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Anti-drug antibodies to brolucizumab and ranibizumab in serum and vitreous of patients with ocular disease

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ABSTRACT.

Purpose: **Postapproval reports of intraocular inflammation (IOI) and occlusive retinal vasculitis following intravitreal brolucizumab are accumulating. A role of anti-drug antibodies (ADAs) to brolucizumab is under current scientific discussion. The purpose of the present study was to measure brolucizumab ADAs in a cross-sectional ophthalmic patient population and to compare the occurrence of brolucizumab ADAs with that of ranibizumab ADAs.**

Methods: **One hundred and ninety-two serum samples and 54 vitreous samples were collected from patients with a range of eye diseases including neovascular age-related macular degeneration (AMD), diabetic retinopathy, retinal vein occlusion, cataract, glaucoma, dry eye disease, macular hole, epiretinal membranes and intraocular lens (IOL) dislocation. Serum and vitreous samples were analysed for immune globuline (Ig) G ADAs to brolucizumab and ranibizumab using indirect enzyme-linked immunosorbent assay (ELISA). Optical Density (OD) was read at 450 nm (wavelength correction at 550 nm) for ADA level measurements.**

Results: **Presence of brolucizumab ADAs was observed in patients with and without prior brolucizumab exposure. Both the frequency of notable ADA signals (OD > 0.1) and the mean ADA signal in serum samples were higher for brolucizumab than for ranibizumab. Two patients who experienced severe IOI and occlusive retinal vasculitis following intravitreal brolucizumab had high brolucizumab ADA serum levels. In one of these two patients, high brolucizumab ADA levels were also found in vitreous. Another patient developed moderate IOI without retinal vasculitis in the presence of low brolucizumab ADA serum levels. Overall, notable brolucizumab ADA levels were less frequent in vitreous than in the corresponding serum samples but with a tendency for higher prevalence in vitreous from patients with diabetic retinopathy.**

Conclusion: **Brolucizumab ADAs occur with significant prevalence in a typical ophthalmic patient population and may represent a risk factor for IOI and occlusive retinal vasculitis following brolucizumab.**

Key words: ADA – anti-drug antibodies – brolucizumab – intraocular inflammation – intravitreal – occlusive vasculitis – ranibizumab – retinal vasculitis – VEGF inhibitors

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Introduction

Exudative age-related macular degeneration (AMD) is one of the leading causes of severe visual impairment or even blindness in the elderly population (Ferris et al. 1984; Congdon et al. 2004; Wong et al. 2014; Klein et al. 2020). A hallmark of exudative AMD is the development of choroidal neovascularization (CNV), which is characterized by pathologic and uncontrolled growth of choroidal vessels. The newly formed vessels are located beneath the retinal pigment epithelium (RPE) or breach the RPE into the subretinal space and are of a fragile and leaky nature. Sight-threatening complications of macular neovascularization include intra- and subretinal fluid, haemorrhages and macular oedema leading to the degeneration of photoreceptors and disciform macular scarring (Grossniklaus & Gass 1998; Ambati et al. 2003; Grossniklaus & Green 2004; Coleman et al. 2008; Jager et al. 2008).

Vascular endothelial growth factor (VEGF) plays a key role in these pathologic processes and the intravitreal injection of VEGF-binding proteins such as aflibercept, bevacizumab, brolucizumab or ranibizumab effectively attenuates neovascularization and microvascular hyperpermeability. Intravitreal VEGF inhibition thus represents the gold standard for treating

exudative AMD and other retinal microangiopathies such as diabetic retinopathy or macular oedema secondary to retinal vein occlusion (Andreoli & Miller 2007; Penn et al. 2008; Park et al. 2017; Pham et al. 2019; Ricci et al. 2020).

Albeit rare, severe adverse events may be associated with intravitreal anti-VEGF treatment including infectious endophthalmitis (Martin et al. 2011; Heier et al. 2012; Kodjikian et al. 2013; Schmidt-Erfurth et al. 2014; Berg et al. 2015). More recently, various case reports and case series have reported an increased risk for non-infectious intraocular inflammation (IOI) and retinal vasculitis with the use of brolucizumab (Baumal et al. 2020; Haug et al. 2020; Jain et al. 2020; Monés et al. 2021; Witkin et al. 2020). The mechanisms behind the increased risk for non-infectious IOI following brolucizumab remain unclear at this point. One possibility may lie in the presence of anti-drug antibodies (ADAs) to brolucizumab. Such ADAs may either be pre-existing or induced by prior exposure to brolucizumab. Binding of ADAs to brolucizumab may lead to the formation of immune complexes that can play a role in both the emergence of IOI and retinal vasculitis (Witkin et al. 2020; Sharma et al. 2020a; Cox et al. 2021). At present, it is unclear whether and to what extent ADAs to brolucizumab exist in the general ophthalmic patient population and whether their existence is associated with an increased risk of IOI following intravitreal brolucizumab injection.

The purpose of this cross-sectional study was to measure ADAs to brolucizumab and ranibizumab in the general at-risk population (*i.e.* patients with exudative AMD or other eye diseases) and to describe ADA levels in patients with and without occurrence of IOI events that have received intravitreal injections.

Materials and Methods

Patients and sample collection

Serum and vitreous samples were collected at the Department of Ophthalmology at the University Medical Center Greifswald (Germany) and at the ophthalmological practice 'Augenärzte an der Pappelallee', Greifswald (Germany) between 01/2020 and 02/2021. In total, 192 serum and 54 vitreous samples were analysed. Ethics approval was obtained from The Ethics Committee at the University Medical Center Greifswald and complied with the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient included in the study. No additional procedures except venous blood sampling were performed for this study. Vitreous samples were obtained only from patients undergoing vitrectomy for medically necessary indications. Samples (serum and/or vitreous where appropriate) were obtained from patients with neovascular AMD, diabetic retinopathy, retinal vein occlusion, cataract, glaucoma, dry eye disease, macular hole, epiretinal membranes and intraocular lens (IOL) dislocation thus representing a wide range of eye diseases not all related to aberrant blood angiogenesis or hyperpermeability and not all treated with anti-VEGF agents. The study population thus represents a cross section of the general patient population seen in an Ophthalmology department.

Serum and vitreous samples were centrifuged for 10 min at 3000 *g* and stored in aliquots at −80°C until analysis.

Enzyme-linked immunosorbent assay (ELISA) for ADA detection

96-well immunoplates (Nunc MaxiSorp flat-bottom, Thermo Fisher Scientific, Rockford, IL, USA) were coated with 1 µg/µl brolucizumab (Beovu, Novartis Pharma GmbH, Nuremberg, Germany) or ranibizumab (Lucentis, Novartis Pharma GmbH, Nuremberg, Germany) in phosphate-buffered saline (PBS) at 4°C overnight. After washing four times with 0.05% Tween-20 (vol/vol) in PBS (PBS-T), wells were blocked with 200 µl blocking buffer (1% BSA in PBS-T (w/vol)) for at least 1 hour at room temperature. After blocking, wells were washed again with PBS-T and incubated with 100 µl patient serum or vitreous samples for 2 hr at room temperature. Serum samples were diluted 1:100 and vitreous samples 1:1 in blocking buffer. After washing, wells were then incubated with HRP-conjugated anti-human IgG-Fc secondary antibody (# 05–4220, Thermo Fisher Scientific) for 1 hour at room temperature. Colorimetric reaction of TMB substrate solution (TMB Substrate Set, Biolegend, San Diego, CA, USA) was stopped with 2N H_2SO_4. Optical density (OD) was read at 450 nm with wavelength correction at 550 nm using a multi-mode microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany). An OD of 0.1 allowed to reliably discriminate a definite signal from faint background staining. Therefore, an OD > 0.1 cut-off was defined as a notable ADA signal.

Statistical analysis

IBM SPSS Statistics V.27 (IBM Corporation, Armonk, NY, USA) was used to statistically analyse data. p-Values <0.05 were considered statistically significant.

Results

Anti-brolucizumab antibodies in serum samples

Across our general study population, including a variety of Ophthalmologic diagnoses as well as three healthy volunteers, we observed several samples with distinct levels of ADAs to brolucizumab (Fig. 1). In 35 of 192 serum samples (18.2%), a notable brolucizumab ADA signal with an OD > 0.1 was measured. Some but not all of these patients with brolucizumab ADAs had received brolucizumab injections in the past (patients who had received brolucizumab are colour coded in red in Fig. 1). In female patients, a notable brolucizumab ADA signal was more frequent than in males (females: $n = 92$; OD > 0.1 in 23.9%; males: $n = 100$; OD > 0.1 in 13.0%; Chi^2 test: $p = 0.05$) and female patients had higher ODs than males in the brolucizumab ADA ELISA (OD females: median = 0.033, interquartile range = 0.085; OD males: median = 0.018, interquartile range = 0.035; Mann–Whitney-*U* test: $p = 0.031$; Fig. S1).

Our study population contains two patients who experienced severe vision loss due to occlusive retinal vasculitis following brolucizumab (patient ID S0084 and S0128; both marked in Fig. 1). The clinical pictures of these patients are shown in Fig. 2. One additional patient presented with moderate IOI and signs of segmented blood flow (boxcar-like filling) in one retinal

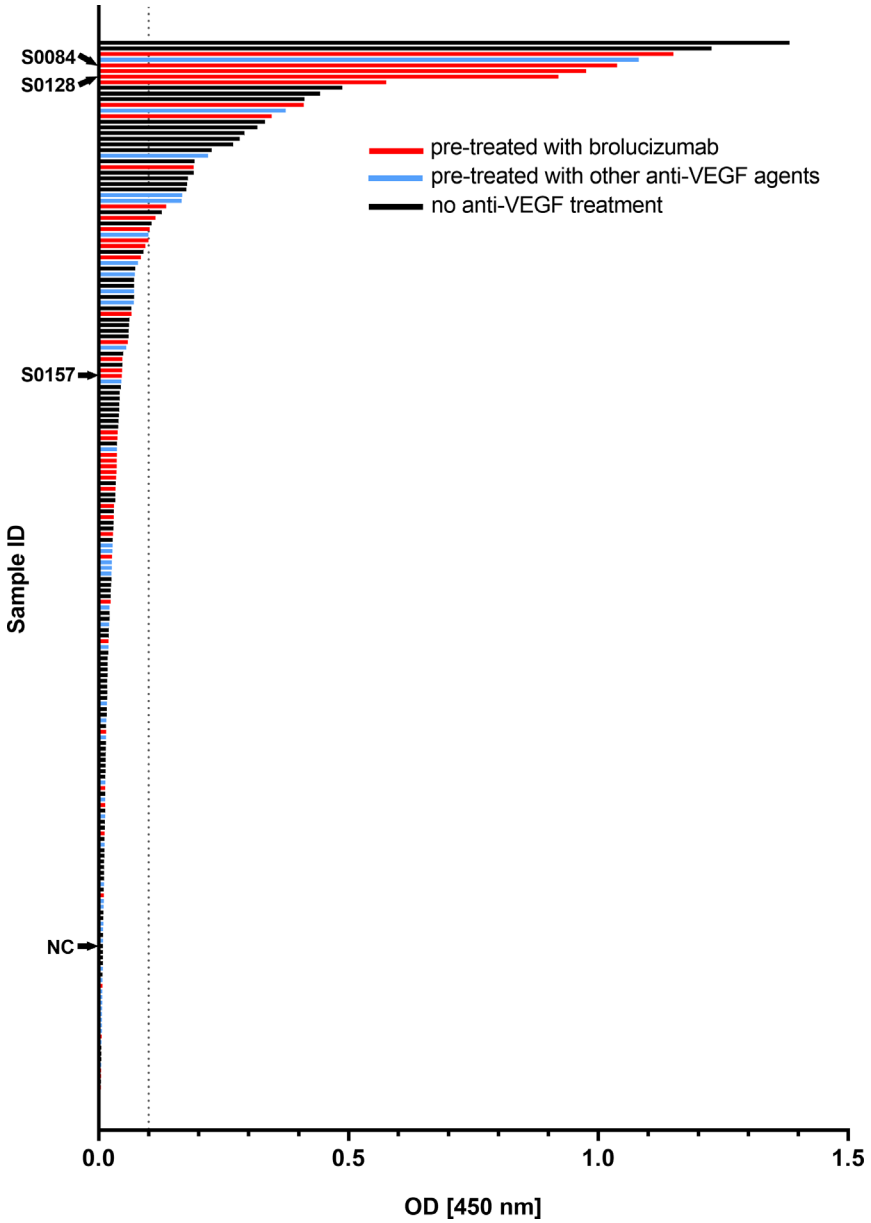

Fig. 1. Brolucizumab anti-drug antibodies (ADAs) in serum samples. Anti-brolucizumab antibody levels are shown for each individual serum sample. Red bars represent patients with prior intravitreal brolucizumab treatment (patients may also have received anti-VEGF agents other than brolucizumab before), blue bars patients with prior intravitreal anti-VEGF treatment, excluding brolucizumab, and black bars patients with no prior anti-VEGF treatment. Codes on the left refer to individual samples; S0084: serum sample from a patient with severe IOI, retinal vasculitis and vascular occlusion following intravitreal brolucizumab and aflibercept injection (taken at time of acute IOI); S0128: serum sample from a patient with severe IOI, retinal vasculitis and vascular occlusion following intravitreal brolucizumab injection (taken 13 weeks after IOI); S0157: serum sample from a patient with moderate IOI following intravitreal brolucizumab injection (taken at time of acute IOI). NC: negative control (no serum; OD = 0.009). Dotted line: OD > 0.1 cut-off defining a notable ADA signal.

vessel, but no vasculitis following brolucizumab injection (S0157, marked in Fig. 1). This patient had a rapid response to intravitreal corticosteroids with full recovery of visual acuity. The clinical picture of this patient is shown in Fig. 3. A review of fundus images from this patient revealed that arterial vessel wall changes had developed under brolucizumab therapy unnoticed by patient and physician prior to the IOI event (Fig. S2).

Anti-brolucizumab antibodies in vitreous samples

Our study population contained 54 patients who underwent vitrectomy for various medical indications. In these patients, we analysed vitreous samples for the presence of anti-brolucizumab antibodies (Fig. 4). Overall, notable brolucizumab ADA signals (OD > 0.1) were slightly less frequent in vitreous samples (7/54 = 13.0%) than in the corresponding serum samples (11/54 = 20.4%) (McNemar's test: p = 0.289). Patient S0084 with severe IOI and retinal vasculitis had high ODs for brolucizumab ADAs both in serum and vitreous (marked in Fig. 4). In other patients, notable ADA serum levels were not always accompanied by notable ADA levels in the vitreous. In the vast majority of our cases (6/7 = 85.7%), vitreous samples with notable brolucizumab ADA levels were obtained from patients who had never received brolucizumab previous to ADA sampling. Only one patient (S0084)

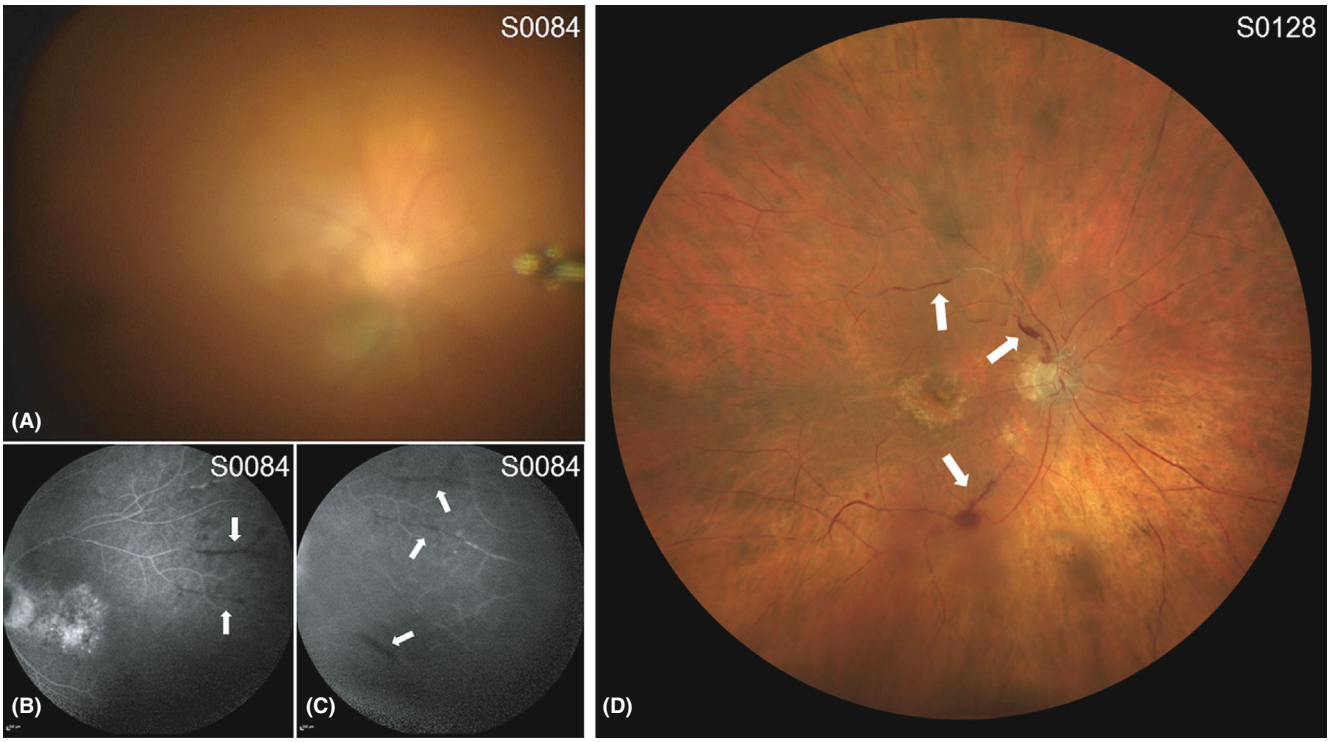

Fig. 2. Clinical images of patient S0084 (A–C) and S0128 (D) who experienced severe IOI and retinal vasculitis in the presence of high brolucizumab ADAs. In patient S0084, vitrectomy was performed in the acute event because infectious endophthalmitis could not be ruled out. Fig. 2A shows the intraoperative picture during vitrectomy. After fundus details became visible, retinal vasculitis was apparent during vitrectomy and later confirmed by fluorescence angiography demonstrating vascular nonperfusion (arrows in Fig. 2B and C). Patient S0128 (Fig. 2D) experienced a similar course with severe IOI, retinal vasculitis and retinal nonperfusion following brolucizumab injection. Arrows in Fig. 2D indicate areas of nonperfusion.

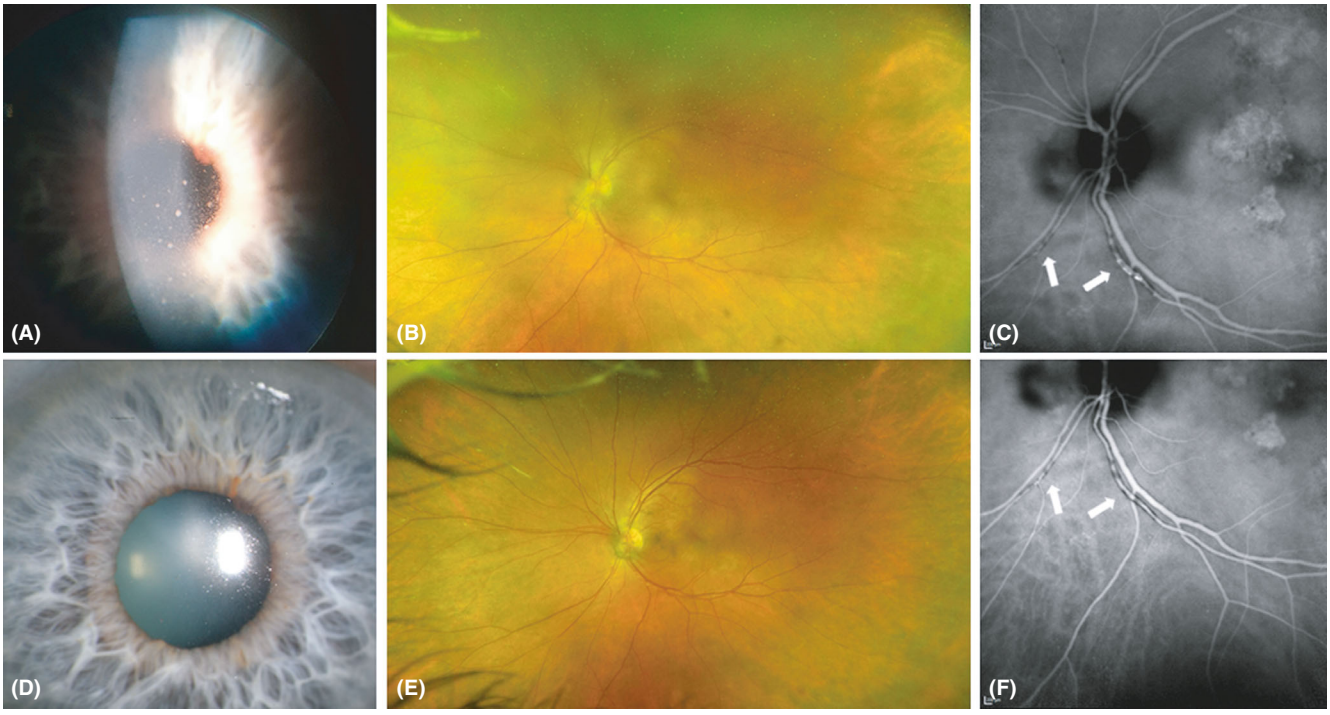

Fig. 3. Clinical images of patient S0157 with lower anti-brolucizumab antibody levels and moderate IOI following intravitreal brolucizumab treatment. (A–C) Moderate IOI four days after repeat intravitreal brolucizumab injection showing anterior chamber cells (A), vitreous haze (B) and retinal vessels with boxcar-like filling (arrows in ICG angiography), but no signs of retinal vasculitis (C); visual acuity was reduced to 20/100. (D–F) Resolved IOI, anterior chamber cells and vitreous haze after four days of systemic high-dose i.v. corticosteroid therapy; visual acuity had returned to 20/20. (A, D) Slit lamp examination. (B, E) Fundus photography. (C, F) ICG angiography. Arrows: boxcar-like filling of retinal vessels on ICG angiography.

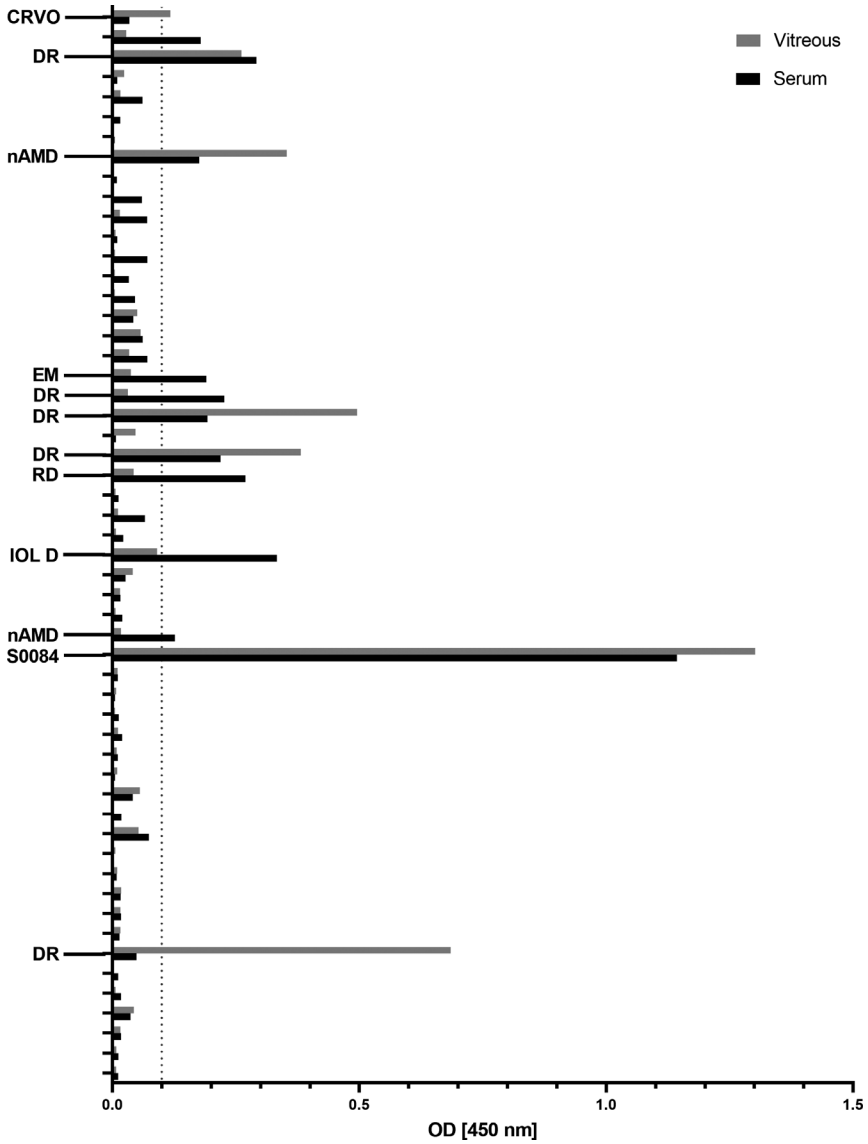

Fig. 4. Brolucizumab anti-drug antibody (ADA) levels in vitreous and serum samples. Grouped bars represent corresponding vitreous (grey) and serum (black) brolucizumab ADA levels from individual patients. Individual patients with notable ADA signals in vitreous and/or serum are marked. Patient S0084 is the only patient who had been exposed to brolucizumab prior to sampling. EM: epiretinal membrane; CRVO: central retinal vein occlusion; nAMD: neovascular AMD (macular haemorrhage without prior anti-VEGF treatment); DR: diabetic retinopathy; IOL D: dislocation of intraocular lens; RD: retinal detachment. Dotted line: OD > 0.1 cut-off defining a notable ADA signal.

received brolucizumab prior to vitreous sampling. We observed a tendency for an increased likelihood of notable vitreal ADA levels in patients with diabetic retinopathy.

Anti-ranibizumab antibodies in serum and vitreous samples

In order to analyse whether ADAs were observable also with other anti-VEGF drugs, we performed anti-ranibizumab antibody ELISAs in our patient cohort. Figure S3 shows that for ranibizumab ADAs, the mean OD over all serum samples, including ranibizumab-treated and other anti-VEGF-treated patients, was very low. Only five patients had notable ODs > 0.1 (5/192 = 2.6% versus 35/192 = 18.2% for brolucizumab ADAs; Chi^2 test: $p < 0.001$), and the highest OD observed for ranibizumab ADAs was 0.544 *vs.* 1.383 for brolucizumab ADAs (the patient with the highest ranibizumab ADA levels is not the same subject as the patient with the highest brolucizumab ADA levels). Across all serum samples, the ODs for ADAs to ranibizumab were significantly lower than for ADAs to brolucizumab (OD ranibizumab ADAs: median = 0.004, interquartile range = 0.008; OD brolucizumab ADAs: median = 0.025, interquartile range = 0.058; Wilcoxon signed-rank test: $p < 0.001$) (Fig. 5).

In vitreous samples, no notable ranibizumab ADA levels were measured. The highest OD for ADAs to ranibizumab measured in vitreous samples was 0.049 (data not shown).

Discussion

In the present study, we analysed the presence of ADAs to brolucizumab and ranibizumab in a broad Ophthalmic cohort of patients with various ocular diseases. We also investigated the correlation of brolucizumab ADAs

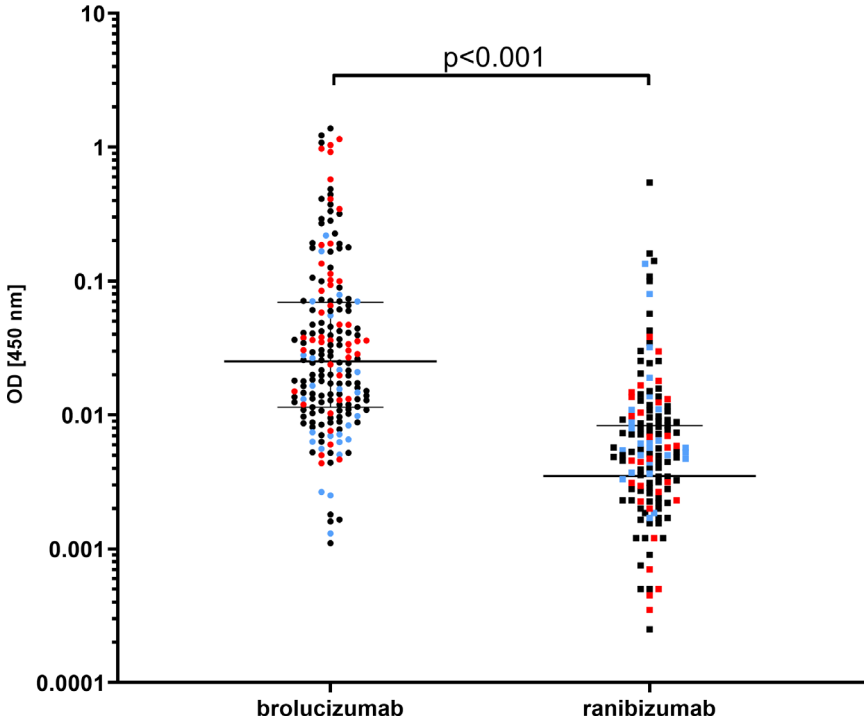

Fig. 5. Comparison of ADA levels between ranibizumab and brolucizumab across all patients. Median ADA levels are significantly lower for ranibizumab compared to brolucizumab. Horizontal bars represent the median with interquartile range. Wilcoxon signed-rank test: $p < 0.001$. OD measured at 450 nm is shown on a logarithmic scale (wavelength correction at 550 nm; blank subtracted). Data points with OD = 0 ($n = 49$ in the ranibizumab group) are not plotted, as the logarithm is not defined mathematically for zero. Red symbols: patients had been not only exposed to brolucizumab prior to sampling, but may have also received other anti-VEGF agents in the past, including ranibizumab. Blue symbols: patients had been not only exposed to ranibizumab prior to sampling, but may have also received other anti-VEGF-agents in the past, excluding brolucizumab. Black symbols: patients with no anti-VEGF treatment prior to sampling.

with IOI reactions following intravitreal brolucizumab treatment.

In our cohort, we observed 3 of 43 (7.0%) patients who experienced moderate ($n = 1$; 2.3%) or severe ($n = 2$; 4.7%) IOI following intravitreal brolucizumab. These observations are comparable to data from the *post hoc* analysis of HAWK and HARRIER phase 3 clinical trials of brolucizumab for neovascular age-related macular degeneration, which demonstrated the incidence of IOI following intravitreal brolucizumab to be about 4.6%, the incidence of IOI with concomitant retinal vasculitis to be about 3.3% and the incidence of IOI, vasculitis and vascular occlusion to be about 2.1% (Monés et al. 2021).

An association between intraocular inflammatory events following intravitreal drug delivery and the presence of ADAs is subject of current scientific discussion and evaluation (Sharma et al. 2019, 2021). Intraocular inflammation was more common in patients who were positive for ADAs to brolucizumab (6%) compared with patients who were negative for ADAs to brolucizumab (2%) (Witkin et al. 2020). A type III hypersensitivity reaction mediated by intravascular IgG/IgM immune complex deposition was proposed to potentially cause the vasculitis, which is a known adverse event to systemic monoclonal antibodies including anti-VEGF agents used for cancer therapy (Baldo 2013; Sharma et al. 2020a, 2021; Cox et al. 2021). The two patients in our study population who experienced severe IOI and occlusive retinal vasculitis following intravitreal brolucizumab injection (S0084 and S0128; Figs 1 and 2) had both high ODs in the anti-brolucizumab antibody ELISA, indicating high serum titres of IgG ADAs to brolucizumab. The patient who experienced only moderate IOI following brolucizumab had only low serum ADA titres to brolucizumab. In this patient, prompt improvement of IOI was achieved with systemic corticosteroids. Our data also show that the presence of ADAs does not necessarily result in IOI or vasculitis as we observed high brolucizumab ADA titres in patients receiving intravitreal brolucizumab without any clinically apparent signs of inflammatory events. Therefore, intraocular inflammatory adverse events including inflammatory vasculitis seem to be a rather complex and multifactorial entity and not solely related to the presence of ADAs. Several medication-specific and delivery-specific factors have been discussed to contribute to inflammatory reactions, including impurities of the drug formulations such as endotoxin remnants and non-human proteins originating from manufacturing processes or the formation of silicone oil-protein complexes due to the siliconized inner wall of syringes used for intravitreal injections (Sharma et al. 2020b; Anderson et al. 2021).

The fact that we observed anti-brolucizumab antibodies in patients who had never received brolucizumab demonstrates the presence of pre-existing antibodies. These cross-reactive antibodies may result from contact with microbial or other foreign antigens sharing similar structures to brolucizumab. Genetic predisposition for specific ADA occurrence can also be considered as human leukocyte antigen (HLA) status is involved in the context of adaptive immune reactivity (Jawa et al. 2013). In our study

population, higher brolucizumab ADA levels were observed in female patients. This is in line with recently published case series implying an increased risk for IOI and retinal vasculitis in female patients receiving brolucizumab (Baumal et al. 2020; Witkin et al. 2020). In the HAWK and HARRIER clinical trials, 36–52% of patients were reported to have pre-existing anti-brolucizumab antibodies and this proportion increased to 53–67% after initiation of intravitreal brolucizumab treatment (Witkin et al. 2020). The use of different assays and setting different cut-offs for notable ADA levels may explain the difference between HAWK and HARRIER and our data.

Both the frequency of notable ADA signals and the mean ADA level were higher for brolucizumab than for ranibizumab in the present study. Non-infectious intraocular inflammation in association with intravitreal ranibizumab was reported to be a rare event, occurring at low rates ranging from 0.005% to 1.9% depending on the study (Anderson et al. 2021), and to be less frequent with ranibizumab than with bevacizumab (Sigford et al. 2015; Knickelbein et al. 2016; Williams et al. 2016; Anderson et al. 2021) or aflibercept (Knickelbein et al. 2016; Souied et al. 2016; Williams et al. 2016; Anderson et al. 2021). Due to the lack of the Fc antibody portion in the ranibizumab molecule, restricted activation of Fc receptors by ranibizumab was hypothesized to be responsible for these differences (Knickelbein et al. 2016; Souied et al. 2016). Brolucizumab does, like ranibizumab, not have an Fc portion, but nevertheless seems to provoke immunogenicity to a larger degree than ranibizumab. Although not statistically significant, it is interesting to note that more patients with prior brolucizumab exposure (12/43 = 27.9%) showed notable ADAs compared with patients who had never been exposed to brolucizumab (23/149 = 15.4%) (Chi^2 test: p = 0.062). Similar results were obtained for alternative cut-offs (p = 0.112 for an OD cut-off of 0.25 and p = 0.09 for an OD cut-off of 0.05). However, this was not observed for ADAs to ranibizumab (1/25 = 4% and 4/167 = 2.4% for exposed *vs.* non-exposed patients; Chi^2 test: p = 0.638) and is also different from data from a phase 3 trial for aflibercept that found ADAs prior to and after aflibercept exposure at comparable rates of 1–3% (Heier et al. 2012). Currently, it is not clear, if the enhanced immunogenicity is related to the brolucizumab molecule itself or to a secondary dose effect. Due to the small size of brolucizumab (26 kDa), elevated molar concentrations can be achieved, allowing for longer intervals between intravitreal injections compared with other anti-VEGF agents (Holz et al. 2016). Higher molar concentrations may however also contribute to an increased formation of silicone oil-protein aggregates that may alter and potentially exacerbate the immunogenicity of brolucizumab (Anderson et al. 2021). Structural similarity of brolucizumab with a relatively common (albeit unknown) immunogen may account for the higher incidence of pre-existing brolucizumab than ranibizumab ADAs in patients not exposed to prior anti-VEGF agent.

Collectively, our data are in line with previous observations and indicate that both pre-existing and induced brolucizumab ADAs may play a role in IOIs following brolucizumab.

The blood–retinal barrier contributes to the ocular immune privilege that protects the inner eye from immunogenic inflammation (Streilein 2003). Data from Wessels et al. (2018) indicate that ADAs are generated systemically and then transported into the eye. Therefore, blood–ocular barrier disturbances may increase the likelihood of ADAs entering the eye. Indeed, we observed high vitreal brolucizumab ADA signals primarily in patients with diabetic retinopathy in which blood–retinal barrier breakdown is a disease characteristic. This observation may be important when treatment of patients with diabetic retinopathy with brolucizumab is considered.

A limitation of the present study is related to the restricted number of cases with IOI or retinal vasculitis following intravitreal brolucizumab, which was about 3 out of 43 patients treated intravitreally with brolucizumab. A statistical correlation of IOI/retinal vasculitis with the presence of ADAs to brolucizumab cannot be drawn from these observations. However, with respect to the only recently arising issue and the overall scarcity of published data, descriptive data presentation is valuable to advance our understanding of possible causative pathways and associations between clinical parameters and outcomes. Future studies are mandatory to investigate whether ADA screening can be an applicable tool to predict patients at risk and to reduce intraocular inflammatory adverse events in response to brolucizumab.

Taken together, in our cross-sectional study population including both brolucizumab-treated and treatment-naïve patients, we detected ADAs to brolucizumab or pre-existing antibodies cross-reacting with brolucizumab. The ADA signal was stronger and more frequent for brolucizumab than for ranibizumab. Taking previous observations and our data into consideration, anti-brolucizumab ADAs may represent a risk factor for IOI and retinal occlusive vasculitis in patients treated with brolucizumab.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Brolucizumab ADA ELISA from serum of female and male patients. OD measured at 450 nm is shown for each serum sample on a logarithmic scale (wavelength correction at 550 nm; blank subtracted). Horizontal bars (red) represent the median with interquartile range. Mann–Whitney U-test: $p = 0.031$

Figure S2. Fundus photography of patient S0157 demonstrates developing segmented blood flow in an arterial vessel (arrows) under intravitreal brolucizumab treatment prior to IOI event (text boxes indicate time and type of anti-VEGF drug injection)

Figure S3. Ranibizumab ADAs in serum samples. Anti-ranibizumab antibody levels for each individual serum sample (OD measured at 450 nm; wavelength correction at 550 nm; blank subtracted). Red bars represent patients with prior intravitreal ranibizumab treatment (patients may also have received anti-VEGF agents other than ranibizumab before), blue bars patients with prior intravitreal anti-VEGF-treatment, excluding ranibizumab, and black bars patients with no prior anti-VEGF treatment. Notable ADA levels for ranibizumab are less frequent compared with brolucizumab. NC: negative control (no serum; OD = 0.003). Dotted line: OD > 0.1 cut-off defining a notable ADA signal. Legends