



# Inhibitory and Agonistic Autoantibodies Directed Against the $\beta_2$ -Adrenergic Receptor in Pseudoexfoliation Syndrome and Glaucoma

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Pseudoexfoliation syndrome (PEXS) and glaucoma (PEXG) are assumed to be caused by a generalized elastosis leading to the accumulation of PEX material in ocular as well as in extraocular tissues. The exact pathophysiology of PEXS is still elusive. PEXG, the most common type of secondary open-angle glaucoma (OAG), is characterized by large peaks of intraocular pressure (IOP) with a progressive loss of the visual field. Agonistic autoantibodies (agAABs) against the  $\beta_2$ -adrenergic receptor (AR) have been shown to be present in sera of patients with primary and secondary OAG and ocular hypertension and are seemingly linked to IOP. In the present study, we investigated the autoantibodies directed against the  $\beta_2$ -AR in sera of patients with PEXS and PEXG. We recruited 15, 10, and 15 patients with PEXG, PEXS, and primary OAG, respectively. Ten healthy individuals served as controls. All patients underwent standard ophthalmological examination with Octopus G1 perimetry. agAABs prepared from serum samples were analyzed in a rat cardiomyocyte-based bioassay for the presence of agAABs. We identified the interacting loop of the  $\beta_2$ -AR and the immunoglobulin G (IgG) subclasses using synthetic peptides corresponding to the extracellular loops of the receptors and enzyme-linked immunosorbent assay, respectively. None of the controls were  $\beta_2$ -agAAB-positive ( $0.2 \pm 0.5$  U). No  $\beta_2$ -agAABs ( $0.2 \pm 0.4$  U), but inhibitory  $\beta_2$ -AABs were observed in 80% of the patients that partially blocked the drug-induced  $\beta_2$ -adrenergic stimulation;  $5.8 \pm 1.7$  U vs.  $11.1 \pm 0.9$  U for clenbuterol in the absence and the presence of sera from patients with PEXS, respectively. Epitope analyses identified the third extracellular loop of the  $\beta_2$ -AR as the target of the inhibitory  $\beta_2$ -AABs, being of IgG3 subtype in PEXS patients. In contrast, patients with PEXG showed  $\beta_2$ -agAABs ( $5.6 \pm 0.9$  U), but no inhibitory ones. The  $\beta_2$ -agAABs levels of patients with PEXG and primary OAG patients ( $3.9 \pm 2.8$  U;  $p > 0.05$ ) were at a similar level. In two cases of

PEXG, the  $\beta_2$ -agAAbs exert synergistic effects with clenbuterol. The activity increased from  $11.5 \pm 0.3$  (clenbuterol only) to  $16.3 \pm 0.9$  U. As autoimmune mechanisms were reportedly involved in the pathogenesis of glaucoma, agonistic and inhibitory  $\beta_2$ -AAbs seem to be a part of this multifactorial interplay.

**Keywords:** autoantibodies, glaucoma, pseudoexfoliation,  $\beta_2$ -adrenergic receptor, imbalanced autonomic theory

## INTRODUCTION

Pseudoexfoliation syndrome (PEXS) is an age-related disorder affecting the extracellular matrix of the whole body (Elhawry et al., 2012; Scharfenberg and Schlotzer-Schrehardt, 2012). It is characterized by the pathological accumulation of extracellular material (i.e., PEX material) in intraocular and extraocular tissues (Ritch and Schlotzer-Schrehardt, 2001; Elhawry et al., 2012). PEX material consists of fibrillar aggregates containing elastic fiber components such as elastin, fibrillin-1, fibulin, microfibril-associated glycoprotein-1 (MAGP-1), latent transforming growth factor  $\beta$  (LTBP)1/2, and cross-linking enzymes such as lysyl oxidase-like 1 (LOXL1) (Schlotzer-Schrehardt et al., 2000; Krumbiegel et al., 2009; Lee et al., 2009; Zenkel and Schlotzer-Schrehardt, 2014; Founti et al., 2015), which can accumulate in the trabecular meshwork, the main outflow pathway of the eye, and may reduce the outflow of aqueous humor and increased intraocular pressure (IOP) (Johnson and Brubaker, 1982). Consequently, PEXS is strongly associated with the development of PEX glaucoma (PEXG) (Schlotzer-Schrehardt et al., 2002; Anastasopoulos et al., 2015). The latter is the most common type of secondary open-angle glaucoma (SOAG) (Ritch, 2001). The pathophysiology of PEXS and PEXG is still elusive (Anastasopoulos et al., 2015; Schlotzer-Schrehardt and Khor, 2021). It is widely assumed that a generalized elastosis, induced by stress, is the principal process in the pathophysiology of PEXS (Schlotzer-Schrehardt et al., 2002; Anastasopoulos et al., 2015; Schlotzer-Schrehardt and Khor, 2021). Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), being increased in aqueous humor of PEXG and PEXS, and oxidative stress were considered to be the main contributors to the disease (Schlotzer-Schrehardt et al., 2001; Schlotzer-Schrehardt, 2010). PEX is reportedly associated with an increased risk of vascular diseases like myocardial infarction, coronary artery disease, aortic aneurysms, stroke, and cerebrovascular disease (Mitchell et al., 1997; Praveen et al., 2011; Yokusoglu, 2012; Gonen et al., 2013; Andrikopoulos et al., 2014; Siordia et al., 2016). Again the underlying mechanisms are not fully understood (Mitchell et al., 1997; Siordia et al., 2016). As with other glaucomas, the main target in treating PEXG is the increased IOP (Schlotzer-Schrehardt et al., 2002; Anastasopoulos et al., 2015). All conservative, laser, or surgical strategies aim to lower IOP up to an individual target level in order to win sighted lifetime (Lusthaus and Goldberg, 2019; Cvenkel and Kolko, 2020). Reduction of IOP by 1 mm Hg is known to prevent the visual field loss of patients by 10% (Leske et al., 2003) and should be made strictly forward (Musch et al., 2011). A main antiglaucomatous medication is timolol, a blocker of the  $\beta_2$ -adrenergic receptor (AR) (Katz et al., 1976; Barnes and Moshirfar, 2021), that reduces the formation of aqueous

humor by up to 50% (Coakes and Brubaker, 1978; Yablonski et al., 1978; Dailey et al., 1982; Topper and Brubaker, 1985; Mccannel et al., 1992; Wayman et al., 1997; Larsson, 2001). As  $\beta_1$ -AR blockers (e.g., betaxolol) reportedly lower IOP less effectively than  $\beta_2$ -AR blockers, the role of the latter in regulating IOP appears to be predominant (Gaul et al., 1989).  $\beta_2$ -ARs are expressed on cells of the ciliary body and the trabecular meshwork, the tissues involved in aqueous humor production and outflow (Wax and Molinoff, 1987; Crider and Sharif, 2002). Cells of the optic nerve and retinal pericytes are further tissues that express  $\beta_2$ -AR (Mantyh et al., 1995; Feher et al., 2005). agAAbs against  $\beta_2$ -AR ( $\beta_2$ -agAAbs) have been detected in the sera of patients with primary open-angle glaucoma (POAG) and SOAG, as well as ocular hypertension (OHT) (Junemann et al., 2018; Hohberger et al., 2019a). These agAAbs were mainly of the immunoglobulin G3 (IgG3) subtype and were directed against the second extracellular loop of the  $\beta_2$ -AR (peptide 181–192) (Junemann et al., 2018). The immunological targeting of the  $\beta_2$ -AR is the basis for the hypothesis of an involvement of  $\beta_2$ -agAAbs in dysregulation of IOP (Zimmerman, 1977). The IOP is the result of production (ciliary body) and outflow (trabecular meshwork, uveosclera) of the aqueous humor (Johnstone et al., 2020; Kaufman, 2020; Sunderland and Sapra, 2021). Clinical data of patients with POAG, showing a reduced IOP after removal of all circulating IgG antibodies by immunoabsorption, support this hypothesis (Junemann et al., 2018). These findings indicated a potential role for autoimmune processes in the dysregulation of IOP. As patients with PEXG typically show an increased IOP with large fluctuations (Heijl et al., 2009), we hypothesize a role of the  $\beta_2$ -agAAbs in the development of PEXG. Various types of agAAbs, directed against the  $\beta_2$ -AR, were described in literature (Wallukat and Wollenberger, 1991; Wallukat, 2002; Junemann et al., 2018): agonistic and inhibitory AAbs activate and block the  $\beta_2$ -AR, respectively. It was the aim of this study to analyze serum samples from patients with PEXS and PEXG for agAAbs against  $\beta_2$ -AR.

## MATERIALS AND METHODS

### Patients and Controls

Fifty subjects were recruited from the Department of Ophthalmology, University of Erlangen-Nuremberg: patients [ $n = 40$ ; PEXG ( $n = 15$ ), PEX ( $n = 10$ ), POAG ( $n = 15$ )] and controls ( $n = 10$ ). All patients underwent a complete ophthalmological examination including slit-lamp biomicroscopy, funduscopy, and measurement of IOP by Goldmann applanation tonometry (Haag Streit, Koenig,

Switzerland). In addition, we performed standard white-on-white full-field perimetry (Octopus 500, G1 protocol; Interzeag, Schlieren, Switzerland).

### PEX Glaucoma

Diagnosis of PEXG was based on an open anterior chamber angle, the presence of PEX material on anterior segment structures, repeated elevated IOP > 21 mm Hg, glaucomatous alterations of the optic disc according to Jonas' classification (Jonas et al., 1988), and perimetric field loss, confirmed at least once.

### PEX Syndrome

Patients with PEXS showed PEX material deposits in the anterior chamber, but without any signs of glaucoma (Scharfenberg and Schlotzer-Schrehardt, 2012; Hohberger et al., 2019b).

### POAG

Diagnosis of POAG was based on the glaucoma criteria for PEXG, yet without any signs of PEX material on ocular structures (Jonas et al., 1988; Barnes and Moshirfar, 2021).

### Controls

Tonometry, slit-lamp inspection, funduscopy, and papillometry were normal in control subjects. No signs of PEX or glaucoma were observed, yet the presence of cataract was allowed.

Blood samples were collected according to clinical all-day life procedures and centrifuged, and sera were stored at  $-80^{\circ}\text{C}$  previous to analysis. All individuals declared informed consent. The study was performed according to the tenets of the Declaration of Helsinki and approved by the Ethic Committee of the University of Erlangen and the Max-Delbrück-Centrum for Molecular Medicine, Berlin-Buch (Y 9004/19, 02.11.2020).

### Cardiomyocyte Bioassay

The cardiomyocyte bioassay was used as described previously (Junemann et al., 2018). Cell culture of cardiac myocytes (heart ventricle of 1–3-day-old Sprague–Dawley rats) was digested with a 0.25% solution of crude porcine trypsin (Serva, Germany). The cells were dispersed and suspended in SM20-I medium (Biochrom, Germany), glutamine (Serva, Germany), which contained streptomycin (HEFA Pharma, Germany), penicillin (Heyl, Germany), hydrocortisone (Merck, Germany), 10% heat-inactivated neonatal calf serum (Gibco, Germany), and fluorodeoxyuridine (Serva, Germany). The cells were seeded with a field density of 160,000 cells/cm<sup>2</sup>. After 24 h, the culture medium was replaced, respectively. Before stimulation, the cardiomyocytes were cultured for 3–4 days ( $37^{\circ}\text{C}$ ). Two hours before the experiment, the medium was renewed. After incubation with the immunoglobulin fractions of the sera of each proband (1:40, for 60 min), the beating rates (BRs) of the cardiomyocytes were counted for 7–10 spontaneously beating cells for 15 s at a heated stage inverted microscope ( $37^{\circ}\text{C}$ ). One unit reflects 4 beats/min (i.e., adrenergic activity). Samples of less than 2 units of adrenergic activity were considered as normal. Differentiation between agonistic and inhibitory  $\beta_2$ -AABs was done after additional incubation with 3  $\mu\text{M}$  clenbuterol; the latter one partially blocked the clenbuterol effect. Application

of ICI-118,551 (2  $\mu\text{L}$  ICI to 2 mL medium in order to avoid dilution) was done for identification of the receptor type ( $\beta_2$ -receptor).

### Preparation of the Serum Immunoglobulins

The IgGs were prepared from sera of patients by direct ammonium sulfate precipitation (final concentration of 40%) overnight at  $4^{\circ}\text{C}$ . Afterward, the precipitates were centrifuged ( $4,000 \times g$ , 30 min). The pellets were dissolved in dialysis buffer (154 mmol/L NaCl, 10 mmol/L  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ , pH 7.2). Precipitating, washing, and dissolving were repeated twice. The samples were dialyzed against phosphate-buffered saline ( $4^{\circ}\text{C}$ , 3 days) prior to application to the cardiomyocyte bioassay. The samples were stored at  $-20^{\circ}\text{C}$ .

### Characterization of the Target Loop of Inhibitory AABs Against $\beta_2$ -AR

Characterization of the target loop was done as described previously (Junemann et al., 2018). Synthetic peptides corresponding to the extracellular loops I, II, and III of the human  $\beta_2$ -AR were used to identify the molecular domain of the inhibitory  $\beta_2$ -AABs:

1. loop I (HILMKMWTFGNFWCEFWT).
2. loop II (HWYRATHQEAINCYANETCCDFFTNQ).
3. loop III (VIQDNLIRKEV).

After shaking 0.5  $\mu\text{g}$  peptide in 50  $\mu\text{L}$  IgG fraction, they were incubated for 1 h at room temperature before adding them to the bioassay (final IgG dilution of 1:40).

### Analysis of IgG Subclass of the Inhibitory AABs Against $\beta_2$ -AR

IgG subclass analysis of the inhibitory AABs was done as described previously (Junemann et al., 2018). IgGs (50  $\mu\text{L}$ ) from selected patients were treated with murine monoclonal anti-human IgG1, IgG2, IgG3, and IgG4 antibodies (3  $\mu\text{L}$ , SEROTEC, Germany). After incubation (1 h, room temperature), each sample was treated with 3  $\mu\text{L}$  of a polyclonal anti-mouse Fc antibodies (1 h) in order to increase the immune complex. Centrifugation of the samples (at 10,000 g) was done, and the supernatants were added to the cardiomyocyte bioassay (see above; dilution of 1:40).

### Statistical Analysis

Statistical calculations were done with Microsoft Excel 2016 and the GraphPad Prism Software version 8.3. Data are shown as means and standard errors of the mean for each group. The Kolmogorov–Smirnov test was run prior to analysis for testing the normal distribution. According to this, the one-way analysis of variance (normally distributed data) and the Kruskal–Wallis test (non-normally distributed data) with a post-test for multiple comparisons were applied, respectively. When only two groups were compared, the Mann–Whitney test or the Student's *t*-test was applied, accordingly.  $p < 0.05$  was

**TABLE 1** | List of abbreviations.

PEXS	Pseudoexfoliation syndrome
PEXG	Pseudoexfoliation glaucoma
OAG	Open-angle glaucoma
POAG	Primary open-angle glaucoma
SOAG	Secondary open-angle glaucoma
OHT	Ocular hypertension
IOP	Intraocular pressure
agAAbs	Autoantibody
$\beta_2$ -AAbs	Autoantibody against the adrenergic $\beta_2$ -adrenoreceptor
$\beta_2$ -agAAbs	Agonistic autoantibody against the adrenergic $\beta_2$ -adrenoreceptor
$\beta_2$ -AR	Adrenergic $\beta_2$ -adrenoreceptor
CB	Clenbuterol
IgG	Immunoglobulin G
MAGP-1	Microfibril-associated glycoprotein-1
LTBP	Latent transforming growth factor $\beta$
LOXL1	Lysyl oxidase-like 1
Transforming growth factor	Transforming growth factor
IL	Interleukin
BR	beating rate

considered significant. A list of all abbreviations can be seen in **Table 1**.

## RESULTS

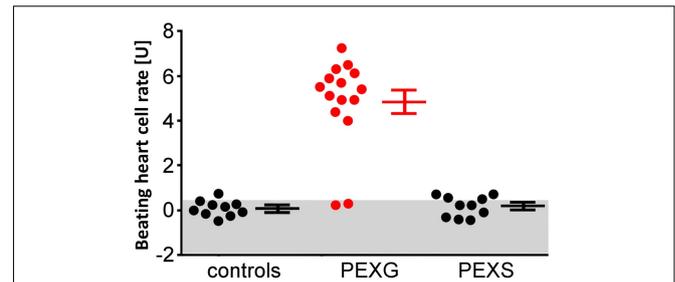
We analyzed the sera of patients with PEXS and PEXG and controls for the presence of agonistic and inhibitory AAbs directed against the  $\beta_2$ -AR. None of the control samples showed a  $\beta_2$ -agAAb seropositivity (neither agonistic nor inhibitory) of  $0.2 \pm 0.5$  U in the cardiomyocyte bioassay. The cutoff was set at mean  $\pm 3$  standard deviation of the controls (1.66 U).

### Sera of Patients With PEXS Contain Inhibitory but No Agonistic $\beta_2$ -AABs

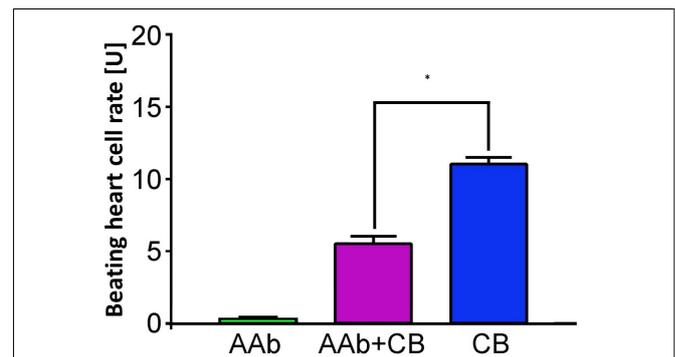
None of the 10 patients with PEXS showed  $\beta_2$ -agAABs (mean =  $0.2 \pm 0.1$  U) (**Figure 1**). Clenbuterol alone increased the BR value of the cardiomyocytes by  $11.1 \pm 0.5$  U. After coincubation of the IgG with 3  $\mu$ M clenbuterol (mean), a decreased BR value was observed in 8 (80%) of 10 PEXS patients ( $5.8 \pm 0.6$  U,  $p < 0.001$ ). This decrease was attributed to the inhibitory  $\beta_2$ -AABs that partially blocked the clenbuterol effect ( $p < 0.001$ ; **Figure 2**).

### The Inhibitory $\beta_2$ -AABs Recognize the Third Extracellular Loop of the $\beta_2$ -AR

Clenbuterol yielded an increase in the BR (mean =  $12.4 \pm 0.2$  U). We performed the epitope analyses employing specific competitive inhibitory peptides of the second and third extracellular loops of the  $\beta_2$ -AR (**Figure 3**). Loop III but not loop II peptides neutralized the inhibitory  $\beta_2$ -AABs. These data indicate that inhibitory  $\beta_2$ -AABs in the sera of patients with PEXS are directed against the third extracellular loop of the  $\beta_2$ -AR.



**FIGURE 1** | Adrenergic activity of  $\beta_2$ -AABs in sera of patients with PEX syndrome (PEXS), PEX glaucoma (PEXG), and controls. Beating heart cell rates were shown for each group [absolute data, mean  $\pm$  standard deviation (SD)]: controls, patients with PEXG and PEXS. One unit (U) reflects 4 beats/min. The values represent the adrenergic activity of  $\beta_2$ -AABs. Samples less than 2 units of adrenergic activity were considered as normal. Increased beating heart cell rate was to be observed for patients with PEXG (red) when compared to patients with PEXS and controls as measured by rat cardiomyocyte beating heart cell rates.



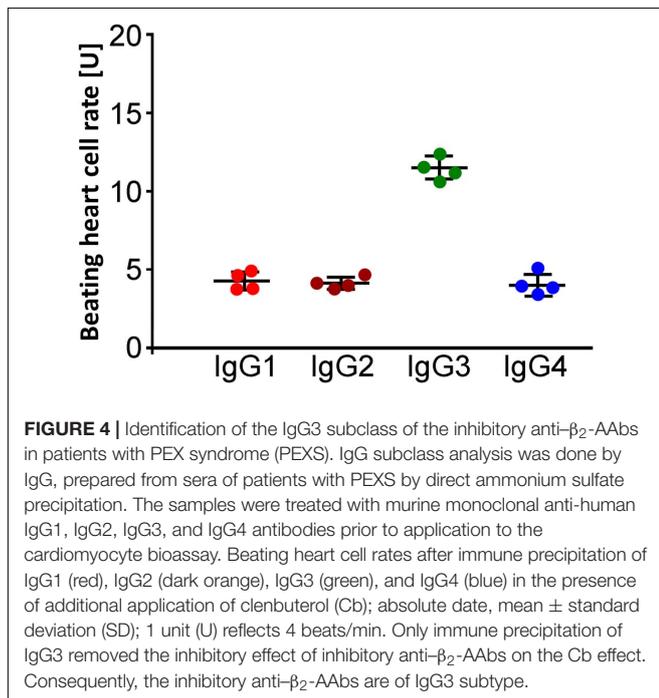
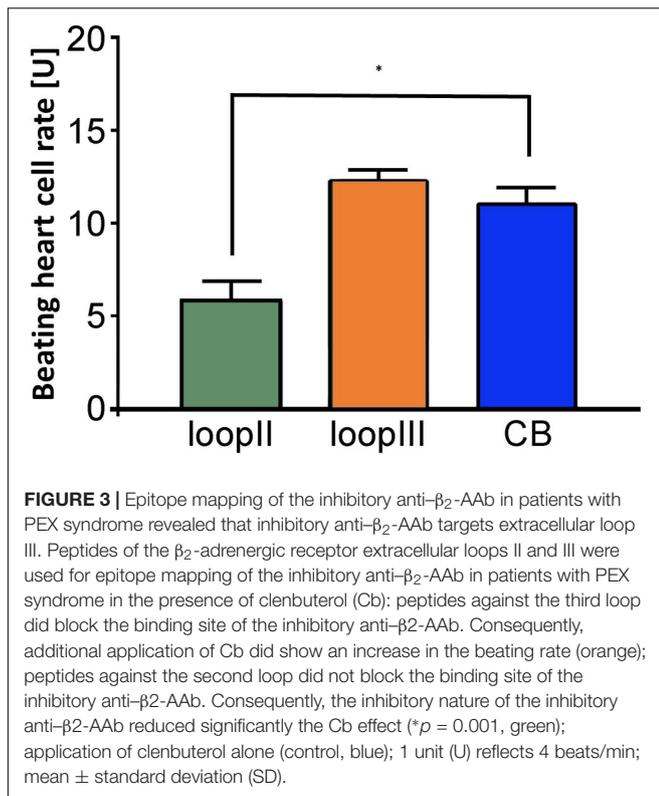
**FIGURE 2** |  $\beta_2$ -AABs in patients with PEXS are of inhibitory nature. Beating heart cell rates after single application of sera of patients with PEXS without (green) and with additional clenbuterol (CB, pink); reference: single incubation with CB (blue); 1 unit (U) reflects 4 beats/min; mean  $\pm$  standard deviation (SD).  $\beta_2$ -AABs inhibit the increased beating heart cell rate, due to the effect of clenbuterol, and are thus of inhibitory nature. \* $p < 0.001$ , with a post-test for multiple comparisons.

### The Inhibitory $\beta_2$ -AABs Are of IgG3 Subclass

The analyses of the IgG subtype were performed by immune precipitation with antibodies against the immunoglobulins IgG1–4. The residual inhibitory  $\beta_2$ -AAB activities after preincubation with IgG subclass-specific antibodies were  $4.3 \pm 0.3$  U (IgG1),  $4.1 \pm 0.2$  U (IgG2),  $11.7 \pm 0.4$  U (IgG3), and  $4.0 \pm 0.3$  U (IgG4), respectively. The activity of the inhibitory  $\beta_2$ -AABs was eliminated by preincubation with anti-IgG3 antibodies. Thus, the inhibitory  $\beta_2$ -AABs are of the IgG3 subclass (**Figure 4**).

### Sera of Patients With PEXG Contain Agonistic, Yet No Inhibitory $\beta_2$ -AABs

In contrast to patients with PEXS and controls, 12 (80%) of 15 of those with PEXG had  $\beta_2$ -agAABs. The mean was  $4.55 \pm 0.6$  U



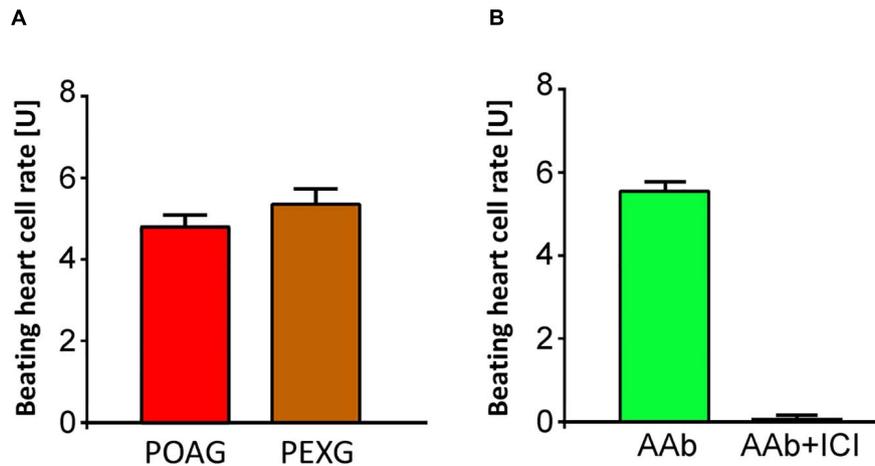
in all the PEXG patients (Figure 5). The BR between patients with PEXG and those with POAG did not differ ( $3.9 \pm 0.7$  U;  $p > 0.05$ ). In contrast, no inhibitory  $\beta_2$ -AABs were to be observed. Figure 6 shows that 2 (13.3%) of the 15 of the patients with PEXG showed synergistic  $\beta_2$ -AABs that markedly increased BR

values after coincubation with  $1 \mu\text{M}$  clenbuterol ( $16.3 \pm 0.6$  U vs.  $11.5 \pm 0.1$  U for clenbuterol alone). Only 1 (6.7%) of 15 PEXG patients showed neither agonistic nor inhibitory  $\beta_2$ -AABs.

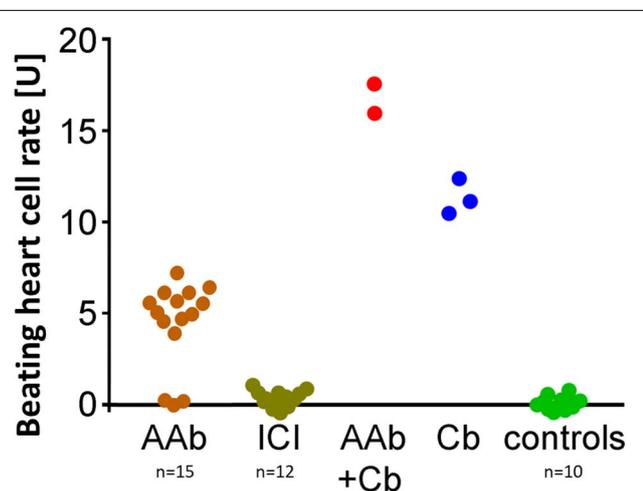
Analysis of IgG subclasses showed that the  $\beta_2$ -agAABs were of the IgG3 subclass (Figure 7), as only precipitation with IgG3 antibodies eliminated the increase in the BR value ( $0.29 \pm 0.1$  U). Consistently, no alterations of the BR were seen after precipitation of IgG1 ( $5.0 \pm 0.3$  U), IgG2 ( $5.0 \pm 0.5$  U), and IgG4 ( $5.1 \pm 0.5$  U). Epitope analyses of  $\beta_2$ -AR showed that the  $\beta_2$ -agAABs were directed against the second extracellular loop of this G-protein-coupled receptor (in full) as described previously (Junemann et al., 2018).

## DISCUSSION

PEXS and PEXG are age-related ocular and systemic diseases associated with the accumulation of abnormal fibrillar material in intraocular and extraocular tissues (Elhaway et al., 2012; Scharfenberg and Schlotzer-Schrehardt, 2012; Schlotzer-Schrehardt and Khor, 2020). With an annual incidence of 1.81%, PEXS is a common condition (Astrom et al., 2007), yet its prevalence varies worldwide (Cashwell and Shields, 1988; Krishnadas et al., 2003; Topouzis et al., 2019). It accounts for 25% and up to 70% of all open-angle glaucoma (OAG), depending on the country (Ritch, 1994; Mitchell et al., 1999; Andrikopoulos et al., 2009). Although PEXS is a major risk factor for glaucoma, it is important to note that only 30–40% of all individuals with PEXS will develop PEXG (Thorleifsson et al., 2007). In longitudinal population-based studies, the probability to develop glaucoma after 10–12 years was found to be 9–15% (Jeng et al., 2007; Topouzis et al., 2019). PEXG is a hypertensive type of glaucoma associated with high levels and fluctuations of the IOP, as well as an increased outflow resistance (Kozobolis et al., 2012; Huchzermeyer et al., 2015). Eyes of patients with PEXS tend to have higher IOP levels as healthy eyes but can be normotensive as well (Koz et al., 2009). Although PEXS is an established risk factor for PEXG (Mitchell et al., 1999; Topouzis et al., 2011), the exact relationship between the two conditions is not well understood (Topouzis et al., 2019). Similarly, it remains unclear why only a proportion of PEXS patients develop glaucoma, whereas the majority of them will not (Anastasopoulos et al., 2015; Schlotzer-Schrehardt and Khor, 2021). In a longitudinal population-based study, statistically significant risk factors for the development of PEXG among PEXS patients included higher levels of IOP, with 26% increased risk per mm Hg, and a history of heart attack (Topouzis et al., 2019). It is also conceivable that an underlying defect in the flow dynamics of the aqueous humor or additional involvement of glaucoma susceptibility genes may predispose to glaucoma development in PEXS eyes (Schlotzer-Schrehardt and Naumann, 1995; Kozobolis et al., 2012; Founti et al., 2015; Huchzermeyer et al., 2015; Schlotzer-Schrehardt and Khor, 2021). It has been suggested that in addition to IOP, further factors contribute to the development of glaucoma in patients with PEXS. These include genetic, epigenetic, environmental, and tissue factors such as increased susceptibility of the lamina cribrosa to



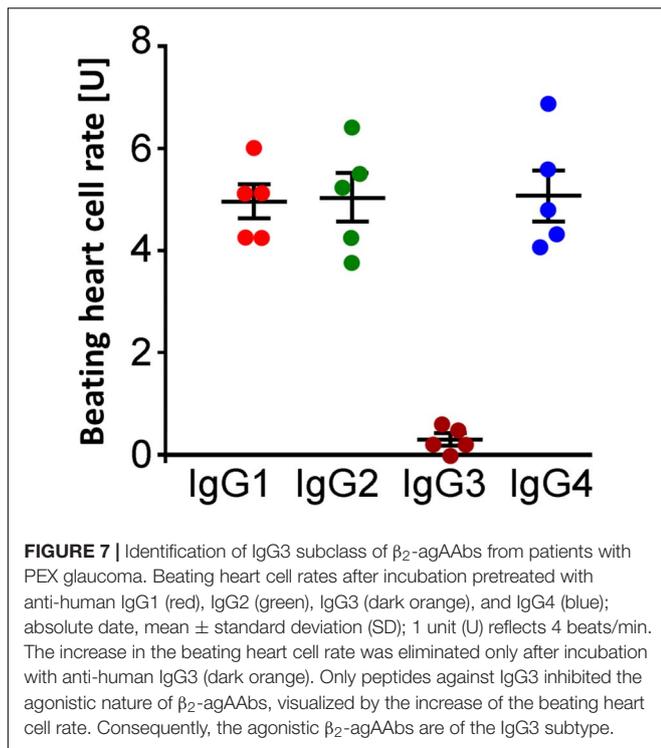
**FIGURE 5 |** Agonistic  $\beta_2$ -agAAbs in sera of open-angle glaucoma. **(A)** Beating heart cell rates after incubation with sera of patients with primary open-angle glaucoma (POAG, red) and PEX glaucoma (PEXG, orange); mean  $\pm$  standard deviation (SD); 1 unit (U) reflects 4 beats/min. An increase of the beating heart cell rate was observed in POAG and PEXG, showing its agonistic effect. Beating heart cell rate of POAG and PEXG did not differ significantly ( $p > 0.05$ ). **(B)** Beating heart cell rates after application of sera without (green) and with ICI-118,551 (black): the agonistic effect of the  $\beta_2$ -AAbs is shown. The agonistic nature of  $\beta_2$ -agAAbs activated the receptor with consequent increase of the beating heart cell rate. These agonistic  $\beta_2$ -agAAbs were observed in sera of patients with open-angle glaucoma.



**FIGURE 6 |** Synergistic  $\beta_2$ -AAbs enhanced the effect of Cb alone and enhanced the agonistic effect of  $\beta_2$ -agAAbs. Beating cell rates after incubation of live rat cardiomyocyte cells with patient sera from PEX glaucoma (PEXG): sera without (orange) and with the  $\beta_2$ -blocker ICI-118,551 (grey-green); synergistic  $\beta_2$ -AAbs (red) after coinubation of the beating heart cells with clenbuterol (Cb); reference: beating heart cell rate after incubation with clenbuterol alone (blue) and of control subjects (green). One unit (U) reflects 4 beats/min. Sera of PEX glaucoma showed synergistic and agonistic  $\beta_2$ -AAbs: agonistic  $\beta_2$ -AAbs were present as beating heart cell rates were increased compared to controls, reaching Cb effect alone. Synergistic  $\beta_2$ -AAbs enhanced the effect of Cb alone and enhanced the agonistic effect of  $\beta_2$ -agAAbs. ICI blocked the effect of the  $\beta_2$ -adrenergic receptor AAbs demonstrating the specificity for the  $\beta_2$ -adrenergic receptor on the beating heart cells.

to participate. Interestingly, there was no difference between the antibody patterns in the aqueous humor of patients with POAG and PEXG. This indicates common pathomechanisms in dysregulation of aqueous humor dynamics and IOP in these glaucoma subtypes (Joachim et al., 2007). Previously, we have identified specific agAAbs affecting the  $\beta_2$ -AR in sera of patients with PEXG, as has been also shown for patients with POAG (Hohberger et al., 2019a). A subsequent study showed that  $\beta_2$ -agAAbs were also present in aqueous humor samples of patients with POAG or PEXG (Hohberger et al., 2020). It has been hypothesized that via an impaired blood-aqueous barrier in PEXS/PEXG, serum proteins including  $\beta_2$ -agAAbs enter the anterior chamber, as observed in a previous study (Schlotzer-Schrehardt and Naumann, 2006). Alternatively, the  $\beta_2$ -agAAbs may be produced locally in ocular tissues. As the  $\beta_2$ -agAAbs already occur in very early stages of glaucoma and OHT [e.g., patients without optic disc alterations, yet increased IOP (OHT)] (Junemann et al., 2018), we were interested to find out if patients with risk of PEXG (e.g., PEXS) harbor  $\beta_2$ -agAAbs. The findings of the present study showed that patients with PEXS lacked any agAAbs. Instead, 80% contained inhibitory  $\beta_2$ -AAbs of the IgG3 subtype, which is directed against the third extracellular loop of the  $\beta_2$ -AR. In contrast, patients with PEXG had  $\beta_2$ -agAAbs, yet no inhibitory ones. Interestingly, synergistic  $\beta_2$ -agAAbs were to be observed in the sera of two patients with PEXG, overtopping the effect of the  $\beta_2$ -adrenergic agonist clenbuterol. The presence of synergistic  $\beta_2$ -AAbs had never been published before. The data of the present study suggest that inhibitory AAbs present in the majority of PEXS patients may contribute to maintenance of normal IOP by reducing aqueous humor production and counterbalance the age-related decline in outflow facility. In addition, we hypothesized that agAAbs may contribute to an increase in IOP by stimulating aqueous humor production in the presence of increased outflow resistance due to abnormal

optic nerve damage (Topouzis et al., 2011; Braunsman et al., 2012; Hohberger et al., 2018; Schlotzer-Schrehardt and Khor, 2021). Autoimmune mechanisms have also been suggested



deposits in the trabecular meshwork. This scenario potentially contributes to the etiopathogenesis of glaucoma.

Immune and autoimmune factors were recently reported to be involved in the pathogenesis of OAG (Tezel et al., 2007; Tezel and Fourth ARVO/Pfizer Ophthalmics Research Institute Conference Working Group, 2009; Joachim et al., 2013; Rieck, 2013; Kamat et al., 2016; Von Thun Und Hohenstein-Blaul et al., 2016; Chen et al., 2018; Hohberger et al., 2019a; Tsai et al., 2019). A spectrum of various autoantibodies, mostly directed against retinal antigens, has previously been detected in the sera of patients with PEXS and PEXG (Dervan et al., 2010; Altintas et al., 2012; Huflejt

et al., 2014; Mazeikaite et al., 2016) and in the aqueous humor of patients with PEXG (Joachim et al., 2007). In the latter study, there were no significant differences in these antibody patterns between PEXG and POAG.  $\beta_2$ -AgAAbs levels of PEXG and POAG were similar as well. The present study showed for the first time inhibitory  $\beta_2$ -AAbs in the sera of patients with PEXS. Inhibitory  $\beta_2$ -AAbs block the function of the  $\beta_2$ -AR. This finding is supported by the clinical feature of a less pronounced mydriasis after application of 10% phenylephrine in PEXS eyes compared to eyes without PEXS (Puska, 2002). As reported in a previous study, the local level of  $\beta_2$ -agAAbs in the aqueous humor was increased compared to the systemic levels (Hohberger et al., 2020). Thus, we propose that adrenergic  $\beta_2$ -AAbs can be locally produced in ocular tissues. The adrenergic agAAbs in aqueous humor are the basis of our hypothesis of inhibitory  $\beta_2$ -AAbs in PEXS: due to blocking of  $\beta_2$ -AR by inhibitory  $\beta_2$ -AAbs, endogenous catecholamine will shift to alternative AR. Consecutively, AR receptors will be uncoupled (acute response), phosphorylated, and internalized (desensitization of AR) (see Wallukat, 2002). Application of phenylephrine induces mydriasis by contraction of the iris dilator in unaffected eyes. This mydriatic effect might be reduced in cells of the iris dilator with desensitized AR.

The exact etiopathogenesis of PEXS and PEXG are still under debate. Two main questions remain unanswered: what is the pathomechanism behind the unilateral clinical manifestation of PEXS/PEXG? And why does only a subgroup of the eyes with PEXS convert to PEXG? It is assumed that the basis of PEXS is a genetic predisposition (e.g., LOXL1). In order to present the clinical features with PEX material on ocular structures, several endogenous and/or exogenous factors are engaged in this complex interplay. Inhibitory, agonistic, and even synergistic  $\beta_2$ -AAbs turned out to be a common feature in PEXS/PEXG patients in samples of the present cohort (80%); thus, we propose that this form of autoimmunity contributes to the clinical characteristics of PEXS/PEXG. Patients with PEXS show inflammatory markers in their sera (Gonzalez-Iglesias et al., 2014) and aqueous humors [e.g., interleukin (IL-6), IL-8]

**TABLE 2 |** Autoantibodies targeting the adrenergic  $\beta_2$ -AR in glaucoma suspects and patients with glaucoma: pseudoexfoliation syndrome (PEXS), pseudoexfoliation glaucoma (PEXG), ocular hypertension (OHT), primary open-angle glaucoma (POAG), secondary open-angle glaucoma (SOAG), and pigment dispersion (PD).

	Clinical characteristics			Serum	Aqueous humor	References
	Increased IOP	Glaucomatous visual field defect	Glaucomatous optic disc			
PEXS	–	–	–	Inhibitory $\beta_2$ -AAbs		Present study
PEXG	+	+	+	Agonistic $\beta_2$ -AAbs		Hohberger et al., 2019a
					Agonistic $\beta_2$ -AAbs	Hohberger et al., 2020
				Synergistic $\beta_2$ -AAbs		Present study
OHT	+	–	–	Agonistic $\beta_2$ -AAbs		Junemann et al., 2018; Hohberger et al., 2019a
Preperimetric POAG	+	–	+	Agonistic $\beta_2$ -AAbs		Hohberger et al., 2019a
POAG	+	+	+	Agonistic $\beta_2$ -AAbs		Junemann et al., 2018
					Agonistic $\beta_2$ -AAbs	Hohberger et al., 2020
SOAG due to PD	+	+	+	Agonistic $\beta_2$ -AAbs		Hohberger et al., 2019a

(Koliakos et al., 2001; Zenkel et al., 2010; Borrás, 2018), arguing for an underlying subclinical inflammation. According to the findings of the present study, we propose that because of this inflammatory status, autoimmune mechanisms occur with the generation of autoantibodies, being inhibitory (PEXS), agonistic, or synergistic (PEXG). The specific adrenergic agAAbs might further increase the variation of each clinical entity. Inhibition or overstimulation of the AR might result in a dysregulation of the homeostatic adrenergic balance. This non-canonical receptor-immune complex interaction inhibits or overactivates the adenylate cyclase, causes receptor internalization (due to overstimulation by  $\beta_2$ -agAAbs), or even enhances AR expression in the sarcolemma membrane (e.g., due to inhibition by inhibitory  $\beta_2$ -AAbs) (Wallukat et al., 2002). If this molecular dysbalance has sufficient “power,” clinical features might occur in patients with PEXS/PEXG (imbalanced autonomic theory; see Szentiványi, 1968).

Inhibitory  $\beta_2$ -AAbs were observed in sera of patients with allergic bronchial asthma for the first time (Wallukat and Wollenberger, 1991). The present study showed inhibitory  $\beta_2$ -AAbs in patients with PEXS as second clinical entity for the first time. Inhibitory  $\beta_2$ -AAbs bind to  $\beta_2$ -AR in a competitive way (Wallukat and Wollenberger, 1991). Thus, their binding site to the third loop seems to be different from the  $\beta_2$ -agAAbs that bound to the first or second extracellular loop of the receptors. After binding, the AR function will be blocked, reducing the activity of adenylate cyclase with consecutive enzymatic dysbalance (Wallukat, 2002; Johnson, 2006). On the contrary, agonistic and even synergistic  $\beta_2$ -AAbs were present in patients with PEXG. As described in previous studies, we propose that  $\beta_2$ -agAAbs contribute to the increased IOP in patients with OAG (Junemann et al., 2018; Hohberger et al., 2019a). The presence of synergistic  $\beta_2$ -AAbs in sera of patients with PEXG argues for the distinct IOP pattern in these eyes. IOP in PEXG is elevated as in all OAG eyes, yet with strongly enhanced diurnal fluctuations and worsened disease progression compared to POAG. This theory might be parallel to the “ $\beta$ -adrenergic theory of the atopic abnormality in bronchial asthma” (Szentiványi, 1968). Szentiványi (1968) hypothesized that decreased  $\beta$ -adrenergic sensitivity results in an autonomic dysbalance, featuring the typical clinical characteristics of asthma. Szentiványi postulated that “its ubiquitous character suggests, therefore, that the ultimate clinical manifestation of the fundamentally same atopic abnormality will be determined by the type of cell primarily involved, that is, by the specialized cell system of the multicellular organism which harbors primarily the postulated enzymatic abnormality.” It would be of interest, if these autoimmune phenomena will change during disease progression: if either one agAAb subtype will disappear and another one appears, or the presence of a specific adrenergic agAAb subtype would potentially influence the clinical characteristics: PEXS (inhibitory  $\beta_2$ -AAbs) or entities with increased IOP [OAG including

PEXG and POAG (Hohberger et al., 2019a, 2020) and OHT (Junemann et al., 2018);  $\beta_2$ -agAAbs, **Table 2**]. A longitudinal follow-up study would be of interest to focus on the pattern of adrenergic AAbs in progressive glaucoma patients. This offers the potential function of  $\beta_2$ -AAbs as novel biomarkers, supporting diagnosis and monitoring of PEXS, PEXG, and POAG. It potentially provides novel therapeutic targets for one of the most serious types of glaucoma (PEXG), as well as the most common glaucoma form (POAG). The data of the present study already enable the use of adrenergic autoantibodies as novel biomarker, differentiating between PEXS and PEXG. Analysis of a potential presence of adrenergic AAbs locally in aqueous humor of PEXS and PEXG would give additional novel hints insight toward locus of production and pathogenesis.

## CONCLUSION

The presence of inhibitory, agonistic, and even synergistic  $\beta_2$ -AAbs in sera of PEXS and PEXG, respectively, argues for an imbalanced adrenergic control of the IOP (imbalanced autonomic theory). Each adrenergic agAAb subtype might contribute to the typical clinical characteristics (PEXS, PEXG). Whereas patients with PEXS showed inhibitory  $\beta_2$ -AAbs, none of these AAbs were to be observed in the sera of PEXG, yielding agonistic and synergistic  $\beta_2$ -AAbs. These observations argue for an involvement of the  $\beta_2$ -AAbs early in the pathogenesis of glaucoma. In addition,  $\beta_2$ -AAbs might offer a novel option as biomarker or therapeutic target in glaucoma.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Erlangen-Nuremberg. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

US-S, RK, MH, and GW conceived the idea. GW performed the experimental work. BH wrote the draft of the manuscript. CM interpreted the results and revised the manuscript. RL revised the manuscript. BH, MH, and LM performed statistical analysis. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** RK as the patent EP1832600A1. MH has the patent EP1832600A1. GW was employed by Berlin Cures GmbH, Berlin, Germany and holds the patent P1832600A1.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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