AUTOIMMUNITY IN UVEITIS AND OTHER CHORIORETINAL DISEASES

J.C.E.M. TEN BERGE

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AUTOIMMUNITY IN UVEITIS AND OTHER CHORIORETINAL DISEASES

Autoimmuniteit in uveïtis en andere chorioretinale ziektebeelden

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Promotoren:	Prof.dr. J.R. Vingerling Prof.dr. A. Rothova
Overige leden:	Prof.dr. P.M. van Hagen Prof.dr. J.H. de Boer Prof.dr. C.J.F. Boon
Copromotor:	Dr. M.W.J. Schreurs

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Uveitis

Uveitis is an inflammation of the vascular layer of the eye (uveal tract), which includes the iris, ciliary body and choroid. However, in practice the term uveitis is usually used as a collective term for any form of intraocular inflammation. Uveitis is a major cause of visual impairment or even blindness. The annual prevalence of uveitis in the western world is increasing and varies between 85-115 cases per 100.000 persons. The incidence is around 25-52 cases per 100.000 person-years with a peak at the age of 25-44 year, affecting predominantly the adult working population.[1,2]

Uveitis is usually classified according the anatomical location of the inflammation into anterior, intermediate, posterior or panuveitis group.[3] The most common location encountered by primary care ophthalmologists is anterior uveitis, whereas the involvement of posterior eye segment is typically referred to tertiary care institutions.[1] In approximately one third of uveitis cases the cause remains elusive (idiopathic uveitis), but the remainder may be either associated with systemic infectious (e.g. syphilis, toxoplasmosis), or an underlying systemic autoimmune or auto-inflammatory diseases.[4-6] In these systemic non-infectious diseases, the eye is usually one of the several organs involved and uveitis might be the first clinical sign of a more widespread systemic disease.

Scleritis represents also an inflammatory disease of the eye, involving predominantly the sclera, but corneal, episcleral and retinal tissue may also be involved. Scleritis can be very severe, painful and result in blindness. As in uveitis, scleritis is sometimes associated with an underlying non-infectious systemic disease such as rheumatoid arthritis or granulomatosis with polyangiitis. Scleritis is formally not a subtype of uveitis, but these ocular inflammations may have similar causes and associations, as well as comparable diagnostic and therapeutic approaches.

Autoimmunity in uveitis

The pathogenesis of uveitis is not fully clarified, but a crucial role of autoimmune reactions has been suggested. Autoimmunity is characterized by an aberrant activity of the immune system directed against the body's own cells and tissues. It occurs when the immune system stops tolerating 'self' antigens and autoreactive cells attack the body's own antigens. An exogenous or endogenous trigger (for example tissue damage) causes activation of the immune system, resulting in production of pathogenic antibodies and/or T-cells directed against ocular antigens. Several theories have been proposed about why this expansion occurs, including molecular mimicry in which autoantigens are mistaken for peptides from micro-organisms.[7]

Insights about autoimmunity in eye-specific disease are limited, especially in ocular diseases without systemic manifestations (e.g. birdshot chorioretinopathy). Indirect evidence for involvement of autoimmunity in uveitis has been provided by induction of autoimmune uveitis after immunization of animals with retinal autoantigens in combination with Freund adjuvant.[8-10] These animal models, representing experimental autoimmune uveitis (EAU), have provided insight into the pathogenesis of human uveitis. In EAU predominantly mice are injected with different antigens (such as S-arrestin and interphotoreceptor retinal binding protein) causing inflammation of intraocular tissue similar to human uveitis. More recently, models with genetic manipulated mouse and spontaneously emerging uveitis, have been developed.[2,5] Similar to the heterogeneity of human uveitis, clinical manifestations of uveitis in animal models may differ and is probably related dose and type of immunization as well as genetic sensibility.

Antiretinal antibodies

The activity of our humoral immune system can be studied in the laboratory by measuring autoantibodies directed against retinal tissue. These so-called antiretinal antibodies (ARAs) can be detected by various laboratory techniques including immunohistochemistry, Western blot and enzyme-linked immunosorbent assay (ELISA). However, a standardized assay to measure ARAs is lacking and results may vary depending on the laboratory tool.[11]

The exact role of ARAs in uveitis and other chorioretinal diseases such as retinitis pigmentosa (RP), age-related macular degeneration (AMD) and glaucoma, was scarcely investigated. It has been suggested that ARAs might be involved in the inciting process of the ocular disease. Another hypothesis addresses a secondary phenomenon of ARAs induced by retinal damage. It has been proposed that ARAs in ocular disease might cause a mild inflammation in the retina and subsequently aggravate and/or prolong the ocular disease.[12,13]

Autoimmune retinopathy

ARAs have been described also in the context of autoimmune retinopathy (AIR). AIR encompasses a spectrum of rare autoimmune diseases that primarily affect retinal cells, and includes cancer-associated retinopathy (CAR), melanoma-associated retinopathy (MAR) and non-paraneoplastic autoimmune retinopathy. The affected patients produce ARAs directed against their own retina, which are thought to play a pathogenic role and being able to attack and destroy retinal cells, leading rapidly to visual loss or even blindness. It is hypothesized that the underlying mechanism of AIR is an immune response to tumor antigens sharing homology with retinal antigens (molecular mimicry).[14,15] AIR has been associated with the presence of various serum ARAs including antibodies directed against recoverin, α-enolase, transducin-α, carbonic anhydrase, arrestin and various other retinal antigens. While the patients with anti-recoverin antibodies frequently suffer from associated cancer and severe loss of rod and cone function, the anti-enolase retinopathy is characteristically associated with cone dysfunction and is also prevalent in patients without

cancer. The autoreactivity to specific antigens and the clinical manifestations of AIR indicate that different antigens might be associated with distinct clinical signs. However, since most AIR patients exhibit multiple antiretinal antibodies, it is not yet known which antibodies are pathologic and clinically relevant, and which represent innocent bystanders.

Current dilemmas

Although humoral autoimmune reactions directed against retinal tissue are thought to play an important role in either initiation or modification of diverse chorioretinal disorders including uveitis, they were not as yet systematically measured and their possible clinical impact in retinal diseases was not examined. It is not known which specific retinal antigens provoke the formation of antibodies (and the repertoire of retinal autoimmune reactions). Furthermore, possible associations between ARAs and clinical characteristics, such as phase of the disease and being on immunosuppressive treatment, were not systematically examined. In addition, determination of ARAs was so far performed predominantly in serum of patients with chorioretinal diseases, which does not give precise information on what is exactly happening within the eye itself. The eye represents an immune-privileged organ, in which the immune reactions might be downplayed and/or different than observed in the peripheral blood. Information on the autoimmune reactions measured in intraocular fluid is scarce and the prevalence of ARAs in chorioretinal diseases has not been determined.

Understanding of autoimmune processes in ocular diseases might help to further elucidate their pathogeneses and may have consequences for the design of new diagnostic and treatment modalities. More detailed insight in the immuno-pathogenesis may be extremely valuable for patients, because the inhibition (or prevention) of inflammation (if present) in specific phases of the ocular disease might beneficially influence the course of disease and hopefully its visual outcome.

AIM AND SCOPE OF THIS THESIS

This thesis aims to assess the presence of humoral autoimmunity in uveitis and other chorioretinal diseases, including AIR, and to gain insight its role. To achieve this, we start by providing an overview of a large series of patients with uveitis and/or scleritis and examine the prevalence of systemic autoimmune and autoinflammatory diseases in this population. We review specific ocular diagnoses and clinical manifestations of patients affected by systemic autoimmune diseases. Further, we critically evaluate the term "autoimmune uveitis" (chapter 2). In chapter 3 we measure the prevalence of common systemic autoantibodies (antinuclear antibodies; ANA) in serum of uveitis patients. Subsequently, we determined the presence of retina specific antibodies (ARAs) in serum of patients with uveitis, AIR and central serous chorioretinopathy (CSC), and

discuss their possible pathogenic role (chapter 4-9). In the last chapters of this thesis we explore the determination of humoral autoimmune reactions in intraocular fluid samples of patients with uveitis (chapter 10). In addition, in chapter 11 we investigate intraocular fluid samples further and determine the presence of ARAs and inflammatory cytokines in intraocular fluid samples of patients with diverse ocular diseases, including RP, AMD, glaucoma and cataract.

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AUTOIMMUNITY IN UVEITIS

Josianne C.E.M. ten Berge, Marco W.J. Schreurs, Paul L.A. van Daele, Aniki Rothova

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ABSTRACT

Purpose: Recent insights into the pathogenesis of immune-mediated diseases proposed a new classification, which includes autoimmune and auto-inflammatory diseases. The prevalence of specific autoimmune and auto-inflammatory diseases in uveitis and/or scleritis is not yet known. In this study we examine the presence of systemic immune-mediated diseases in patients with uveitis and/or scleritis and put a special emphasis on autoimmune disorders by reporting on their clinical manifestations and visual prognosis.

Methods: In this retrospective study we reviewed data of 1327 patients presenting with uveitis and/or scleritis between January 2010 and July 2016 at the Erasmus Medical Center Rotterdam, the Netherlands. All patients with non-infectious uveitis and/or scleritis were classified according to novel criteria for immune-mediated diseases. Various clinical data, including visual acuity, of patients with uveitis of autoimmune origin were registered during five-year follow-up.

Results: The origin of uveitis was in 5% (62/1327) autoimmune, in 15% (197/1327) auto-inflammatory and in 14% (180/1327) mixed autoimmune/auto-inflammatory. Patients with classical autoimmune connective tissue disease (N=17) suffered mostly from rheumatoid arthritis and granulomatosis with polyangiitis and exhibited predominantly scleritis (53%). After five years of follow-up none of the eyes of these patients developed legal blindness (visual acuity of <0.1). The visual acuity in patients with uveitis associated with autoimmune neuro-ophthalmological diseases (multiple sclerosis and neuromyelitis optica; N=27) remained stable over time.

Conclusion: Uveitis and scleritis of autoimmune origin were observed in 5% of the total series. The term autoimmune uveitis should not be used as a synonym for intraocular inflammation of non-infectious origin.

INTRODUCTION

Uveitis is a potentially blinding ocular disease of multiple causes. It may be associated with various systemic infectious and non-infectious diseases. Various non-infectious uveitis cases are caused by an underlying systemic autoimmune or auto-inflammatory disease.[1-3]

The label "autoimmune uveitis" is commonly (and in our view unjustly) used for all types of uveitis associated with a systemic disease. Autoimmune diseases are characterized by an aberrant activity of the immune system directed against the body's own cells and tissues. Recent advances in the understanding of the pathogenesis of immune-mediated diseases proposed a new classification, which includes autoimmune and auto-inflammatory diseases.[4-6] The prevalence of these specific diseases in patients with uveitis and scleritis has not yet been assessed.

In this study, we examine the presence of autoimmune diseases, according to current classification of autoimmune- and auto-inflammatory diseases in a large series of patients with uveitis and/or scleritis. Further, we report on the prevalence, clinical features and visual prognosis of patients with autoimmune uveitis.

METHODS

At the department of Ophthalmology at the Erasmus Medical Center Rotterdam (a tertiary referral center), we conducted a retrospective study in patients with uveitis and/or scleritis to examine the prevalence of associated diseases. All patients presenting with uveitis and/or scleritis between January 2010 and July 2016 were identified. A total of 1327 patient files were reviewed. This study was performed in accordance with the Declaration of Helsinki and in agreement with our institutional regulations and after approval of our institutional review board.

Clinical data of patients were collected, and included patient demographics (age, gender and race), specific diagnoses and anatomical location of uveitis. Furthermore, all patients were divided according to specific cause or association with systemic diseases into following groups: infectious origin, associated with a non-infectious systemic disease, clinically established ocular syndrome, masquerade syndrome and idiopathic types.[7] To be classified as infectious uveitis, either microbiological proof for presence of specific pathogens in ocular fluids or evidence of active systemic infection was required. Patients with a positive IGRA test in the presence of otherwise unexplained uveitis were classified as of unknown origin and further specified as IGRA positive uveitis of unknown cause.

Patients with uveitis associated with a non-infectious systemic disease were further classified into four groups based on recent classification of their degree of autoimmunity: autoimmune, mixed autoimmune / auto-inflammatory, auto-inflammatory or not classified.[4-6,8-10] Additional clinical data of patients with uveitis and autoimmune disease were collected, and included the manifestation at the time of first presentation (ocular versus non-ocular). Data regarding visual acuity (of both eyes) and use of systemic immunosuppressive medication were collected at onset of uveitis, and during follow-up at 1, 3 and 5 years. In patients with sympathetic ophthalmia also both eyes were included for visual acuity outcomes. Furthermore, we registered ocular complications including the presence of cystoid macular edema (CME) and optic neuropathy.

All patients underwent a standardized diagnostic investigation protocol according to the localization of the inflammation.[7] This protocol included erythrocyte sedimentation rate (ESR), blood counts, serum angiotensin-converting enzyme levels, serology for syphilis and Lyme disease, interferon gamma release assay (IGRA) test (QuantiFERON–TB Gold In-Tube test) and radiologic chest imaging. In patients with scleritis, anterior uveitis or panuveitis presence of Human Leukocyte Antigen (HLA) B27 was determined. Depending on the clinical manifestations, additional examinations were performed (tailored approach). Patients with juvenile idiopathic arthritis were screened for the presence of antinuclear antibodies (ANA). In patients with scleritis anti-neutrophil cytoplasmic antibodies (ANCA), anti-citrullinated protein antibodies (ACPA) and rheumatoid factor were determined. All diagnoses were made according to current diagnostic and internationally accepted criteria.

RESULTS

The causes and associations in the entire uveitis series are depicted in Table 1. Specific causes of uveitis and/or associations with systemic diseases were found in the majority of patients (62%, 820/1327), of which 186/1327 (14%) were of infectious origin and 438/1327 (33%) were associated with non-infectious systemic diseases.

The classification of systemic non-infectious diseases according to current classification of autoimmune- and auto-inflammatory diseases is indicated in Table 2. An association with a systemic disease of established autoimmune origin was identified in 4% (59/1327), which was lower than the percentage of patients with auto-inflammatory (15%, 197/1327) and mixed autoimmune-/auto-inflammatory diseases (14%, 180/1327). The association with classic autoimmune connective tissue diseases was even lower, (1%, 17/1327). The most commonly associated autoimmune disease was MS (24 patients) followed by VKH (14 patients). In our series, sarcoidosis (38% presumed and 62% biopsy proven) represented the most common non-infectious systemic disease associated with uveitis (13%, 172/1327).

Three patients did not show association with systemic autoimmune disease, but had ocular inflammation of autoimmune origin, specifically sympathetic ophthalmia, resulting in a total of 62 patients (5%) affected by autoimmune uveitis and/or scleritis.

Table 1. Causes and association of uveitis and scleritis patients

	Number (%)	
Total	**1327**	**(100%)**
Infections	**186**	**(14%)**
Toxoplasma gondii	52	(4%)
Rubella virus	23	(2%)
Cytomegalovirus	23	(2%)
Varicella zoster virus	22	(2%)
Herpes simplex virus	19	(1%)
Remainder	47	(4%)
Non-infectious systemic diseases	**438**	**(33%)**
Sarcoidosis	172	(13%)
HLA-B27 associated (without (defined) systemic disease)	52	(4%)
Juvenile idiopathic arthritis	48	(4%)
Remainder	166	(13%)
Clinical ocular entity	**98**	**(7%)**
Birdshot chorioretinopathy	53	(4%)
Fuchs' uveitis syndrome*	12	(1%)
Presumed ocular histoplasmosis syndrome	7	(1%)
Remainder **	26	(2%)
Masquerade syndrome	**70**	**(5%)**
Benign	49	(4%)
Malignant	21	(2%)
Miscellaneous	**28**	**(2%)**
Toxic	20	(2%)
Post traumatic	8	(1%)
Unknown ǂ	**507**	**(38%)**
IGRA positive	46	(3%)

* Rubella virus negative or not investigated

** Includes two patients with immune recovery uveitis and three patients with sympathetic ophthalmia

ǂ In three patients the cause of uveitis might have been attributed to their diabetes mellitus

Most patients with uveitis and/or scleritis of autoimmune origin were initially referred to an ophthalmologist (57%; 33/58, 5/63 missing data). Other patients were referred the departments of neurology (19%, 11/58), internal medicine (22%, 13/58) or otolaryngology (2%, 1/58). The median time between the moment of first presentation of complaints and making the definite diagnosis was 365 (0-8234) days.

Table 2. Overview of non-infectious systemic diseases associated with uveitis and scleritis

	Number (%)*	
Total	**438**	**(33%)**
Autoimmune diseases, total	**59**	**(4%)**
Multiple sclerosis	24	(2%)
Vogt-Koyanagi-Harada disease	14	(1%)
Granulomatosis with polyangiitis	7	(<1%)
Rheumatoid arthritis	4	(<1%)
Systemic lupus erythematosus	4	(<1%)
Neuromyelitis optica	3	(<1%)
Sjögren syndrome	1	(<1%)
Autoimmune hepatitis	1	(<1%)
Systemic sclerosis	1	(<1%)
Mixed autoimmune / auto-inflammatory, total	**180**	**(14%)**
HLA-B27 associated (without (defined) systemic disease)	52	(4%)
Juvenile idiopathic arthritis	48	(4%)
Ankylosing spondylitis**	28	(2%)
Behçet syndrome	26	(2%)
Psoriatic arthritis**	9	(1%)
Reactive arthritis	8	(1%)
Tubulointerstitial nephritis and uveitis	5	(<1%)
Relapsing polychondritis	4	(<1%)
Auto-inflammatory diseases, total	**197**	**(15%)**
Sarcoidosis	172	(13%)
Inflammatory bowel diseases**	18	(1%)
Systemic vasculitis, ANCA negative‡	6	(<1%)
Muckle-Wells syndrome	1	(<1%)
Not classified, total	**2**	**(<1%)**
Graft versus host disease	1	(<1%)
Immune deficiency	1	(<1%)

* Percentage of total patients with uveitis (N=1327)

** In these groups, 26/28 patients with ankylosing spondylitis, 6/9 patients with psoriatic arthritis and 4/18 patients with inflammatory bowel diseases were HLA B27 positive.

‡ Includes three patients with arteritis temporalis and one patient with Kawasaki disease

Uveitis of autoimmune origin affected all segments of the eye (Table 3). The most common location for autoimmune uveitis was the vitreous and peripheral retina (intermediate uveitis; 31%, 19/62), followed by panuveitis (27%, 17/62). Intermediate uveitis was present only in (the majority of) patients with MS (70%, 19/24). Patients with connective tissue disease, specifically patients with rheumatoid arthritis (RA) and granulomatosis with polyangiitis (GPA), exhibited predominantly scleritis (53%, 9/17). Uveitis associated to other autoimmune diseases, including VKH disease and sympathetic ophthalmia (SO), was most frequently associated with panuveitis (86%, 12/14 and 100%, 3/3, respectively). Posterior uveitis was present in all patients with systemic lupus erythematosus (100%, 4/4) and neuromyelitis optica (100%, 3/3), and was seldom seen in other autoimmune uveitis entities.

Table 3. Clinical characteristics in uveitis and scleritis of autoimmune origin

	No. of patients	Anterior	Inter-mediate	Posterior	Panuveitis	Scleritis**	CME	Optic neuropathy
Total	**62***	**13% (8)**	**31% (19)**	**16% (10)**	**27% (17)**	**15% (9)**	**24% (15)**	**44% (27)**
Neuro-ophthalmological diseases	**27**	**11% (3)**	**70% (19)**	**15% (4)**	**4% (1)**	**0% (0)**	**33% (9)**	**41% (11)**
Multiple sclerosis	24	13% (3)	79% (19)	4% (1)	4% (1)	0% (0)	38% (9)	33% (8)
Neuromyelitis optica	3	0% (0)	0% (0)	100% (3)	0% (0)	0% (0)	0% (0)	100% (3)
Connective tissue diseases	**17**	**24% (4)**	**0% (0)**	**24% (4)**	**0% (0)**	**53% (9)**	**0% (0)**	**12% (2)**
Granulomatosis with polyangiitis	7	14% (1)	0% (0)	0% (0)	0% (0)	86% (6)	0% (0)	14% (1)
Rheumatoid arthritis	4	25% (1)	0% (0)	0% (0)	0% (0)	75% (3)	0% (0)	0% (0)
Systemic lupus erythematosus	4	0% (0)	0% (0)	100% (4)	0% (0)	0% (0)	0% (0)	25% (1)
Systemic sclerosis	1	100% (1)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Sjögren syndrome	1	100% (1)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Other	**18**	**6% (1)**	**0% (0)**	**11% (2)**	**89% (16)**	**0% (0)**	**33% (6)**	**78% (14)**
Vogt Koyanagi Harada disease	14	0% (0)	0% (0)	14% (2)	86% (12)	0% (0)	29% (4)	93% (13)
Autoimmune hepatitis	1	100% (1)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Sympathetic ophthalmia	3	0% (0)	0% (0)	0% (0)	100% (3)	0% (0)	33% (1)	0% (0)

* Includes 59 patients with uveitis associated with systemic autoimmune disorders and three additional patients with sympathetic ophthalmia

** Four patients with scleritis had a combination of scleritis and uveitis anterior

Abbreviation: CME = cystoid macular edema

The prevalence of complications in patients with uveitis of autoimmune origin is presented in Table 3. CME was present in 24% (15/62) and was most frequently observed in patients with MS (38%, 9/24), VKH disease (29%, 4/14) and SO (33%, 1/3). None of the patients with uveitis/scleritis associated with connective tissue disease exhibited CME. The optic disk was involved in 44% (27/62) of patients with uveitis of autoimmune origin. Almost all patients with VKH had involvement of the optic disk (93%, 13/14). In patients with neuro-ophthalmological diseases, the optic disk was involved in 41% (11/27), predominantly in patients with neuromyelitis optica (100%, 3/3). Involvement of the optic disk in other entities of autoimmune uveitis was only occasionally observed.

Overall, visual prognosis was favorable as none of the patients developed bilateral visual acuity of less than 0.1 after five years of follow-up. At onset, visual acuity less than 0.1 in at least one eye was present in 8/46 (17%) patients. The overall prevalence of a visual acuity of <0.1 in at least one eye remained stable over the first five years of follow-up (Table 4). Visual acuity <0.1 in at least one eye in patients with uveitis associated with neurological diseases varied between 6% and 13% and did not change over time. Patients with VKH disease improved; 25% of these patients started with a visual acuity of <0.1 in at least one eye, but after five years this percentage was reduced to none.

The use of systemic immunosuppressive medications and/or systemic corticosteroids for systemic and/or ocular inflammation during the first five years of follow-up in patients with uveitis of autoimmune origin reached 76% (Table 4). The majority of patients with uveitis associated with connective tissue disease were successfully treated with systemic immunosuppressive medication and/or systemic corticosteroids during the follow-up period of five years (75% - 83%). In the group of patients with neuro-ophthalmological diseases immunosuppressive systemic medication was least frequently used (37% during year one and 56% during year 3-5).

Table 4. Visual acuity during a five-year follow-up of patients with uveitis or scleritis of autoimmune origin

	No. of patients	Visual acuity < 0.1 in at least one eye**			
		At onset	At 1 year	At 3 years	At 5 years
Total	**62***	**17% (8/46)**	**15% (5/39)**	**16% (5/32)**	**13% (3/23)**
Neuro-ophthalmological diseases	**27**	**10% (2/20)**	**6% (1/17)**	**13% (2/16)**	**13% (2/16)**
Multiple sclerosis	24	6% (1/17)	0% (0/14)	7% (1/14)	7% (1/15)
Neuromyelitis optica	3	33% (1/3)	33% (1/3)	100% (1/1)	100% (1/1)
Connective tissue diseases	**17**	**7% (1/15)**	**0% (0/10)**	**0% (0/5)**	**0% (0/2)**
Granulomatosis with polyangiitis	7	0% (0/6)	0%(0/4)	0% (0/1)	NA
Rheumatoid arthritis	4	0% (0/3)	0% (0/3)	0% (0/1)	0% (0/1)
Systemic lupus erythematosus	4	25% (1/4)	0% (0/3)	0% (0/2)	NA
Systemic sclerosis	1	0% (0/1)	NA	0% (0/1)	0% (0/1)
Sjögren syndrome	1	0% (0/1)	NA	NA	NA
Other	**18**	**45% (5/11)**	**33% (4/12)**	**27% (3/11)**	**20% (1/5)**
Vogt Koyanagi Harada disease	14	25% (2/8)	11% (1/9)	0% (0/7)	0% (0/3)
Autoimmune hepatitis	1	NA	NA	0% (0/1)	0% (0/1)
Sympathetic ophthalmia	3	100% (3/3)	100% (3/3)	100% (3/3)	100% (1/1)

* Includes 59 patients with uveitis associated with systemic autoimmune disorders and three additional patients with sympathetic ophthalmia in whom both eyes were included for visual acuity outcomes.
** Not all data available

DISCUSSION

Our results show that uveitis and/or scleritis of autoimmune origin was identified in 5% (62/1327) of all patients. The most common autoimmune disease in patients with uveitis was MS (39%, 24/62), followed by VKH disease (23%, 14/62) and GPA (11%, 7/62). Scleritis was only observed in patients with uveitis associated with connective tissue diseases (RA and GPA), and intermediate uveitis was present only in patients with MS. Optic neuropathy was the most frequent complication (44%, 27/62). Use of systemic immunosuppressive treatment was frequent (up to 76%), and visual outcomes were favorable as none of the patients developed permanent bilateral visual acuity of less than 0.1 and only 13% of patients with uveitis of autoimmune origin developed unilateral visual acuity < 0.1 after five years of follow-up.

Autoimmune diseases are classically defined by the Witebsky's postulates: 1. presence of an autoantibody or cell-mediated autoimmune reaction, 2. identification of a corresponding autoantigen, and 3. an analogous autoimmune response inducible in an experimental animal model with development of a similar disease.[11] These postulates have been revisited in 1993 by Rose and Bona, which resulted in three types of evidence to establish an autoimmune origin: direct proof, indirect proof and circumstantial evidence.[12] Reproducing the disease by transfer of autoantibody or auto-reactive T-cells, or documenting the involvement of immunological reactions after immunization with the autoantigen provides (in)direct proof of autoimmunity. Associations with other autoimmune diseases, favorable response to immunosuppression or other distinctive clinical clues such as presence of autoantibodies represent circumstantial evidence.

Autoimmune diseases predominantly involve the adaptive immune system, and are characterized by the production of autoantibodies and / or auto-reactive T-cells that recognize specific cells or tissues.[4] Various autoantibodies are specific for individual autoimmune diseases, although their exact role in the pathogenesis of the disease is often unknown. Since diverse autoantibodies (e.g. antinuclear antibodies) appear also in healthy subjects, the mere presence of autoantibodies does not always indicate the presence of an autoimmune disease.[13] Self-directed inflammation by auto-inflammatory diseases is caused by an over-activity of the adaptive and/or innate immune system, without specific identification of auto-reactive B- and T-cell responses (e.g. Crohn disease).[4] Recent classification of inflammatory diseases into autoimmune, mixed and auto-inflammatory diseases takes these differences into account.[4-6,8-10]

The eye is an immune privileged organ, which indicates that immune responses to foreign- and self-antigens are suppressed or inhibited.[14-16] This phenomenon prevents ocular damage and preserves vision. Features that contribute to the mechanism of ocular immune privilege include the blood-retina barrier, decreased lymphatic drainage, and soluble factors with immunosuppressive properties in aqueous humor known as the anterior chamber associated immune deviation.

Autoimmune reactions against retinal antigens have repeatedly been suggested to play a crucial role in diverse clinical uveitis entities. Direct evidence for an autoimmune pathogenesis has been described in cancer-associated retinopathies by reproducing the disease after transfer of auto-antibodies.[17,18] In uveitis however definite proof of autoimmune reactions and inciting antigens is very limited. Secondary contribution of autoimmune reactions has been proposed to play a role diverse uveitis entities, including intraocular infections.[19] Indirect evidence for autoimmunity in uveitis has been provided by induction of autoimmune uveitis after immunization of animals with retinal antigens and Freund adjuvant.[20,21] These animal models, so called experimental auto-immune uveitis (EAU), have provided insight into the immuno-pathogenesis of human uveitis. In EAU predominantly mice are injected with different antigens (such as S-arrestin and interphoto-receptor retinoid-binding protein) causing inflammation of intraocular tissue similar to human uveitis. Other animal models induced autoimmune uveitis by transfer of retina specific T-cells. For autoimmunity in human uveitis only circumstantial evidence was reported, for example by an increasing number of T-helper 17 cells during active uveitis and scleritis, and a decreasing number during treatment.[22] So far, human autoimmune uveitis was only proven in uveitis when it is part of a systemic autoimmune disease and is highly suspected in sympathetic ophthalmia. It is not unlikely that other ocular entities (e.g. birdshot chorioretinopathy) might also be of autoimmune origin, but direct evidence for an autoimmune pathogenesis is lacking.

Despite the high number of patients included in our series from a tertiary center, our study has certain limitations. First, a bias to a more severe uveitis population is evident and is valid for most studies from tertiary centers. Furthermore, it should be noted that our hospital represents one of the national referral centers for sarcoidosis, which is probably in part responsible for a somewhat higher prevalence of ocular sarcoidosis in our series (13%, 172/1327).[23] In addition, at 5-year follow-up a significant number of patients with autoimmune uveitis was lost to follow-up (65%, 40/62). The most probable explanation is that in the majority of these cases uveitis stabilized or diminished and the patients were referred back to the ophthalmologists in peripheral centers. Last, it cannot be ruled out that uveitis appearing with a systemic disease represent an epiphenomena and is not associated with the systemic disease, although this is highly unlikely.

In conclusion, autoimmune uveitis is a rare diagnosis, which comprises 5% of our large uveitis/scleritis population. It is feasible that secondary autoimmune reactions might play a role in some uveitis entities (e.g. infections), as a consequence of damage and subsequent exposure of (so far hidden or altered) retinal/choroidal antigens. Clinicians caring for uveitis patients should be aware of the variety of diagnoses and the high prevalence of uveitis associated to sarcoidosis. In our view, the term autoimmune uveitis should be reserved for intraocular inflammations of confirmed autoimmune origin and should not be used as a synonym for non-infectious uveitis.

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3

ANTINUCLEAR ANTIBODY PROFILING IN UVEITIS

Josianne C.E.M. ten Berge, F. Groen-Hakan, Aniki Rothova, Marco W.J. Schreurs

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ABSTRACT

Purpose: Antinuclear antibody (ANA) profiling plays an important role in diagnosis of various autoimmune and autoinflammatory diseases. ANA is associated with the development of uveitis in children and its poor prognosis. In contrast, the diagnostic value of ANA in work-up of adults with uveitis is debatable. The aim of this study is to assess the diagnostic value of ANA profiling in adult patients with uveitis.

Methods: In this prospective study, we assessed the presence of ANA in serum of 105 consecutive adult patients with uveitis. In samples positive for ANA, ANA titer, ANA subtypes and staining patterns on IIF were also determined. Clinical data from uveitis patients were collected and statistical analyses were performed to relate laboratory results to clinical data of the patients.

Results: A positive ANA result was observed in 29/105 (28%) patients with uveitis, and the median ANA titer was 160. Positive ANA titers were associated with longer duration of uveitis (p=0.037). No other associations were found between the presence of ANA, ANA titer or ANA staining pattern and specific diagnosis and various clinical characteristics of uveitis (all p-values > 0.05).

Conclusion: A positive ANA was found in 28% of patients with uveitis. The ANA profile was not distinctive for specific causes or clinical manifestations of uveitis. The diagnostic value of ANA assessment in the adult uveitis population is limited.

INTRODUCTION

Uveitis is a clinical syndrome, which can be associated with different causes, including infections and systemic diseases. The pathogenesis of most uveitis entities in not clarified, although the immune system has been considered to play a major role. Various uveitis entities are associated with autoimmune and autoinflammatory diseases.[1]

Antinuclear antibodies (ANA) are antibodies directed against a variety of nuclear antigens, and can be detected in patients with autoimmune diseases. The presence of ANA is not specific for disease, since it has also been observed in the healthy population (predominantly women and elderly).[2] In the past, ANA were determined in all patients with uveitis for diagnostic screening purposes, but this approach has been abandoned since its diagnostic value in adult patients with uveitis shown to be limited.[3] In contrast, in patients with juvenile idiopathic arthritis (JIA) the presence of ANA has been demonstrated to be valuable, because its presence increases the risk of developing uveitis.[4,5]

In the last decades, the analysis of ANA has been improved and various subtypes and staining patterns are being determined. Profiling of ANA has been proven to play a significant role for diagnostic purposes in various diseases, including systemic lupus erythematosus, Sjögren syndrome and systemic sclerosis.[6,7] The diagnostic relevance of the ANA profile and its possible association with clinical features in uveitis are not known. The aim of this study is to assess the presence, subtypes and titers of ANA in adult patients with uveitis of different etiologies and evaluate its possible value for diagnostic screening in uveitis.

METHODS

We conducted a prospective study at the department of Ophthalmology, Erasmus MC, University Medical Center Rotterdam and determined ANA profile in 105 consecutive adult patients with uveitis who underwent a standardized screening protocol for the cause of their uveitis between January 2016 and July 2017. The study was performed in accordance with the Declaration of Helsinki and in agreement with the institutional regulations and approval of our institutional review board.

In addition to ANA screening, all patients underwent a diagnostic screening protocol, which was related to the location of uveitis (according to the Standardization of Uveitis Nomenclature (SUN) Working Group) and included chest radiography, erythrocyte sedimentation rate, blood counts, serum angiotensin converting enzyme levels, serology for syphilis and Lyme disease and interferon gamma release assay test (QuantiFERON–TB Gold In-Tube test). Patients with anterior

uveitis or panuveitis were also tested for presence of human leucocyte antigen-B27. A tailored approach was applied for further examinations.

Data from included patients were collected from medical charts and registered were patients' demographics (age, gender and race), definitive diagnosis of uveitis as well as ocular characteristics (laterality, duration and activity of uveitis), use of systemic immunomodulating medications and ANA characteristics (presence, titer, staining pattern and ANA subtype).

Screening for ANA in serum samples from included patients was performed by indirect immunofluorescence (IIF) according to standard protocol. In short; HEp-2 cells (Inova, San Diego, CA) were incubated with 1:80 diluted serum samples for 30 minutes, and after being washed, the slides were incubated for 30 minutes with goat anti-human IgG conjugated with fluorescein isothiocyanate with propidium iodide for counterstaining (Inova, San Diego, CA) to label antibodies. ANA titers of 1:80 or higher were considered positive and in these samples the ANA pattern and exact ANA titer were also analyzed. ANA patterns were classified according to international consensus and include only nuclear and mitotic patterns, whereas cytoplasmic HEp-2 staining was considered negative.[8] In ANA positive samples, further identification for detection of anti-extractable nuclear antigens (anti-ENA antibodies) and anti-double stranded DNA (anti-dsDNA) was performed by EliA (Thermo Fisher Scientific/Phadia, Freiburg, Germany), ELISA (Inova, San Diego, CA) and/or LIA (Euroimmun, Lübeck, Germany). The ENA-panel consisted of anti-SS-A, anti-SS-B, anti-RNP, anti-Smith (anti-Sm), anti-CenpB, anti-Scl-70, and anti-Jo-1.

Statistical analyses were performed to evaluate the presence and characteristics of ANA in uveitis patients. Continuous variables were described by median and range, and categorical variables were summarized by percentages (proportions). We used Fisher's exact test for categorical data and Mann Whitney U test, Kruskall-Wallis 1-way ANOVA test and Spearman's Rank Correlation for continues variables. All statistical analyses were performed using SPSS software (version 22.0, Chicago, IL). A p-value of <0.05 was considered statistically significant and all tests were two-sided.

RESULTS

The clinical characteristics of included uveitis patients are shown in Table 1. The majority of patients were female (69/105, 66%) and the median age was 51 years. Most patients had an active uveitis (77/105 73%) and did not use systemic immunosuppressive medication (96/105, 91%) during blood sampling.

Positive ANA results were observed in 29/105 (28%) of patients with uveitis. The presence of ANA was equally distributed between genders and no association was observed between age and

presence of ANA. Most ANA positive samples were observed in idiopathic uveitis (18/55, 33%) and no positive ANA were seen in patients with uveitis classified as a clinical ocular syndromes (e.g. birdshot chorioretinopathy; Table 1). Prevalence of ANA was higher in patients with anterior uveitis (12/28, 43%) compared to other locations, but this difference did not reach significance. A visual acuity of <0.5 was observed in 7/26 (27%) ANA positive patients and in 22/82 (27%) of patients without ANA. Positive ANA titers were associated with longer duration of uveitis (p=0.037). All other clinical characteristics of uveitis were not significantly associated to the presence of ANA (all p-values > 0.05). Activity of uveitis could not be related to ANA presence (p=0.90).

Table 1. Presence of antinuclear antibodies (ANA) in uveitis patients

	All uveitis	ANA positive	ANA negative
Total	105 (100%)	29/105 (28%)	76/105 (72%)
Median age in years (range)	51 (19-88)	49 (21-87)	52 (19-88)
Gender			
Male	36/105 (34%)	11/36 (31%)	25/36 (69%)
Female	69/105 (66%)	18/69 (26%)	51/69(74%)
Race			
Caucasian	73/105 (70%)	18/73 (25%)	55/73 (75%)
Non-Caucasian	32/105 (30%)	11/32 (34%)	21/32 (66%)
Cause of uveitis			
Clinical ocular syndrome	6/105 (6%)	0/6 (0%)	6/6 (100%)
Immune mediated systemic disease	15/105 (14%)	3/15 (20%)	12/15 (80%)
Infection	16/105 (15%)	5/16 (31%)	11/16 (69%)
Masquerade	13/105 (12%)	3/13 (23%)	10/13 (77%)
Idiopathic	55/105 (52%)	18/55 (33%)	37/55 (67%)
Laterality of uveitis			
Unilateral	49/105 (47%)	13/49 (27%)	36/49 (73%)
Bilateral	56/105 (53%)	16/56 (29%)	40/56 (71%)
Median duration of uveitis in years* (range)	1 (0-50)	1 (0-50)	0 (0-19)
Location of uveitis			
Anterior	28/105 (27%)	12/28 (43%)	16/28 (57%)
Intermediate	9/105 (9%)	2/9 (22%)	7/9 (78%)
Posterior	36/105 (34%)	9/36 (25%)	27/36 (75%)
Panuveitis	20/105 (19%)	4/20 (20%)	16/20 (80%)
Sclero-/kerato-uveitis	12/105 (11%)	2/12 (17%)	10/12 (83%)
Activity of uveitis			
Active	77/105 (73%)	21/77 (27%)	56/77 (73%)
Quiet	28/105 (27%)	8/28 (29%)	20/28 (71%)
Immunocompromised **			
Yes	14/105 (13%)	5/14 (36%)	9/14 (64%)
No	91/105 (87%)	24/91 (26%)	67/91 (74%)

*p = 0.037

** Use of immunosuppressive medications, malignant disorder or HIV with CD4 cell count of <300 during blood collection

The median ANA titer (within ANA positive uveitis patients) was 160 and ranged from 80 to 640 (Table 2). Clinical features of uveitis were not associated to ANA titer (all p-values > 0.05). The ANA pattern was classified as nuclear in 23/25 (92%) of patients and as mitotic in the remaining 2/25 (8%) patients. A speckled ANA pattern was the most frequent observed pattern (13/29, 45%), followed by a homogeneous ANA (9/29, 31%). The speckled pattern was most frequently observed in uveitis with an unknown cause (9/13, 69%). The distribution of ANA patterns was however not characteristic for specific causes, locations or clinical manifestations of uveitis. Two patients were anti-dsDNA positive and one patient was positive for anti- ribonucleoprotein (anti-RNP); none exhibited any signs of autoimmune systemic disorder on examination by immunologist and all three were (so far) classified as uveitis of unknown origin.

Table 2. Antinuclear antibody (ANA) characteristics of uveitis population (N=29)

	Number (%)
Total ANA positive uveitis	29/105 (28%)
Median ANA titer	160 (80-640)
ANA patterns	
Speckled	13/29 (45%)
Homogeneous	9/29 (31%)
Nucleolar	3/29 (10%)
Speckled + nucleolar	2/29 (7%)
Centriole	1/29 (3%)
Mitotic spindle apparatus	1/29 (3%)
Anti-double stranded DNA	2/29 (7%)
Anti-extractable nuclear antigens (ENA) (anti- ribonucleoprotein (RNP))	1/29 (3%)

DISCUSSION

Our prospective study shows that ANA is positive in 28% of patients with uveitis. No significant associations were found between the presence of ANA, ANA titer, or specific ANA patterns and various clinical characteristics of uveitis, including specific diagnoses or activity of intraocular inflammation.

The prevalence of 28% is higher than in the age-matched healthy population.[9-11] The prevalence of ANA in healthy population varies between 8-17% and tends to be higher in female individuals and elderly.[9-11] In our series, the relationship between age, gender and positive ANA could not be confirmed, probably due to the limited number of patients in various age groups. Although the ANA prevalence in our study was higher compared to the healthy age-matched population, no clinical relevance for the work-up of uveitis in adult patients could be identified. Therefore, routine

ANA determination as a part of the diagnostic testing of uveitis patients cannot be recommended. Determining ANA should however be performed in cases with signs suggesting specific systemic diseases such as systemic lupus erythematosus.

Three decades ago, approximately 14% prevalence of ANA in uveitis population was observed in a setting similar to our series.[3,12] This percentage is lower compared to the 28% ANA positivity found in our cohort and might reflect the changing spectrum of uveitis entities over time. Further it is possible that referral pattern might also play a role. In line with previous studies we did not find associations between specific uveitis entities, their characteristics and ANA, with exception of a borderline association between ANA positivity and longer duration of uveitis. Although our study is prospective, it includes a limited number of patients and therefore we cannot exclude that a specific uveitis entity could be associated with ANA.

The prevalence of positive ANA in autoimmune- and autoinflammatory diseases varies widely. Almost all patients with systemic lupus erythematosus (SLE) are ANA positive and in systemic sclerosis and rheumatoid arthritis ANA prevalence varies between 30%-70%.[13] Therefore, one could expect a higher ANA prevalence in uveitis associated with systemic non-infectious disorders. However, in our series only 3/15 (20%) of the patients with systemic immune mediated disorders were ANA positive. Interestingly, ANA was more prevalent in patients with infectious uveitis (5/16, 31%) than in other uveitis entities. A transiently positive ANA test was previously noted in systemic infectious diseases.[14] The relationship between ANA and ocular infections has not been specified in earlier studies.

Reactivity to specific ENA discriminates between various types of systemic autoimmune diseases and plays herein also a prognostic role. For example, in SLE, antibodies directed against the Sm antigen are specific for the disease and presence of anti-Topo-I antibodies is associated with more severe course of systemic sclerosis.[15] We identified 3/29 (10%) with positive anti-ENA in our ANA positive patients but found no associations with uveitis characteristics, including its severity. This low proportion of anti-ENA positivity is not surprising, since still many anti-ENA specificities are not known. Usually anti-ENA antibodies occur more frequently in patients with high ANA titers.[16] The low to moderate ANA titers in our study appears in agreement with the small proportion of anti-ENA presence. The distribution of ANA patterns in our cohort seems similar compared to previous studies on ANA positive samples.[17]

Our findings in adult uveitis population differ from uveitis in children. Specifically, in JIA-associated uveitis the proportion of ANA positivity has been described in up to 86%.[3,12] Presence of ANA in JIA has been documented to impose a significant risk for the development of uveitis.[18] The most common pattern of ANA in JIA-uveitis is (partly) homogeneous (86%) and no specific anti-ENA have been identified.[18]

In conclusion, positive serum ANA was observed in 28% of adult patients with uveitis. Specific associations between ANA positivity, ANA titer and ANA subtype, and ocular characteristics of uveitis were not identified. Based on our results, we do not recommend including ANA for the screening purposes of patients with uveitis.

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4

PREVALENCE AND CLINICAL IMPACT OF ANTIRETINAL ANTIBODIES IN UVEITIS

Josianne C.E.M. ten Berge, Marco W.J. Schreurs, Jacolien Vermeer,
Magda A. Meester-Smoor, Aniki Rothova

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ABSTRACT

Purpose: To determine the prevalence of serum antiretinal antibodies (ARAs) among patients with uveitis and establish their clinical relevance.

Methods: This prospective study assessed the presence of ARAs by indirect immunofluorescence (IIF) using primate retina in 126 patients with uveitis and 60 healthy controls. Clinical data of uveitis patients were collected from medical charts and included the classification of uveitis, cause of uveitis or its association with systemic disease, stage and activity of uveitis and specific retinal features. Correlations between the presence of specific ARAs and various clinical characteristics were analyzed.

Results: The presence of ARAs was observed in 49/104 (47%) of patients with uveitis and in 10/59 (17%) of healthy controls ($p<0.001$). Staining of the nuclear layers and the photoreceptors were both more often observed in patients with uveitis compared to healthy controls ($p=0.002$ and $p=0.047$, respectively). No specific associations were found between the presence of serum ARAs and various clinical characteristics.

Conclusion: Serum ARAs were more frequent in patients with uveitis compared to healthy controls, but their clinical role remains elusive. The assessment of intraocular production of specific ARAs may provide further insight into the role of ocular autoantibodies in diverse uveitis entities.

INTRODUCTION

Ocular autoimmunity characterized by the presence of multiple antiretinal antibodies (ARAs) has been documented in auto-immune retinopathy (AIR), including carcinoma associated retinopathy, melanoma associated retinopathy or non-paraneoplastic autoimmune retinopathy.[1-3] In addition, retinal autoimmune reactions are considered to play an important role in the pathogenesis of diverse retinal and uveo-retinal disorders. Multiple serum ARAs have been observed in various cohorts of patients with diverse uveitis entities but were also observed in up to 62% of the healthy population (Table 1).[4-13] In addition, the decrease of serum ARAs in patients with exudative age-related macular degeneration following treatment with bevacuzimab injections has been described.[14] Previous reports hypothesized that retinal damage caused by inflammation might induce a secondary formation of autoantibodies and cellular auto-immune responses which might subsequently contribute to continuation, recurrence rate and/or aggravation of the original inciting process. The precise sequence of events that might result in autoimmune attack of retinal cells is not yet elucidated.

Uveitis is an inflammatory process of the uvea and is a major cause of blindness, resulting in 10% of all cases of blindness. Uveitis can be caused by infection, systemic inflammatory disease, trauma or malignancy, however the etiology of uveitis remains unknown for up to 50% of the cases. Usually uveitis is classified according to its localization in the eye; anterior, intermediate, posterior or panuveitis.[15] The eye is an immune privileged organ, because of its blood-retina barrier and the absence of lymphatic drainage. Furthermore, the introduction of foreign antigens into the anterior chamber of the eye can induce a tolerance to the foreign antigen, called the anterior chamber associated immune deviation (ACAID). Absence of these features might enhance intraocular inflammation and subsequent loss of vision.

The clinical relevance of serum ARAs in uveitis is still unknown. Previous studies on serum ARAs in uveitis lacked clinical data such as ocular features, activity of uveitis and use of medications (with the exception of a Polish publication).[7] Furthermore, only small cohorts of specific uveitis entities were analyzed using S-antigen, interphotoreceptor retinoid-binding protein (IRBP) or crude human or bovine retinal extract. The identification of autoimmune processes in uveitis will help to elucidate the pathogenesis, and will also aid in the development of new diagnostic and treatment modalities.

In this study, we investigate the presence of serum ARAs in 126 patients with uveitis and 60 healthy controls and correlate their clinical manifestations to laboratory findings.

Table 1. Survey of literature on retinal autoimmune reactions in uveitis

Article (author - year)	Method	Cases: Positive for unspecified ARA / all (%)	Controls: Positive for unspecified ARA / all (%)	Specific antigens: % of positivity
Walscheid - 2014	IIH	Juvenile idiopathic arthritis (JIA) associated uveitis: 42/89 (47%)	- JIA without uveitis: 48/72 (67%) - Idiopathic anterior uveitis: 7/58 (12%) - Healthy: 12/23 (52%)	n.a.
Cursino - 2010	ELISA	- Ocular toxoplasmosis: 30/30 (100%) - Noninfectious uveitis: 50/50 (60%)	Healthy (with/without anti-toxoplasma antibodies): 106/500 (21%)	S-antigen: 100% of cases; 43% of controls positive IRBP: 100% of cases; 87% of controls
Kubicka – 2004*	IIF	Idiopathic posterior uveitis: 49/50 (98%)	Healthy: (not specified)/50	Not specified
Kubicka – 2002*	IIF	Active idiopathic posterior uveitis: 40/50 (80%)	Cases 1 year after immunosuppressive therapy: 10/50 (20%)	n.a.
Whittle – 1998	IIF, ELISA	Toxoplasma retinochoroiditis: 34/36 (94%)	- Healthy (with/without anti-toxoplasma antibodies): 6/16 (38%) - Idiopathic retinal vasculitis: 3/12 (25%)	S-antigen: 75% of cases; 63% of healthy controls; 75% of vasculitis controls
Lelij - 1990	ELISA	n.a.	n.a.	S-antigen and IRBP: 100% of cases (onchocerciasis); 100% of healthy controls
Forrester - 1989	ELISA	n.a.	n.a.	S-antigen: 100% of cases (uveitis); 100% of healthy controls
Chan - 1985	ELISA, ABC technique	Vogt-Koyanagi-Harada, Behcet's disease, sympathic ophthalmia: positive reaction in all groups	n.a.	S-antigen: 0% of cases
Stanford - 1988	IIF	Retinal vasculitis (22 Behcet's disease, 3 sarcoidosis, 1 JIA, 26 no systemic disease): 35/52 (67%)	n.a.	n.a.

* Possibly based on the same cohort of patients

Abbreviations: ARA = antiretinal antibodies, ELISA = enzyme-linked immuno sorbent assay, IIF = indirect immunofluorescence, IRBP = interphotoreceptor retinoid-binding protein, ABC = Avidin-biotin-peroxidase complex technique, IIH = indirect immunohistochemistry, JIA = juvenile idiopathic arthritis, n.a. = not applicable

METHODS

Patients and sample collection

Blood samples from 126 patients with uveitis were prospectively collected from April 2013 until November 2014 at the department of Ophthalmology, Erasmus MC, University Medical Center Rotterdam and stored at minus 80°Celsius at the biobank of our department. All patient signed informed consent. Our laboratory assessments were performed in May and June 2015. The study was performed in accordance with the Declaration of Helsinki and in agreement with the institutional regulations and approval of our institutional review board. Patients were classified according to SUN classification of their anatomical locations.[16] All patients with uveitis underwent a standardized diagnostic protocol according to the localization of the inflammation. This protocol included radiologic chest examination erythrocyte sedimentation rate, blood counts, serum angiotensin-converting enzyme levels, serology for syphilis and Lyme disease as well as interferon gamma release assay (IGRA) test (QuantiFERON–TB Gold In-Tube test). In those with anterior and panuveitis Human Leukocyte Antigen (HLA)-B27 testing was also performed. According to the clinical manifestations, additional examinations were performed (tailored approach). Serum samples of 60 presumably healthy individuals (blood bank donors) were used as controls (gender and age unknown).

Data collection

Clinical data of uveitis patients were collected from medical data files and included patient demographic and ocular characteristics such as age, gender, onset and duration of uveitis, cause of uveitis or association with systemic disorder, anatomical location and activity of uveitis, visual acuity, use of immunosuppressive medications, laterality, presence of retinal lesions, vasculitis, cystoid macular edema (CME) and presence of glaucoma.

Detection of ARAs using indirect immunofluorescence (IIF)

Initial screening of sera was performed using primate retinal tissue. Cryosections manufactured by Euroimmun (Lubeck, Germany) were left unfixed. The quality of the cryosections of primate retinal tissue was checked with immunohistochemistry and staining with DAPI. Serum was diluted 1:100 with phosphate phosphate buffered saline (PBS), pH 7.8. Retinal tissue was then incubated with the diluted serum from a patient for 30 minutes at room temperature. Sections were washed in stagnant PBS (pH 7.8) for 15 minutes. After the washing, sections were incubated with goat-anti-human IgG conjugated with fluorescein isothiocyanate that was provided in the previous mentioned Euroimmun kit for 30 minutes at room temperature to label the bound antibodies. The sections were washed again in stagnant PBS (pH 7.8) for 15 minutes. Embedding medium was placed onto the cryosections and were covered with a cover glass. The positive control consisted of retinal tissue incubated with 1:100 diluted serum of an antinuclear antibody (ANA) positive patient, which also allowed visual identification of the different retinal layers. Retinal tissue incubated with PBS or with 1:100 diluted serum of a healthy control were used as negative controls.

Detection of ANA using IIF

All sera showing any kind of antiretinal immunoreactivity by IIF were analyzed for the presence of antinuclear antibodies. ANA detection was performed by IIF using Hep-2 cells (Inova, San Diego, California). Serum samples were diluted 1:80 with PBS (pH 7.8). Hep-2 cells were incubated with the diluted serum for 30 minutes at room temperature. After washing in PBS with continuous stirring, slides were incubated for another 30 minutes with goat anti-human IgG conjugated with fluorescein isothiocyanate with propidium iodide for counterstaining (Inova, San Diego, California) to label specifically bound antibodies. Subsequently, slides were washed and embedded.

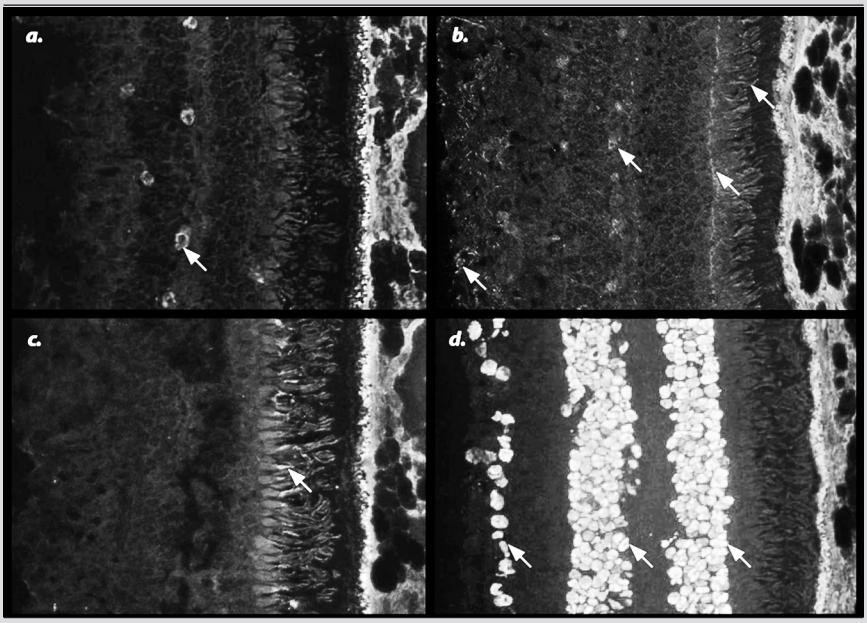
Evaluation of laboratory results

All slides were evaluated on a fluorescence microscope (20x magnification) by two independent observers. Specific retinal layers (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, and rods and cones layer) were separately evaluated for presence of fluorescent staining and the location of staining was noted. In addition, specific repetitive patterns of staining were noted (Figure 1); band A (fluorescence between the inner nuclear layer and the outer plexiform layer) and band B (fluorescence between the outer nuclear layer and the photoreceptors). In case both ANA and ARA were positive, a sample was scored as 'unknown' because this combination does not allow a proper discrimination between the presence or absence of ARAs (by possible masking of ANAs). These samples (n= 23) were excluded from the final analyses.

Statistical analysis

Chi-square tests and Mann Whitney U-tests were employed to evaluate differences in autoantibody presence between groups and to determine possible associations between clinical characteristics and presence of ARAs. $P<0.05$ was considered as statistically significant.

Figure 1. Examples of different staining patterns on IIF of patients with uveitis



Indirect immunofluorescence test on primate retinal tissue incubated with serum of patients with uveitis. Fluorescein isothiocyanate (FITC) labeled antiretinal antibodies (arrows) react upon incubation with serum of the patients with staining of: a. specific cells in the outer nuclear layer; b: photoreceptors, band B, bipolar cells and parts of the ganglion cell layer; c. outer segments of the photoreceptors; and d. nuclei in the inner nuclear layer, outer nuclear layer and ganglion cell layer (this serum was positive for antinuclear antibodies).

RESULTS

Patient characteristics

The general characteristics of the patients are indicated in Table 2. The median age of patients was 54 years and the majority was female (84/126, 67%). The median duration of uveitis at sample collection was 4 years with a wide range of 0 - 57 years. Most patients were diagnosed with posterior uveitis (45/126, 36%) or panuveitis (38/126, 30%; Table 2), and most cases were bilateral (101/126, 80%). Uveitis was associated with a systemic disease in 40/126 patients (32%), of which most patients had sarcoidosis. An established clinically defined ocular syndrome was seen in 20/126 (16%) of the cases with uveitis, with birdshot chorioretinopathy as the most common diagnosis (N=16). During sample collection more than half of the patients had an active uveitis (74/126, 59%) and immunosuppressive treatment was administered in 45/126 patients (36%).

Table 2. General and ocular characteristics of uveitis patients (N=126)

Characteristic	Study participants
Male-to-female ratio	**42 - 84 (33% - 67%)**
Median age in years (range)	**54 (16 – 80)**
Median duration of uveitis in years (range)	**4 (0 – 57)**
Location uveitis	
Anterior uveitis	19/126 (15%)
Intermediate uveitis	18/126 (14%)
Posterior uveitis	45/126 (36%)
Panuveitis	38/126 (30%)
Scleritis	6/126 (5%)
Specific cause or association	
Association with systemic disease	**40/126 (32%)**
Sarcoidosis	10/40 (8%)
Multiple sclerosis	8/40 (6%)
Inflammatory bowel disease	4/40 (3%)
HLA B27 associated uveitis	3/40 (2%)
Psoriasis	3/40 (2%)
Miscellaneous group	12/40 (2%)
Clinical ocular syndrome	**20/126 (16%)**
Birdshot chorioretinopathy	16/126 (13%)
Miscellaneous group	4/126 (3%)
Proven ocular infection	**8/126 (6%)**
Toxoplasmosis	4/126 (3%)
Miscellaneous group	4/126 (3%)
Unknown cause	**58/126 (46%)**
Unknown	49/126 (39%)
Unknown with latent tuberculosis infection	9/126 (7%)
Active uveitis	**74/ 126 (59%)**
Systemic immunosuppressive treatment	**45/126 (36%)**
Retinal lesions	**59/126 (47%)**
Vasculitis	**16/126 (13%)**
Cystoid macular edema	**33/126 (26%)**
Bilateral uveitis	**101/126 (80%)**
Glaucoma	**22/126 (18%)**
Visual acuity in the worst eye	
≥ 0.5	66/126 (52%)
≥ 0.1 & < 0.5	35/126 (28%)
< 0.1	25/126 (20%)

Serum ARAs in uveitis and healthy controls

A positive retinal IIF staining was observed in 71/126 (56%) patients with uveitis and in 11/60 (18%) healthy controls ($p<0.001$; Table 3). ANAs were positive in 22/126 (17%) cases and in 1/60 (2%) of the healthy controls; in these ANA-positive subjects the presence of ARAs was scored as 'unknown'. After adjustment of the results for the presence of ANAs, 49/104 (47%) patients with uveitis and in 10/59 (17%) of the healthy controls ($p<0.001$) had ARAs.

Several retinal staining patterns were recognized in patients with uveitis. Specifically staining of nuclear layers (29/98, 30%), the photoreceptors (19/104, 18%) and band A and/or band B were more often observed in patients with uveitis compared to healthy controls (5/58 (9%), 3/59 (5%) and 2/59 (3%); p=0.002, p=0.018 and p=0.027, respectively).

Table 3. Antiretinal antibodies (ARAs) and antinuclear antibodies (ANAs) in uveitis patients and healthy controls

	Uveitis (N=126)	Healthy controls (N=60)	p-value
Any retinal staining	**71/126 (56%)**	**11/60 (18%)**	**< 0.001**
ARA considered unknown due to ANA+	22/71 (31%)	1/11 (10%)	
Retina specific staining	**49/104 (47%)**	**10/59 (17%)**	**< 0.001**
Nuclear layers*	29/98 (30%)	5/58 (9%)	0.002
Photoreceptors	19/104 (18%)	3/59 (5%)	0.018
Band A and/or band B	15/104 (14%)	2/59 (3%)	0.027

* Presence of retina specific staining could be determined in some cases with ANA positivity, since photoreceptors and band A/B were not masked by ANA. Therefore the number of patients with ARA is higher in the analysis of staining of the retina, photoreceptors and band A and/or band B compared to staining of the nuclear layers.

Serum ARAs and clinical characteristics of uveitis

We observed no significant associations between the presence of any retinal IIF staining or specific patterns of ARAs and various demographic and clinical manifestations, specifically the age of patients; cause, duration, location or laterality of uveitis; presence of retinal lesions, vasculitis, cystoid macular edema or glaucoma; use of immunosuppressive treatment; or history of carcinoma (Table 4). Furthermore, the presence of ARAs did not differ between patients with specific chorioretinal lesions when comparing peripheral multifocal chorioretinitis, birdshot- lesions and focal retinitis. Several borderline associations were noted such as the presence of total ARAs and uveitis in remission (Table 4).

Staining of photoreceptors was not associated with any clinical manifestation of uveitis. Staining of the nuclear retinal layers was less prevalent in patients with birdshot chorioretinopathy (0/11%) and more prevalent in patients with acute multifocal placoid pigment epitheliopathy (2/3,67%) compared to other causes of uveitis. Staining of band A/B was more often seen in the female population (20%), patients with uveitis caused by more rare systemic diseases (50%) and in visually compromised (visual acuity of < 0.1) patients (32%). No other relationships between clinical manifestations and staining of the nuclear layers or band A/B were observed.

Table 4. Presence of antiretinal antibodies (ARAs) and staining patterns of ARAs on indirect immunofluorescence (adjusted for presence of antinuclear antibodies) in relation to clinical characteristics in patients with uveitis

	Presence of ARAs in uveitis	Staining patterns of ARAs in uveitis**		
		Nuclear layers*	Photo-receptors	Band-A/-B
Total	49/104 (47%)	29/98 (30%)	19/104 (18%)	15/104 (14%)
Gender	**p>0.05**	**p>0.05**	**p>0.05**	**p=0.044**
Male	14/38 (37%)	7/36 (19%)	5/38 (13%)	2/38 (5%)
Female	35/66 (53%)	22/62 (36%)	14/66 (21%)	13/66 (20%)
Age	p>0.05; n/a	p>0.05; n/a	p>0.05; n/a	p>0.05; n/a
Duration uveitis	p>0.05; n/a	p>0.05; n/a	p>0.05; n/a	p>0.05; n/a
Location uveitis	**All p>0.05**	**All p>0.05**	**All p>0.05**	**All p>0.05**
Anterior uveitis	8/16 (50%)	4/16 (25%)	4/16 (25%)	3/16 (19%)
Intermediate uveitis	8/16 (50%)	5/15 (33%)	3/16 (18%)	3/16 (19%)
Posterior uveitis	20/38 (53%)	11/36 (31%)	5/38 (13%)	4/38 (11%)
Panuveitis	11/30 (37%)	7/27 (26%)	7/30 (23%)	5/30 (17%)
Scleritis	2/4 (50%)	2/4 (50%)	0/4 (0%)	0/4 (0%)
Etiology of uveitis ǂ	**All p>0.05**	**All p>0.05**	**All p>0.05**	**All p>0.05**
Association with systemic disease	14/32 (44%)	8/29 (28%)	7/32 (22%)	5/32 (16%)
Clinical ocular syndrome	8/16 (50%)	2/15 (13%)	4/16 (25%)	2/16 (13%)
Proven ocular infection	3/7 (43%)	2/7 (29%)	1/7 (14%)	1/7 (14%)
Unknown cause	24/49 (49%)	17/47 (36%)	7/49 (14%)	7/49 (14%)
Activity uveitis	**p=0.022**	**p>0.05**	**p>0.05**	**p>0.05**
Active	23/61 (38%)	14/59 (24%)	10/61 (16%)	9/61 (15%)
Remission	26/43 (61%)	15/39 (39%)	9/43 (21%)	6/43 (14%)
Systemic immuno-suppressive treatment	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	17/37 (46%)	7/32 (22%)	8/37(22%)	8/37 (22%)
No	32/67 (48%)	22/66 (33%)	11/67 (16%)	7/67 (10%)

Retinal lesions	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	25/49 (51%)	16/47 (34%)	8/49 (16%)	6/49 (12%)
No	24/55 (44%)	13/51 (26%)	11/55 (20%)	9/55 (16%)
Vasculitis	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	8/15 (53%)	4/13 (31%)	5/15 (33%)	4/15 (27%)
No	41/89 (46%)	25/85 (29%)	14/89 (16%)	11/89 (12%)
Cystoid macular edema	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	8/24 (33%)	7/24 (29%)	1/24 (4%)	2/24 (8%)
No	41/80 (51%)	22/74 (30%)	18/80 (23%)	13/80 (16%)
Laterality uveitis	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Unilateral uveitis	7/22 (32%)	5/22 (23%)	2/22 (9%)	13/82 (16%)
Bilateral uveitis	42/82 (51%)	24/76 (32%)	17/82 (21%)	2/22 (9%)
Glaucoma	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	7/18 (39%)	4/18 (22%)	3/18 (17%)	2/18 (11%)
No	42/86 (49%)	25/80 (31%)	16/86 (19%)	13/86 (15%)
History of carcinoma	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Yes	7/15 (47%)	2/13 (15%)	3/15 (20%)	2/15 (13%)
No	42/89 (47%)	27/85 (32%)	16/89 (18%)	13/89 (15%)
Visual acuity in the worst eye		**All p>0.05**	**All p>0.05**	
≥ 0.5	p>0.05; 27/55 (49%)	17/53 (32%)	10/55 (18%)	p>0.05; 6/55 (11%)
≥ 0.1 & < 0.5	p=0.034; 8/27 (30%)	4/26 (15%)	4/27 (15%)	p> 0.05; 2/27 (7%)
< 0.1	p>0.05; 14/22 (64%)	8/19 (42%)	5/22 (23%)	p=0.016; 7/22 (32%)

* Presence of retina specific staining could be determined in some cases with ANA positivity, since retinal ANA staining did not mask photoreceptors and/or band A/B. Therefore the number of patients with ARA is higher in the analysis of staining of the retina, photoreceptors and band A and/or band B compared to retinal staining of the nuclear layers.

** Some staining pattern were present in combinations

ǂ Sub-analysis of specific etiologic entities within the indicated groups did not show any additional positive correlations

DISCUSSION

Our results show that serum ocular antibodies, specifically ARAs, are more prevalent in patients with uveitis as compared to healthy controls (47% versus 17%, p<0.001). However, specific associations between the presence of serum ARAs (or typical staining patterns on IIF) and clinical ocular characteristics were not observed so far, except for the association between patients with a visual acuity of less than 0.1 and the presence of positive staining of band A/B (p=0.013). Furthermore, some marginal associations were noted, though this probably a coincidence occurring due to multiple comparisons in a small cohort (in perspective to number of tested variables). Our study includes a heterogeneous population of uveitis patients in which major groups of etiology are represented. Due to the limited number of patients in specific subgroups, it cannot be fully excluded that some associations were missed and that in a larger population of patients with specific uveitis entities the associations between clinical manifestations and serum ARA's could be found.

The involvement of autoimmunity in uveitis has repeatedly been implicated and the term autoimmune uveitis was sometimes even used as a synonym for all non-infectious types of uveitis. The proposed autoimmune pathogenesis is often being explained by mimicry between the ocular and other infectious or non-infectious antigens, but actual autoimmune processes in uveitis have seldom been proved. The autoimmune reactions against S-antigen, the best-known uveitogenic antigen, were examined and found to be involved in the pathogenesis of infectious uveitis.[5,10,11] In several animal models with monkeys and rats, vaccination with retinal S antigen induced an ocular disease similar to human uveoretinitis.[17-19]

The results of our study on the prevalence of serum ARAs in uveitis are in line with previous reports. Cohorts with different specific entities of uveitis have been analyzed for the presence of ARAs in serum, including cohorts of patients with juvenile associated uveitis, idiopathic retinal vasculitis, toxoplasmosis, onchocherciasis, or a variation of noninfectious or idiopathic posterior uveitis (Table 1). The presence of any ARA in serum was noted in approximately 70% in patients with retinal vasculitis and up to 100% in patients with toxoplasmosis.[5,10,12] Since the aforementioned studies usually lacked clinical data and information on laboratory techniques the exact comparison with previous results is not possible. In our series, we have chosen to use (commercially available) primate retinal tissue to investigate the presence of ARAs by IIF. Primate tissue from an evolutionary perspective closely resembles human tissue and is also used for diagnostic purposes. Furthermore, in human retinal tissue certain antigens may induce higher background staining, including blood group-associated antigens. In addition, the use of commercially available tissues may facilitate future comparisons of results.

The presence of diverse patterns on IIF indicates that different ARAs are produced by individual patients. However, IIF does not allow the precise identification of ARAs, i.e. their antigen speci-

ficity. To clarify the type and complexity of the ARAs detected in our study, specific IIF patterns must be correlated to specific retinal antigen reactivity. In the past, patterns of specific ARAs have been determined, however, IIF showed different patterns in various studies even with the use of purified antibodies.[20-23] The cause of this inconsistency could be explained by the use of variable tissues and reagents and the recognition of different epitopes. More useful techniques for confirmation of specific type of ARA and determination of multiple ARAs consist of Western Blot and/or ELISA. [3,24]

The higher prevalence of serum ARAs in uveitis remains unexplained as well as their possible clinical role. Hypothetically, the presence of serum ARA's (and absence of their clinical impact) might be due to local tissue damage, leakage of (altered) retinal antigens into the circulation and subsequent reaction of the immune system and production of diverse serum ARAs. This implicates that the production of serum ARAs might develop due to the general response of the immune system to ocular tissue damage. The systemic production of coincidental ARAs might be responsible for the lack of associations between the serum ARAs and ocular clinical manifestations in parallel to the presence of multiple autoantibodies, which are commonly observed in systemic autoimmune disorders and sometimes in healthy elderly population. In addition, the absence of ARAs in a subset of uveitis patients indicates that probably not antibodies but cell-mediated autoimmune reactions might play a primary role in the development of presumed autoimmunity in uveitis.

Further, the levels of antibodies in the serum reflect their total systemic production and are probably not influenced by the additional production of specific antibodies within the eye. This phenomenon has also been observed in the diagnosis of infectious uveitis in which local production of specific antibodies within the eye rather than serum levels is regarded as a proof of the ocular infection (determined with a Goldmann-Witmer coefficient.[25] The importance of locally produced antibodies has also been shown in cerebrospinal fluid for the central nervous system, another immune privileged organ.[26] The local production of ARAs might show an entirely different pattern and clinical importance than ARAs found in the peripheral circulation and has - to our knowledge - so far not been examined. Further research assessing the intraocular levels of specific ARAs might elucidate the clinical relevance of autoimmune processes directed against the retinal tissue in uveitis.

Serum ARAs were observed in the majority of patients with uveitis and were more frequent compared to healthy controls. The cause of enhanced serum ARA production in patients with uveitis is currently not known. Specific associations between the presence of serum ARAs (or typical staining patterns on IIF) and clinical ocular characteristics were not identified. The role of presumed autoimmune reactions in uveitis could be further investigated by assessment of intraocular fluids within the eye instead of peripheral blood samples.

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5

SERUM AUTOANTIBODY PROFILING OF PATIENTS WITH PARANEOPLASTIC AND NON-PARANEOPLASTIC AUTOIMMUNE RETINOPATHY

Josianne C.E.M. ten Berge, Joost van Rosmalen, Jacolien Vermeer, Cecilia Hellström, Cecilia Lindskog, Peter Nilsson, Ulrika Qundos, Aniki Rothova, Marco W.J. Schreurs

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ABSTRACT

Purpose: Although multiple serum antiretinal autoantibodies (ARAs) have been reported in patients with paraneoplastic and non-paraneoplastic autoimmune retinopathy ((n)pAIR), not all retinal antigens involved in (n)pAIR are specified. This study aims to serologically identify patients with presumed (n)pAIR through determination of both known and unknown ARAs by autoantibody profiling.

Methods: An antigen suspension bead array using 188 different antigens representing 97 ocular proteins was performed to detect ARAs in serum samples of patients with presumed (n)pAIR (N=24), uveitis (N=151) and cataract (N=21). Logistic regressions were used to estimate the associations between ocular antigens and diagnosis. Validation of interphotoreceptor matrix proteoglycan 2 (IMPG2) and recoverin antigens was performed by immunohistochemistry and immunoblot, respectively.

Results: Samples of patients with presumed (n)pAIR exhibited a broad spectrum of ARAs. We identified retinal antigens that have already been described previously (e.g. recoverin), but also identified novel ARA targets. Most ARAs were not specific for (n)pAIR since their presence was also observed in patients with cataract or uveitis. High titers of autoantibodies directed against photoreceptor-specific nuclear receptor and retinol-binding protein 3 were more common in patients with presumed (n)pAIR compared to uveitis (p=0.015 and p=0.018, respectively). The presence of all other ARAs did not significantly differ between groups. In patients with presumed (n)pAIR, anti-recoverin autoantibodies were the most prevalent ARAs. Validation of bead array results by immunohistochemistry (anti-IMPG2) and immunoblot (anti-recoverin) showed concordant results in (n)pAIR patients.

Conclusions: Patients with (n)pAIR are characterized by the presence of a broad spectrum of ARAs. The diagnosis of (n)pAIR cannot be based on the mere presence of serum ARAs, as these are also commonly present in uveitis as well as in age-related cataract patients.

INTRODUCTION

Paraneoplastic and non-paraneoplastic autoimmune retinopathy ((n)pAIR) is a rare blinding retinal disorder of unknown pathogenesis. It is presumed that antiretinal autoantibodies (ARAs) are involved in the pathogenesis of (n)pAIR and damage ocular tissue causing poor visual outcome. Symptoms associated with (n)pAIR are progressive visual loss (most often bilateral), visual field loss frequently associated with a ring scotoma or loss of the peripheral field, and decreased amplitudes on electroretinogram (ERG).[1-4]

Paraneoplastic autoimmune retinopathy (pAIR) includes two subgroups: cancer associated retinopathy (CAR) and melanoma associated retinopathy (MAR). In pAIR the presence of the same auto-antigens in both retinal tissue and malignant tissue has previously been described (e.g. recoverin).[5-7] The presence of ARAs however is not conclusive for the diagnosis of (n)pAIR, since several ARAs were also reported in patients with other ocular disorders and individuals without ocular disease.[8] Nevertheless, ARAs are considered to support the diagnosis of (n)pAIR, which is often difficult to confirm by clinical symptoms only.[9]

Multiple serum ARAs have regularly been reported in affected patients (Table 1), although not all retinal autoantibodies involved in the pathogenesis of (n)pAIR are known and information regarding their exact pathological roles is lacking.[10] Further, a gold standard for the determination of ARAs is missing.[11-13] The optimal approach for the determination and specification of ARAs is currently unknown. Different techniques, including indirect immunofluorescence, western blot and enzyme-linked immunosorbent assay (ELISA), have been used for the detection of ARAs; however, results and conclusions differ and cannot be reliably compared.

Currently, antigen bead arrays are being used to profile autoantibody reactivity in body fluids.[38] With this technique, very small volumes of body fluids can be tested for IgG reactivity across hundreds of samples towards hundreds of different antigens. This technique has already successfully been used for the analysis of autoantibodies in serum and cerebrospinal fluid.[39-41]

Our study aimed to serologically identify patients with presumed (n)pAIR through determination of ARAs. For this purpose, we used a bead array-based multiplex assay for autoantibody profiling using 188 ocular antigens representing 97 different retinal proteins.

Table 1. Previously described antiretinal autoantibodies in serum of patients with paraneoplastic and non-paraneoplastic autoimmune retinopathy [1,14,15]

Antigen	Associated with			Location in retina	Size (kDa)
	CAR	MAR	npAIR		
Recoverin [16]	x	x	x	Inner segments and nuclei of photoreceptor cells, outer plexiform layer	23
α - Enolase [17]	x	x	x	Inner segments of the cone cells, Müller cells and ganglion cell layer	46
Carbonic anhydrase II [18]	x	x	x	Ganglion cell layer, inner nuclear layer, outer segments of photoreceptors	30
Heat shock cognate protein 70 [19]	x	x	x	N/A	65
Transducin α (guanine nucleotide-binding protein G(t) subunit alpha-1) [20]	x	x	x	Outer and inner segments of photoreceptor cells, cytoplasm of ganglion cells	40
Transducin β (guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β-1)* [21]	x	x		Photoreceptor cells, ganglion cell layer	35
Arrestin (S-antigen) [22,23]		x	x	Photoreceptor cells	48
Interphotoreceptor binding protein (retinol binding protein 3) [24-26]		x	x	Outer and inner segments of photoreceptor cells	141
Rhodopsin [27,28]		x	x	Rod photoreceptor cells	40
Photoreceptor-cell-specific nuclear receptor [29]	x			Outer nuclear layer	44.7
Müller-cell-specific antigen [30]		x	x	N/A	35
Transient receptor potential cation channel subfamily M, member 1 [31-34]	x	x	x	Bipolar cells	182
Tubby-like protein 1 [35]	x		x	Photoreceptor cells	78
Bestrophin-1 [36]		x		Basal lateral membrane of retinal pigment epithelium	68
Aldolase A and C [15]		x	x	Ganglion cell layer, inner nuclear layer (aldolase C)	39
Glyceraldehyde 3-phosphate dehydrogenase [37]	x	x	x	Rod outer segments	30 and 36

Abbreviations: CAR = cancer associated retinopathy, MAR = melanoma associated retinopathy, npAIR = non-paraneoplastic autoimmune retinopathy

METHODS

Sample collection and patient selection

Serum samples were either collected during routine diagnostic analysis for the presence of anti-recoverin autoantibodies in the Laboratory of Medical Immunology of the Erasmus University Medical Center between April 2013 and August 2015 or were obtained from biobank of our department. The study was approved by the local ethical committee from the Erasmus University Medical Center (Medical Ethics Committee Erasmus MC) and adhered to the tenets of the Declaration of Helsinki. The ethical committee decided that no informed consent of patients was required for the use of the remainder of the diagnostic material, as the samples were anonymized and the patients were not subjected to additional risk or procedures. Samples which were obtained from the biobank (for which an approval of the ethical committee was obtained) included signed informed consent from all participants. All whole blood samples were centrifuged after at least 30' clotting time at 3,000 rpm for 10 minutes, and serum was stored at -80°C.

According to the recently published report on the nomenclature of (n)pAIR, the general term autoimmune retinopathy (AIR) is recommended to indicate the non-paraneoplastic autoimmune retinopathy (npAIR) subtype. In our present series we indicate the specific subtype(s) of AIR (pAIR, npAIR or (n)pAIR) to prevent any misunderstanding regarding nomenclature.[9] The diagnosis of presumed (n)pAIR was made if the patients fulfilled all of the following inclusion criteria: 1. visual complaints, 2. markedly decreased amplitudes on ERG, 3. visual field loss, and 4. no alternative explanation for their ocular disorder. In addition, patients with genetically proven retinitis pigmentosa or a family history of retinitis pigmentosa were excluded. A total of 17 patients fulfilled the criteria indicated above and were included in this study. Patients fulfilling the criteria without a malignancy were indicated as presumed npAIR (N=9), and patients with a malignancy were indicated as patients with presumed pAIR (N=8).

An additional group of presumed pAIR (CAR or MAR) patients (N=7) in whom ERG or visual field tests were not performed (choice of the patient or poor general condition), but who fulfilled all other inclusion criteria was included separately. An additional required criterion for these patients comprised the development of a malignancy before or within 3 months after presentation with ocular problems.

We collected various clinical data of the patients with presumed (n)pAIR, including patient demographics (age and gender) and ocular characteristics such as complaints of photopsia, complaints of nyctalopia, subjective or objective problems with colour-vision, unilateral or bilateral visual problems and the presence of a malignancy in the medical history or during follow-up.

Controls consisted of two groups: 21 serum samples from cataract patients without retinal damage and 151 samples from patients with uveitis of different causes. Patients with age related cataract were included as controls rather than healthy people, as this disorders does not involve retina nor exhibits retinal damage, and represents a clinical setting in which the tests might be employed. Samples of control patients were collected at the department of Ophthalmology of the Erasmus University Medical Center between February 2009 and April 2015. Patient demographics (age and gender) and known malignancies of these patients were registered.

Antigen suspension bead array

Autoantibody profiling was performed in all serum samples from patients with presumed (n) pAIR (N=24), uveitis (N=151) and cataract (N=21). Antigens used for the autoantibody profiling were selected based on potential relevance to ocular diseases according to literature and previous positive retinal immunohistochemistry staining, resulting in 188 antigens (human protein fragments) representing 97 unique proteins. The protein fragments were produced within the Human Protein Atlas and designed to represent unique parts of each target protein.[42,43] Protein fragments were 20-150 amino acids long (median 78 aa) and produced in Escherichia coli, with an affinity tag consisting of six histidines and an albumin binding domain from streptococcal protein G (His6ABP) (Supplementary Table). Immobilization onto color-coded magnetic beads was conducted as described previously.[39] In short, diluted antigens were covalently coupled to activated carboxy groups on color coded polystyrene beads (MagPlex, Luminex Corp.) by undirected amine coupling. In addition to the selected protein targets, one bead identity was used for immobilization of anti-human IgG (positive control), one for Epstein-Barr virus nuclear antigen 1 (second positive control), one for His6ABP (negative control, to monitor binding to the affinity tag) and one bead identity went through the coupling process without addition of antigen (second negative control, to monitor binding to bare beads). After incubation, the coupled beads were washed and stored in a blocking reagent before combining all bead identities to create a bead array in suspension. Samples were distributed across 96-well microtiter plates, together with triplicate aliquots of a sample pool and a buffer blank in each plate for determination of the intra- and inter-reproducibility. Serum samples were diluted 1:250 in assay buffer before being mixed with the bead array. Incubation was performed at room temperature for 2 hours followed by detection of the IgG reactivity by a fluorophore conjugated anti-human IgG Fab fragment and measured in a FlexMap3D instrument (Luminex Corp.).

Recoverin immunoblot

For validation purposes, samples that tested positive for anti-recoverin autoantibodies on the antigen bead array, and all samples from patients with presumed (n)pAIR, were analysed on a recoverin specific immunoblot (Euroimmun AG, Lubeck, Germany). Membrane strips coated with recombinant human recoverin were incubated with a sample buffer for 5 minutes. After aspiration of the sample buffer, the membrane strips were incubated with diluted serum samples for 30

minutes on a shaking platform. Subsequently membrane strips were washed three times, incubated with secondary antibodies (enzyme conjugated anti-human IgG), washed again for three times and stained with a substrate solution which was capable of promoting an enzymatic colour reaction. To identify positive reactions, assessment of visible bands was performed relative to the included control. Results from the antigen suspension bead array and the recoverin specific immunoblot were compared.

Immunohistochemistry of interphotoreceptor matrix proteoglycan 2 on human retina tissue

Another method for validation was performed with the antigens of interphotoreceptor matrix proteoglycan 2 (IMPG2). Polyclonal antibodies affinity purified against the IMPG2 antigens no. 214 and 205 were used as antigens for immunization of rabbits to generate polyclonal antibodies for immunohistochemistry on normal human tissues, in order to determine retina specificity and cell type expression. The antibodies were applied on tissue microarrays (TMAs) containing samples from 45 different human tissues, including retina from two individuals. TMAs from human tissues were generated essentially as previously described.[44] The TMAs contained 1 mm diameter formalin-fixed and paraffin-embedded tissue cores from 45 different histologically normal tissue types, including two samples of human eye: one male 75 years and one female 54 years. All samples were received from the Department of Pathology, Uppsala University Hospital, Sweden, approved by the local Research Ethics Committee (Uppsala, Sweden, Ups 02–577). Four-micrometer sections were cut from the TMA blocks, mounted on adhesive slides and baked at 60 °C for 45 min. TMA slides were then deparaffinised in Neo-Clear® (Merck Millipore, Darmstadt, Germany), followed by hydration in graded alcohols and blocking for endogenous peroxidase in 0.3 % hydrogen peroxide. For antigen retrieval, slides were immersed and boiled in Citrate buffer®, pH6 (Lab Vision, Freemont, CA) for 4 min at 125 °C and then allowed to cool to 90 °C. Automated immunohistochemistry was performed essentially as previously described, using an Autostainer 480 instrument® (Lab Vision).[44] Affinity purified polyclonal antibodies towards IMPG2 (HPA008779, antigen number 205, diluted 1:250 and HPA015907, antigen number 214, diluted 1:2500, both Atlas Antibodies AB) and a dextran polymer visualization system (UltraVision LP HRP polymer®, Lab Vision) were incubated for 30 min each at room temperature. Slides were developed for 10 min using Diaminobenzidine (Lab Vision) as chromogen. All incubations were followed by rinse in Wash buffer® (Lab Vision) for 5 min. The slides were counterstained in Mayers hematoxylin (Histolab) and cover slipped using Pertex® (Histolab) as mounting medium. Digital whole slide high-resolution images were captured with a 20× objective using an AperioScanScope XT Slide Scanner (Aperio Technologies, Vista, CA).

Data analysis

Continuous variables were summarized using medians and ranges, and categorical variables were summarized using percentages. Patient demographics were compared between diagnosis groups using Mann Whitney U tests for continuous data and Fisher's exact tests for categorical

data. All data from the antigen suspension bead array were represented as ratios (antigen specific reactivity over patient background (represented by the His6ABP negative control bead)). A ratio of >2 was considered positive and a ratio of >25 was considered highly positive for the presence of ARAs. Logistic regressions with correction for age and gender were performed to analyse differences between the diagnosis groups ((n)pAIR versus uveitis and (n)pAIR versus cataract) for both ratio's. In the logistic regression analyses, confidence intervals of the estimated odds ratios were calculated using a profile likelihood method, and the differences between groups were tested using a likelihood ratio test. To adjust for the multiple comparisons of the different antigens, a Bonferroni correction was used for the p-values of the logistic regression analyses, so that only p-values ≤ 0.0002 were considered statistically significant in these analyses. Intra- and inter-assay reproducibility was calculated with the coefficient of variation using the technical replicates within and between plates, based on the pooled serum samples.

The distribution of age, gender and the most prevalent ARAs (using the cut-off values for the ratio of 2 and 25) were compared between the subtypes of AIR (pAIR, npAIR) using Mann Whitney U tests for continuous data and Fisher's exact tests for categorical data. The number of different ARAs per patient in highly positive titres were counted and compared between groups using a linear-by-linear association chi-square test. The association between the number of ARAs and age was assessed using Spearman's rank correlation coefficient. All statistical tests were two-sided and used a significance level of 0.05. The analyses were performed using SPSS and R.[45]

RESULTS

Patient characteristics

Characteristics of the patients with presumed (n)pAIR (N=24) are specified in Table 2. The median age of patients was 67 years, with a range of 27-86 years. The majority of the patients were female (17/24, 71%). Most patients had bilateral visual complaints (21/24, 88%), and photopsia, nyctalopia and colour vision problems were noted frequently (12/19, 63%; 11/13, 85%; 9/11, 82%). A malignancy was seen in 15/24 (63%) patients (indicative for pAIR: CAR or MAR), of whom 8/24 (33%) patients had a malignancy in the past and 7/24 (29%) patients developed a malignancy during follow-up. The most frequently diagnosed malignancy was a lung carcinoma (6/15; 40%). A total of 9/24 (38%) patients did not have a malignancy and were diagnosed with presumed npAIR. Comparison of patient demographics (age and gender) between groups showed that patients with uveitis were significantly younger than patients with AIR (p<0.001). Gender did not differ between groups.

Table 2. General characteristics of patients

Patient characteristics	(n)pAIR (N=24)	Uveitis (N=151)	Cataract (N=21)
Gender (male-female)	7 (29%) - 17 (71%)	63 (42%) – 88 (58%)	10 (48%) – 11 (52%)
		p=0.271 **	*p=0.233* **
Age in years (median; min-max)	67; 27-86	49; 17-86	69; 48-83
		p<0.001 **	*p=0.339* **
Bilateral visual complaints	21/24 (88%)		
Complaints of photopsia	12/19 (63%) *		
Complaints of nyctalopia	11/13 (85%) *		
Colour-vision problems	9/11 (82%) *		
Presence of malignancy (pAIR)	15/24 (63%)		
Malignancy in history	8/24 (33%)		
Malignancy during follow-up	7/24 (29%)		

* Data not available for all patients

** p-value of comparison with (n)pAIR patients

Abbreviations: (n)pAIR = non-paraneoplastic and paraneoplastic autoimmune retinopathy

Antigen suspension bead array: highly positive titres of ARAs (ratio > 25)

Patients with presumed (n)pAIR were characterized by the presence of a wide spectrum of ARAs (Figure 1 and Supplementary Figure). There was no specific ARA associated with a majority of patients with presumed (n)pAIR. In patients with presumed (n)pAIR, anti-recoverin autoantibodies were the most prevalent ARAs (12.5%). The presence of anti-recoverin autoantibodies was not fully specific for (n)pAIR, since high titres were also present in sporadic patients with cataract (4.8%; p = 0.351) or uveitis (1.3%; p = 0.061). Further, no association between the presence of anti-recoverin autoantibodies and a malignancy was found. High titre autoantibodies to photoreceptor-specific nuclear receptor and retinol-binding protein 3 were more prevalent in patients with (n)pAIR than in patients with uveitis (p=0.015 and p=0.018, respectively; p-values were not significant after applying correction for multiple testing). Autoantibodies towards IMPG2 (antigen number 205) were prevalent with highly positive titres in two patient samples with (n)pAIR (8.3%) and with lower prevalence in uveitis patients (2%). The results of the most prevalent ARAs present in high titres (ratio > 25) in patients with presumed (n)pAIR are shown in Table 3. The ARAs (in highly positive titres) indicated in Table 3 were only found in patients with presumed pAIR with the exception of two patients with presumed npAIR (one patients with npAIR was positive for high titres of antibodies against progressive rod-cone degeneration protein and one patient for high titres of Cbp/p300-interacting transactivator 1). The prevalence of high-titre ARAs (from Table 3), age and gender did not significantly differ between patients with npAIR and pAIR (all p > 0.05).

Table 3. Prevalence of antiretinal autoantibodies in paraneoplastic and non-paraneoplastic autoimmune retinopathy, uveitis and cataract *

		Ratio > 25						
		Prevalence of ARAs			(n)pAIR vs uveitis		(n)pAIR vs cataract	
Antigen number	Antigen	(n) pAIR	Uveitis	Cataract	OR; 2.5% - 97.5%	p-value	OR; 2.5% - 97.5%	p-value
225	Recoverin **	12.5% (3/24)	1.3% (2/151)	4.8% (1/21)	6.3; 0.91 - 54.7	0.061	2.98; 0.32 - 6.49	0.351
303	Progressive rod-cone degeneration protein	12.5% (3/24)	8.6% (13/151)	14.3% (3/21)	2.01; 0.39 - 8.21	0.370	1.21; 0.19 - 8.23	0.837
205	Interphoto-receptor matrix proteoglycan 2	8.3% (2/24)	2.0% (3/151)	0% (0/21)	3.82; 0.46 - 6.43	0.195	NA	0.062
207	Photoreceptor-specific nuclear receptor **	8.3% (2/24)	0% (0/151)	0% (0/21)	NA	0.015	NA	0.062
378	G protein-coupled receptor kinase 7	8.3% (2/24)	8.6% (13/151)	0% (0/21)	1.10; 0.16 - 4.77	0.905	NA	0.081
245	Serotonin N-acetyl-transferase	4.2% (1/24)	0% (0/151)	0% (0/21)	NA	0.126	NA	0.251
296	Retinol-binding protein 3 **	4.2% (1/24)	0% (0/151)	0% (0/21)	NA	0.018	NA	0.272
335	Cbp/p300-interacting transactivator 1	4.2% (1/24)	0.6% (1/151)	0% (0/21)	7.07; 0.27 - 7.13	0.203	NA	0.377
239	Retinitis pigmentosa 1-like 1 protein	0% (0/24)	0.6% (1/151)	0% (0/21)	NA	0.537	NA	NA
325	Sodium / potassium / calcium exchanger 1	0% (0/24)	1.3% (2/151)	0% (0/21)	NA	0.674	NA	NA
270	Pigment epithelium-derived factor	0% (0/24)	0% (0/151)	0% (0/21)	NA	NA	NA	NA

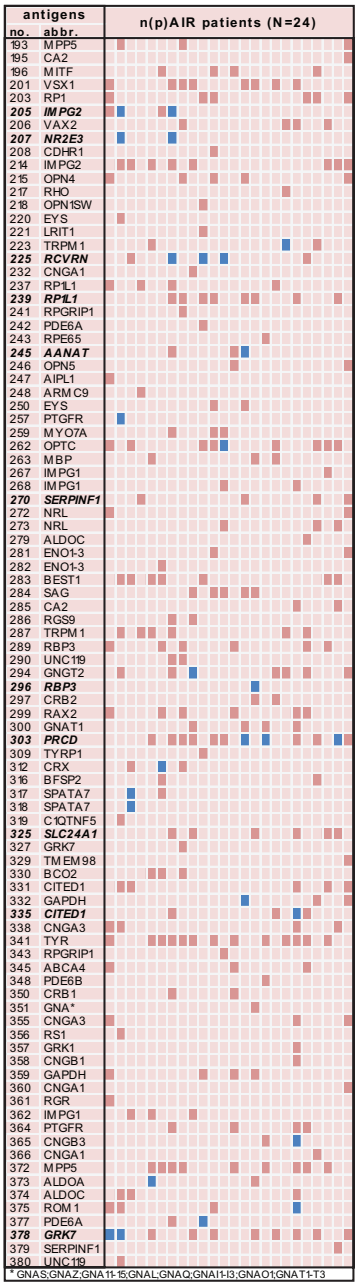
** Calculation of OR was not possible in case of an ARA prevalence of 0 in either group*

*** ARAs which have been described also in previous studies as autoantibodies associated with (n)pAIR*

Ratio >2						
Prevalence of ARAs			(n)pAIR vs uveitis		(n)pAIR vs cataract	
(n)pAIR	Uveitis	Cataract	OR; 2.5% - 97.5%	p-value	OR; 2.5% - 97.5%	p-value
20.8% (5/24)	11.9% (18/151)	14.3% (3/21)	2.21; 0.64 - 6.87	0.199	1.30; 0.25 - 7.63	0.753
50.0% (12/24)	34.4% (52/151)	57.1% (12/21)	1.83; 0.73 - 4.58	0.195	0.73; 0.20 - 2.50	0.611
16.7% (4/24)	25.2% (38/151)	19.0% (4/21)	0.59; 0.16 - 1.75	0.354	1.28; 0.244 - 6.95	0.766
8.3% (2/24)	1.3% (2/151)	0% (0/21)	5.15; 0.55 - 49.67	0.141	NA	0.062
41.7% (10/24)	55.6% (84/151)	57.1% (12/21)	0.67; 0.26 - 1.69	0.399	0.47; 0.12 - 1.72	0.253
12.5% (3/24)	0% (0/151)	4.8% (1/21)	NA	0.003	3.72; 0.39 - 84.11	0.262
4.2% (1/24)	2.6% (4/151)	4.8% (1/21)	2.65; 0.12 - 22.72	0.456	0.71; 0.03 - 19.31	0.815
16.7% (4/24)	6.0% (9/151)	9.5% (2/21)	6.52; 1.43 - 28.34	0.017	1.22; 0.17 - 10.56	0.842
33.3% (8/24)	15.2% (23/151)	23.8% (5/21)	3.54; 1.23 - 10.02	0.020	1.90; 0.47 - 8.75	0.375
25.0% (6/24)	17.9% (27/151)	4.8% (1/21)	1.41; 0.46 - 3.93	0.530	8.67; 1.13 - 188.88	0.037
16.7% (4/24)	10.6% (16/151)	0% (0/21)	1.64; 0.41 - 5.48	0.456	NA	0.030

Abbreviations: (n)pAIR = non-paraneoplastic and paraneoplastic autoimmune retinopathy, ARAs = antiretinal antibodies, OR = odds ratio, NA = not available

Figure 1. Spectrum of antiretinal autoantibodies in patients suspected of paraneoplastic and non-paraneoplastic autoimmune retinopathy



Blue: highly positive titer for the presence of antiretinal antibodies. Dark red: positive titer for the presence of antiretinal antibodies.

The number of highly positive ARAs present in individual patients is shown in Table 4. A higher number of different ARAs per patient was most prevalent in patients with presumed (n)pAIR and least present in patients with cataract. Three or more different ARAs were present in 29% of the patients with presumed (n)pAIR, compared to 24% of the patients with uveitis and 14% of the patients with cataract. The number of highly positive ARAs did not show any statistical differences between presumed (n)pAIR and uveitis (p=0.457) or cataract (p=0.385). Furthermore, there was no correlation between the number of ARAs and age (p=0.926).

Table 4. Number of highly positive antiretinal autoantibodies per patient

No. of highly positive ARAs (ratio > 25)	(n)pAIR (N=24)	Uveitis (N=151)	Cataract (N=21)
0	37.5% (9/24)	45.7% (69/151)	52.4% (11/21)
1	33.3% (8/24)	29.8% (45/151)	33.3% (7/21)
2	12.5% (3/24)	13.2% (20/151)	4.8% (1/21)
3	12.5% (3/24)	7.9% (12/151)	4.8% (1/21)
4	4.2% (1/24)	2.6% (4/151)	0% (0/21)
5	0% (0/24)	0.6% (1/151)	4.8% (1/21)

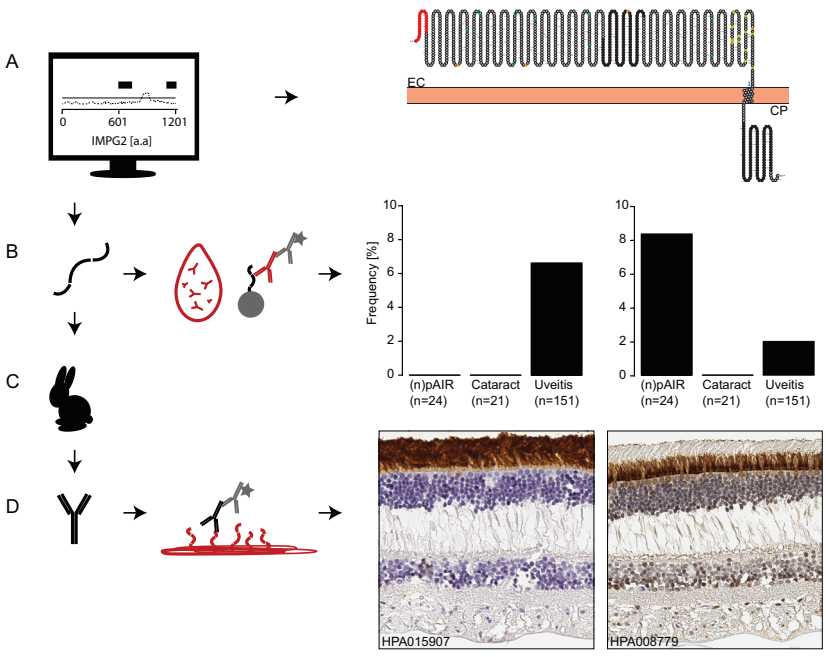
Abbreviations: (n)pAIR = non-paraneoplastic and paraneoplastic autoimmune retinopathy, ARAs = antiretinal antibodies

Antigen suspension bead array: positive titres of ARAs (ratio > 2)

The samples of patients with presumed (n)pAIR as well as both control cohorts exhibited a broad spectrum of positive ARAs (Figure 1 and Supplementary Figure). None of the ARAs were specific for presumed (n)pAIR only. Autoantibodies directed against serotonin N-acetyltransferase, cbp/p300-interacting transactivator 1 and retinitis pigmentosa 1-like 1 protein were more prevalent in patients with presumed (n)pAIR than in patients with uveitis (p=0.003, p=0.017 and p=0.020; p-values were not significant after applying correction for multiple testing). When comparing the serum of patients with presumed (n)pAIR to the serum of patients with cataract, in presumed (n)pAIR autoantibodies directed against sodium/potassium/calcium exchanger 1 and pigment epithelium-derived factor were more often present (p=0.037 and p=0.030). The presence of most ARAs indicated in Table 3 was predominantly found in patients with presumed pAIR (CAR or MAR), but (often less frequently) also in patients with presumed npAIR. The prevalence of low-titre ARAs (from Table 3) was not significantly different between patients with npAIR and pAIR (p > 0.05).

The coefficient of variation based on replicates of the serum pools within and across plates (indicating the intra- and inter-reproducibility) ranged between 5 and 23% (median = 13%) for all 188 antigens. ARAs were present in all patients with presumed (n)pAIR and consequently all fulfilled the recent criteria for the diagnosis of (n)pAIR.[9]

Figure 2. Screening and validation for interphotoreceptor matrix proteoglycan 2



(Left panel, A-D) The path from antigen design and generation to autoantibody screening in serum and secondly protein expression in retinal tissue. (A) Two antigens representing non-overlapping regions with either an extracellular or cytoplasmic location of IMPG2 were selected for recombinant protein expression (antigen 214 and 205 respectively, amino acids highlighted in black). (B) Detection of auto-antibody reactivity with IMPG2 antigens using the antigen suspension bead array. Ratios > 25 are displayed per disease group for antigen 214 (left) and 205 (right). (C) The antigens were further used as antigens for immunization of rabbits to generate polyclonal antibodies. (D) Antibodies HPA015907 and HPA008779, affinity purified against antigens 214 and 205, were applied for immunohistochemical staining of human retina tissue. Both antibodies specifically showed cytoplasmic staining of cells in the photoreceptor layer in the retina (D, right). The antibody targeting the CP region of IMPG2 (HPA008779) stained only the inner segment of the photoreceptor layer, while HPA0015907 stained both inner and outer segment.
Abbrevations: EC; extracellular, CP; cytoplasmic, IMPG2; Interphotoreceptor matrix proteoglycan 2.
Color annotation for central panel: black; Human Protein Atlas antigens and antibodies, red; human sample serum and tissue and grey; assay consumables suspension bead array and labelled detection antibodies.

Recoverin immunoblot

Anti-recoverin autoantibodies on immunoblot were positive in 3 out of 24 (12.5%) patients with (n) pAIR. These positive results were in accordance with the positive high titre results on the antigen suspension bead array. Occasional discrepancy between the recoverin immunoblot and the antigen suspension bead array (using a high cut-off value, ratio > 25) was found in the controls (3 patients positive in antigen suspension bead array while negative on recoverin immunoblot).

IMPG2 expression in human retina tissue

The antigens towards IMPG2 (antigen number 214 and 205) represent two non-overlapping domains of IMPG2, located either extracellularly or in the cytoplasm (Figure 2A).[46] Antibodies directed against antigens 214 and 205, showed staining exclusively in cells in the photoreceptor layer of the retina. The antibodies targeting the cytoplasmic region of IMPG2 (against antigen number 205) stained only the inner segment of the photoreceptor layer, while HPA0015907 (antibodies against antigen number 214) stained both inner and outer segment (Figure 2D).

DISCUSSION

Our study shows that patients with (n)pAIR are characterized by the presence of a broad spectrum of various ARAs. We identified ARAs that have already been described in previous studies, such as anti-recoverin autoantibodies, but also identified new retinal targets. Our findings illustrate that serum ARAs are not only present in patients with (n)pAIR, but also in patients with cataract and uveitis. Though some ARAs appeared to be specific for (n)pAIR, their prevalence and consequently their sensitivity as markers for (n)pAIR were low. This autoantibody screening using 188 antigen provides insight into the autoimmune repertoire of patients with (n)pAIR and a base for further validation with independent methods for protein analysis and independent sample cohorts.

A gold standard for the determination of ARAs is currently lacking.[11] Different techniques are being used, hampering the comparison of results from various laboratories.[47] Moreover, the mere presence of ARAs does not provide any information on the role of this specific antibody. In addition, information on clinical relevance of the specific ARAs and their pathological titres are lacking. A combination of different ARAs was observed in some cases and therefore their individual effects on retinal tissue could not be distinguished.

In our study, we performed statistical analyses using different cut-off levels. By using a high cut-off value, a ratio > 25, false positive results were minimized and retinal targets with a high specificity for (n)pAIR were found. The low cut-off value, a ratio of >2 (indicating ARAs with at least twice the reactivity of the negative control), was used for a more sensitive approach, limiting the exclusion of possibly relevant ARA targets with a lower titre. However, with both cut-off values, no ARAs were found eligible for diagnostic purposes. Some ARAs were specific for (n)pAIR, but had low prevalence while others were more frequently identified but lacked specificity.

In concordance with previous findings, positive results of serum anti-recoverin autoantibodies were not only observed in patients with presumed (n)pAIR, but also in patients with uveitis and cataract. Furthermore, no association between the presence of autoantibodies directed against recoverin and the presence of a malignancy was found. A discrepancy between the antigen

suspension bead array results for anti-recoverin and the anti-recoverin immunoblot was found in three control patients (one with cataract and two with uveitis). The difference in results could be explained by the different techniques used for determination of ARAs imposing differences in analytical performance. Possibly, the number and/or availability of recoverin antigenic epitopes differed between the antigen suspension bead array and the immunoblot technique. The protein fragments we used in this study to screen for autoantibody reactivity in serum were designed to represent unique parts of each target protein. The binding of the autoantibodies towards their target may be influenced by the protein folding of antigens and may differ in comparison to full-length protein arrays or peptide arrays. Both linear and conformational epitopes, recognized by some ARAs, might be missed for some proteins, preventing recognition by certain autoantibodies.

The identification of new ARAs in (n)pAIR is in line with findings from previous studies using Western blot analysis for the determination of ARAs.[11,48] Although many ARAs have already been identified, several studies have described so far unknown retinal autoantibodies presumably damaging retinal tissue and causing loss of vision.[14] In our study, we were able to identify novel ARAs possibly associated with (n)pAIR, e.g. serotonin N-acetyltransferase. Serotonin N-acetyltransferase plays a role in melatonin synthesis and is expressed only in the pineal gland and retina.[49] Autoantibodies directed against serotonin N-acetyltransferase have to our knowledge not been described in (n)pAIR so far. Another novel, although unspecific ARA found in this study is anti-G protein-coupled receptor kinase 7. Interestingly, it has been suggested previously that G protein-coupled receptor kinases in cancer cell lines are functionally associated with recoverin.[50] Moreover, protein IMPG2 was identified as an ARA and reactivity towards the cytoplasmic protein region (antigen number 205) was associated with (n)pAIR. Autoantibody reactivity towards a second antigen representing an extracellular region of IMPG2 was in contrast present in serum from uveitis patients. In short, ARAs targeting IMPG2 were identified using antigen arrays in serum samples and a retina specific protein expression of IMPG2 identified using immunohistochemistry in healthy human tissue.

Our present study focused on the autoantibodies prevalent in serum, which reflects systemic production and is probably not influenced by potential (additional) production or accumulation of specific autoantibodies within the eye. Analysis of local, intraocular retinal autoantibodies might show an entirely different pattern and may differ in clinical importance compared to retinal autoantibodies found in the peripheral circulation. The importance of locally produced autoantibodies has already been shown in cerebrospinal fluid for the central nervous system. In addition, it is unknown which autoantibodies penetrate from the circulation, through the blood retina barrier, into the retina and cause a local inflammation. Further research addressing the intraocular presence of specific retinal autoantibodies might elucidate the clinically relevant autoimmune processes directed against the retinal tissue in (n)pAIR.

A gold standard for the definitive diagnosis of (n)pAIR is currently lacking. Also in this study, the diagnosis of presumed (n)pAIR was based on clinical symptoms. To compensate for this inaccuracy, we used strict inclusion criteria and selected a uniform cohort of patients with unexplained visual loss, visual field defects and decreased or absent ERG while other diagnostic possibilities leading to this configuration of clinical characteristics were (so far as possible) excluded. The presence of ARAs was found in all our patients with presumed (n)pAIR and therefore all fulfilled the criteria for the diagnosis of (n)pAIR.[9]

Although the mere presence of ARAs supports the diagnosis of (n)pAIR, it has been stated that there are no specific ARAs which would be exclusive for (n)pAIR and none of the ARAs were identified to be of higher diagnostic value than other ARAs.[4,9,14] Our results are in full agreement with this statement. Proof for the definitive diagnosis of (n)pAIR is still missing and even the presence of ARAs is not specific for (n)pAIR, which has been illustrated by the finding of ARAs in control groups and healthy individuals.[8,51,52]

In conclusion, our study identified a heterogenous reactivity pattern of ARAs in serum of patients with (n)pAIR, although the presence of ARAs was not discriminatory between (n)pAIR, cataract and uveitis and exhibited a low sensitivity. Therefore, the diagnosis of (n)pAIR cannot be based on the mere presence of serum ARAs and such presence thus warrants careful interpretation. The determination of ARAs in intraocular fluid might provide more insight into the pathogenesis of (n)pAIR and might indicate more sensitive and specific diagnostic tools. Therefore, future research on the prevalence of ARAs in ocular fluid represents an important next step.

SUPPLEMENTARY DATA

Supplementary Table. Amino acid sequence and uniprot ID of ocular antigens used for the autoantibody profiling
https://doi.org/10.1371/journal.pone.0167909.s001

Supplementary Figure. Spectrum of antiretinal autoantibodies in patients suspected of paraneoplastic and non-paraneoplastic autoimmune retinopathy, uveitis and cataract
https://doi.org/10.1371/journal.pone.0167909.s002

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6

ERNSTIGE VISUSDALING DOOR AUTO-IMMUUN RETINOPATHIE

SEVERE VISUAL LOSS CAUSED BY AUTOIMMUNE RETINOPATHY

Josianne C.E.M. ten Berge, Marco W.J. Schreurs, Diana Dufour-van den Goorbergh, Pauline M. de Witte, Mary J. van Schooneveld, Aniki Rothova

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SAMENVATTING

Achtergrond: Auto-immuunretinopathie (AIR) is een zeldzaam ziektebeeld dat onder andere als een paraneoplastisch syndroom kan voorkomen. AIR is geassocieerd met de aanwezigheid van anti-retinale antistoffen. Er wordt verondersteld dat deze antistoffen schade aan de retina veroorzaken, met progressieve visusdaling als gevolg.

Casus: Een 74-jarige man kwam bij de oogarts met ernstige, progressieve visusdaling, zonder noemenswaardige afwijkingen bij het standaard oogheelkundig onderzoek. Het elektroretinogram was kenmerkend voor functieverlies van de fotoreceptoren. In het serum werden anti-retinale antistoffen tegen recoverine aangetoond. Na doorverwijzing naar de internist vanwege de verdenking op een paraneoplastische AIR, werd een longcarcinoom gediagnostiseerd. De diagnose 'carcinoom-geassocieerde paraneoplastische AIR' werd hiermee bevestigd.

Conclusie: De verdenking op een paraneoplastische AIR is hoog bij een onbegrepen visusdaling, ook bij patiënten zonder bekende maligniteit. Recent zijn in Nederland laboratoriumtechnieken geïmplementeerd voor de bepaling van de anti-retinale antistof tegen recoverine, waardoor het stellen van de diagnose 'AIR' beter mogelijk is.

ABSTRACT

Background: Autoimmune retinopathy (AIR) is a rare disorder which may present as a paraneoplastic syndrome. AIR is associated with the presence of antiretinal antibodies. These antibodies are assumed to cause damage to the retina, resulting in progressive vision loss.

Case description: A 74-year-old man visited the ophthalmologist with a serious, progressive loss of vision, without any noteworthy abnormalities at routine ophthalmological examination. The electroretinogram was characteristic of loss of photoreceptor function. Anti-retinal antibodies against recoverin were detected in serum. After referral to an internist on account of a suspected diagnosis of paraneoplastic AIR, the patient was diagnosed with a lung carcinoma, confirming the diagnosis of cancer-associated paraneoplastic AIR.

Conclusion: An unexplained loss of vision is highly suggestive of paraneoplastic AIR, even in patients without a known malignancy. Laboratory techniques for the detection of the antiretinal antibody against recoverin have recently been implemented in the Netherlands, facilitating the diagnosis of AIR.

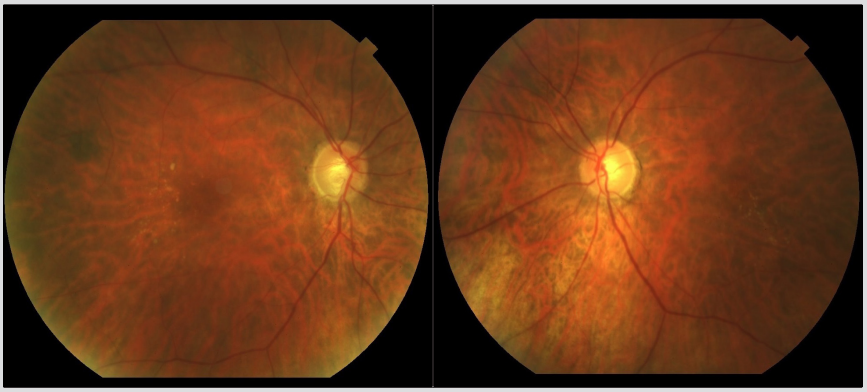
INTRODUCTIE

Auto-immuunretinopathie (AIR) omvat een zeldzame groep immuungemedieerde ziektebeelden die blijvende visusdaling tot gevolg kunnen hebben. AIR is geassocieerd met de aanwezigheid van anti-retinale antistoffen. Er wordt verondersteld dat deze antistoffen betrokken zijn bij de pathogenese van AIR. AIR kan als een paraneoplastische aandoening voorkomen, waarbij onderscheid wordt gemaakt tussen carcinoom-geassocieerde AIR en melanoom-geassocieerde AIR. Ook bestaat er non-paraneoplastische AIR; deze diagnose wordt per exclusionem gesteld.[1-4] In dit artikel bespreken wij een man met ernstige visusdaling als gevolg van carcinoom-geassocieerde AIR bij een longcarcinoom, met anti-retinale antistoffen in zijn serum.

ZIEKTEGESCHIEDENIS

Patiënt A, een 74-jarige blanke man, kwam bij de oogarts in verband met ernstige, progressieve, bilaterale visusdaling sinds enkele weken. Tevens had hij last van nachtblindheid en gekleurde lichtsensaties. In de voorgeschiedenis had patiënt goed gereguleerde diabetes mellitus type 2 en primair openkamerhoekglaucoom, waarvoor hij adequaat werd behandeld. De visus van het rechter oog was 1/300 (handbewegingen op 1 meter afstand) en van het linker oog 0,2. De oogdrukken waren normaal en aan het voorsegment werden geen afwijkingen geconstateerd. In fundo was de papil rechts nauwelijks afwijkend en links was de papil glaucomateus geëxcaveerd; de arteriën waren vernauwd, maar er was geen diabetische retinopathie. In de maculae waren enkele drusen zichtbaar. Dit is op oudere leeftijd niet ongebruikelijk (Figuur 1).

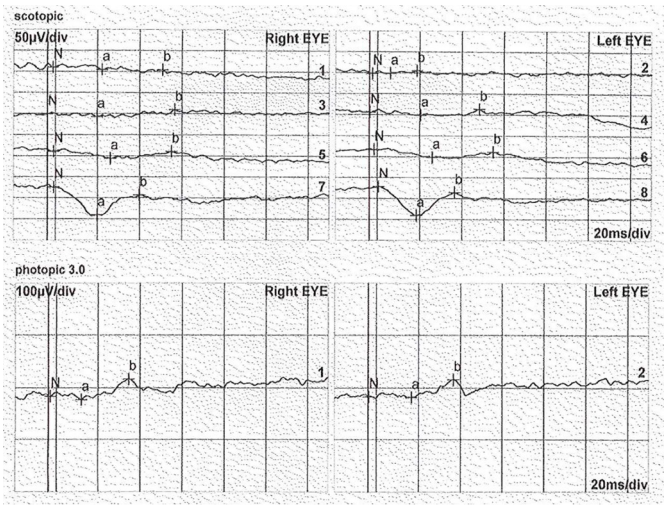
Figuur 1. Fundusfoto van patiënt A



De papil rechts is nauwelijks afwijkend en de papil links is ruimer geëxcaveerd. De arteriën zijn nauw en er zijn enkele drusen in de maculae zichtbaar.

Patiënt werd opgenomen op de afdeling Neurologie voor aanvullend onderzoek. Differentiaal diagnostisch dachten wij aan een intracranieel proces of een vasculaire oorzaak. Op de CT- en MRI-scan van de hersenen en met duplexechografie van de carotiden zagen wij echter geen noemenswaardige afwijkingen. Het laboratoriumonderzoek liet evenmin afwijkingen zien die pasten bij een infectie. Beeldvorming van het netvlies middels optische coherentietomografie toonde beschadiging van de fotoreceptoren. Het fluorescentie-angiogram gaf geen aanwijzingen voor een neuritis optica of een opticusinfarct. Op het elektroretinogram (ERG), waarbij de functie van de fotoreceptoren van de retina wordt gemeten, zagen wij echter sterk verlaagde fotopische en scotopische responsies; dit is een maat voor de functie van respectievelijk de kegeltjes en staafjes (Figuur 2). Op grond van deze bevinding en de klinische symptomen dachten wij aan AIR.

Figuur 2. Elektroretinogram (ERG) van het linker en het rechteroog van patiënt A

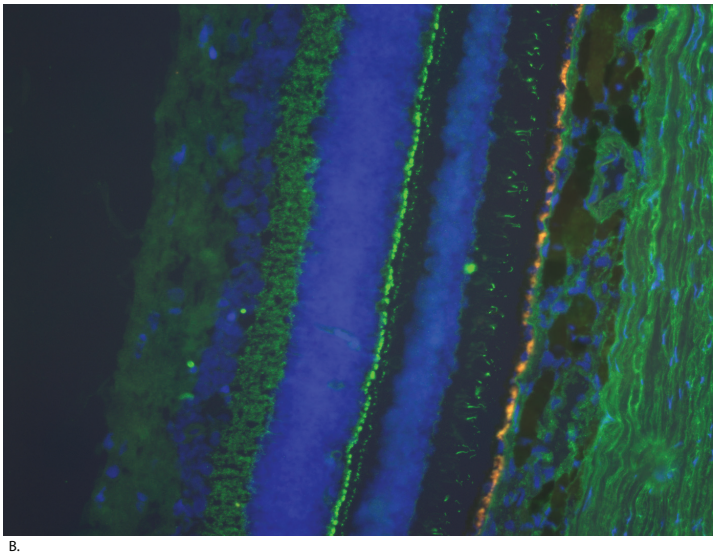


Hierbij wordt de elektrische activiteit van de retina geregistreerd als reactie op een stimulus (licht). De hoogte van de amplitude geeft de mate van respons weer. Zowel de fotopische (functie van de kegels) als de scotopische (functie van de staven) responsies zijn fors verlaagd in beide ogen.

Het serologisch onderzoek naar anti-retinale antistoffen bleek positief voor antistoffen tegen recoverine. Hiermee werd de diagnose 'AIR' bevestigd (Figuur 3). Patiënt kreeg prednison oraal, 80 mg/dag gedurende 3 dagen en vervolgens 45 mg/dag, om de visus te verbeteren. Deze behandeling had geen resultaat. Daarnaast werd patiënt door de oogarts verwezen naar de internist voor onderzoek naar een eventuele onderliggende maligniteit. De internist stelde vast dat patiënt een grootcellig neuro-endocrien carcinoom in stadium IIIA had. Hij kreeg chemotherapie en radiotherapie, waarop de tumor sterk in grootte afnam, maar dit leidde niet tot een verbetering van zijn visus. Op de CT-scan die 7 maanden na het begin van de therapie ter controle werd gemaakt, was slechts een resttumor zichtbaar. Er waren geen aanwijzingen voor metastases. Vanwege de blijvende ernstige visusdaling werden patiënt visuele hulpmiddelen en begeleiding aangeboden. Patiënt woonde 6 maanden na het stellen van de diagnose longcarcinoom thuis, in redelijk goede conditie.

Figuur 3. Recoverine auto-antistoffen: immunoblot en immunofluorescentie

A.



B.

(A) De immunoblot toont een sterke reactie van het serum van de patiënt op de test strip welke met het antigeen recoverine is gecoat. (B) Bij (indirecte) immunofluorescentie op een coupe van de retina (primaat) is bij het serum van de patient in het groen (FITC) een sterke fluorescentie te zien van gedeeltes van de fotoreceptoren en een gedeelte van de buitenste plexiforme laag, waarin in beide gevallen recoverine aanwezig is. De blauwe aankleuringen (DAPI) zijn de celkernen in de verschillende lagen van de retina.

BESCHOUWING

AIR kenmerkt zich door bilaterale, progressieve visusdaling die vaak gepaard gaat met lichtflitsen en andere visuele sensaties, gezichtsvelddefecten, nachtblindheid, kleurzienstoornissen en paracentrale of centrale scotomen.[1-6] Bij fundoscopie worden meestal geen of geringe afwijkingen gevonden; sommige patiënten tonen een bleke papil en dunne retinale vaten. Het ziektebeeld is bilateraal aanwezig, maar kan zich asymmetrisch presenteren.

Volgens de huidige hypothese wordt carcinoom-geassocieerde AIR veroorzaakt door immunologische kruisreactiviteit tussen tumorantigenen en retinale antigenen.[1-5] De aanwezigheid van circulerende anti-retinale antistoffen ondersteunt de diagnose 'AIR'. Sommige anti-retinale antistoffen kunnen ook voorkomen bij gezonde individuen, zij het in lage titer.[1-5,7] Verder zijn er patiënten met AIR zonder aantoonbare retinale auto-antistoffen. Van het gehele spectrum van verschillende anti-retinale antistoffen zijn de bepalingen van anti-recoverine en anti-enolase het meest gebruikelijk voor het stellen van de diagnose AIR.[4]

Visusklachten als paraneoplastisch verschijnsel

Visusklachten zijn meestal het eerste symptoom bij patiënten met carcinoom-geassocieerde AIR.[3-5] Pas nadat de verdenking op carcinoom-geassocieerde AIR is ontstaan, wordt de patiënt naar de internist doorverwezen en een carcinoom ontdekt. De latentietijd tussen carcinoom-geassocieerde AIR en de diagnose van een carcinoom varieert van weken tot jaren.[1,3-5] Bij patiënten met melanoom-geassocieerde AIR zijn de visusklachten met fotopsieën vaak het eerste teken van metastasering van een reeds bekend melanoom.[1,3-5] Onze patiënt werd in enkele weken zo goed als blind. Gezien zijn ernstige klachten, zonder duidelijke afwijkingen bij fundoscopie of aanwijzingen voor andere oorzaken, was de verdenking op AIR hoog. Een afwijkend ERG en de aanwezigheid van anti-retinale antistoffen ondersteunden deze diagnose.

AIR wordt meestal gezien als paraneoplastisch syndroom bij diverse soorten carcinomen. Het is daarom belangrijk verder onderzoek te verrichten naar een mogelijke maligniteit. Onze patiënt had een longcarcinoom, een van de typen carcinomen die gepaard gaan met paraneoplastische AIR. Andere carcinomen die met carcinoom-geassocieerde AIR kunnen voorkomen, zijn gynaecologische en hematologische maligniteiten, long-, mamma-, prostaat-, en coloncarcinoom.[1,3-5] De behandeling bestaat uit reductie van de tumorgrootte door middel van radiotherapie, chemotherapie of chirurgie.[1-3] Ook immunomodulatoren zouden een positief effect kunnen hebben op de visus.[8] Door de beperkte behandelingsmogelijkheden en de blijvende schade aan de retina is de prognose voor de visus vaak slecht.

Het ERG is cruciaal voor de diagnostiek naar AIR, maar de diagnostiek blijft lastig. Tot voor kort was het niet mogelijk om anti-retinale antistoffen in Nederland te laten bepalen. Op de afdeling

Immunologie van het Erasmus MC in Rotterdam is recent de diagnostiek naar de anti-retinale antistof tegen recoverine geïmplementeerd; deze methode bestaat uit indirecte immunofluorescentie en immunoblot. Tot op heden is het alleen in Duitsland mogelijk anti-retinale antistoffen tegen enolase te bepalen; bepalingen van andere antistoffen worden alleen in het kader van onderzoek verricht. In de nabije toekomst verwacht men meerdere anti-retinale antistoffen te kunnen bepalen, waaronder antistoffen tegen enolase, transducine en bipolaire cellen. Deze diagnostiek, die ook met ELISA kan worden uitgevoerd, zal naar verwachting voor routinebepalingen ter beschikking komen.

CONCLUSIE

Auto-immuunretinopathie is een zeldzame, snel progressieve oogaandoening waarbij anti-retinale antistoffen voorkomen die vermoedelijk destructie van de retina veroorzaken, met blijvende visusdaling als gevolg. De verdenking op paraneoplastische AIR is hoog bij een onbegrepen visusdaling bij patiënten met of zonder een maligniteit. Omgekeerd dient bij patiënten met AIR of de verdenking daarop onderzoek naar een mogelijke maligniteit verricht te worden. De anti-retinale antistof tegen recoverine kan sinds kort bepaald worden in het Erasmus MC, ter ondersteuning van de diagnose 'AIR'. Een visusdaling door AIR is vaak ernstig en blijvend en behandeling met immunosuppressiva is niet altijd toereikend. Mogelijk bieden nieuwe behandelingen die de vorming van antistoffen kunnen beïnvloeden, zoals rituximab, meer mogelijkheden in de toekomst.

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ANTIRETINAL ANTIBODIES IN CENTRAL SEROUS CHORIORETINOPATHY: PREVALENCE AND CLINICAL IMPLICATIONS

Josianne C.E.M. ten Berge*, Elon H. C. van Dijk*, Marco W.J. Schreurs, Jacolien Vermeer, Camiel J. F. Boon, Aniki Rothova

* These authors contributed equally

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ABSTRACT

Purpose: To investigate the possible role of autoimmune reactions directed against retinal tissue in central serous chorioretinopathy (CSC), by analyzing the presence of serum antiretinal antibodies (ARAs) and establishing their clinical relevance.

Methods: Sixty-three CSC patients were included and clinical characteristics were collected. Serum samples of all CSC patients, 101 uveitis patients, and 60 healthy donors were analysed for the presence of ARAs by indirect immunofluorescence. Furthermore, all CSC serum samples were analysed on Western blot. Correlations between laboratory findings and clinical features of CSC were determined by logistic regression.

Results: ARAs were present in 54% of the CSC patients, in 46% of uveitis patients (p=0.153), and in 17% of healthy controls (p<0.001). The majority of ARAs in CSC were directed against photoreceptors (27%), which occurred significantly more often compared to uveitis patients (15%, p=0.039) and to healthy controls (5%, p=0.003). No associations between clinical CSC characteristics and the presence of ARAs were found.

Conclusion: Serum ARAs are present in more than half of the CSC patients, and especially ARAs directed against photoreceptors were detected more frequently compared to both healthy controls and uveitis patients. Further research is warranted to unravel the role of ARAs in the pathogenesis of CSC.

INTRODUCTION

Central serous chorioretinopathy (CSC) is a specific and relatively common early-onset chorioretinal disease that primarily affects the macula. In CSC, a subretinal fluid (SRF) leakage through a dysfunctional retinal pigment epithelium (RPE) leads to detachment of the neuroretina.[1-3] A prolonged neuroretinal detachment in the macula causes permanent central visual loss due to photoreceptor atrophy.[1,2] Such a loss of visual acuity, sometimes associated with image distortion and loss of colour and contrast vision, may have a high impact on a patient's personal and professional life. Early diagnosis and treatment is desirable to try to improve the visual outcome and quality of life.[1,3-12]

The exact pathogenesis of CSC is currently obscure; presumably CSC occurs due to dysfunction of the RPE with hyperpermeability, swelling, and leakage of the underlying choroid.[3,13] Moreover, the optimal treatment for CSC is unknown.[14] CSC is up to six times more common in men (estimated mean annual age-adjusted incidence: 9.9 per 100,000) compared to women (estimated incidence: 1.7 per 100,000).[15] CSC is associated with the use of steroid containing medication, with odds ratios up to 37.1, as well as with endogenous hypercortisolism.[16-19] Familial occurrence of CSC has been described and recent studies have found evidence of genetic associations in CSC patients, including genetic polymorphrisms in the CFH gene, the ARMS2 gene, the C4b gene, and the CD5 gene.[20-27]

Although little is known about the exact cause of CSC, a role of the immune system via the complement system has been suggested based on associations found in complement-related genes. Recent evidence suggests a role for antiretinal antibodies (ARAs) in uveitis and age-related macular degeneration.[28-31] A systematic study on presence and the possible role of ARAs in CSC is currently lacking.

We hypothesize that damage to the RPE outer blood-retinal barrier in CSC may result in a secondary formation of ARAs, which may affect the clinical course of CSC. In this study, we set out to investigate the presence of serum ARAs in CSC patients and to analyse a possible correlation of ARAs with the clinical characteristics of CSC.

METHODS

Patient and data selection

In this study, we included 63 Caucasian patients with chronic CSC who visited the outpatient clinic of the Department of Ophthalmology of the Leiden University Medical Center, Leiden, the Netherlands. The diagnosis of chronic CSC was based on ophthalmic examination and multimodal imaging, including fundoscopy, optical coherence tomography (OCT) using either the

spectral-domain OCT or the Cirrus OCT device, fundus autofluorescence, fluorescein angiography (FA), and indocyanine green angiography. The diagnosis of chronic CSC was based on the presence of all of the following criteria: serous SRF on OCT, ≥1 area of multifocal diffuse leakage or irregular RPE window defects on FA, and corresponding hyperfluorescent areas on indocyanine green angiography. Patients diagnosed with acute CSC were excluded from this study, since it is currently unclear if acute CSC and chronic CSC represent a continuum or are separate disease entities. Patients with evidence of other retinal diagnoses, choroidal neovascularisation, and/or polypoidal choroidal vasculopathy were also excluded. Clinical data of CSC patients were collected from medical data files and included patient demographics (age, gender, and (family) history) and ocular characteristics comprising stage, duration, activity and laterality of CSC, use of steroids or immunosuppressive medications, presence of intraretinal edema, treatment for CSC, and central retinal thickness (distance from the outer part of the ellipsoid zone to the inner part of the internal limiting membrane; CRT). The CRT of the affected eye was selected; if both eyes were affected the right eye was included. Diffuse CSC was characterized by the presence of >5 disc areas of hyperfluorescent RPE changes or leakage on FA. Serum samples of 101 patients with uveitis (intermediate uveitis, posterior uveitis or panuveitis) were used as disease controls, since a higher prevalence of ARAs in serum in this cohort has been described previously.[30] Serum of 60 blood bank donors (gender and age unknown) was used as (presumed) healthy controls. The study adhered to the tenets of the Declaration of Helsinki. Approval of the ethics committee and institutional review board was obtained.

Detection of ARAs using indirect immunofluorescence (IIF)

Initial screening of sera for ARAs was performed as described previously.[30] In short, cryosections of primate retinal tissue generated by Euroimmun (Lubeck, Germany) were left unfixed and incubated with 1:100 diluted serum for 30 minutes at room temperature. Sections were washed in stagnant phosphate-buffered saline (PBS) and incubated with goat-anti-human IgG conjugated with fluorescein isothiocyanate (FITC) for 30 minutes at room temperature. Thereafter, sections were washed in stagnant PBS and embedded. The positive control consisted of retinal tissue incubated with 1:100 diluted serum of an antinuclear antibody (ANA) positive patient; for the negative controls we used incubation with PBS and 1:100 diluted serum of a healthy control.

Detection of ANA using IIF

All sera showing staining of the retinal nuclear layers (outer/inner nuclear layer, ganglion cell layer) on IIF were subsequently analysed for the presence of ANAs. ANA detection was performed by IIF using HEp-2 cells (Inova, San Diego, California, United States), as described before.[30] In summary, HEp-2 cells were incubated with 1:80 diluted serum for 30 minutes at room temperature. After washing in PBS with continuous stirring, slides were incubated for another 30 minutes with FITC-conjugated goat anti-human IgG with propidium iodide (Inova, San Diego, California, United States). Subsequently, slides were washed and embedded.

Evaluation of IIF results

All slides were evaluated with a fluorescence microscope (20x magnification) by two independent observers. Specific retinal layers (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, and rods and cones layer) were evaluated for the presence of fluorescent staining including intensity of the staining. When both ANA (on HEp-2-cells) and ARA (on primate retinal tissue) had an equivalent intensity of staining, a sample was scored as 'unknown' because this combination does not allow a proper discrimination between the presence and absence of ARAs, by possible masking due to ANAs. These samples were excluded from the final analyses.

Western blot analysis

All CSC patients were evaluated for the presence of ARAs using Western blot analysis. Healthy human retinal protein extract was obtained after retina tissue homogenization in PBS. Tissue fragments were removed by centrifugation and the supernatant was frozen at -80°C until use. Retinal tissue extract was fractionated by SDS-polyacrylamide gel electrophoresis and separated proteins were subsequently transferred to nitrocellulose membranes. The membranes were blocked by incubation with 5% non-fat dry milk and incubated with serum of CSC patients or with appropriate control serum at a dilution of 1:100 in 5% non-fat dry milk in tris-buffered saline (TBS) overnight. After multiple washes with 0.1% TBS, membrane bound human IgG was identified by horseradish peroxidase-conjugated F(ab') 2 goat anti-human IgG (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at a dilution of 1:5000 in 5% non-fat dry milk in TBS-Tween. Reactivity was visualized using enhanced chemiluminescence.

Statistics

First, descriptive analyses were performed to obtain information on the characteristics of the CSC patients. Second, logistic regression with correction for age and gender was employed to evaluate if the presence of ARAs is associated to CSC. Finally, logistic regression with correction for age and gender was used to identify any clinical characteristics that were possibly associated with the presence of ARAs. $P<0.05$ was considered statistically significant. All analyses were performed with IBM SPSS Statistics version 21.

RESULTS

Patient characteristics

All patient characteristics are shown in Table 1. A total of 63 chronic CSC patients (56 male, 7 female), with a mean age of 51 ± 9 years (range, 31-72 years) was included in this study. The median duration of CSC at the time of blood collection was 585 days (range, 3-7832 days). Diffuse CSC was present in 11/62 patients (18%; data not available for one patient). In 38/48 CSC patients

(79%; data not available for 15 patients) SRF was present at the moment of blood collection, indicating active CSC. The mean CRT was 134 ± 37 µm (range, 57-226 µm). Previous to blood collection, no treatment for CSC was given in 35/63 patients (56%), whereas 13/63 patients (21%) had received either micropulse or focal laser treatment and 10/63 patients (16%) had received photodynamic therapy.

Table 1. Clinical characteristics of 63 patients with central serous chorioretinopathy at the moment of blood collection

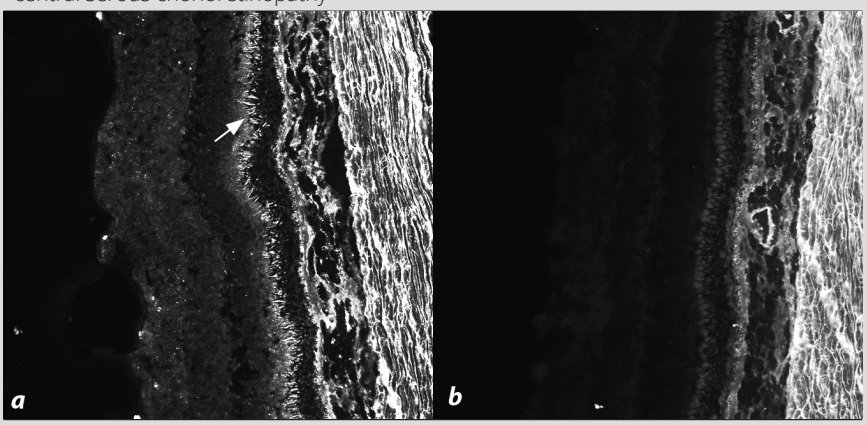
Patient characteristics	
Male-to-female ratio	8:1
Mean age (SD), in years	51 (9)
Median duration of CSC (min-max), in days*	585 (3-7832)
CSC characteristics	**Number (%)**
Stage **	
Focal CSC	51/62 (82%)
Diffuse CSC	11/62 (18%)
Active CSC **,‡	38/48 (79%)
Bilateral CSC during follow-up ‡	32/63 (51%)
Recurrent CSC **, ‡	26/62 (42%)
Familial CSC	2/63 (3%)
Presence of intraretinal edema during follow-up	10/63 (16%)
Mean central retinal thickness (SD), in micrometers **	134 (37) (46 patients)
Previous treatment for CSC	
Photodynamic therapy	10/63 (16%)
Micropulse or focal laser treatment	13/63 (21%)
Other treatments or combinations of treatment	5/63 (8%)
No treatment	35/63 (56%)
Systemic corticosteroids medication	
Never	33/63 (52%)
During diagnosis of CSC	30/63 (16%)
< 3 months before the diagnosis of CSC	7/63 (11%)
After or > 3 months before the diagnosis of CSC	13/63 (21%)
Use of systemic corticosteroids at the moment of blood collection	9/63 (14%)
Use of systemic immunosuppressive medication (excluding steroids) **	1/49 (2%)
Comorbidities **	
Autoimmune diseases	3/59 (5%)
Malignancies	4/59 (7%)

* *Duration of CSC: interval between the initial diagnosis of CSC and the moment of blood collection*
** *Data not available for all patients*
‡ *Presence of subretinal fluid is considered to be the indication of 'active' CSC*
Abbreviations: CSC = central serous chorioretinopathy, SD = standard deviation

ARAs on IIF in CSC patients

The IIF results are summarized in Table 2. Positive staining of the retina on IIF was present in 35/63 CSC patients (56%). After adjustment for retinal staining due to ANA, the presence of ARAs was confirmed in 32/59 CSC patients (54%). Among different staining patterns observed, staining of the photoreceptors was the most prevalent pattern in CSC patients (17/63; 27%; Figure 1). Other observed staining patterns in serum of CSC patients included staining of nuclear layers (N=8), fluorescence between the inner nuclear layer and the outer plexiform layer (N=7), fluorescence between the outer nuclear layer and the photoreceptors (N=3), and/or staining of the inner plexiform layer (N=2). Nine CSC patients had a combination of different staining patterns on IIF.

Figure 1. Staining patterns on indirect immunofluorescence from serum of patients with central serous chorioretinopathy



Serum of central serous chorioretinopathy patients was tested for the presence of antiretinal antibodies using indirect immunofluorescence with primate retinal tissue. Presence of antiretinal antibodies was visualized by labelling with fluorescein isothiocyanate (FITC). Panel A shows staining of the photoreceptors (arrow); panel B shows no retinal staining (absence of antiretinal antibodies).

The prevalence of ARAs in CSC patients was higher than in healthy controls (10/59; 17%; p<0.001). In addition, staining of the photoreceptors occurred in a lower percentage of healthy controls (5%) compared to CSC patients (p=0.003). In patients with uveitis, any positive staining of the retina (after correction for ANA presence) was observed in 39/84 patients (46%), which was not significantly different from CSC patients (p=0.153). In contrast, specific staining of the photoreceptors in uveitis patients (15%) was less prevalent compared to CSC patients (p=0.039). No significant differences in the prevalence of staining of other specific retinal layers between CSC patients and the two control groups were observed.

Clinical characteristics of CSC in relation to presence of ARAs on IIF

The presence of ARAs on IIF was higher in the six female CSC patients (100%) compared to the 57 male CSC patients (46%; p=0.024; Table 3). Staining of the photoreceptors in the samples of female and male CSC patients (57% versus 23%) did not differ (p=0.078). All other clinical characteristics of CSC (including age at onset, duration of CSC, stage of CSC, activity of CSC, unilateral versus bilateral CSC, recurrence of CSC, familial occurrence of CSC, CRT, presence of intraretinal edema, previous CSC treatment, and systemic steroid use during confirmation of the diagnosis of CSC) were not significantly associated with the presence of ARAs or specific staining of the photoreceptors. Masked assessment of the OCT images of the 17 CSC patients with photoreceptor staining on IIF and of 17 CSC patients without photoreceptor staining on IIF (randomly selected) showed no remarkable differences in signs of retinal damage.

ARAs on Western blot in patients with CSC

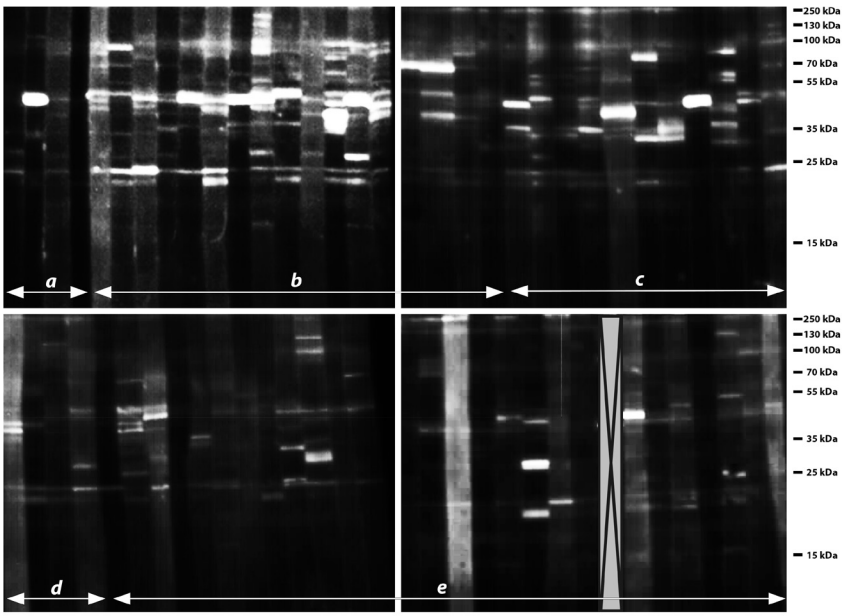
Multiple ARAs were observed in the majority of CSC patients (Figure 2). The most abundant ARA reactivity on Western blot was observed with those CSC patients showing staining of the photoreceptors on IIF, when compared to patients without retinal staining patterns as well as to patients with other staining patterns on IIF. The most prevalent ARAs on Western blot in CSC patients showing staining of the photoreceptors on IIF had molecular weights of approximately 24 kDa and 45 kDa. However, these antibodies were not entirely specific for samples with photoreceptor staining, since they were also present in CSC patients showing no staining of the photoreceptors on IIF, and even in patients with no staining at all.

DISCUSSION

We show that serum ARAs were present in 54% of patients with chronic CSC and in 17% of healthy controls. No significant difference in the prevalence of overall ARAs in serum was found between CSC patients and uveitis patients (46%), although staining of the photoreceptors occurred more often in CSC patients (27%) than in uveitis patients (15%). No differences in the presence of ARAs were observed when CSC patients were subdivided into specific subgroups based on clinical characteristics.

The multiple staining patterns on IIF in individual CSC patients indicate the presence of diverse ARAs, which was confirmed by Western blot analysis. Also, within patients who were solely showing staining of the photoreceptors on IIF, Western blot indicated the presence of diverse antibodies directed against the retina. This suggests that diverse retinal antibodies are associated with photoreceptor staining.

Figure 2. Reactivity on Western blot from serum of patients with central serous chorioretinopathy



Serum of patients with central serous chorioretinopathy was evaluated for the presence and approximate size of antiretinal antibodies using Western blot analysis with retinal protein extract. The results are sorted based on staining patterns on indirect immunofluorescence : a. nuclear staining (antinuclear antibodies (ANA) negative); b. photoreceptor staining; c. diverse staining patterns; d. nuclear staining (ANA positive); e. no staining.

The reason for the higher prevalence of ARAs in CSC, especially those directed against photoreceptors, and their clinical significance is currently not clear. Possibly, chronic retinal damage caused by the presence of SRF accumulation, associated with a breakdown of the RPE (which normally constitutes the outer blood-retinal barrier), may trigger formation of ARAs through an inflammatory reaction of the immune system.[32] In the past, ARAs have been shown in autoimmune retinopathy, in which ARAs are suggested to have pathogenic properties. Elevated serum ARAs were also observed in other chorioretinal diseases (e.g. uveitis, macular degeneration, retinitis pigmentosa) as well as in the healthy population.[28-30,33-44] Here, an immune response to ocular tissue damage has been previously suggested to play a role in aggravation of the various retinal diseases.[42,45] Similarly, autoreactive immunologic responses have been found in patients with proven infectious uveitis, in which the primary cause (infection) presumably incited the secondary formation of antibodies, possibly due to tissue damage.[46] Interestingly, recent genetic studies in CSC have found an association with genetic variants in the complement system, an essential part of the innate immune system.[25,47] However, it is unclear if there is a primary role of the

Table 2. Presence of antiretinal antibodies in patients with central serous chorioretinopathy and control groups

	CSC (N=63)	Uveitis (N=101)	P-value	Odds ratio (95% CI)	Healthy controls (N=60)	P-value*	Odds ratio (95% CI)*
Staining of any retinal layer(s)	32/59 (54%)**	39/84 (46%)	0.153	1.806 (0.803-4.063)	10/59 (17%)	< 0.001	5.807 (2.479-13.606)
Staining of photoreceptors	17/63 (27%)	15/101 (15%)	0.039	2.864 (1.054-7.786)	3/60 (5%)	0.003	7.022 (1.938-25.442)

* Data not adjusted for age and gender

** In four patients, both antinuclear antibodies and antiretinal antibodies were present, and these patients were therefore excluded from final analysis. Exclusion of these samples did not affect the results of the statistical analysis.

Abbreviation: CSC = central serous chorioretinopathy

Table 3. Presence of antiretinal antibodies in relation to clinical characteristics of central serous chorioretinopathy

	Staining of any retinal layer(s) (N=32/59)	P-value	Staining of photoreceptors (N=17/63)	P-value
Gender				
Male	26/53 (49%)	0.018 **	13/56 (23%)	0.078 **
Female	6/6 (100%)*		4/7 (57%)	
Mean age (SD), in years	51 (9)	0.675 **	50 (9)	0.739 **
Median duration of CSC (min-max), in days ‡	261 (3-7832)	0.299	530 (3-6570)	0.446
Stage‡‡				
Focal CSC	23/47 (47%)	0.066	13/51 (26%)	0.405
Diffuse CSC	8/11 (73%)		3/11 (27%)	
Activity, #**				
Active	20/36 (56%)	0.782	12/38 (32%)	0.573
Not active	4/9 (44%)		2/10 (20%)	
Laterality of SRF during follow-up				
Unilateral	15/30 (50%)	0.496	9/31 (29%)	0.645
Bilateral	17/29 (59%)		8/32 (25%)	

Recurrence‡‡				
Recurrent CSC	11/24 (46%)	0.393	5/26 (19%)	0.379
First CSC episode	21/34 (62%)		12/36 (33%)	
Familial				
Familial CSC	1/2 (50%)	0.949	1/2 (50%)	0.454
Not familial CSC	31/57 (54%)		16/61 (26%)	
Mean central retinal thickness (SD), in micrometers‡‡				
	125 (32) (24 patients)	0.091	121 (39) (14 patients)	0.080
Intraretinal edema during follow-up				
Present	7/9 (78%)	0.222	4/10 (40%)	0.288
Absent	25/50 (50%)		13/53 (25%)	
Treatment: photodynamic therapy (PDT)				
PDT	6/10 (60%)	0.665	3/10 (30%)	0.804
No(t solely) PDT	26/49 (53%)		14/53 (26%)	
Treatment: (focal and/or micropulse) laser				
Laser treatment	7/13 (54%)	0.956	6/13 (46%)	0.053
No(t solely) laser treatment	25/46 (54%)		11/50 (22%)	
Use of corticosteroids				
During or < 3 months before CSC diagnosis	25/49 (51%)	0.674	12/53 (23%)	0.138
No current use	7/10 (70%)		5/10 (50%)	

* After the study was completed, three additional samples from female patients with CSC were analyzed and all showed absence of ARAs on IIF, resulting in an ultimate percentage positive ARAs among females of 67% (6/9), which is not distinct from male CSC patients (26/53; 49%).
** Determined by χ^2 test (gender) or Mann Whitney U test (age)
‡ Duration of CSC: from CSC diagnosis to the moment of blood collection
‡‡ Data not available for all patients
# Presence of subretinal fluid is considered to be the indication of 'active' CSC
Abbreviations: CSC = central serous chorioretinopathy, SD = standard deviation

immune system in the pathogenesis of CSC. The mere presence of serum autoantibodies does not necessarily indicate an autoimmune basis of the disease. Moreover, ARAs were not found in all patients and exhibited a wide variety.

The presence of ARAs in serum of CSC patients might be hypothetically explained by their immune predisposition. CSC can occur in patients with various immune-mediated diseases including membranoproliferative glomerulonephritis and systemic lupus erythematosus.[48,49] It is unclear whether CSC in these patients is only caused by glucocorticoid treatment prescribed for these conditions or if it is also influenced by the presence of inflammatory disease.[22] Another potential systemic mechanism in the development of ARAs could be the mimicry between ocular antigens and microbial proteins, brought up by a previous systemic infection.[50] However, the presence of antecedent infectious disorders in CSC has not previously been assigned as its cause. A higher prevalence of Helicobacter pylori infections in CSC patients has been described, although a firm association has never been proven.[14,51,52] Moreover, autoimmune responses would be rather expected in a female-dominated disease. In CSC, particularly men between the age of 25 and 55 are affected, analogous to the phase of life in which highest androgen levels can be detected, suggesting a possible role of this hormone within the development of CSC. In our study, the role of the immune system in CSC seemed to be most important in female CSC patients, as ARAs occurred in all six initially included female CSC patients. However, after our study was completed, we had the possibility to analyse three extra samples from female patients with CSC. All three samples showed absence of ARAs on IIF, resulting in similar occurrences of ARAs in male and female CSC patients (Table 3).

So far it is unclear whether ARAs are involved in the pathogenesis of CSC or whether their presence represents a secondary epiphenomenon. As CSC is mainly a disease of the choroid and RPE, experiments assessing antibodies directed against the choroid and RPE could give more insight to the pathogenesis.[1-3,13] The assessment of intraocular ARAs might help to clarify the possible pathogenic role of ARAs.[30]

The prevalence of ARAs in serum of blood bank donors of 17% is similar to the prevalences of ARAs in healthy controls in previous studies.[29,30,43,45,46,53] One could argue that some of the blood donors might have ocular conditions which could also lead to the formation of ARAs. However, this would not affect our results as we found a difference in the prevalences of ARAs even with our blood bank controls.

In conclusion, serum ARAs are more common in CSC patients than in healthy controls. No clear association between the presence of ARAs and clinical CSC characteristics could be identified in the current cohort. To unravel the possible involvement of autoimmune reactivity in the pathogenesis of CSC, further research is warranted.

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ANTIRETINAL AND ANTINUCLEAR ANTIBODIES IN UVEITIS WITH LATENT AND ACTIVE TUBERCULOSIS

Rina La Distia Nora, Josianne C.E.M. ten Berge, Aniki Rothova, Marco W.J. Schreurs

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The pathogenesis of uveitis in the setting of active and latent tuberculosis (TB) is not entirely clarified. Next to genuine infection, an important part of pathogenesis was attributed to (auto)immune reactions initiated by Mycobacterium tuberculosis (Mtb).[1] Infection with Mtb can be associated with the production of diverse serum autoantibodies.[2] Herein, we investigate the influence of (latent) TB on the presence of serum antinuclear and antiretinal autoantibodies (ANA and ARA) in Indonesian patients with uveitis.

Blood samples from patients with uveitis associated with active (not yet treated) pulmonary TB (N=10) and uveitis of unknown cause (N=85) were collected from June 2014 until May 2015. Classification of patients was performed according to SUN classification, and specific diagnoses were determined after the basic work-up for uveitis as indicated in our previous publication.[3] The diagnosis of active pulmonary TB was based on clinical and/or microbiological and radiological findings.[3] This study was performed with the approval of the local medical ethical committee.

All patients underwent QuantiFERON-Tb Gold (QFT) (Cellestis Inc., Carnegie, Australia). Screening for the presence of ANA using HEp-2 cells (Inova, San Diego, California) and the presence of ARA using primate retinal tissue (Euroimmun, Lubeck, Germany) was performed by indirect immunofluorescence as described before.[4] Logistic regressions with correction for age and gender were performed using SPSS to analyse differences in the presence of ANA and ARA between the diagnosis groups.

Patients with uveitis of unknown cause were divided according to their QFT results in 58 patients with latent TB (QFT positive) and 27 patients without evidence of prior TB exposure (QFT negative). All QFT positive patients were assessed by the pulmonologist and examined for the possible presence of pulmonary and extrapulmonary TB, but no cases of extrapulmonary involvement were found. The group with an unknown cause of uveitis and latent TB consisted of more female patients compared to the other groups (72% vs 30% in uveitis in the setting of active pulmonary TB group and 41% in the uveitis of unknown cause and QFT negative group, p-value 0.044) and older age patients (mean age 46 years vs 40 years in uveitis in the setting of active pulmonary TB group and 39 years in uveitis of unknown cause and QFT negative group, p-value 0.003). The median QFT value in patients with uveitis of unknown cause and latent TB was 5.0 IU/ml, and in patients with known tuberculosis induced uveitis 1.7 IU/ml.

Patients' serum ANA and ARA results are shown in Table 1. Patients with uveitis and either active or latent TB were characterized by high prevalence of systemic autoreactivity (ANA positive). In contrast, a higher proportion of organ-specific autoreactivity (ARA positive) was found in uveitis patients without evidence of any previous contact with Mtb. Induction of ANA, reported previously for active TB, apparently also occurs in latent TB.[5] Interestingly, organ-specific autoreactivity (ARA) appears to be suppressed in both active and latent TB, however, the local production of

ARA in ocular fluid samples was not investigated in this series. The presence of serum autoantibodies, directed against endothelial cells, and their decrease following treatment was reported in age-related macular degeneration.[6] Unfortunately, we have no samples of our patients after they completed the treatment. Further studies are warranted to dissect the pathogenesis of this selective systemic induction of autoreactivity in uveitis patients as a result of Mtb infection and its implication on disease course.

Table 1. Prevalence of antinuclear and antiretinal antibodies in tuberculosis induced uveitis and uveitis of unknown cause with positive or negative QuantiFERON–TB Gold outcomes

	ANA positive	ARA positive
Total	18/95 (19%)	39/87 (45%)
1. Uveitis in the setting of active pulmonary TB	5/10 (50%)	4/9 (44%)
2. Uveitis of unknown cause, QFT positive	12/58 (21%)	19/52 (37%)
3. Uveitis of unknown cause, QFT negative	1/27 (4%)	16/26 (62%)
p-value: 1 vs. 2 vs. 3	0.023	0.049
p-value: 1 vs. 2	>0.05	>0.05
p-value: 2 vs. 3	>0.05	0.014
p-value: 1+2 vs. 3	0.03	0.021

Serum ANA were more prevalent in patients with TB-induced uveitis (50%) than in patients with uveitis of unknown cause with latent TB (21%) or without latent TB (4%; p=0.023). The prevalence of ARA was higher in QFT negative patients with unknown uveitis cause (62%) than in QFT positive uveitis cases (p=0.049). Prevalence of ARA or ANA did not differ between TB-induced uveitis and QFT positive uveitis of unknown cause.
Abbreviations: ANA = antinuclear antibodies, ARA = antiretinal antibodies, OR = odds ratio, QFT = QuantiFERON–TB Gold test

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ANTIRETINAL ANTIBODIES IN MEXICAN CHILDREN WITH SEVERE PARS PLANITIS

Luz Elena Concha-del Río, Maria de Lourdes Arellanes-García, Marco W.J. Schreurs, Aniki Rothova, Josianne C.E.M. ten Berge

Submitted for publication

ABSTRACT

Pars planitis (PP) is a subtype of intermediate uveitis and a particularly severe form of PP has been described in pediatric Mexican patients. Although the pathogenesis of PP is not clarified, autoimmune reactions have been suggested to play a role. This study investigates the presence and possible clinical role of serum antiretinal antibodies (ARAs) of Mexican children with PP (N=16) and age-matched controls with strabismus (N= 19). All samples were tested for the presence of ARAs by indirect immunofluorescence using primate retinal tissue. Serum ARAs were noted in 57% (8/14) of PP patients and in 40% (6/15) of controls (p=0.356), and no correlation was found between the presence of ARAs and clinical characteristics of PP. Insight in the cellular component of the immune system and / or analyses of intraocular fluids of PP eyes might provide further insight in the pathogenesis of PP.

INTRODUCTION

Pars planitis (PP) is characterized predominantly by vitreous inflammation, with or without vitreous condensations ("snowballs"), pars plana exudates ("snowbanks") in at least one eye, and peripheral retinal vasculitis. An exceptionally severe form of PP has been reported in children with uveitis from Mexico.[1-3] The clinical course of PP is usually chronic with exacerbations and has great on patients' well-being and quality of life. Despite the numerous reports on intermediate uveitis, the specific cause and pathogenesis of PP are unknown.[4] PP is considered to be of autoimmune origin, but possible inciting antigens are so far entirely unknown. In this short report, we assess the presence of antiretinal antibodies (ARAs) in serum of in young patients with PP from Mexico and evaluate their possible clinical role.

METHODS

We prospectively collected serum samples from 16 patients with PP and 19 age-matched children with strabismus who served as controls, from the Eye Inflammatory Disease Clinic at Hospital "Dr. Luis Sánchez Bulnes", Asociación Para Evitar la Ceguera in Mexico. Clinical data were collected from medical data files (Table 1). The study was performed in accordance with the Declaration of Helsinki and in agreement with the institutional regulations and approval of local institutional review boards.

Table 1. Clinical characteristics of patients with pars planitis

Characteristic	Number (%)
Age onset (median; interquartile range)	8; 6-10
Gender (male:female)	9:7
Bilateral pars planitis	13/15 (87%)$_1$
Anterior chamber inflammation	13/16 (81%)
Anterior synechiae	2/16 (13%)
Posterior synechiae	6/16 (38%)
Cyclitic membrane	6/16 (38%)
Snowballs	16/16 (100%)
Snowbanking	7/15 (47%)*
Phlebitis	15/15 (100%)*
Systemic treatment**	14/16 (88%)

* Data not available for all patients
** Prednisone and/or systemic immunosuppressive therapy

Screening of serum samples for the presence of ARAs was performed by indirect immunofluorescence using primate retinal tissue (Euroimmun, Lubeck, Germany), as described before.[5] The determination of ARAs was not possible in 2 patients and 4 controls, due to positivity of antinuclear antibodies which mask the possible presence of ARAs. Mann Whitney U tests for continuous data and Chi-square tests for categorical data were performed to analyse differences on the presence of ARAs between the diagnosis groups and specific clinical characteristics of PP.

RESULTS AND DISCUSSION

Serum ARAs were found in 57% (8/14) of PP patients and in 40% (6/15) of controls (p=0.356). Staining of photoreceptors was the most frequently observed pattern with similar prevalence in both groups. The presence of ARAs was not associated to any clinical characteristic of PP. The high prevalence of ARAs in our young control patients without retinal disease (40%) is remarkable and much higher than the 17% observed in the general population in Europe.[6] The possible explanation might be a higher exposure to exogenous antigens such as infections in Mexican children. Despite the high prevalence of 57% of serum in young patients with PP, our results suggest that ARAs are not the primary cause of the ocular disease, but possibly represent a secondary phenomenon.

In conclusion, the prevalence of serum ARAs is similar in pediatric PP and controls from Mexico. Further assessment of potential infectious agents and insight in the cellular component of the immune system and/or assessment of intraocular ARAs of PP eyes might provide further insight in the pathogenesis of PP.

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AUTOANTIBODY PROFILING IN INTRAOCULAR FLUID OF PATIENTS WITH UVEITIS

Josianne C.E.M. ten Berge, Marco W.J. Schreurs, Joost van Rosmalen, Aniki Rothova

Submitted for publication

ABSTRACT

A high prevalence of serum antiretinal antibodies (ARAs) in patients with uveitis has been previously described, though their clinical role remains elusive. Assessment of intraocular ARAs may provide further insight into the pathogenesis of diverse uveitis entities. In this study we investigate the prevalence of multiple specific anti-ocular antibodies (AOcAs), including ARAs, in intraocular fluid of patients with uveitis. Autoantibody profiling with 188 different ocular antigens was performed by a multiplex immunoassay with intraocular fluid samples of 76 patients with uveitis. Clinical data from uveitis patients were collected and statistical analyses were executed to evaluate associations between intraocular AOcAs and clinical characteristics. Controls consisted of 19 intraocular fluid samples from cataract patients. A spectrum of 22 different AOcAs was present in higher levels in patients with uveitis than in controls ($p \leq 0.05$), but in moderately elevated titers (<2x). High elevations of intraocular AOcAs in uveitis (>5x compared to cataract) were observed in varicella zoster virus-induced uveitis, multiple sclerosis-associated uveitis and patients with unexplained uveitis but positive quantiferon test. Presence of macular edema was associated with increased intraocular levels of tyrosinase antibodies. Our results show that patients with uveitis are characterized by the presence of a broad spectrum of moderately elevated levels of intraocular AOcAs, and high intraocular AOcA levels were found in several specific uveitis entities. This study favors secondary production of AOcAs and not their inciting role.

INTRODUCTION

Uveitis is a severe ocular disease, which can result in permanent blindness. Uveitis has multiple causes including infections and shows strong associations with various systemic immune-mediated diseases. The pathogenesis of most uveitis entities is not fully understood, but the immune system plays a crucial role; especially the development of autoimmune intraocular reactions in non-infectious uveitis has been repeatedly proposed, but scarcely proven. Serum antibodies directed against retinal tissue were more prevalent in patients with uveitis compared to healthy controls.[1] It has been hypothesized that the antiretinal antibodies (ARAs) in uveitis might either incite the ocular disease or represent a secondary epiphenomenon induced by retinal damage. The possible pathogenic role of these ARAs is unknown, although it has been suggested that ARAs might aggravate and/or prolong the ocular disease.[2-4]

The eye is an immune privileged organ and intraocular ARAs might show an entirely different profile than ARAs found in the peripheral blood. This phenomenon has been shown previously in infectious uveitis, in which local production of specific antibodies is regarded as indirect proof of the intraocular infection.[5] Similarly, in neurological diseases including autoimmune encephalitis and multiple sclerosis the importance of analysis of cerebrospinal fluid has been previously proven.[6, 7] Currently, antigen bead arrays are successfully being used for the analysis of autoantibodies. This technique enables analysis of very small volumes (such as intraocular fluids) towards hundreds of different antigens and its potential for ARA detection has been suggested.[8]

Herein, we perform an autoantibody profiling of intraocular fluid samples of patients with diverse uveitis entities and assess the prevalence of antibodies directed against 188 different ocular antigens as potential targets, and relate the results to clinical manifestations of uveitis.

METHODS

Remainders of diagnostic intraocular fluid samples from 76 patients with uveitis were collected from the Laboratory of Virology of the Erasmus MC, University Medical Center Rotterdam between February 2009 and April 2015. Intraocular fluid samples of 19 patients with cataract stored in the biobank in the same time period were used as controls. All intraocular fluid samples were stored at -80°C. This study was approved by the local ethical committee from the Erasmus University Medical Center (Medical Ethics Committee Erasmus MC) and adhered to the tenets of the Declaration of Helsinki.

All uveitis patients were classified according to the localization of uveitis using the Standardization of Uveitis Nomenclature SUN criteria.[9,10] Patients underwent a standardized diagnostic protocol

based on this anatomical site of inflammation. The protocol included chest radiography, erythrocyte sedimentation rate, blood counts, serum angiotensin-converting enzyme levels, serology for syphilis and Lyme disease and interferon gamma release assay (IGRA) test (QuantiFERON–TB Gold In-Tube test). In patients with anterior uveitis or panuveitis Human Leukocyte Antigen (HLA)-B27 testing was also performed. According to the clinical uveitis manifestations, further examinations were added (tailored approach). Specific diagnoses were determined after the various diagnostic procedures were completed. The diagnosis of intraocular infections was always confirmed by polymerase chain reaction (PCR) and/or Goldmann-Wittmer coefficient in intraocular fluid. The diagnosis of Fuchs uveitis syndrome (FUS) was based on clinical characteristics. The diagnosis of sarcoidosis was either histologically proven or based on chest imaging in patients with otherwise unexplained uveitis . All other specific diagnoses were made according to current diagnostic criteria.

Clinical data of patients with uveitis were collected from medical data files. We registered age, gender, location of uveitis and specific cause of uveitis. Further, we registered the following characteristics at the moment of sample collection: duration of uveitis, presence of cystoid macular edema (CME), presence of vasculitis, use of systemic immunosuppressive medication and activity of uveitis.

Autoantibody profiling with 188 different ocular antigens, representing 97 unique ocular proteins, was performed on intraocular fluid samples from patients with uveitis (N=76) and cataract (N=19; Supplementary Table). Antigens were selected based on potential relevance to ocular diseases according to literature or previous retinal immunohistochemistry staining. The used antigens were protein fragments produced within the Human Protein Atlas, designed to have low homology to other human proteins and expressed in Escherichia coli with an affinity tag consisting of six histidines and an albumin-binding domain from streptococcal protein G (His6ABP).[11,12] A multiplex assay, previously validated by immunoblot and immunohistochemistry, was performed as described before with minor alterations.[13] In short, the antigens were covalently coupled to color-coded magnetic beads to create a bead array. The samples were diluted 1:10 in assay buffer (0.1% PBS-Tween20, 3% BSA, 160 µg/ml His6ABP), let to pre-block potential antibodies towards the ABP-domain for 1 hour in room temperature, and subsequently incubated O/N in room temperature with the bead array. Interactions were fixated with 0.2% PFA for 10 min before incubation for 30 min with a fluorophore conjugated anti-human IgG Fab fragment. A FlexMap3D instrument (Luminex Corp.) was used to acquire a read-out. The autoantibody profiling was performed at the SciLifeLab Autoimmunity Profiling Facility in Stockholm, Sweden.

For the statistical analyses, continuous variables were summarized using medians and ranges, and categorical variables were summarized using percentages. Patient demographics were compared between diagnosis groups using Mann Whitney U tests for continuous data and Fisher's

exact tests for categorical data. Linear regressions with correction for age and gender were performed to analyse differences between the diagnosis groups (uveitis or specific uveitis entities versus cataract). In addition subgroup analyses of these linear regressions were performed to compare specific uveitis entities with cataract. To analyse differences within the uveitis group we performed linear regressions with age, gender, location of uveitis (reference category: anterior uveitis), uveitis entity (reference category: uveitis associated to sarcoidosis), uveitis activity, systemic immunosuppressive therapy, presence of CME and presence of vasculitis as independent variables. Some specific uveitis entities were not analysed as separate groups (uveitis associated with herpes simplex virus (HSV), Sjögren syndrome, HLA B27-associated uveitis and birdshot chorioretinopathy), because their numbers were too small. All regression analyses were performed using the natural logarithm of the measured median fluorescent intensities as dependent variable. Relative (fold) increases of differences in levels of AOcAs were calculated by exponentiating the estimated regression coefficients. The linear regressions were performed with a robust MM-type estimation method, to account for the fact that the model residuals were not normally distributed.[14] We used the signal intensities as an indication for the levels of intraocular AOcAs. To adjust for the multiple comparisons of the different antigens, a Bonferroni correction was applied (all p-values were multiplied by 188). P-values of ≤0.05 were considered statistically significant. All statistical tests were two-sided. The analyses were performed using SPSS and R (version 3.3.1), with the robustbase package for the robust linear regressions.[15]

RESULTS

The median age of the 76 included uveitis patients was 48 years, and 46% of patients were males. Median duration of uveitis was two years (ranging from zero to 36 years), and 51 (67%) cases were active during sample collection. Patients' characteristics are shown in Table 1. The most prevalent cause of uveitis in our cohort was sarcoidosis (14/76, 18%). Nine patients with sarcoidosis-associated uveitis were biopsy proven and in five patients the diagnosis was based on radiologic criteria. Varicella zoster virus (VZV)-induced uveitis included 4 patients with retinitis and 3 with anterior uveitis. Cataract patients were older than the uveitis patients (median age of 69 years, p=0.0001) and 42% were males (p>0.05).

The levels of 22 different intraocular AOcAs were higher in uveitis than in controls (all p≤0.05 as determined by linear regression), but with moderately increased titers (up to 2x compared to controls). The most significant results were observed in the AOcA levels specific for RPE-retinal G protein-coupled receptor (number 169; p=0.0031) and retinol dehydrogenase 8 (number 160; p= 0.0042). Levels of intraocular AOcAs varied greatly between different uveitis entities. Intraocular AOcAs with at least 5-fold increased titers (compared to cataract controls) were observed in three specific entities: VZV-induced uveitis, multiple sclerosis (MS)-associated uveitis and patients

with unexplained uveitis but with a positive quantiferon test (all p<0.05; Table 2 and Figure 1). Intraocular levels of all AOcAs in FUS (with or without an intraocular rubella virus infection) were similar to cataract controls (all p>0.05).

Table 1. Characteristics of uveitis patients (N=76)

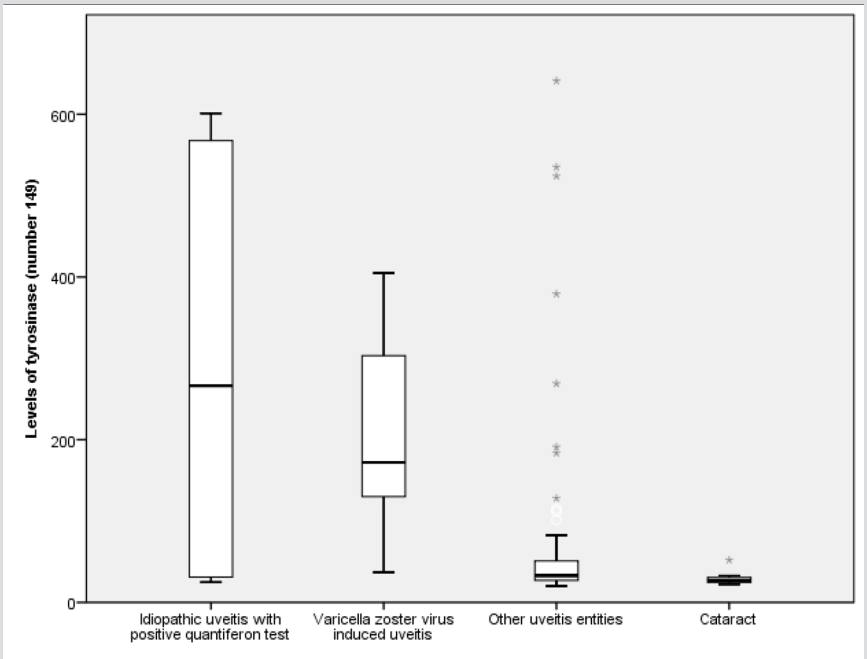
Characteristic	Number (%)
Male-to-female ratio	35 - 41 (46% - 54%)
Median age in years (range)	48 (17 - 86)
Median duration uveitis in years (range)	2 (0 – 36)
Location	
Anterior	26 (34%)
Intermediate	10 (13%)
Posterior	23 (30%)
Panuveitis	17 (22%)
Specific cause or association	
Infectious	36 (47%)
Rubella	12 (16%)
Toxoplasma gondii	8 (11%)
Varicella zoster virus	7 (9%)
Cytomegalovirus	7 (9%)
Herpes simplex virus	2 (3%)
Systemic disease	24 (32%)
Sarcoidosis	14 (18%)
Multiple sclerosis	8 (11%)
Sjögren syndrome	1 (1%)
HLA B27 positive	1 (1%)
Ocular lymphoma	5 (7%)
Clinical entity	3 (4%)
Birdshot chorioretinopathy	2 (3%)
Fuchs uveitis syndrome	1 (1%)
Unknown	8 (11%)
Quantiferon positive	6 (8%)
Active uveitis	51 (67%)
Presence of cystoid macular edema	19 (25%)
Presence of vasculitis	16 (21%)
Systemic immunosuppressive treatment	16 (21%)

Table 2. Prevalence of anti-ocular antibodies in specific uveitis entities (N=76) compared to cataract (N=19)*

Antigen no.	Antigen name	Short name	p-value**	Fold increase
Varicella zoster virus induced uveitis vs. cataract				
186	G protein-coupled receptor kinase 7	GRK7	<0.00001	31.7
80	Neural retina-specific leucine zipper protein	NRL	<0.00001	11.3
149	Tyrosinase	TYR	<0.00001	6.4
104	Retinol-binding protein 3	RBP3	<0.00001	5.6
Multiple sclerosis associated uveitis vs. cataract				
102	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2	GNGT2	<0.00001	25.1
107	Retina and anterior neural fold homeobox protein 2	RAX2	<0.00001	12.3
33	Recoverin	RCVRN	<0.00001	8.5
153	Retinal-specific ATP-binding cassette transporter	ABCA4	<0.00001	5.4
87	Fructose-bisphosphate aldolase C	ALDOC	<0.00001	5.23
Idiopathic uveitis with positive quantiferon test vs. cataract				
149	Tyrosinase	TYR	<0.00001	18.1
164	Retinoschisin	RS1	<0.00001	8.3
11	Oxygen-regulated protein 1	RP1	<0.00001	7.8
143	Cbp/p300-interacting transactivator 1	CITED1	<0.00001	7.7

* P-values for comparison of intraocular AOcA levels between specific uveitis entities and cataract were determined by robust linear regression. Only significant p-values ($p<0.05$) with fold increases >5 are shown in this table.
** after Bonferroni correction for multiple testing

Figure 1. Levels of intraocular tyrosinase (number 149) in different uveitis entities and in cataract



* Extreme outliers: <(25th percentile -/- 3x interquartile range) or >(75th percentile +/+ 3x interquartile range)

The AOcA levels within the uveitis population and their associations with clinical characteristics are shown in Table 3. Significant associations between high levels of specific AOcAs and VZV-induced uveitis, MS-associated uveitis or unexplained uveitis with positive quantiferon test were identified. Presence of CME was associated with higher titers of tyrosinase (number 79, p=0.0026), although with a moderate increased titer compared to uveitis patients without CME (1.4x). Active uveitis had lower levels of most AOcAs, but these results did not reach significance after Bonferroni correction. No differences in levels of AOcAs were found between all other clinical characteristics of uveitis.

Table 3. Presence of anti-ocular antibodies compared within uveitis (N=76) in relation to uveitis characteristics*

Antigen no.	Antigen name	Short name	p-value**	Fold increase
Specific uveitis entities				
Varicella zoster virus induced uveitis				
104	Retinol-binding protein 3	RBP3	0.0080	3.8
134	Bestrophin-2	BEST2	0.0054	2.2
Multiple sclerosis associated uveitis				
107	Retina and anterior neural fold homeobox protein 2	RAX2	0.0001	4.6
86	Age-related maculopathy susceptibility protein 2	ARMS2	0.0043	2.9
34	Visual pigment-like receptor peropsin	RRH	0.0372	2.4
185	Rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit alpha	PDE6A	0.0006	2.2
Idiopathic uveitis with positive quantiferon test				
139	Cbp/p300-interacting transactivator 1	CITED1	0.0008	3.7
143	Cbp/p300-interacting transactivator 1	CITED1	0.0220	3.4
Clinical characteristics				
Presence of cystoid macular edema				
79	Tyrosinase	TYR	0.0026	1.4

* P-values for comparison of intraocular AOcA levels within the uveitis cohort were determined by robust linear regression. Specific uveitis entities were compared with sarcoidosis associated uveitis as reference category. Only significant p-values with the highest fold increases are shown in this table.
** after Bonferroni correction for multiple testing

DISCUSSION

Our study documents the presence of a broad spectrum of ocular autoantibodies in intraocular fluids of patients with uveitis patients. Significant differences between the levels of intraocular AOcAs in uveitis and cataract were identified. However, levels of intraocular AOcAs in uveitis were only moderately increased (up to 2x compared to cataract), which may have been caused by the merger of heterogeneous autoantibody profiles of different uveitis entities. Comparison of specific uveitis entities with cataract showed various high intraocular AOcA levels (>5x) in VZV-induced uveitis, MS-associated uveitis and idiopathic uveitis with positive quantiferon test. Patients with CME exhibited moderately increased titers of anti-tyrosinase.

VZV-induced uveitis was one the entities which was associated with high levels of AOcAs. Specifically, antibodies directed against retinol-binding protein 3 (RBP-3) were present in high levels (compared to cataract, and other uveitis entities). RBP-3, also known as interphotoreceptor retinoid-binding protein (IRBP) is found primarily in the interphotoreceptor matrix of the retina and it is thought to transport retinoids between the retinal pigment epithelium (RPE) and the photoreceptors. RBP-3 antibodies have been previously observed in some ocular diseases, including autoimmune retinopathy, macular telangiectasia type 2 and age related macular degeneration.[16-18] The RBP-3 antigen has been found to be highly uveitogenic in multiple animal models.[19,20] RBP-3 might represent a non-specific target involved in the pathogenesis different ocular diseases. Another highly prevalent antibody in VZV-induced uveitis was directed against bestrophin-2 (Best-2). Within the bestrophin family, bestrophin-1 (Best-1) is the most investigated and described ocular antigen. Mutations in the BEST gene may cause bestrophinopathies (such as Best's disease), and antibodies directed against the Best-1 protein are involved in vitelliform paraneoplastic retinopathy.[21,22] The Best-2 antigen is, in contrast to Best-1, not found in the RPE, but in the ciliary body.[23] The Best-2 antigen is involved in regulation of intra-ocular pressure by antagonizing the formation of aqueous humour.[24,25] The relationship between Best-2 antigen and uveitis has not yet been reported. Interestingly, all four patients with retinitis as well as one patient with VZV-induced anterior uveitis exhibited high titres of Best-2. These patients had no eye pressure associated problems.

In patients with MS (with and without uveitis), the presence of AOcAs in serum and cerebrospinal fluid has been repeatedly noted.[26-29] In our series, we identified several specific AOcAs in high titres in intraocular fluids in MS-associated uveitis, namely recoverin and fructose-bisphosphate aldolase C. These two AOcAs were previously related to cancer associated retinopathy, and aldolase C was also observed in patients with diabetic retinopathy.[30,31] Another target found in high titres in MS-associated uveitis was surprisingly age-related maculopathy susceptibility protein 2 (ARMS2). ARMS2 is involved in complement activation and its gene was repeatedly reported in AMD.[32,33] An over-activated complement system has also been noted in MS lesions by immuno-

histochemistry.[34-36] It has been suggested that the complement may play a secondary role in the pathogenesis of MS by aggravating the course of disease.

Multiple reports propose the involvement of the immune system in uveitis associated with latent tuberculosis infection (LTBI).[37] The current hypotheses include a low-grade infection, an immunological reaction induced by the tuberculosis bacilli, or a combination of both. High levels of intraocular AOcAs in uveitis with LTBI favour the involvement of immune reactions in the pathogenesis of LTBI associated uveitis.

Interestingly, CME was associated with antibodies directed against tyrosinase. Tyrosinase is a peptide of the melanocytes, a protein that within the eye is mainly located in the RPE and was previously described in serum of patients with Vogt-Koyanagi-Harada (VKH) disease.[38] It is thought that the pathogenesis of VKH disease involves autoreactive T-cells directed against tyrosinase, possibly triggered by molecular mimicry.[39,40] Tyrosinase involvement in CME might be in part due to damage of RPE, which plays a crucial role in the pathogenesis of CME. Previously, it has been noted that use of eye drops toxic for melanocytes also causes increased activity of tyrosinase.[41] It might be that an immune response directed against tyrosinase is indicative for ocular diseases that affect the pigmented layers within the eye.

The spectrum of multiple AOcAs found in different uveitis entities suggest a secondary production of AOcAs in uveitis rather than its inciting role. The presence of AOcAs in these patients might be explained by a secondary formation induced by ocular tissue damage. Ocular antigen release by leakage through the blood-retina-barrier might cause exposure to the immune system and activate the production of systemic AOcAs, which then diffuse back into the eye. Intraocular AOcAs may contribute to the aggravation of uveitis and have a modulating role in the course of the disease. In contrast, an inciting role of intraocular AOcAs has been proposed in the pathogenesis of autoimmune retinopathies (including cancer-associated- and melanoma-associated retinopathies), but the possible intraocular production of AOcAs in patients with AIR was not yet investigated.[42] However, details on the pathogenicity of most AOcAs are lacking and the presence of natural intraocular autoantibodies is unknown.[43,44] The mere presence of AOcAs does not automatically relate to a disease. The absence of a standardized and validated method for AOcA testing complicates the determination of the possible role of specific AOcAs.[8,45]

In the present study, we have chosen for the use of continuous values of the signal intensities from the multiplex assay for our statistical analyses, since this approach is more sensitive than the use of dichotomous values. One could argue that by using continuous data, outcomes might have been influenced by background signals, which cannot be distinguished from signals from measured antibodies bound to the beads. However, the use of dichotomous data has similar limitations as this approach demands a specific cut-off determination, which similarly is influenced

by the background signal and would be in consequence arbitrary. Despite this drawback of our laboratory method, it has given us the opportunity to analyze a great number of antigens in a very small amount of material and indicates the potentially relevant ocular targets. Our study includes measurements of intraocular fluids without simultaneous analyses of AOcAs in serum. Comparison of intraocular AOcAs levels with the levels of AOcAs in serum might indicate which AOcAs are indicative of intraocular production and possibly clinically relevant.[5] Exploration of simultaneously collected serum and intraocular fluid for potential interesting ocular autoantibodies that were observed in this study could provide valuable information. The multiplex assay, which we used for the antibody profiling, however does not allow direct comparison of AOcA levels between serum and intraocular fluid.

In conclusion, our results show moderately increased levels of a broad spectrum of AOcAs in multiple uveitis entities. High levels of specific AOcAs were observed in intraocular fluid of patients with VZV-induced uveitis, MS-associated uveitis and idiopathic uveitis with positive quantiferon test. The results of this study may serve as a platform for future exploration in the pathogenesis of specific uveitis entities.

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SUPPLEMENTARY DATA

Supplementary Table. Amino acid sequence and uniprot ID of ocular antigens used for the autoantibody profiling

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11

INTRAOCULAR CYTOKINE PROFILE AND AUTOIMMUNE REACTIONS IN RETINITIS PIGMENTOSA, AGE-RELATED MACULAR DEGENERATION, GLAUCOMA AND CATARACT

Josianne C.E.M. ten Berge, Zainab Fazil, L. Ingeborgh van den Born,
Roger C.W. Wolfs, Marco W.J. Schreurs, Willem A. Dik*, Aniki Rothova*

These authors contributed equally as last authors

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ABSTRACT

Purpose: To analyze intraocular cytokine levels and prevalence of intraocular antiretinal antibodies (ARAs) in patients with retinitis pigmentosa (RP), age-related macular degeneration (AMD), glaucoma and cataract, and correlate the results to clinical manifestations.

Methods: We collected intraocular fluid samples from patients with RP (N=25), AMD (N=12), glaucoma (N=28) and cataract (N=22), and serum samples paired with the intraocular fluids from patients with RP (N=7) and cataract (N=10). Interleukin(IL)-1β, IL-1ra, IL-2, IL-6, IL-6rα, IL-7, IL-8, IL-10, IL-17A, IL-23, thymus- and activation-regulated chemokine (TARC), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) were measured using a multiplex assay. ARA detection was performed by indirect immunofluorescence.

Results: Increasing age was associated with increasing levels of IL-6, IL-8, TNF-α and VEGF. All patient groups exhibited distinct profiles of intraocular cytokines. Intraocular levels of IL-8 were highest in patients with AMD and glaucoma. Cataract patients exhibited high intraocular levels of IL-23. Intraocular levels of IL-2, IL-6, MCP-1 and PlGF in RP patients exceeded the levels of serum, indicating intraocular production. Intraocular ARAs were found in only one patient with AMD.

Conclusions: Increased levels of inflammatory cytokines in intraocular fluid of patients with originally non-inflammatory ocular diseases show that intraocular inflammation is involved in their pathogenesis of these entities. Moreover, we show that increasing age is associated with increasing levels of intraocular cytokines, and conclude that future studies on intraocular mediators should be corrected for age of patients.

INTRODUCTION

Retinitis pigmentosa (RP), age related macular degeneration (AMD) and glaucoma are ocular diseases that can cause irreversible damage, resulting in reduced visual acuity and blindness. Treatment options are often limited; so far, no cure is available for these diseases and only some of their complications can be prevented or treated. The pathogenesis of RP, AMD and glaucoma is not fully clarified, but a growing body of evidence documents the involvement of the immune system. Further insights into the role of immune activation could lead to potential new therapeutic modalities for these blinding diseases.

Recent studies identified autoantibodies directed against retinal tissue in serum of patients with RP, AMD or glaucoma.[1,2] An association between antiretinal antibodies (ARAs) in serum and macular edema has been observed in RP.[3] Decrease of serum ARA levels were reported after intravitreal anti-vascular endothelial growth factor (VEGF) therapy in AMD.[4] Also, a variant of the complement factor H (CFH) gene, which causes uncontrolled complement activation, has been linked to AMD.[2] Presence of ARAs in aqueous humor and serum have been observed in patients with glaucoma and in neurodegenerative damage of the optic nerve.[5] Further, elevated levels of different chemokines, including monocyte chemotactic protein 1 (MCP-1) and interleukin IL-8, as well as the pro-inflammatory cytokine IL-6, have been described in aqueous humor of RP, AMD and glaucoma.[6-11]

Autoimmune reactions against retina, choroid and/or retinal pigment epithelium (RPE) might contribute to continuation and/or aggravation of some of these initially non-inflammatory ocular diseases.[2,12] However, the role of autoimmune reactions within the eye and comparison of inflammatory reactions between different degenerative ocular diseases has been scarcely addressed.

Herein, we investigate specific cytokine-, chemokine- and growth factor levels, and presence of ARAs in intraocular fluid samples in patients with RP, AMD, glaucoma and cataract, and relate the laboratory outcomes to clinical manifestations.

METHODS

Sample collection

In this cross-sectional study, we obtained intraocular fluid samples from 87 patients with RP, AMD, glaucoma and cataract (controls) from the biobank at the Erasmus University Medical Center and the biobank of the Rotterdam Eye Hospital. Ocular fluids within the biobank were collected during the beginning of a cataract extraction. The study was approved by the local ethics committee from the Erasmus University Medical Center (Medical Ethics Committee Erasmus MC) and the

ethics committee from the institutional research board from the Rotterdam Eye Hospital, and adhered to the tenets of the Declaration of Helsinki. All intraocular fluid samples were stored at -80°C. Serum samples paired with intraocular fluids of 7 patients with RP and 10 patients with age related cataract were also obtained from the biobank.

Patient and data collection

The diagnosis of RP was based on clinical characteristics such as night blindness, visual field constriction, retinal abnormalities observed through fundoscopy, and/or electroretinographic changes confirming the presence of RP-related photoreceptor damage. The diagnosis of AMD was carried out through clinical examination and optical coherence tomography. Diagnosis of glaucoma was based on the clinical presentation with high intraocular pressure, optic nerve damage and/or on characteristic visual field loss. Participants suffering from ocular comorbidity or from a combination of included ocular diseases were excluded.

Clinical characteristics of all patients were collected. For RP patients, the presence of cystoid maculopathy (CM) was assessed as: 1). no CM, 2). any prior CM, and/or 3). current CM (<4 weeks prior to sample collection). For patients with AMD, differentiation between exudative and dry AMD was made and treatment with anti-VEGF was noted: 1). no anti-VEGF medication, 2). any prior use of anti-VEGF medication and/or 3). current use of anti-VEGF medication (<4 weeks prior to sample collection). Patients with glaucoma were classified by the type of their glaucoma. Prescription of antihypertensive eye drops and filtrating surgery prior to sample collection were registered.

Cytokine analysis

Measurement of interleukins (IL-1 β, IL-1ra, IL-2, IL-6, IL-6rα, IL-7, IL-8, IL-10, IL-17A, IL-23), thymus- and activation-regulated chemokine (TARC), MCP-1, tumor necrosis factor-alpha (TNF-α), placental growth factor (PlGF) and VEGF was performed with a Luminex multiplex bead immunoassay system (R&D Systems Europe, Ltd; UK). The selection of the cytokine panel was based on potential relevance according to previous reports and/or possible targets for treatment options.[7,8,11,13,14] The assays were performed according to the manufacturer's instructions with exception of one additional dilution step within the standards (in total 7 standard dilutions). Fifty µL of undiluted intraocular fluid samples were transferred to the plate, with exception of intraocular fluid samples with insufficient amount of material (N =16), which were diluted to a total volume of 50 µL. Serum samples were diluted two-fold according to the manufacturer's standard protocol. Measurements were performed on a Bio-Plex MAGPIX machine and data was analyzed using Bio-Plex Manager MP software.

Antiretinal antibody analysis

Presence of ARAs was assessed by indirect immunofluorescence (IIF) using primate retinal tissue (Euroimmun) and evaluated as described before in ten Berge et al.[15] IIF was conducted with

intraocular fluids samples with sufficient volume available, and on all serum samples. Samples that displayed nuclear staining on retinal tissue were also analyzed in a routine IIF antinuclear antibody (ANA) screening test using HEp-2 cells (Inova).

Statistical analysis

Data from the Luminex immunoassay were analyzed both as continuous data as well as categorical data. For the continuous analyses, values below the lower limit of detection were replaced by the lowest value of the reference curve. For categorical analyses, we used the lowest value of the reference curve as cut-off point. Continuous variables were summarized using medians and ranges and categorical variables were summarized using percentages. Logistic regression for categorical data and linear regressions for continuous data with age, gender and diagnosis in the model were assessed to compare laboratory outcomes between diagnosis groups. Statistical analyses were performed using IBM SPSS Statistics, version 21 and a p-value of <0.05 was considered as statistically significant.

RESULTS

Patient characteristics

Aqueous humor samples were obtained from a total of 87 patients: RP (N =25), AMD (N =12), glaucoma (N =28) and cataract (N =22). Serum samples obtained simultaneously with intraocular fluids samples were available from 17 patients: RP (N =7) and cataract patients (N =10). Gender distribution did not differ between groups, but age differed significantly (p<0.001); specifically patients with AMD were older and patients with RP were younger (Table 1). The AMD group consisted of 10 patients with dry and 2 patients with exudative AMD. The classification of glaucoma included primary open angle glaucoma (POAG, N =22), narrow-angle glaucoma (N =4), normal tension glaucoma (N =1) and glaucoma secondary to pigment dispersion syndrome (N =1).

Table 1. Patient characteristics

	Retinitis pigmentosa	Age-related macular degeneration	Glaucoma	Cataract	p-value
Total number of patients	N=25	N=12	N=28	N=22	
Median age in years (range)	51 (25-86)	84 (68-94)	73 (50-88)	66 (17-80)	< 0.001
Gender (male)	12/25 (48%)	3/12 (25%)	13/28 (46%)	8/22 (36%)	n.s.

Abbreviation: n.s.= not significant (p>0.05)

Prevalence of intraocular cytokines

The prevalence of cytokines, chemokines and growth factors in intraocular fluid is summarized in the Supplementary Table. Gender did not influence the prevalence of cytokines. The prevalence of intraocular IL-6 and TNF-α increased with age (p=0.012 and p=0.002, respectively). IL-2 and MCP-1 were present in all intraocular fluid samples, while IL-1 β and IL-17A were undetectable in all intraocular fluid samples. TARC was detected in intraocular fluid of patients with RP, AMD and glaucoma, but not in cataract patients. Differences in presence of cytokines in intraocular fluids between ocular diseases were however not significant. No associations were found between clinical characteristics (such as duration of disease or treatment) and the mere presence of cytokines, chemokines or growth factors in intraocular fluid.

Prevalence of cytokines in paired intraocular and serum samples

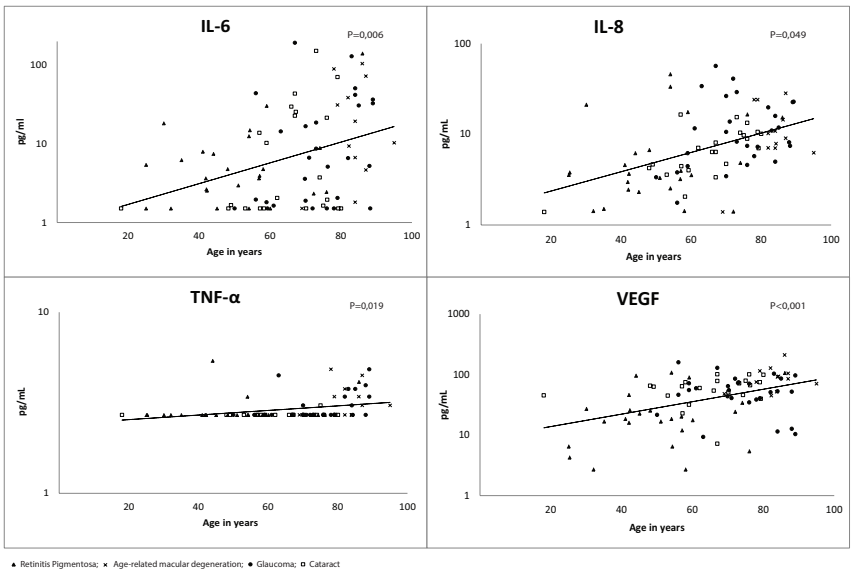
A similar cytokine profile was found in serum and intraocular fluid in RP patients, except for IL-2 and IL-6, which were more often present in the intraocular fluid samples, and IL-1ra and TARC, which were more frequently observed in serum. Presence of serum cytokines was not different between RP patients and cataract patients.

Levels of intraocular cytokines

Linear regressions using specific diagnosis, age and gender in the model showed that intraocular levels of IL-6, IL-8, TNF-α and VEGF correlated positively with age (p=0.009, p=0.049, p=0.019 and p<0.001, respectively; Figure 1). Gender showed no association with intraocular cytokine levels. Different cytokine profiles were observed for RP, AMD, glaucoma and cataract, specifically intraocular levels of IL-6rα (p=0.019), IL-8 (p=0.032), and IL-23 (p<0.004) differed between the studied ocular diseases (Figure 2). RP patients were characterized by low levels of intraocular IL-8 and IL-23. Intraocular IL-8 levels were highest in patients with AMD and glaucoma. Cataract patients had high levels of IL-23. Intraocular levels of IL-6rα were higher in patients with RP or glaucoma than in patients with AMD or cataract. VEGF levels were highest in intraocular fluids of AMD patients and lowest in RP, though the differences did not reach significance after correction for age (Table 2).

RP patients who ever had CM during their disease course, exhibited lower intraocular IL-2 levels than RP patients without CM (p=0.042). Glaucoma patients treated with antihypertensive eye drops displayed lower intraocular IL-6 levels compared to glaucoma without this treatment modality (p<0.001). Intraocular IL-8 levels were higher in glaucoma patients who underwent surgical treatment (p=0.035).

Figure 1. Levels of intraocular cytokines in relation to age



P-values were determined by linear regression with adjustment for diagnosis and gender

Levels of cytokines in paired intraocular and serum samples

IL-2, IL-6, MCP-1 and PlGF levels were higher in intraocular fluid than in serum in both RP ($p<0.001$, $p=0.001$, $p<0.001$ and $p=0.006$) and cataract ($p<0.001$, $p=0.047$, $p<0.001$ and $p<0.001$). Moreover, cataract patients had higher levels of IL-23 in intraocular fluid than serum ($p=0.043$). In contrast, intraocular IL-23 levels in RP were lower than the serum levels ($p<0.001$). Intraocular levels of all other cytokines, chemokines and growth factors in intraocular fluid did not exceed serum levels. Levels of serum cytokines were not different between RP patients and cataract patients.

Prevalence of antiretinal antibodies

ARAs were not detected in intraocular fluid samples from RP (N = 21), glaucoma (N =18), and cataract (N =16). In one patient with AMD (1/8, 13%) intraocular ARA were detected. Serum ARAs were detected in 5/7 (71%) patients with RP and 6/10 (60%) patients with cataract. All samples with nuclear staining on retinal tissue (N =7) were negative for ANA.

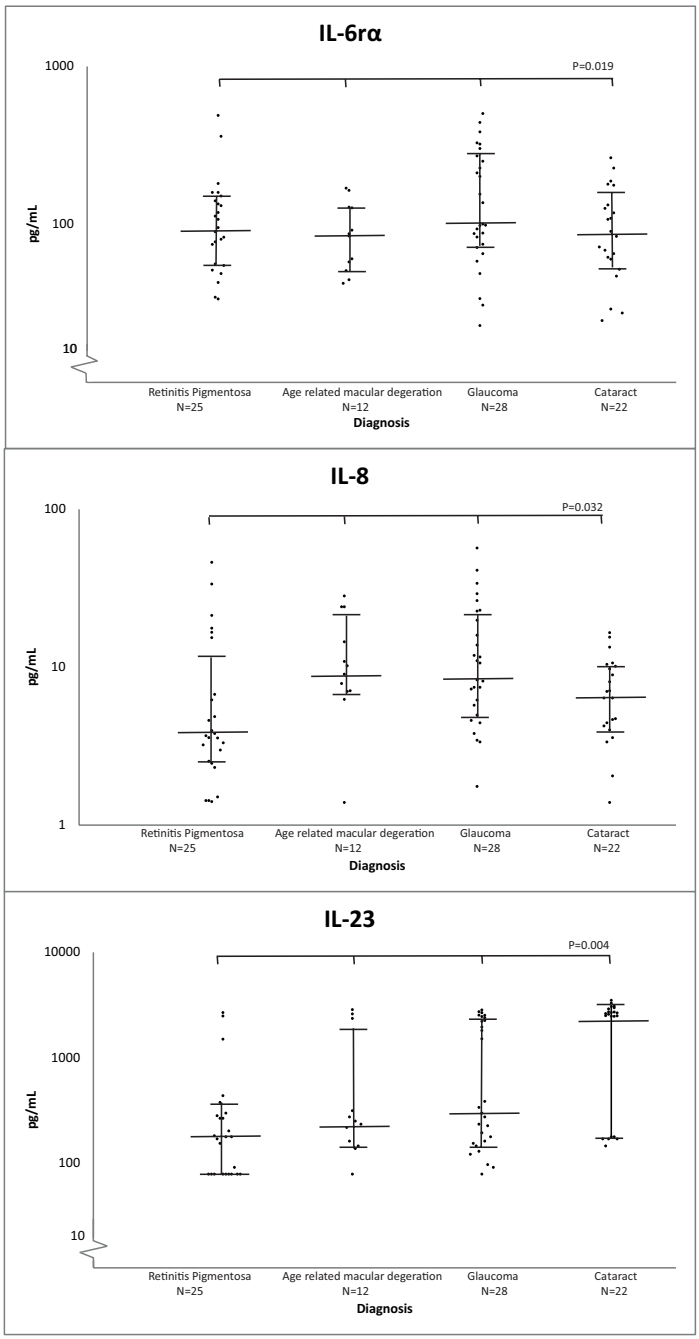
Table 2. Levels of cytokines in different ocular disease

	Cytokines (median, ranges)	Retinitis pigmentosa			Age-related macular degeneration
		IOF	Serum	p-value	
Cytokines	**IL-1β**	5 (5-5)	5 (5-5)	n.s.	5 (5-5)
	IL-1ra	33 (9-3673)	576 (426-2436)	n.s.	121 (9-1169)
	IL-2	292 (142-899)	33 (33-93)	<0.001	282 (205-1727)
	IL-6rα	95 (33-489)	41505 (39074-50271)	<0.001	85 (42-169)
	IL-6	4 (1-140)	1 (1-1)	0.001	14 (1-104)
	IL-7	3 (1-6)	4 (1-7)	n.s.	2 (1-7)
	IL-10	3 (3-5)	3 (3-3)	n.s.	3 (3-8)
	IL-17A	8 (8-8)	8 (8-8)	n.s.	8 (8-8)
	IL-23	176 (77-3611)	812 (539-919)	0.001	240 (77-2844)
	TNF-α	2 (2-5)	2 (2-3)	n.s.	3 (2-4)
Chemokines	**IL-8**	3 (1-46)	6 (2-21)	n.s.	9 (1-28)
	TARC	28 (28-57)	180 (101-681)	<0.001	28 (28-37)
	MCP-1	891 (339-1805)	256 (201-451)	<0.001	638 (256-1035)
Growth factors	**PlGF**	6 (0-26)	1 (0-1)	0.006	6 (0 -13)
	VEGF	18 (2-108)	52 (18-107)	0.020	88 (45-210)

Abbrevations: IOF = intraocular fluid, n.s.= not statistically significant (p>0.05)

Glaucoma	Cataract			p-value (comparison of IOF)
	IOF	Serum	p-value	
5 (5-5)	5 (5-5)	5 (5-5)	n.s.	n.s.
65 (9-876)	124 (9-2357)	608 (328-1043)	n.s.	n.s.
271 (151-1371)	275 (212-1101)	136 (15-262)	<0.001	n.s.
99 (22-502)	87 (24-263)	44327 (34714-58859)	<0.001	0.019
5 (1-192)	2 (1-151)	1 (1-3)	0.047	n.s.
3 (1-12)	1 (1-9)	5 (2-7)	0.004	n.s.
3 (3-7)	3 (3-8)	5 (3-24)	n.s.	n.s.
8 (8-8)	8 (8-8)	10 (8-25)	n.s.	n.s.
315 (77-2828)	2592 (144- 3493)	750 (510-945)	0.043	0.004
2 (2-4)	2 (2-3)	2 (2-4)	n.s.	n.s.
9 (1-56)	6 (1-16)	7 (1-12)	n.s.	0.032
28 (28-57)	28 (28-28)	262 (62-470)	<0.001	n.s.
731 (264-3495)	618 (249-1920)	320 (120-478)	<0.001	n.s.
5 (0-13)	5 (2-12)	1 (0-2)	<0.001	n.s.
54 (9-159)	63 (7-101)	73 (18-126)	n.s.	n.s.

Figure 2. Levels of intraocular cytokines in relation to ocular diagnosis



P-values were determined by linear regression with adjustment for age and gender

DISCUSSION

Our study describes different pro-inflammatory intraocular cytokine profiles in RP, AMD, glaucoma and cataract, and reveals a positive correlation between intraocular cytokine levels and increasing age. Interestingly, intraocular fluid samples from cataract patients displayed the highest levels of IL-23. Though intraocular levels of VEGF were highest in AMD patients, after age correction no significant differences were found between the various diagnostic groups. Comparison of paired serum and intraocular fluid samples of RP patients showed that intraocular levels of IL-2, IL-6, MCP-1 and PlGF exceeded the serum levels, suggesting a local production. Intraocular ARAs were absent in nearly all samples.

Although a genetic mutation is the cause of RP, inflammation was suggested to have a (secondary) role in the disease pathogenesis.[2,16,17] It has been reported that RP patients with CM exhibited more often ARAs in their peripheral blood.[18] Conform these findings, in our series we observed ARAs in all RP patients with CM. However, in contrast to serum, ARAs were not at all detected in intraocular fluids of patients with RP.

Previous reports on intraocular cytokines in RP show higher levels of IL-6, IL-8, MCP-1 and TARC in RP compared to cataract.[11,19] We observed also intraocular presence of these mediators in RP patients, but their levels were not elevated compared to other groups. This discrepancy may be explained by the low number of included patients in our study, or possible differences in disease stage and/or extent of degeneration. Yet RP patients, like glaucoma patients, had higher intraocular levels of soluble IL-6rα (sIL-6rα) compared to cataract. sIL-6Rα interacts with IL-6, forming the IL-6/sIL-6Rα complex, which subsequently induces IL-6 trans-signaling by binding cell membrane expressed gp130.[20] IL-6 trans-signaling is recognized to enhance IL-6 activity under inflammatory conditions and moreover to inhibit intra-ocular T-cell apoptosis in uveitis, which likely exacerbates or prolongs the disease process.[21-23] Further we observed a significant association between lower levels of intraocular IL-2 (a growth factor for regulatory T-cells) in RP patients who had CM. This may indicate a deregulated immune function, such as loss of tolerance, affecting the clinical manifestation of the disease and the formation of serum ARAs as observed in this and other studies. Intraocular VEGF levels were lowest in the RP group, which is in line with the rare presence of retinal neovascularization in RP.

Inflammation was implicated in the development and progression of AMD.[24] So far, most previous studies investigated intraocular fluids of exudative AMD, demonstrating high levels of inflammatory mediators, including IL-6, IL-8, MCP-1 and VEGF.[6,25] However, it is still unknown whether these cytokines play a role in the primary pathogenesis of AMD or represent a secondary result of the disease process. We investigated patients with mainly dry AMD and observed higher intraocular IL-8 compared to cataract and RP. Previous studies revealed that elevated (intraocular) levels of

IL-8 and IL-8 gene polymorphisms were associated with angiogenesis.[26,27] IL-6 and VEGF reached highest levels in the AMD group, though not significantly different compared to other diagnosis groups. According to previous studies, these mediators have been implicated in angiogenesis, and decrease during treatment with anti-VEGF agents.[8,28] Retinal neuroprotective effects of VEGF have also been described, yet data on this matter are inconclusive and may be dependent on the VEGF variant, disease (stage) or experimental model used.[29,30]

In glaucoma the role of immune reactions is not known and could be either pathogenic or neuroprotective. In our study patients with glaucoma, who consisted mainly of POAG, were characterized by high intraocular levels of IL-8, consistently with previous findings.[31-34] IL-8 is a main chemoattractant for neutrophils which have been found to accumulate in the trabecular meshwork in POAG.[35] The highest levels of IL-8 were found in glaucoma patients who underwent surgical treatment prior to the surgical procedure during intraocular sample collection. This suggest that higher levels of IL-8 might be explained by immune activation in response to (surgically inflicted) tissue damage. Increased TNF-α levels have been reported in intraocular fluid, the trabecular meshwork, optic head and the retina of glaucoma patients, however in our study intraocular TNF-α appeared undetectable in most cases.[33,35-39] This discrepancy may have resulted from differences in laboratory techniques and specific patient groups. So far, to our knowledge, only sporadic studies are available on the effect of anti-TNF medication in glaucoma, which show an increase of fibrosis after surgical treatment.[40]

In our study, cataract was generally characterized by lower levels of pro-inflammatory mediators compared to other studied diseases, with the exception of IL-23. IL-23, produced by dendritic cells/myeloid cells, is well known for its key role in several autoimmune diseases via the IL-23/IL-17axis and associated pathological Th17 development.[41,42] Despite the presence of IL-23 in most intraocular fluids analyzed in our study, IL-17A was never detected. Interestingly, some studies report immunosuppressive effects of IL-23 within tumor microenvironments by suppressing lymphocyte effector function and enhanced production of immune-regulatory cytokines.[43-45] Also in a model of experimental autoimmune uveitis it was found that IL-23 receptor expressing gd T cells can exert immune suppressive effects due to their ability to bind IL-23.[46] Although the mechanism by which IL-23 can mediate immune suppressive effects clearly requires further study, it is tempting to speculate that the low intraocular IL-23 levels observed in patients with RP, AMD and glaucoma may reflect diminished immune protection of the eye. Intraocular IL-23 levels revealed no association with serum levels nor age of patients.

Aging is associated with development of a chronic state of low-grade tissue inflammation that also involves the retina, and is associated with increased susceptibility to multiple diseases, including glaucoma and AMD.[47-50] In support of this, we observed a gradual increase of the intraocular levels of IL-6, IL-8, TNF-α and VEGF with increasing age. A positive correlation between intraocular

cytokine levels and age has been reported in two previous studies.[31,34] As a consequence of this correlation, all of our results are adjusted for the age of patients. However, systematic corrections for age have not been performed in previous studies, which may have affected the interpretation of these findings. It should thus be kept in mind that studies on intraocular cytokine profiling without age adjustment (and without age matched control groups) may show age-related bias rather than disease-associated differences.

Intraocular levels of IL-2, IL-6, MCP-1 and PlGF were higher than serum levels of patients with RP and cataract. The higher intraocular levels of inflammatory components may suggest local production, possibly by infiltrated immune cells or resident cells, such as retinal pigment epithelial cells.[51,52] These findings may contribute to the understanding of the pathogenesis of RP and development of new treatment possibilities. In contrast, the low occurrence of intraocular ARAs suggests a negligible role of such antibodies in disease pathogenesis of RP. ARAs detected in serum from RP and cataract patients may represent a reflection of altered blood retinal barrier properties.

In conclusion, the expression of inflammatory cytokines within the eye was strongly influenced by the age of patients, which shows that the correction for age is necessary in future studies on intraocular mediators. Differences in intraocular cytokines profiles were observed between originally non-inflammatory ocular diseases, suggesting involvement of inflammation, however complex pathways with multiple signaling functions make a diagnostic role rather impossible. The role of immune reactions in basically non-inflammatory ocular diseases might influence the clinical manifestations and severity of ocular changes.

SUPPLEMENTARY DATA

Supplementary Table. Prevalence of cytokines in different ocular disease

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SUMMARY AND CONCLUSIONS

SUMMARY

Autoimmune reactions have been implicated in various ocular diseases but their presence and pathogenic role have been scarcely proven. Autoimmunity was especially linked to uveitis (a collective term for any intraocular inflammation) and autoimmune retinopathy (AIR), but also to retinitis pigmentosa (RP), age-related macular degeneration (AMD) and glaucoma. Obviously, if autoimmunity plays a principal role in these diseases, the downregulation of autoimmune reactions might bring a beneficial effect to affected patients. The work presented in this thesis aims to assess the presence of humoral autoimmunity in uveitis and other chorioretinal diseases, including AIR, and to clarify its potential role in their pathogenesis. This summary presents the main findings of this thesis.

Chapter 1 provides a general introduction addressing the presence and the role of humoral autoimmunity in uveitis and other chorioretinal diseases. Autoimmunity is characterized by activity of the immune system directed against the body's own cells and tissues. The immune system stops tolerating 'self' antigens, and autoreactive antibodies and/or T-cells attack the body's own antigens. Convincing evidence exists that our immune system is able to react against our own retina and can produce antiretinal antibodies (ARAs). These ARAs can presumably attack and destroy retinal cells, and rapidly leading to visual loss or even blindness. Such reactions of the immune system have long been considered to play a major pathogenic role in uveitis. In other ocular diseases, such as RP, AMD and glaucoma, the formation of ARAs has also been observed and reported. However, the exact pathogenic role of antiretinal humoral autoimmunity in uveitis and other ocular diseases is not yet known. One current hypothesis includes the primary role of ARAs in the initiation of the chorioretinal diseases; another possibility addresses the secondary role of ARAs and proposes that the formation of ARAs in any ocular disease might cause a mild inflammation in the retina, that might negatively influence the course of the disease (e.g. by development of chronicity or enhancement of progression of retinal damage).

In **Chapter 2** we determine the prevalence of systemic immune-mediated diseases in a large cohort of 1327 patients with uveitis and/or scleritis, and put a special emphasis on prevalence of autoimmune and auto-inflammatory diseases according to novel findings in immune mediated diseases. In contrast to the widespread belief, it appeared that autoimmune uveitis is a rare diagnosis, which comprises only 5% (62/1327) of the total uveitis/scleritis population. The most common autoimmune diseases observed in patients with uveitis were multiple sclerosis (1.8%, 24/1327) and Vogt Koyanagi Harada disease (1.1%, 14/1327). Patients with classical autoimmune connective tissue disease (N=17) suffered mostly from rheumatoid arthritis and granulomatosis with polyangiitis and exhibited predominantly scleritis (53%, 9/17). Optic neuropathy was the most frequent complication of autoimmune uveitis (44%, 27/62), and was predominantly observed in MS and Vogt Koyanagi Harada disease. Interestingly, the visual acuity in autoimmune diseases with uveitis remained stable during 5-year follow-up, possibly as a result of adequate treatment

with systemic immunomodulatory medication. None of the patients with autoimmune uveitis/scleritis developed permanent bilateral visual acuity of less than 0.1. In this study we emphasize that the term "autoimmune uveitis" should be reserved exclusively for intraocular inflammations of confirmed autoimmune origin and should not be used as a synonym for all non-infectious uveitis cases.

In **Chapter 3** we prospectively evaluate the presence of antinuclear antibody (ANA) and the diagnostic value of ANA profiling in 105 adult patients with uveitis. ANAs are antibodies directed against a variety of nuclear antigens, and can be detected in elevated levels in serum of patients with genuine autoimmune diseases such as systemic lupus erythematosus, but can also be prevalent in other disease types and in the general population, predominantly in women and elderly. Our study showed that 28% of patients with uveitis is positive for ANA whilst only 8-17% of the healthy population is considered ANA positive. ANA positivity was more prevalent in patients with infectious uveitis (31%) than patients with systemic immune mediated diseases (20%). We did not observe any relationship between the presence of ANA, ANA titer, or specific ANA patterns and specific diagnoses or clinical characteristics of uveitis. The discrimination of autoimmune systemic diseases in uveitis by ANA profiling seems not possible, however this cannot be entirely excluded since we included a limited number of patients in the specific diagnostic groups. Based on our findings, we concluded that ANA profiling in the adult uveitis population has limited diagnostic value and that ANAs are probably not involved in the pathogenesis of uveitis.

Chapter 4 describes the prevalence and possible clinical relevance of ARAs in serum in a prospective study of 126 patients with different uveitis entities and compares it with 60 blood bank donors. Screening for the presence of ARAs was performed by indirect immunofluorescence (IIF) using primate retinal tissue. Although IIF does not allow antigenic specification of ARAs, this technique is very sensitive and therefore represents a useful screening assay. In our study, serum ARAs were observed in 47% of patients with uveitis and were more frequent in uveitis when compared with healthy controls (17%, $p<0.001$). Specific associations between the presence of serum ARAs or its subtypes (based on typical staining patterns on IIF) and clinical ocular characteristics of uveitis were not observed. These results suggest that serum ARAs reflect a secondary response of the immune system to damaged ocular tissue, and probably have no inciting role in uveitis.

In **Chapter 5** we attempt to determine the possible diagnostic role of both known and unknown ARAs in patients with AIR. AIR encompasses a group of rare diseases most commonly presenting as paraneoplastic syndromes in combination with various malignancies. Multiple serum ARAs have been reported in AIR, and it was assumed that ARAs are directly involved in retinal destruction and rapidly progressive loss of vision. So far, however, only few retinal antigens in AIR were specified and studied. To investigate this more extensively, we performed a multicenter study in cooperation with the SciLifeLab Autoimmunity Profiling Facility in Stockholm (Sweden). Serum

samples of 24 patients with presumed AIR were examined for the presence of antibodies against multiple retinal antigens by autoantibody profiling and compared to 151 uveitis patients and 21 cataract controls. Antigens used for the autoantibody profiling consisted of 188 peptide fragments derived from 97 unique ocular proteins produced within the Human Protein Atlas of the Swedish laboratory and profiling was performed by a multiplex bead array based immunoassay. We found that patients with presumed AIR were characterized by the presence of multiple serum ARAs, and that the spectrum of ARAs differed widely among individual patients with AIR. Autoantibodies directed against the retinal antigen recoverin were the most prevalent ARAs in patients with presumed AIR (13%). Despite the high levels of multiple ARAs in AIR, our findings clearly show that serum ARAs are also present in patients with cataract and uveitis, and consequently the specificity of ARAs as markers for AIR was low. We concluded that the diagnosis of AIR cannot be based on the mere presence of (multiple) serum ARAs.

In **Chapter 6** we demonstrate the medical history of a patient with paraneoplastic AIR. The patient presented with progressive severe visual loss in both eyes. He also suffered from loss of his peripheral vision, and in his serum high levels of ARAs directed against recoverin were detected. After referral to an internist he was diagnosed with lung carcinoma. The patient was treated with chemo-/radiotherapy and high dosage of corticosteroids, unfortunately without any effect on the visual performance of the patient. A lung carcinoma is the most commonly associated malignancy in paraneoplastic AIR, however any type of malignancy can be involved in this disease. Usually both eyes are affected, but unilateral cases also have been described. Visual complaints may precede detection of the malignancy, which makes the role of the ophthalmologist vital. Treatment options in cancer-associated AIR are limited and visual prognosis is often very poor.

In **Chapter 7** we investigate the presence and possible role of ARAs directed against retinal tissue in central serous chorioretinopathy (CSC). Although little is known about the exact cause of CSC, genetic variants in the complement system of patients with CSC indicate a possible role of the immune system. We retrospectively analysed serum of 63 patients with CSC for the presence of ARAs by IIF and Western blot with retinal protein extract and compared those to 101 uveitis patients, and 60 healthy donors. The Western blot allowed us to approximate the antigen size of observed ARAs, which indicates the presence of specific ARAs. We detected ARAs in 54% of patients with CSC compared to 46% of patients with uveitis ($p>0.05$) and 17% of healthy controls ($p<0.001$). Interestingly, the ARAs in CSC were more often directed against the photoreceptors (27%) than in uveitis (15%, $p=0.039$), however staining patterns on retinal tissue were divers, indicating the presence of various ARAs. Western blot analysis showed multiple ARA reactive antigens in retinal extract in the majority of patients with CSC. This heterogeneity and variable presence of ARAs in CSC make a primary role of ARAs in CSC unlikely. Furthermore, the mere presence of serum autoantibodies does not necessarily indicate an autoimmune basis of the disease and shows that, similar to uveitis, ARAs might be secondary and induced by the retinal damage.

In **Chapter 8** we retrospectively explore the prevalence of systemic and retinal autoantibodies in patients from Indonesia with uveitis and either active or latent tuberculosis (TB). In Indonesia, uveitis associated with active systemic and latent TB is highly prevalent. The pathogenesis of uveitis in the setting of latent TB is not clarified. One of the hypotheses presumes the immune reaction to mycobacterial antigens and their molecular mimicry with retinal antigens. We investigated the presence of ARAs and ANAs in 95 Indonesian patients with uveitis classified into 3 groups: uveitis associated with active TB (N=10), with latent TB (N=58) and without evidence for prior or current TB (N=85). We found that patients with uveitis and either active or latent TB were characterized by a low proportion of ocular-specific auto-reactivity (ARAs) and a high prevalence of systemic auto-reactivity (ANA), compared to uveitis patients who had no evidence of previous contact with *M. tuberculosis* ($p=0.03$ and $p=0.021$). The lower prevalence of ARAs in serum does not point toward an autoimmune pathogenesis in uveitis associated with latent or active tuberculosis, however assessment of intraocular ARAs might show an entirely different pattern and clinical relevance of autoimmune processes in uveitis with active or latent TB.

In **Chapter 9**, we perform a retrospective study and assess the presence of ARAs in young Mexican patients with severe pars planitis (N=16) and compare the results to Mexican age-matched controls (N=19). An exceptionally severe form of pars planitis of unknown origin is observed in pediatric Mexican patients. Despite the extensive examinations, the cause of this disorder is not clarified. Pars planitis is considered to be of autoimmune origin, but the exact pathogenesis is not known. Investigation of serum samples of pars planitis patients for the presence of ARAs showed a prevalence of 57%, however a comparable prevalence (40%) was observed in Mexican control children with strabismus. This high prevalence of ARAs in young control patients could be the result of a higher exposure to exogenous antigens (e.g. infections). Similar to the Netherlands, these results suggest that ARAs are not the primary cause of uveitis, but possibly represent a secondary phenomenon. Nevertheless, a cellular autoimmune component or investigation of intraocular material might still point in the direction of an autoimmune pathogenesis in pars planitis.

In the last chapters we left the serological analysis and explore the determination of humoral autoimmune reactions in intraocular fluid. **Chapter 10** is a retrospective study that investigates intraocular fluid samples from 76 patients with uveitis and 19 cataract controls for the presence of ARAs. In collaboration the SciLifeLab Autoimmunity Profiling Facility in Stockholm (Sweden), we performed a multiplex immunoassay using the same 188 specific ocular antigens as in chapter 5. We found a spectrum of 22 different ARAs in higher levels in patients with uveitis compared to cataract controls ($p \leq 0.05$), but in moderately elevated titers. High elevations (5-fold increased titers compared to cataract controls) of multiple ARAs were observed in three specific uveitis entities: varicella zoster virus-induced uveitis, multiple sclerosis-associated uveitis and patients with unexplained uveitis but positive quantiferon test (all $p<0.05$). Presence of macular edema was associated with high intraocular levels of tyrosinase antibodies ($p=0.0026$). The spectrum of

multiple ARAs in intraocular fluid of patients with uveitis favors a secondary production rather than their inciting role and implicate that uveitis in the setting of latent TB infection might be joint with intraocular autoimmune reactions. The results of this study may provide a platform for future exploration of specific ocular antigens in the pathogenesis of uveitis.

Inflammatory autoimmune processes were not only assumed to play a role in the pathogenesis of uveitis, but also in various originally non-inflammatory ocular diseases such as RP, AMD and glaucoma. Therefore, in **Chapter 11** we retrospectively determine the levels of 15 intraocular cytokines and the prevalence of intraocular ARAs in patients with RP (N=25), AMD (N=12), glaucoma (N=28) and cataract (N=22), and correlate the laboratory results to clinical manifestations. In addition, we studied serum samples paired with the intraocular fluids from patients with RP (N=7) and cataract (N=10). We observed that increasing age was associated with increasing levels of multiple intraocular cytokines and other mediators, and detected distinct cytokine profiles for each group of diagnoses. Specifically, intraocular levels of IL-6rα ($p=0.003$), IL-8 ($p=0.032$), and IL-23 ($p<0.004$) differed between the studied ocular diseases. In RP patients, intraocular levels of IL-2, IL-6, MCP-1 and PlGF exceeded the serum levels, which indicates an active intraocular production and involvement of these mediators in RP. Intraocular ARAs were found in only one patient with AMD, in contrast to serum ARAs that were detected in a majority of studied entities. The differences in cytokine profiles between ocular diseases, suggest a role of immune activity in the pathogenesis of these originally non-inflammatory ocular diseases. The involvement of immune reactions might contribute to continuation and/or aggravation of the disease.

CONCLUSIONS

Our results do not show any evidence for a primary role of retina specific autoantibodies in the initiation of uveitis and other chorioretinal diseases. The presence of serum ARAs in ocular diseases might be explained by a secondary formation induced by ocular tissue damage. Though higher levels of serum ARAs were found in patients with ocular disorders than in control population, we found no associations with specific disorders or clinical manifestations of studied ocular diseases. Moreover, our findings indicate that serum ARAs probably do not have an inciting role in AIR, and that the diagnosis of AIR cannot be based on the mere presence of serum ARAs.

The spectrum of intraocular ARAs in uveitis indicate some involvement of humoral autoimmunity, predominantly in three specific disorders: varicella zoster virus-induced uveitis, multiple sclerosis-associated uveitis and patients with unexplained uveitis but positive quantiferon test. In addition, intraocular autoantibodies directed against tyrosinase have been associated with the presence of cystoid macular edema. Intraocular ARA production in AIR, especially in cancer associated retinopathies, would be an interesting topic for future research and could help clarify its pathogenesis and provide novel treatment opportunities.

APPENDICES

SAMENVATTING
DANKWOORD
ABOUT THE AUTHOR
PHD PORTFOLIO
LIST OF PUBLICATIONS

SAMENVATTING

Het immuunsysteem lijkt een belangrijke rol te spelen in de pathogenese van verschillende oogziekten. Met name bij uveïtis, een inwendige oogontsteking, lijkt het immuunsysteem betrokken te zijn, maar ook bij andere oogaandoeningen zoals retinitis pigmentosa (RP), leeftijdsgebonden maculadegeneratie (AMD) en glaucoom wordt een rol van het immuunsysteem verondersteld. Verder inzicht in de rol van het immuunsysteem bij netvliesaandoeningen kan in de toekomst als mogelijk aangrijpingspunt dienen voor gerichtere behandelingen van diverse oogaandoeningen. Het werk dat in dit proefschrift wordt gepresenteerd, heeft als doel de potentiële pathogene rol van (humorale) auto-immuun reacties bij uveïtis en enkele andere netvliesaandoeningen verder op te helderen. In deze samenvatting worden de belangrijkste bevindingen van dit proefschrift beschreven.

In **hoofdstuk 1** wordt een algemene introductie gegeven over auto-immuniteit in uveïtis en andere oogaandoeningen. Auto-immuniteit wordt gekenmerkt door activiteit van het immuunsysteem gericht tegen eigen cellen en weefsels van het lichaam. Het immuunsysteem stopt met het tolereren van lichaamseigen antigenen, en auto-antistoffen (humorale immuniteit) en/of auto-reactieve T-cellen (cellulaire immuniteit) vallen het eigen lichaam aan. Er zijn aanwijzingen dat het immuunsysteem zich onder bepaalde omstandigheden tegen het eigen netvlies kan richten. Hierbij worden zogenaamde anti-retinale antistoffen (ARA's) geproduceerd die mogelijk schade aan het netvlies kunnen aanbrengen. Tot op heden zijn de bewijzen voor de aanwezigheid van ARA's bij oogaandoeningen schaars en is de rol van humorale auto-immuun reacties in de pathogenese van uveïtis en andere oogaandoeningen onbekend. Diverse theorieën over ARA's en hun mogelijke effect bij oogaandoeningen zijn ontwikkeld. Een van de hypotheses veronderstelt dat ARA's specifieke oogaandoeningen initiëren; een andere hypothese suggereert dat ARA's slechts een bijproduct zijn en de reeds bestaande ziekte nadelig kunnen beïnvloeden, bijvoorbeeld door het ontwikkelen van een chronisch of progressief beloop van de oogaandoening.

In **hoofdstuk 2** bepalen we de aanwezigheid van systemische immuun-gemedieerde ziektebeelden in een cohort van 1327 patiënten met uveïtis en/of scleritis (ontsteking van de harde oogrok). We leggen hierbij nadruk op de prevalentie van auto-immuniteit en auto-inflammatie in uveïtis. Het bleek dat auto-immuun uveïtis een zeer zeldzame diagnose is, welke slechts bij 5% (62/1327) van de totale uveïtis-/scleritis-populatie voorkomt. De meest voorkomende auto-immuunziekten bij patiënten met uveïtis waren multiple sclerose (MS; 1,8%, 24/1327) en de ziekte van Vogt-Koyanagi-Harada (VKH; 1,1%, 14/1327). Bij patiënten met systemische auto-immuun collageenziekten zoals reumatoïde artritis en granulomatose met polyangiitis kwam scleritis relatief veel voor (53%, 9/17). Optische neuropathie (een beschadiging van de oogzenuw) was de meest voorkomende complicatie van auto-immuun uveïtis (44%, 27/62), en werd voornamelijk gezien bij MS en de ziekte van VKH. De visus van patiënten met auto-immuun uveïtis bleef gedurende 5 jaar follow-up

stabiel en niemand ontwikkelde een permanente bilaterale gezichtscherpte van minder dan 0.1, mogelijk door de adequate behandeling met systemische immunomodulerende medicatie. In dit onderzoek benadrukken wij dat de term 'auto-immuun uveïtis' uitsluitend gebruikt mag worden voor inwendige ontstekingen met bewezen karakteristieken van auto-immuun reacties, en het geen synoniem is voor een niet-infectieuze uveïtis.

In **hoofdstuk 3** evalueren we de aanwezigheid en diagnostische waarde van antinucleair antistoffen (ANA) bij 105 volwassen patiënten met uveïtis. ANA's zijn antilichamen gericht tegen verschillende delen van de eigen celkern (nucleus). Deze kunnen worden gedetecteerd in een verhoogde titer in het bloed van patiënten met auto-immuunziekten, zoals systemische lupus erythematosus. Daarnaast kunnen ANA's ook voorkomen bij de algemene bevolking, met name bij vrouwen en ouderen. Uit onze studie bleek dat 28% van de patiënten met uveïtis positief is voor ANA, wat hoger is dan de in de literatuur beschreven prevalentie van de ANA positieve gezonde bevolking (8-17%). Een positieve ANA in het bloed werd vaker geobserveerd bij patiënten met een infectieuze uveïtis (31%) dan bij patiënten met systemische immuun-gemedieerde ziekte (20%). We hebben geen verband gevonden tussen de aanwezigheid van ANA, de ANA titer of een specifiek ANA patroon en specifieke diagnoses of klinische kenmerken van uveïtis. Hierdoor lijkt het onderscheiden van de verschillende soorten uveïtis door middel van ANA-analyse niet mogelijk, maar dit kan niet volledig worden uitgesloten gezien het beperkt aantal patiënten in de subgroepen. Op basis van onze bevindingen concludeerden we dat ANA-analyse in de volwassen uveïtis populatie een beperkte diagnostische waarde heeft en dat ANA's waarschijnlijk niet betrokken zijn bij de pathogenese van uveïtis.

Hoofdstuk 4 is een prospectieve studie waarin de prevalentie en mogelijke rol van ARA's in bloed van 126 patiënten met uveïtis wordt vergeleken met 60 bloedbankdonoren. Middels indirecte immunofluorescentie (IIF) met primaat retinaal weefsel werd serum getest op de aanwezigheid van ARA's. Hoewel het met IIF niet mogelijk is om ARA's te specificeren, is het een zeer gevoelige techniek, wat het een bruikbare screeningsassay maakt. In onze studie werden ARA's aangetoond bij 47% van de patiënten met uveïtis, wat significant vaker was dan bij de gezonde controles (17%, $p < 0{,}001$). Specifieke verbanden tussen de aanwezigheid van ARA's en klinische oculaire kenmerken van uveïtis werden niet waargenomen. Onze resultaten suggereren dat ARA's in het bloed geen primaire rol spelen in het ontstaan van uveïtis, en waarschijnlijk een secundaire reactie zijn van het immuunsysteem op het reeds beschadigde oogweefsel.

In **hoofdstuk 5** bepalen we de mogelijke diagnostische rol van verschillende specifieke ARA's bij patiënten met auto-immuun retinopathie (AIR). AIR omvat een zeldzame groep immuun-gemedieerde oogziektes, die meestal als een paraneoplastische aandoening voorkomen in combinatie met een maligniteit. AIR is geassocieerd met de aanwezigheid van verschillende ARA's, waarbij wordt verondersteld dat deze antistoffen schade aan het netvlies veroorzaken en tot progressief

visusverlies leiden. Tot nu toe zijn slechts enkele ARA's bij AIR gespecificeerd en is de rol van deze ARA's bij AIR niet volledig opgehelderd. In dit onderzoek hebben wij in samenwerking met SciLifeLab Autoimmunity Profiling Facility in Stockholm (Zweden) serum van 24 patiënten met AIR onderzocht de op aanwezigheid van 188 verschillende ARA's. Middels een multiplex immunoassay werden bloedmonsters getest en vergeleken met serum van 151 patiënten met uveïtis en 21 patiënten met cataract. Uit de resultaten bleek dat individuele patiënten met AIR zeer verschillende ARA's in hun serum hadden. Er werden ARA's gevonden die reeds vanuit de literatuur bekend waren (zoals anti-recoverine), maar er werden ook een aantal nieuwe ARA's geïdentificeerd. Tevens bleek uit onze resultaten dat ARA's ook voorkomen bij patiënten met uveïtis en cataract, waardoor de specificiteit van ARA's voor de diagnose AIR laag was. We concludeerden dat de diagnose van AIR niet kan worden gesteld op enkel de aanwezigheid van ARA's in het serum.

In **hoofdstuk 6** tonen we een casus van een patiënt met een paraneoplastische AIR. De patiënt presenteerde zich op de polikliniek oogheelkunde met ernstig visusverlies in beide ogen. In het serum van de patiënt werden anti-retinale antistoffen tegen recoverine aangetoond. Na doorverwijzing naar een internist werd hij gediagnosticeerd met longcarcinoom, waarmee de diagnose carcinoom geassocieerde AIR werd bevestigd. De patiënt werd behandeld met chemo- en radiotherapie en een hoge dosis corticosteroïden, helaas zonder enige verbetering van zijn visus. Een longcarcinoom is de meest voorkomende maligniteit die is geassocieerd met een paraneoplastische AIR, maar elke vorm van een maligniteit kan bij deze ziekte betrokken zijn. Vaak zijn bij AIR beide ogen aangedaan, maar in zeldzamere gevallen presenteert de ziekte zich asymmetrisch of zelfs unilateraal. Visusklachten kunnen het eerste symptoom zijn van een paraneoplastische AIR, waardoor de rol van de oogarts van groot belang is bij het diagnosticeren van de maligniteit. De behandelingsmogelijkheden bij een paraneoplastische AIR zijn beperkt en de visuele prognose is vaak erg slecht.

In **hoofdstuk 7** onderzoeken we de aanwezigheid en mogelijke rol van ARA's in centrale sereuze chorioretinopathie (CSC). Bij CSC is er sprake van vochtlekkage in het netvlies. Hoewel weinig bekend is over de exacte oorzaak van CSC, wordt een mogelijke rol van het immuunsysteem verondersteld. We hebben retrospectief serum onderzocht van 63 patiënten met CSC op de aanwezigheid van ARA's middels IIF en Western blot, en de resultaten vergeleken met 101 uveïtis patiënten en 60 bloedbankdonoren. ARA's werden bij 54% van de patiënten met CSC gedetecteerd, in vergelijking met 46% van de uveïtis patiënten ($p>0.05$) en 17% van de bloedbankdonoren ($p <0.001$). ARA's in CSC waren vaker gericht tegen de fotoreceptoren dan ARA's bij uveïtis ($p=0.039$). Zowel de IIF als Western blot analyse toonde multipele ARA's bij de meerderheid van de patiënten met CSC aan, die tussen de patiënten zeer divers waren. De heterogeniteit van ARA's in CSC maakt dat het onwaarschijnlijk is dat ARA's een primaire rol spelen bij CSC, eveneens het geval is met ARA's in serum bij uveïtis.

In **hoofdstuk 8** onderzoeken we de prevalentie van systemische en retinale auto-antistoffen (ANA en ARA) bij patiënten uit Indonesië met uveïtis en actieve of latente tuberculose (TB). In Indonesië is uveïtis geassocieerd met actieve systemische of latente TB een veel voorkomende oogaandoening. De pathogenese van uveïtis en een latent TB is nog niet opgehelderd en verondersteld wordt dat een auto-immuun reacties geïnitieerd door *M. Tuberculosis* hierin een belangrijke rol spelen. We hebben de prevalentie van ARA's en ANA's onderzocht bij 95 Indonesische patiënten met 3 verschillende soorten uveïtiden: uveïtis geassocieerd met actieve TB (N = 10), met latente TB (N = 58) en zonder (latente) TB (N = 85). Het bleek dat patiënten met uveïtis en een actieve of latente TB vaker systemische antistoffen (ANA's) en minder vaak retinale autoantistoffen (ARA's) hadden, ten opzichte van patiënten met uveïtis zonder (latente) TB ($p = 0,03$ en $p = 0,021$). De lagere prevalentie van ARA's in het serum bij patiënten met uveïtis en een (latente) TB wijst niet in de richting van een auto-immuun pathogenese van dit ziektebeeld, echter zou onderzoek van intra-oculair materiaal een totaal ander inzicht kunnen bieden.

In **hoofdstuk 9** voeren we een retrospectieve studie uit naar de prevalentie van ARA's bij jonge Mexicaanse patiënten met ernstige pars planitis (N=16) ten opzichte van Mexicaanse leeftijdsgenoten (N=19). Pars planitis is een idiopathische vorm van uveïtis, die gekarakteriseerd wordt door voornamelijk ontsteking van het glasvocht. Een uitzonderlijk ernstige vorm van pars planitis wordt waargenomen bij kinderen in Mexico. Ondanks eerder uitgebreid onderzoek naar de pathogenese van pars planitis, waarbij wordt verondersteld dat het ziektebeeld auto-immuun zou zijn, is de exacte oorzaak van deze aandoening niet opgehelderd. In ons onderzoek naar de prevalentie van auto-immuun reacties vonden we ARA's bij 57% van de kinderen met pars planitis, maar een vergelijkbare prevalentie (40%) werd waargenomen bij Mexicaanse kinderen met strabismus. De hoge prevalentie van ARA's bij de controle patiënten kan het gevolg zijn van een hogere blootstelling aan exogene antigenen (bijvoorbeeld infecties). Deze resultaten suggereren dat ARA's niet de primaire oorzaak zijn van pars planitis, evenals de resultaten in Nederland met uveïtis patiënten laten zien. Een rol van auto-immuniteit bij het ontstaan van pars planitis kan echter niet worden uitgesloten, aangezien cellulaire auto-immuun reacties en het van intra-oculair materiaal niet onderzocht zijn. Deze zouden alsnog op een auto-immuun pathogenese van pars planitis kunnen duiden.

In de laatste hoofdstukken van dit proefschrift hebben we ons geconcentreerd op de bepaling van humorale auto-immuun reacties in intra-oculair vocht. **Hoofdstuk 10** is een retrospectieve studie waarin intra-oculair vocht van 76 patiënten met uveïtis en 19 cataractcontroles wordt onderzocht op de aanwezigheid van 188 verschillende ARA's. In samenwerking met de SciLifeLab Autoimmunity Profiling Facility in Stockholm (Zweden), hebben we een multiplex immunoassay uitgevoerd. We vonden 22 verschillende ARA's in hogere titers bij patiënten met uveïtis in vergelijking met controles met cataract ($p \leq 0,05$), maar de titers waren zwak verhoogd. Hoge titers van verschillende ARA's (5-voudige verhoogde titers in uveïtis in vergelijking met cataractcontroles) werden

waargenomen bij drie specifieke uveïtis-entiteiten: varicella zoster-virus geïnduceerde uveïtis, multiple sclerose geassocieerde uveïtis en patiënten met onverklaarde uveïtis en een positieve quantiferon test (alle p <0,05). De aanwezigheid van cystoïd macula oedeem was geassocieerd met hoge intra-oculaire titers van tyrosinase antistoffen (p = 0,0026). Het brede spectrum van verschillende intra-oculaire ARA's in patiënten met uveïtis suggereert eerder een secundaire productie dan een initiërende rol bij uveïtis. De resultaten van deze studie kunnen als platform dienen voor toekomstige onderzoek naar specifieke oculaire antigenen in de pathogenese van uveïtis.

Inflammatoire auto-immuun processen werden niet alleen verondersteld een rol te spelen bij de pathogenese van uveïtis, maar ook bij verschillende oorspronkelijk niet-inflammatoire oogziekten zoals RP, AMD en glaucoom. In **hoofdstuk 11** bepalen we de prevalentie en titers van 15 verschillende intra-oculaire cytokines en de prevalentie van intra-oculaire ARA's bij patiënten met RP (N = 25), AMD (N = 12), glaucoom (N = 28) en cataract (N = 22) en trachten we de laboratoriumresultaten aan de klinische manifestaties te correleren. Daarnaast wordt er serum onderzocht dat gepaard met intra-oculair vocht werd afgenomen van patiënten met RP (N = 7) en cataract (n = 10). We hebben geconstateerd dat een toenemende leeftijd geassocieerd was met stijgende concentraties van meerdere intra-oculaire cytokines, en we detecteerden dat de concentraties van een aantal cytokines tussen de onderzochte groepen verschilden, zijnde: IL-6rα (p = 0,003), IL-8 (p = 0,032) en IL-23 (p <0,004). Bij patiënten met RP waren de titers van IL-2, IL-6, MCP-1 en PlGF hoger in het oog dan in het bloed, wat een aanwijzing kan zijn voor een actieve intra-oculaire productie van deze mediatoren. Intra-oculaire ARA's werden gevonden bij slechts één patiënt met AMD, in tegenstelling tot serum ARA's, welke werden gedetecteerd in de meerderheid van de onderzochte patiënten. De verschillen in cytokine profielen tussen de onderzochte patiëntengroepen suggereren een rol van het immuunsysteem bij de pathogenese van deze oorspronkelijk niet-inflammatoire oogziekten. Mogelijk dragen deze immuunreacties bij aan een verergering van een reeds bestaande aandoening.

Concluderend, onze resultaten geven geen aanwijzingen voor een primaire rol van ARA's bij het initiëren van uveïtis en andere chorioretinale aandoeningen. De aanwezigheid van serum ARA's bij oogaandoeningen kan worden verklaard door een secundaire vorming van antistoffen welke wordt geïnduceerd door oogweefselschade. Hoewel bij patiënten met oogaandoeningen hogere titers van ARA's in serum werden gevonden dan bij de gezonde populatie, vonden we geen associaties met specifieke oogheelkundige klinische manifestaties. Tevens wijzen onze bevindingen erop dat ARA's in serum bij AIR zeer waarschijnlijk geen initiërende rol hebben, en dat de diagnose van AIR niet kan worden gebaseerd op enkel de aanwezigheid van ARA's in het bloed.

Het spectrum intra-oculaire ARA's bij uveïtis duidt op een rol van het humorale auto-immuun systeem bij de pathogenese bij uveïtis, voornamelijk bij 3 specifieke entiteiten: varicella zoster-vi-

rus geïnduceerde uveïtis, multiple sclerose geassocieerde uveïtis en patiënten met onverklaarde uveïtis en een positieve quantiferon test. Daarnaast zijn autoantistoffen gericht tegen tyrosinase geassocieerd met de aanwezigheid van cystoïd macula oedeem. Intra-oculaire ARA's bij AIR, in het bijzonder bij paraneoplastische retinopathie, zouden een interessant onderwerp zijn voor toekomstig onderzoek en zouden kunnen bijdragen aan het verduidelijken van de pathogenese AIR en het ontwikkelen van nieuwe behandelingsmogelijkheden.

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ABOUT THE AUTHOR

Josianne Carina Elvire Maria ten Berge was born on the 8th of March, 1988 in Delft, the Netherlands. She grew up in Hoek van Holland and graduated from secondary school at the ISW Gasthuislaan, 's-Gravenzande in 2006. After studying one year Liberal Arts and Sciences at the University of Utrecht, she transferred to the Erasmus University Rotterdam to study Medicine. She obtained her medical degree in February 2014, and subsequently began her PhD study described in this thesis under supervision of Prof. dr. A. Rothova (Ophthalmology) and dr. M.W.J. Schreurs (Clinical Immunology). In February 2018, she will start her residency in Ophthalmology at the Department of Ophthalmology at the Erasmus Medical Centre, headed by Prof. dr. J.R. Vingerling. Since 2007 Josianne runs together with her sister wine shop 'Vini Sardi' located in the center of The Hague.

PHD PORTFOLIO

Name PhD student:	J.C.E.M. ten Berge
Erasmus MC Department:	Ophthalmology
PhD period:	March 2014 – January 2018
Promotors:	Prof. dr. J.R. Vingerling, Prof. dr. A. Rothova

Courses and workshops

2014 Work with endnote
2014 Systematic Literature Retrieval in PubMed
2014 Research Integrity
2014 PhD Day Erasmus Medical Center 2014
2014 The Basic Introduction Course on SPSS
2015 Biomedical English Writing and Communication
2015 PhD Day Erasmus Medical Center 2015
2015 Fundus Auto Fluorescence in Practice
2015 LVAO Training Day on Intra-ocular Inflammation and Uveitis
2016 Diagnostics and Treatment of Uveitis
2017 Coaching medical students (as part of University Teaching Qualification)

Presentations

2014 Department of Ophthalmology, Erasmus MC: '*Autoimmune retinopathy*'
2014 Department of Immunology, Erasmus MC: '*Autoimmune retinopathy*'
2014 Uveitis workgroup: '*Autoimmune retinopathy*'
2016 Department of Immunology, Erasmus MC: '*The eye under attack: Impact of autoimmunity on chorioretinal disorders*'
2017 Department of Ophthalmology, Erasmus MC: '*Uveitis: autoimmune or not?*'
2017 Department of Ophthalmology, Erasmus MC: *'Autoimmunity in uveitis and other chorioretinal disorders'*
2016 Department of Immunology, Erasmus MC: '*Autoimmunity in uveitis and other chorioretinal disorders*'

(Inter)national conferences

2014 Dutch Ophthalmology PhD Students 3rd conference (DOPS), Nijmegen, The Netherlands
2014 ARVO-Ned, Utrecht, The Netherlands: '*Antibodies in malignancy-associated eye diseases* (oral presentation)
2014 'Eilanddagen Oogheelkunde' Congress, Schiermonnikoog, The Netherlands
2015 Dutch Ophthalmology PhD Students (DOPS) 4th conference, Nijmegen, The Nether-

lands: '*The eye under attack: Impact of autoimmunity on chorioretinal disorders'* (oral presentation)

2015 Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering, Groningen, The Netherlands: '*Antiretinal antibodies in uveitis, autoimmune retinopathy and the healthy population'* (oral presentation)

2015 'Eilanddagen Oogheelkunde' Congress, Schiermonnikoog, The Netherlands

2015 Nederlandse Vereniging voor Immunologie (NVvI) Annual Meeting, Noordwijkerhout, The Netherlands: '*Prevalence and clinical impact of anti-retinal antibodies in uveitis'* (poster presentation)

2016 Dutch Ophthalmology PhD Students (DOPS) 5th conference, Nijmegen, The Netherlands: *Serum antibody profiling of patients with autoimmune retinopathy* (poster presentation)

2016 Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering, Maastricht, The Netherlands: *Serum antibody profiling of patients with autoimmune retinopathy'* (oral presentation)

2016 'Eilanddagen Oogheelkunde' Congress, Schiermonnikoog, The Netherlands

2016 9th International Symposium on Uveitis (ISU), Dublin, Ireland: *'Serum antibody profiling of patients with paraneoplastic and non-paraneoplastic autoimmune retinopathy'* (oral presentation)

2017 Dutch Ophthalmology PhD Students (DOPS) 6th conference, Nijmegen, The Netherlands

2017 Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering, Maastricht, The Netherlands: *Uveitis: autoimmune or not?* (oral presentation)

2017 'Eilanddagen Oogheelkunde' Congress, Schiermonnikoog, The Netherlands

2017 14th Congress of the International Ocular Inflammation Society (IOIS), Lausanne, Switzerland: '*Autoantibody profiling in intraocular fluid of patients with uveitis*' (oral presentation)

Teaching activities

2017 Supervising research master 4th year medical student

2017 – current Coaching of medical bachelor students

2017 Lecture for 2nd year medical students (in association with immunologist): '*Autoimmune diseases of the eye*'

Other academic activities

2014 – current Weekly clinical work at uveitis outpatient department of ophthalmology, Erasmus MC

2014 – current Uveitis workgroup meetings, Utrecht, The Netherlands

2014 – current Weekly clinical ophthalmology-immunology seminars, Erasmus MC

2014 – current Bimonthly clinical ophthalmology seminars, Erasmus MC

LIST OF PUBLICATIONS

Ten Berge JC, Schreurs MW, Van Daele PL, Rothova A. Autoimmunity in uveitis. Accepted for publication in Acta Ophthalmologica

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