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Autoantibodies detection in patients affected by autoimmune retinopathies

M.R. CECCARINI[1], M.C. MEDORI[2,3], K. DHULI[2], S. TEZZELE[2], G. BONETTI[1,2], C. MICHELETTI[2], P.E. MALTESE[2], S. CECCHIN[2], K. DONATO[4,5], L. COLOMBO[6], L. ROSSETTI[6], G. STAURENGHI[7], A.P. SALVETTI[7], M. OLDANI[7], L. ZICCARDI[8], D. MARANGONI[9], G. IAROSSI[10], B. FALSINI[11], G. PLACIDI[11], F. D'ESPOSITO[12,13], F. VIOLA[14,15], M. NASSISI[14,15], G. LEONE[15], L. CIMINO[16,17], L. DE SIMONE[17], V. MASTROFILIPPO[17], T. BECCARI[1], M. BERTELLI[2,4,5]

[1]Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy
[2]MAGI'S LAB, Rovereto, Trento, Italy
[3]Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy
[4]MAGI EUREGIO, Bolzano, Italy
[5]MAGISNAT, Atlanta Tech Park, Peachtree Corners, GA, USA
[6]Department of Ophthalmology, ASST Santi Paolo e Carlo Hospital, University of Milan, Milan, Italy
[7]Eye Clinic, Department of Biomedical and Clinical Science, Luigi Sacco Hospital, University of Milan, Milan, Italy
[8]IRCCS Fondazione Bietti, Rome, Italy
[9]Eye Clinic, Department of Medical, Surgical Sciences and Health, University of Trieste, Trieste, Italy
[10]Ophthalmology Unit, Bambino Gesù Children's Hospital, IRCSS, Rome, Italy
[11]Ophthalmology Unit, Fondazione Policlinico Universitario "A. Gemelli" IRCCS/Università Cattolica del S. Cuore, Rome, Italy
[12]Imperial College Ophthalmic Research Group (ICORG) Unit, Western Eye Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom
[13]Eye Clinic, Department of Neurosciences, Reproductive Sciences and Dentistry, University of Naples Federico II, Naples, Italy
[14]Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy
[15]Department of Ophthalmology, Fondazione IRCCS Cà Granda, Clinica Regina Elena, Milan, Italy
[16]Department of Surgery, Medicine, Dentistry and Morphological Sciences, with Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy
[17]Ocular Immunology Unit, Azienda USL-IRCCS, Reggio Emilia, Italy

Abstract. – OBJECTIVE: Autoimmune retinopathies (ARs) encompass a spectrum of immune diseases that are characterized by the presence of autoantibodies against retinal proteins in the bloodstream. These autoantibodies (AAbs) lead to a progressive and sometimes rapid loss of vision. ARs commonly affect subjects over 50 years of age, but also rare cases of kids under 3 years of age have been reported.

PATIENTS AND METHODS: In this study, 47 unrelated Caucasian patients were enrolled. All subjects showed negative cancer diagnoses and negative results in their genetic screenings. We studied 8 confirmed retinal antigens using Western blotting analysis, with α-enolase followed by carbonic anhydrase II being the two most frequently found in the patients' sera.

RESULTS: Nineteen patients were positive (40.4%), thirteen uncertain (27.7%), and fifteen were negative (31.9%). Their gender did not correlate with the presence of AAbs (p=0.409).

CONCLUSIONS: AAbs are responsible for retinal degeneration in some cases, while in others, they contribute to exacerbating the progression of the disease; however, their detection is crucial to reaching a better diagnosis and developing more effective treatments for these conditions. Moreover, finding good biomarkers is important not only for AR monitoring and prognosis, but also for helping with early cancer diagnosis.

Key Words:
Autoimmune retinopathies, Autoantibodies, Western-blotting, Alpha-enolase, Recoverin, Rhodopsin, Heat Shock Protein 27, Glyceraldehyde-3-phosphate dehydrogenase, Carbonic Anhydrase 2.

Corresponding Author: Maria Chiara Medori, Ph.D; e-mail: chiara.medori@assomagi.org

Introduction

Retinopathy can be defined as the damage to the retina, the light-sensitive tissue at the back of the eye that is responsible for vision. There are several types of retinopathies caused by inflammatory, infectious, vascular, or degenerative mechanisms or associated with systemic diseases, among which one of the most common is diabetic retinopathy[1,2]. In recent years, many researchers have focused their attention on autoimmune retinopathies (ARs), a rare and, unfortunately, poorly understood condition[3] that occurs when the immune system attacks healthy cells in the retina, leading to vision loss[4]. The presence of autoantibodies (AAbs) in the patients' serum is established, but their exact role is still unknown; they are probably caused by an overactive immune response to antigens in the retina.

The prevalence of autoimmune diseases in the general population is around 7-9% and seems to affect more women than men[5]. One of the most crucial goals in autoimmune diagnosis could be to identify if AAbs are involved in the pathogenic process. Autoantigens are always self-proteins or molecules that are normally present in the body and are recognized by the immune system as "self". However, in some cases, the immune system can mistakenly recognize autoantigens as foreign or nonself, leading to an autoimmune response and the destruction of healthy tissues – in this case, retinal tissue. The correct interpretation for ARs could be the tight link between the retina and thymus[6,7]. In fact, the retina contains a huge number of proteins that are expressed also in the thymus and other secondary lymphoid tissues[8]. In the thymus, the process of negative selection is a crucial step in the development of self-tolerance[9]. During this process, developing T cells that recognize autoantigens expressed in the thymus are eliminated or suppressed. This helps in preventing the development of autoimmune diseases, by ensuring that only T cells that recognize foreign antigens are able to leave the thymus and enter the bloodstream[10].

AAbs against retinal proteins can be generated after 3 possible trigger occurrences[11]:

1. Antitumor response: Both malignant and benign cancers are able to induce an immune response because tumoral antigens have been exposed to the antigen-presenting cells and induced the production of AAbs against epitopes that cross-react with retinal proteins[12-14]. These types of retinopathies are called "cancer-associated retinopathies" (CARs) or "melanoma-associated retinopathies" (MARs).

2. Anti-microbial infection: A putative similarity between proteins found in pathogens and in the retina can cause this reaction. An example of cross-reaction occurs in the glycolytic pathway, which has important metabolic functions in both microbial and retinal cells[15].

3. Retinal injury: This mechanism can occur due to a variety of factors, such as trauma, inflammation, vascular disorders, and degenerative diseases. Causative mutations may induce cellular stress in photoreceptors, and their death by apoptosis produces metabolic debris, which subsequently may lead to autoimmunization.

The exact AAbs involved in these conditions are not yet fully known, but different AAbs have been identified in independent studies. AAbs against glycolytic enzymes – including aldolase, alpha-enolase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase – were found in the serum of patients with retinal diseases, and their elevated titers suggested a possible correlation with pathogenicity[15,16]. Other potential target proteins have been investigated with interesting results, such as recoverin, rhodopsin, heat shock protein 27 (HSP27), one of Rab-related proteins (Rab6A), and carbonic anhydrase II (CA2)[17].

AR symptoms may include blurred vision, loss of peripheral vision, night blindness, and visual distortion. The condition can progress rapidly and can lead to severe and permanent vision loss if left untreated. Diagnosing ARs is often challenging, as there is no single definitive test for this condition. However, a thorough medical history, comprehensive eye exam, and specialized tests – such as electroretinography (ERG), Humphrey Visual Fields (HVF), Optical Coherence Tomography (OCT), visual acuity and color vision testing – can help in the diagnosis[18].

Overall, ARs are complex and challenging conditions that require specialized care from an experienced healthcare team. First of all, it is important to promptly seek medical attention, to help prevent the progression of the condition and preserve visual function: early diagnosis followed by a correct treatment may prevent widespread retinal degeneration and, sometimes, permanent vision loss. Treatments for ARs typically involve the use of immunosuppressive drugs (such as corticosteroids, methotrexate, or mycophenolate mofetil) to help suppress the overactive immune response[19]. In some cases, intravenous immunoglobulin therapy (IVIg) or plasmapheresis may also be used. Recently, the use of Rituximab (Rituxan, Genentech, South San Francisco, CA, USA), a monoclonal

IgG antibody that depletes B cells, was deeply investigated in both non-paraneoplastic (ARs) and paraneoplastic (CARs and MARs) cases[20,21]. The combination of these therapies could probably deliver promising outcomes for those patients[22].

Patients and Methods

Materials

The employed BSA (Bovine Serum Albumin) was purchased from Invitrogen (Milan, Italy). SDS (Sodium Dodecyl Sulphate) was purchased from Carlo Erba (Reagenti Srl, Milan, Italy). NZY Color Protein molecular Weight Marker II was used in SDS-PAGE (Lisboa, Portugal). Recombinant human proteins expressed in mammalian cells against alpha-enolase (ENO1), recoverin (RCVRN), and rhodopsin (RHO), together with monoclonal horseradish peroxidase (HRP)-conjugated rabbit anti-human secondary antibody, were purchased from MyBioSource (San Diego, CA, USA). Heat shock protein 27 (HSP27), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and carbonic anhydrase 2 (CA2) recombinant proteins expressed in *E. Coli* were from Sigma (Saint Louis, USA). Arrestin-3 (Arr3) and transducin alpha-2 chain (GNAT2), produced in wheat germ, were bought from Biotechne (Minneapolis, MN, USA). Immunoreactive bands were visualized using the Amersham Cytiva ECL Prime Chemiluminescent Substrate (Rainham, Essex, UK).

Ethical Statement and Inclusion Criteria

The study was approved by the Ethics Committee of Azienda Sanitaria dell'Alto Adige (Ethikkomitee Südtiroler Sanitätsbetrieb, Italy), Prot. No. 0122029-BZ (22/11/2016). All research process was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

A written informed consensus was obtained from all patients or their guardians, and a unique alphanumeric code was assigned to each of them to protect their anonymity.

The samples (47 patients) used in this study were collected from January 2017 to November 2020, and stored at a temperature of -80°C. Sample collection was undertaken by several Italian research centers. We selected only unrelated patients of Caucasian origin who met the following criteria: progressive loss of vision, visual field defects, abnormal rod and/or cone responses to ERG, and negative cancer diagnosis. All patients did not have cancer-associated retinopathy (CAR) and, moreover, showed negative results in the genetic screening for inherited retinal dystrophies.

Western Blotting Analysis

A 10% polyacrylamide containing sodium-dodecyl sulfate (SDS-PAGE) gel (1 mm well-thick) was prepared fresh every time in reducing conditions.

For each recombinant human protein (listed in Table I), 1 µg was prepared using Laemmli Buffer 1X (BIO-RAD, 2000, CA, USA) with beta-mercaptoethanol and kept for 10 minutes at 100°C[23].

Electrophoresis was performed at constant voltage (100 V) for about 1 hour and a half. The transfer was carried out in a 0.45 mm nitrocellulose membrane for 1 hour at +4°C in a transfer buffer, as previously described[24]. At the end, before blocking with BSA 5% O/N at +4°C, Ponceau Red staining was performed to verify the proper transfer of markers and proteins (**Supplementary Figure 1**).

Patient serum was diluted 1:200 in BSA 2%, then it was left for 1 hour at room temperature (RT) in the oscillator MPM Instruments s.r.l. Bernareggio- Italia) set at 80 rpm. After 3 washes with PBS-Tween 0.2% (10 minutes each), HRP-conjugated anti-human Ig (1:500,000 final dilution in BSA 2%) was incubated for 1 hour in oscillation at RT, in dark condition. Again, 3 washes with PBS-Tween 0.1%, (10 minutes each) were done. ECL Cytiva Amersham Prime was left in contact with the membrane for 5 minutes; at the end, band acquisition and densitometric analysis were performed by Chemidoc Imagequant LAS500 – Ge Healthcare-Life Science (Milan, Italy), as previously standardized[25].

As a positive control, we used a human serum, previously analyzed by Casey Eye Institute (Ocular Immunology Laboratory, Oregon Health & Science University, Portland, USA), supervised by Dr. Grazyna Adamus. As a negative control, we omitted serum and applied directly after BSA blocking solution the secondary antibody, according to previously published methods[26].

Statistical Analysis

Data were checked for normality, outliers, and missing data. No imputation of missing data was performed.

Chi-square test was used to compare categorical variables. Correlation between variables was identified with Pearson correlation analysis and partial correlation analysis. The statistical analysis was performed with R-software (The R

Foundation for Statistical Computing, Vienna, Austria). Data were considered statistically significant if the *p*-value was lower than 0.05.

Results

The AAbs found in the serum are believed to play an important role in the development of ARs, in particular for the proteins expressed in the retina tissue. Using Western blotting analysis, we investigated eight different possible antigens, as reported in Table I. All the proteins were examined by SDS-PAGE and Coomassie Blue staining to ensure their purity and integrity.

Demographic characteristics for the whole cohort (n=47) are reported in Table II. Subjects over 30 years of age represented the majority (89.4%); only three young adults (22, 25, and 26 years of age) and two rare cases of young children (a female, 9-year-old, and a male, 10-year-old) were registered in the study. We divided all samples into three different groups, based on the WB results: positive patients, with more than one AAbs in the sera (**Supplementary Figure 2**); dubious patients, with only one AAb (usually ENO1 or CA2); and negatives, all those without AAbs. In

Table I. List of proteins used in the study.

Protein	Name	Cat number	Expression Host	Molecular Weight	Company
ENO1	Recombinant Human Alpha-enolase	MBS953222	Mammalian cell	51 kDa	MyBioSource
RCVRN	Recombinant Human Recoverin	MBS965646	Mammalian cell	27 kDa	MyBioSource
RHO	Recombinant Pig Rhodopsin	MBS1157518	Mammalian cell	32 kDa	MyBioSource
HSP27	Heat Shock Protein 27	H8158	*E. coli*	27 kDa	SIGMA
GAPDH	Glyceraldeide-3-phosphate deydrogenase	SRE0024	*E. coli*	37 kDa	SIGMA
CA2	Carbonic Anhydrase 2	SRP6484	*E. coli*	29.2 kDa	SIGMA
ARR3	Recombinant Human Arrestin-3 GST (N-term)	H00000407-P01	Wheat germ	70 kDa	Biotechne
GNAT2	Recombinant Human GNAT GST	H00002780-P01	Wheat germ	62 kDa	Biotechne

Table II. Demographic characteristics of patients and autoantibodies (AAbs) presence are reported first as a total cohort (n=47); in the two columns on the far right they are divided by gender: male (n=13) and female (n=34).

Patients' characteristics	Total Cohort	Males	Females
Number	47	13	34
Age	49.0 (9-75)	46.2 (10-73)	50.1 (9-75)
Familiarity	2	0	2
Diagnosis			
Retinitis pigmentosa	16	4	12
Leber's congenital amaurosis	3	1	2
Macular dystrophy	3	0	3
Cone-rod dystrophy (CORD)	2	1	1
AAbs presence			
Positive	19 (40.4%)	7 (53.8%)	12 (35.3%)
Doubtful	13 (27.7%)	2 (15.4%)	11 (32.4%)
Negative	15 (31.9%)	4 (30.8%)	11 (32.4%)

Table III. Proteins presence in the total cohort (n=47) and in the patients, as divided by gender.

Proteins presence	Total Cohort (n=47)	Male (n=13)	Female (n=34)
ENO1	24 (51.1%)	6 (46.2%)	18 (52.9%)
RCVRN	5 (10.6%)	3 (23.1%)	2 (5.9%)
RHO	7 (14.9%)	2 (15.4%)	5 (14.7%)
HSP27	6 (12.8%)	2 (15.4%)	4 (11.8%)
GAPDH	8 (17.0%)	3 (23.1%)	5 (14.7%)
CA2	20 (42.6%)	7 (53.8%)	13 (38.2%)
ARR3	4 (8.5%)	1 (7.7%)	3 (8.8%)
GNAT2	7 (14.9%)	2 (15.4%)	5 (14.7%)

the end, 19 patients were positive (40.4%), 13 uncertain (27.7%), and 15 negative (31.9%). The gender did not match with the presence of AAbs (p=0.409).

As reported in Table III, the most frequent AAbs were against anti-α-enolase (anti-ENO1, 51.1%), followed by anti-carbonic anhydrase II (anti-CAII, 42.6%) and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GADPH, 17%). These results follow the same trends of AAbs found in different subgroups of cancer patients, recently published by Adamus et al[26]. Anti-ENO1, anti-CAII, and anti-GAPDH are the three most frequently found AAbs in the patients' serum, also when divided by gender. Moreover, in this case, gender did not match with the presence of different AAbs (p=0.289). An interesting finding was the presence of recoverin (RCVRN) and heat shock protein 27 (HSP27) in 10.6% and 12.8% of the patients, respectively.

Anti-enolase retinopathy is a protean autoimmune retinopathy that characteristically presents with cone dysfunction. We confirmed this association with ENO1 positivity and Cone-rod dystrophy (CORD) in the two cohorts of patients[27].

Discussion

Retinopathies can lead to permanent vision loss. Regular eye examinations are important for monitoring and managing retinopathy, but a specific genetic screening and AAbs detection could be fundamental to predict the progression. Retinopathies can pose unique diagnostic challenges, accurate genetic diagnosis can pave the way for targeted therapies and personalized management options to enhance the lives of patients[28-32]. In this paper, we stressed the importance of AAbs detection because, first of all, it can precede the clinical onset of autoimmune diseases; moreover, it may provide important information for molecular diagnosis and specific medical care.

Before the year 2000, the scientific community had agreed that AAbs alone might have minor effects on healthy subjects, but their presence in damaging conditions – such as inflammation or cancer treatment – may have enormous cytotoxic effects[33]. This theory is probably going to change over the years: in fact, the penetration of AAbs into living cells seems to play a critical role in healthy individuals, participating in the pathogenesis of diverse autoimmune diseases[34].

AAbs follow-up tests (every 6 months) are needed in a broad range of diseases and, in the specific case of retinopathies, they could be extremely useful as a biomarker of disease activity associated with vision worsening. It is important to keep in mind that AAbs – especially those against glycolytic enzymes, such as enolase, aldolase, and GADPH – are more significantly elevated in patients than in healthy controls, but their presence alone is not synonymous with pathology. In other words, AAbs positivity should not be used as an exclusive marker for diagnosis, but it should be considered in retinal degeneration as a possible trigger of disease progression.

The hypothesis that AAbs act as a stress to photoreceptor cells is now deeply accepted, and multiple AAbs circulating in the serum can promote antibody-mediated retinal degeneration by blocking their functions[17]. Probably new AAbs such as AAbs against RCVRN, one of the first autoantigens found in CAR (recently discovered) can be detected[35,36] in the AR patients' serum. Similar results were recently obtained with AAbs *vs.* HSP27, a molecular chaperone with neuroprotective activity able to regulate the apoptosis process, which was detected[37,38] with high incidence in different diseases but not in healthy controls. Anti-RCVRN and anti-HSP27 could be good biomarkers not only for AR monitoring and prognosis but also for helping with early cancer diagnosis[39].

Limitations

Finally, we limited our investigation to 8 autoantigens that were identified and verified, but being this a work-in-progress research, we expect a larger number of AAbs to be characterized as having a role in autoimmune retinopathy.

Conclusions

The management of retinopathies, which can lead to irreversible vision loss, hinges on early detection and treatment. While treatment options like laser therapy, medication injections, and surgery exist, their effectiveness remains a subject of ongoing research. Regular eye examinations are essential for monitoring retinopathies, but our paper emphasizes the potential significance of autoantibody (AAbs) detection as a predictive tool, not only for autoimmune diseases but also for personalized medical care.

The next challenge will be the construction of a bigger library of verified AAbs, to help the medical community in the management of patients affected by such conditions.

Conflict of Interest
The authors declare no conflict of interest.

Authors' Contributions
Conceptualization, MB; methodology, MRC; investigation, MCM, CM, LC, LR, GS, APS, MO, LZ, DM, GI, BF, GP, DFE, FV, MN, GL, LC, LDS, and VM; data curation, MRC; writing-original draft preparation, MRC, KDhuli, GB; writing-review and editing, MCM, ST, KDonato, CM, PEM, SC, LC, LR, GS, APS, MO, LZ, DM, GI, BF, GP, FE, FV, MN, GL, LC, LS, VM, and TB; project administration, TB and MB; funding acquisition, MB. All authors have read and agreed to the published version of the manuscript.

Informed Consent
All subjects gave their informed consent for inclusion before they participated in the study.

Availability of Data and Materials
The data are within the test or in the supplementary materials document.

Ethics Approval
The study was approved by the Ethics Committee of Azienda Sanitaria dell'Alto Adige (Ethikkomitee Südtiroler Sanitätsbetrieb, Italy), Prot. No. 0122029-BZ (22/11/2016). All research process was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

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