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Analysis of autoantibody repertoires in sera of patients with glaucoma

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PURPOSE. *Glaucoma is the second cause of blindness worldwide. It is usually considered a neurodegenerative disease. There is evidence that an autoimmune mechanism is involved in the development of glaucoma in some patients. The aim of this study was to analyze the IgG autoantibody repertoires in sera of glaucoma patients and healthy subjects.*
METHODS. *A total of 82 patients were divided into four groups: healthy volunteers without any ocular disorders (CO, n=30), patients with primary open-angle glaucoma (POAG, n=19), ocular hypertension (OHT, n=16), and normal tension glaucoma (NTG, n=17). All groups were matched for age and gender. The sera of these patients were tested against Western blots of retinal antigens. Immunodetection was done using 4-chloro-1-naphthol staining. The autoantibody patterns were digitized and subsequently analyzed by multivariate statistical techniques.*
RESULTS. *All patients showed different, complex staining patterns of autoantibodies against retinal antigens. There was an increase in the number of peaks in sera of patients with primary open-angle glaucoma (POAG) compared to healthy subjects (CO). Including all peaks the analysis of discriminance revealed a statistically significant difference between the patterns of POAG compared to all other groups ($p<0.01$). Sera of normal tension glaucoma (NTG) had no statistically different autoantibody pattern compared to those of control subjects.*
CONCLUSIONS. *In this study, we demonstrated a difference in the IgG autoantibody patterns of primary open-angle glaucoma patients compared to healthy subjects. However, the patterns were not significantly different in normal tension glaucoma compared to control subjects. (Eur J Ophthalmol 2003; 13: 752-8)*

KEY WORDS. *Autoantibodies, Glaucoma, Retinal antigen, Western blots*

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INTRODUCTION

Glaucoma is one of the leading causes of irreversible blindness worldwide (1). It is a group of ocular disorders that are responsible for loss of retinal ganglion cells, atrophy of the optic nerve, and gradual loss of visual field (2). Glaucoma is usually considered a neurodegenerative disease, but there is also evidence that an autoimmune mechanism is involved in the development of this disease in a subset of patients. The most common form of glaucoma is called primary open-angle glaucoma (POAG), where optic nerve damage and high intraocular pressure (IOP) are found. But there are also a number of patients with glaucoma (about 30%) who have never had increased intraocular pressure. This form of glaucoma is commonly called normal tension glaucoma (NTG). An autoimmune mechanism is perhaps not only involved in patients who

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have never had elevated intraocular pressure, but also in patients with primary open-angle glaucoma. Recent studies suggest antibodies against retinal antigens, such as heat shock proteins (HSPs) (3), rhodopsin (4), gamma-enolase (5) (γ-enolase), glutathione-S-transferase (GST) (6), tumor necrosis factor-alpha (TNF-α) (7), and gamma-synuclein (γ-synuclein) (8), are possible neurodegenerative factors in glaucomatous eyes. Heat shock proteins have been closely studied and therefore several HSP-antibodies against ocular antigens have been detected such as HSP-90 (90 kilo Dalton), HSP-70 (70 kDa), HSP-60 (60 kDa), and small heat shock proteins (25 to 30 kDa) antibodies. In patients with glaucoma elevated serum autoantibodies against small heat shock proteins have been found (9). These small heat shock proteins include α-crystallins (αA-crystallin and αB-crystallin subunits) and HSP-27 (10). Also serum autoantibodies that cross-react with glycosaminoglycans can play a significant role in specific injury in patients with systemic autoimmune diseases and are also found in patients with glaucoma. Glycosaminoglycans (GAGs) play an important role as membrane proteins and add to the strength and elasticity of the optic nerve head. GAGs can be considered activity markers in autoimmune disease (11). Besides the possibility that autoantibodies are a pathologic factor in the development of glaucoma, it is also possible that autoantibodies are the result of stress induced to retinal ganglion cells due to glaucoma (12). This stress may be ischemia, mechanical stress due to high intraocular pressure, a high level of amino acids, or toxic products from high nitric oxide synthethase production in neurons. In this study we examined the specific IgG autoantibody pattern against retinal antigen in patients with glaucoma (POAG and NTG) and patients with ocular hypertension to show the differences between glaucoma patients and healthy subjects.

MATERIALS AND METHODS

Patients

The study was performed in accordance with the Declaration of Helsinki on Biomedical Research involving human subjects. We included four groups of patients in this study. Nineteen patients with intraocular pressure (IOP) greater than 21mmHg and visual field loss were placed in the primary open-angle glaucoma (POAG, mean age 67.0 SD±11) group. They had open angles and the absence of alternative causes of optic neuropathy (e.g. infection, inflammation, ischemic disease, and compressive lesions). Sixteen patients had IOP greater than 21mmHg but no visual field loss and therefore fulfilled the criteria of ocular hypertension (OHT, mean age 59.6 SD±14). Seventeen patients with visual field loss but normal IOP (= less than 24mmHg) are normal tension glaucoma (NTG, mean age 70.6 SD±9) patients. Thirty healthy age matched volunteers, with no history of ocular disorders, no pathologic fundus, and no elevated IOP, were chosen as control group (CO, mean age 69.8 SD±11). All patients were matched for age and gender. Exclusion criteria for all groups were: acute attack of glaucoma, diabetes mellitus and retinopathy, retinal detachment, and retinal vascular obliteration. Intraocular pressure was determined by Goldmann applanation tonometer and visual field was examined by Goldmann perimeter.

Western blot

After giving their informed consent blood was taken from all patients and healthy control subjects. Those blood samples were centrifuged at 1000 g for ten minutes. The serum was stored at -23°C for later examination. We dissected retinas from bovine eyes and homogenized the retinas in sample buffer (1M Tris, pH 7.5; 10% SDS; DTT; bromphenol blue, pH 6.8). The samples were centrifuged at 15000RPM for one hour. Then they were cooked and centrifuged several times afterwards. The pellet was discarded and the supernatant was stored for later analysis. The retina extracts were used for 13.5% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) using a MultiGel-Long (Biometra, Goettingen, Germany). After electrophoresis the gels were transferred onto nitrocellulose membranes Protran BA 83 (Schleicher and Schuell, Dassel, Germany) by using a Semi-Dry Blotter (Biometra, Goettingen, Germany). After blotting the membranes for one hour the quality of the transfer was checked by staining the nitrocellulose membranes with PonceauS solution (Sigma, Munich, Germany). The blots were then blocked with blocking buffer (5% non fast dry milk with Tween20 in phosphate-buffered saline (PBS)) for one hour. The nitrocellulose was cut into strips and one strip was used per pa-

tient. The strips were incubated over night with patient serum (1:40 dilution, in washing buffer (0.5% non fast dry milk with Tween20 in PBS)). After washing the strips with Tris-buffered saline (TBS) several times, they were incubated with secondary antibody: peroxidase-conjugated Immuno Pure® Goat Anti-Human IgG (H+L) (diluted 1:500, Pierce, Illionois, USA) for one hour. After several washing steps the bands were developed by staining with 0.05% 4-chloro-1- naphthol (Sigma, Munich, Germany) with 0.015% hydrogen peroxide in 20% methanol in TBS for 20 minutes. Molecular weights were estimated for each band based on the distance migrated for ten known molecular weight standards (BenchMark, Invitrogen, Karlsruhe, Germany).

Data analysis

The data were acquired using a color flatbed scanner (Epson GT-9000, Epson Germany, Duesseldorf, Germany). Digital image analysis and evaluation of Western blots was done using BioDocAnalyze (Biometra, Goettingen, Germany), which created densitometry data of the blots showing the grey-intensity values versus relative mobility (Rf-values). BioDocAnalyze evaluated the height, area, and molecular weight of all peaks of the densitographic data file. The maximum extinction (band intensity of an individual blot) was set to 100%. The other extinction values of this blot were transformed into relative percentage, thus reducing the influence of different absolute staining intensities of individual blots. Therefore, the antigen-antibody reactions were analyzed by Western blotting followed by a digital image analysis. Based on the densitographs of each Western blot, multivariate statistical techniques were used to detect differences in the distribution of antibodies against retinal antigen in patient sera. The densitographic data, such as peak height, localization, and area under the curve is exported as data vectors to Statistica® (Statsoft, Tulsa, Arizona, USA) and the statistical calculations were then performed by Statistica®. These data vectors are subsequently calculated by multivariate analysis of discriminance. Recently, this technique has been successfully used in myasthenia gravis, Graves' disease (endocrine ophthalmopathy) and experimental uveitis (13-15). This technique (digital analysis of Western blots) allows quantitative screening of many autoantibodies or antigens at the same time. The analysis of discriminance can test the null hypothesis if the patterns of clinical groups derive from the same population or if they are statistically significantly different. Furthermore, the analysis can quantify the difference between the overall differences of the average group patterns using statistical distance measure parameters (e.g. Mahalanobis distances) and can demonstrate the similarity or dissimilarity in antibody patterns for each patient. This is done by a so called canonical analysis by the canonical roots. The canonical roots provide information about the antibody profiles by ordering them in the discriminant space. In simple words: if the canonical roots of patterns are very similar, the patterns were found to be similar by the analysis of discriminance (13-15). A similar approach was taken by Petriocin et al for the detection of serum proteins in patients with ovarian cancer. They used Seldi-TOF mass spectroscopy (surface-enhanced laser desorption and ionization, by Ciphergen, Freemont, CA, USA) to detect signs of ovarian cancer in patient sera (16). A complex proteomic serum pattern was found which can be used a biomarker for ovarian cancer. They got a positive-predictive value of about 94 percent. This serum proteomic pattern analysis might be used in medical screenings as a supplement to diagnostic work-up and assessments.

RESULTS

All patient and control sera showed a different and complex staining pattern of IgG autoantibodies against retinal antigen. Figure 1 shows some of the Western blots of autoantibodies of patient sera against retinal antigen. There are many 4-chloro-1-naphthol stained bands visible in all three patient groups (POAG, NTG, and OHT) as well as in healthy control subjects (CO), sometimes more than 20 bands. We found no correlation between age of the patients and peaks or between gender and peaks. Figure 2 shows a Western blot of a patient with primary open-angle glaucoma on the left side and on the right side the densitograph of the blot of the same patient can be seen. In the densitograph scanner units (U =optic density) were plotted against relative mobility (Rf-values). We looked at a molecular weight up to 177 kDa. Including all peaks the analysis of discriminance revealed a statistically significant difference between the patterns of POAG compared to all other groups

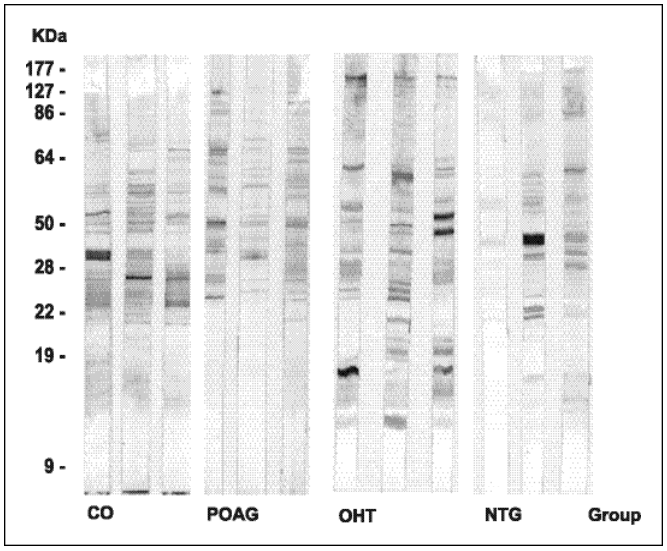

Fig. 1 - *Western blots of IgG autoantibody repertoires in patient sera made visible by 4-chloro-1-naphthol staining. There is a complex pattern of antibodies in all four groups: healthy subjects (CO), patients with primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), and ocular hypertension (OHT). Each lane was incubated with patient serum (diluted 1:40) against bovine retina over night. Secondary antibody, peroxidase-conjugated goat anti-human IgG (diluted 1:500), was applied after one hour.*

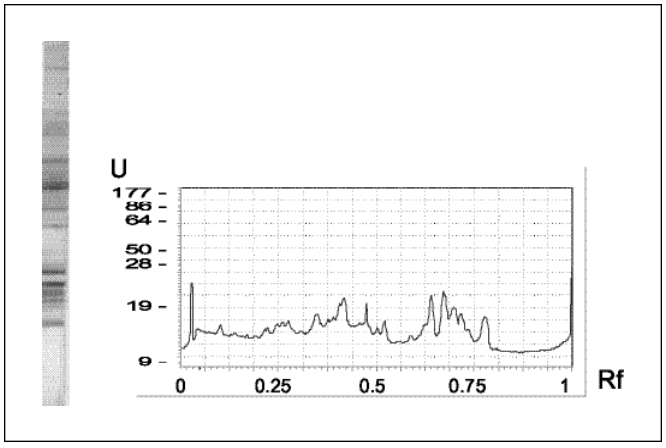

Fig. 2 - *On the left side you can see the 4-choro-1-naphthol stained strip of a patient with primary open-angle glaucoma (POAG). And on the right side the densitograph of the Western blot of this patient. The densitograph scanner units (U =optic density) were plotted against relative mobility (Rf-values).*

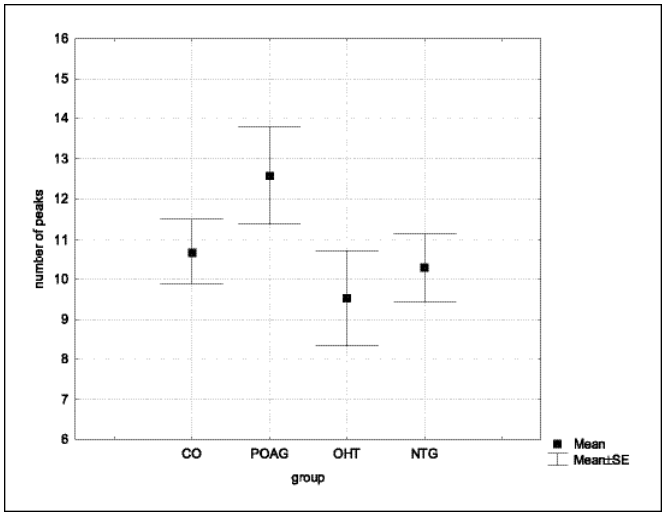

Fig. 3 - *Mean number of peaks and standard errors in sera of patients with primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), and ocular hypertension (OHT) compared to healthy volunteers (CO). The number of peaks was significantly higher in patients with POAG (mean = 12.6±SE) compared to the number of peaks in CO subjects (mean = 10.6±SE). Patients with ocular hypertension (OHT, mean = 9.9±SE) had a slightly lower number of peaks than healthy subjects. Whereas no significant difference in the number of peaks of patients with normal tension glaucoma (NTG, mean = 10.1±SE) compared to healthy volunteers was found.*

(p=0.0021). The patterns of NTG and OHT patients had no statistically different pattern compared to those of control subjects. The number of peaks of Western blots against retinal antigens was increased in sera of POAG patients compared to all other groups (Fig. 3). The number of peaks was significantly higher in patients with POAG (mean = 12.6±SE) then in CO subjects (mean = 10.6±SE). Patients with OHT (mean = 9.9±SE) had a slightly lower number of peaks than controls. Whereas no significant difference in the number of peaks of patients with NTG (mean = 10.1±SE) compared to healthy volunteers was found. Against retinal antigens, several molecular weight regions could be demonstrated that are elevated in the sera of POAG patients e.g., at approx. 14, 18, or 45 kDa. At 18 kDa, there was a higher antibody titer in both POAG and NTG. Furthermore, there are several regions with complex reactivity in all groups or even much lower reactivity in one of the glaucoma groups compared to all other groups. The POAG group shows a decreased level of antibodies e.g. at about 25 kDa and the NTG shows a decrease at approximately 60 kDa compared to all other groups (Fig. 4). The multivariate analysis of discriminance which includes the complete complex banding pattern of each Western blot in the calculation, revealed a significant difference between all groups. (Wilks' lambda= 0.15; $p < 0.01$) for retinal antigens. The analysis can provide some more

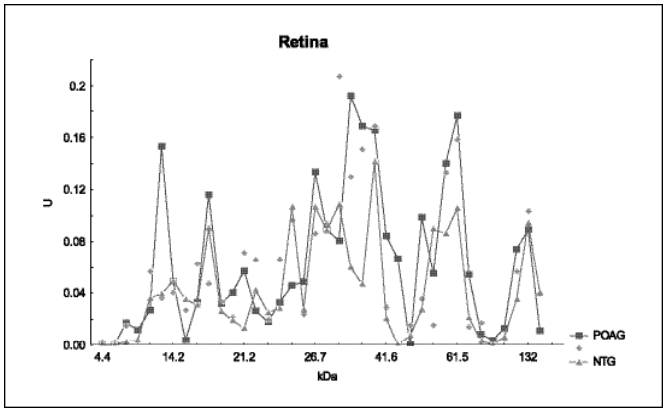

Fig. 4 - *The mean antigen-antibody reactivity of all groups was plotted against the corresponding molecular weight of the retinal antigens. Complex staining patterns could be found in all four groups: control subjects (CO), patients with primary open-angle glaucoma (POAG), with ocular hypertension (OHT), and with normal tension glaucoma (NTG).*

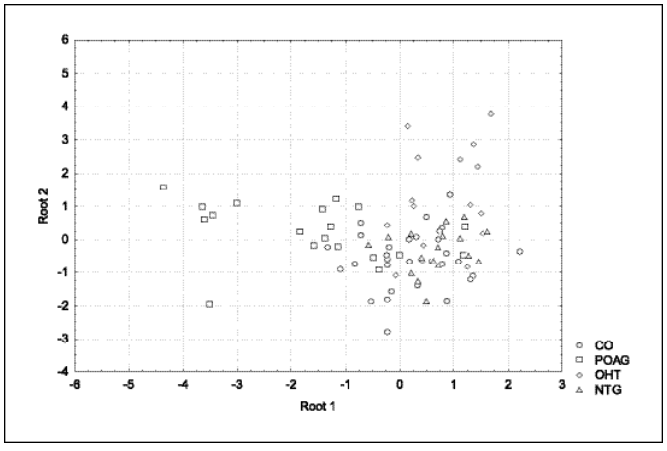

Fig. 5 - *Canonical roots of serum IgG autoantibodies of patients with primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), ocular hypertension (OHT), and healthy volunteers (CO). These canonical roots are derived from discriminant analysis and were plotted for each single patient. This two-dimensional graph shows the similarity of the different patient groups. The closer the points to each other, the more similar the autoantibody patterns are on the Western blots. A clear separation between POAG patients and the CO group can be seen, whereas the canonical roots of NTG patients are close to those of CO subjects. OHT patients show a slight separation from the control group.*

details about the discriminance power i.e., a measure of the overall similarity between the groups. The POAG group was the most different from all other groups ($p<0.0001$; distance POAG-CO=5.78). Furthermore, the NTG group revealed the smallest difference from the control group (NTG-CO=1.9; OHT-CO=3.11). Figure 5 shows the canonical roots of serum IgG antibodies of the four different groups. Canonical roots show the similarity of different patients. The closer the points to each other, the more similar are the autoantibody patterns of the Western blots of these patients. A different pattern of autoantibodies in patients with POAG compared to the control group is detectable. The quality of separation of these two groups could be seen very clearly in Figure 5. The OHT patient group shows only a minor separation from the control group. The antibody repertoire of NTG and OHT patients is also more similar to healthy subjects than that of POAG patients.

DISCUSSION

Several antibodies against retinal antigens, such as heat shock proteins (3), rhodopsin (4), gamma-enolase (5), glutathione-S-transferase (6), tumor necrosis factor α (7), gammasynuclein (8), and glycosaminoglycans (11) have already been detected. These observations suggest an autoimmune involvement in the case history of glaucomatous patients. Their glaucoma compromises an organ-specific autoimmune disease, which leads to retinal ganglion cell destruction. Heat shock proteins for example are implicated in the development of a number of human autoimmune disorders like glaucoma (3). Increased autoantibodies against heat shock proteins may accompany glaucomatous optic neuropathy as something like an epiphenomenon of retinal ganglion cell injury or increased stress for retinal cells (17). There is also the possibility that heat shock proteins are pathogenic factors and therefore contribute to the disease progression in glaucomatous patients. Monoclonal antibodies to HSP-27 induce degradation of actin cytoskeleton and cause apoptotic cell death (= programmed cell death) in human retina (9). In our study the number of peaks was significantly higher in patients with POAG then in CO subjects, whereas patients with OHT had a slightly lower number of peaks than controls. And we found no significant difference in the number of peaks of patients with NTG compared to controls. But finding no difference in the general number of peaks in NTGs does not mean that we did not find an increase in some

specific autoantibodies (e.g. at about 24kDa, Fig. 4). This could be an increase in α-B crystallin as detected previously in other studies (10). The elevated antibody peak at about 14 kDa in the POAG pattern corresponds to an antibody peak found by Surgucheva et al (8). In this study an antibody peak at 14kDa was detected in sera of glaucoma patients and identified as γ-synuclein.

It is known that natural autoantibodies occur even in the sera of healthy subjects. The variety of these auto-antigens is very complex (18). Therefore it is very important to look at the whole autoantibody repertoire of a person to possibly predict the persons' risk for glaucoma. Right now the role of autoantibodies in the pathogenesis of glaucoma is not fully resolved, but finding a specific antigen or antibody pattern could provide a possibility to judge the progression of the disease in a glaucoma patient. This is especially important because many people are unaware that they do have the disease. Glaucoma is sometimes called the "silent blinder", because no noticeable symptoms are recognized by the patients before they lose their central vision (19). Therefore a screening method is necessary where glaucoma can be detected before loss of vision has already occured.

Through the method used in this study we found a very complex IgG antibody pattern in patients with glaucoma and healthy subjects. Differences between patients with POAG and healthy subjects (CO) could be found and quantified. We could demonstrate a statistically significant difference between the autoantibody repertoires of patients with POAG compared to CO. POAG patients showed an increased level of autoantibodies which is perhaps not only due to apoptotic cell death in retinal ganglion cells of glaucomatous patients. Maybe a correlation between elevated intraocular pressure and an increased number of autoantibodies will also be found. Furthermore, we could detect a slightly decreased level of autoantibodies in patients with ocular hypertension. Although no significant difference in the number of peaks of the NTG group compared to controls was found, the NTG antibody pattern is quite different from the CO pattern (Fig. 4). These differences in the autoantibody repertoire underscore the significance of a possible autoimmune involvement in glaucoma.

Besides the possible involvement of an autoimmune mechanism in optic nerve damage in patients with glaucoma a protective factor of the immune system in glaucomatous disease is also considered. Signal pathways of the immune system regulate cell death in response to stress conditions in retinal neurons, including ischemia, mechanical stress from high intraocular pressure, nitric oxide (20) or glutamate. Nitric oxide (NO) is a physiologic mediator that is also used by retinal ganglions cells. Glutamate is elevated to toxic levels in the vitreous body and aqueous humor of glaucoma patients (21). Glutamate is a neurotransmitter and found in high levels in the central nervous system and especially in the retina. High levels of glutamate can be toxic to neurons and lead to apoptosis. In both glaucoma groups POAG and NTG axons die through apoptotic cell death. Postmortem studies of the retinas of NTG patients show a significant loss of retinal ganglion cells and their axons compared to control subjects (22). In studies of eyes of people with POAG retinal ganglion cell death through apoptosis has also been found (23). The glaucomatous eyes have a higher frequency of TUNEL-positive cells compared to control subjects. This may also be a reason for the elevation of autoantibodies in glaucoma patients.

It could also be possible that antibodies develop as a result of glaucoma. Schwartz et al developed an optic nerve crush model to find further prove of an autoimmune mechanism involvement in glaucoma development (24). In this model not only primary but also secondary loss of retinal ganglion cells was visible. This may explain the fact that loss of retinal ganglion cells continues in patients with glaucoma receiving therapy that lowers intraocular pressure (25). These patients show a progression of retinal ganglion cell loss even at a lower intraocular pressure.

In this study a significant increase in the number of IgG autoantibody peaks in sera of patients with primary open-angle glaucoma compared to healthy subjects could be found. NTG patients had no increased number compared to those of control subjects, which may be in contrast to other studies where an increased level of antibodies against a specific antigen was found in NTG patients. But, as shown in Figure 4, we did find a different antibody pattern in both glaucoma groups compared to the control group. Maybe an autoimmune mechanism is not only involved in glacoma patients with normal intraocular pressure but also in patients with increased oc-

ular pressure (POAG). The increased level of autoantibodies could lead to elevated intraocular pressure by interfering with the outflow of aqueous humor. A stronger confirmation of the thesis of an autoimmune mechanism in glaucoma is still necessary and requires further studies.

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