the evaluation of CFH immunostaining in this layer. CFHR3 signal was found only in the microglia, while CFHR1 was expressed in the choriocapillaris. Our study is a first step towards understanding the complicated interplay between genetic factors and the structural changes that appear in the human retina with the aging process. In summary, underpinning the expression of these components is a vital key in understanding the pathogenesis of AMD, but further research is needed to unveil the potential involvement of these modulators in new diagnostic strategies and innovative complement-based immunotherapy.

Conflict of interests

The authors declare that they have no conflict of interests.

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