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Biomarkers for glaucoma: from the lab to the clinic

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Abstract

Glaucoma, a leading cause of irreversible blindness worldwide, is often not diagnosed until many years after disease onset. Early and objective diagnostic measures are yet missing. Besides the main risk factor, an elevated intraocular pressure (IOP), age, sex, and ethnicity are known to affect disease progression and severity. Furthermore, oxidative stress, elevated glutamate concentrations, and an autoimmune component are considered possible risk factors. We could identify several potential proteomic biomarkers in glaucoma and examine distinct changes in the glaucomatous human retina proteome. Using an experimental autoimmune glaucoma animal (EAG) model we could demonstrate an IOP-independent loss of retinal ganglion cells (RGC), which is accompanied by antibody depositions and increased levels of microglia. In a different animal model we showed that intermittent IOP elevations provoke neurodegeneration in the optic nerve and the retina and elicit changes of IgG autoantibody reactivities. The correlation between neuronal damage and changes in autoantibody reactivity suggests that autoantibody profiling could be a useful biomarker for glaucoma. *In vivo* studies on neuroretinal cells and porcine retinal explants demonstrated a protective effect of antibodies (eg, anti-GFAP) on RGC, which seems to be the result of reduced stress levels in the retina. We conclude that the absence of some autoantibodies in glaucoma patients reflects a loss of the protective potential of natural autoimmunity and may thus encourage neurodegenerative processes. Concluding, autoantibody profiles resemble useful biomarkers for diagnosis, progression and severity of glaucoma. Future longitudinal studies will help to improve early detection and enable better monitoring of disease progression.
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Introduction

The term glaucoma subsumes a group of optic neuropathies that share characteristic morphological changes within the retinal nerve fiber layer and the optic nerve head which are associated with a slow and progressive retinal ganglion cell (RGC) death and visual field loss.[1] Glaucoma is the most common cause for irreversible blindness and the second leading cause of blindness worldwide.[2] The most frequent glaucoma form in the Western world is the primary open-angle glaucoma (POAG).[3,4] Although the risk for POAG rises with the elevation of intraocular pressure (IOP) and an elevated IOP is the most common known risk factor for glaucoma, most patients with ocular hypertension will not develop glaucoma.[5] It is assumed that risk factors others than IOP are mainly involved in the onset of the condition, especially depicted in glaucoma patients with statistically normal pressure levels less than 21 mm Hg, the so called normal tension glaucoma (NTG).[6] Beside age, sex, and ethnicity,[7] events such as oxidative stress,[8] systemic and ocular vascular factors,[9] elevated glutamate concentration[10] or nitric oxide levels,[11] or an autoimmune component[12,13] are considered possible risk factors. It is also likely that a combination of multiple risk factors increases the possibility of developing glaucoma and may influence its severity and other phenotypic characteristics.[14] By now, elevated IOP is the only risk factor that can be treated, for example, with medications or glaucoma surgery. Control of IOP early in the disease process has been shown to delay or even arrest glaucoma progression and the resultant visual field loss.[15,16]

The diagnosis of glaucoma requires a detailed examination of optic disc structure and visual field, assessing both structure and function of the eye. Unfortunately, most potential screening tests have an estimated specificity of approximately 85%[17] resulting in an insufficient

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predictive power. Hence, most patients have suffered from glaucoma for over 10 years without knowing and as many as half of RGC and their axons can be lost before first pathological changes can be detected.[18,19] Several epidemiological studies have shown that at least half of glaucoma patients remain undiagnosed in developed countries[20] and nine out of 10 worldwide.[7] Especially the early diagnosis of the disease has an important role, since so far the threat of blindness can only be prevented by timely treatment through lowering the IOP. This underlines the strong demand for additional diagnostic options and disease or treatment monitoring, for example, by use of biomarkers. A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention, and has valuable applications in disease detection and monitoring of health status.[21]

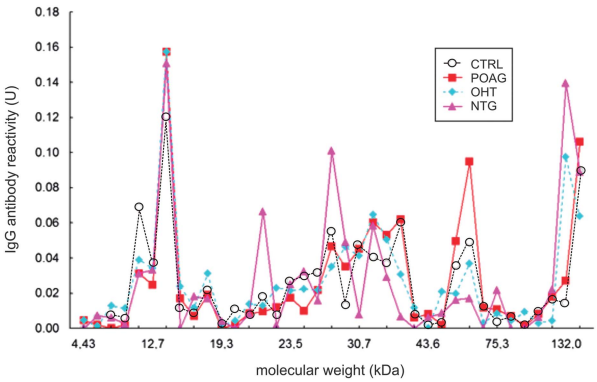


Figure 1 Antibody profiles against optic nerve antigens in different glaucoma groups. Antibody (IgG; immunoglobulin G) profiles against optic nerve antigens in two glaucoma groups (primary open-angle glaucoma (POAG) and normal tension glaucoma (NTG) in comparison to healthy controls (CTRL). The *x* axis shows the molecular weight in kilodalton (kDa) and the *y* axis the IgG antibody reactivity (U). Complex IgG antibody profiles could be found in all groups. The antibody profiles of the glaucoma groups were significantly different from controls.[28] (Courtesy of Molecular Vision)

Identification of potential glaucoma biomarkers from clinical studies

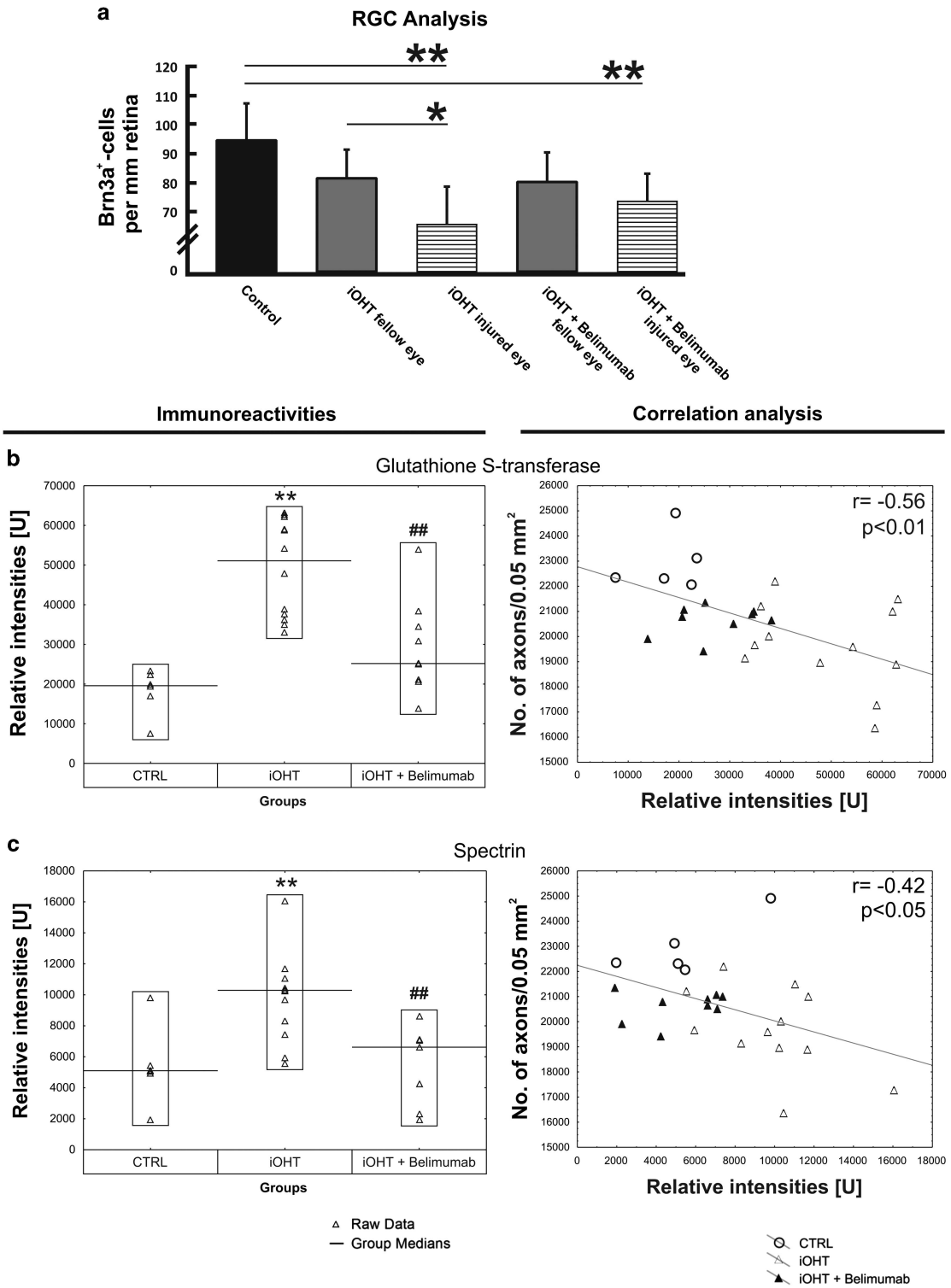
By now, several proteomic markers, for example, crystallins,[22,23] heat shock protein 60 (HSP 60) and HSP 90, myotrophin, apolipoprotein B and apolipoprotein E, endothelial leukocyte adhesion molecule-1, myoblast determination protein 1, myogenin, vasodilator-stimulated phosphoprotein, ankyrin-2[24] and transthyretin,[25] just to mention a few, have been identified as potential biomarkers in POAG. In a recently published study[26] we examined the human retina proteome of glaucoma patients by state-of-the-art mass spectrometry (MS) method. Distinct proteomic changes could be observed in 10% of proteins and support the involvement of three functional classes in glaucomatous processes: mitochondrial, stress and nucleus proteins, indicating an impairment of energy metabolism, stress response and gene expression alterations in the course of retinal neurodegenerative processes. Beside an increase of stress related proteins we also found a decrease of new glaucoma-related candidates, highlighting new molecular players ADP/ATP translocase 3 (ANT3), PC4 and SRFS1-interacting protein 1 (DFS70) and methyl-CpG-binding protein 2 (MeCp2) associated with glaucoma. Moreover, these candidates could be validated by Accurate Inclusion Mass Screening and immunostaining, and supported for the RGC layer by laser capture microdissection,[26] giving direction for future glaucoma research projects.

Autoimmune involvement in glaucoma pathogenesis

Several clinical and experimental studies point toward a possible autoimmune involvement in the pathogenesis of glaucoma. Disease-specific changes in complex expression levels of immunoglobulin G (IgG) autoantibodies against

Figure 2 Analysis in intermittent ocular hypertension model (iOHT). (a) The survival of RGC was analyzed as the number of Brn3a-positive cells per mm retina in naso-temporal cross-sections of the eye in control animals (CTRL), animals that received unilateral intermittent ocular hypertension (iOHT), and animals with unilateral intermittent ocular hypertension which received B lymphocyte inhibitor Belimumab treatment (iOHT+Belimumab). A significant loss of RGC was observed in injured eyes of iOHT group and iOHT+B lymphocyte inhibitor Belimumab group, to hinder B lymphocyte activation, (both $P<0.01$, horizontal lines) compared with control eyes (black). Furthermore, a significant difference in damage was observed between fellow and injured eye of iOHT group ($P<0.05$. Significant values are indicated as follows: $^{*}P<0.05$, $^{**}P<0.01$). (b and c) Quantification of different antigen reactivities The left side shows the immunoreactivities in relative intensities per group against glutathione S-transferase (b) and spectrin (c). Each triangle represents one animal, and the black line indicates the group median. Compared with the relative intensity of CTRL, iOHT was significantly upregulated ($^{**}P<0.01$) for all investigated antigens. Compared with iOHT, all immunoreactivities of iOHT+Belimumab were significantly downregulated ($^{\#\#}P<0.01$). Scatterplots of the number of optic nerve axons in a distinct area (no. of axons/0.05 mm2) against relative intensities of glutathione S-transferase (b) and spectrin (c), are shown on the right side. The gray fitting line shows the negative linear dependence between the number of axons per 0.05 mm2 and the relative intensities. Significant values are indicated as follows: $^{**}P<0.01$ compared with control group, $^{\#\#}P<0.01$ compared with iOHT group. (Adapted from Gramlich *et al*[43])

ocular tissue could be detected in sera and aqueous humor of glaucoma patients suffering from different glaucoma forms: NTG, POAG, or patients with ocular hypertension (Figure 1).[27–32] Furthermore, these autoantibody profiles are very stable and could be found in study populations from different continents.[32] Especially worth mentioning is the fact that not only elevated antibody reactivities could be detected in the



glaucoma groups, but also a reduction of some antibody levels could be observed. Analyses in numerous glaucoma patients showed for example up-regulation of autoantibodies against alpha-fodrin,[32] HSP70[33] or myelin basic protein (MBP)[29] and downregulation of antibodies against αB-Crystallin or Vimentin,[33] just to name a few.

To answer the question whether elevated antibody reactions can induce an autoaggressive RGC loss in animal models, an experimental autoimmune glaucoma animal (EAG) model has been developed. After being systemically immunized with biomarkers identified in clinical studies, the animals developed antibodies against the applied proteins, for example HSP60,[34,35] HSP27,[35] or MBP[36] resulting in glaucomatous damage with distinct retinal RGC loss. Subsequent EAG studies focused on the pathomechanism of immunizations with retinal and optic nerve associated antigens and showed that the IOP-independent RGC loss in EAG depends on the specificity of the antigen and leads to altered serum antibody patterns.[37] Moreover, RGC loss after immunization with retinal antigens is accompanied by antibody depositions and increased levels of microglia.[38–40]

IOP and immunity

To further examine a possible link between elevated IOP and immune response we used a recently established intermittent ocular hypertension model,[41,42] where a silicone loop is attached around the limbus of the eye to temporarily increase the IOP. After the manipulation, the IOP values return to baseline levels, which demonstrates that this model does not cause angle-closure glaucoma.[43] We found evidence that intermittent IOP elevations are sufficient to provoke neurodegeneration in the optic nerve and the retina and elicit changes of IgG autoantibody reactivities.[43] Unilateral intermittent IOP elevations led to a significant decrease of RGCs (Figure 2a) and their axons in the optic nerve, accompanied by bilateral microglia activation and IgG autoantibody depositions in the retina. Furthermore, a systemic immune response due to RGC loss in association with an increased IOP could be shown with a significant increase of serum IgG reactivities as well as IgG immunoreactivities against glutathione S-transferase (Figure 2b), spectrin (alpha-fodrin, respectively; Figure 2c), and transferrin.[43] The additional immunomodulation using the B lymphocyte inhibitor Belimumab failed to ameliorate axonal and RGC survival and had very little effect on deactivation of microglia, and reduction of IgG autoantibody depositions despite lowering the total IgG serum concentration. However, the question remains whether such *in vivo* results are transferable to humans and how the circumstances in human glaucomatous patients are.

Nevertheless, the correlation between neuronal damage and changes in autoantibody reactivity suggests that autoantibody profiling could be useful as a biomarker for glaucoma.[43]

Neuroprotective effects of antibodies on retinal explants

Our previous *in vitro* studies showed that antibodies downregulated in glaucoma patients, for example, antibodies against glial fibrillary acidic protein (GFAP), 14-3-3 or gamma (γ)-synuclein, have protective effects on immortalized neuroretinal cells and increased their viability under stress conditions.[44–46] This neuroprotective effect could be traced back to altered anti-apoptotic protein expressions in the mitochondrial apoptosis pathway for gamma (γ)-synuclein antibody or anti-14-3-3,[44,45] or to altered proteins of the actin cytoskeleton pathway for GFAP antibody.[46]

In subsequent experiments we used an adolescent retinal organ culture from pigs and incubated the retinal explants with different antibodies for 24 h (anti-GFAP, anti-γ-synuclein, and anti-myoglobin antibody as a control). In accordance with previous results, immunohistochemical analysis revealed a protective effect of anti-γ-synuclein and anti-GFAP antibodies on RGC survival (Figure 3) and demonstrated the uptake of γ-synuclein antibodies, as well as the binding of GFAP antibodies to the cell membrane of Müller cells.[47] Furthermore, mass spectrometric results showed

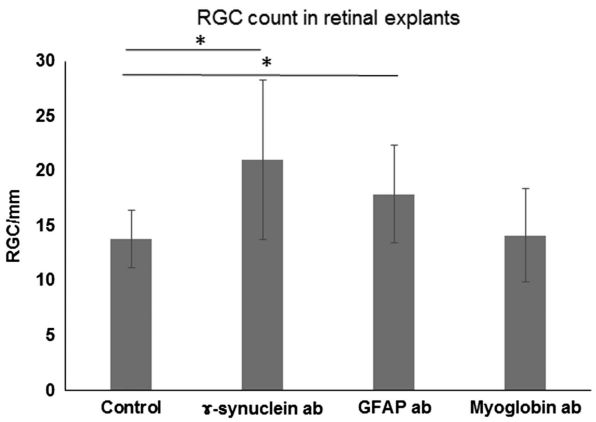


Figure 3 Effect of antibodies on the number of RGC in retinal explants from adolescent pigs. Retinal explants were cultivated either with control medium without additional antibodies (ab) (control) or with additional γ-synuclein antibodies or glial fibrillary acidic protein (GFAP) antibodies for 24 h, or with anti-myoglobin antibodies, serving as an isotype-matched control antibody (*ex vivo* 27 h). The Quantification of RGC/mm: revealed significantly increased RGC numbers in both the retinae incubated with GFAP antibodies as well as retinae incubated with γ-synuclein antibodies. $^{*}P<0.05$; $^{**}P<0.01$ (error bars are SD; Adapted from Bell *et al*[47])

increased levels of glutamine synthetase in antibody-treated retinal explants. Glutamine synthetase is expressed exclusively in Müller cells and serves as a specific Müller cell marker. Müller cells are essential for RGC survival and have an important role in the recycling of neurotransmitters, homeostasis of the extracellular environment, removal of metabolic end products, as well as the supply with neurotrophic and antioxidant factors.[48] The strong Müller cell involvement was found associated with reduced endoplasmic reticulum stress response, and a redistribution of glutamine synthetase localization in direction of the end feet of the Müller cells towards the inner retinal layer. These findings suggest that anti-GFAP and anti-γ-synuclein antibodies have a protective effect on RGC which seems to be the result of reduced stress levels in the retina.[47] Possibly the absence or the loss of some autoantibodies in glaucoma patients (eg, anti-GFAP) reflects a loss of natural protective autoimmunity, thus encouraging neurodegenerative processes and making the RGC more vulnerable for stress factors.[12]

Human glaucoma and autoimmunity

Beside the consistent antibody profile in sera (Figure 1), aqueous humor and tears of glaucoma patients,[27–33] we found evidence that autoantibodies are accumulated in the retinae of glaucoma patients and that the local immune homeostasis might be affected.[49] More detailed, the IgG autoantibody deposits were accompanied by IgG-positive plasma cells (Figure 4) and occurred in pro-inflammatory conditions with increased levels of tumor necrosis factor-α, interleukin-6 and interleukin-8, which seems to be maintained locally by immune-competent cells like microglia.[49] We suggest that microglia activation might provide the increased levels of tumor necrosis factor-α and interleukin-8 in glaucomatous retina after activation by autoantibody depositions, maintain antibody-dependent cell-mediated cytotoxicity and regulate the pro-inflammatory homeostasis along with the recruitment of plasma cells.[49] These findings comply with results from EAG where immunization led to an antigen specific, complex systemic immune response, which included the development of autoreactive antibodies against retinal and optic nerve epitopes with an increasing and time-dependent severity.[37] The loss of RGCs in EGA model is also accompanied by IgG autoantibody deposits on cells in the RGC layer and often found in co-localization with activated microglia cells.[38,39]

Improvement of glaucoma detection

Despite of these results, it is still unclear whether the changes found in autoantibodies are pathogenic or merely a consequence of glaucomatous optic neuropathy.[12] However, the distinct changes in antibody profiles could be used as biomarkers for the detection of glaucoma. And indeed, using a targeted antigen microarray we were able to differentiate sera of glaucoma patients with POAG from non-glaucomatous controls based on antibody profiles with a sensitivity and specificity of $>93\%$ (Figure 5).[50] This represents an enormous diagnostic precision when compared to established screening tests with an estimated specificity of ~85%.[17] Furthermore, the correlation between damage and changes in the autoantibody reactivity found in the intermittent ocular hypertension model suggests that autoantibody profiling could also be useful as biomarkers for progression and severity of the disease.[43] To further answer this question longitudinal studies are required that allow monitoring of glaucoma progression and permit the staging of the disease in correlation with antibody patterns. The analysis of longitudinal antibody profiles could also be useful to

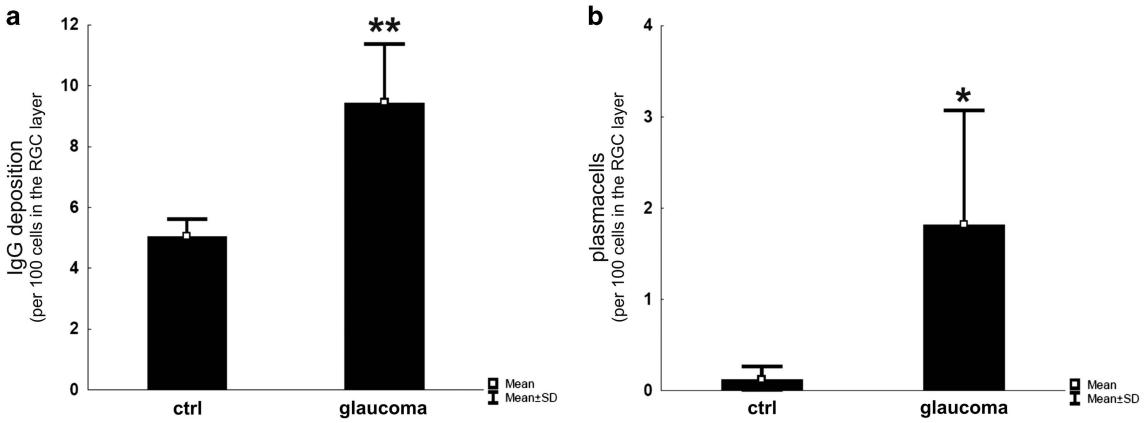


Figure 4 Quantification of IgG depositions and plasma cells in cross-sections of human glaucomatous and healthy retina. (a) The number of IgG deposits in relation to the number of remaining cells is significantly increased. (b) Based on morphological features, plasma cells were only detectable in the glaucomatous group. $*P = 0.05$; $**P = 0.01$. (Adapted from Gramlich *et al*[49])

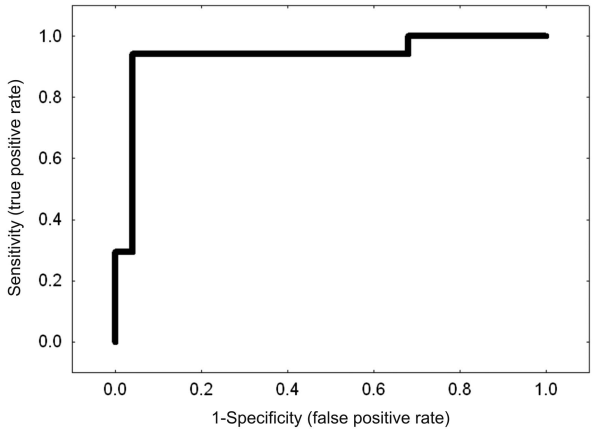


Figure 5 Sensitivity and specificity of autoantibody patterns for diagnosis. Receiver operating characteristic for comparison of autoantibody reactivities in serum samples of primary open-angle glaucoma patients (POAG; $N=20$) and non-glaucomatous controls ($N=13$) using specific antigen microarrays in combination with an artificial neural network (X-axis: 1-specificity, Y axis: sensitivity, $R=0.93$). The sensitivity and specificity for a discrimination of prospective glaucoma and control subjects was 93%. (Reprinted from Boehm *et al*,[50] with permission from Elsevier).

detect glaucoma at an early stage before clinical signs appear.

In the future, a quick and easy to handle immunological test based on antibody responses could be used for diagnosis and screening purposes. Additionally, the altered antibody reactions could possibly offer new immunological targets for innovative treatment options

Conflict of interest

The authors declare no conflict of interest.

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