Immunoproteomic analysis of serological autoantibodies in glaucoma

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DECLARATION I

Parts of this dissertation have been published in scientific journals and been presented on international conferences.

Publications

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Conference contributions

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Funke, S., **Beutgen, V.M.**, Bechter, L., Schmelter, C., Perumal, N., Pfeiffer, N., Grus, F.H., An in-depth view towards the trabecular meshwork proteome. DOG (Deutsche Ophthalmologische Gesellschaft) annual meeting 2018, Bonn / Germany. Poster.

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DECLARATION II

This dissertation was elaborated at the Experimental and Translational Ophthalmology, Department of Ophthalmology, University medical center of the Johannes Gutenberg – University Mainz under the supervision of Prof. Dr. Dr. F.H. Grus. The doctoral thesis is designed as a cumulative dissertation and is based on the following publications and manuscripts:

1. **Beutgen, V. M.**, Perumal, N., Pfeiffer, N., Grus, F. H., Autoantibody Biomarker Discovery in Primary Open Angle Glaucoma Using Serological Proteome Analysis (SERPA). *Frontiers in immunology* 2019, *10*.
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2. **Beutgen, V. M.**, Schmelter, C., Pfeiffer, N., Grus, F. H., Autoantigens in the trabecular meshwork and glaucoma-specific alterations in the natural autoantibody repertoire. *Clinical & Translational Immunology 2020; 9: e1101.*
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3. **Beutgen, V. M.**, Pfeiffer, N., Grus, F. H., Subtype-specific alterations in the serological autoantibody profile of open-angle glaucoma patients.
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ABSTRACT

As a neurodegenerative disease, glaucoma is not curable with currently available treatments. Nowadays, only the prevention of further vision loss by delaying disease progression is possible. The focus for glaucoma management lies in the lowering of the intraocular pressure by eye drops, laser treatment or surgery. A timely start of treatment is crucial to prevent glaucoma-caused blindness. Therefore, early diagnosis plays a particularly important role.

The pathomechanisms of glaucoma are not conclusively discovered, but different studies have shown an involvement of immunological processes in the pathogenesis of glaucoma. One aspect of glaucoma pathogenesis is related to processes of humoral immunity, resulting in altered serological levels of autoantibodies. In this scope, altered abundance of several specific autoantibodies to ocular antigens, in retina and optic nerve, were found in serum and aqueous humour of glaucoma patients.

The trabecular meshwork (TM) is a critical site in glaucoma pathogenesis, too. It is subjected to major structural changes in the course of the disease. Dysregulation of extracellular matrix deposition, the actin cytoskeleton and cell-cell / cell-matrix connections enhance tissue rigidity and thereby increase aqueous humour outflow resistance. This lead to the hypothesis that these major structural and biomechanic alterations in the glaucomatous TM are accompanied by shifts in the abundance of autoantibodies in natural autoimmunity as well. The aim of this doctoral thesis was the identification of antigens in the TM and the investigation of their possible association with glaucoma, using different immunoproteomic techniques.

The potential of these autoantibodies as diagnostic biomarker candidates was also evaluated. The research focused on primary open-angle glaucoma (POAG) as the most common form of the disease, however, altered levels of serological antibodies were also analysed for other forms of open-angle glaucoma. Although, it remains unclear if these changed autoantibody levels are cause or consequence of glaucoma, the identification of antibodies to self-antigens in serum is a promising approach towards the discovery of diagnostic and prognostic biomarkers. These biomarkers could facilitate the decision making upon treatment options of ophthalmologists and promote early diagnosis of the disease.

With the application of "serological proteome analysis" (SERPA) and ensuing protein microarray analysis, three new biomarker candidates were identified. Autoantibodies to Caldesmon, Voltage-dependent anion-selective channel protein 2 and Phosphoglycerate mutase 1 showed significantly higher levels in POAG patients ($p < 0.05$) in comparison to a non-glaucomatous control group. Antibody levels of these markers and to two previously known glaucoma-related autoantigens, mitochondrial

60 kDa heat shock protein and Vimentin, were used to classify POAG patient from controls via artificial neural networks. With the panel of these potential biomarkers it was possible to classify the groups with a sensitivity of 81% and a specificity of 93%.

In another approach "mass spectrometry-based antibody-mediated identification of autoantigens" (MS-AMIDA) was used for initial autoantibody profiling. Sixty-six autoantigens were identified as targets of the natural autoantibodies. Twentyone of these showed an association with POAG. Subsequent protein microarray validation revealed a significant ($p < 0.05$) increase of autoantibodies to Threonine--tRNA ligase 1, Complement component 1 Q subcomponent-binding protein and Paraneoplastic antigen Ma2 in POAG patients compared to a non-glaucomatous control group. Furthermore, an association of the identified glaucoma-related autoantigens with the "platelet-derived growth factor receptor β" pathway that is known to be involved in glaucoma-related fibrosis in the trabecular meshwork was discovered.

Data retrieved from an explorative microarray experiment analysing sera of POAG, normal tension glaucoma (NTG) and pseudoexfoliation glaucoma (PEXG) patients versus a control group, revealed altered levels of serological autoantibodies in all three manifestations of OAG. The analysis showed especially low levels of anti-clathrin antibodies in sera of PEXG patients and significantly higher levels of β-2 adrenergic receptor antibodies in POAG and NTG patients.

In summary, the results in this doctoral thesis provide novel biomarker candidates and give new insights into the characteristics of autoantibodies in glaucoma., whilst demonstrating these to be part of natural immunity. Furthermore, pathways with an enrichment of immunogenic proteins were identified, which delivers additional findings to our knowledge of the natural autoimmunity. These results form the basis for further analysis of autoantibodies in health and disease.

ZUSAMMENFASSUNG

Das Glaukom ist eine neurodegenerative Erkrankung, die mit den derzeit verfügbaren Mitteln nicht heilbar ist. Gegenwärtig ist lediglich die Verhinderung von weiterer Verschlechterung des Visus durch Verzögerung des Fortschreitens der Krankheit möglich. Der Fokus im Krankheitsmanagement beim Glaukom liegt auf der Reduktion des intraokulären Drucks mittels Augentropfen, Laserbehandlung oder operativen Eingriffen. Ein frühzeitiger Start der Behandlung ist essenziell, um glaukombedingte Erblindung zu verhindern. Aus diesem Grund spielt die Glaukom-Früherkennung eine besonders wichtige Rolle.

Die Pathomechanismen des Glaukoms sind noch nicht abschließend erforscht, jedoch haben verschiedene Studien eine Beteiligung immunologischer Prozesse in der Pathogenese gezeigt. Ein Aspekt der Glaukompahtogenese steht in Verbindung zur humoralen Immunantwort, die eine Veränderung der serologischen Autoantikörperspiegel bedingt. In diesem Zusammenhang wurden einige Autoantikörper spezifisch für diverse okuläre Antigene, in Retina und Sehnerv, in veränderter Menge in Serum und Kammerwasser von Glaukompatienten gefunden.

Das Trabekelmaschenwerk (TM) ist auch eine kritische Stelle in der Glaukompathogenese. Das TM ist im Verlauf der Krankheit großen strukturellen Veränderungen ausgesetzt. Eine Dysregulation der extrazellulären Matrixablagerungen, des Aktin-Zytoskeletts und der Zell-Zell / Zell-Matrix-Verbindungen erhöht die Gewebestarrheit und erhöht dadurch den Abflusswiderstand des Kammerwassers. Dies führte zu der Hypothese, dass diese wesentlichen strukturellen und biomechanischen Veränderungen im glaukomatösen TM auch mit Verschiebungen in der Menge von Autoantikörpern bei der natürlichen Autoimmunität einhergehen. Ziel dieser Doktorarbeit war die Identifizierung von Antigenen im TM und die Untersuchung ihrer möglichen Assoziation mit dem Glaukom unter Verwendung verschiedener immunoproteomischer Techniken.

Das Potenzial dieser Autoantikörper als Biomarker-Kandidaten wurde ebenfalls evaluiert. Die Forschung konzentrierte sich auf das primäre Offenwinkel-Glaukom (POWG) als der häufigsten Ausprägung der Krankheit, wobei veränderte Spiegel von serologischen Antikörpern auch in anderen Formen des Offenwinkel-Glaukoms untersucht wurden. Es bleibt jedoch weiterhin unklar, ob die veränderten Autoantikörperspiegel Ursache oder Folge der Glaukomerkrankung sind. Dennoch ist die Identifizierung von Antikörpern gegen Selbstantigene im Serum ein vielversprechender Ansatz zur Entwicklung von diagnostischen und prognostischen Biomarkern. Solche Biomarker können die Entscheidungsfindung von Ophthalmologen über die passende Behandlung erleichtern und eine frühe Diagnose der Krankheit fördern.

Durch die Anwendung einer serologischen Proteom Analyse (SERPA) und anschließender Analyse mittels Microarray konnten drei neue Biomarker-Kandidaten identifiziert werden. Autoantikörper gegen Caldesmon, Voltage-dependent anion-selective channel protein 2 und Phosphoglycerate mutase 1 zeigten signifikant höhere Level in POWG Patienten ($p < 0.05$) im Vergleich zu einer nicht-glaukomatösen Kontrollgruppe. Antikörperspiegel dieser Marker, sowie zwei weiterer zuvor bekannter Glaukom-bezogener Autoantigene, mitochondrial 60 kDa heat shock protein und Vimentin, wurden verwendet um POWG Patienten von der Kontrollgruppe mittels künstlichen neuralen Netzwerken zu klassifizieren. Mit dem Panel dieser potenziellen Biomarker war es möglich die Gruppen mit einer Sensitivität von 81% bei einer Spezifität von 93% zu unterscheiden.

In einem weiteren Ansatz zu dieser Untersuchung, wurde die Technik der Massenspektrometrie-basierten, Antikörper-vermittelten Identifizierung von Autoantigenen (MS-AMIDA) zum initialen Autoantikörper-Profiling eingesetzt. Damit konnten 66 Autoantigene als Ziele von natürlichen Autoantikörpern identifiziert werden. Einundzwanzig davon wiesen einen Bezug zum POWG auf. Die anschließende Protein-Microarray Analyse zeigte signifikant erhöhte Autoantikörperspiegel gegen Threonine--tRNA ligase 1, Complement component 1 Q subcomponent-binding protein und Paraneoplastic antigen Ma2 im Serum von POWG Patienten im Vergleich zur nicht-glaukomatösen Kontrollgruppe. Weiterhin wurde eine Verbindung der Glaukom-assoziierten Autoantigenen zum Platelet-derived Growth Factor Receptor β – Signalweg entdeckt, der dafür bekannt ist an der Glaukom-bedingten Fibrose des TM beteiligt zu sein.

Daten, die aus einem explorativen Microarray-Experiment gewonnen wurden, bei dem Seren von POAG-, NTG- und PEXG-Patienten im Vergleich zu einer Kontrollgruppe analysiert wurden, zeigten auch bei Manifestationen des primären Offenwinkelglaukomes veränderte Spiegel an serologischen Autoantikörpern. Die Analyse zeigte besonders niedrige Spiegel an Anti-Clathrin-Antikörpern in Seren von PEXG-Patienten und signifikant höhere Spiegel an β-2-adrenergen Rezeptor-Antikörpern bei POAG- und NTG-Patienten.

Zusammenfassend liefern die Ergebnisse dieser Dissertation neue Biomarker-Kandidaten, sowie neue Erkenntnisse über die Eigenschaften von Autoantikörpern beim Glaukom, aber auch im Rahmen der natürlichen Immunität. Darüber hinaus wurden Signalwege mit einer außergewöhnlichen Häufigkeit immunogener Proteine identifiziert, die zusätzliche Erkenntnisse zu unserem Wissen über die natürliche Autoimmunität beitragen. Diese Ergebnisse bilden die Grundlage für weitere Analysen von Autoantikörpern im gesunden und kranken Organismus.

INTRODUCTION

Blindness

The evolutional acquisition of the ability to see implied a major advantage in terms of natural selection. Eyesight favoured the search for food and the avoidance of environmental dangers or predators. A sudden loss of this ability is fatal in a natural environment. Through our cultural and technical advances in our developed society, blindness no longer means the demise of an individual. Nonetheless, the loss of vision is a significant restriction in the life of an affected person. Beneath the obvious difficulties in managing everyday life, blindness can lead to severe depression and the tendency to commit suicide is not uncommon among blind people [1]. There are a plethora of possible causes for the loss of vision. Besides injuries and genetic mutations, ophthalmic diseases constitute the most important reason for blindness. The characterization and investigation of eye disorders on a molecular level is the pursuit of basic research in the field of ophthalmology. The gained knowledge is the foundation for the development of new treatment options that help to improve disease management. A common cause for irreversible blindness is represented by a group of neurodegenerative eye diseases summarized under the term glaucoma, which is the research field of this dissertation.

Glaucoma

Glaucoma is acknowledged as optic neuropathy that is characterised by a progressive loss of retinal ganglion cells (RGCs) and their axons in the optic nerve. The clinically relevant signs of glaucoma are a characteristic optic nerve head (ONH) cupping that is accompanied by progressive visual field loss. If left untreated, the accumulating damage will eventually lead to complete loss of vision.

Several known forms of the disease can be generally divided into types with an open iridocorneal angle and a closed angle. These forms are referred to as ‘open-angle glaucomas’ and ‘angle-closure glaucomas’ respectively. In a second instance, the subtypes are categorized according to the circumstances of their development as ‘primary’ or ‘secondary’ disease forms. A selection of glaucoma forms relevant in this doctoral thesis is shown in **Table 1**.

Table 1 Overview of open-angle - glaucoma forms relevant in this doctoral thesis.

Classification	Name	Abbreviation	Main Characteristics
Primary	Primary open-angle glaucoma	POAG	Open-angle; IOP ≥21mmHg; ONH cupping
	Normal-tension glaucoma	NTG	Open-angle; ONH cupping
Secondary	Pseudoexfoliation glaucoma	PEXG	Accumulation of pseudoexfoliation material in the anterior chamber
Other	Ocular hypertension	OHT	Repeatedly measured elevated IOP without glaucomatous damage

The major risk factor for glaucoma is a high intraocular pressure (IOP) [2-4]. Patients showing permanently increased IOP without showing signs of glaucoma suffer from the condition termed ocular hypertension (OHT). OHT patients have a chance of ~10% to convert to glaucoma in the course of five years [5]. In contrast, around 30% of glaucoma patients never show an elevated IOP that is thus referred to as normal-tension glaucoma (NTG) [6]. Other factors have been identified to favour the development of glaucoma. Among these, advanced age (above 60 years of age) [3,4], ethnicity [7] and a family history of glaucoma [8] are the most significant.

An elevated IOP is still considered the most critical risk factor for the development of glaucoma. Elevated IOP is defined as a pressure of ≥ 21mmHg. The designation was defined as the upper boundary of normal IOP by adding two times the standard deviation to the population mean IOP (15.5mmHg). If left untreated, an increased IOP was shown to convert to glaucoma in 9.5% of the affected patients over five years [9]. Causes for increased IOP, however, can vary between different forms of glaucoma. While the outflow of aqueous humour (AH) in primary open-angle glaucoma (POAG) is impaired by molecular changes in the trabecular meshwork (TM) concerning augmentation of the actin cytoskeleton and excessive production of extracellular matrix (ECM), the outflow resistance in pseudoexfoliation glaucoma (PEXG) is highly elevated by the accumulation of pseudoexfoliation (PEX) material. However, glaucomatous damage to the optic nerve can also occur independent from high IOP [10] and alterations in the TM, as known from NTG. On the other hand, patients with ocular hypertension do not necessarily develop glaucoma. This indicates that other mechanisms besides the mechanical stress induced by high IOP need to be

considered as pathogenesis driving factors. These will be elaborated in a later chapter.

Globally, the prevalence of the different forms of glaucoma is not consistent among all ethnic groups. A population-based study in the United States showed a higher prevalence of glaucoma in the black population in comparison to white residents [7]. The highest prevalence for POAG was observed in Africa (4.2% of the population aged 40 – 80 years) whilst showing the lowest frequency among the Asian population (**Figure 1**). In contrast, the highest prevalence for primary angle-closure glaucoma (1.09% of the population aged 40 – 80 years) can be found in Asia [11].

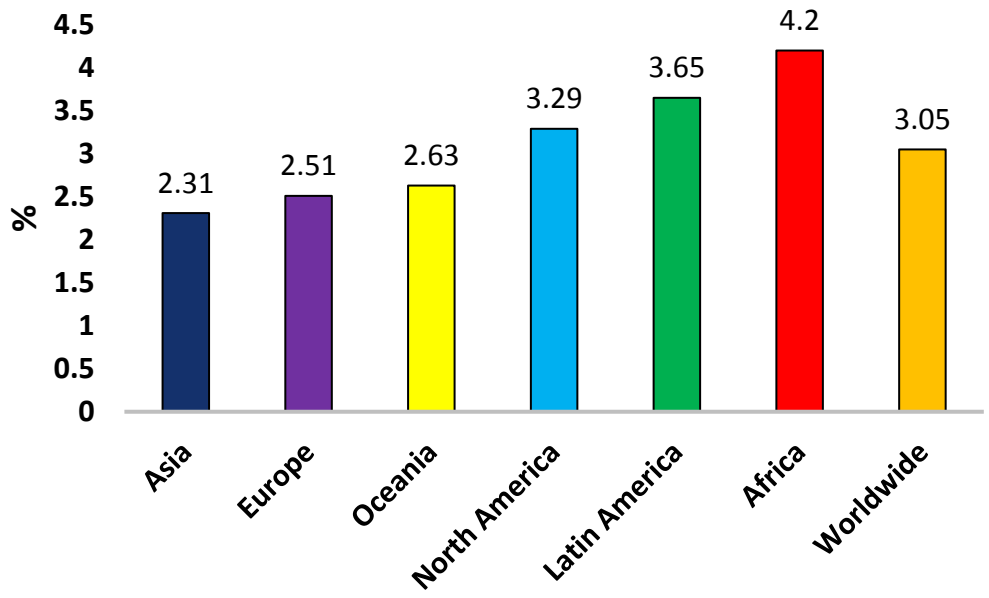

Figure 1 Prevalence of POAG in different regions worldwide in 2013. Incidence is shown as a percentage. Data derived from [11].

Next to an elevated IOP, increasing age is the most important risk factor for glaucoma, with 64.3 million affected people at the age of 40 – 80 worldwide (as of 2013)[11]. Although the decay of retinal nerve cells also occurs with an average loss of 5000 - 9000 RGC per year [12,13] as an inevitable consequence of the ageing process, the progression of cell death is highly accelerated in glaucoma patients.

According to predicitions for Germany, the number of people aged 67 and older will increase by 32% from 2020 to 2040 [14]. The increase in the elderly population is also reflected by the estimated 47% increase of glaucoma cases worldwide among 40 –

80 year-olds from 2020 to 2040 [11]. From 2010 to 2050, the number of people in the US with glaucoma is expected to increase by 133%, from 2.7 million to 6.3 million [15]. The demographic change will also lead to increased costs for health care and represents a major challenge in the future, not only in the field of ophthalmology.

DIAGNOSTICS

The slow progression of the disease often leaves patients unaware of their illness and thus undetected for enhanced periods[10]. The Barbados Eye Study showed that 53% of 125 study participants with an open-angle glaucoma (OAG) were not aware of their disease [16]. Because of the asymptomatic onset, professional help is often firstly sought in advanced stages of the disease. An earlier diagnosis could highly improve the effectiveness of respective treatment approaches. As of today, a combination of various diagnostic tools is used to examine the hallmarks of glaucoma pathogenesis. The cup-to-disc ratio is evaluated by ophthalmoscopy to assess the advancement of excavation of the optic disc as a measure of progressive loss of RGCs. The loss in cellularity can also be assessed regarding the thickness of the retinal nerve fibre layer by optical coherence tomography (OCT). The standard procedure for testing the progression of glaucoma-related loss of vision is the visual field test by perimetry. For more precise determination of the glaucoma form, the assessment of IOP by tonometry is essential. Further, the status of the iridocorneal angle is observed by gonioscopy and allows the distinction of an open- or closed-angle form of glaucoma. The combination of the outcome of these tests allows the ophthalmologist to diagnose the presence and manifestation of glaucoma. Sometimes these diagnostic parameters cannot deliver unambiguous results. Patients occasionally cannot undoubtedly be diagnosed with "definite" or no glaucoma. These patients are then categorised as "glaucoma suspects" and follow up examinations are necessary to monitor possible progression of disease hallmarks. These circumstances call for more objective and sophisticated criteria for early and explicit glaucoma diagnosis. The establishment of serological biomarkers can be a promising diagnostic tool. Especially the measurement of circulating autoantibodies in serum and other body fluids can deliver valuable insights into disease states. This approach has already been taken in other neurodegenerative diseases [17,18], and previous studies also proposed the potential of autoantibodies as glaucoma biomarkers [19]. The identification and analysis of glaucoma-related autoantibodies and the judgment on their feasibility as disease biomarkers are covered in this dissertation and are presented in the following chapters.

TREATMENT

As of today, there is no cure for glaucoma. Vision loss caused by damage to the ON cannot be recovered. Much research is conducted with the aim of efficient neuroprotection [20-22] and regeneration [23], but the field is stil in its beginnings. At present, no causative treatment for the disease is available. Currently, the only effective treatment option for POAG is the lowering of IOP and thereby (in the majority of patients) preventing its progression. The reduction of IOP also slows disease progression in NTG patients. This can be achieved by topical application of diverse classes of IOP lowering drugs (e.g. β-blockers, prostaglandins, α-2 adrenoreceptor agonists) provided as eye drops. These classes affect different mechanisms to lower IOP. Prostaglandins facilitate the AH drainage via the uveoscleral outflow pathway that is only responsible for 10 – 20% of the outflow under physiological conditions [24], while β-blockers reduce the AH production in the ciliary body. Surgical options are also available to increase AH outflow. The most widely used technique is the trabeculectomy, where a small portion of the TM is removed, allowing the AH todrain under the conjunctiva. All these approaches are merely able to decrease the speed of disease progression and do not represent a cure. Furthermore, IOP lowering procedures are not effective for every patient. Therefore, several new treatment options are currently under investigation. These involve approaches to achieve neuroprotection [22] or even regeneration of optic nerve cells [23]. Especially research projects investigating the protective role of antibodies have been implemented in pre-clinical cell culture [25-28] and animal models [29]. Clinical trials involving human subjects to confirm the observed neuroprotective effects of the applied anitbodies are still outstanding.

GLAUCOMA PATHOGENESIS

Glaucoma is considered a neurodegenerative disease with a multifactorial pathogenesis. The most striking correlation in glaucoma is the relationship between increased IOP and RGC death. An elevated IOP is the major risk factor for POAG concerning 70% of the diseased individuals [6]. It is based on a misbalance of AH production in the ciliary body and its efflux via the TM to Schlemm's canal (**Figure 2**). The TM is responsible for about 90% of the outflow of aqueous humour from the anterior chamber, the remaining 10% drain via the uveoscleral outflow pathway. The tissue shows smooth muscle-like properties and is actively involved in the AH outflow and IOP modulation by contractile movements. The TM comprises three different regions (**Figure 2 b**). AH outflow through the tissue starts from the uveal meshwork, adjacent to the anterior eye chamber, further to the corneoscleral and finally the juxtacanalicular region [30]. The inner endothelial wall builds a border to Schlemm's canal. Here, the AH outflow follows either the transcellular or the intercellular route [30]. Outflow resistance is caused by the cells and ECM of the juxtacanalicular meshwork, which are constantly

remodelled to adapt to changes in IOP. The most common cause for an elevated IOP is a defect in these functions of the TM. Alteration of Transforming growth factor receptor β (TGFβ) signalling has been identified as one of the major causes for these pathological changes. TGFβ levels were found to be elevated in the AH of glaucoma patients. This leads to an activation of the downstream signalling effector 'connective tissue growth factor' (CTGF) that causes an augmentation of the actin cytoskeleton, ECM proteins and associated integrins [31] (**Figure 2 c**). Another mechanism increasing tissue stiffness is the formation of cross-linked actin networks (CLANs), which is inducible by TGFβ [32] (**Figure 2d**). Induced TM fibrosis increases the rigidity of the tissue and, thus, increases outflow resistance [33]. Increasing age, the second most important risk factor for glaucoma, is also proportional to the tissue stiffness in the TM [34]. However, the molecular mechanisms of IOP regulation in health and disease are not entirely understood.

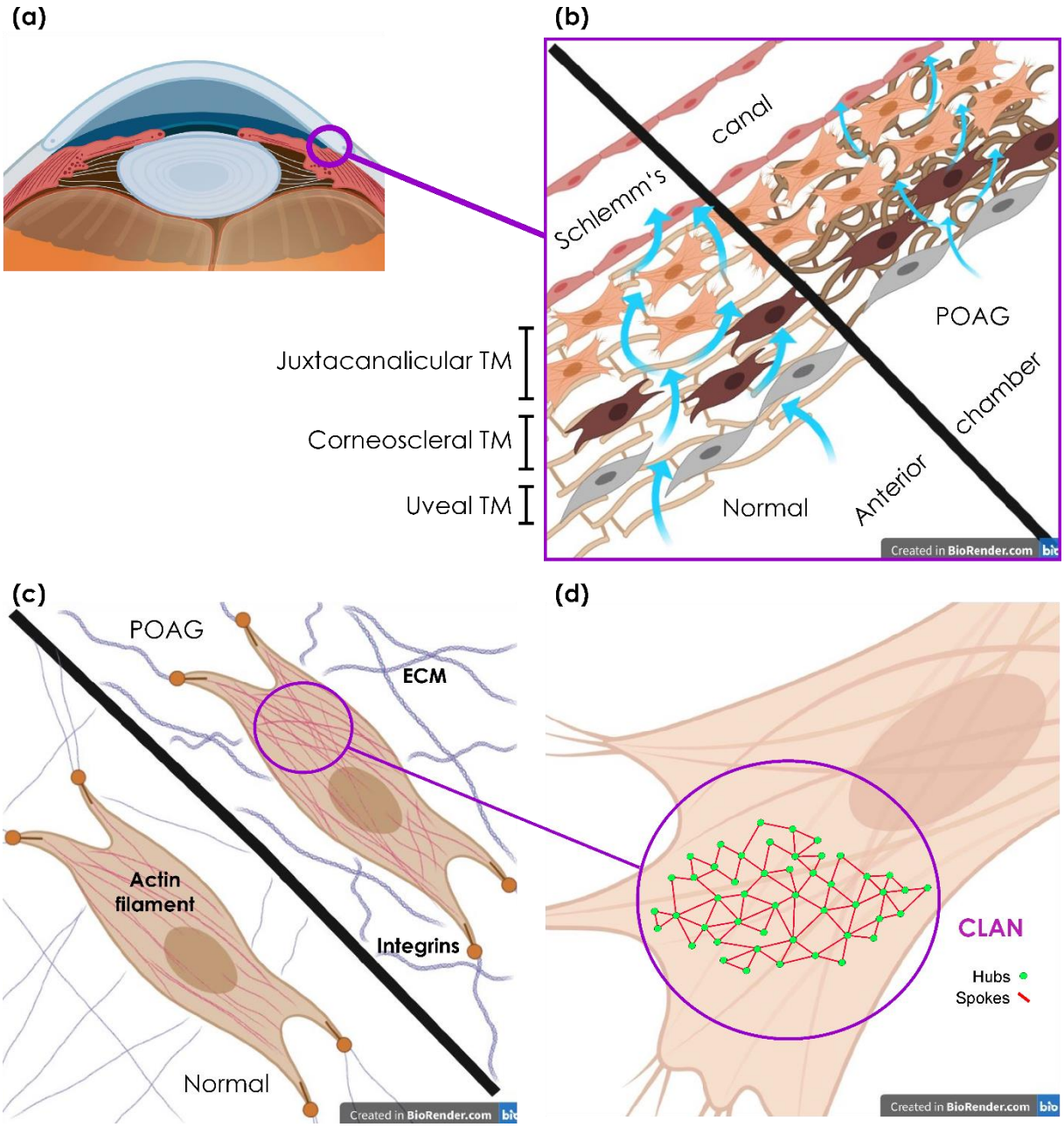

Figure 2 POAG-related alterations in the TM. Schematic representation, resources from 'Biorender.com'. **(a)** Anterior eye with cornea, anterior chamber, iris, lense and vitrious. The TM is located in the iridiocorneal angle, adjacent to Schlemm's canal and the scleral spur. **(b)** The TM consists of three different regions: The uveal, corneoscleral and juxtacanalicular TM. The juxtacanalicular portion provides the highest outflow resistance. AH flows from the anterior chamber through the intercellular space towards the inner endothelial wall of Schlemm's canal (indicated by blue arrows). The AH can drain into Schlemm's canal via an intercellular or a transcellular route. The TM in POAG provides an overall increased outflow resistance, hence, the AH outflow is impeded, causing an increase of IOP. The glaucomatous TM shows increased tissue stiffness caused by an augmentation of the ECM and actin cytoskeleton, as well as altered contractility of TM cells. **(c)** TM cells of the juxtacanalicular region. During POAG pathogenesis, several alterations occur in the TM cell. Accumulation of ECM and the remodelling of the actin cytoskeleton are majorly associated with increased outflow resistance. **(d)** Reorganization of the actin cytoskeleton has been reported to involve formation of cross-linked actin networks (CLANs) that are inducible by TGFβ2. These three-dimensional structures consist of various hubs and spokes, able to increase TM cell stiffnes and thereby outflow resistance.

The neurodegeneration in glaucoma is mainly driven by apoptosis of RGCs [35]. The ON exits the eye through the lamina cribrosa (LC), a mesh-like tissue with similar characteristics as the TM [36]. In the event of an elevated IOP, mechanical stress is applied to the LC and the tissue is compressed. This eventually leads to axonal damage to the RGCs and impaired axonal transport. The resistance of the LC tissue decreases with age, making it more susceptible to damage from elevated IOP [37].

Another noteworthy cause of glaucoma is the so-called astronauts' glaucoma [38]. Triggered by exposure to a low gravity environment in space flight, astronauts are prone to develop a lower cerebrospinal fluid pressure (CSFP). CSFP and IOP interrelate at the level of the LC. This can lead to LC compression and axonal damage even though the IOP is at a normal level. This threat, however, is not only observed in astronauts but can also occur in the general population, as a very low CSFP can also lead to glaucomatous damage to the ON. This is especially the case for patients suffering from NTG and is hypothesised to be the main influence in the etiopathogenesis of this glaucoma subtype [39,40]. This also reinforces the importance of the biophysical balance in the interplay of AH production, outflow and susceptibility to axonal injury dependent on LC resistance. The measurement of the CSFP, however, is not conducted in a routine examination in glaucoma diagnostics, as it demands surgical intervention and complications can not be excluded.

In around 10% of the glaucoma cases, a genetic mutation can be associated with the disease [41]. The most important gene locus identified is the MYOC gene. MYOC (myocilin) mutations are often found in juvenile open-angle glaucoma [42] but are also found in 2 - 4% of adult POAG onset [43]. Other genes linked to glaucoma are OPTN [44], CAV1/2 [45] and CDKN2BAS [46].

Apart from genetic factors and various forms of impaired ocular pressure homeostasis, other factors play an important role in the progression of glaucoma. This is a necessary conclusion as the disease continues to progress in some patients even though the IOP could be significantly reduced [47]. This was observed in several major trials as the Early Manifest Glaucoma Trial (EMGT) [48] or the Advanced Glaucoma Intervention Study (AGIS) [49]. Additional to that, many individuals with OHT never develop any glaucomatous damage. The conversion rate for OHT patients to actual glaucoma is only around 1 – 2% per year [5]. These circumstances highly support the necessity for additional components to be effective for the development of glaucoma.

Being regarded as a multifactorial disease, other aspects in the glaucoma pathogenesis have shown to be influential. These comprise vascular factors [50], including retinal ischemia and reperfusion damage, mitochondrial dysfunction [51] and glutamate excitotoxicity [52]. Scientific evidence has accumulated showing the involvement of the immune system in glaucoma pathogenesis. This manifests in the

occurrence of neuroinflammation and in alterations in the adaptive immunity, described in the following chapter.

INVOLVEMENT OF IMMUNITY IN GLAUCOMA PATHOGENESIS

Various aspects of the immune system in glaucoma have been analysed. The manifestation of such involvement shall be elucidated in this chapter. As these topics are of relevance to this thesis, the focus lies on glaucoma-related affection of immune cells and involvement of autoantibodies.

The immune system is the organisms defence line against exogenous pathogens of various forms. It consists of innate and adaptive immunity, providing defence on a broad unspecific base, as well as targeted protective mechanisms. These comprise a cellular and the humoral immune response. Beneath its purpose to defend the organism from pathogens, the immune system can act on cells and molecules of the organism itself, which is referred to as autoimmunity.

In general, autoimmunity describes the response of the immune system against endogenous self-antigens. Involvement of autoimmune processes in glaucoma pathogenesis have previously been described. On one hand, cellular immunity is involved, as alterations in the lymphocyte repertoire of glaucoma patients were observed. This includes changed serum levels of different T cell types like specific $CD4^+$ T cells and regulatory T cells (T_{reg}). T_{reg} cells have a suppressive function on the immune system and are suggested to be responsible for the induction and maintenance of peripheral tolerance [53]. The number of T_{reg} cells is increased in POAG demonstrating a connection between cellular and humoral immunity [54]. Evidence supports the hypothesis that these $CD4^+$ $CD25^+$ cells are possible regulators of antibody-producing B cells [55]. However, the distribution of $CD4^+$ and $CD8^+$ T cells, as well as T helper cells shows no differences in glaucomatous and non-glaucomatous serum samples [56]. Increased levels of HSP60 and HSP27-specific $CD4^+$ T cells were observed in the sera of a mouse glaucoma model following the transient elevation of IOP, and were also increased in sera of POAG and NTG patients [57]. In the mouse IOP model, T cells were able to infiltrate the retina and cause sustained neurodegeneration preceeding the initial damage. The authors linked the occurrence of these T cells to a cross-reaction of highly conserved heat shock proteins in humans and bacteria of the commensal microflora that activate T cells through molecular mimicry [57]. They also evaluated the involvement of B cell immunity in RGC degeneration but could only observe attenuated effects in comparison to the T cell mediated impact. In contrast, autoantibody mediated RGC loss was detectable in experimental autoimmune glaucoma (EAG) animal models immunized with HSP27, HSP60 or myelin basic protein [58-60]. Here, the authors attest RGC loss primarily to autoantibody mediated effects, however, aspects of cellular immunity were not investigated. In addition, merely the

correleation with increased autoantibody levels were attested, without providing their evidence for causality.

In summary, in both cases, T and B cell-mediated immunity, an elevated IOP was shown as a trigger for persisting neuronal damage, caused by T cell infiltration in the retina and autoantibodies targeting retinal antigens [57,61]. However, RGC loss can also be observed independently of IOP in immunized animals showing elevated autoantibody levels to retinal antigens [58-60].

Under the paradigm of the 'horror autotoxicus', shaped by Paul Ehrlich, autoimmunity was long regarded to be inevitable of pathological nature. The field of immunology, however, has evolved from these strict perceptions and now acknowledges the presence of natural antibodies as part of physiological immunity. Also in glaucoma, they seem to play an important role in its pathogenesis.

For the case of humoral immunity, evidence has shown that antibodies of the IgA and IgG class, but especially of the IgM class, are abundant in human body fluids even under physiological conditions as part of the natural autoimmunity. The cause and purpose for their existence are not revealed in conclusion but they are attested several other functions beyond mere self-destructive characteristics. Those natural autoantibodies are polyreactive and show a mild affinity for their respective antigens. In 1963, Boyden firstly described these antibodies in sera of healthy individuals and shaped the term "natural autoantibodies" (cited in [62]). IgM class autoantibodies are known to be generated by B1 Lymphocytes during orthogenesis, while IgG class antibodies are produced via T cell-dependent activation of B2 cells [63]. As part of the innate immune system, natural autoantibodies serve as a front line in the defence against pathogens before adaptive immunoglobulins are produced [64]. The natural antibody repertoire is also assumed to have protective effects, especially in the prevention of autoimmune disease and for decreasing inflammatory processes [65,66]. This, for instance, was shown in mouse models, where depletion of IgM led to an increment of pathogenic IgG that resulted in lupus-like symptoms [67]. Natural autoantibodies also play a role in the clearance of cellular debris of apoptotic cells and thereby ensure homeostasis of the organism [68]. The involvement of autoantibodies in pathologies other than autoimmune diseases is especially well examined in cancer. Several tumour-associated autoantibodies have been identified in relation to different forms of malignancies. Apart from the more obvious oncoviral and cancer-specific antigens, a series of protein alterations is held responsible for the immunogenicity of the tumour-associated autoantibodies. These include mutations, overexpression and post-translational modifications [69]. Alterations in the IgG class autoantibody repertoire were also found in neurological disorders such as Parkinson's and Alzheimer's disease, as well as in the blood of multiple sclerosis patients [70]. While the number of IgM

autoantibodies and their respective B1 cell levels decrease with increasing age, the amount of IgG autoantibodies show a linear increment with age [70,71].

Up to now, a plethora of different studies showed alterations of the natural autoantibody repertoire of open-angle glaucoma patients. Wax et al. were the first to report elevated anti-HSP60 autoantibodies in NTG patients [72]. In the following years, more alterations in the autoantibody profiles of glaucoma patients were found in serum and AH. These include altered levels of anti-Vimentin [73,74], anti-a-Fodrin [75], anti-GFAP [74], anti-MBP [74], anti-γ-Enolase [76,77], anti-γ-Synuclein antibodies [25] and more. **Table 2** lists the so far identified antigens of IgG class autoantibodies showing altered serological levels in comparison to non-glaucomatous controls. In addition, the novel autoantibodies discovered in the course of this dissertation are contained.

Table 2 Glaucoma-related autoantigens (n.a. = 'not available'). Antigens discovered and/or validated through the efforts of this dissertation are highlighted in bold letters.

Protein name	**Gene ID**	**Glaucoma subtype**	**Method**	**References**
HSP60	HSPD1	NTG	ELISA	72
HSP27	HSPB1	POAG, NTG	Microarray, ELISA	19,78
HSP70	HSPA1	NTG	WB+MS	73
a A-crystallin	CRYAA	NTG	ELISA	78
a B-crystallin	CRYAB	NTG	WB+MS, ELISA	73,78
β-L-crystallin	CRYB	POAG	Microarray	19
Annexin 5	ANXA5	POAG	Microarray	19
Ubiquitin	(isoform not specified)	POAG	Microarray	19
Glial fibrillary acidic protein	GFAP	POAG,	Microarray	19,74
14-3-3	(isoform not specified)	POAG	Microarray	26
a Fodrin	SPTAN1	NTG	ELISA	75
Phosphatidylserine	n.a.	NTG	ELISA	79
Gamma enolase	ENO2	POAG, NTG	WB + Edman sequencing	76,77
Glycosaminoglycans	n.a.	POAG, NTG	ELISA	80
Vimentin	VIM	NTG, POAG	WB+MS	73,74
Myelin basic protein	MBP	POAG	Microarray, WB+MS	19,74

Protein name	Gene ID	Glaucoma subtype	Method	References
Retinaldehyde-binding protein	RLBP1	OHT	WB+MS	[81]
Glutathione-S-transferase	GST	NTG, POAG	WB+MS	[82]
Retinal S-antigen	SAG	POAG/OHT/NTG	WB+MS	[81]
Histone H4	H4	POAG	WB+MS	[81]
a 1 antitrypsin	SERPINA1	POAG	Microarray	[19]
Gamma synuclein	SNCG	POAG	Microarray	[25]
Voltage-dependent anion-selective channel protein 2	**VDAC2**	POAG	SERPA & Microarray	[83]
Caldesmon	**CALD1**	POAG	SERPA & Microarray	[83]
Phosphoglycerate mutase 1	**PGAM1**	POAG	SERPA & Microarray	[83]
Threonine--tRNA ligase 1, cytoplasmic	**TARS1**	POAG	MS-AMIDA & Microarray	[84]
Complement component 1 Q subcomponent-binding protein, mitochondrial	**C1QBP**	POAG	MS-AMIDA & Microarray	[84]
Paraneoplastic antigen Ma2	**PNMA2**	POAG	MS-AMIDA & Microarray	[84]
β-2 adrenergic receptor	**ADRB2**	POAG / NTG	Functional Assay // Microarray	[85,86] // Beutgen et al. (unpublished Publication 3)
Clathrin	**CLTA/B/C**	PEXG	Microarray	Beutgen et al. (unpublished Publication 3)

The cause for the occurrence of these autoantibodies is not entirely clarified. Their role, origin and causality of their alterations in the pathogenesis of glaucoma still needs to be specified comprehensively. However, several studies including animal and cell culture models have provided valuable information about the possible effects of the deranged autoantibodies in glaucoma. Tezel and Wax showed that autoantibodies to HSP27 are internalized to retinal cells and lead to retinal neurodegeneration via

apoptosis [87]. Serological autoantibodies to ADRB2 were found in connection to an elevated IOP in POAG and OHT [85]. Moreover, autoantibodies showing decreased levels in glaucoma patients (anti-GFAP and anti-SNCG antibodies) mediated neuroprotective effects in retinal cell and organ culture, which appear to be lost in glaucoma etiopathogenesis [28]. Although an elevated IOP can initiate retinal neurodegeneration and provoke alterations of the serological autoantibody repertoire, these effects cannot be eliminated by inhibition of the B cell response [61]. This indicates, that B cell immunity might play only a minor role in glaucomatous neuronal damage, whereas the T cell response to retinal antigens appears as a major driving force in persisting RGC and axonal destruction [57]. Therefore, a though autoantibodies may not be causative for glaucoma onset, altered immune reactions triggered by an elevated IOP could have synergetic effects that amplify disease progression.

Despite the unresolved origin and effects of glaucoma-specific autoantibodies, they might serve as valuable disease biomarkers and facilitate glaucoma diagnostics. In a previous study, POAG patients could be classified from non-glaucomatous subjects with good precision (area under roc curve = 0.93) by measuring their autoantibody profiles in a protein microarray approach with subsequent deployment of an artificial neuronal network algorithm [19]. Thus it can be assumed that the identification of new antibodies to self-antigens in serum is a promising approach towards the discovery of new diagnostic biomarkers.

Immunoproteomics in Biomarker discovery

According to the definition of the National Institute of Health, a biomarker is "a characteristic used to measure and evaluate objectively normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention". When looking at biomarkers on a molecular level, current research reaches out in multiple directions. The main aspects encompass Genomics, Proteomics, Metabolomics as well as Lipidomics. In each one of these fields, disease-specific alterations may occur that could be useful as diagnostic biomarkers. Another major field in the discovery of disease biomarkers, which our research-group takes special interest in, is immunoproteomics. Aim of immunoproteomics is the identification and mapping of different proteomic actors of the immune system in its physiological state, as well as in pathological conditions. Especially serological antibodies are already widely used as biomarkers in various diseases, such as Rheumatoid arthritis [88], several autoimmune diseases [89,90], cardiovascular disease [91] and multiple types of cancer [92], where specific autoantibodies to tumour antigens can be found. Recent findings also suggest their usability as biomarkers in other non-autoimmune diseases such as the neurodegenerative disorders Alzheimer's [18,93] and Parkinson's disease [94,95]. Diagnosis of

these neurological diseases was attempted by the analysis of an autoantibody biomarker panel, consisting of 10 – 50 features. With this approach, the discrimination of patients and healthy subjects was possible with high accuracy. We therefore believe that that serological autoantibodies could also be efficiently used as biomarkers in the diagnosis of glaucoma.

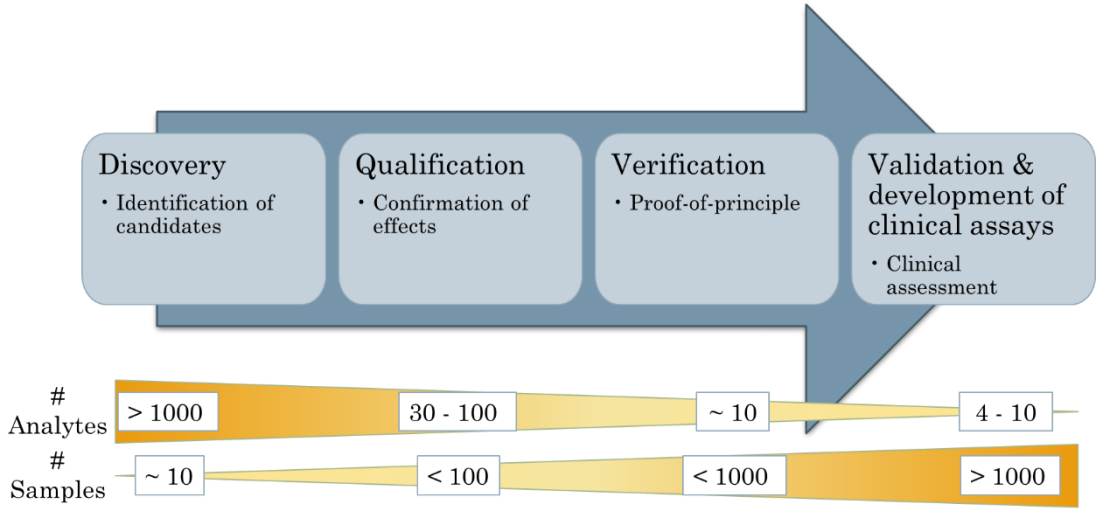

Figure 3 Biomarker pipeline.

The discovery of biomarkers suitable for clinical routine passes through different stages, summarized as the "biomarker pipeline" (**Figure 3**). The first step is the discovery of candidate biomarkers. At first, the number of possible candidates is very high and the methods used for initial profiling only allow a low through-put of samples. This discovery stage of the biomarker pipeline has the purpose of identifying targets that seem promising and should be further examined. To this end, several different immunoproteomic methods have been developed. Techniques such as "serological identification of antigens by recombinant expression cloning" (SEREX) [96], "serological proteome analysis" (SERPA) [97], "luciferase immunoprecipitation systems" (LIPS) [98] or "antibody-mediated identification of autoantigens" (AMIDA) [99] are frequently applied at this stage. In the qualification phase, the putative biomarkers are evaluated using orthologous methods. With the reduced number of candidates more samples can be tested at a time. After passing the qualification phase, these candidates are evaluated in several hundred samples, ideally from various study populations. Here, the usage of high-throughput methods, such as the microarray technology, are most feasible (**Figure 4**).

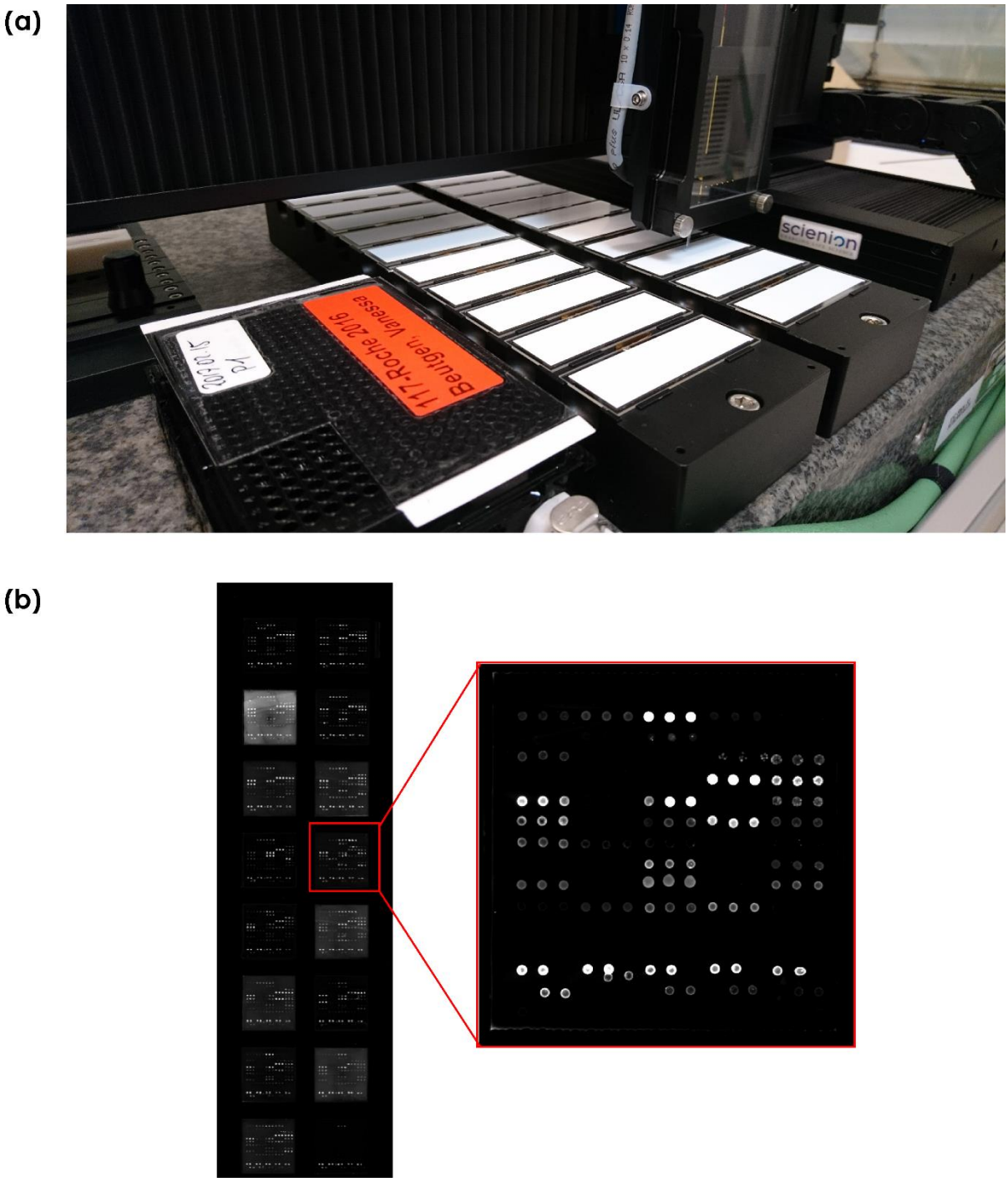

Figure 4 Customized in-house protein microarray production. **(a)** Array slides are printed using a piezo-electric non-contact dispenser (SciFlexarrayer; Scienion). **(b)** Example of scanned antigen microarray after incubation with serum and fluorophor-labelled anti-human IgG secondary antibody. Antigens were printed in triplicates. Each array can contain 70 – 90 antigens to be tested simultaneously.

The microarray setup used in our lab enables the simultaneous analysis of up to 90 features, measured in triplicates. Over 250 samples can be analysed in one batch, requiring minimal volumes. Through several quality control steps and normalization

strategies, we achieve small coeficients of variation. These characteristics make the protein microarray an ideal platform for biomarker studies. The end result ideally is the development of clinical assays and their validation in extensive clinical studies. These assays can be provided in various format as point-of-care devices such as lateral flow assays, but also other formats requiring more extensive laboratory equipment are possible options. The development of precise biomarker-based diagnostic tests for glaucoma is a work in progress. The research in this dissertation comprises the first two steps of the biomarker pipeline and identifies promising new candidates that can be evaluated in further studies in clinically applicable assays.

Overall, further analyses of the physiological and pathological autoantibody repertoire via immunoproteomic techniques and high-throughput approaches are mandatory as a step towards more personalized diagnostic test that allows easy and individual monitoring of disease states. Also, it can help to characterized different diseases more accurate and perhaps define them in a new holistic way in the scope of systems medicine.

AIMS OF THE PROJECT

The numbers of glaucoma patients are steadily increasing and this, as well as other age-related diseases, will be a progressively higher burden to the national health care systems. The disease remains asymptomatic often until later stages and many people are not aware of their disease. The current diagnostic tools require ophthalmic professionals, and are time and cost intensive. The growing number and increasing age of the population will lead to an overall increased workload for medical professionals in general. Therefore, new diagnostic approaches need to be evaluated to overcome these challenges. The availability of easy monitorable diagnostic biomarkers is essential to promote early diagnosis and thereby reduce the prevalence of blindness. Suitable biomarkers can help to reduce the costs of disease management and reduce personnel expenditure of ophthalmic specialists.

In the past years, a multitude of different studies have shown an involvement of immunological components in the pathogenesis of glaucoma. Foremost, changes in specific autoantibody levels have been described. These alterations in immunity bear the potential as useful indicators of disease state and might be used as biomarkers in glaucoma diagnostics. The abundance of specific autoantibodies can predict the presence and state of a disease, as shown not only in autoimmune diseases but also in cancer and cardiovascular disease. This project aimed to identify and analyse new potential autoantibody biomarkers for the diagnosis of glaucoma and also gain new insights into autoantibodies in health and disease. To achieve this, autoantibodies to antigens originating in the TM were identified, and their biomarker potential was evaluated. Combinations of immunoproteomic techniques, including sophisticated mass-spectrometric analysis and a high-throughput protein microarray platform, enable the identification of potential autoantibody biomarkers for the diagnosis of OAG. The identification of antibodies to self-antigens in serum that can be collected with minimal-invasive blood-sampling is a promising approach to the discovery of diagnostic and prognostic biomarkers.

RESULTS
Publication 1

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Autoantibody Biomarker Discovery in Primary Open Angle Glaucoma Using Serological Proteome Analysis (SERPA)

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Glaucoma is an optic neurological disorder and the leading cause of irreversible blindness worldwide, with primary open angle glaucoma (POAG) as its most prevalent form. An early diagnosis of the disease is crucial to prevent loss of vision. Mechanisms behind glaucoma pathogenesis are not completely understood, but disease related alterations in the serological autoantibody profile indicate an immunologic component. These changes in immunoreactivity may serve as potential biomarkers for glaucoma diagnostics. We aimed to identify novel disease related autoantibodies targeting antigens in the trabecular meshwork as biomarkers to support early detection of POAG. We used serological proteome analysis (SERPA) for initial autoantibody profiling in a discovery sample set. The identified autoantibodies were validated by protein microarray analysis in a larger cohort with 60 POAG patients and 45 control subjects. In this study, we discovered CALD1, PGAM1, and VDAC2 as new biomarker candidates. With the use of artificial neural networks, the panel of these candidates and the already known markers HSPD1 and VIM was able to classify subjects into POAG patients and non-glaucomatous controls with a sensitivity of 81% and a specificity of 93%. These results suggest the benefit of these potential autoantibody biomarkers for utilization in glaucoma diagnostics.

Keywords: autoantibodies, biomarker, glaucoma, immunoproteomics, microarray, trabecular meshwork

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INTRODUCTION

Glaucoma describes a group of optic neuropathies and is the leading cause of irreversible blindness. Primary open angle glaucoma (POAG) is the most common form of the disease (1), with a prevalence of 3.05% in people aged 40–80 years worldwide (2). Characteristic for glaucoma is the loss of retinal ganglion cells (RGCs) and their axons by apoptosis, leading to constant impairment of the visual field and finally complete loss of sight. Alongside old age, a major risk factor for this disorder is an elevated intraocular pressure (IOP), leading to the onset of glaucoma in 10% of affected people over the course of 5 years (3). As of today, the reduction of the IOP is the only therapy available that can prevent glaucoma progression. Cause of the IOP elevation is a deficiency in the drainage of aqueous humor (AH) from the eye via the trabecular meshwork (TM). Under normal conditions, TM cells also show fibroblast like properties, allowing them to contract and actively modulate outflow resistance. The TM in POAG is characterized by an extensive accumulation of extracellular matrix (4), the reorganization of the actin cytoskeleton (5, 6) and

a decreased cellularity. This causes an overall increase of tissue rigidity leading to diminishing capability to adapt to changing IOP levels. Malfunction of the AH outflow facility finally causes an increase of IOP above physiological levels, inducing mechanical stress to the optic nerve head. The constant stress to the RGCs is thought to be one major factor in glaucoma pathogenesis, leading to apoptosis of the nervous cells (7). Albeit, molecular mechanisms behind pathological changes in the TM are not fully understood.

The subtle onset of the disease usually leads to a late diagnosis, since visual field defects are often first detectable by visual field testing when 25–35% of RGCs are already lost (8). This clearly shows the need for new diagnostic tools, capable of an early detection of reliable markers in easy accessible body fluids, like tear or serum.

Previous studies indicate an autoimmune component to the pathogenesis of glaucoma (9–11). Autoantibodies show altered levels in serum and aqueous humor of glaucoma patients. The quantity of autoantibodies against various heat shock proteins (11–14), α-fodrin (10), γ-enolase (15), vimentin (11), myelin basic protein (16), glial fibrillary acidic protein (16), and more have been found to be altered in a disease specific manner. Increased and decreased autoantibody reactivities could be detected, indicating an autoaggressive potential along with the loss of putative neuroprotective effects (17) of some autoantibodies, which could contribute to the pathogenesis of the disease.

With the goal of improving the diagnostic potential of serological biomarkers for glaucoma diagnosis, we sought to find new immunological biomarkers for POAG. To this end we used a serological proteome analysis (SERPA) approach. This technique has already been successfully applied for the identification of potential autoantibodies in other diseases, such as diabetes type 1 (18), multiple sclerosis (19), and different forms of cancer (20–22). Validation of the findings from the discovery stage SERPA are achieved by the use of the protein microarray technique, a high-throughput method for the simultaneously analysis of multiple samples (23, 24).

This work includes the discovery stage and the early validation stage of the biomarker pipeline (25) in search of reliable diagnostic biomarkers for POAG. Although several candidate biomarkers have been suggested in other studies, their diagnostic power is not sufficient yet. Based on its role in the elevation of IOP in the course of the disease, we expect to find novel autoantibodies directing target proteins in the TM.

MATERIALS AND METHODS

Sera

All samples were collected in accordance to the Declaration of Helsinki on biomedical research involving human subjects.

Abbreviations: ANN, artificial neural network; AUROCC, area under ROC curve; IOP, intraocular pressure; MS, mass spectrometry; MLP, multilayer perceptron; POAG, primary open angle glaucoma; POCD, point-of-care device; RGC, retinal ganglion cells; ROC, receiver operator characteristic; SERPA, serological proteome analysis; TM, trabecular meshwork.

TABLE 1 | Descriptive statistics of the study population.

	POAG	CTRL
DISCOVERY PHASE		
Sample size	6	6
Gender (m/f)	3/3	3/3
Disease stage grading (mild/advanced)	3/3	No grading
Mean age (±SD)	66.17 ± 10.22	67 ± 11.45
VALIDATION PHASE		
Sample size	60	45
Gender (m/f)	30/30	23/22
Disease stage grading (mild/advanced)	22/38	No grading
Mean age (±SD)	62.75 ± 12.20	63.31 ± 15.32

Written informed consent was obtained from each subject to use serum samples for research purposes. The study was approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz [Vote: 827.228.11 (7770)]. The subjects included in this study underwent an ophthalmic examination at the ophthalmic department of the university medical center in Mainz, Germany. For the initial screening using SERPA, six POAG samples were compared to six age and gender matched controls. In the microarray analysis, 60 sera from POAG patients and 45 sera from controls were analyzed. Inclusion criteria for POAG subjects was a diagnosis based on the European Glaucoma Society guidelines (26), according to characteristic optic nerve appearance and clinical parameters with elevated IOP, optic nerve cupping and/or characteristic visual field defects. The samples used in this study were randomly selected from available samples, only ensuring an even distribution of male and female patients using stratified random sampling with all available cases as sampling frame. Samples for the SERPA screening and for the microarray analysis were independently drawn from the same sampling pool. As control, a group of non-glaucomatous, age and gender matched subjects was used. This group comprises patients with other eye diseases and eye-healthy volunteers. Subjects with a reported autoimmune disease or other forms of glaucoma were excluded from the analysis. Other systemic diseases were not an exclusion criterion. In detail, the control group comprises 25 subjects with other eye diseases, 5 subjects with a systemic disease, 6 subjects with systemic and another eye disease, as well as 9 healthy subjects. Characteristics of the study population in the discovery and validation stage are demonstrated in **Table 1**. The POAG group includes patients with mild/early (MD < -6 db; 36.7%) and advanced (MD > -6 db; 63.3%) glaucomatous visual field defects, as observed by perimetry. Thereby, early POAG was regarded as a subgroup of special interest. All blood samples were allowed to clog for 30 min and were centrifuged for 10 min at 4°C and 1,000 g. The supernatant was collected and serum samples were stored at −80°C until further use.

TM Tissue Procurement

Porcine eyes are a commonly used *ex vivo* model for ophthalmic research with a morphology analog to the human eye (27, 28)

and therefore were used as protein source for the experiments in this study. The eye balls were collected from a local abattoir, immersed in phosphate buffered saline (PBS, Sigma) and kept on ice during transition. Eyes were processed within 5 h after enucleation. TM tissue was dissected according to Bachmann et al. (29). Briefly, eyes were opened with an equatorial cut, the posterior segment and the vitreous were discarded. Lens, iris, and ciliary body were removed with a forceps by a gentle pull. The anterior segment of the eye was washed with PBS to remove remaining iris pigment. The following steps were executed under a dissecting microscope. After removing the remnants of the pectinate ligaments, two incisions were made, flanking the TM. One was set adjacent to the line of Schwalbe, another one adjacent to the scleral spur, leading to exposure of the TM. The tissue was excised using fine scissors, avoiding contaminations with sclera and neighboring tissue. Since preparation of this tissue is a delicate task, contaminations with other tissues cannot entirely be ruled out. TM tissue of five eyes was pooled in one tube and stored in lysis buffer at −80°C until further processing.

Protein Extraction and Precipitation

TM tissue of five eyes was disrupted, using a sonicator, in 100 µL lysis buffer [125 mM Tris-HCl, pH 7; 100 mM NaCl; 0.1% Triton-X 100; 0.1% Tween 20; 0.5% Protease Inhibitor Cocktail (Sigma)]. After 1 h incubation on ice, tissue lysates were centrifuged at 20,000 g and 4°C. Soluble proteins were collected in a new vial, the cell pellet was resuspended in fresh lysis buffer and centrifuged at 20,000 g and 4°C for additional two times. Supernatants were pooled in one tube.

Seventy-five microliters of 72% trichloroacetic acid were added to the soluble protein fraction, and incubated for 30 min on ice. Precipitated protein was centrifuged at 20,000 g and 4°C for 30 min. The supernatant was discarded and the protein pellet was washed one time with HPLC grade water and two times with Acetone. After the last washing step, the pellet was air dried and resuspended in resolubilization buffer (8M Urea; 400 mM Tris; 4% CHAPS). Total extracted protein amount was determined using a BCA Assay kit (Thermo Scientific).

2D PAGE

For first dimension protein separation, according to the isoelectric point, 7 cm NL pH 3–10 IPG-strips (GE Healthcare) were used. One microliter Protease Inhibitor Cocktail (Sigma), 1 µL Bromophenol blue, 1.25 µL 2M dithiothreitol (DTT) and 2.5 µL IPG buffer (pH 3–10) were added to 100 µg TM protein. Volume was adjusted to 125 µL by adding resolubilization buffer + 0.12% DeStreak Reagent (GE Healthcare). Samples were incubated at 4°C with light agitation for 30 min, applied to IPG strip holders together with the respective IPG strips. Isoelectric focusing protocols were used as previously described (30). Briefly, proteins were allowed to rehydrate for 2 h at room temperature, followed by a 12 h step at 20 V and 20°C. Afterwards proteins were focused by increasing voltage gradually over 1 h to 500 V and holding this voltage for another hour. Afterwards voltage was increased again using a gradient to 1,000 V in 0.5 h and staying at this voltage for one additional hour. This step was followed by a 0.5 h gradient to 4,000 V and 2 h a holding step at 4,000 V. The final focusing step included voltage increase to 8,000 V during a 2 h gradient followed by a 2 h holding step.

After focusing, IPG strips were equilibrated for 2 × 15 min with slight agitation in 10 mL equilibration buffer (6M Urea; 75 mM Tris; 2%SDS; 34.5% Glycerol) containing 100 mg DTT for first equilibration and 250 mg iodoacetamid for the second.

For the second dimension SDS-PAGE, 4–12% precast gradient gels were used (NuPage, Novex; Bis-Tris gels) with MES running buffer (NuPage, MES SDS running buffer, Invitrogen). The IPG strip was briefly washed in MES buffer prior to application to the gel. The strip was fixed by adding a 0.5% Agarose solution, stained with bromophenol blue. Proteins were separated at 150 V for approximate 2 h.

Gels for the creation of the fusion image spot map and preparative gels were stained using the Invitrogen colloidal blue staining kit.

2D Western Blot

Proteins from the 2D gels were transferred for 2 h to a nitrocellulose membrane using a wet blot system (Mini Trans-Blot Cell; Bio Rad) using a standard Towbin buffer (31). Proteins were fixed and transfer efficiency was validated by ponceau S staining (Thermo Scientific). Unspecific binding sites on the blot were blocked by incubation of the membrane in 4% non-fat dry milk in TBST (Tris buffer saline + 0.5% Tween 20) for 1 h at room temperature. After washing the membrane three times with TBST, it was incubated with patients serum (1:40 in TBST) overnight under slight agitation at 4°C. After three more washing steps, the membranes were incubated with an anti-human IgG Fc HRP secondary antibody (Goat anti-human IgG H&L (HRP); Abcam) for 1 h at room temperature with slight agitation. Following three additional washing steps with TBST, the detection of the antibody reaction was achieved by a colorimetric approach, incubating the blots with a 3,3′,5,5′-tetramethylbenzidine substrate solution (1-Step™ TMB-Blotting Substrate Solution; Thermo Fisher Scientific) for 30 s. The reaction was quenched by washing the blots in HPLC grade water.

Imaging

Stained gels and western blots were imaged using a flatbed scanner (Epson V600). Images were scanned as 16 bit grayscale files. Spot detection, creation of the 2D fusion map and quantitative analysis was achieved by using the software Delta2D (Decodon). Spot quantities were analyzed as relative quantity of the spot, excluding background, where the total quantity of all spots on a 2D western blot is 100%. Thereby, the bias from different absolute staining intensities on individual blots is minimized.

Total Protein Stain

After imaging, the western blot membranes were washed for 5 min in water and another 5 min in PBS. Afterwards, the membranes were incubated in PBST (PBS + 0.5% Tween 20) for 1 h. After washing three times for 2 min in water, the membranes were incubated for 2 h in colloidal gold staining solution (Colloidal Gold Total Protein Stain, Biorad). Following

three further washing steps with water, the membranes were allowed to dry and scanned using a flatbed scanner system.

LC-ESI-MS/MS

Spots of interest were cut out of three colloidal blue stained gels using a scalpel. Each protein spot was pooled with its corresponding spot from two other identical gels to allow the detection of less abundant proteins by MS. In-gel digestion was carried out according to the protocols of Shevchenko et al. (32). Gel pieces were destained, reduced and alkylated before tryptic digestion of the proteins. Prior to MS analysis, the samples were cleaned up using C18 Resin ZipTip pipette tips (Millipore), as described earlier (30, 33). The LC-system consisted of a Rheos Allegro pump (Thermo Scientific, Rockford, USA) and a PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland), as described elsewhere (34). The system comprised of a 30 × 0.5 mm BioBasic C18 column (Thermo Scientific). Solvent A was LC-MS grade water with 0.1% (v/v) formic acid and solvent B was LC-MS grade acetonitrile with 0.1% (v/v) formic acid. The gradient was run for 90 min per gel spot as follows: 0–50 min, 10–35% B; 50–70 min, 35–55% B; 70–75 min, 55–90% B; 75–80 min, 90% B; 80–83 min, 90–10% B; and 83–90 min, 10% B. Continuum mass spectra data were acquired on an ESI-LTQ-Orbitrap-XL MS (Thermo Scientific, Bremen, Germany). The LTQ-Orbitrap was operated in a data-dependent mode of acquisition to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from *m/z* 300 to 2,000) were acquired in the Orbitrap with a resolution of 30,000 at *m/z* 400 and a target automatic gain control (AGC) setting of 1.0×10^6 ions. The lock mass option was enabled in MS mode and the polydimethylcyclosiloxane (PCM) *m/z* 445.120025 ions were used for internal recalibration in real time (35). The five most intense precursor ions were sequentially isolated for fragmentation in the LTQ with a collision-induced dissociation (CID) fragmentation, the normalized collision energy (NCE) was set to 35% with activation time of 30 ms with repeat count of 10 and the dynamic exclusion segment was disabled. The resulting fragment ions were recorded in the LTQ.

The acquired continuum MS spectra were analyzed by Thermo Proteome Discoverer software (ver. 1.1.0.263; Thermo scientific). The tandem MS spectra were searched against Uniprot database (combined *Homo sapiens* and *Sus scrofa*, date:20.04.2018) using settings with peptide mass tolerance of ±50 ppm, fragment mass tolerance of ±0.5 Da and peptide charge state 1+ to 4+, using MASCOT server version 2.2.7. FDR for peptide and protein identification was set to 0.01. Carbamidomethylation of cysteine was set as a static modification, while protein oxidation of methionine were defined as dynamic modifications, enzyme: trypsin and maximum number of missed cleavages: 1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner (36) repository with the dataset identifier PXD011752.

Microarray Fabrication

Microarrays were manufactured in our lab using a non-contact piezo-dispenser (SciFLEXARRAYER S3, Scienion, Berlin, Germany). The selected candidate antigens were purchased as recombinant human proteins (**Table S1**) and spotted in triplicates onto nitrocellulose coated microarray slides (AVID Oncyte, 16 Pad NC slides, Grace Biolabs, Bend, Oregon, USA). As positive and negative control spots, human IgG (Sigma), secondary antibody conjugated to alexa flour 647 and spotting buffer (PBS) were included on each array. The spotting process was carried out in a humidity chamber with humidity set to 60%. To allow optimal immobilization of the proteins on the microarray surface, slides were kept on the spotter platform to dry overnight prior to incubation.

Microarray Incubation and Image Acquisition

Slides were incubated using incubation chambers (ProPlate Multiwell chambers, Grace Biolabs, Bend, USA), that divide the slide in 16 subarrays. The following incubation steps were performed at 4°C on an orbital shaker. To decrease background signals, the arrays were incubated with blocking buffer (Super G, Grace Biolabs, Bend, Oregon, USA) for 1 h. After removing the blocking buffer, residual buffer was washed away three times with phosphate-buffered saline containing 0.5% Tween-20 (PBST). The arrays then were incubated with 100 µL serum samples in a 1:250 dilution in PBS overnight. One subarray on each slide was incubated with PBS to serve as a negative control. Slides were washed again three times with PBST followed by incubation for 1 h at room temperature with a secondary anti-human antibody conjugated with Alexa flour 647 (Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, 109-605-008, Jackson Immunoresearch) in a 1:500 dilution in PBS. After the incubation with the secondary antibody, the slides were washed two times with PBST and two times with ultrapure water. The microarray slides were dried for 2 min in a vacuum concentrator (SpeedVac, Thermo Scientific, Waltham, Massachusetts, USA).

Slides were scanned with a high resolution confocal laser scanner (428 Array Scanner, Affymetrix, Santa Clara, California, USA), yielding 16 bit TIFF images. Spot intensities were quantified with the image analysis software Imagene (Imagene 5.5, BioDiscovery Inc., Los Angeles, California, USA). Spots with poor quality were manually flagged and removed from further analysis.

Microarray Data Pre-procession

Local background was subtracted from the median spot intensities. Negative background subtracted intensities were set to zero. Low quality spots were flagged and excluded from analysis. To correct for the unspecific binding of the secondary antibody, the signal of the negative control included on each slide was subtracted from each spot. The replicate spot intensities were averaged, yielding one mean fluorescence intensity. To correct for technical variability, the signal intensities were normalized to the IgG control spots on each subarray by median centering. To this end, the IgG median signal intensities were divided by the overall IgG signal median, yielding a normalization factor. All signal intensities on one subarray were multiplied with their correspondent normalization factor. The resulting

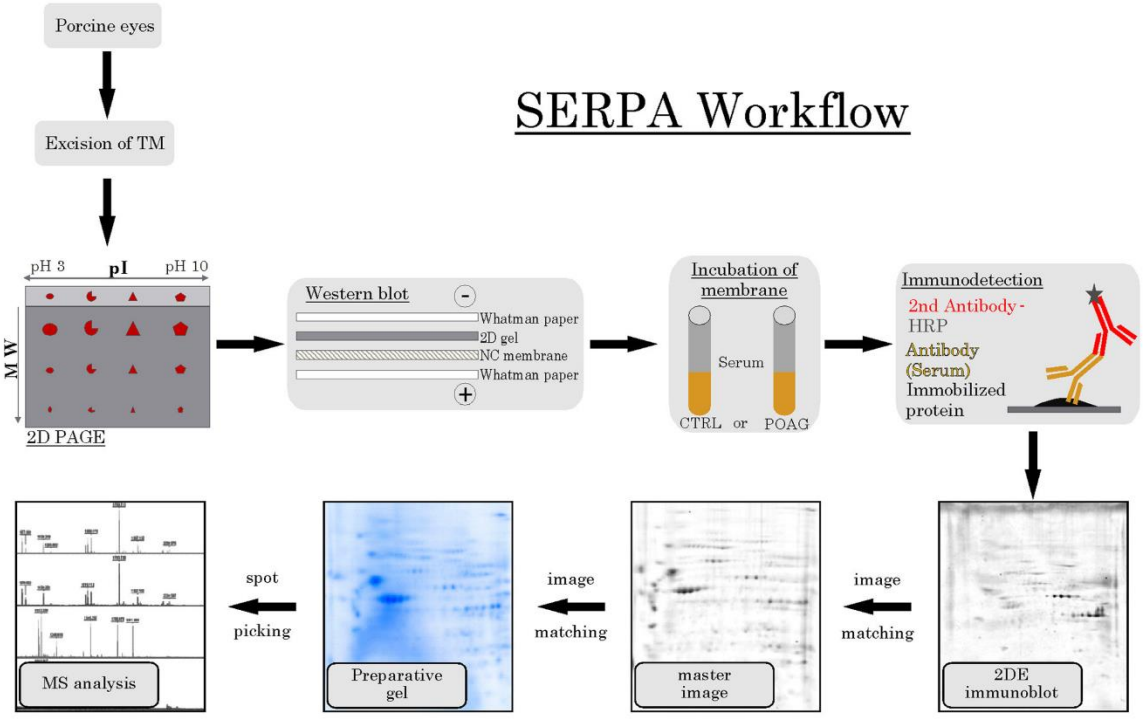

FIGURE 1 | SERPA workflow. TM tissue was excised from porcine eyes and analyzed by 2DE PAGE, separating the proteins according to their isoelectric point (pI) and their molecular weight (MW). Next, proteins were transferred to a nitrocellulose (NC) membrane using a wet blot system. Then, the membranes containing the immobilized protein were incubated with either CTRL or POAG serum, using the contained autoantibodies as primary antibodies. As secondary detection antibody, anti-human antibodies conjugated to horse radish peroxidase (HRP) were used and visualized by a colorimetric detection. The resulting 2DE immunoblots were matched to their corresponding spots on a colloidal blue stained master image, computed as average pattern from three independent 2DE gels. Spots of interest were then identified on preparative gels and prepared for MS analysis.

normalized fluorescence intensities (NFI) were forwarded to statistical analysis.

Statistical Analysis

Normality of the obtained data was assessed using one-sample Kolmogorov–Smirnov (K-S) test. Data obtained by SERPA was normally distributed (K-S test not significant at alpha = 0.05), therefore, differences in spot quantities in the SERPA discovery phase were assessed using student's *t*-test. Some variables in the microarray data did not follow a normal distribution (K-S $p < 0.05$) and consequently changes in autoantibody levels were assessed using Mann–Whitney *U*-test. A *p*-value of <0.05 was considered statistical significant. In order to obtain a more robust statistic and reduce the influence of outliers in the microarray data set, values below the 5th and above the 95th percentile in each group were excluded from analysis. Kruskal–Wallis H test was used for multiple group comparisons. ROC curves were computed using artificial neural networks (ANN). The multilayer perceptron (MLP) networks were trained by randomly using 70% of the cases as training set and 30% as test set. ROC curves and AUROCC values refer to test performance only on the test set. Missing data in the microarray data set was replaced by the respective group mean for the ANN model building. The statistical data analysis was performed using Statistica (Statistica 13, Statsoft, Tulsa, Oklahoma, USA).

RESULTS

Discovery of Candidate Autoantigens by SERPA

The aim of this analysis was the identification of potential autoantibodies to antigens in the TM. In this discovery phase study, serum of six POAG patients and six age and gender matched controls (CTRL) were analyzed for autoreactive antibodies against proteins derived from porcine TM tissue lysates. To this end, we used SERPA as a widely used immunoproteomic method for the identification of serological autoantibodies (**Figure 1**). TM tissue lysates were separated by 2DE and transferred onto nitrocellulose membranes. Membranes were subsequently incubated with individual serum samples from the POAG or the control group. As shown in **Figure 2**, a broad range of antigen-autoantibody-complexes were detected in both

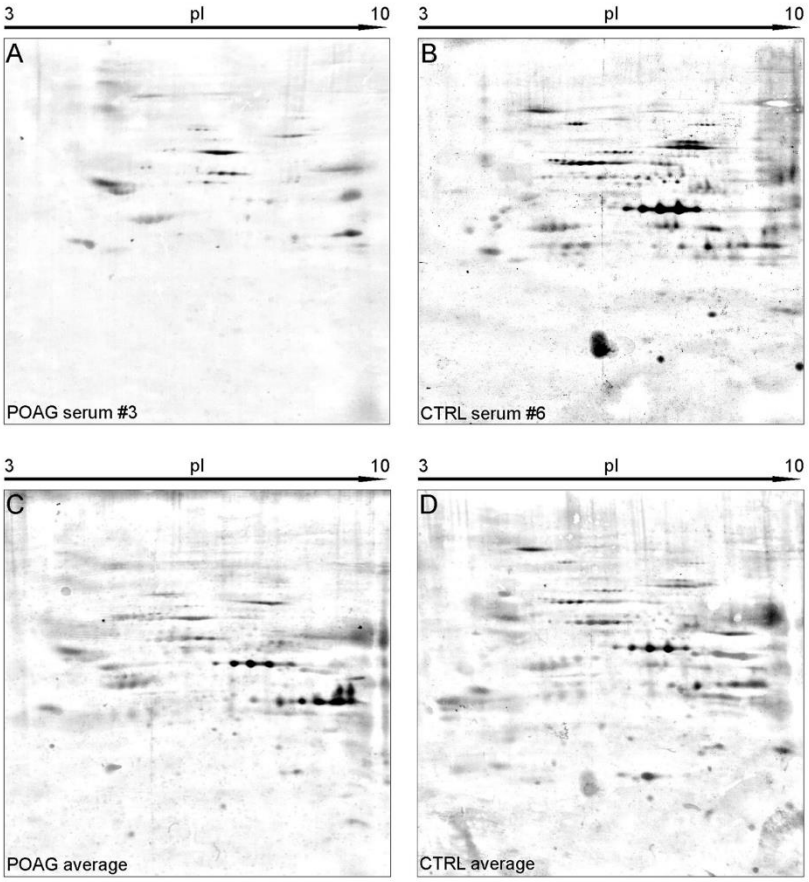

FIGURE 2 | Representative SERPA immunoblots. **(A,B)** Show 2DE immunoblots of individual sera form either POAG **(A)** or CTRL group **(B)**. **(C,D)** Show the average pattern for the POAG and the CTRL group computed from all immunoblots by Delta2D. A high abundance of diverse autoantibodies could be detected in both groups. Interesting spots were selected upon their different gray intensity between the two groups and forwarded to MS analysis.

groups, but autoantibody profiles also strongly varied amongst individuals. To find differences in the autoantibody repertoire of the two groups, protein spots on the immunoblots were analyzed and spot intensities were quantified using Delta2D (Decodon). Raw spot quantities were normalized to the equalized total signal intensities of the immunoblots to prevent bias from blot incubation. A spot map was created of three colloidal blue stained 2DE gels using TM tissue lysates. In Delta2D images of these gels were computed to obtain a single master image showing the average spot pattern of the three gels (**Figure 3A**). Spots that showed differences between groups in the quantity of their average gray value (**Figure 3B**) and could be visually identified on colloidal blue stained gels were selected for identification by MS. The spots of interest from the 2DE western blots were matched with the correspondent spot on the spot map. To help the identification of the spots on the spot map, a total protein stain using colloidal gold was applied to the western blot membranes. This allowed for the application of the image warping option in Delta2D, were spot pattern of two different images can be aligned. This enabled a reliable identification of the immunoreactive spots on the preparative gels used for the MS analysis. The spots of interest were cut out in three preparative gels and pooled to increase the protein amount for MS analysis. Spots were identified by MS analysis as shown in **Table 2**.

A total of 16 autoantigens could be identified by LC-ESI-MS/MS. They all showed variations in mean autoantibody levels between groups, with in- and decreased signal intensities, but only one spot showed significant changes (**Table 3**). The spot identified as phosphoglycerate mutase 1 (PGAM1) showed significant differences between groups ($p = 0.03$), as analyzed by student's t-test. Other autoantigens that have not yet been described in the context of glaucoma were identified as ATP synthase subunit alpha (ATP5A1), Caldesmon (CALD1), Voltage-dependent anion-selective channel protein 2 (VDAC2), and L-lactate dehydrogenase A (LDHA) but failed to show significant group differences. Furthermore, some

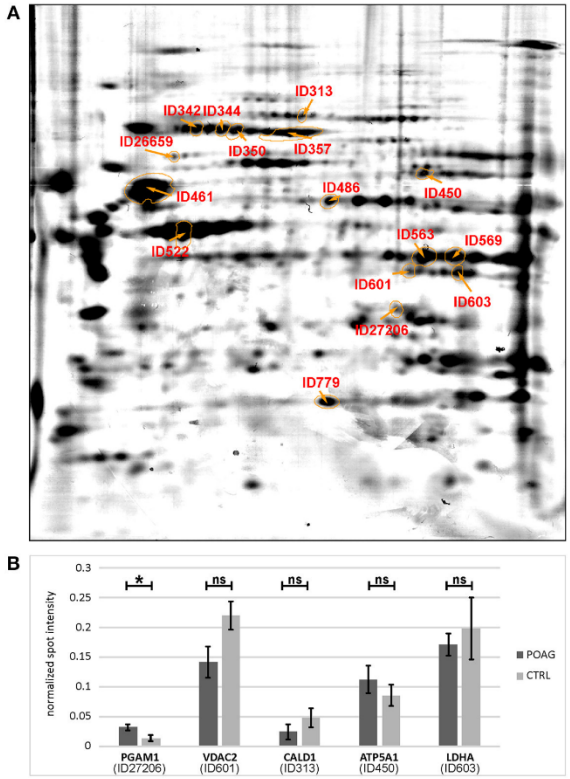

FIGURE 3 | Quantification of spot intensities and protein identification. **(A)** The picture shows a spot map computed of three individual colloidal blue stained 2DE gels, reflecting an average image of the porcine TM proteome. Spots of interest were labeled with IDs and identified by MS analysis **(Table 2)**. **(B)** Comparison of immunoblot spot intensities between groups. Normalized spot intensities are shown for PGAM1, VDAC2, CALD1, ATP5A1, and LDHA. Asterisk indicates significance at $p < 0.05$, ns, not significant.

previous identified glaucoma autoantigens including heat shock protein 60 dDA (HSPD1) (12, 13), heat shock protein 70 kDa (HSPA1B) (11), vimentin (VIM) (11), α-enolase (ENO1) (37) and superoxide dismutase 1 (SOD1) (38) could be detected (**Table 2**). Considering the caveats of the colorimetric detection used in this approach and the low statistical power of this discovery screening approach, it has been decided to include all detected autoantigens in the microarray validation step to analyze the respective autoantibody levels in a method providing a higher dynamic range and capable to process a larger number of samples. Thus, this enables the detection of smaller effects that might be missed in the initial discovery.

Protein Microarray Analysis for the Validation of Putative Autoantibodies

To analyze the autoantibody distribution of the detected candidates in a larger sample size, customized microarray slides were fabricated using commercially available recombinant

TABLE 2 | Identified 2DE spots by MS analysis.

Spot ID	Gene name	Protein	Accession	Sequence coverage (%)	# Peptides	# AAs	MW [kDa]	calc. pI	Score	Total peptide intensity/spot (%)
ID313	CALD1	Caldesmon	Q05682	1.51	3	793	93.2	5.66	124.08	100.00
ID342	HSPA7	Putative heat shock 70 kDa protein 7	P48741	3.54	8	367	40.2	7.87	348.76	100.00
ID344	HSPA2	Heat shock-related 70 kDa protein 2	P54652	23.63	147	639	70.0	5.74	6070.68	26.78
ID350	HSPA1B	Heat shock 70 kDa protein 1B	Q6S4N2	28.71	169	641	70.1	5.82	7349.82	32.62
ID357	ALB	Serum albumin	P08835	44.15	287	607	69.6	6.49	13530.87	99.25
ID450	ATP5A1	ATP synthase subunit alpha, mitochondrial	P80021	20.25	83	553	59.7	9.19	3511.75	85.46
ID461	VIM	Vimentin	P02543	52.15	412	466	53.6	5.12	18264.69	66.19
ID486	ENO1	Alpha-enolase	P06733	20.74	99	434	47.1	7.39	5076.76	68.38
ID522	ACTB	Actin, cytoplasmic 1	P60712	33.33	243	375	41.7	5.48	12218.77	50.79
ID563	ANXA2	Annexin A2	P19620	56.64	380	339	38.5	6.93	15255.93	52.92
ID569	ANXA2	Annexin A2	P19620	53.98	248	339	38.5	6.93	10170.05	70.66
ID601	VDAC2	Voltage-dependent anion-selective channel protein 2	P45880	24.83	69	294	31.5	7.56	3231.96	70.09
ID603	LDHA	L-lactate dehydrogenase A chain	P00339	32.83	86	332	36.6	8.07	2434.55	45.75
ID779	SOD1	Superoxide dismutase [Cu-Zn]	P04178	29.41	46	153	15.9	6.52	1699.91	94.41
ID26659	HSPD1	60 kDa heat shock protein, mitochondrial	P10809	24.43	118	573	61.0	5.87	5271.25	95.14
ID27206	PGAM1	Phosphoglycerate mutase 1	P18669	35.43	100	254	28.8	7.18	4999.49	80.05

TABLE 3 | Quantification of normalized signal intensities from SERPA immunoblots and statistical significance (*p*-value *t*-test).

	POAG mean	CTRL mean	POAG *SD*	CTRL *SD*	*t*-value	*p*-value
PGAM1	0.03	0.01	0.01	0.01	2.50	0.03
VDAC2	0.14	0.22	0.07	0.06	−2.20	0.05
CALD1	0.02	0.05	0.03	0.04	−1.16	0.27
ATP5A1	0.11	0.09	0.06	0.04	0.92	0.38
HSPA7	0.33	0.20	0.33	0.13	0.87	0.40
ANXA2 (ID563)	0.61	1.01	0.21	1.11	−0.85	0.42
ENO1	0.17	0.38	0.08	0.60	−0.84	0.42
HSPD1	0.09	0.06	0.08	0.03	0.79	0.45
SOD1	0.23	0.38	0.14	0.62	−0.59	0.57
ALB	0.81	0.55	1.28	0.35	0.49	0.63
LDHA	0.17	0.20	0.04	0.13	−0.48	0.64
ANXA2 (ID569)	0.53	0.42	0.61	0.34	0.39	0.71
HSPA2	0.18	0.21	0.07	0.17	−0.30	0.77
VIM	1.27	1.09	1.29	0.74	0.28	0.78
ACTB	0.44	0.39	0.29	0.30	0.27	0.80

Mean values and standard deviation (SD).

proteins (**Table S1**). This approach yields fluorescence intensities for each tested autoantigen that correlate with the serological autoantibody level. Autoantibody levels of the selected candidates were analyzed in serum samples of 60 POAG patients and compared to an age and gender matched control group of 45 individuals. The received data was pre-processed and normalized to IgG control spots on each subarray. Descriptive statistics of the obtained data are shown in **Table 4**. Kolmogorov-Smirnov one-sample test for normality indicated, that the data is not normally distributed. Therefore, non-parametric Mann-Whitney *U* test was applied to analyze the data for alternating autoantibody distributions in POAG patients and the control group. This analysis revealed a significant increase of autoantibody levels against VIM, HSPD1, PGAM1, VDAC2, and CALD1 ($p < 0.05$, **Figures 4A–E**) in POAG compared to the control group. To investigate, whether the presence of other eye or systemic diseases has an influence on the tested autoantibody levels, we further analyzed the control group. To this end we divided the control group into four subgroups with regard to other diseases, comprising subjects with "other eye disease" ($n = 25$), "systemic disease" ($n = 5$), "systemic + other eye disease" ($n = 6$) and "none" ($n = 9$). Kruskal–Wallis *H*-test revealed no significant ($p \geq 0.05$) difference among these subgroups.

To evaluate the potential of the identified markers to discriminate between POAG and non-glaucomatous subjects we used ANNs. The MLP network was trained using a training set consisting of 70% randomly selected samples from the microarray data set of these five markers. To assess the classification power, the network was deployed on the remaining 30% of the data, the test set. Specifications of the ANN can be found in **Table S2**.The trained network was able to classify the POAG patients from the control group with a sensitivity of 81% and a specificity of 93% (**Figure 4F**). With an area under the roc curve (AUROCC) of 0.875 the test yields a good accuracy.

It is further of special interest to enhance glaucoma diagnostics at an early stage of disease progression, when glaucomatous visual field damage is still minor. Therefore, a subgroup of POAG subjects, being at an early disease stage with mild visual field defects, was analyzed separately. The autoantibody levels of PGAM1, VDAC2, CALD1, and VIM are significantly higher in the subgroup of patients with mild POAG ($p < 0.05$) (**Figures 5B–E**). HSPD1 autoantibody levels showed no significant difference in mild POAG (**Figure 5A**). Kruskal-Wallis H test however, revealed a significant change in HSPD1 autoantibodies between different POAG gradations ($p = 0.017$). The mean level increases with visual field damage progression (**Figure 5F**).

DISCUSSION

Glaucoma diagnostic as it is today is time and cost intensive. Only experienced ophthalmic medical staff can evaluate glaucomatous damages of the optic nerve and even then, the diagnosis often is a matter of subjective interpretation. Clinical signs of the damage regularly only show, when the disease is far in progression. In most cases, 25–35% of the retinal ganglion cells underwent apoptosis before glaucoma is first diagnosed. To help the medical staff with the assessment of glaucoma and to provide a fast and objective test that can be included in routine examinations, reliable biomarkers need to be established in easy monitorable body fluids, such as tear or serum. Regarding autoantibody levels as potential biomarkers fulfilling these requirements, there is the caveat that no single marker is specific enough to classify healthy subjects from diseased. As already shown in cancer diagnostics (39–41), a set of markers needs to be established to enable sufficient discrimination, since fluctuations of autoantibody levels can occur in higher magnitude, even in healthy individuals. In an earlier study, the data of nine autoantibody biomarkers in serum and aqueous humor samples was used to train an ANN. The created network was able to classify the subjects in the test set with an AUROCC of 0.93 (38). But the translation of this biomarkers to an actual diagnostic test to be used in clinical routine is no trivial task, since multiplexing analysis of autoantibody concentration in a point-of-care device (POCD) with up to nine analytes has not been established yet. The challenge now is to find a set of biomarkers that is small enough to be deployed in a POCD but robust enough to allow testing with high specificity and sensitivity.

In this study, our goal was to identify novel glaucoma specific biomarkers by SERPA. To this end, we analyzed sera of six POAG patients and six non-glaucomatous individuals. With this approach, we were able to identify 16 autoantigens, but only PGAM1 autoantibodies showed significant elevated levels in POAG. To validate the observations made in this discovery phase, the identified candidates were subjected to a protein microarray analysis. Sera of 60 POAG patients and 45 controls were tested for their autoantibody levels against these target antigens. Here, VDAC2, CALD1, and PGAM1 autoantibodies have shown to be elevated in serum of POAG patients and also in the subgroup of POAG patients with an early disease stage.

TABLE 4 | Normalized fluorescence intensities (NFI) obtained by protein microarray analysis.

	CTRL mean	POAG mean	CTRL SE	POAG SE	CTRL valid *N*	POAG valid *N*	*p*-value
CALD1	212.16	430.47	28.54	76.10	35	45	0.023
HSPD1	9684.98	13267.04	1263.34	1210.67	41	55	0.015
ATP5A1	233.12	271.94	67.09	126.36	12	21	0.385
VIM	2171.83	2853.60	260.29	193.24	41	57	0.014
ENO1	353.22	393.12	70.63	71.74	22	33	0.858
VDAC2	291.00	404.68	29.06	33.37	36	55	0.038
LDHA+B	91.98	106.99	17.09	25.53	17	25	0.839
SOD1	280.79	340.55	38.76	37.20	38	52	0.299
ANXA2	5229.18	5594.91	499.77	337.74	30	41	0.200
PGAM1	7475.67	9186.97	676.15	558.01	39	52	0.018

Mann–Whitney U-test for evaluation of group differences. Mean values and standard error (SE).

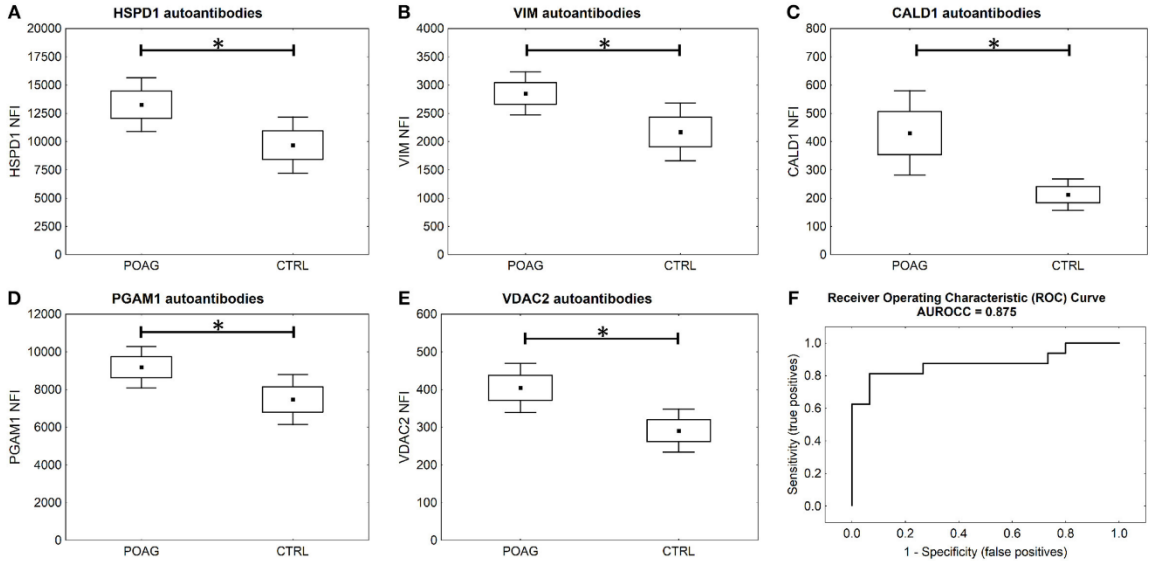

FIGURE 4 | Microarray results and ROC curve. **(A–E)** Microarray analysis shows significant higher serological levels of HSPD1, VIM, CALD1, PGAM1, and VDAC2 antibodies in POAG (*U* test $p < 0.05$). Small squares indicate mean fluorescent intensities, boxes present the standard error of the mean (SE), whiskers show 1.96 times SE. $^{*}\triangleq p < 0.05$; ns $\triangleq$ "not significant" **(F)** ROC curve for five autoantibody-marker panel by use of ANNs.

Therefore, they might be used as novel serological biomarkers that improve early glaucoma diagnosis.

VDAC2 is a voltage-dependent anion channel in the outer mitochondrial membrane. It is a ∼30 kDa transmembrane protein (42) and is important for the translocation of small molecules across the membrane. The porin does not only act as channel for ATP and ions but can also release the apoptosis inducing cytochrome c by interacting with proteins of the Bcl-2 family (43, 44). Although, the mechanisms behind RGC death in glaucoma are not yet understood entirely, it is suggested, that the vast majority of RGCs undergo apoptosis, thereby underlining a potential role for this mitochondrial porin in the pathogenesis. Moreover, apoptosis triggered by various mechanisms can also be observed in the glaucomatous TM (45). VDAC2 was also found to be upregulated in glaucomatous rat RGCs (46), strongly demonstrating the role of apoptosis in RGC death. The deregulation of VDAC2 is a possible trigger for the onset of autoimmunity, hence the elevation of VDAC2 antibodies might be the result of the overexpression of this protein, as well as apoptotic events (47, 48). Antibodies to VDAC can also be found in sera of autistic children and have shown to induce apoptosis in human neuroblastoma cells (49), suggesting that the presence of these autoantibodies could not only be a mere expression of the pathological changes, but also enforces their progression in a synergetic manner.

Caldesmon, the gene product of CALD1, acts as a modulator of the actomyosin network by interacting with actin and cadherin, involved in cell-cell and cell-matrix adhesions. It

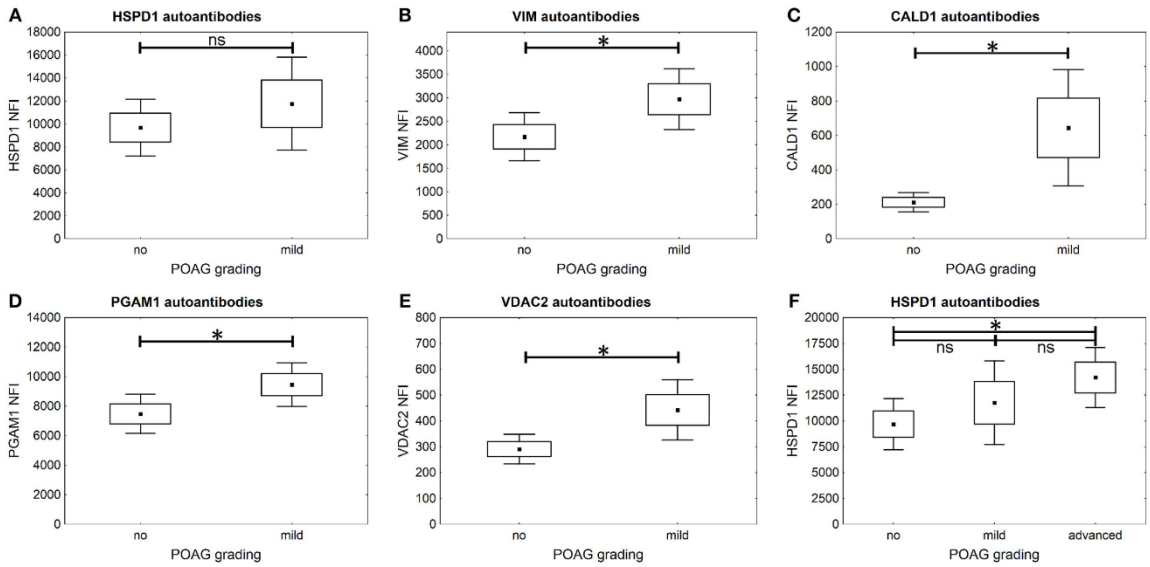

FIGURE 5 | Comparison of autoantibody levels in mild POAG and CTRL (no). Small squares indicate mean fluorescent intensities, boxes present the standard error of the mean (SE), whiskers show 1.96 times SE. $^{*}\triangleq p < 0.05$; ns $\triangleq$ "not significant" **(A–E)** The microarray analysis shows that autoantibodies to VIM **(B)**, CALD1 **(C)**, PGAM1 **(D)**, and VDAC2 **(E)** already show significantly higher levels in early POAG ($p < 0.05$), but not autoantibodies to HSPD1 **(A)** ($p = 0.38$). **(F)** Kruskal–Wallis *H*-test shows significant differences in HSPD1 autoantibody levels between different POAG gradations. The mean HSPD1 autoantibody levels increase with glaucoma progression and show significant differences between CTRL (no) and advanced POAG ($p = 0.013$).

regulates the cell contractility in a Ca^{2+}–dependent manner. CALD1 overexpression in TM cells leads to actin cytoskeleton reorganization and in high abundance to disruption of adherens junctions (50). These molecular changes cause a relaxation of the tissue and thereby an increase of TM outflow facility. Therefore, CALD1 has been proposed for gene therapy of glaucoma (50, 51). Reorganization of the actin cytoskeleton has been observed in glaucomatous TM (5, 6) leading to increased tissue rigidity and failure to actively regulate IOP. Thus, we hypothesize that a participation of CALD1 deregulation in these processes cannot be ruled out. Pathologic changes in CALD1 expression, incomplete protein folding or post-translational modification could induce autoimmunity to this antigen. However, the effects and the origin of the identified CALD1 autoantibodies remain elusive and need to be addressed in further studies.

PGAM1 encodes the protein phosphoglycerate mutase 1 and is involved in the glycolytic pathway. Here it acts as an enzyme catalyzing the reaction from 3-phosphoglycerate to 2-phosphoglycerate (52). Autoantibodies to PGAM1 have been found increased in multiple sclerosis and neuromyelitis optica and have been suggested as marker for neuroinflammatory diseases (53, 54). Inflammatory processes are also prevalent in glaucoma as shown in experimental glaucoma models and in clinical studies, as reviewed by Russo et al. (55) and Soto et al. (56). This leads to the hypothesis, that PGAM1 autoimmunity can be triggered by neuroinflammatory conditions also appearing in glaucoma pathogenesis.

Levels of HSPD1 and VIM antibodies have already been described to be altered in glaucomatous diseases. Antibodies against the 60 kDa heat shock protein, encoded by HSPD1, were amongst the first to be detected in connection with glaucoma (12, 13) and are consistently increased throughout different study populations (57). The results obtained in the present study further confirm these findings. The increased VIM antibody levels detected in the present study however, are inconsistent with an earlier study from 2008 (16), in which antibody levels against optic nerve antigens were found to be downregulated in POAG and normal tension glaucoma using western blot analysis.

Overall, training of ANNs using a 5-biomarker-panel of the three new biomarker candidates with HSPD1 and VIM yielded a test that was able to classify POAG from CTRL with 81% sensitivity at 93% specificity and an AUROCC of 0.875.

The findings of this study are a further step to the development of a glaucoma rapid test that will identify early stage POAG showing only minor clinical signs. This would allow diagnostic testing not only for trained glaucoma specialists and would therefore be suitable for general health examinations. This can promote an early detection of the disease and allow an earlier start of treatment, thereby preventing glaucoma progression and preserve patients from severe vision loss. Autoantibodies as serological markers are accessible using minimal invasive methods and therefor provide a promising approach to enhance diagnostics.

Although the alteration of autoantibody levels to several ocular antigens in glaucoma has also been shown in different

other studies (9–16, 58), it remains elusive, whether they are causative for pathological changes or just a byproduct that arises from them. Some studies provide evidence that endogenous antibodies can enter living cells and even promote or protect from apoptosis (59–61). Also, accumulation of IgG in the TM has been shown (62) and antibody levels in the aqueous humor are widely consistent with serum levels (38). The specific effects of autoantibodies to this tissue however, have not yet been analyzed. The hypothesis of the detected autoantibodies being an epiphenomenon seems to be more obvious. The TM in POAG suffers from a loss in cellularity (63) but the mechanisms of cell loss have not been clarified conclusively. Besides autophagy and necrosis, apoptosis has been discussed to induce cell death in glaucomatous TM (64, 65). An increased number of apoptotic and (postapoptotic) necrotic events can be a trigger for the emergence of autoantibodies to intracellular proteins (48). The cleavage of cellular components during apoptosis can even lead to the generation of neoepitopes (66). Furthermore, disease related post-translational modifications of the proteins can be an additional trigger for the occurrence of highly specific autoantibodies (67). In this case, the autoantibodies are an epiphenomenon of the damage of the tissue and/or induced by related modifications of the antigen. Both possibilities are supported by multiple circumstantial evidences and the concluding proof, of which mechanism is applicable in POAG remains a task for future studies. But regardless of their origin or effect, autoantibodies may serve as valuable disease biomarkers, as has also been demonstrated in other neurodegenerative diseases as Parkinson's disease (68) or Alzheimer's disease (69, 70). However, an important task for future evaluation studies to improve the reliability of the identified biomarkers is their investigation in other chronic and acute diseases. Also, it needs to be defined if they have a role in the natural autoantibody repertoire to gain further insights into their function in health and disease.

CONCLUSION

With VDAC2, PGAM1, and CALD1, new autoantibodies have been identified in association with POAG by the application of immunoproteomic methods. A combination of these and former known autoantibodies have shown to be suitable biomarker candidates for the diagnosis of POAG, even in early disease stages. The application of advanced bioinformatic analysis using artificial neural networks improves the value of these biomarkers by building models based on a panel of five candidate biomarkers. Applied in a rapid test, available not only to glaucoma specialists, they could help to promote early POAG detection and reduce the probability of severe vision loss by timely onset of treatment. To this end however, the evaluation of test results in larger, more heterogeneous study populations is necessary. Likewise, the search for even better biomarkers and biomarker combinations needs to continue, to enable the development of reliable rapid test immunoassays as a new standard tool in glaucoma diagnostics.

DATA AVAILABILITY

The MS dataset is publicly available at the PRIDE database (https://www.ebi.ac.uk/pride/archive/) with the identifier PXD011752.

AUTHOR CONTRIBUTIONS

VB planned the experimental design of this study, performed SERPA and microarray analysis, interpreted the data, and wrote the manuscript. NaP performed mass spectrometric analysis. FG and NaP critically revised the manuscript. FG and NoP contributed to conception of the study and provided resources.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00381/full#supplementary-material

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Publication 2

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ORIGINAL ARTICLE

Autoantigens in the trabecular meshwork and glaucoma-specific alterations in the natural autoantibody repertoire

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Abstract

Objectives. Primary open-angle glaucoma (POAG) is a neurodegenerative disorder leading to a gradual vision loss caused by progressive damage to the optic nerve. Immunological processes are proposed to be involved in POAG pathogenesis. Altered serological autoantibody levels have been frequently reported, but complete analyses of the natural autoantibodies with respect to disease-related alterations are scarce. Here, we provide an explorative analysis of pathways and biological processes that may involve naturally immunogenic proteins and highlight POAG-specific alterations. **Methods.** Mass spectrometry-based antibody-mediated identification of autoantigens (MS-AMIDA) was carried out in healthy and glaucomatous trabecular meshwork (TM) cell lines, using antibody pools purified from serum samples of 30 POAG patients and 30 non-glaucomatous subjects. Selected antigens were validated by protein microarray ($n = 120$). Bioinformatic assessment of identified autoantigens, including Gene Ontology (GO) enrichment analysis and protein–protein interaction networks, was applied. **Results.** Overall, we identified 106 potential autoantigens [false discovery rate (FDR) < 0.01], from which we considered 66 as physiological targets of natural autoantibodies. Twenty-one autoantigens appeared to be related to POAG. Bioinformatic analysis revealed that the platelet-derived growth factor receptor beta (PDGFRB) pathway involved in TM fibrosis was particularly rich in POAG-related antigens. Antibodies to threonine-tRNA ligase (TARS), component 1 Q subcomponent-binding protein (C1QBP) and paraneoplastic antigen Ma2 (PNMA2) showed significantly ($P < 0.05$) higher levels in POAG patients as validated by protein microarray. **Conclusion.** This study provides new insights into autoimmunity in health and glaucoma. Bioinformatic analysis of POAG-related autoantigens showed a strong association with the PDGFRB pathway and also increased levels of PNMA2, TARS, and C1QBP autoantibodies in the serum of POAG patients as potential glaucoma biomarkers.

Keywords: autoantigen, biomarker, glaucoma, immunoproteomics, natural autoantibodies, trabecular meshwork

INTRODUCTION

Primary open-angle glaucoma (POAG) is an optic neuropathy generally characterised by an elevated intraocular pressure (IOP) above 21 mmHg,[1] apoptosis of retinal ganglion cells and gradual loss of vision.[2] In addition to old age,[3] genetic predisposition[4,5] and decreased central corneal thickness,[6] a high IOP is the major risk factor for POAG,[7] but alone it is not sufficient for its onset. An elevated IOP in POAG is caused by an increased outflow resistance of the aqueous humour, triggered by pathological changes in the trabecular meshwork (TM). Outflow resistance in the TM is primarily caused by the cells and extracellular matrix of the juxtacanalicular region that is subject to constant remodelling to maintain aqueous humour efflux. In the glaucomatous eye, signalling pathways regulating extracellular matrix deposition, the actin cytoskeleton and cell–cell/cell–matrix connections are dysregulated.[8,9] Transforming growth factor β levels in aqueous humour are increased, leading to a rise in its downstream effector, connective tissue growth factor. This matricellular protein promotes the excessive accumulation of extracellular matrix proteins in the extracellular space, causing the TM tissue to be denser and thereby decreasing outflow.[10]

Furthermore, cross-linking of the actin cytoskeleton in the TM increases. This limits the ability of the tissue to actively respond to changes in IOP by actomyosin-mediated contraction of TM cells. The β-integrin-induced formation of cross-linked actin networks increases the overall rigidity of the tissue and exacerbates the resistance in the TM outflow pathway.[11] The stiffness of the tissue also accumulates with age,[12] elevating the chances of drainage failure. The TM in glaucoma is about 20 times more rigid than in healthy tissue,[13] reflecting enormous changes in TM biomechanics during the course of the disease. However, in addition, another form of open-angle glaucoma without increased pathological IOP exists. This particular subgroup is known as normal-tension glaucoma and comprises between 20% and 61% of all open-angle glaucomas.[14] This also suggests that other pathomechanisms are involved in the generation of glaucomatous damage to the optic nerve. In the case of normal-tension glaucoma, a weakness of the lamina cribrosa has been proposed, making the retinal ganglion cells more prone to mechanical damage, even under physiological IOP.[15] The optic nerve leaves the posterior eye through this mesh-like tissue which has similar properties to the TM. Therefore, as the LC provides mechanical support to the optic nerve, compression and distortion of this structure may be involved in the damage to retinal ganglion cell axons in glaucoma.[16,17]

Another factor that has been forwarded by previous studies is a possible involvement of immunological mechanisms. Several research groups already found altered levels of autoantibodies (AAbs) against diverse ocular antigens.[18–23] Interestingly, not only increased but also downregulated AAb reactivities have been identified,[24–26] suggesting a complex alteration of the natural autoantibody repertoire. Many scientists have abandoned the paradigm of Paul Ehrlich's 'horror autotoxicus', which links AAbs only to pathological effects. The physiological immunome most likely fulfils more tasks than a direct defence against invading pathogens as part of humoral immunity. Instead, the immune system is also involved in homeostasis and clearance of cellular debris.[27] The impairment of these functions can likely favor the development or progression of diseases.

Up to now, the human immunome has not been well explored. In this study, we looked at antigens in TM cells and their serological AAbs, as well as their function, to identify pathways involved in the emergence of autoantigens. Furthermore, we searched for hints of any altered immunogenicity of these proteins in POAG and validated an increase in AAbs against of paraneoplastic antigen Ma2 (PNMA2), threonine-tRNA ligase (TARS) and complement component 1 Q subcomponent-binding protein (C1QBP) by antigen microarray.

RESULTS

Identification of autoantigens in human TM cells using MS-AMIDA

Mass spectrometry-based antibody-mediated identification of autoantigens (MS-AMIDA) was

used to detect serological AAbs to antigens in human TM cells. The workflow comprises the isolation of IgGs from human non-glaucomatous serum (for characteristics, see Table 1, MS-AMIDA CTRL group) and their cross-linking to protein G-coated magnetic beads. These beads were subsequently incubated with TM cell lysates from immortalised human cell lines. The putative autoantigens bind to the immobilised antibodies, and after on-bead tryptic digestion, they were identified by high-resolution MS. The IgG repertoire of non-glaucomatous subjects against antigens from healthy (HTM) and glaucomatous TM (GTM) cells was tested. Also, negative controls, without IgG, and a mock control were included, which was incubated only with lysis buffer instead of cell lysate. An overview of the experimental design is depicted in Table 2.

With this approach, we identified 157 proteins in all groups, which were reproducibly found in all three replicates of at least one experimental group, with high confidence [false discovery rate (FDR) < 0.01]. Fifty-one of these proteins were also found in the negative control and mock control groups and were excluded from further analysis. We considered the remaining 106 proteins as potential autoantigens (Supplementary table 1). To elucidate the natural autoantibody repertoire detectable with this method, the control experimental group, incubated with HTM cell lysates ('CH'), was evaluated with special interest. In this group, 66 antigens were identified (Table 3). The analysis of the intensity-based absolute quantification (iBAQ) values revealed that 12 antigens contribute to 67.3% of all identified proteins (Figure 1). The most abundant antigens were histone H4 with 14.4% and histone H2B type 1-L (9.7%), followed by the histone H4-binding partner protein SET with 8.2%. Other antigens among the top 10 most abundant were proteins from the tubulin beta chain (TBB6 6.5% and TBB4B 5.9%) and 40S ribosomal proteins (RS16 4.6%, RS2 3.3%, RS24 2.6%). Forty of the (auto)-antibody-captured proteins showed relative low abundance with $< 1\%$. A complete list with all iBAQ values can be found in Table 3.

Table 1. Characteristics of the study population

	POAG	CTRL
MS-AMIDA		
Samples (*n*)	30 (3 pools)	30 (3 pools)
Sex (m/f)	17/13	15/15
Mean age (±SD)	63.93 ± 9.13	64.5 ± 9.82
Eye surgery	None	None
Autoimmune disease	No reported	No reported
Other eye disease	Cataract (9 of 30)	Cataract (7 of 30)
Microarray validation		
Samples (*n*)	120	120
Sex (m/f)	65/55	65/55
Mean age (±SD)	67.25 ± 10.95	67.17 ± 11.89

Characterisation of natural autoantigens by GO enrichment and pathway analysis

Gene Ontology (GO) enrichment was analysed using DAVID (https://david.ncifcrf.gov/) to get an overview of the characteristics of the identified targets of the natural AAbs in the CH experiment group. Figure 2 shows significantly enriched GO terms among the autoantigens for biological process (A), cellular component (B) and molecular function (C).

The analysis showed that the antigens are profoundly involved in several processes associated with translation- and RNA-related processes. Furthermore, the detected immunogenic proteins are involved in virus-associated processes, as well as cell–cell adhesion. Highlighting the molecular functions of the autoantigens, nearly all of them showed the ability to bind poly (A) or other forms

Table 2. Experimental design for the initial MS-AMIDA profiling

ID	Sample name	IgG source	Protein source	Comment
NC1	Negative control 1	None	Healthy TM cells	Unspecific binding to beads
NC2	Negative control 2	None	Glaucomatous TM cells	Unspecific binding to beads
MA	Mock A	POAG	Lysis buffer	Mock control; 3 replicates
MB	Mock B	CTRL	Lysis buffer	Mock control; 3 replicates
PH	POAG/HTM	POAG	Healthy TM cells	3 replicates
PG	POAG/GTM	POAG	Glaucomatous TM cells	3 replicates
CH	CTRL/HTM	CTRL	Healthy TM cells	3 replicates
CG	CTRL/GTM	CTRL	Glaucomatous TM cells	3 replicates

Table 3. Autoantigens identified in HTM cells, captured by autoantibodies of CTRL sera (Group ID: 'CH')

No.	Entry name (*_HUMAN)	UniProt accession	Protein name	Sequence coverage (%)	No. of peptides	No. of unique peptides	iBAQ (%)
1	FLNA	P21333	Filamin-A	11	20	16	1.24
2	TBB6	Q9BUF5	Tubulin beta-6 chain	46.6	15	6	6.51
3	SYEP	P07814	Bifunctional glutamate/proline-tRNA ligase	11	12	12	1.15
4	SET	Q01105	Protein SET	19.3	3	3	8.23
5	TBB4B	P68371	Tubulin beta-4B chain	51.7	17	1	5.88
6	SYIC	P41252	Isoleucine-tRNA ligase, cytoplasmic	12.1	12	12	0.99
7	FAS	P49327	Fatty acid synthase	4.8	7	7	0.44
8	TERA	P55072	Transitional endoplasmic reticulum ATPase	12.8	6	6	1.14
9	H4	P62805	Histone H4	35.9	4	4	14.39
10	XRCC5	P13010	X-ray repair cross-complementing protein 5	23.8	8	8	2.28
11	RS2	P15880	40S ribosomal protein S2	18.8	4	4	3.09
12	H2B1L	Q99880	Histone H2B type 1-L	19	2	2	9.69
13	HNRPF	P52597	Heterogeneous nuclear ribonucleoprotein F	11.3	3	2	4.65
14	RS16	P62249	40S ribosomal protein S16	14.4	2	2	4.6
15	SYTC	P26639	Threonine-tRNA ligase, cytoplasmic	11.1	7	7	1.77
16	PLPHP	O94903	Pyridoxal phosphate homeostasis protein	17.5	3	3	1.81
17	HS90B	P08238	Heat shock protein HSP 90-beta	20.9	10	7	1.41
18	ATPA	P25705	ATP synthase subunit alpha, mitochondrial	15.9	5	5	1.56
19	SERPH	P50454	Serpin H1	14.1	4	4	1.6
20	NPM	P06748	Nucleophosmin	12.6	3	3	3.29
21	MCM7	P33993	DNA replication licensing factor MCM7	7.4	4	4	0.55
22	CCD47	Q96A33	Coiled-coil domain-containing protein 47	14.3	4	4	2.03
23	ADT2	P05141	ADP/ATP translocase 2	14.4	4	2	1.69
24	SYRC	P54136	Arginine-tRNA ligase, cytoplasmic	17.7	10	10	0.74
25	IF4A1	P60842	Eukaryotic initiation factor 4A-I	27.8	6	6	1.69
26	IQGA1	P46940	Ras GTPase-activating-like protein IQGAP1	2.8	2	2	0.1
27	HNRPK	P61978	Heterogeneous nuclear ribonucleoprotein K	7.8	3	3	1.01
28	EIF3L	Q9Y262	Eukaryotic translation initiation factor 3 subunit L	3	1	1	0.54
29	SYK	Q15046	Lysine-tRNA ligase	8.9	4	4	0.5
30	SYDC	P14868	Aspartate-tRNA ligase, cytoplasmic	7.8	4	4	0.66
31	SRP68	Q9UHB9	Signal recognition particle subunit SRP68	12.1	5	5	0.51
32	TBA1A	Q71U36	Tubulin alpha-1A chain	46.1	17	1	1.07
33	RS24	P62847	40S ribosomal protein S24	9	1	1	2.63
34	RL15	P61313	60S ribosomal protein L15	5.9	1	1	1.3
35	EIF3E	P60228	Eukaryotic translation initiation factor 3 subunit E	2.9	1	1	0.45
36	EIF3F	O00303	Eukaryotic translation initiation factor 3 subunit F	5.3	1	1	0.76
37	SYQ	P47897	Glutamine-tRNA ligase	9	5	5	0.36
38	TCPZ	P40227	T-complex protein 1 subunit zeta	7.2	2	2	0.47
39	MYO1C	O00159	Unconventional myosin-Ic	1.2	1	1	0.05
40	EIF3A	Q14152	Eukaryotic translation initiation factor 3 subunit A	3.5	4	4	0.16
41	DEST	P60981	Destrin	10.3	1	1	1.63
42	EIF3B	P55884	Eukaryotic translation initiation factor 3 subunit B	3.4	1	1	0.26
43	SYMC	P56192	Methionine-tRNA ligase, cytoplasmic	1.2	1	1	0.33
44	NSUN2	Q08J23	tRNA (cytosine(34)-C(5))-methyltransferase	3.9	2	2	0.43
45	COPG1	Q9Y678	Coatomer subunit gamma-1	4.9	2	2	0.2
46	MCM3	P25205	DNA replication licensing factor MCM3	2.7	2	2	0.13
47	PSMD2	Q13200	26S proteasome non-ATPase regulatory subunit 2	4.5	2	2	0.12
48	OLA1	Q9NTK5	Obg-like ATPase 1	3.8	1	1	0.19
49	RS5	P46782	40S ribosomal protein S5	13.7	1	1	0.9
50	ACTN1	P12814	Alpha-actinin-1	3.8	3	3	0.27
51	PYRG1	P17812	CTP synthase 1	2.7	1	1	0.19
52	PPIL4	Q8WUA2	Peptidyl-prolyl cis-trans isomerase-like 4	8.7	2	2	0.34
53	NU160	Q12769	Nuclear pore complex protein Nup160	1.3	1	1	0.06
54	P5CS	P54886	Delta-1-pyrroline-5-carboxylate synthase	4.5	2	2	0.14

(Continued)

Table 3. Continued.

No.	Entry name (*_HUMAN)	UniProt accession	Protein name	Sequence coverage (%)	No. of peptides	No. of unique peptides	iBAQ (%)
55	TKT	P29401	Transketolase	6.9	2	2	0.21
56	DCTN1	Q14203	Dynactin subunit 1	3.7	3	3	0.08
57	CLH1	Q00610	Clathrin heavy chain 1	0.5	1	1	0.07
58	PRP8	Q6P2Q9	Pre-mRNA-processing-splicing factor 8	0.9	1	1	0.03
59	DX39A	O00148	ATP-dependent RNA helicase DDX39A	4.9	2	2	0.31
60	TIM50	Q3ZCQ8	Mitochondrial import inner membrane translocase subunit TIM50	2.8	1	1	0.4
61	CPSF7	Q8N684	Cleavage and polyadenylation specificity factor subunit 7	5.3	1	1	0.25
62	SPT5H	O00267	Transcription elongation factor SPT5	1.7	1	1	0.07
63	IMB1	Q14974	Importin subunit beta-1	1	1	1	0.06
64	RT35	P82673	28S ribosomal protein S35, mitochondrial	3.1	1	1	0.17
65	TGFI1	O43294	Transforming growth factor beta-1-induced transcript 1 protein	4.3	1	1	0.11
66	DDX46	Q7L014	Probable ATP-dependent RNA helicase DDX46	1	1	1	0.05

Proteins were reproducibly identified in all three replicates. MaxQuant iBAQ values were used to calculate protein abundance.

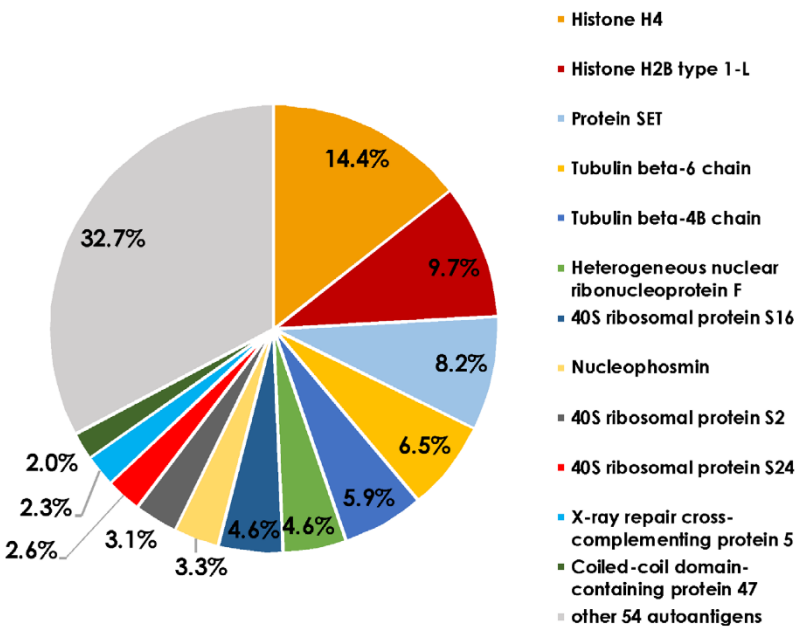

Figure 1. iBAQ values of captured autoantigens using CTRL serum and HTM cells. The iBAQ values are proportional to the molar quantity of the respective antigen measured by mass spectrometry. These proportions represent the abundance of the associated autoantibodies in natural autoimmunity. The diagram shows the distribution of different autoantigens from healthy TM cells captured by antibodies in the control sera (n = 3). The percentage of total peptide intensities is shown.

of RNA. Also, the binding of cadherin in cell–cell adhesion represented a frequent function among the autoantigens captured by antibodies from the control sera. We also found marked enrichment of the immunogenic proteins in the cellular components membrane, cytosol and extracellular exosomes.

Identification of POAG-related autoantigens

The involvement of immunological processes has been frequently shown for several neurodegenerative diseases and for the ocular neuropathy, POAG. To identify antigens that are related to POAG, we analysed autoantibody

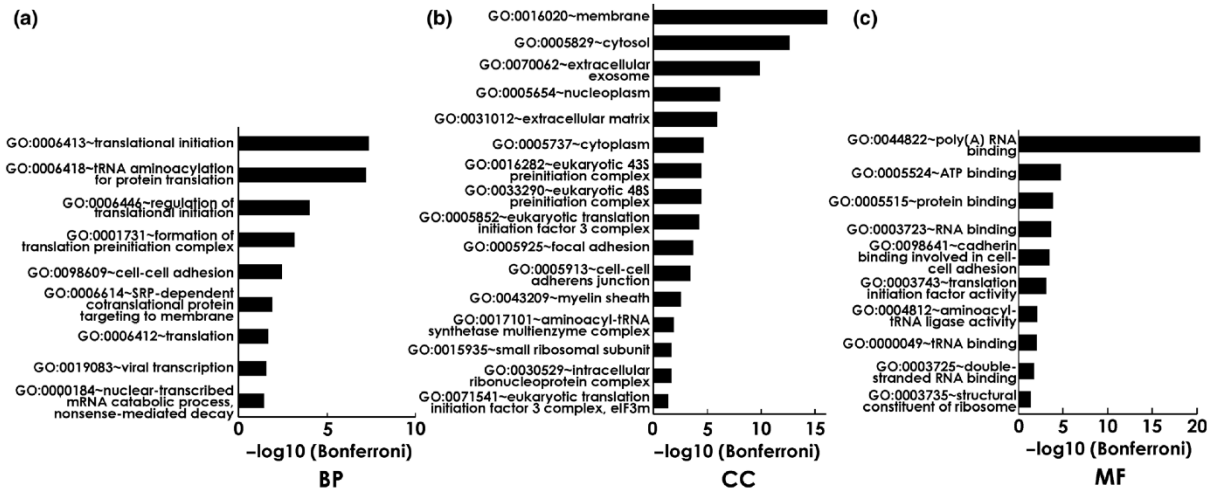

Figure 2. GO enrichment analysis of autoantigens from HTM captured by autoantibodies in CTRL sera (n = 3), representing natural autoimmunity. Negative $\log_{10}$-transformed P-values of significantly enriched GO terms ($P < 0.05$ with Bonferroni correction) are shown in **(a)** biological process (BP), **(b)** cellular component (CC) and **(c)** molecular function (MF).

abundance in the sera of POAG patients and a non-glaucomatous control group (Table 1). To this end, the proteins identified by MS-AMIDA were quantified using MaxQuant label-free quantification (LFQ) intensities. The experimental groups CH and PH (Figure 3a) and also CG and PG (Figure 3b) were analysed for significant differences in autoantibody levels. These were tested using Student's *t*-test with permutation-based FDR (< 0.01) to adjust for multiple testing. The test, however, revealed no significant alterations. Principal component analysis revealed that there is a higher association of the autoantibody levels with the TM cell line used than with the antibody source (Figure 3c). Component 1 and Component 2 explain 68.11% of the variance in the data (see also scree plot in Supplementary figure 1). The influence of single autoantibody levels on the components can be evaluated by observing the component coefficients in Supplementary table 2. Component 1 is mainly influenced by EIF5A1, H4, C1QBP and PNMA2, whereas Component 2 is mainly influenced by NUCL, NSUN2, HS90B and ACTN1. Hierarchical clustering of these antigens based on Euclidean distances (Figure 4a) supports the findings from the principal component analysis (Figure 3c). The experimental groups cluster together based on the specific TM cells, rather than the serum source. The antigens showed two clusters with 11 antigens having lower levels in the glaucomatous TM cells and 27 with increased levels. Therefore, we also examined the differences in the captured autoantigens between GTM and HTM cells. Here, 38 (auto)-antibody-captured proteins were found at significantly different levels (Supplementary table 3). We wanted to use a larger sample size to analyse whether different autoantibody serum levels caused these observed changes. To select suitable candidates for further validation, antigens were not only analysed for significant hits but also their fold change (Figure 4b). Only antigens with an FDR < 0.01 and a $\log_2$ fold change of > 2 were considered eligible. A total of 21 autoantigens passed these criteria (Table 4). From the subset of the 21 eligible autoantigens, nine targets were chosen for further validation by a protein microarray analysis.

Characterisation of POAG-related autoantigens

To investigate in which biological processes these possibly POAG-related autoantigens are involved, we used DAVID's functional annotation clusters and also Metascape enrichment and pathway analysis. DAVID identified six functional clusters among 19 of the 21 targets analysed using GO terms, UniProt (UP) keywords and UP sequence features (seq feature) (Figure 5). These clusters comprise cell–cell adhesion and junctions (cluster 1), nucleotide and ATP-binding (cluster 2), cytoplasmic proteins with different isoforms

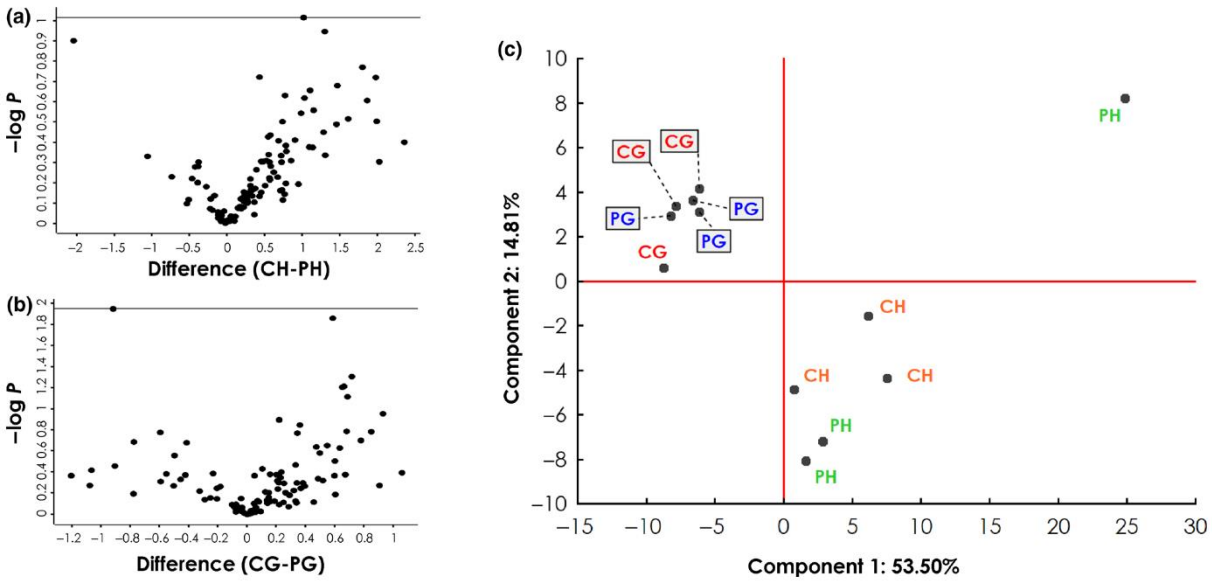

Figure 3. Analysis of autoantigen abundance in different experiment groups (IDs: PH, CH, PG, CG). Detailed information about the experimental groups can be found in Table 2. **(a, b)** Volcano plots showing negative $\log_{10}$-transformed *P*-values (*t*-test with permutation-based FDR to correct for multiple testing; *y*-axis) against the differences of the means ($\log_2$; *x*-axis) of two groups. **(a)** Comparison of antigens from healthy TM cell lysates captured by antibodies from control (CH; $n = 3$) and POAG sera (PH; $n = 3$). **(b)** Comparison of antigens from glaucomatous TM cell lysates captured by antibodies from control (CG; $n = 3$) and POAG sera (PG; $n = 3$). **(c)** Principal component analysis, including all four experiment groups, reveals that most of the variance between the groups can be attributed to the cell line used rather than the antibody source.

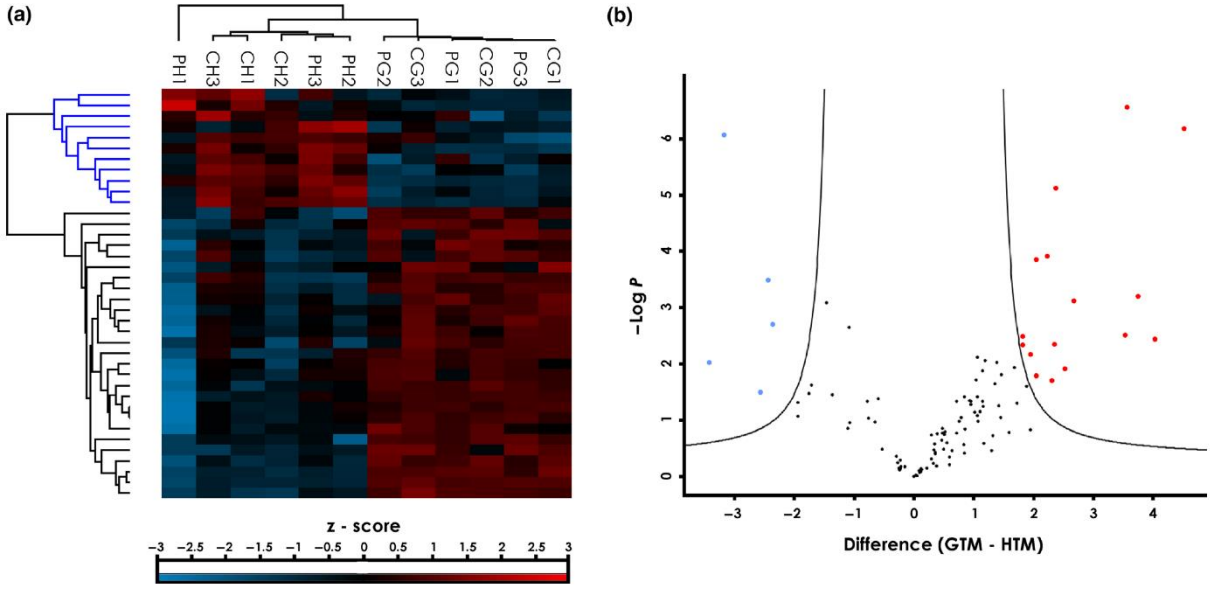

Figure 4. Statistical analysis of captured autoantigens from HTM and GTM cells. **(a)** Hierarchical clustering of antigens in single samples with significantly different abundance between the cell lines HTM and GTM (38 proteins; *t*-test with permutation-based FDR < 0.01). Clustering is based on *z*-score-transformed LFQ intensities. **(b)** Volcano plot for candidate selection. Highlighted dots represent proteins with significant differences between cell lines and a $\log_2$ fold change of 2 or more, to ensure relevant effect size. Blue dots represent antigens with lower levels and red dots antigens with higher levels in the GTM samples than HTM samples.

Table 4. Candidate biomarkers with *P*-value < 0.05 and $\log_2$ fold change of > 2 ($\log_2$ differences of the means)

UniProt accession	Entry name	Gene	Protein	−Log(*P*-value)	Difference (GTM – HTM)
P62805	H4_HUMAN	HIST1H4A	Histone H4	2.028	−3.428
Q08J23	NSUN2_HUMAN	NSUN2	tRNA (cytosine(34)-C(5))-methyltransferase	6.063	−3.172
P13010	XRCC5_HUMAN	XRCC5	X-ray repair cross-complementing protein 5	1.496	−2.564
P12814	ACTN1_HUMAN	ACTN1	Alpha-actinin-1	3.484	−2.437
P26639	SYTC_HUMAN	TARS	Threonine-tRNA ligase, cytoplasmic	2.706	−2.362
Q6UVK1	CSPG4_HUMAN	CSPG4	Chondroitin sulphate proteoglycan 4	2.490	1.816
Q02878	RL6_HUMAN	RPL6	60S ribosomal protein L6	2.340	1.819
Q14566	MCM6_HUMAN	MCM6	DNA replication licensing factor MCM6	2.164	1.950
Q96AB3	ISOC2_HUMAN	ISOC2	Isochorismatase domain-containing protein 2	3.856	2.043
Q9NTK5	OLA1_HUMAN	OLA1	Obg-like ATPase 1	1.786	2.053
Q00005	2ABB_HUMAN	PPP2R2B	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	3.909	2.226
P10809	CH60_HUMAN	HSPD1	60 kDa heat shock protein, mitochondrial	1.705	2.305
P42704	LPPRC_HUMAN	LRPPRC	Leucine-rich PPR motif-containing protein, mitochondrial	2.342	2.350
O00159	MYO1C_HUMAN	MYO1C	Unconventional myosin-Ic	5.121	2.368
P63104	1433Z_HUMAN	YWHAZ	14-3-3 protein zeta/delta	1.907	2.527
P46940	IQGA1_HUMAN	IQGAP1	Ras GTPase-activating-like protein IQGAP1	3.117	2.680
Q07021	C1QBP_HUMAN	C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial	2.513	3.536
Q9ULC4	MCTS1_HUMAN	MCTS1	Malignant T-cell-amplified sequence 1	6.556	3.560
P27708	PYR1_HUMAN	CAD	CAD protein	3.204	3.754
P63241	IF5A1_HUMAN	EIF5A	Eukaryotic translation initiation factor 5A-1	2.437	4.028
Q9UL42	PNMA2_HUMAN	PNMA2	Paraneoplastic antigen Ma2	6.185	4.521

(cluster 3), mitochondria-associated proteins (cluster 4), DNA-binding proteins (cluster 5) and transcription (cluster 6). The analysis with Metascape revealed eight enriched GO and Kyoto Encyclopaedia of Genes and Genomes (KEGG) terms (Figure 6a and b). The target antigens were enriched in translation, mRNA transport, cell junction assembly, positive regulation of apoptotic process and establishment of protein localisation to membrane, as well as viral carcinogenesis pathways, G2/M checkpoints and the platelet-derived growth factor receptor beta (PDGFRB) pathway. The protein–protein interaction (PPI) network shows only antigens that are known to interact with at least one other of the identified targets (Figure 6c). This analysis showed that 15 of the 21 targets are connected in the PPI network. Of these, the Metascape molecular complex detection (MCODE) algorithm identified one densely connected network component, consisting of the proteins CAD, HSPD1, YWHAZ, OLA1 and EIF5A. The independent enrichment analysis for this MCODE component revealed an enrichment of the five antigens in translation, peptide biosynthetic process and the PDGFRB pathway (Figure 6d).

Validation of selected targets by protein microarray

We chose alpha-actinin-1 (ACTN1), serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform (PPP2R2B), mitochondrial complement component 1 Q subcomponent-binding protein (C1QBP), malignant T-cell-amplified sequence 1 (MCTS1), paraneoplastic antigen Ma2 (PNMA2), Ras GTPase-activating-like protein IQGAP1 (IQGAP1), unconventional myosin-1c (MYO1C) and cytoplasmic threonine-tRNA ligase (TARS) to be analysed by protein microarray. Even though it is not a new target, heat shock protein 60 (HSP60) has been included as a well-defined glaucoma-related antigen to serve as a positive control. The targets were obtained as recombinant proteins and were used to prepare customised microarray slides in our laboratory. Slides were hybridised with sera from 120 POAG patients and 120 non-glaucomatous controls (Table 1, microarray validation). With this method, the samples were analysed for their antigen-autoantibody reactions. Incubation with a fluorescent dye-labelled anti-human IgG antibody yielded signal intensities that

Figure 5. DAVID functional annotation clusters of potentially POAG-related autoantigens. Nineteen out of the 21 included proteins showed an enrichment in at least one cluster. Enrichment scores: cluster 1 (2.54), cluster 2 (1.7), cluster 3 (1.59), cluster 4 (1.32), cluster 5 (1.02) and cluster 6 (0.39). The analysis includes Gene Ontology terms (GO terms), UniProt keywords (UP keywords) and UniProt sequence features (UP seq feature). Green indicates enrichment in the cluster for the respective protein.

were proportional to the concentration of the respective autoantibody. After data pre-processing and normalisation, statistical tests and analysis were conducted. The results of the Mann–Whitney *U*-test are listed in Table 5. This approach identified significant increased autoantibody levels to PNMA2, TARS, C1QBP and HSPD1 (Figure 7). Signals representing the binding of ACTN1, IQGAP1 and MYO1C AAbs were at the detection limit of this method and therefore could not be quantitatively evaluated.

Correlation with disease-related clinical parameters

We wanted to examine further whether the detected increase in PNMA2, TARS, C1QBP and HSPD1 AAbs showed any relationship to clinical hallmarks of POAG pathogenesis. Calculation of Spearman's rank-order correlation coefficients was used to detect correlations of the AAbs with cup disc ratio (CDR), visual field defect ('mean deviation'; MD) and IOP (Table 6). The correlation coefficients reveal a weak negative correlation of PNMA2 and HSPD1 AAbs with CDR ($R = -0.29$ and $R = -0.28$), a moderate negative correlation of HSPD1 AAbs with visual field defect ($R = -0.42$) and a weak positive correlation between TARS AAbs and visual field defect ($R = 0.34$). None of the AAbs showed a relation to IOP. To investigate whether these AAbs show significant alterations in other systemic or eye diseases, we divided the control group into the categories, other eye disease ($n = 39$), systemic disease ($n = 18$), other eye + systemic disease ($n = 40$) and healthy subjects ($n = 23$). Kruskal–Wallis ANOVA for multiple group comparison revealed no significant difference ($P > 0.05$) for PNMA2, TARS, C1QBP and HSPD1 AAbs among these groups.

Evaluation of diagnostic potential

The establishment of reliable biomarkers for the objective diagnosis of POAG is still a crucial task in ophthalmic research. Therefore, we wanted to evaluate whether validated glaucoma-related AAbs to PNMA2, TARS, C1QBP and HSPD1 hold the potential to serve as biomarker candidates.

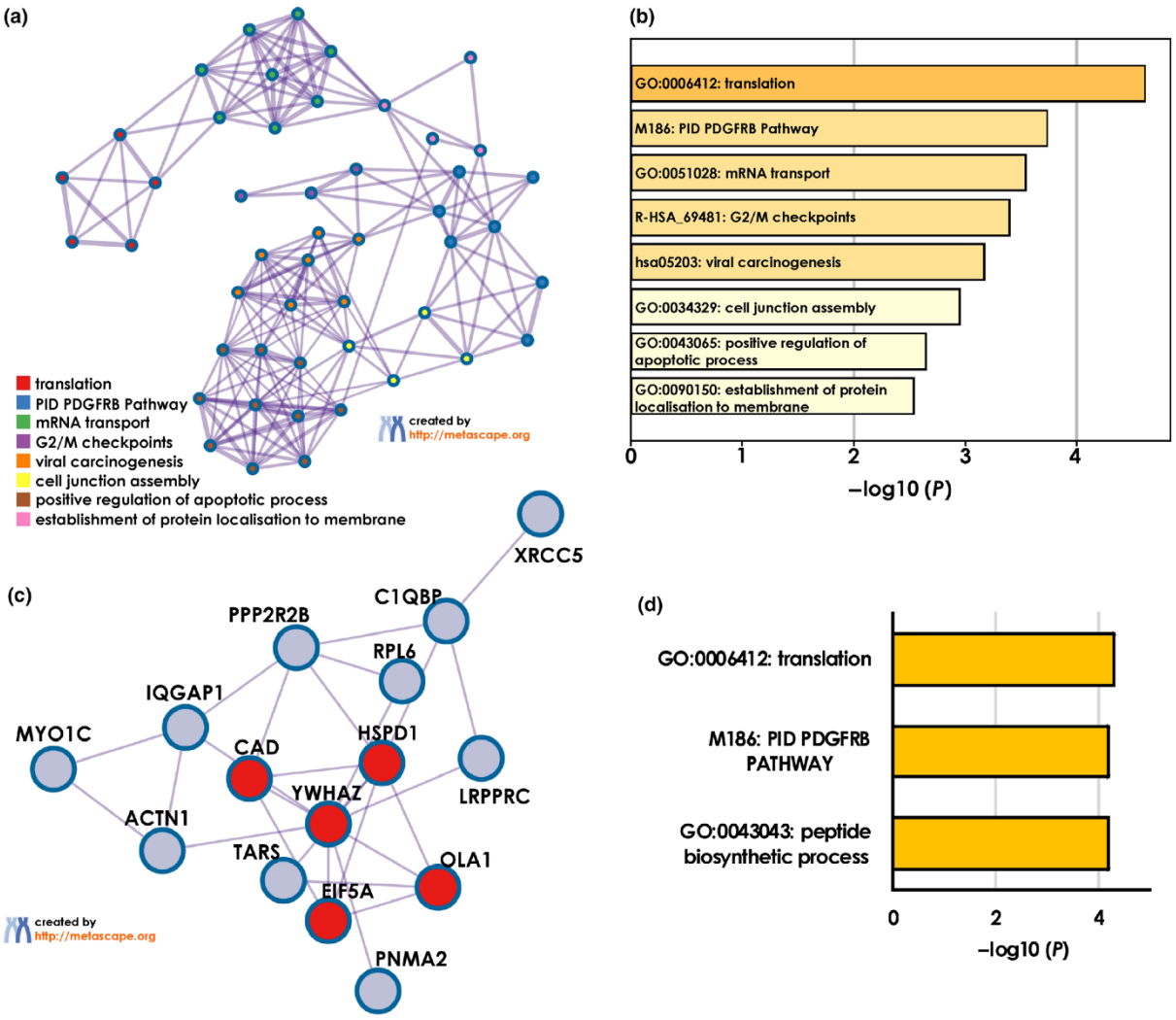

Figure 6. Metascape analysis of POAG-related autoantigens. **(a)** Network of enriched GO and KEGG terms coloured by cluster. **(b)** Enriched GO and KEGG terms from **(a)** with corresponding *P*-values. **(c)** Protein–protein interaction network with densely connected components (red) identified by the MCODE algorithm. **(d)** Enrichment analysis for MCODE cluster only.

Table 5. Results of microarray validation

	Rank Sum POAG	Rank Sum CTRL	*U*	*Z*	*P*-value	*Z* adjusted	*P*-value
PPP2R2B AAbs	14 078	14 842	6818	−0.710	0.477	−0.712	0.477
TARS Aabs	16 741	12 179	4919	4.242	0.000	4.258	< 0.001
C1QBP Aabs	15 714	13 206	5946	2.332	0.020	2.333	0.020
PNMA2 Aabs	16 032	12 888	5628	2.923	0.003	2.930	0.003
MCTS1 Aabs	15 015	13 905	6645	1.032	0.302	1.034	0.301
HSPD1 Aabs	16 793	12 127	4867	4.338	0.000	4.350	< 0.001

Comparison of autoantibody levels in POAG and CTRL samples (n = 120) assessed by a two-sided Mann–Whitney *U*-test.

For this purpose, we applied a random forest algorithm to the microarray data set to seek a potential classification of POAG patients and non-glaucomatous controls. The data set was randomly divided into a training (n = 165) and a test set (n = 75). The resulting random forest model was

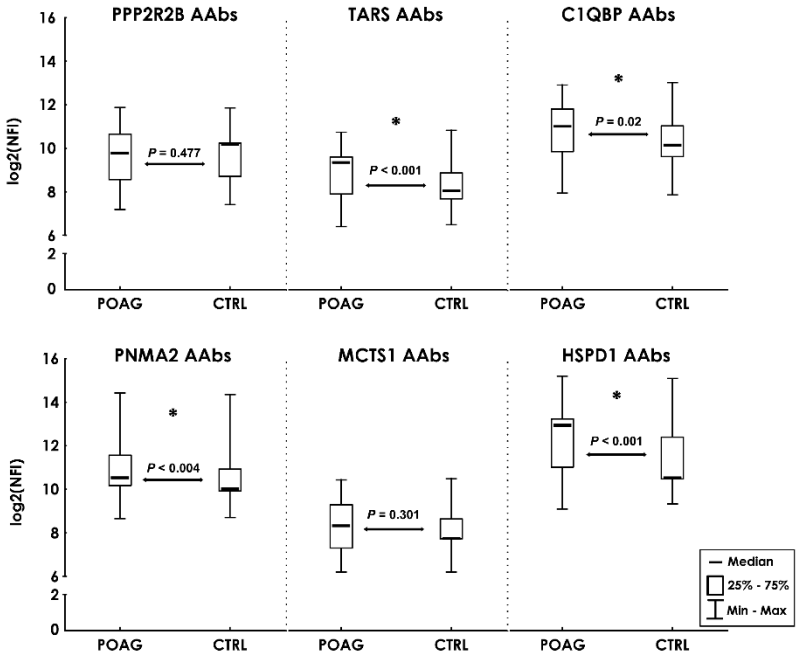

Figure 7. Results of the microarray validation. Comparison of autoantibody levels in POAG (n = 120) and CTRL (n = 120) serum. Median log_2-transformed normalised fluorescence intensities. Median intensities with 25–75% percentiles (box) and min/max values (whiskers). P-values from a Mann–Whitney U-test. Asterisks mark significant differences at $P < 0.05$.

Table 6. Spearman rank correlations of autoantibodies in POAG patients and glaucoma-related clinical parameters: cup disc ratio (CDR); visual field defects ('mean deviation', MD); intraocular pressure (IOP). Spearman correlation coefficients and corresponding P-values.

Clinical parameter	Autoantibody	Valid N	Spearman R	t (N-2)	P-value
CDR	C1QBP	82	0.082	0.732	0.466
	PNMA2	82	−0.286	−2.673	0.009
	TARS	82	0.098	0.884	0.379
	HSPD1	82	−0.284	−2.648	0.010
MD	C1QBP	55	0.113	0.826	0.413
	PNMA2	55	−0.155	−1.140	0.260
	TARS	55	0.335	2.586	0.012
	HSPD1	55	−0.421	−3.376	0.001
IOP	C1QBP	114	−0.120	−1.279	0.203
	PNMA2	114	0.091	0.964	0.337
	TARS	114	0.085	0.905	0.367
	HSPD1	114	0.037	0.396	0.693

able to correctly classify 30 out of 38 POAG patients and 33 out of 37 controls (Figure 8). This translates to a sensitivity of 79% at 89% specificity with an overall accuracy of 84%.

DISCUSSION

Antibodies to self-antigens are mostly associated with immune disorders such as systemic lupus erythematosus[28,29] or Hashimoto's disease,[30] but are also frequently found in conditions that are not considered autoimmune. Antibodies to tumor antigens are present in the blood of cancer patients,[31] and AAbs are even found in neurological disorders such as Alzheimer's[32] or Parkinson's disease.[33] In recent years, evidence has increased that AAbs are not only involved in pathology, but are also a part of physiologic processes. An important goal for the better understanding of the role of immunological

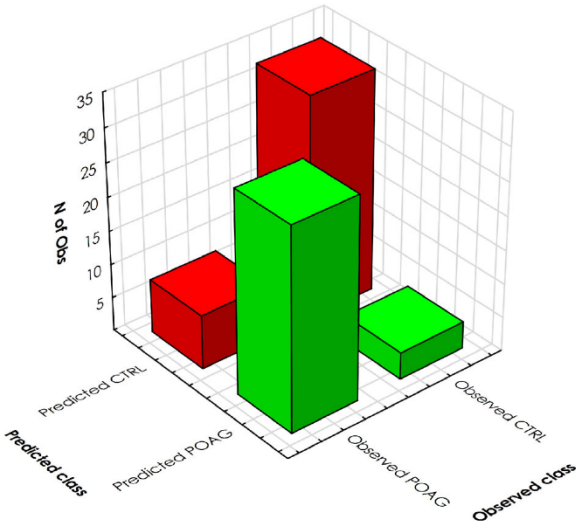

Figure 8. Random forest classification. Prediction is plotted using test samples not used for training (n = 75) only. Bars represent the number of predicted (POAG in green; CTRL in red) and observed cases. The random forest algorithm classified POAG samples and CTRL samples with a sensitivity of 79%, a specificity of 89% and 84% accuracy.

processes in health and disease is to link the natural AAbs to specific biological processes and signalling pathways. Also, the formation of autoantigens in relation to diseases has not been elucidated conclusively. So far, several causes for proteins to become immunogenic have been discussed. These comprise the hypothesis of molecular mimicry,[34] but alterations in post-translational modification, protein overexpression, alternative splicing and the generation of neo-epitopes in the course of apoptosis have also been proposed.[35–38] These proposals also infer that differences in circulating autoantibody abundances might reflect molecular changes. However, most identified autoantigens have not been studied for the underlying reason for their immunogenicity. Although the possible involvement of the immune system in glaucoma pathogenesis has been investigated in many studies, the qualitative characterisation of the involved antigens and AAbs is incomplete. The specific conditions under which an antigen can lead to a modified autoimmunity are also unclear. There is a further need to understand the nature and function of the natural autoantibody repertoire as a whole. That will also significantly help to understand the changes that occur under different disease states. In this study, we investigated the autoantibody repertoire against TM proteins as part of natural autoimmunity as well as POAG-related alterations. This exploratory study also aims to stimulate the formation of hypotheses that would help to explain the properties of the natural immunome and the implications of autoimmunity in glaucoma.

We analysed autoantigens in healthy and glaucomatous TM cells that were captured by serological antibodies of POAG and non-glaucomatous controls using MS-AMIDA. At first, we wanted to identify targets of the naturally occurring AAbs and link them to biological processes and signalling pathways. To this end, we performed GO enrichment analysis with the 66 proteins captured by antibodies isolated from non-glaucomatous sera. The GO analysis involved the evaluation of three different categories, 'biological process', 'molecular function' and 'cellular component'. This analysis showed that there is a strong association of the antigens targeted by naturally occurring AAbs with translational processes as part of protein biosynthesis. Interestingly, several enzymes of the family of tRNA ligases were identified as immunogenic proteins. Many proteins representing subunits of the aminoacyl-tRNA synthetase multienzyme complex were also enriched in different cellular components. However, the AAbs also directly targeted epitopes of the 40S ribosomal subunit. This was also reflected by the strong enrichment of RNA-binding properties in the identified antigens.

Furthermore, the antigens were highly enriched in the cellular components, 'membrane' and 'cytosol'. They were, however, also located in 'extracellular exosomes' and the 'nucleoplasm'. That the autoantigens are also involved in intranuclear processes was also reflected by their enrichment in poly (A)-RNA-binding properties and nuclear-transcribed mRNA processing in the control mechanism of nonsense-mediated decay. Another study on the overall immunogenicity of autoantigens could also show a frequent enrichment of autoantigens with a function of RNA and protein binding and also a strong association with ribosomes and spliceosomes.[39] The abundance of antigens associated with viral transcription-related processes may be because the cell lines used in this experiment were transformed by simian virus 40[40] that could be responsible for changes in cellular transcription proteins.

The most abundant antigens identified were nuclear proteins. At 23.3%, histone proteins were the most common, followed by the histone-

binding protein SET. Anti-histone antibodies have been linked to the autoimmune disease systemic lupus erythematosus,[28] but our results indicate that they might also be involved in natural autoimmunity. AAbs to ribonucleoproteins are associated with a spectrum of rheumatic diseases.[41] It is presumed that RNA-binding proteins can activate B cells and thereby enable AAb production by interaction of the RNA with TLR-7 or TLR-8.[42] Since we have also found these antibodies in individuals who are not suffering from an autoimmune disease (according to anamnesis), there is also the probability that anti-ribonucleoprotein antibodies are also part of the natural autoantibody repertoire. This also suggests that other criteria would need to be met for these AAbs to become pathogenic.

The second goal of this study was to investigate possible alterations in the immunoproteome in POAG patients. To this end, we compared the binding of serological antibodies, purified from POAG patients and non-glaucomatous controls, to proteins derived from GTM and HTM cells. The autoantigens, identified and quantified by LFQ, showed more pronounced alterations in the groups with a different protein source (GTM vs HTM) than in the CTRL vs POAG sera groups. Antibody reactivity seems to be more influenced by possibly disease-related antigen properties than by mere abundance of the antibodies. This could indicate that not only the antibodyome is changed during disease, but that natural AAbs bind with higher affinity to antigens with disease-related alterations. Our data do not provide further insights into the nature of these alterations, but the occurrence of neo-epitopes, specific altered post-translational modifications or truncations are possible changes that could enhance the target binding of physiological AAbs. These explanations need to be evaluated through additional research, so the implications we can make from these results are merely theoretical. A hypothesis that needs further validation by subsequent experiments is that not only quantitative but also qualitative differences between natural and disease-related AAbs may play a role. Hints that support such assumptions come from other studies that show the disease-specific accumulation of distinct complementarity determining region peptides (CDR peptides) of the highly variable region of AAbs in glaucoma and other diseases.[43–45] From these findings, it seems likely that not only alterations of the antigen but also of the AAbs themselves can lead to the onset of autoimmune processes. This raises an interesting question for future studies that aim to analyse whether alterations of either antibody or target protein can influence the binding properties. Also, the possible ability of these alterations to promote undesirable effects that may accompany or even accelerate pathological processes needs to be investigated.

The PPI network and pathway enrichment analysis of the POAG-related autoantigens using Metascape revealed that especially those proteins involved in translation and the PDGFRB pathway show altered immunogenicity. Also, the five connected proteins, HSPD1, CAD, YWHAZ, EIF5A and OLA1, as identified by MCODE, are strongly enriched in these molecular processes. PDGF signalling has been linked to fibrotic diseases and is implicated in the proliferation of cells with myofibroblast-like properties.[46] TM cells also show similar characteristics to contractile myofibroblasts that express alpha-smooth muscle actin (a-SMA).[47] Fibrosis also involves an accumulation of ECM, which was also observed in the glaucomatous TM.[48] The hypothesis of an involvement of fibrotic processes in POAG-related modulations of TM that could be mediated by increased levels of transforming growth factor β2 (TGFβ2) in the aqueous humour has already been discussed.[49,50] The MCODE cluster of AAbs potentially reflects POAG-specific alterations of the PDGFRB pathway. However, a causative or synergetic effect of these AAbs in mediating autoimmune-driven fibrosis in glaucomatous TM cannot be excluded. Thus, an investigation of the effects of AAbs on TM cells seems to be required. In a previous study from our group, a retinal ganglion cell line was incubated with serum or serological antibodies alone, derived from POAG patients or non-glaucomatous controls.[51] In this experiment, no changes in cell viability were observed. However, a change in protein expression towards a pro-apoptotic state occurred when incubated with POAG serum or isolated POAG IgG, but not after incubation with control serum. We assume that the antibodies would exert similar effects on TM cells. Nonetheless, this would be an interesting subject for further studies, especially as the interaction of autoantibodies with targets in the PDGFR pathway of TM cells could give new insights into their role in glaucoma pathogenesis.

In other studies, which examined autoantibodies in glaucoma, but also in other neurodegenerative

diseases such as Alzheimer's or Parkinson's disease, a disease-specific change in the levels of various serum autoantibodies could be shown. We presume that an altered interplay of antigens and autoantibodies underlies both qualitative and quantitative changes that must be taken into account. Therefore, we wanted to analyse whether the antibody levels to the antigens identified here also show a change in abundance that can be detected by a more sensitive method. To analyse the relative abundance of the serological AAbs to selected targets in glaucomatous and non-glaucomatous subjects, we used a protein microarray approach as a high-throughput method. Even though specific attributes of the AAbs and their respective antigens seem to play a significant role in the characteristic of their interaction, many previous studies have also shown that disease-related alterations of natural autoimmunity can also be related to the levels of the serological AAbs. The evaluation of these quantitative differences is especially valuable in disease diagnostics, as they are easier to measure in clinical tests. We used recombinant human proteins, produced with a wheat germ expression system, generating proteins without post-translational modifications, to achieve a standardised experiment set-up. The analysis of sera from 120 POAG and 120 non-glaucomatous subjects revealed an increase of anti-C1QBP, anti-TARS, anti-PNMA2 and anti-HSPD1 AAbs in the POAG group. While antibodies to HSPD1 (mitochondrial 60 kDa heat shock protein) have frequently been observed in association with glaucoma,[18,52–54] AAbs to C1QBP, TARS and PNMA2 have not yet been described in the disease context.

Component 1 Q subcomponent-binding protein (C1QBP) is a multifunctional protein and putative receptor for component 1Q. This function leads to the inhibition of the C1 complex and thereby to suppression of complement activation. It is also involved in the regulation of mRNA splicing via its RNA-binding capacity. Our results showed upregulated levels of anti-C1QBP antibodies in the serum of POAG patients. Although AAbs to C1QBP have not yet been described in glaucoma, activation of the complement system has been discovered in the retinal ganglion cells of glaucomatous eyes.[55] C1QBP AAbs could presumably prevent the inhibition of C1 complex formation by blocking the C1QBP effector sites.

Our study also showed increased levels of autoantibodies to threonyl-tRNA synthetase (TARS). TARS is an enzyme functioning as the ligase of threonine to its respective tRNA. Neither TARS AAbs nor the antigen itself has been investigated in the context of glaucoma. It cannot be clearly stated what causes the formation of these AAbs and whether they are involved in disease-related tissue damage. Antibodies to different other tRNA synthetases are often found in patients with inflammatory myositis, especially Jo-1 AAbs.[56] Although these myositis-specific AAbs are far better characterised, researchers did not propose the reason for their occurrence, or the implications for the disease.[57] The characterisation of the origin and the properties of the TARS AAbs remains a task for further studies.

The paraneoplastic antigen 2 (PNMA2) is known to be linked to paraneoplastic disorders and is also related to neurodegenerative processes. AAbs are frequently found in cancer patients, as tumor cells are suspected of producing abnormal amounts of different members of the PNMA family.[58] These proteins are also naturally expressed by neuronal cells. The presence of this protein in TM cells is conceivable, since this tissue is likely to have originated from the neural crest.[59] Also, other PNMA proteins could be identified in porcine TM.[60] It is assumed that AAbs to PNMA2 are produced as a consequence of protein overexpression in cancer cells. But it cannot be excluded that other properties of the antigen are responsible for its immunogenicity as well. The PNMA2 protein seems to be prone to an autoimmune reaction, which could also be relevant to other neurodegenerative diseases such as glaucoma. However, the AAbs do not seem to drive the progression of paraneoplastic neurologic disease, since their depletion did not result in relief of the symptoms, nor can the injection of the AAbs induce the disease.[61] It could be an interesting topic for further studies to investigate the specific role of PNMA family members in the pathogenesis of glaucoma.

The identified AAbs seem to be associated with POAG but are mostly not exclusive to glaucoma. This is also true for several glaucoma-related AAbs previously identified. For example, antibodies to HSP60, vimentin and annexin A5 have not only been reported in association with glaucoma,[18,23,25,52,54] but are also frequently found in the sera of cancer patients.[62,63] Also, the antigens are not TM-specific. Therefore, they might not be specific enough to serve as

biomarker candidates. Although the application of random forest algorithms in this study enabled classification of POAG patients with a sensitivity of 79% at 89% specificity, a translation of these finding to be used in diagnostics might not be feasible and therefore needs additional evaluation. As observed with other autoantibody disease markers, variability in AAb levels in the population can be very high. Biomarkers often lack specificity since the putative disease markers are also widespread among subjects without the respective disease.[64] The establishment of reliable biomarkers requires further knowledge about their roles in glaucoma and their distribution in the general population.

If the occurring autoantibodies are an epiphenomenon of the pathological changes in the TM, alterations in the involved proteins would necessarily occur beforehand. It has been shown that pathways, especially those regulating extracellular matrix deposition, the actin cytoskeleton and cell–cell/cell–matrix connections, are altered in glaucomatous TM. Several factors are thought to play a role in these changes, including increased expression of TGFβ. It seems quite possible that a single occurrence of elevated IOP could lead to proteomic alterations and consequently altered autoantibody profiles. In a previous study, our group was able to highlight changes in autoantibody levels after acute angle-closure glaucoma attack, an event accompanied by a sudden increase of IOP and probable substantial damage to the optic nerve, in patients without previous glaucoma history.[65]

Although our study provides new insights into the properties and POAG-related changes of the natural autoantibody repertoire, some limitations shall be pointed out. The proteomic analysis involved in the MS-AMIDA approach requires a large amount of protein sample material. The availability of human TM tissue samples is limited, and primary cell cultures are also restricted in the amount of material they can yield. For the purpose of our explorative study, we used the most practical TM cell lines. These, however, have the disadvantage of being transformed by simian virus 40 that alters the cellular transcription machinery. Also, the donors of the TM cells were not the same age (GTM cells from a 74-year-old POAG patient and HTM cells from an 18-year-old donor). This makes further evaluation of our findings using more appropriate TM protein sources mandatory.

CONCLUSION

Overall, we could provide new insights into the natural AAb repertoire and highlight pathways which appear to harbour immunogenic proteins. PPI and pathway analysis of POAG-related autoantigens showed a strong association with the PDGFRB pathway, among others. This pathway is assumed to play an essential role in TM fibrosis, and the related AAbs could play an active role in the pathology or not. Lastly, we could confirm the presence of increased levels of AAbs against PNMA2, TARS and C1QBP in the serum of POAG patients that deserve to be further analysed as potential glaucoma biomarkers.

METHODS

Sera

The blood samples in this study were collected in accordance with the Declaration of Helsinki on biomedical research involving human subjects. Written informed consent was obtained from each subject. The ethics committee of the Landesärztekammer Rheinland-Pfalz approved the usage of the samples (Vote: 827.228.11 (7770)). All subjects included in this study received an ophthalmic examination at the Department of Ophthalmology of the University Medical Center in Mainz, Germany. POAG patients were diagnosed according to the guidelines of the European Glaucoma Society,[66] based on elevated IOP, visual field defects and optic nerve cupping. The control group consists of age- and sex-matched non-glaucomatous subjects. In the discovery phase using MS-AMIDA, 30 serum samples of POAG patients and 30 non-glaucomatous controls were analysed. Subjects with other eye diseases (except cataract), known autoimmune diseases (as evaluated from anamnesis) and previous eye surgeries were excluded to reduce the possible effects of other conditions on the serum antibody levels. With these exclusion criteria, we wanted to exclude bias from immunologic reactions that are not glaucoma-related or might be a cause of the mechanical disruption of the ocular tissue caused by surgical intervention. This might lead to an exchange of intracellular proteins with blood or aqueous humour that otherwise would not be in contact under physiological or disease-related conditions and could influence the conclusions drawn from the experiments. In the large-scale microarray validation, 120 POAG samples were compared to 120 non-glaucomatous, age- and sex-matched control samples. Here, the exclusion criteria were abandoned, except for other glaucoma types in the control group. The characteristics of the study population can be found in Table 1.

IgG isolation

Mass spectrometry-based antibody-mediated identification of autoantigens was conducted using 30 POAG and 30

control serum samples (Table 2). Samples were pooled, creating three sample pools (10 samples/pool) for each group. IgG was isolated from 2 mL of pooled serum samples by affinity purification, using the NAb Protein G Spin Kit (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions.

Cell culture

As the source for TM proteins, the immortalised human TM cell line HTM5 and the glaucomatous TM cell line GTM3 were used. Both cell lines were a kind gift of Professor Abbot F. Clark and colleagues [North Texas Eye Research Institute (NTERI), Fort Worth, TX, USA]. The cell lines are derived from human TM as described elsewhere.[40,67] Cells were grown in DMEM low glucose medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% foetal calf serum (Gibco, Life Technologies, Carlsbad, USA), 1% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) and 1% 200 mM L-alanyl-L-glutamine (Biochrom, Berlin, Germany). The TM cells were cultured in an incubator at 37°C and 5% CO_2. Media were changed every 2–3 days. On confluence, cells were collected using cell scrapers. Cell pellets were snap-frozen in liquid nitrogen and stored at −20°C until further use.

Cell lysis

Cell pellets were incubated for 30 min on ice with lysis buffer, modified after Alhamdani *et al.*[68] [20 mM HEPES pH7.9, 1 mM MgCl2, 5 mM EDTA, 1 mM PMSF, 0.5% Triton X-100, 0.5% NP40, 0.25% ASB-14, 0.25% CHAPS (Carl Roth, Karlsruhe, Germany), 0.5% Protease Inhibitor Cocktail, 0.5% Phosphatase Inhibitor Cocktail (reagents obtained from Sigma-Aldrich, Steinheim, Germany, unless otherwise stated)] with occasional vortexing. Additionally, cells were mechanically disrupted using an ultrasonic probe. The cells were sonicated in short burst, with occasionally cooling of the samples on ice. After an additional incubation for 15 min on ice, the cells were centrifuged at 20 000 *g* and 4°C. Afterwards, the supernatant was collected, and protein concentration was determined using a BCA assay kit (Thermo Scientific, Frankfurt, Germany).

Immunoprecipitation

For the immunoprecipitation (IP) of autoantigens from TM lysates, isolated IgG from serum was cross-linked to magnetic Sepharose beads with Protein G as ligand (Protein G Mag Sepharose; GE Healthcare, Freiburg, Germany). The IP was carried out using a modified protocol, based on instructions from the manufacturer. One hundred microlitres of bead slurry was incubated with 2 mg of isolated IgG for 1 h at 4°C on a rotation incubator. Unbound IgG solution was removed, and beads were washed with TBS [Tris (Carl Roth)-buffered saline; pH 7.5]. For the chemical cross-linking of the antibodies, beads were first equilibrated in triethanolamine solution [TEA (Sigma-Aldrich, Steinheim, Germany); 200 mM; pH 8.9] before incubation with dimethyl pimelimidate dihydrochloride (Sigma-Aldrich, Steinheim, Germany; 50 mM in 200 mM TEA; pH 8.9) for 30 min with slow rotation at room temperature. Beads were washed with TEA solution following 30-min incubation in 100 mM ethanolamine (Sigma-Aldrich, Steinheim, Germany; pH 8.9). The beads were washed once with elution buffer [0.1 M Glycine (AppliChem, Darmstadt, Germany)-HCl (Carl Roth), 2 M urea (Carl Roth); pH 2.9] and two times with TBS, to reduce unspecific binding. The beads covalently bound to the antibodies were incubated overnight with 2 mg TM protein (1 mg mL^{-1}) at 4°C on a rotation incubator. After removing unbound TM proteins, the beads were washed three times with TBS and then four times with 100 mM ammonium bicarbonate solution (ABC; Sigma-Aldrich, Steinheim, Germany).

On-bead digestion

For the preparation of the samples prior to MS analysis, on-bead tryptic digestion of the precipitated proteins was used, as described in Ref.[69] To this end, 30 µL of trypsin solution (Promega, Madison, WI, USA; diluted in 100 mM ABC) was directly added to the beads following incubation for 15 min at room temperature with occasional vortexing. After overnight incubation at 37°C, supernatant was collected and stored at 4°C. Another 30 µL of trypsin solution was added to the beads before incubation for additional 4 h at 37°C. The supernatant was separated from the magnetic beads, and both digests were pooled. Formic acid (Merck, Darmstadt, Germany) was added to a final concentration of 5%. The samples were then dried in a vacuum concentrator. Prior to MS analysis, samples were diluted in 0.1% trifluoroacetic acid (TFA; Merck) in HPLC-grade water (AppliChem) and purified using SOLAµ SPE plates (HRP 2 mg mL^{-1} 96-well plate; Thermo Scientific, Rockford, IL, USA) using the manufacturer's protocol with slight modifications.[70] In brief, the plate was activated with 150 µL acetonitrile (ACN; AppliChem) and equilibrated with 0.1% TFA solution. Samples were loaded three consecutive times on the plates, followed by two washing steps with 0.1% TFA. Peptides were eluted twice with 25 µL 60% ACN. All samples were lyophilised by vacuum centrifugation (SpeedVac, Thermo Scientific, Waltham, MA, USA) and stored at −20°C until MS analysis.

LC-ESI-MS/MS

Following on-bead tryptic digestion, the samples were analysed using an LC-ESI-MS/MS system (LTQ Orbitrap XL; Thermo Scientific, Rockford, IL, USA).[60,71] The samples were first solubilised in 10 µL of 0.1% TFA. The LC system consisted of a 30 × 0.5 mm BioBasic C18 column (Thermo Scientific, Rockford, IL, USA) and a Rheos Allegro pump (Thermo Scientific, Rockford, IL, USA). A PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland) was used to inject 6 µL of the samples into the system, followed by a solvent gradient. The gradient was run for 120 min per sample: 0–40% solvent B (0–40 min), 40–80% solvent B (40–80 min), 80–100% solvent B (80–100 min), 100–80% solvent B (100–110 min), 80% solvent B (110–120 min) (solvent A: LC-MS-grade water (AppliChem) + 0.1% (v/v) formic acid; solvent B: LC-MS grade ACN + 0.1% (v/v) formic acid). The LC system was coupled to an electrospray ionisation (ESI)–LTQ–Orbitrap XL MS (Thermo Scientific, Bremen, Germany)

for the acquisition of the mass spectra data. The system was operated in a data-dependent mode of acquisition to switch between Orbitrap-MS and LTQ-MS/MS acquisition automatically. The detection range was set to 300–2000 m/z with a resolution of 30 000. Parameters for dynamic exclusion were set to a repeat count of 1, a repeat duration of 30 s, with an exclusion list size of 50 and exclusion duration of 90 s. Collision-induced dissociation (CID) fragmentation was used to isolate the five most intense precursor ions for fragmentation in the LTQ. Activation time was set to 30 ms with a repeat count of 10. Normalised collision energy (NCE) was set to 35%.

Mass spectrometry spectra were analysed using MaxQuant (version 1.5.3.30; Max Planck Institute of Biochemistry, Martinsried, Germany). The spectra were searched against UniProt reviewed protein database (*homo sapiens*; 11.02.2019). Mass tolerance was set to ±20 ppm for precursor ions and ±0.5 Da for fragmentation. Carbamidomethylation of cysteine was set as fixed modification, N-terminal acetylation and oxidation of methionine as variable modifications. Trypsin was chosen as the digestive enzyme, and a maximum of two missed cleavages were allowed. Identification was based on a minimum peptide length of 7 with a FDR < 0.01. Proteins were quantified according to their peptide intensities using MaxQuant label-free quantification (LFQ).

Antigen microarray analysis

Selected autoantigen candidates were purchased as recombinant proteins with no post-translational modifications (wheat germ expression system). All antigens are listed in Supplementary table 4. The arrays were produced in our laboratory using a non-contact array printer (SciFLEXARRAYER S3, Scienion, Berlin, Germany). The selected antigens were spotted in triplicate onto nitrocellulose-covered glass slides (AVID Oncyte, 16 Pad NC slides, Grace Bio-Labs, Bend, OR, USA). A human IgG mix (Sigma-Aldrich, St. Louis, MO, USA) and PBS (Life Technologies, Paisley, UK) were included as positive and negative control spots. The spotting procedure was carried out in a humidity chamber at 60% humidity. After the array spotting, slides were allowed to dry on the spotter platform overnight. Array hybridisation was performed using 16-well incubation chambers (ProPlate Multiwell chambers, Grace Bio-Labs). All incubation steps were carried out on an orbital shaker at 4°C. At first, the arrays were incubated for 1 h with a blocking buffer (Super G, Grace Bio-Labs) to reduce background signals. Then, the blocking buffer was discarded and the residual buffer was removed by washing the slides with phosphate-buffered saline containing 0.5% Tween-20 (PBST; Sigma-Aldrich, Steinheim, Germany) three times. Afterwards, the arrays were incubated with 100 µL diluted serum samples (1:250 in PBS) overnight. PBS-only negative controls were included on each slide. Next, slides were again washed three times with PBST followed by incubation with an anti-human antibody conjugated with a fluorophore (Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG, Fcγ fragment-specific, 109-605-008, Jackson ImmunoResearch, West Grove, PA, USA) as secondary antibody at a 1:500 dilution in PBS for 1 h. After this step, the arrays were washed twice with PBST and twice with ultrapure water. The slides were subsequently dried for 2 min in a vacuum centrifuge concentrator.

Array images were acquired as 16-bit TIF files using a high-resolution confocal laser scanner (428 Array Scanner, Affymetrix, Santa Clara, CA, USA). The image analysis software Imagene (Imagene 5.5, BioDiscovery Inc., Los Angeles, CA, USA) was used to quantify spot intensities. Poor-quality spots were manually flagged and removed from the analysis.

Microarray data pre-procession

Net signal intensities were calculated by subtraction of local background intensity. Signals reaching negative values after background subtraction were treated as missing data. Signals derived from the negative control included on each slide were subtracted from each spot to take unspecific binding of the secondary detection antibody into account. Intensities from the triplicate spots were averaged, resulting in one mean fluorescence intensity. All signals were then normalised to the IgG control spots included on each subarray by median centring to reduce intra-slide variability and batch effects. Therefore, IgG median signal intensities were divided by the overall IgG signal median to attain a normalisation factor for each subarray. All further analyses are based on these normalised fluorescence intensities (NFI). To ensure robustness of the data set and reduce the influence of outliers, values below the 5th and above the 95th percentile were set as missing data. Targets with more than 25% missing data overall were not included in the statistical analyses. Missing data of targets with < 25% missing values were imputed using the k-nearest neighbour (KNN) algorithm.

Statistical analysis

The statistical analysis of the MS data was performed with Perseus (version 1.6.2.3; Max Planck Institute of Biochemistry). Before statistical analysis, proteins were filtered, and potential contaminants, reverse hits and proteins only identified by site were excluded. Also, proteins needed to be identified in all three replicates in at least one group. Proteins that were also identified in any of the negative controls were excluded as well. Missing data have been imputed from normal distribution using the inbuilt algorithm to enable statistical testing. Differences in protein abundance were assessed with the Student's *t*-test using a permutation-based FDR of < 0.01 as a threshold for statistical significance to correct for multiple testing. Principal component analysis (PCA) was used to show the influence of the protein source on the data variability. Selected target proteins (based on significant altered levels in HTM and GTM samples) were displayed on a heat map with hierarchical clustering based on Euclidean distance using *z*-score-transformed LFQ intensities. For PCA and clustering analysis, missing data were replaced by random values drawn from a normal distribution.

Microarray statistics were calculated using Statistica (Statistica 13, StatSoft, Tulsa, OK, USA). Microarray data were evaluated for normality using the Shapiro–Wilk test. The data did not follow a normal distribution ($P < 0.05$),

and so, the non-parametric Mann–Whitney *U*-test was used for hypothesis testing. Spearman's rank correlation coefficients were used to evaluate the correlations of autoantibody levels and clinical parameters. Kruskal–Wallis non-parametric ANOVA was used for multi-group comparisons. A *P*-value of < 0.05 was considered statistically significant. A random forest classification algorithm was used to evaluate the diagnostic potential of the identified, disease-related AAbs. Here, 165 cases were used as the training set and 75 samples as the test set.

Pathway and enrichment analysis

Gene Ontology enrichment analysis was done using DAVID (http://david.abcc.ncifcrf.gov/home.jsp).[72,73] GO terms with a *P*-value < 0.05 after Bonferroni adjustment were considered as significantly enriched. Additionally, DAVID functional annotation clusters were used to characterise putative POAG-related autoantigens. The analysis was carried out with whole *homo sapiens* proteome as background. Metascape (http://metascape.org)[74] was used for further pathway and enrichment analysis of identified autoantigens. Also, Metascape's molecular complex detection (MCODE) algorithm was used to detect connected network components.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Publication 3 (manuscript)

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Subtype-specific alterations in the serological autoantibody profile of open-angle glaucoma patients

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ABSTRACT

Evidence for immunologic contribution to glaucoma pathophysiology is steadily increasing in ophthalmic research. Particularly, an altered abundance of circulating autoantibodies to ocular antigens is frequently observed. Here, we report an analysis of autoantibody abundancies to selected antigens in sera of open-angle glaucoma patients, subdivided into normal-tension glaucoma (N=31), primary open-angle glaucoma (N=43) and pseudoexfoliation glaucoma (N=45), versus a non-glaucomatous control group (N=46). Serum samples were analysed by protein microarray, including 38 antigens. Differences in antibody levels were assessed by ANOVA. Eleven serological antibodies showed significantly altered levels among the four groups ($P<0.05$), which can be used to cluster the subjects in groups consisting mainly of PEXG, control or POAG/NTG samples. Among the altered autoantibodies, anti-Clathrin and anti-β-2 adrenergic receptor antibodies were identified as most important subgroup predictors, enhancing prospective glaucoma subtype prediction. As a second aim, we wanted to gain further insights into the characteristics of previously identified glaucoma-related antigens and their role in glaucoma pathogenesis. To this end, we used the bioinformatics toolset of Metascape to construct protein-protein interaction networks and GO enrichment analysis. Glaucoma-related antigens were significantly enriched in 13 biological processes, including mRNA metabolism, protein folding, blood coagulation and apoptosis, proposing a link of glaucoma-associated pathways to changes in the autoantibody repertoire. In conclusion, our study provides new aspects of the involvement of natural autoimmunity in glaucoma pathomechanisms and promotes advanced opportunities towards new diagnostic approaches.

INTRODUCTION

Glaucoma is a group of progressive neurodegenerative diseases of the optic nerve with variating forms of manifestation. An open-angle glaucoma (OAG) manifests as atrophy of the optic nerve and resulting vision loss while a normal iridiocorneal angle is maintained. The disease subtypes are assumed to have multifactorial pathogenesis, but few factors can be considered as significant hallmarks. Primary open-angle glaucoma (POAG), the most common form, is characterised by a typical optic nerve cupping, caused by the death of

retinal ganglion cells (RGCs) and their axons, along with an increased intraocular pressure (IOP) that is mainly caused by dysregulations in the trabecular meshwork (TM) [1-3]. An elevated IOP can also be observed in secondary glaucoma forms. In pseudoexfoliation syndrome (PEX), affected patients have a higher chance to develop pseudoexfoliation glaucoma (PEXG) [4]. PEX is the most frequent reason for secondary open-angle glaucoma [5]. Here, the high IOP is caused by an accumulation of pseudoexfoliation material in the anterior angle chamber, blocking the aqueous humour (AH) outflow in the TM [6]. Another form of OAG that develops independently from increased IOP is normal-tension glaucoma (NTG), as reviewed in [7]. Here, glaucomatous damage can be observed despite what is generally considered as physiological IOP (<21mmHG). This implicates that other disease mechanisms other than mere mechanical stress are likely to be involved in this subtype. One component adding to the possible disease mechanisms has been identified to be of an immunological kind. Signs of neuroinflammation and alterations in the innate immunity have been described in the context of glaucoma [8-12]. One aspect here is also the disease-specific alteration of the natural autoantibody repertoire, as has been shown in different studies over the past years [13-16]. Several alterations in specific serological autoantibodies targeting ocular antigens have been recently identified. Thus, we wanted to further investigate whether the identified autoantibodies also show specifically altered levels in different subtypes of glaucoma, as already indicated in earlier studies of our research group [17-19]. To this end, we analysed sera from POAG, NTG and PEXG patients in comparison to a non-glaucomatous control group using protein microarray, also to identify potential markers that allow discrimination between OAG subtypes.

In this context, we also wanted to explore common characteristics of previously identified glaucoma-related antigens and investigate their relation to glaucoma pathogenesis. Autoantibodies to several ocular antigens have been frequently found by different groups using various methods, but they have not been regarded in a holistic approach. To close this gap we searched previously detected glaucoma-related antigens in the literature and analysed their interactions and connections with the disease using bioinformatic tools.

RESULTS

Analysis of autoantibody levels in open-angle glaucoma subtypes

In this study, we used a set of 38 antigens associated with glaucoma and other neurodegenerative diseases for the preparation of antigen microarrays (**Supplementary file**

S1). We analysed the IgG autoantibody reactivity to these antigens in glaucoma patients, comprising NTG (N=31), POAG (N=43), and PEXG (N=45), and a non-glaucomatous control group (CTRL; N=46). To identify alterations in antibody reactivity among these groups, we performed an ANOVA with consecutive post hoc test (Tukey's HSD test for unequal N). **Figure 1 A** shows a heat map for the eleven significantly ($P<0.05$) altered autoantibody levels in either group. Post hoc testing revealed significant differences as follows: Antibodies to MYO1C and ENO1 show decreased levels in NTG patients compared to PEXG. HSP27 autoantibodies are elevated in PEXG compared to POAG. Levels of CRYGS antibodies are higher in PEXG patients than in control subjects and POAG patients. FN1 antibodies are decreased in POAG sera compared to PEXG. Serological MBP antibodies show significantly lower levels in POAG compared to control and PEXG. NTG and POAG patients show higher levels of ADRB2 autoantibodies and PEXG patients decreased levels of CLTA/B/C autoantibodies. These results are also available in **Supplementary file S2**. Measurements of autoantibody reactivity to ACTN1, MECP2, PROK, TGFB1I1, TTR and VIM were not possible because the protein spots did not pass quality requirements (maximum of 25% missing values) or signals were below the detection limit.

Using k means clustering on all cases, three major clusters could be identified. Cluster 1 is mainly comprised of CTRL cases (40.66%), Cluster 2 contains a majority of NTG and POAG cases (34.62% and 51.92%), whereas Cluster 3 mostly contains PEXG cases (86.36%) (See also **Table 1**). To investigate, which autoantibody levels have the biggest impact on the clustering, we used a feature selection algorithm (Statistica Feature Selection and Variable Screening) to find the best predictors. We identified the antibodies to the β-2 adrenergic receptor (ADRB2) as the most important predictor (Chi-square = 95.86; $P<0.01$), followed by antibodies to Clathrin (Chi-square = 57.36; $P<0.01$) (**Figure 1 B**). ANOVA and Tukey's HSD post hoc test revealed that ADRB2 autoantibody levels are especially high in NTG and POAG patients (**Figure 1 C**) and that Clathrin autoantibody levels are significantly decreased in the serum of PEXG patients compared to the other groups (**Figure 1 D**).

Correlation of autoantibodies with clinical features

Major hallmarks of glaucoma pathogenesis are IOP, visual field defects (measured as ('mean deviation' (MD)) and the cupping of the optic nerve that is assessed as the cup-disc ratio (CDR). We performed a Pearson correlation analysis to examine whether the serological antibody levels show an association with these clinical parameters (**Table 2**).

The results reveal a weak negative correlation of CLTA/B/C autoantibodies with IOP (r = -0.25), a weak positive correlation of MYO1C (r = 0.329) and a weak negative correlation of ADRB2 autoantibodies (r = -0.307) with visual field defect. Also a weak positive correlation of ADRB2 and CLTA/B/C (r = 0.192 and r = 0.225) and a weak negative correlation of CRYGS autoantibodies (r = -0.285) with CDR was disclosed.

Bioinformatic analysis of established glaucoma-related autoantigens

Starting with a literature search, we identified 28 antigens that have been identified as targets to glaucoma related autoimmunity by different groups and methods (**Table 3**). To get an overall impression of the characteristics of theses previously identified glaucoma-related antigens, we conducted a protein-protein interaction analysis, as well as a GO term enrichment analysis using Metascape. We found that 22 of the 28 antigens had at least one interaction partner among the tested proteins (**Figure 2a**). A strong network was identified by Metascapes' Molecular COmplex DEtection algorithm (MCODE) with exceptionally high number of interactions for six of the antigens. These antigens are HSPA1A, HSPD1, YWHAZ, ENO2, PGAM1 and VDAC2. The GO enrichment analysis for all 28 antigens revealed 13 terms describing biological processes as significantly enriched. The most significantly enriched processes were regulation of mRNA process, protein folding, blood coagulation and apoptosis (**Figure 2b**). The GO enrichment analysis for the MCODE cluster of antigens only, revealed a significant enrichment of three biological processes. These comprise apoptotic mitochondrial changes, nucleobase-containing catabolic processes and heterocycle catabolic processes (**Figure 2c**). Regarding the cellular components of the MCODE cluster of antigens, three compartments were enriched (**Table 4**). Four of the six antigens are located in the myelin sheath and / or in mitochondria. Also, 50% of these antigens are to be found in the extracellular space.

DISCUSSION

In this study, we analysed serological autoantibodies in sera of OAG patients and non-glaucomatous controls by means of an antigen microarray approach. We found altered levels of antibodies to ADRB2, ATP5A1, ENO1, MYO1C, CLTA/B/C, CRYGS, HSP27, FN1, TARS and MBP using ANOVA. Although the Pearson correlation analysis revealed few significant correlations between some autoantibody levels and glaucoma hallmarks, the relationships

are very weak. With a correlation coefficient of $|r|<0.3$ the correlations can be considered negligible. Thus, it can be concluded that the abundance of the analysed antibodies is not essentially dependent on alterations of IOP, MD or CDR and *vice versa*. Although, some biological processes involved in pathological changes influencing IOP, and advancing neurodegeneration leading to increasing CDRs could have stronger dependencies leading to the observed statistical significance, but this cannot be deduced from the data at hand.

We analysed, whether these autoantibodies might serve as disease markers to classify non-glaucomatous subjects and OAG subtypes. An earlier study of our research group already found significant differences between the antibody profiles of POAG and NTG, also in comparison to a control group, indicating their diagnostic value for OAG subtype discrimination [19]. Another previous study showed significant differences between the antibody profiles to retinal antigens in aqueous humour of patients with PEXG and a control group [17]. This study also compared the antibody profiles of POAG and PEXG patients. Although these two forms of OAG have vast differences in their pathogenesis, they showed no significant differences in their antibody profiles between each other. For a potential diagnostic discrimination of various glaucoma subtypes, suitable biomarker candidates need to be identified. Here, we achieved to find two putative autoantibody biomarkers that show significantly altered serological levels in PEXG compared to the other most common OAG types, POAG and NTG. Using a predictor screening algorithm, we identified antibodies to CLTA/B/C (Clathrin) and ADRB2 (β-2 adrenergic receptor) as the most important predictors.

Clathrin is a protein majorly involved in the formation of vesicles associated with clathrin-mediated endocytosis (reviewed in [20]). Not much is known about the association of clathrin with glaucoma. However, northern blot analysis of gene expression patterns in PEX patients has shown an increase in clathrin expression in the lens epithelium [21]. Generally, it has been discussed that uptake of circulating antibodies is mediated by clathrin-mediated endocytosis that opens the opportunity of autoantibodies to cause various effects on an intracellular level [22, 23]. Antibodies to clathrin, however, have not been reported in relation to PEXG. Circulating antibodies are hypothetically attributed to many different functions, including agonistic and antagonistic receptor effects or activation of the complement cascade [24]. Agonistic as well as antagonistic effects are possible. The aggregation of exfoliation material in the anterior chamber has vision-threatening effects

in PEX patients. Polymorphisms in the LOXL1 gene contribute to the accumulation of elastic myofibrils and extracellular matrix that impair the physiological function of the TM, leading to increased IOP and severe damage to the optic nerve as a consequence [25]. Possibly, an altered clathrin-mediated extracellular matrix turnover in PEXG patients plays a role in the pathogenesis. Thus, the decreased autoantibody levels might be a consequence or precursor of this condition. Further studies are necessary to investigate the causalities of clathrin and anti - clathrin antibodies in PEXG.

The β-2 adrenergic receptor is a cell surface protein that mediates the activation of adenylate cyclase by connection to G proteins. These receptors are expressed in the TM and in the ciliary body [26, 27] and are directly involved in AH homeostasis. They have long been the target for therapeutic β adrenoceptor antagonists capable of lowering IOP in POAG patients by reducing the formation of AH in ciliary epithelial cells and increasing the outflow via the TM [28]. Circulating antibodies to ADRB have been found in Alzheimer's [29] and in cardiovascular disease [30, 31]. Recently, agonistic ADRB2 IgG3 class autoantibodies have been detected in POAG and OHT patients using a cardiomyocyte bioassay and inhibitory ADRB2 peptides [32, 33]. With our work, we could confirm an increase of anti-ADRB2 autoantibodies in the sera of POAG patients and also found them elevated in NTG. However, we did not find elevated levels in the secondary PEX glaucoma [33]. Our results further empower the significant role for ADRB2 in glaucoma pathogenesis that also involves immunological manifestations.

Autoantibodies to ocular antigens that show altered levels in glaucoma patients have already been described by numerous different studies conducted by various research groups. To learn more about the nature of the autoantigens identified in the past, we implemented a holistic bioinformatic analysis to investigate the relations between these glaucoma antigens. We assumed that the examination of how they connect to each other in the context of the disease could help for a better understanding of synergies between autoimmune effects and other disease mechanisms. We found 13 biological processes enriched among the glaucoma-related antigens. Proteins were most highly enriched in regulation of mRNA processes, followed by protein folding, blood coagulation and apoptosis. Pathological changes in these processes were found in OAG patients and we assume a glaucoma – related association between alterations of these processes and corresponding autoantibody levels. Deregulations of RNA metabolism associated processes are a common feature of neurodegenerative diseases [34]. Alterations in the

regulation of some glaucoma-related mRNAs were also reported in TM cells of glaucoma patients [35], showing a possible relation to altered autoantibody abundance. The enrichment of antigens that are part of the biological process of protein folding arises mainly from overrepresentation of molecular chaperons. Disturbed protein folding can cause formation of protein aggregates that, when accumulating, can induce the unfolded protein response (UPR), which protects the cell from endoplasmic reticulum (ER) stress [36]. ER stress in turn can lead to apoptosis if the capacities of the UPR are exceeded. ER stress - induced apoptosis is common in neurodegenerative diseases and also occurs in glaucoma pathogenesis [37]. Abnormal blood coagulation was described in POAG patients in form of age-dependent spontaneous platelet aggregation [38] and platelet activation was also assumed to be involved in glaucoma pathomechanisms [39]. Glaucoma-related autoantibodies were shown to especially reflect the platelet-derived growth factor receptor pathway [16]. Whether the detected autoantibodies partake in the deregulation of these processes or act as possible countermeasures cannot be answered here. We can however assume a strong influence of these alterations on the serological autoantibody repertoire, presumably reflecting the antigenic status.

Findings from the PPI network suggest a special role in glaucoma associated immunity for the six antigens clustered by the MCODE algorithm. These antigens are mainly associated with mitochondria and apoptotic mitochondrial changes. In general, apoptosis is considered the main mechanism of RGC demise in glaucoma [40] and previous studies already emphasized the importance of mitochondrial dysfunction as an aspect in glaucoma pathogenesis, which is also considered a major cause of cell death of RGCs [41, 42] . Pro-apoptotic alterations in the mitochondrial proteome have already been observed in retinal cells after incubation with serum of POAG patients [43]. This indicates that serological autoantibodies are capable to promote disease progression and persistent neuronal damage. Future work should further investigate the connection and molecular mechanisms of a possible antibody-mediated impairment of mitochondria in RGCs.

Additionally, four of the six MCODE clustered antigens are also located in myelin sheaths. Conventionally, glaucoma is not considered as a demyelinating disorder. Recent investigations however found evidence that demyelination could play a role in the mechanisms causing neuronal damage [44]. Although there is some inconsistency in data, suggesting that this could not be true for all cases [45]. Additionally, a study in DBA/2J mice has shown that early insults of the optic nerve occur in proximity to the lamina cribrosa and

that axon segments in the lamina and more distal undergo degeneration [46]. Retinal ganglion cells are unmyelinated until they pass through the lamina cribrosa, but the myelinated axons beyond might be targeted by glaucoma-related antibodies. Autoantibodies to proteins of the myelin sheath are also known form other diseases such as multiple sclerosis and neuromyelitis optica. Here, antibodies against myelin-associated proteins (e.g. anti - myelin oligodendrocyte glycoprotein, anti-myelin basic protein [47] and anti-aquaporin 4) are frequently found in patients. Their role in the pathogenesis is not finally clarified yet. Though it is assumed that they have the potential to inflict axonal damage and inflammatory demyelination, which would also apply to the optic nerve cells distal to the lamina cribrosa [47, 48]. This gives rise to the hypothesis that damages of the optic nerve sheath mediated by antibodies could also be capable to cause neuronal damage in glaucoma. On the other hand, IgG autoantibodies have been described to be involved in the debris clearance after injury of neuronal cells of the peripheral nerve system, thereby supporting axon regeneration [49]. However, regardless of the effect of the IgG class autoantibodies, their occurrence is strongly linked to injuries of nerve cells and their axons. The antibody-mediated effects affecting glaucoma pathogenesis have not yet been elucidated and will require further investigation in future studies.

Overall, these results contribute to the assumption that serological autoantibodies can reflect pathological changes in the affected sites of the eye. Glaucoma-related autoantigens are enriched in biological processes with a strong link to previously described pathomechanisms. The causality of autoantibody genesis and corresponding pathological events, however, still remains a conundrum. Nonetheless, the observation of IgG level alterations in serum can be exploited for the monitoring of the disease and usage for diagnostic purposes. The detection of significantly altered autoantibody levels to clathrin and the β-2 adrenergic receptor promotes prospective diagnostic glaucoma-subtyping, especially for the discrimination of PEXG from other forms of OAG.

MATERIALS AND METHODS

Sera

Sample collection was carried out in accordance with the Declaration of Helsinki on biomedical research involving human subjects. Written informed consent was obtained

from each subject. The study was approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz. All subjects included in this study received an ophthalmic examination at the department of ophthalmology of the university medical center in Mainz, Germany. Glaucoma patients were diagnosed according to the guidelines of the European Glaucoma Society [50]. Visual field defects were determined using OCTOPUS 101 Perimeter (Haag-Streit, Wedel, Germany) or Humphrey Visual Field analyser (Carl Zeiss Meditec, Dublin, CA). Correlation analysis was only run with OCTOPUS data sets, to avoid systematic bias. The control group consists of non-glaucomatous subjects. According to the anamnestic query, none of the included subjects suffered from Alzheimer's or Parkinson's disease. One subject of the non-glaucomatous control group reported suffering from multiple sclerosis. Glaucoma patients were diagnosed based on glaucomatous optic disc damage and typical visual field defects. All patients had open iridiocorneal angles. PEXG was diagnosed when patients showed PEX material in at least on eye. PEXG and POAG patients showed an IOP >21mmHg, while IOP in NTG patients was <21mmHg. The demographics of the study population can be found in **Table 5**.

Antigen Microarray analysis

Recombinant or purified proteins selected for the antigen microarray are listed in **file S1**. The arrays were produced in our lab, as described elsewhere [14]. Array hybridization was performed using 16-well incubation chambers (ProPlate Multiwell chambers, Grace Biolabs, Bend, USA). All incubation steps were carried out on an orbital shaker at 4°C. Arrays were incubated for one hour with a blocking buffer (Super G, Grace Biolabs, Bend, Oregon, USA). Then, the blocking buffer was removed, and the slides were washed three times with phosphate-buffered saline containing 0.5% Tween-20 (PBST). Subsequently, the arrays were incubated with 100µL serum in a 1:250 in PBS overnight. As negative control PBS only arrays were included on each slide. Next, slides were washed three times again with PBST followed by incubation with an anti-human antibody conjugated with a fluorophore (Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, 109-605-008, Jackson Immunoresearch) as secondary antibody diluted 1:500 in PBS for one hour. Next, the arrays were washed twice with PBST and twice with ultrapure water. Lastly, the slides were dried for two minutes in a vacuum centrifuge concentrator (SpeedVac, Thermo Scientific, Waltham, Massachusetts, USA).

Array images were acquired as 16-bit TIF file using a high-resolution confocal laser scanner (428 Array Scanner, Affymetrix, Santa Clara, California, USA). The image analysis software Imagene (Imagene 5.5, BioDiscovery Inc., Los Angeles, California, USA) has been used to quantify spot intensities. Poor quality spots have been manually flagged and removed from the analysis.

Microarray data pre-procession

Net signal intensities were calculated by subtraction of local background intensity. Signals reaching negative values after background subtraction were treated as missing data. Negative control signal intensities were subtracted from each spot. Intensities from the triplicate spots were averaged, yielding one mean fluorescence intensity. All signals were then normalised to the IgG control spots included on each subarray by median centring to reduce intra – slide variability and batch effects. Therefore, IgG median signal intensities were divided by the overall IgG signal median to obtain a factor for each subarray. All further analyses are based on these normalised fluorescence intensities (NFI). To ensure the robustness of the dataset and reduce the influence of outliers, values below the 5th and above the 95th percentile in each group were set as missing data. Targets with overall more than 25% missing data were not eligible for statistical analyses. Missing data of targets with less than 25% missing values was imputed using the k-nearest-neighbour (KNN) algorithm. Grand mean normalisation was applied for all arrays.

Statistical analysis

Statistical analyses were performed using Statistica (Statistica 13, Statsoft, Tulsa, Oklahoma, USA). One-way ANOVA with consecutive Tukey's HSD post hoc test for unequal n was performed to determine significant alterations in antibody levels among the study groups. Pearson's correlation coefficients were used to evaluate correlations of autoantibody levels and clinical parameters. A *P*-value of < 0.05 was considered statistically significant. Multinomial and binomial logistic regression was used to evaluate the potential of serological autoantibody levels to discriminate between glaucoma subtypes and non-glaucomatous controls. Heat mapping and k means clustering was implemented using Morpheus (https://software.broadinstitute.org/morpheus/).

Protein-Protein interaction networks and Gene Ontology enrichment analysis

Gene Ontology enrichment analysis and the protein-protein interaction network was conducted in Metascape (http://metascape.org) [51]. Also, Metascape's molecular complex detection (MCODE) algorithm was used to detect densely connected network components.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURES AND TABLES

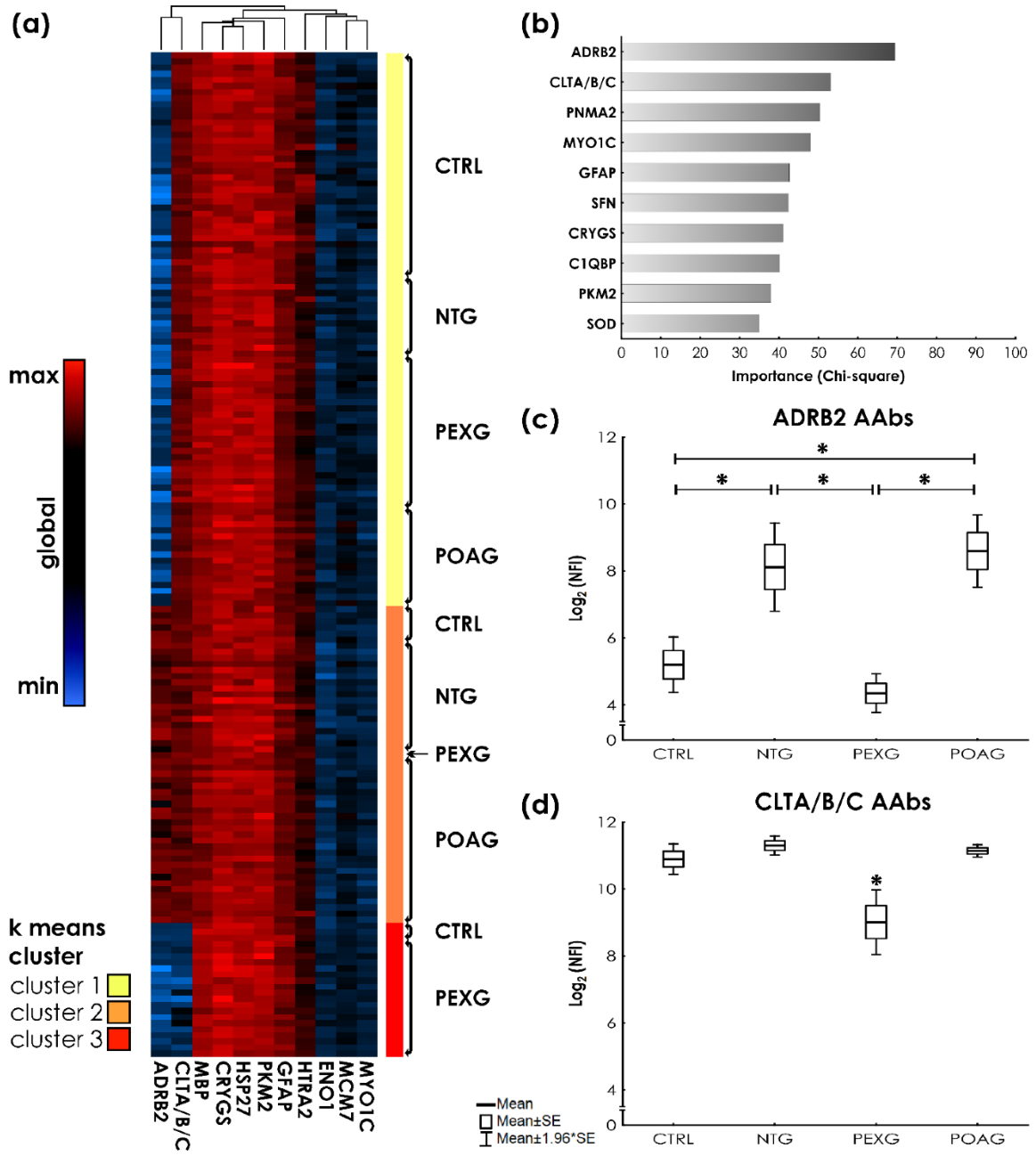


Figure 1 K means clustering and predictor screening. **(a)** Heat map showing autoantibody reactivities in glaucoma subtypes and control samples. **(b)** Predictor screening of significantly altered autoantibody levels. The most important predictor for group affiliation are ADRB2 autoantibodies, followed by CLTA/B/C antibody levels. **(c)** and **(d)** ANOVA with HSD unequal N post hoc test. It is shown that ADRB2 autoantibody levels are especially high in NTG and POAG patients. CLTA/B/C autoantibody levels are significantly decreased in serum of PEXG patients compared to the other groups.

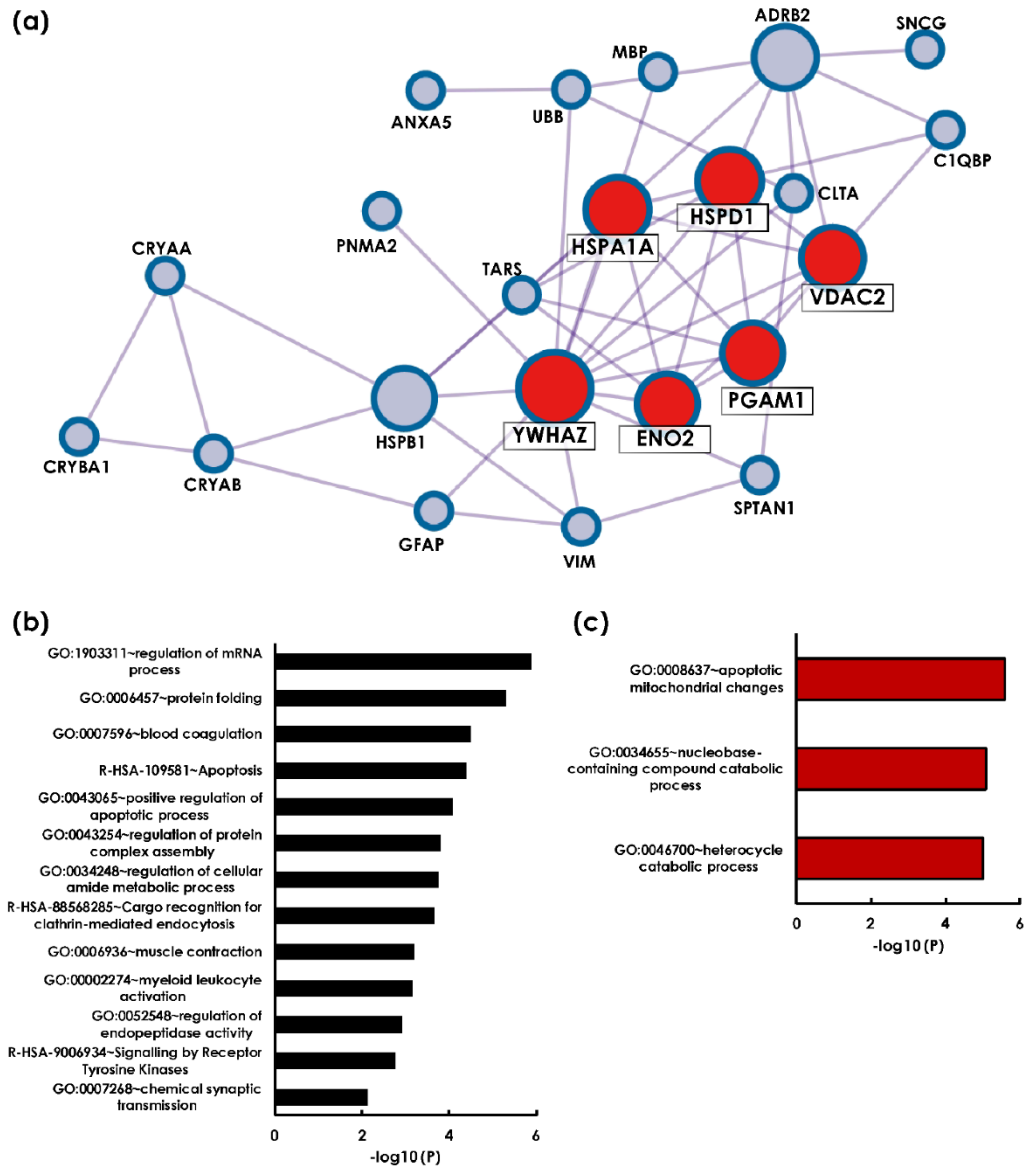


Figure 2 Holistic bioinformatics analysis of previously established glaucoma-related autoantigens. **(a)** Protein-protein interaction network. 22 of the 28 glaucoma-related antigens have at least one interaction partner among each other. Especially strong interactions were observed for six antigens (HSPA1A, HSPD1, YWHAZ, VDAC2, PGAM1, ENO2), that were identified by the Metaspcape algorithm MCODE (marked with red spots). **(b)** GO analysis of 28 glaucoma-related antigens. Shown are significantly enriched GO terms of biological processes and reactome gene sets. **(c)** GO analysis of MCODE cluster antigens only.

Table 1 Distribution of glaucoma cases and controls in the three k means clusters. Cluster 1 mainly contains cases of the control group (40.66%). Cluster 2 is dominated by POAG (51.92%) and NTG cases (34.62%). Cluster 3 predominantly contains PEXG cases (86.36%).

%	CTRL	NTG	PEXG	POAG	Sum
Cluster 1	40.66	14.29	27.47	17.58	100
Cluster 2	11.54	34.62	1.92	51.92	100
Cluster 3	13.64	0	86.36	0	100
Cases	27.88	18.79	27.27	26.06	100

Table 2 Pearson correlation analysis. Table shows correlation coefficients (r). Marked entries (red) are significant at $p<0.05$. Analysis was carried out with all available cases.

	IOP (N=160)	*MD (N=77)*	*CDR (N=113)*
MYO1C	0.091	0.329	-0.022
MCM7	-0.049	0.118	0.146
ENO1	0.063	0.056	-0.099
PKM2	-0.053	-0.104	-0.074
HTRA2	-0.105	0.056	0.042
GFAP	-0.086	-0.056	-0.108
HSP27	-0.004	0.028	-0.134
CRYGS	-0.036	-0.171	-0.285
CLTA/B/C	-0.250	0.074	0.225
ADRB2	0.011	-0.307	0.192
MBP	0.015	-0.018	-0.156

Table 3 Glaucoma-related antigens identified in different previous studies.

Protein	Gene ID	References
HSP60	HSPD1	[52]
HSP27	HSPB1	[13, 53]
HSP70	HSPA1	[54]
Alpha A-crystallin	CRYAA	[53]
Alpha B-crystallin	CRYAB	[54]
β-L-crystallin	CRYBA1	[13]
Annexin 5	ANXA5	[13]
Ubiquitin	Isoform not specified; UBB used for analysis	[13]
Glial fibrillary acidic protein	GFAP	[13, 18]

14-3-3	Isoform not specified; YWHAZ used for analysis	[55]
Alpha Fodrin	SPTAN1	[56]
Gamma enolase	ENO2	[57, 58]
Vimentin	VIM	[54]
Myelin basic protein	MBP	[13, 19]
Retinaldehyde-binding protein	RLBP1	[18]
Glutathion-S-transferase	GST	[59]
Retinal S-antigen	SAG	[18]
Histone H4	H4	[18]
Alpha 1 antitrypsin	SERPINA1	[13]
Gamma synuclein	SNCG	claimed in [60]
Voltage-dependent anion-selective channel protein 2	VDAC2	[14]
Caldesmon	CALD1	[14]
Phosphoglycerate mutase 1	PGAM1	[14]
Threonine--tRNA ligase 1, cytoplasmic	TARS1	[16]
Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	[16]
Paraneoplastic antigen Ma2	PNMA2	[16]
Beta-2 adrenergic receptor	ADRB2	This study + [32]
Clathrin	CLTA/B/C	This study

Table 4 Cellular components of MCODE clustered antigens. DAVID GO enrichment analysis.

GO term	Cellular component	Count	%	P Value	Antigens	List Total	Pop Hits	Pop Total	Fold Enrichment
GO:0043209	myelin sheath	4	66.67	<0.001	ENO2, PGAM1, HSPD1, VDAC2	6	152	18224	79.930
GO:0005739	mitochondrion	4	66.67	0.003	YWHAZ, HSPA1A, HSPD1, VDAC2	6	1331	18224	9.128
GO:0005615	extracellular space	3	50	0.047	YWHAZ, ENO2, HSPD1	6	1347	18224	6.765

Table 5 Demographics of study population.

	N	*m/f*	*age (min - max)*
CTRL	46	27/19	66.96 (34 - 83)
NTG	31	15/16	68.29 (49 - 80)
PEXG	45	21/24	70.47 (52 - 86)
POAG	43	25/18	66.37 (24 - 81)

SUPPLEMENTARY FILES

S1 Supplementary file 1: Recombinant and purified proteins on antigen microarray.

#	*Gene name*	*Protein name*	*Supplier*
1	ACTA1	Actin, bovine	Sigma-Aldrich
2	ACTN1	Alpha-actinin-1	abnova
3	ADRB2	β-2 adrenergic receptor	Sigma-Aldrich
4	ANP32B	Acidic leucine-rich nuclear phosphoprotein 32 family member B	abnova
5	ATP5A1	ATP synthase subunit alpha, mitochondrial	Aviva systems biology
6	C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial	abnova
7	CALD1	Caldesmon	Abcam
8	CCDC 42	Coiled-coil domain-containing protein 42	sino biological
9	CLTA/B/C	Clathrin, bovine	Sigma-Aldrich
10	COPA	Coatomer subunit alpha	abnova
11	CRYGS	Gamma-crystallin S	Abcam
12	DDX46	Probable ATP-dependent RNA helicase DDX46	creative biomart
13	ENO1	Alpha-enolase	Abcam
14	FLNA	Filamin-A	abnova
15	FN1	Fibronectin	Sigma-Aldrich
16	GFAP	Glial fibrillary acidic protein	US Biological
17	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	abnova
18	HSP27	Heat shock protein beta-1	Enzo Life Science
19	HSP70	Heat shock 70 kDa protein 1A	Abcam

20	HTRA2	Serine protease HTRA2, mitochondrial	Novoprotein
21	IQGAP1	Ras GTPase-activating-like protein IQGAP1	abnova
22	MBP	Myelin basic protein, bovine	Sigma-Aldrich
23	MCM7	DNA replication licensing factor MCM7	abnova
24	MCTS1	Malignant T-cell-amplified sequence 1	abnova
25	MECP2	Methyl-CpG-binding protein 2	Novoprotein
26	MYO1C	Unconventional myosin-Ic	abnova
27	PKM2	Pyruvate kinase PKM	Abcam
28	PNMA2	Paraneoplastic antigen Ma2	abnova
29	PPP2R2B	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	abnova
30	PROK	Proteinase K from *Tritirachium album*	Sigma-Aldrich
31	SERPINA1	Alpha-1-antitrypsin	Sigma-Aldrich
32	SFN	14-3-3 protein sigma	Enzo Life Science
33	SOD	Superoxide dismutase [Cu-Zn]	Sigma
34	TARS	Threonine--tRNA ligase 1, cytoplasmic	abnova
35	TGFB1I1	Transforming growth factor beta-1-induced transcript 1 protein	Abnova
36	TTR	Transthyretin	Sigma-Aldrich
37	VDAC2	Voltage-dependent anion-selective channel protein 2	Abcam
38	VIM	Vimentin	ProGen

S2 Supplementary file 2: Post hoc test of significant ANOVA results. HSD test for unequal N was applied. Tables show respective P values.

Group	Unequal N HSD; Variable: **TARS** Marked differences are significant at p < .05000			
	{1} M=8.7990	{2} M=8.5626	{3} M=9.0065	{4} M=8.6279
CTRL {1}		0.609110	0.564100	0.714459
NTG {2}	0.609110		0.096795	0.986617
PEXG {3}	0.564100	0.096795		0.088558
POAG {4}	0.714459	0.986617	0.088558	

Group	Unequal N HSD; Variable: **MYO1C** Marked differences are significant at p < .05000			
	{1} M=6.6287	{2} M=6.1787	{3} M=6.6590	{4} M=6.3098
CTRL {1}		0.072993	0.997300	0.171809
NTG {2}	0.072993		0.047885	0.894804
PEXG {3}	0.997300	0.047885		0.112821
POAG {4}	0.171809	0.894804	0.112821	

Group	Unequal N HSD; Variable: **ATP5A1** Marked differences are significant at p < .05000			
	{1} M=5.0681	{2} M=4.1752	{3} M=4.9130	{4} M=4.8922
CTRL {1}		0.063276	0.954673	0.937633
NTG {2}	0.063276		0.170479	0.191554
PEXG {3}	0.954673	0.170479		0.999883
POAG {4}	0.937633	0.191554	0.999883	

Group	Unequal N HSD; Variable: **ENO1** Marked differences are significant at p < .05000			
	{1} M=5.9747	{2} M=5.5878	{3} M=6.3246	{4} M=5.8914
CTRL {1}		0.250242	0.182295	0.964800
NTG {2}	0.250242		0.002430	0.467160
PEXG {3}	0.182295	0.002430		0.065213
POAG {4}	0.964800	0.467160	0.065213	

Group	Unequal N HSD; Variable: **HSP27** Marked differences are significant at p < .05000			
	{1} M=13.202	{2} M=13.309	{3} M=13.624	{4} M=13.113
CTRL {1}		0.954156	0.061692	0.953942
NTG {2}	0.954156		0.410668	0.773043
PEXG {3}	0.061692	0.410668		0.015179
POAG {4}	0.953942	0.773043	0.015179	

Group	Unequal N HSD; Variable: **CRYGS** Marked differences are significant at p < .05000			
	{1} M=13.626	{2} M=13.706	{3} M=14.166	{4} M=13.473
CTRL {1}		0.979238	0.007610	0.806636
NTG {2}	0.979238		0.107872	0.660463
PEXG {3}	0.007610	0.107872		0.000294
POAG {4}	0.806636	0.660463	0.000294	

Group	Unequal N HSD; Variable: **FN1** Marked differences are significant at p < .05000			
	{1} M=11.918	{2} M=12.190	{3} M=12.329	{4} M=11.744
CTRL {1}		0.672770	0.170452	0.828869
NTG {2}	0.672770		0.940177	0.253074
PEXG {3}	0.170452	0.940177		0.020839
POAG {4}	0.828869	0.253074	0.020839	

Group	Unequal N HSD; Variable: **CLTA/B/C** Marked differences are significant at p < .05000			
	{1} M=11.009	{2} M=11.280	{3} M=8.8645	{4} M=11.180
CTRL {1}		0.946577	0.000008	0.976257
NTG {2}	0.946577		0.000013	0.997028
PEXG {3}	0.000008	0.000013		0.000008
POAG {4}	0.976257	0.997028	0.000008	

Group	Unequal N HSD; Variable: **ADRB2** Marked differences are significant at p < .05000			
	{1} M=4.6822	{2} M=8.2928	{3} M=4.2590	{4} M=9.0180
CTRL {1}		0.000011	0.895742	0.000008
NTG {2}	0.000011		0.000008	0.748979
PEXG {3}	0.895742	0.000008		0.000008
POAG {4}	0.000008	0.748979	0.000008	

Group	Unequal N HSD; Variable: **MBP** Marked differences are significant at p < .05000			
	{1} M=12.860	{2} M=12.576	{3} M=13.178	{4} M=12.367
CTRL {1}		0.403290	0.152225	0.007066
NTG {2}	0.403290		0.005307	0.662126
PEXG {3}	0.152225	0.005307		0.000008
POAG {4}	0.007066	0.662126	0.000008	

Additional Data

The data presented in this chapter has not been published previously but provides important additional information for the discussion of the results in this dissertation. Methods and results are presented in this chapter and are discussed in the respective sections of the chapter "Comprehensive discussion".

GENE-DISEASE ASSOCIATION ENRICHMENT OF GLAUCOMA-RELATED ANTIGENS

Introduction

To characterize the yet identified glaucoma-related antigens further, a search for similarities among these proteins was conducted. It was searched for gene-disease association (GAD) as an additional functional annotation.

Method

Enrichment GAD term enrichment was analysed with DAVID (https://david.ncifcrf.gov/home.jsp) [100]. Whole human proteome was defined as the background for the analysis.

As input for the analyses, Uniprot IDs from target proteins were used as displayed in **Table 3**.

Table 3 Input for enrichment analyses.

GENE ID	UNIPROT ID
HSPD1	P10809
HSPB1	P04792
HSPA1A	P0DMV8
CRYAA	P02489
CRYAB	P02511
CRYBA1	P05813
ANXA5	P08758
UBB	P0CG47
GFAP	P14136
YWHAZ	P63104
SPTAN1	Q13813
ENO2	P09104
VIM	P08670
MBP	P02686
RLBP1	P12271

GENE ID	UNIPROT ID
GST	P09488
SAG	P10523
H4	P62805
SERPINA1	P01009
SNCG	O76070
VDAC2	P45880
CALD1	Q05682
PGAM1	P18669
TARS	P26639
C1QBP	Q07021
PNMA2	Q9UL42
ADRB2	P07550
CLTA	P09496

Results

The GAD disease classes "Vision" and "Neurological" were significantly enriched among the input proteins (**Table 4**). The antigens are also associated with "Aging", "Immune", "Infection", as well as "Normal variation", "Reproduction" and "Renal", but were not significantly enriched.

Table 4 DAVID functional annotation. Gene annotations of respective glaucoma-related antigens. GAD (gene-disease association) disease classes from GAD database curated from genetic association studies. Homo sapiens proteome as background.

Term: GAD disease class	Count	%	PValue	Genes	List Total	Fold Enrichment	Benjamini
VISION	7	25.0	0.001	P12271, P07550, P10523, P0DMV8, P09488, P02489, P05813	24	5.491	0.018
NEURO-LOGICAL	13	46.4	0.002	P45880, O76070, P07550, P02511, P10809, P08670, P14136, P0DMV8, P01009, P09488, P09104, P04792, P63104	24	2.431	0.015
AGING	6	21.4	0.023	P07550, P02511, P08670, P14136,	24	3.421	0.130

Term: GAD disease class	Count	%	PValue	Genes	List Total	Fold Enrichment	Benjamini
				P0DMV8, P09488			
IMMUNE	11	39.3	0.024	P07550, P02511, Q05682, P10809, P10523, P02686, P0DMV8, P01009, P09488, P08758, P04792	24	1.991	0.102
INFECTION	9	32.1	0.027	P07550, P10809, P02686, P0DMV8, P45880, Q07021, P01009, P09488, Q13813	24	2.258	0.093
NORMAL-VARIATION	4	14.3	0.062	P07550, P10809, P01009, P09488	24	4.181	0.174
REPRO-DUCTION	5	17.9	0.086	P07550, P0DMV8, P01009, P09488, P08758	24	2.815	0.206
RENAL	6	21.4	0.098	P07550, P02686, P0DMV8, P01009, P09488, P08758	24	2.288	0.208

GENE ENRICHMENT ANALYSIS OF MOST ABUNDANT NATURAL AUTOANTIBODIES

Introduction

In Publication 2 [84] molar quantities of identified antigens of the natural immunity were measured by mass spectrometry using MaxQuant iBAQ values. Here, a gene enrichment analysis was used to determine, which biological processes are significantly represented by the twelve most abundant autoantibodies. To reveal pathways that contain antigens that are targeted by the majority of all natural autoantibodies. The results described here are discussed in the section "Natural autoantibodies in health and disease" of the chapter "Comprehensive discussion".

Method

The Metascape analysis was carried out on April 24th 2020 using standard settings (https://metascape.org/) [101]. A detailed description of the settings can be found in the Appendix under "Pathway and Process Enrichment Analysis". Input for the analysis were the target antigens of the twelve most abundant autoantibodies as identified by iBAQ values in Publication 2 [84], **Figure 1**.

Results

The analysis revealed a significant enrichment of the terms "cellular response to stress", "DNA conformation change", "cell cycle" and "negative regulation of organelle organization" (**Figure 5**).

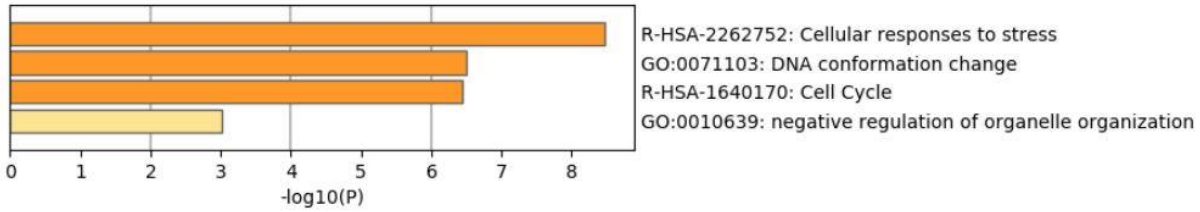


Figure 5 Enriched terms among the 12 most abundant natural autoantibodies identified by MS-AMIDA.

METASCAPE ANALYSIS OF NATURAL AUTOANTIBODIES

Introduction

Metascape is an online tool for the identification of protein-protein interaction networks and gene ontology (GO) enrichment analysis of a provided list of proteins. Here, a Metascape analysis was carried out using the 66 antigens being identified as part of natural immunity in Publication 2, Table 3. The results described here are discussed in the section "Natural autoantibodies in health and disease" of the chapter "Comprehensive discussion".

Method

The Metascape analysis was carried out on April 23th 2020 using standard settings (https://metascape.org/) [101]. A detailed description of the settings can be found in the Appendix under "Settings for Metascape analysis".

Results

The Metascape analysis revealed several enriched pathways and cellular processes. The Top 20 most significantly enriched terms are displayed in **Figure 6**. The results are shown in an interaction network showing connections between the enriched terms (**Figure 6 a**). The terms that were most significantly enriched among the 66 antigens are "Translation", "Translation initiation complex formation", "Metabolism of RNA and the Cellular response to stress (**Figure 6 b**). These terms also appear in close connection in the interaction network, indicating a strong interlacing of these processes.

The protein-protein interaction enrichment analysis revealed that 64 of the 66 antigens show a direct connection to at least one of the other antigens (**Figure 7 a**). Across this network, the MCODE algorithm revealed four clusters of densely functional connected antigens (**Figure 7 b**). Antigens in these clusters are connected via direct interactions and their role in a common pathway or cellular process. Cluster MCODE_1 contains 18 proteins that are related to cell cycle and actin/tubulin folding processes (**Figure 7 c**). MCODE_2 comprises 8 proteins set in cellular processes involved in translation (**Figure 7 d**). The third cluster, MCODE_3, contains 4 antigens involved in mRNA splicing and pre-RNA processing (**Figure 7 e**). Lastly, MCODE_4 includes 3 antigens that are part of the multisynthetase complex related to tRNA aminoacylation (**Figure 7 f**).

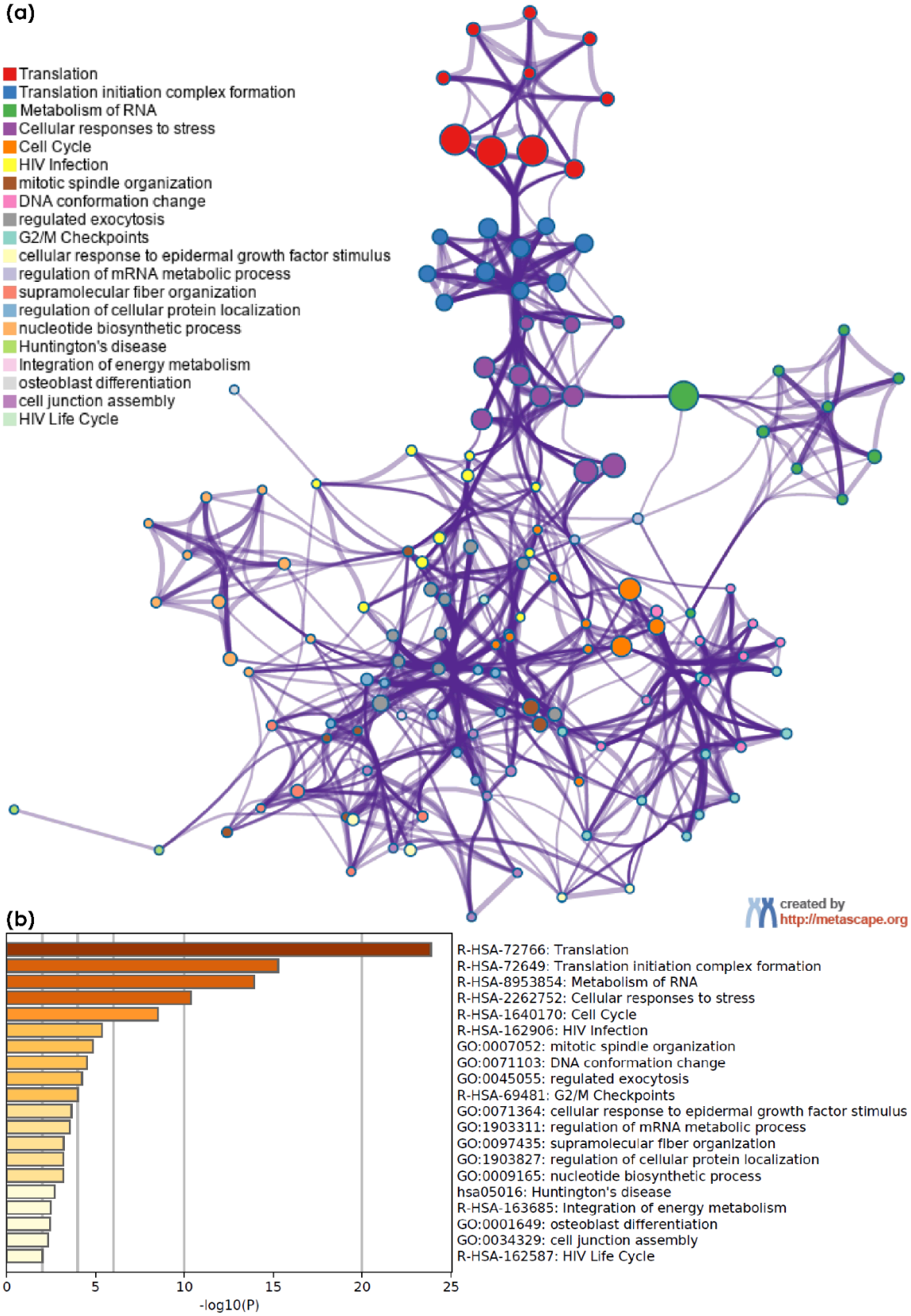
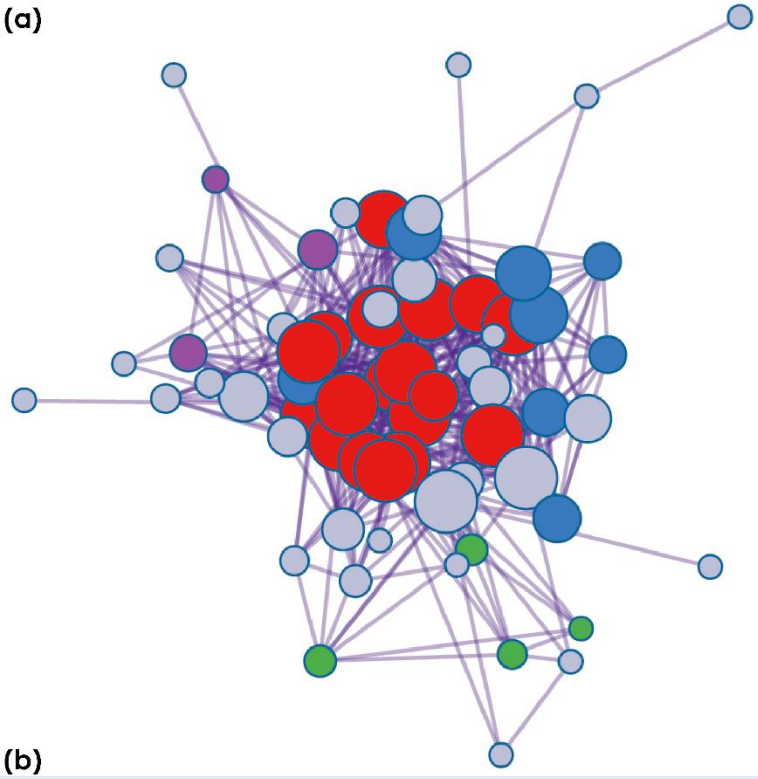
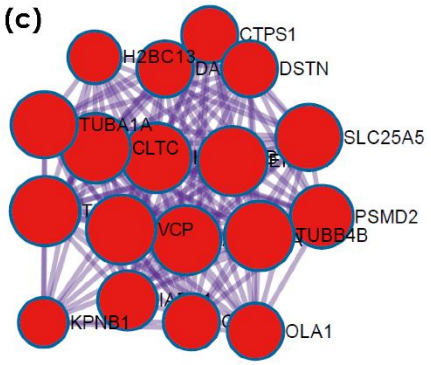


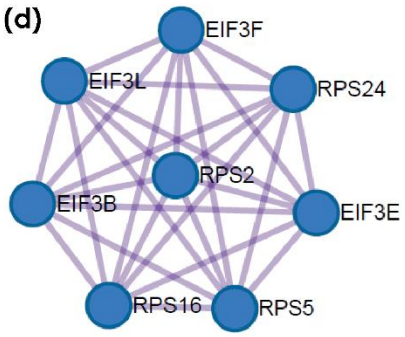
Figure 6 Cytoscape network of Top 20 enriched terms. **(a)** Proteins coloured by cluster ID, where nodes that share the same cluster ID are typically close to each other; **(b)** Bar graph of enriched terms across the list of the 66 input proteins, coloured by p-values.

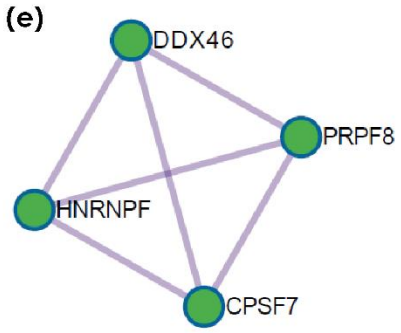


(b)

Color	MCODE	GO	Description	Log10(P)
	MCODE_1	R-HSA-8852276	The role of GTSE1 in G2/M progression after G2 checkpoint	-8.6
	MCODE_1	R-HSA-389960	Formation of tubulin folding intermediates by CCT/TriC	-8.6
	MCODE_1	R-HSA-389957	Prefoldin mediated transfer of substrate to CCT/TriC	-8.4
	MCODE_2	R-HSA-72695	Formation of the ternary complex, and subsequently, the 43S complex	-21.7
	MCODE_2	R-HSA-72649	Translation initiation complex formation	-21.2
	MCODE_2	R-HSA-72702	Ribosomal scanning and start codon recognition	-21.2
	MCODE_3	R-HSA-72163	mRNA Splicing - Major Pathway	-8.5
	MCODE_3	R-HSA-72172	mRNA Splicing	-8.4
	MCODE_3	R-HSA-72203	Processing of Capped Intron-Containing Pre-mRNA	-8.0
	MCODE_4	CORUM:3040	Multisynthetase complex	-10.2
	MCODE_4	R-HSA-379716	Cytosolic tRNA aminoacylation	-9.1
	MCODE_4	R-HSA-379724	tRNA Aminoacylation	-8.3







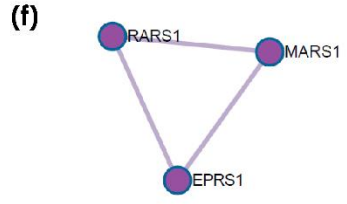


Figure 7 Protein-protein interaction network and MCODE components. **(a)** Protein-protein interaction network. **(b)** List of densely connected network components identified by the MCODE algorithm based on interaction and significant enrichment in GO terms. **(c)** MCODE_1 cluster; **(d)** MCODE_2 cluster; **(e)** MCODE_3 cluster; **(f)** MCODE_4 cluster.

COMPREHENSIVE DISCUSSION

Overview of results

OAG is an increasing threat to the ever-ageing population in developed countries and treatment is limited to decrement of IOP by medical or surgical interventions. These treatments, however, are no cure but can merely delay or stop disease progression. To prevent major vision loss, IOP lowering therapies must be induced as soon as possible. This can only be achieved when diagnostic tools are available that can detect early stages of the disease. Many people are not aware of their disease and suffer from glaucoma-related accelerated RGC degeneration that could be prevented with an early diagnosis and treatment start. New diagnostic tools should be inexpensive and easy to use, so they can find utility as a point-of-care device in precautionary examinations. This would facilitate disease management and facilitate the decision making of clinicians concerning the handling of each patient. The development of autoantibody biomarkers could be the basis for diagnostic tests that can fulfil these criteria. Once reliable biomarker candidates are found, they can be implemented into immunological assays, for example in the format of lateral flow tests that can be easily implemented to clinical routine examinations. Furthermore, there is growing evidence showing that serological autoantibodies are not only able to indicate the mere presence of a disorder but can also give insights into disease progression. This can deliver important information for clinicians to adapt the respective treatment as needed. To make autoantibody profiles available for diagnostics is a huge chance to improve health care and open another path towards personalized medicine making an individual treatment of each patient possible.

The analyses in this piece of research focused around antigens in the TM. The TM is involved in 90% of the aqueous humour outflow from the anterior chamber of the eye. In glaucoma with high IOP, it underlies massive structural and functional modifications that overall lead to increased stiffness of the tissue. The so caused dysfunctionality of the TM is contributing to an impeded drainage of AH and finally leads to an increased IOP. The exact molecular mechanisms underlying IOP-mediated neurodegeneration of RGCs are not investigated conclusively. Regarding the involvement of the immune system in glaucoma pathogenesis, hypotheses arouse that a breach of the retina-blood-barrier is mediated by the rise of the IOP. This, in turn, could trigger B and T cell-mediated autoimmunity, which is also held responsible for sustaining neurodegeneration in retinal cells even after IOP returns to normal values [57]. Autoantigens in the TM have not been investigated so far, although the presence of antigen-presenting cells in this tissue indicates an important role in the ocular immune response [102], especially as also other types of immune cells (B cells, natual killer T cells, mast cells, macrophages) were found in the TM[103]. As an important site in glaucoma

pathogenesis, the TM was assumed an interesting study object to further elucidate immunological mechanisms in glaucoma.

The aim of the research described in this dissertation was the profiling of new autoantibody biomarker candidates to antigens in the TM in the serum of OAG patients and gathering new information on the general serological autoantibody repertoire as an additional goal. This was achieved with different immunoproteomic techniques using SERPA (Publication 1), MS-AMIDA (Publication 2) and protein microarrays (Publications 1 – 3).

In the first approach, covered in Publication 1, elevated levels of anti – VDAC2, anti – CALD1 and anti – PGAM1 antibodies were found elevated in the serum of POAG patients. This was observed in an initial profiling step using SERPA, followed by a validation of promising candidates via protein microarray. The newly detected candidates were used in a biomarker panel together with anti-HSPD1 and anti-VIM autoantibodies, which are already known putative markers from previous studies. These are discussed in detail in Publication 1. With the application of an artificial neural network, POAG patients could be classified from non-glaucomatous subjects with a specificity of 93% at 81% sensitivity.

In the second approach, a more holistic tactic not only identifying glaucoma-specific biomarker candidates but also giving a more detailed impression of the natural autoantibody repertoire to TM antigens was chosen. Using MS-AMIDA, 66 antigens were identified as physiological targets of autoantibodies, whereas 21 self-antigens appeared in relation to POAG. These antigens comprise proteins that are significantly enriched in the PDGFRB pathway that is possibly involved in TM fibrosis during POAG pathogenesis (Publication 2, Figure 6). The alteration of autoantibody levels in sera of POAG patients possibly reflect pathological changes in this pathway, but could also be directly involved. Among the POAG-related antibodies, anti – TARS, anti – C1QBP and anti – PNMA2 autoantibodies showed significantly higher levels in POAG patients compared to the control group and therefore where evaluated as putative glaucoma biomarkers. The application of a random forest algorithm with the microarray data set enabled a classification of POAG patients with an accuracy of 84%.

To further investigate whether serological antibodies also vary in different OAG subgroups, serum samples of POAG, NTG and PEXG patients in comparison to a non-glaucomatous control group were analysed by protein microarray. Here, the levels of ten serological autoantibodies were significantly altered among all groups. Particularly outstanding results were obtained for anti – ADRB2 and anti – Clathrin autoantibody levels. ADRB2 autoantibodies were significantly more abundant in sera of NTG and POAG patients, whereas anti – Clathrin antibodies were particularly low in patients suffering from PEXG. With k means clustering, patients were grouped in three clusters according to their autoantibody profile. The first cluster primarily represented control

samples, the second NTG + POAG patients and the third PEXG patients. This indicates a potential for the detected antibodies to classify glaucoma subgroups and should be evaluated in a larger study group. For an insight into the common characteristics of previously identified glaucoma-related autoantibodies in this and other studies, a protein-protein-interaction analysis, as well as a gene onthology (GO) enrichment analysis, was carried out. The analysis revealed a densely connected network of six antigens by Metascapes' MCODE algorithm. These proteins are HSPA1A, HSPD1, YWHAZ, ENO2, PGAM1 and VDAC2. For these, a strong GO enrichment for the cellular components 'myelin sheath' and 'mitochondria' was revealed. Several previous studies provide evidence suggesting an important role of these sites in glaucoma pathogenesis.

Overall, the work described in this dissertation revealed eight potential glaucoma biomarkers that appeared promising as targets for further validation studies and possible implementation in clinical assays. Also, an antibody-based classification of glaucoma sub-groups seems possible. Serological antibodies covered by the data in the publications of this doctoral thesis appeared to be closely linked to pathological processes and sites in glaucoma pathogenesis. However, information on whether these autoantibodies are causative or an epiphenomenon of glaucoma can not be drawn from the data obtained. Different aspects of the results of the studies included in this dissertation, as well as their methodological drawbacks and short-comings, will be elaborated in the following sections.

Immunoproteomic methods for autoantibody profiling

In this thesis, different immunoproteomic methods were applied to screen serum samples for glaucoma specific alterations in the natural autoantibody profile. The biggest catch for all available profiling techniques is the presence of a large amount of diverse natural IgG autoantibodies, compared to disease-specific antibodies that might only represent small population of the total IgG. Therefore, application of different methods seems best to increase chances for their detection. Serological proteome analysis (SERPA) and mass spectrometry-assisted autoantibody-mediated identification of antigens (MS-AMIDA) were applied as explorative methods in the initial discovery step. These mass spectrometry-based techniques are very time and resources consuming and therefore can only be applied to relatively small sample sizes. This leads to a limited statistical significance of the results making further validation steps mandatory. To fulfil this demand, the autoantibody candidates from the early discovery phase were forwarded to a first validation step, taking advantage of the protein microarray technology. This high-throughput method allows for the simultaneous analysis of multiple targets in hundreds of samples, and a high number of biological replicates could be tested for their autoantibody reactivity against the selected antigen targets. Biomarker candidates, which pass through this stage are promising targets for further evaluation in orthologous methods that are closer to clinical routine testing, such as the often used ELISA. Such final candidate verification paves the way for development of specific clinical tests, ideally to be utilized as a point-of-care device. Although the methods used in this dissertation fit well to the demands of the biomarker pipeline, they have some limitations. To cover this in the context of this work, the methods shall be examined and discussed below.

SERPA is a classical immunoproteomic method for the detection of disease-specific autoantibodies. The method is a combination of 2D polyacrylamide gel electrophoresis (PAGE), western blotting and mass spectrometric identification of proteins. It has been used for the detection of biomarker candidates in several diseases like cancer [104-106], diabetes [107] or multiple sclerosis [108]. However, some known disadvantages come with 2D western blotting. On the one hand, the proteins that can be analysed with this method are restricted to a defined pH range, allowing only the display of proteins with corresponding isoelectric points and 2D PAGE also restricts the size of the analysable proteins. This implies that proteins with high or low isoelectric points, as well as large proteins can not be investigated by this method. Furthermore, SERPA favours the detection of high abundant proteins. Protein spots of high-level proteins can mask low abundant protein spots and prevent their identification by mass spectrometry. This can bias the results towards high abundant proteins and aggravates the detection of autoantibody reactivity to low-abundant antigens. Another drawback is the circumstance that only linear, non-conformational epitopes

can be detected with this method since proteins are denatured during sodium dodecylsulfate-PAGE. This infers that many natural epitopes are destroyed and the reactivity of serological autoantibodies might be a false negative. On the other hand, linear proteins could present epitopes that would never occur in a physiological state and therefore might represent neo-epitopes, which could induce false-positive signals. Results obtained by such an experimental setup therefore should be reviewed carefully and demand further validation steps. Besides these methodological flaws, the method also reveals the heterogeneity of autoantibody repertoires, as there are enormous differences between individual immunoblots, which was also observed in the study included in this doctoral thesis [83]. This indicates high individual diversity of natural autoantibodies. These results are in line with the findings of other researchers, showing that the number of serological IgG varies strongly among individuals [70].

Another approach for the *de novo* profiling of serological autoantibodies was the MS-AMIDA. The idea of the AMIDA methodology was first described by Gires et al. in 2004 [99] using extracted autoantibodies as bait in an immunoprecipitation experiment, followed by 2D PAGE analysis and mass spectrometric protein identification. This approach has been performed and modified thereafter by various research gruops. The method has been applied, inter alia, for the identification of novel autoantibodies in systemic lupus erythematosus [91], immune thrombocytopenic purpura [109] and cancer [99]. As this immunoproteomic approach requires a large amount of source protein for the immunoprecipitation step, to enable the detection of low-abundant antigens, the usage of tissues with highly limited availability such as the TM are challenging. TM tissue biopsies are only available through trabeculectomy surgeries or as post-mortem material and trabeculectomy samples are too small for comprehensive proteomic studies. Post-mortem human eyes for research purposes are scarce, as the eye bulbs are often donated to be used for corneal transplants, and therefore are not available for research purposes. Immortalized human cell lines yielding unlimited source material can bridge this gap. Using cell lysates, however, makes the method prone to allogenic cross-reactions [110] and viral transfection of the cells might introduce virus antigens to the cells and bias the results.

In the different experiments presented within this thesis, partly different antigens were observed in the AMIDA and SERPA analysis. Of the 15 distinct antigens identified in the SERPA analysis only three were also identified in the MS-AMIDA approach with high confidence. The remaining were either not detected (7 antigens), were not detected with high confidence and reproducibility (4 antigens) or another isoform was detected (1 antigen). This is most likely be caused by different protein sources used for the experiments and usage of different protein extraction methods. For MS-AMIDA, human immortalised cell lines where used, whereas porcine TM tissue lysates were used for SERPA. Other groups have also observed differences in immunoreactivity investigated with the same immunoproteomic approach based on the protein source that was

used for the analysis [104]. Here, immunoblots inucubated with the same serum samples but using different cell lines and tissues as antigenic extracts failed to detect every antigen in every setup.

Both profiling methods used are laborious and allow only a small number of samples to be tested in one experiment. Validation of the obtained results is necessary with orthologous methods and in larger sample sizes. To this end, the application of a targeted, high-throughput approach is unavoidable. The protein microarray technique has evolved in the last decades and enables the simultaneous analysis of a high number of samples and targets with high sensitivity by requiring only a small amount of sample. This method comprises the immobilisation of recombinant or purified proteins on a solid surface. Usually, microarray slides with different coatings are used for that purpose. There are many different surface coatings available, with nitrocellulose (NC), NHS and epoxy polymers being the most common ones. In the studies included in this thesis, NC slides were used for all array experiments. The binding of proteins to NC membranes is based on hydrophobic interactions and this immobilisation can affect the tertiary structure of the proteins. This implies that the antigens in microarray experiments do not necessarily retain their natural conformation. As already mentioned for SERPA experiments, this might destroy conformational epitopes and on the other hand expose unphysiological linear epitopes [111]. Furthermore, NC as a microarray surface leads to a randomized orientation of the printed proteins, which can introduce poor reproducibility and low sensitivity of the assay [112]. The method is also prone to the introduction of batch effects, making comparability and reproducibility of the arrays a challenging task. To tackle this methodological flaw, several normalisation methods have been suggested by different research groups, like Loess normalisation, mean/median centring or the use of standard samples in two-colour arrays as internal control [113,114]. These normalisation strategies, however, have their drawbacks and might introduce new biases to the data [114]. At this time point, no consensus of the best normalisation strategy for antigen microarray experiments has been reached. Our approach to this problem was the normalisation of signal intensities to an IgG standard included on each subarray using median centring. To avoid large batch effects, only single batch experiments were performed and samples were run on consecutive days. Another element that needs to be considered is that the method requires high-quality recombinant proteins. Ideally, antigens should be used as functional full-length proteins and be soluble in a buffer that does not interfere with the microarray spotter. In our case, using a non-contact piezoelectric array dispenser, buffer composition is especially essential to the success of array production. Buffers with high viscosity or high concentrations of detergent make the solution hard to handle for the array spotting system. Furthermore, post-translational modifications (PTMs) can have a major impact on the binding affinity of the antibodies. But even though, production of recombinant proteins

containing relevant eukaryotic PTMs is possible, prediction, to which extent the pathologic target antigens underwent disease-specific PTM alterations is not possible. Relevant antigens could also carry other forms of modifications, like truncations, mutations, conformational changes etc., that arise with pathology. All these details can hardly be covered by reasonable means. Although, the use of recombinant proteins carrying a biotin carboxyl carrier protein tag from baculovirus-insect cell expression systems seems to be a good solution to some of the known difficulties. This modification promotes correct protein folding and allows uniformly oriented immobilisation of proteins on the array surface, increasing sensitivity and reproducibility [115]. However, the accessibility of suitable, commercially available proteins is limited and represents the main reason for high-quality arrays to be expensive. Peptide arrays could be a solution to this. Usually, oligomeric peptides are cheaper in production and are also easier to handle experiment-wise although, due to the loss of tertiary protein structure in these linear peptides, the arrays might suffer from a loss of sensitivity. Following initial discovery and early validation steps, an epitope screening of candidate antigens could reveal promising targets to be analysed *via* peptide arrays. This opens up auspicious opportunities for the usage in clinical diagnostics.

Characteristics of self-antigens in glaucoma-specific immunity

A great variety of different studies by different research groups, including ours, have identified glaucoma-related autoantibodies by various immunoproteomic methods. A summary can be found in **Table 2**. Undeniably, it is of great interest to investigate the specific role of these autoantibodies in the course of the disease. It has not yet been possible to answer the question whether the antibodies are a direct cause or a consequence of the pathological alterations. With glaucoma being a multifactorial and complex disease, such investigations are no trivial task. An important aspect to enhance our understanding of the immunological processes that are involved in glaucoma is the comprehensive characterisation of the identified disease-related antigens. One inevitable question concerns possible similarities among the detected self-antigens. Answering this question is crucial to elucidate the causes of an autoantibody response, possibly as part of natural immunity. Backes et al. analysed the properties of antigens from different publicly available datasets, including data from healthy individuals and cancer patients [116]. They found that highly evolutionary conserved proteins that are part of cellular structures are particularly prone to become immunogenic and elicit an antibody response. Specific sequence motifs were enriched among the self-antigens, including coiled-coil motifs, ELR motifs, and Zinc finger DNA-binding motifs. Also, immunogenic proteins often showed a binding capability to nucleic acids, including RNA, and were enriched in the cellular components ribosome or splicosome. Another study found a high immunogenic potential of evolutionarily conserved proteins and linked these to a targeted immune reaction against prokaryotic organisms of the commensal flora caused by molecular mimicry [57].

The existence of such common characteristics have not been investigated for glaucoma-related antigens. GO enrichment and protein-protein interaction network analyses were carried out for currently known verified glaucoma-related autoantigens in Manuscript 3 of this thesis (**Publication 3**) and more specifically for identified TM antigens in a more explorative setting in **Publication 2**. In general, glaucoma-related antigens showed significant enrichment in biological processes including mRNA regulation and apoptosis on different levels. This shows a strong relation with glaucoma pathogenesis, especially as apoptosis is widely recognised as the main pathway for RGC demise in glaucoma [117,118]. Furthermore, antigens were enriched in pathways resulting in nucleotides and nucleic acids breakdown (GO:0034655 nucleobase-containing compound catabolic process). When considering the connection of the corresponding genes with known disease-relevant genes, a significant association with neurological or eye related diseases was apparent (**Table 4**). Additionally the results showed that ageing and immune processes are among the associated disease classes. A strong connection of the antigens with diseases of this spectrum, clearly

allows the characterisation of glaucoma as an age-related, neurodegenerative eye disease with probable involvement of immunity in its pathogenesis.

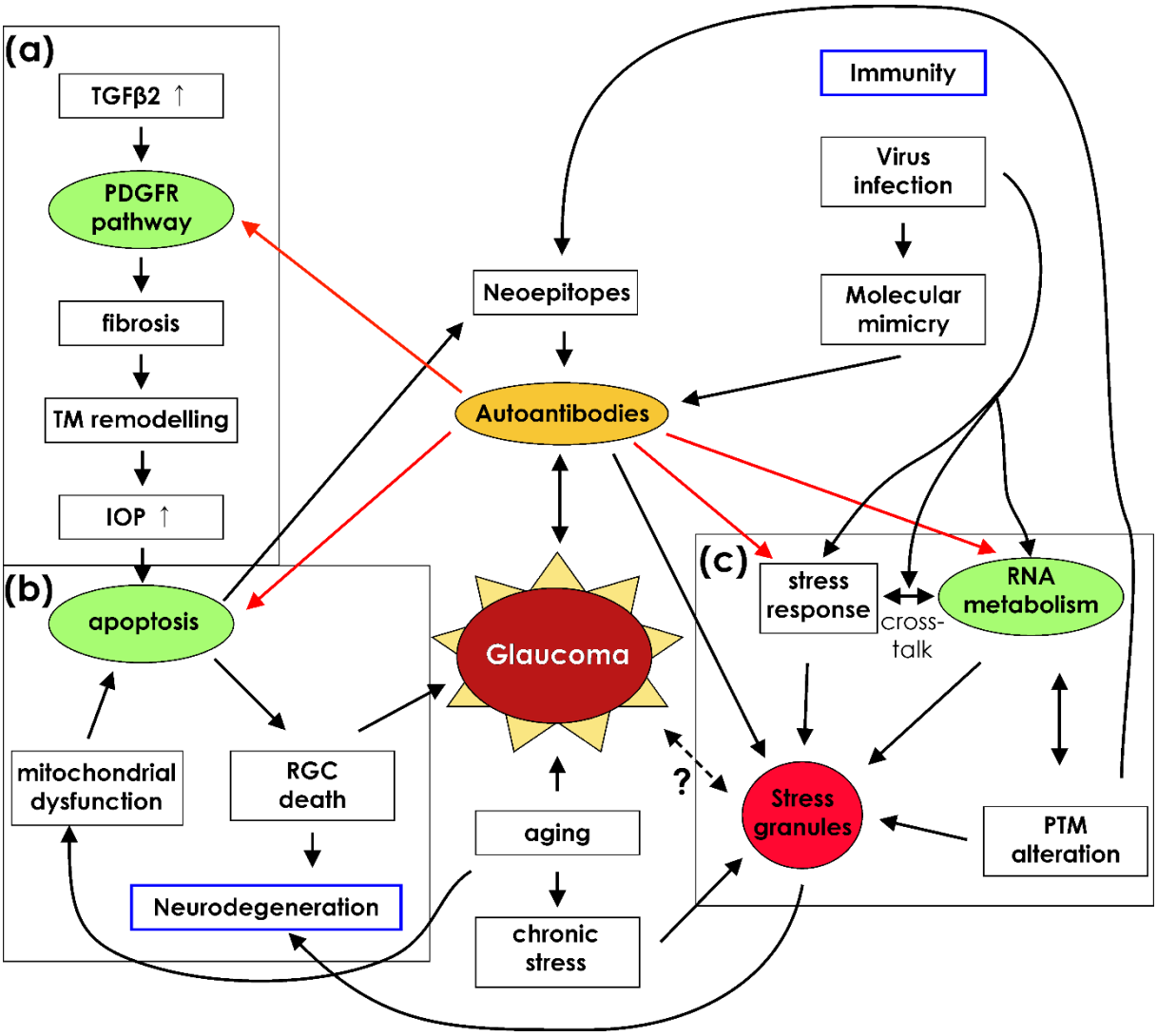


Figure 8 Schematic model of biological processes involved in glaucoma pathogenesis mentioned in this dissertation. Pathways that are overrepresented by glaucoma-related antigens are highlighted in green circles. **(a)** Patients with OAG show increased levels of TGFβ2 in AH [119]. This multifunctional protein can induce PDGFR expression, augmenting the PDGFR pathway, which is known to be implicated in tissue fibrosis [120]. An overrepresentation of antigens belonging to this pathway was discovered as targets of glaucoma associated autoantibodies in this thesis, while other researchers found that some autoantibodies might induce PDGFR signalling [121]. Fibrosis impairs TM function, increasing AH outflow resistance [33], which ultimately results in an increased IOP [122]. **(b)** High IOP is related to RGC death by apoptosis [117]. Also, mitochondrial dysfunction in glaucoma mediates ROS production, leading to caspase-independent apoptosis of RGCs [123]. Autoantibodies to apoptosis-related pathways were identified in relation to glaucoma within this doctoral thesis. Apoptosis is known to produce neoepitopes, favouring the occurrence of autoantibodies [124] that on the other hand are involved in clearance of apoptotic cells via complement activation [68]. **(c)** Autoantibodies to antigens involved in the cellular stress response and the RNA metabolism have been associated with glaucoma within this thesis. Especially heat shock proteins and other small chaperonins involved in the stress response are targeted by serological autoantibodies. Newly discovered were the autoantibodies targeting proteins of the RNA metabolism. Virus infections can cause

alterations of the RNA metabolism in favour of virus protein production and trigger the cellular stress response, which also induces crosstalk between both pathways, attenuating mRNA translation [125]. Stress response, particularly unfolded protein response, is active in glaucoma, as endoplasmic reticulum stress is largely promoting TM and RGC demise [126]. The formation of stress granules is associated with both pathways and is also linked to chronic stress related to aging processes. Stress granules are related to neurodegeneration in other diseases [127] and can contain target proteins for serological autoantibodies [128]. Alteration of PTMs of RNA-binding proteins regulates stress granule dynamics [129], but PTM alterations can also generally introduce the formation of neoepitopes. Whether there is a direct implication for stress granules in glaucoma pathogenesis remains to be determined, although few research hints in this direction [130,131].

When now regarding solely antigens derived from the TM, a strong association of these proteins with processes of translation, mRNA transport and the PDGFRB pathway became apparent. Here, too, mRNA-related processes emerged as targets for autoantibodies, thereby representing signalling pathways that indicate a particular susceptibility to mRNA-associated proteins to become immunogenic. A cellular structure that could be related to these findings is an assembly of ribonucleoproteins (RNP) clusters that contain mRNA and a congregation of various mRNA – binding proteins, ribosomal units as well as decay enzymes and scaffold proteins, which are known as cytoplasmic RNA granules [132]. They function as storage for translationally silenced mRNA and regulate mRNA translation or decay. These cytoplasmic features also can be put into relation with glaucoma pathogenesis, as mutations in RNA granule components have been reported in glaucoma and cataract [131]. More precisely, loss-of-function mutations in the Tudor domain-containing 7 protein (TDRD7), a post-transcriptional mRNA regulator, were linked to glaucoma, as TDRD7 null mice develop characteristically elevated IOP and optic nerve damage. The authors further concluded that a lack of TDRD7 could lead to impaired protection from stress in the TM and thereby inducing a glaucoma phenotype. Another study also found a link to disturbed mRNA regulation, as glaucoma-associated alterations in the decay of some cytokine mRNAs in human TM cells have been suggested [133]. The authors found that H_2O_2 stressed TM cells stabilize IL6 and IL8 mRNA via the binding of Hu antigen R (HuR). HuR is a known component of stress granules, a class of RNA granules inducible by different stressors. The location of HuR to distinct cytoplasmic foci suggested a possible formation of stress granules in TM cells exposed to oxidative stress. This could be of relevance as the AH of glaucoma patients shows less antioxidative capacities in comparison to healthy eyes [134]. Although the formation of stress granules in glaucomatous TM has not been conclusively proven yet, their assembly under these pathological circumstances seems very likely. Furthermore, transforming growth factor β (TGFβ), showing increased levels in AH of glaucoma patients, was revealed to induce the formation of RNA processing bodies, another class of RNA granules [135]. Stress granules additionally contain proteins that are not directly involved in RNA

regulation. The TNF receptor-associated factor 2 (TRAF2) is localized in stress granules mediated by its binding to eIF4G [132]. TRAF2 is involved in the regulation of cell survival and apoptosis but is also required for IgM to IgG antibody switching. In this context, the analysis of the IgM/IgG ratio of antibodies to specific antigens in glaucoma patients appears to be an interesting field of investigation that could deliver further insights. Further, the RNA-binding protein Optineurin, which is also associated with glaucoma, is co-localized with classical components of stress granules [136]. This suggests a connection between stress granules, autoantibodies and glaucoma, especially since stress granules have already been described in connection with other neurodegenerative diseases [127].

The here presented results are not the first to report RNA granules as targets for serological autoantibodies. Johnson et al. already reported stress granules and RNA processing bodies as antibody targets in systemic sclerosis [128]. Overall, these findings strongly suggest that RNA granules could be a possible target for autoantibodies in glaucoma, which should be examined further in future studies.

Farther, the PDGFRB pathway, harbouring significantly enriched amounts of self-antigens, shows a direct connection to pathological alterations in glaucomatous TM. In particular, this pathway is involved in fibrotic processes increasing TM tissue rigidity by excessive accumulation of ECM components [137]. This eventually leads to an impeded aqueous humour efflux that is the characteristic precursor for an elevated IOP in glaucoma. Further, abnormal blood coagulation was reported in POAG patients and a possible involvement of the observed age-dependent spontaneous aggregation of platelets was discussed as part of glaucoma pathogenesis [138]. An enrichment of glaucoma-related antigens in blood coagulation underpins the influence of deregulated biological processes on the autoantibody repertoire, as also discussed in **Publication 3**.

Concluding from the findings of these studies, it seems that most signalling pathways that show enrichment of self-antigens have a strong relation to known dysfunctions or pathological alterations in glaucoma pathogenesis. Pathological changes in the PDGFRB pathway, mRNA and apoptosis regulating processes are reflected by alterations in the serological autoantibody repertoire. Targets of glaucoma-related autoantibodies have clear connections to diseases in the neurological, vision and immune spectrum. This can either indicate that these molecular changes induce an altered antibody response or that the autoantibodies are primarily involved in the occurrence of such pathological alterations. However, the former does not preclude possible synergetic effects that might arise through the autoantibody response. The exact mechanisms remain unsettled for now and should be addressed by future investigations.

Altogether, this enforces the hypothesis that autoantibodies can be seen as reporter molecules for altered cellular processes and are surrogates for pathological changes on a molecular level. This, in turn, makes them excellent candidates as biomarkers for glaucoma diagnosis or even for monitoring the course of the disease.

Autoantibody biomarkers for glaucoma diagnostics

In this day and age, tests for autoantibodies are commonly used for the diagnosis of autoimmune diseases. The detection of autoantibodies to disease-specific antigens is an important hallmark of many classical autoimmune diseases like Hashimoto's thyroiditis or type 1 diabetes. Different types of radioimmunoassays and ELISAs are applied for this purpose. Beyond that, a plethora of studies on tumour-associated antibodies is emerging, investigating autoantibodies as potential early biomarkers for various types of cancer [139]. Autoantibodies have been suggested as potential biomarkers for the diagnosis of neurodegenerative diseases as Parkinson's and Alzheimer's disease [17,18,95]. Previous work of our and other research groups revealed glaucoma-specific changes in the natural autoantibody repertoire (**Table 2**). Many of these glaucoma-related autoantibodies have been reported with increased levels compared to non-glaucomatous controls, but levels of some autoantibodies were found to be decreased. These serological antibodies have been suggested as viable biomarkers for early glaucoma detection. Potential biomarkers were identified and evaluated for their diagnostic potential and it was also shown that their levels deviate already in an early stage of POAG [83]. The usage of advanced classification models allowed us to discriminate glaucoma patients from non-glaucomatous controls with a specificity of 89 – 93% at a sensitivity of 79 – 81% [83,84], confirming earlier studies achieving sensitivity and specificity rates of approximately 93% [19]. A comparison to the accuracy of conventional diagnostic tools like OCT, GDx, FDT and HRT, which have a reported mean sensitivity of 78.8% - 84.7% at a mean specificity of 82.8% - 91.2%, demonstrates the huge potential that autoantibody biomarkers offer for glaucoma diagnostics when used in clinical practice [140].

Our studies have shown that the predictive value of single autoantibody biomarkers is very limited as they only have low sensitivity. However, a combination of different autoantibody biomarkers to a panel can highly increase the overall accuracy of disease classification. Advanced computational models such as artificial neuronal networks and random forest algorithms are a promising approach to reveal the full potential of the autoantibody biomarkers in this context. The application of such models can enable good discrimination potential of a selected autoantibodypanel of rather weak stand-alone predictors. Such complex classification algorithms usually require large sample sets with 1000+ cases to compute reliable models. With the given resources, this was not possible in the studies included in this dissertation. The sample sizes in the SERPA study were 60 in the POAG and 45 in the control group. For the MS-AMIDA study, groups of 120 cases each were analysed. As this is still far from the recommended size of sample sets, the studies should rather be regarded as early stage of the biomarker pipeline and proof-of-principle studies. Whether the observed accuracy of the putative biomarkers can also be achieved with large sample sets needs to be evaluated. Generally, higher number of cases enables better model

building and predictions, and has the potential to improve the accuracy of disease classification.

One big advantage of autoantibody tests for diagnosis is that they can eventually be provided as inexpensive formats like lateral flow tests. These can be used as point-of-care devices, applicable in routine examinations to promote early detection of glaucoma. The achievement of such tests is of utmost importance, as no curative treatment for the disease exists and the immediate start of IOP-lowering therapies is the only way to prevent vision loss. The test for serological autoantibodies requires minimal sample amounts and only minimally invasive blood sampling is necessary. The translation of an autoantibody biomarker panel to such assay formats, however, remains a challenging task. The so far identified biomarker candidates are considered part of the natural autoimmunity and therefore are also present in healthy subjects. Disease relevant changes only occur on a quantitative base, which makes it necessary to quantify the serological levels of the autoantibodies. So far, this has not been achieved in a multiplexed quick test format.

As already mentioned, elevated IOP is considered as a trigger for the onset of neurodegenerative events in RGCs, also via autoimmune processes [57,61]. This indicates that pathological changes in the TM, which are causative for the increase of IOP, preceded the damage of RGCs. As shown in Publication 1, the detected biomarker candidates already showed significantly altered levels in mild forms of POAG. This, in turn, designates glaucoma-related antibodies to TM antigens as potential predictive markers and changes in autoantibody levels could generally be early reporters of deregulated biological processes.

However, autoantibody-based diagnostic tests suffer from a general lack of specificity, since many of the identified glaucoma-related autoantibodies are also described in relation to other diseases. For instance, autoantibodies to a-enolase were reported to be found in 20% of autoimmune biomarker studies [141]. Besides this, other glaucoma-related autoantibodies appear frequently in several diseases. For instance, autoantibodies to HSPD1 have also been detected in patients with different malignancies [104,106], and anti-VIM antibodies were also found in cancer patients [104,106], as well as in subjects with idiopathic pulmonary fibrosis [142] or type 1 diabetes [107] and antibodies to a-fodrin were also detected in the serum of patients with Sjögren's syndrome [143]. In this context, the citrullination of proteins seems to increase their immunogenicity, as shown for a-enolase and vimentin in rheumatoid arthritis [144]. Furthermore, autoantibodies showing strong relations to diseases are also frequently detected in the studies control groups. This could indicate another flaw in many immunoproteomic studies, which is caused by the inconsistent definition of the studies control groups. Sometimes, patients suffering from autoimmune or neurodegenerative diseases are excluded [19,83], as well as subjects with any kind of other systemic diseases

[73,145]. Of course, this creates an unrealistic setup for the clinical application of autoantibodies as diagnostic biomarkers. The exclusion of patients having such features eleviates the detection of putative markers but also limits their potential to discriminate different diseases of the same spectrum. However, the circumstance that many of the detected autoantibodies are frequently detected in different diseases and also control groups in various studies indicates that they are part of the natural autoantibody repertoire, which will also be discussed in the following section. Natural autoimmunity is suggested to have, among other roles, regulatory functions, for example through receptor activation by ligand mimicking [146] or regulating clearance of apoptotic cells by binding to specific membrane antigens [147], and therefore their levels must be maintained at an equilibrium. A dysfunction of the homeostasis of natural autoantibodies could render the individual susceptible to pathologies. As can also be seen in the studies included in this dissertation, not only the presence but also the abundance and even the antibody properties appear to be affected in a disease-dependent manner.

Another aspect, regarding levels of a specific antibody being altered in multiple diseases, could be that the autoantibodies are representative for pathological processes, rather than the specific disease. Autoantibodies in sera of glaucoma patients are significantly enriched among proteins in the PDGFR pathway. However, these antibodies not only occurred in glaucoma samples with an indicated relation to TM fibrosis, but autoantibodies to PDGRF were also reported in patients suffering from systemic sclerosis, displaying fibrotic alterations as major hallmark [121]. There also seems to be a bigger overlap of TM antigens with antigens detected in relation to systemic sclerosis [128]. This could indicate that the autoantibodies are not exclusively disease-specific but rather an expression of single molecular alterations that in sum lead to the symptoms characterizing the disease. Autoantibodies found in association with different diseases could represent a molecular connection between these disorders, for instance when sharing similar symptoms.

This all proves the necessity of using an antibody biomarker panel for a potential diagnostic test, as the markers need to represent several affected biological processes that are characteristic for glaucoma pathogenesis. This seems to be mandatory as autoantibody biomarkers can be similar in other neurodegenerative or autoimmune diseases introducing another caveat to the implementation of autoantibody-based quick tests. As many of the suggested glaucoma biomarkers seem to be part of natural immunity, quantification, rather than determination of absence or presence of the antibodies is required. In combination with the need for multiplexed testing of the aforementioned panel of autoantibody biomarkers, the development of a reliable quick test that can be used with minimal equipment is a demanding task. Although advances are continuously made towards this goal, a lot of work and effort is still needed to achieve it.

A good approach to solve the problems discussed for autoantibody biomarkers might be the implementation of a tactic involving 'multi-omics' [148]. For instance, glaucoma patients do not only show alterations of the serological autoantibody repertoire but also in other proteins and their genome. Igarashi et al. reported significantly lower levels of the brain-derived neurotrophic factor in serum of POAG and NTG patients [149], but also proteins related to oxidative stress (e.g. glutathione peroxidase and superoxide dismutase) or endothelin-1 show altered levels in the blood of glaucoma patients and have been discussed as potential biomarkers [150]. Furthermore, miRNAs have also been suggested as viable biomarker candidates [151]. To fully exploit the potential of all novel serological biomarkers, a combination of different marker types in an omics approach could yield the best results.

Concluding, the scientific work described in the publications included in this dissertation revealed several novel serological autoantibodies, whose abundance is related to the glaucoma disease stage. After an initial discovery step, using either SERPA or MS-AMIDA, six candidate biomarkers passed the early qualification phase of the biomarker pipeline (**Figure 3**). The high-throughput data obtained by microarray analysis enabled the application of classification algorithms, with which computational models were build that could classify glaucomatous from non-glaucomatous subjects with high sensitivity and specificity. Additionally, two markers for the discrimination of OAG subclasses were identified. For the next steps in the biomarker pipeline, eight biomarker candidates (Anti-VDAC2, anti-PNMA2, anti-CALD1, anti-TARS, anti-C1QBP, anti-PGAM1, anti-ADRB2 and anti-CLTA autoantibodies) need to be tested in larger study cohorts, with 1000+ participants, ideally from different study sites. The data collected in such a study can be used to finally validate the antibody biomarkers and pave the way for clinical assay development.

Natural autoantibodies in health and disease

Natural autoantibodies are antibodies against endogenous antigens that occur whithin a physiological state of an organism. In general, the features of the physiological autoantibody repertoire is not well characterized yet, but include antibodies of the IgG, IgA and IgM class. Abundance of serological IgG to numerous antigens was reported [70], but clinical implications of this phenomenon are not sufficiently examined. Especially bioinformatics approaches defining overrepresented pathways or cellular components among the antigens, in health and disease, are not widely used. The slightly better-investigated isotype comprises antibodies of the IgM class. They are regarded to be involved in cellular homeostasis and even have been attributed protective effects, as described in the introduction in greater detail. The function and origin of IgG class autoantibodies, however, is less well investigated. These antibodies are abundant in the serum of healthy subjects and their total repertoire is complex, involving reactivities to hundreds of different self-antigens [70]. It has been hypothesized that the occurrence of IgG class autoantibodies can be regarded as an adaptive process to clear cellular debris from pathology-specific damage and thereby assist tissue homeostasis. To understand how certain proteins become immunogenic, it is of importance to characterize these antigens and search for similarities that are possibly involved in their immunogenicity. Similar to classic proteomic analyzes, immunoproteomics searches for disease-related deviations of the immune system from its physiological state. This is not limited to the investigation of antibodies, but this aspect is the focus of our research. To understand the changes of autoantibody levels in pathology it is also necessary to determine and characterize the physiological state of the autoantibody repertoire as a whole.

To investigate the physiological state of the autoantibody-ome of IgG class antibodies to targets in the TM, the MS-AMIDA approach was used identifying 66 antigens that can be assigned to the natural autoantibody repertoire [84]. The molar quantities of the antigens identified with this approach were measured by mass spectrometry using MaxQuant iBAQ values. Proportionality of the amount of captured antigen with the abundance of circulating autoantibodies can be assumed. The results show that twelve proteins make up 67.3% of all identified antigens (**Publication 2, Figure 1**). Thereby it can be assumed that these represent the twelve most abundant circulating, serological autoantibodies. Taking a closer look at these high abundant antibodies, using a gene enrichment analysis, it was revealed, that the target antigens are most significantly enriched in "cellular response to stress" (see chapter "Gene enrichment analysis of most abundant natural autoantibodies"; **Figure 5**). GO enrichment analysis of all 66 identified targets of natural autoantibodies showed that processes of Translation, Metabolism of RNA and Cellular response to stress are represented by the antigens highly significant (see chapter "Metascape analysis of natural autoantibodies"; **Figure 6**). A deeper analysis based on protein interaction and

pathway affiliation of the antigens using Metascape's MCODE algorithm revealed four clusters (see chapter "Metascape analysis of natural autoantibodies"; **Figure 7**). Three of these clusters contain proteins enriched in different cellular processes involved in translation and RNA metabolism. Regarding these results, it appears that natural autoantibodies predominantly target self-antigens involved in these processes.

That is in line with the findings of other groups. For instance, Backes et al. analysed data on self-antigens from different studies and found an enrichment of the characteristics of RNA and DNA binding [116]. Their sequence-based analysis revealed an enrichment of RNA binding motifs among the antigens and they are often part of or associated with ribosomes and spliceosomes. The authors concluded that ribosome-associated proteins are evolutionary highly conserved and therefore, molecular mimicry originating from defence to pathogens might play an essential origin for their occurrence. Molecular mimicry in the occurrence of autoantibodies has been suggested to be induced by bacteria of the commensal micro-flora [57] or as a consequence of virus infections [152]. Autoantibodies to RNA – associated antigens have also previously been reported in several autoimmune diseases like systemic lupus, Sjögren's syndrome and other connective tissue diseases [153].

Cross-talk occurs between cellular stress response and RNA metabolism, especially in response to virus infections that are also involved in the mechanism of molecular mimicry [125]. The occurrence of mainly RNA metabolism- and stress response - associated autoantibodies could therefore represent a connection to an anti-virus response. Picornaviruses, for example, can alter the RNA metabolism of its host to facilitate virus reproduction [154]. Here, autoantibodies could occur as a countermeasure to proteins of the hijacked cellular processes. Exposure to viruses is common and on average a history of infections with 10 viral species per person was reported [155]. Virus infections could have major effects on host immunity indicating that past viral infections could significantly shape the serological autoantibody repertoire. Another indication for this concept is the observation that IgG levels linearly increase with age which could relate to the increasing number of virus infections a person has gone through. A comprehensive analysis of the correlations between virus infections and the individual autoantibody fingerprint could deliver new insights into this context.

In general, weak affinity to self-antigens is widely considered as a normal feature of natural immunity, whereas high-affinity autoantibodies are regarded as a cause for autoimmune pathologies. Under this premise, it seems conceivable that the majority of autoantibodies are present at all times, either in low abundance but also with low-affinity to their respective targets. Beyond this, slight alterations of target antigens, like altered PTMs, but also mutations, truncations or failures of protein folding, can cause an amplification of antibody-antigen-binding properties. These changes of binding affinity can cause pathological alterations or synergetic effects in disease progression.

Most immunoproteomic techniques only refer to antibody abundance but do not determine their binding affinity. Possibly, antibodies with higher affinity can be detected with higher abundance, as the equilibrium is shifted in favour of association instead of dissociation. For example, the microarray technology measures the quantity of binding at a defined time point, which in turn is dependent on antibody quantity in the analysed sample as well as their binding affinity.

These arguments promote the hypothesis, that the involvement of autoantibodies in various disease-related processes may mainly be driven by alterations of natural autoantibody affinity rather than the occurrence of newly introduced autoantibodies. This is supported by the findings in Publication 2 [84] where differences in autoantibody reactivity were higher between healthy and glaucomatous antigen source as between the used antibody sources (non-glaucomatous vs. POAG). The immune system then could distinguish between "normal" and "abnormal" state of the organism and assure maintenance of homeostasis. This concept is also promoted by Poletaev et al. [156]. They suggest that the immune system, including the natural autoantibody repertoire, permanently screens the recent antigenic situation in the organism, to distinguish between physiological and pathological conditions to eventually induce compensatory processes [156]. Thus the natural autoantibody repertoire can monitor the physiological state of the organism and can undergo alterations as a response to changed antigenic properties. This could deliver explanations for the prevalence of autoantibodies in various diseases to components of disease-related pathways, like the PDGFR pathway in TM fibrosis in POAG.

Overall, these features of the antibody-ome render it highly valuable for prognostic and diagnostic purposes. However, human autoantibody-ome is far from being deciphered. More studies need to be conducted that analyse the antibody repertoire in wholistic approaches to depict a complete image of the natural autoantibody repertoire in health and disease.

CONCLUSION & OUTLOOK

Glaucoma is a growing threat to eye health, leading to increasing impairment of vision especially in elderly people. With the ever-ageing population, particularly in western countries, advances in disease management are necessary to mitigate the growing burden on the healthcare system. There is a demand for easy-to-use and inexpensive tools for a routine examination of people belonging to the glaucoma risk group. A reliable test, that detects glaucoma in the early stages of the disease can help to prevent vision loss due to glaucoma and help clinicians make correct choices for their treatment. Early detection of glaucoma is not only beneficial for the patient but could reduce overall health care expenses and prevent unnecessary medictation. Specific disease biomarkers play a major role in this approach. In glaucoma, autoantibodies to retinal antigens are altered in sera and AH of affected patients. These features have previously been suggested as autoantibody biomarkers for the diagnosis of OAG. Their origin and their role in glaucoma pathogenesis, however, remained unclear.

The research of this thesis aimed to further characterize the physiological autoantibody repertoire and investigate the potential involvement in POAG pathogenesis, thereby focusing on putative biomarker candidates among the altered autoantibody reactivities. The emphasis of the investigation was on the antigens of the TM. Findings of the immunoproteomic analyses proofed anew a clear involvement of autoimmunity in the pathogenesis of OAG.

The *de novo* profiling of serological autoantibodies in patients with glaucoma but also non-glaucomatous subjects showed a highly complex and heterogeneous picture. Autoantibodies against a multitude of different proteins were observed in every individual. The autoantibody-antigen reactivity, however, was altered for some proteins in the glaucoma groups and were evaluated as potential disease biomarkers.

The antigens identified in this work, as well as antigens discovered in previous studies, showed a distinct relation to glaucoma-related processes and showed a distinct enrichment in the GAD disease classes comprising vision and neurological associated characteristics. This suggests that the autoantibody repertoire could serve as a surrogate of the state of the antigenic proteins. How this is regulated and what exactly triggers can be held responsible for altered autoantibody levels and characteristics remains a task for future studies. With systems and personalized medicine as future perspectives in medical research, the usage of this phenomenon can be exploited for diagnostic, prognostic and predictive purposes. The exact origin and function of the detected autoantibodies though remain elusive and any hypotheses are rather speculative and need to be supported by more evidence that could not be provided in the scope of this thesis.

Although the autoantibody-ome in health and disease has not yet been deciphered, the evaluation of the putative autoantibody biomarkers for glaucoma diagnostics showed great potential for the usage of clinical tools based on this feature. Throughout this thesis, six novel glaucoma biomarker candidates were identified and passed the early evaluation step of the biomarker pipeline. Levels of antibodies to VDAC2, PGAM1, CALD1, PNMA2, TARS and C1QBP were elevated in the serum of patients with POAG. **Publication 1** shows that the levels of the therein detected candidate autoantibody biomarkers were significantly increased already in a mild stage of POAG, increasing their value as potential early diagnostic markers. There is also evidence, that it is possibile to classify different open-angle glaucoma subtypes based on serological autoantibody levels, which was also implicated in earlier studies of our research group [81,157]. The results obtained in Publication 3 show autoantibodies to Clathrin and the β-2 adrenergic receptor especially elevated in PEXG or NTG/POAG patients.

However, the predictive power of single autoantibodies is questionable. No single biomarker candidate investigated in this thesis showed sufficient accuracy. It is mandatory to use a panel of several autoantibody biomarkers in combination with advanced classification algorithms for the development of a reliable diagnostic test. In this thesis, artificial neural networks and random forest algorithms were applied to the retrieved data sets. This allowed a classification with a specificity of 89 – 93% at a sensitivity of 79 – 81%. An important task for future studies though, is the investigation of the predictive potential of autoantibodies, regarding the conversion of OHT patients to glaucoma. This could eventually facilitate decisions on treatment options and prevent unnecessary medications.

A next step towards the deployment of a diagnostic test is the evaluation of a panel of these putative biomarkers in a large study cohort, ideally from different study sites under the application of diverse methods. Should the biomarkers also overcome this stage, a distinct approach for the development of an actual diagnostic test to be used in clinical routine can be undertaken. The ideal outcome of this undertaking is the deployment of a test in the form of a point-of-care device that fulfils the criteria of cost-effectiveness and usability with minimal equipment and professional experience.

Beyond the scope of the characterization of autoantibody profiles in relation to glaucoma, the IgG autoantibody repertoire was analysed more holistically. Especially the results obtained in Publication 2 give new insights into the characteristics of the general autoantibody repertoire in a physiological state of the organism. Autoantibodies of individuals are highly complex and heterogenous, which could be well observed in the 2D Western Blots in Publication 1. Each tested serum revealed a unique pattern of autoantibody reactivities. Up to now, litte data exists on natural occurring autoantibodies, especially not in holistic approaches, yielding a

comprehensive map of physiological antibodies of each immunoglobulin class. In this thesis, 66 antigens as targets of natural immunity were identified with high confidence. These antigens were further characterized in terms of abundance, protein-protein interactions and GO enrichment analysis. The results showed a strong association of the antigens to the cellular stress response and RNA metabolism. Whether the respective autoantibodies have an effect on or are affected by these pathways can not be concluded from the data at hand but opens up an interesting research topic for future studies.

The course of autoantibodies in health and disease is a research field that is not widely studied and leaves a plethora of unanswered questions. A lot of work and effort needs to be spent to gain more insights into the functions of the immune system aside from the defence against pathogens. A better understanding of these processes will also enable us to make better use of the information we gather on alterations of the autoantibody repertoire in diseases. This can not only improve their usage as diagnostic tools but could also open up new treatment options. The optimal goal should be the implementation of personalized medicine in everyday life and the monitoring of the natural autoantibody repertoire has the potential to contribute to this aim. It is still a long way to go before achieving this ambitious goal but the benefits to be expected justify all endeavours.

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ABBREVIATIONS

AH	aqueous humour
CSFP	cerebrospinal fluid pressure
EAG	experimental autoimmune glaucoma
ECM	extracellular matrix
ELISA	enzyme-linked Immunosorbent Assay
FDT	frequency doubling technology perimetry
GDx	GDx-Scanning-Laser-Polarimetrie
GO	gene onthology
HRT	Heidelberg Retina Tomograph
IOP	intraocular pressure
LC	lamina cribrosa
LIPS	luciferase immunoprecipitation systems
MS-AMIDA	mass spectrometry-assisted autoantibody-mediated identification of antigens
NC	nitrocellulose
NTG	normal tension glaucoma
OAG	open-angle glaucoma
OCT	optical coherence tomography
OHT	ocular hypertension
ON	optic nerve
PAGE	polyacrylamid gel electrophoresis
PDGFR	platelet-derived growth factor receptor
PEX	pseudoexfoliation
PEXG	pseudoexfoliation glaucoma
POAG	primary open-angle glaucoma
PTM	post translational modification

RGCs	retinal ganglion cells
RNP	ribonucleoproteins
SEREX	serological identification of antigens by recombinant expression cloning
SERPA	serological proteome analysis
TM	trabecular meshwork

APPENDIX

Settings for Metascape analysis

The following explanation for the settings of the Metascape analysis was taken over from the Metascape analysis output page (https://metascape.org/).

Pathway and Process Enrichment Analysis

For each given gene list, pathway and process enrichment analysis have been carried out with the following ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways and CORUM. All genes in the genome have been used as the enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the accumulative hypergeometric distribution, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testings. Kappa scores are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.

Cytoscape clusters

To further capture the relationships between the terms, a subset of enriched terms have been selected and rendered as a network plot, where terms with a similarity > 0.3 are connected by edges. We select the terms with the best p-values from each of the 20 clusters, with the constraint that there are no more than 15 terms per cluster and no more than 250 terms in total. The network is visualized using Cytoscape5, where each node represents an enriched term and is coloured first by its cluster ID (Figure 2.a) and then by its p-value (Figure 2.b). These networks can be interactively viewed in Cytoscape through the .cys files (contained in the Zip package, which also contains a publication-quality version as a PDF) or within a browser by clicking on the web icon. For clarity, term labels are only shown for one term per cluster, so it is recommended to use Cytoscape or a browser to visualize the network in order to inspect all node labels. We can also export the network into a PDF file within Cytoscape, and then edit the labels using Adobe Illustrator for publication purposes. To switch off all labels, delete the "Label" mapping under the "Style" tab within Cytoscape, and then export the network view.

Protein-protein Interaction Enrichment Analysis

For each given gene list, protein-protein interaction enrichment analysis has been carried out with the following databases: BioGrid, InWeb_IM, OmniPath. The resultant

network contains the subset of proteins that form physical interactions with at least one other member in the list. If the network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm has been applied to identify densely connected network components. Pathway and process enrichment analysis have been applied to each MCODE component independently, and the three best-scoring terms by p-value have been retained as the functional description of the corresponding components.

Contribution to manuscripts

PUBLICATION 1

Autoantibody Biomarker Discovery in Primary Open Angle Glaucoma Using Serological Proteome Analysis (SERPA)

Vanessa Beutgen planned the experimental design of this study, performed SERPA and microarray analysis, interpreted the data, and wrote the manuscript. Natarajan Perumal performed mass spectrometric analysis. Franz Grus and Natarajan Perumal critically revised the manuscript. Franz Grus and Norbert Pfeiffer contributed to the conception of the study and provided resources.

PUBLICATION 2

Autoantigens in the trabecular meshwork and glaucoma-specific alterations in the natural autoantibody repertoire

Vanessa Beutgen planned the experimental design of this study, performed MS-AMIDA experiments, bioinformatics and microarray analysis, interpreted the data, and wrote the manuscript. Carsten Schmelter assisted with mass spectrometric measurements and analysis. Franz Grus and Carsten Schmelter critically revised the manuscript. Franz Grus and Norbert Pfeiffer contributed to the conception of the study and provided resources. English proofreading was performed by Peter Taylor.

PUBLICATION 3

Subtype-specific alterations in the serological autoantibody profile of open-angle glaucoma patients

Vanessa Beutgen planned the experimental design of the microarray, performed experiments, bioinformatic analysis, interpreted the data and wrote the manuscript. Franz Grus critically revised the manuscript. Franz Grus and Norbert Pfeiffer contributed to the conception of the study and provided resources.

Declaration of academic honesty

I declare, this doctoral thesis has not previously been submitted to any other university for an academic degree. So far, no comparable graduation procedure in the doctoral subject biology has been successfully or unsuccessfully completed. This scientific work was independently written by me and only the sources and tools indicated were used. No third-party help was used. This scientific work was carried out in accordance with the principles of good scientific practice under the basic regulations of the Johannes Gutenberg University Mainz.

Mainz, 12. November 2020

(Vanessa M. Beutgen)