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Biomechanical Properties of the Internal Limiting Membrane after Intravitreal Ocriplasmin Treatment

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Key Words
Internal limiting membrane · Ocriplasmin · Vitreomacular traction

Abstract
Purpose: To assess the stiffness of the human internal limiting membrane (ILM) and evaluate potential changes of mechanical properties following intravitreal ocriplasmin injection for vitreomacular traction. ***Methods:*** This is an interventional comparative case series of 12 surgically excised ILM specimens consecutively obtained from 9 eyes of 9 patients after unsuccessful pharmacologic vitreolysis with ocriplasmin. During the same time period, 16 specimens from 13 other eyes without ocriplasmin treatment were harvested during vitrectomy and served as controls. All patients presented with macular holes or vitreomacular traction and underwent vitrectomy with ILM peeling either with or without brilliant blue (BB) staining. All specimens were analyzed using atomic force microscopy with scan regions of 25 × 25 µm. In all specimens, both the retinal side and vitreal side of the ILM were analyzed. ***Results:*** Atomic force microscopy revealed no significant differences in elasticity of ILM specimens removed from eyes with or without ocriplasmin treatment. Undulated areas of the retinal side presented stiffer than the vitreal side of the ILM. Topographical mapping of both the vitreal and retinal side of the ILM showed no apparent alteration of the morphology in ocriplasmin-treated eyes compared to untreated eyes. Staining with BB resulted in an increase of tissue stiffness. ***Conclusions:*** Intravitreal injection of ocriplasmin does not change biomechanical properties of the human ILM. There is no evidence of a potential enzymatic effect of ocriplasmin interfering with the stiffness of this basement membrane.

Introduction

Ocriplasmin (Jetrea®, ThromboGenics, Leuven, Belgium) is an unspecific and recombinant truncated form of the human serine protease plasmin that exerts proteolytic activity on laminin and fibronectin, both major components of the vitreoretinal interface [1]. Pharmacologic vitreolysis with ocriplasmin was recently approved for the treatment of patients with symptomatic vitreomacular adhesion and vitreomacular traction (VMT), including when associated with full-thickness macular holes ≤400

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μm in diameter [2, 3]. Release of VMT can be achieved by pharmacologic vitreolysis in up to 70% of cases after a single intravitreal injection of ocriplasmin into the vitreous cavity (0.125 mg/0.1 ml), as confirmed by subgroup analyses of eyes with VMT [4, 5].

Compared to pars plana vitrectomy with peeling of the internal limiting membrane (ILM), which was the only treatment option until recently, intravitreal ocriplasmin injection provides a generally well-tolerated therapeutic intervention for patients with VMT [6, 7]. There are numerous potential advantages of pharmacologic vitreolysis over surgical vitrectomy, such as avoidance of surgical risks, faster visual rehabilitation, and induction of a complete posterior vitreous detachment without remnants of vitreous cortex collagen on the ILM.

However, some concerns were raised regarding the safety profile of ocriplasmin. A more detailed analysis of safety data from phase 2 and 3 trials as well as clinical case reports and small case series revealed acute panretinal structural and functional abnormalities after ocriplasmin treatment that do not seem to be related to the induction of complete posterior vitreous detachment alone. One may hypothesize that under the light of this information, ocriplasmin may somehow affect deeper retinal cellular structures or components relevant for retinal integrity. In this case, ocriplasmin would have to penetrate the ILM. Given the enzymatic properties of ocriplasmin, one may assume that ocriplasmin also affects known ILM components such as laminin and fibronectin. This effect would likely have an impact on the biomechanical properties of the ILM, which is a very rigid tissue whose biomechanical rigidity or stiffness resembles articular cartilage.

Atomic force microscopy (AFM) is a well-established technique to measure tissue rigidity of basement membranes such as the ILM on a nanoscale level [8–11]. In the present experimental study, we used this technique to quantify potential changes of biomechanical properties of the ILM following ocriplasmin treatment for tractional maculopathies such as macular holes or VMT syndrome.

Materials and Methods

This is an interventional comparative case series of 12 surgically excised ILM specimens consecutively obtained from 9 eyes of 9 patients after unsuccessful pharmacologic vitreolysis with ocriplasmin who underwent vitrectomy with ILM peeling at the Department of Ophthalmology, Ludwig Maximilian University, and at the Herzog Carl Theodor Eye Hospital, Munich between May 2014 and January 2015. The Institutional Review Board and the Ethics Committee of the Ludwig Maximilian University, Munich approved the retrospective review of the patients' data as well as the preparation and analysis of the patients' specimens (No. 471-14). This study was conducted according to the tenets of the Declaration of Helsinki.

Six eyes showed persistent macular holes and 3 eyes presented with persistent symptomatic VMT as confirmed by high-resolution optical coherence tomography. Five of 6 eyes with macular holes were seen with resolution of VMT and progression of the macular hole diameter after ocriplasmin injection. Macular surgery followed ocriplasmin injection after a mean period of 4.1 months (range 1–10 months). Eyes with macular holes underwent surgery after a mean period of 2.3 months (range 1–3 months), which was significantly earlier than eyes with VMT. Pharmacologic vitreolysis with ocriplasmin was applied according to the guidelines of the German Ophthalmological Society, the Retina Society and the Professional Association of German Ophthalmologists for therapeutic intravitreal application of ocriplasmin.

During the same time period, 16 specimens from 13 other eyes without ocriplasmin treatment were harvested during vitrectomy and served as controls. In this control group, we included 4 specimens of 4 eyes with idiopathic macular holes and 12 specimens from 9 eyes with VMT.

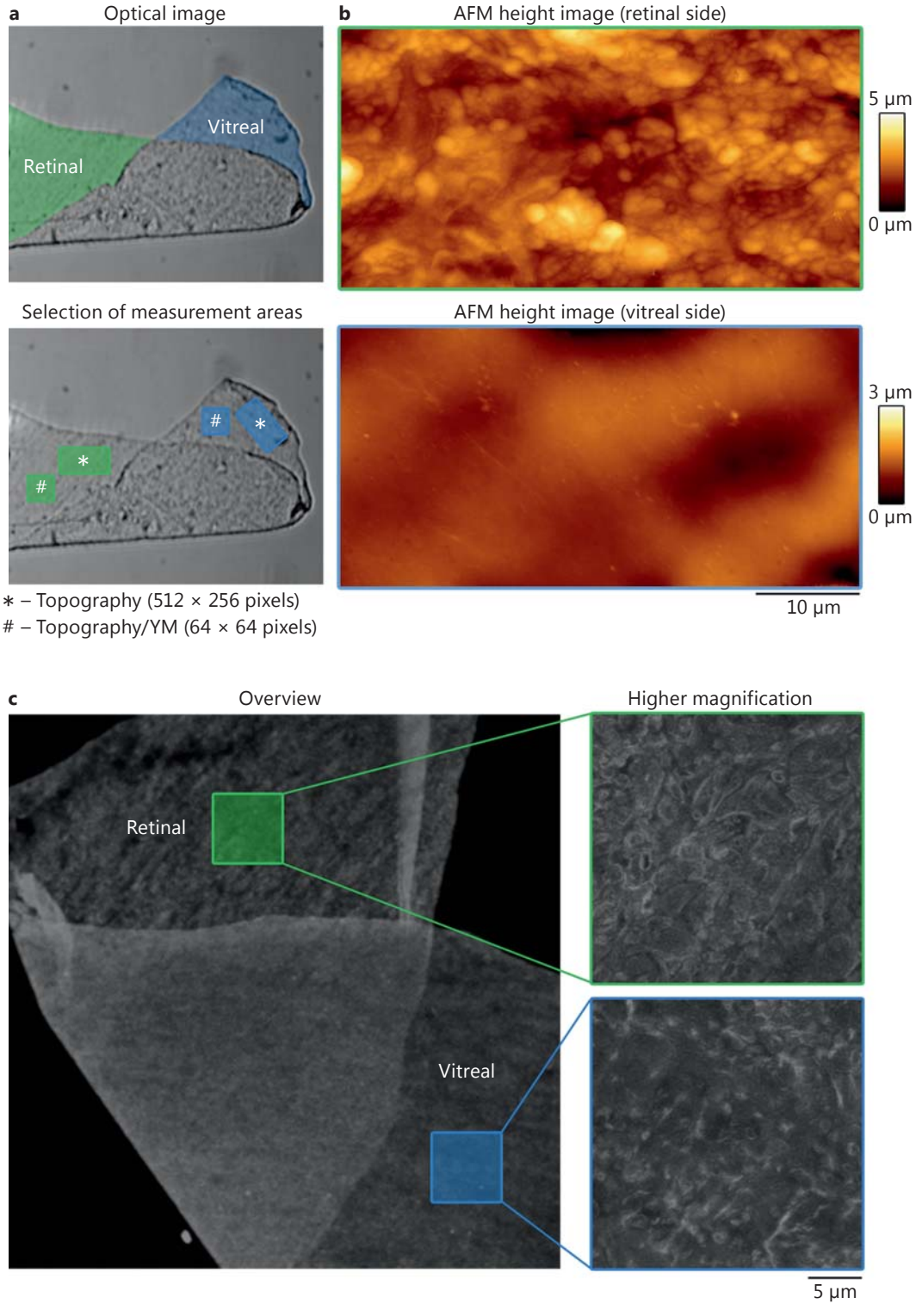
In 3 eyes with ocriplasmin treatment, 2 specimens were harvested during vitrectomy, respectively. In these cases, the first specimen was removed without intravitreal staining of the ILM during surgery, the second specimen was removed with usage of vital dye brilliant blue (BB). Similarly, in 3 eyes of the control group without ocriplasmin treatment, 2 specimens per eye were removed during vitrectomy as illustrated above.

Surgical Procedure and Specimen Removal

The surgical technique consisted of a 23-gauge 3-port pars plana vitrectomy with peeling of the ILM. All patients were operated by 3 experienced surgeons. Vitrectomy was performed as follows: before opening the infusion line, the status of the posterior hyaloid was determined using a plano-concave contact lens. If the vitreous was attached or partially attached to the retina, posterior vitreous detachment was induced by suction with the vitrectomy probe over the optic disc or in the midperiphery. The posterior hyaloid was detached from the retina and excised up to the periphery.

To improve the precision of ILM peeling and to avoid incomplete removal, brilliant blue G (BBG; 0.2 ml, brilliant peel; Geuder, Heidelberg, Germany) was used to visualize the ILM in 7 eyes with previous ocriplasmin treatment and in 8 eyes without ocriplasmin treatment. All other eyes were operated without intraoperative staining of the ILM. In a subgroup of eyes with BB staining, the first ILM specimen was removed without BBG, and the second specimen was removed with usage of BB. In all other eyes, only 1 specimen was harvested. If applied, the dye solution was injected onto the vitreomacular interface and immediately washed out. No other dye was applied. Peeling of the ILM was performed using end-gripping Eckardt forceps. If necessary, removal of the ILM was performed en bloc with epiretinal tissue.

Surgery was completed by checking the retinal periphery for breaks. In eyes with small macular holes, the vitreous cavity was perfused with a mixture of 15% C2F6 following a fluid-air exchange. In eyes with persistent vitreomacular adhesion, balanced salt solution was left in the vitreous cavity at the end of the procedure. The removed ILM specimens were harvested and immediately placed onto glass slides without fixation.



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Atomic Force Microscopy

For AFM analysis, a NanoWizard® 3 AFM (JPK-Instruments, Berlin, Germany) mounted on an inverted optical microscope (Carl Zeiss, Jena, Germany) was used, which allows the selection of the exact scanning area by visualizing the ILM with a 10× objective (fig. 1a). Specimens were mounted on microscopy slides and subjected to AFM measurements to outline topography and elasticity. For each specimen, measurement areas were selected from the optical image where the membrane was clearly seen blank without epiretinal cells or collagen (fig. 1a). The typical morphology of the rough retinal and rather smooth vitreal side was also outlined by AFM height images and laminin staining (fig. 1b, c). Throughout all experiments, the AFM was operated in QI mode, a force spectroscopy-based imaging mode that allows for simultaneous measurements of topography and elasticity of the specimens.

Pyramidal-shaped D tips of Si_3N_4 cantilevers (MLCT, Bruker, Mannheim, Germany) with a nominal spring constant of 0.03 N/m were used. The spring constant k was determined using the thermal noise method [12]. Samples were measured under physiological conditions at 37°C in PBS. For overview pictures, 25 × 50 µm wide scanning areas were chosen with a resolution of 512 × 256 pixels (fig. 1a, *). For elasticity maps, 25 × 25 µm wide scanning areas (3 different areas/side) where chosen from the optical image and resolution was set to 64 × 64 pixels (fig. 1a, #). For each pixel, information on height (for generation of topography maps) and elasticity (for calculation of the Young's modulus, YM) were recorded. In total, 3 areas with 4,096 measurements each were obtained for each specimen. A Z-length of 2,000 nm, an extending and retracting speed of 100 µm/s (overview maps) or 10 µm/s (elasticity maps), an additional retract of 200 nm and a relative set point of 0.2 nN was applied to avoid sample alterations due to mechanical strain. For determination of YM, the JPK analysis software was applied, which calculates YM using the Bilodeau formula for pyramidal indenter of the Hertz model [13, 14].

Due to simultaneous delineation of topography and elasticity, sample orientation with differentiation between the vitreal and the retinal side of the ILM could be specified during measurements. Additional tests to confirm orientation of the specimens were performed after AFM measurements. All specimens were fixed with 2% paraformaldehyde solution and processed for indirect immunostaining with mouse anti-human laminin antibody (clone 4C7; Dako, Hamburg, Germany) and a Cy3-coupled goat anti-mouse (Dianova, Hamburg, Germany) secondary antibody. Image capturing was performed using an SP5 confocal microscope equipped with a Plan Apo 20× NA 0.7 and a Plan Apo 63× NA 1.4 objective (both Leica, Mannheim, Germany; fig. 1c).

Fig. 1. a Optical image of peeled ILM. The ILM is partially flipped over to expose the vitreal and retinal side. The retinal side is marked in green, the vitreal side in blue (upper picture). Scanning areas (green/blue rectangles) were first selected for identification of the respective side of the ILM (50 × 25 µm, 512 × 256 pixels, *) and subsequently for topography and elasticity measurements on both sides (25 × 25 µm, 64 × 64 pixels, #). **b** Rough and undulated retinal and smooth vitreal ILM side. **c** ILM stained for laminin for fluorescence microscopy. Again, rough retinal and smooth vitreal sides are clearly distinguishable.

Data Analysis and Statistics

All surgically excised specimens were processed blinded and analyzed by 2 investigators independently according to 4 groups as follows: (1) specimens after ocriplasmin treatment removed with intravitreal usage of BB; (2) specimens after ocriplasmin treatment removed without intravitreal staining of the ILM; (3) specimens without ocriplasmin treatment removed with intravitreal usage of BB, and (4) specimens without ocriplasmin treatment removed without intravitreal staining of the ILM.

Images and YM fit were processed using the JPK data processing software. Further analysis was done using Adobe Photoshop CS5. Data values were compared using one-way ANOVA with Kruskal-Wallis and Tukey's multiple comparison test in Prism (Graphpad Software, La Jolla, Calif., USA). Statistical significance was presumed for $p < 0.05$. Data are displayed as mean ± standard error of the mean.

Results

AFM revealed no significant differences in elasticity of ILM specimens removed from eyes with or without ocriplasmin treatment. Specimens removed from eyes with ocriplasmin treatment but without staining with BB displayed a slightly but not significantly increased YM of 34.18 ± 8.47 kPa on the vitreal and 148.11 ± 27.29 kPa on the retinal side of the ILM. In contrast, in untreated control group eyes, the ILM demonstrated a YM of 16.29 ± 3.34 kPa on the vitreal and 122.76 ± 26.64 kPa on the retinal side (fig. 2a, YM). According to topography and elasticity measurements, undulated areas of the retinal side presented stiffer (fig. 2a, YM, white arrows). Topographical mapping of both the vitreal and retinal side of the ILM showed no apparent alteration of the morphology in ocriplasmin-treated eyes compared to eyes without ocriplasmin injection before vitrectomy (fig. 2a). Similarly, no alterations in topography were found in the comparison of unstained ILM peeling and ILM peeling with BB.

When comparing ILM specimens removed with or without intravitreal application of BB, there was a difference in YM between specimens removed with or without BB. The intravitreal application of BB for easy and safe ILM peeling resulted in an increased YM, which was especially prominent on the vitreal side (fig. 2b). In the ocriplasmin-untreated eyes, the use of BB resulted in a significant increase in YM of 16.29 ± 3.34 kPa on the vitreal side and 122.76 ± 26.64 kPa on the retinal side. In unstained eyes, the YM measured 68.17 ± 18.74 kPa on the vitreal side and 152.1 ± 17.46 kPa on the retinal side of the ILM. In ocriplasmin-treated eyes, the increase after BB staining was less pronounced with a YM of 34.18 ± 8.47

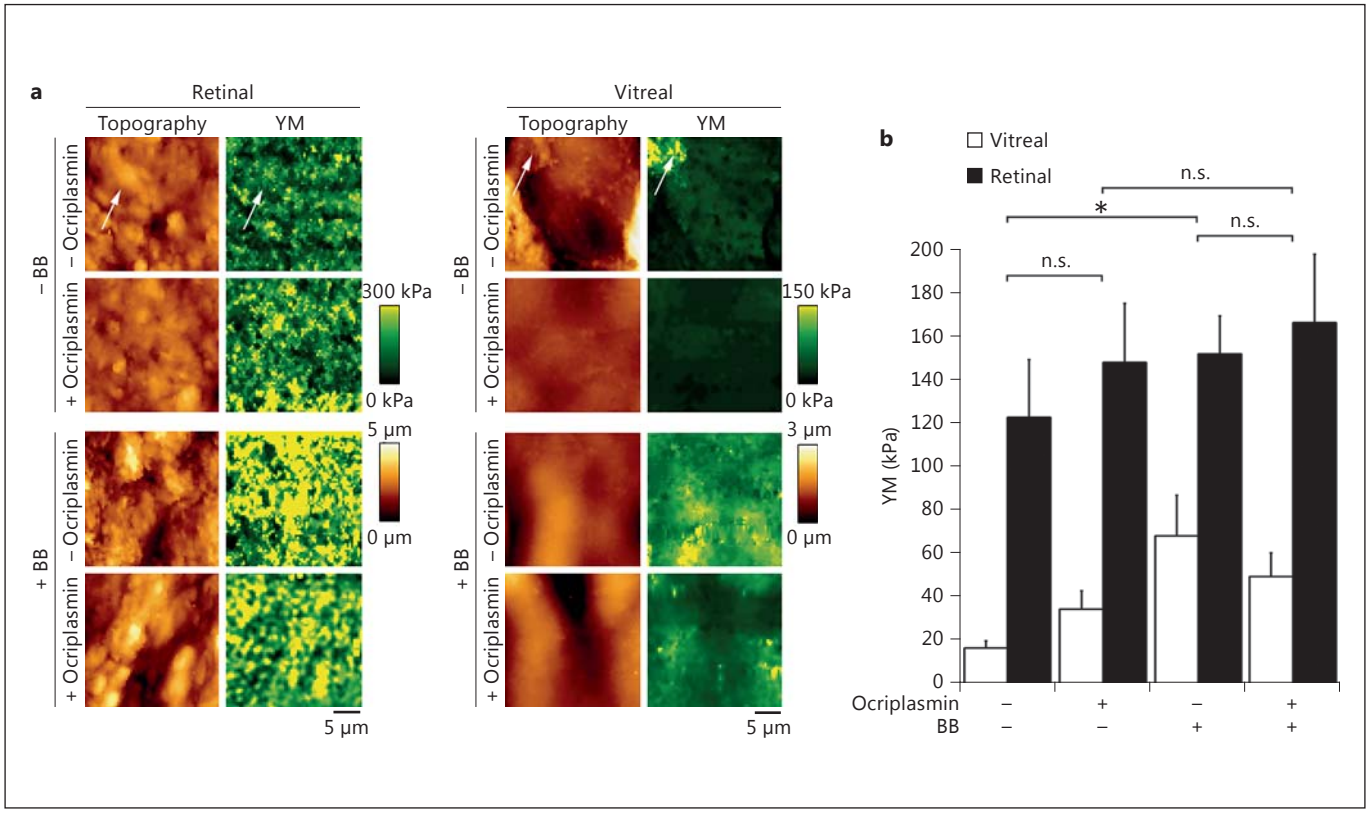

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Fig. 2. a Topography and elasticity maps of the retinal (left panels) and vitreal (right panels) side of the ILM reveal no morphological or elasticity changes due to ocriplasmin treatment. Topography and YM correlate in several areas (white arrows). BB increases YM in both control and ocriplasmin-treated groups. **b** Average of all maps within the respective groups for both ILM sides with error bars representing standard error of the mean. * $p < 0.05$.

kPa on the vitreal side and 148.11 ± 27.29 kPa on the retinal side in unstained ILM, compared to a YM of 49.42 ± 10.89 kPa on the vitreal side and 166.65 ± 31.49 kPa on the retinal side of the ILM in eyes with BB-assisted ILM peeling (fig. 2a, b).

To study the effect of BB-assisted ILM peeling in eyes with ocriplasmin, we investigated specimens from 3 eyes who underwent vitrectomy with peeling of a first unstained ILM specimen followed by application of BB with peeling of a second stained ILM specimen. Topography and elasticity analysis were performed in both specimens as illustrated above. YM before staining was set to 1.0 (fig. 3a). Topography maps confirm that morphology of the ILM is not altered by BB application. Furthermore, a slight but not significant increase of elasticity was evident comparing both sides before and after BB application in these eyes (fig. 3b).

Discussion

Since laminin constitutes a family of glycoproteins that are integral parts of the structural scaffolding of basement membranes [15–18] and ocriplasmin exerts proteolytic activity on laminin, the purpose of this study was to investigate the enzymatic effect of ocriplasmin on biomechanical properties of the ILM. We included ILM specimens removed from eyes that underwent vitrectomy with ILM peeling with and without ILM staining using BBG after unsuccessful ocriplasmin treatment and compared these to specimens removed from eyes that had no ocriplasmin injection prior to surgery.

The present study is in line with and confirms the result of our previous report using AFM measurements following ILM staining using vital dyes [10]. In accordance with our previous investigation, we confirmed a stiffening effect of the ILM following BB application, which

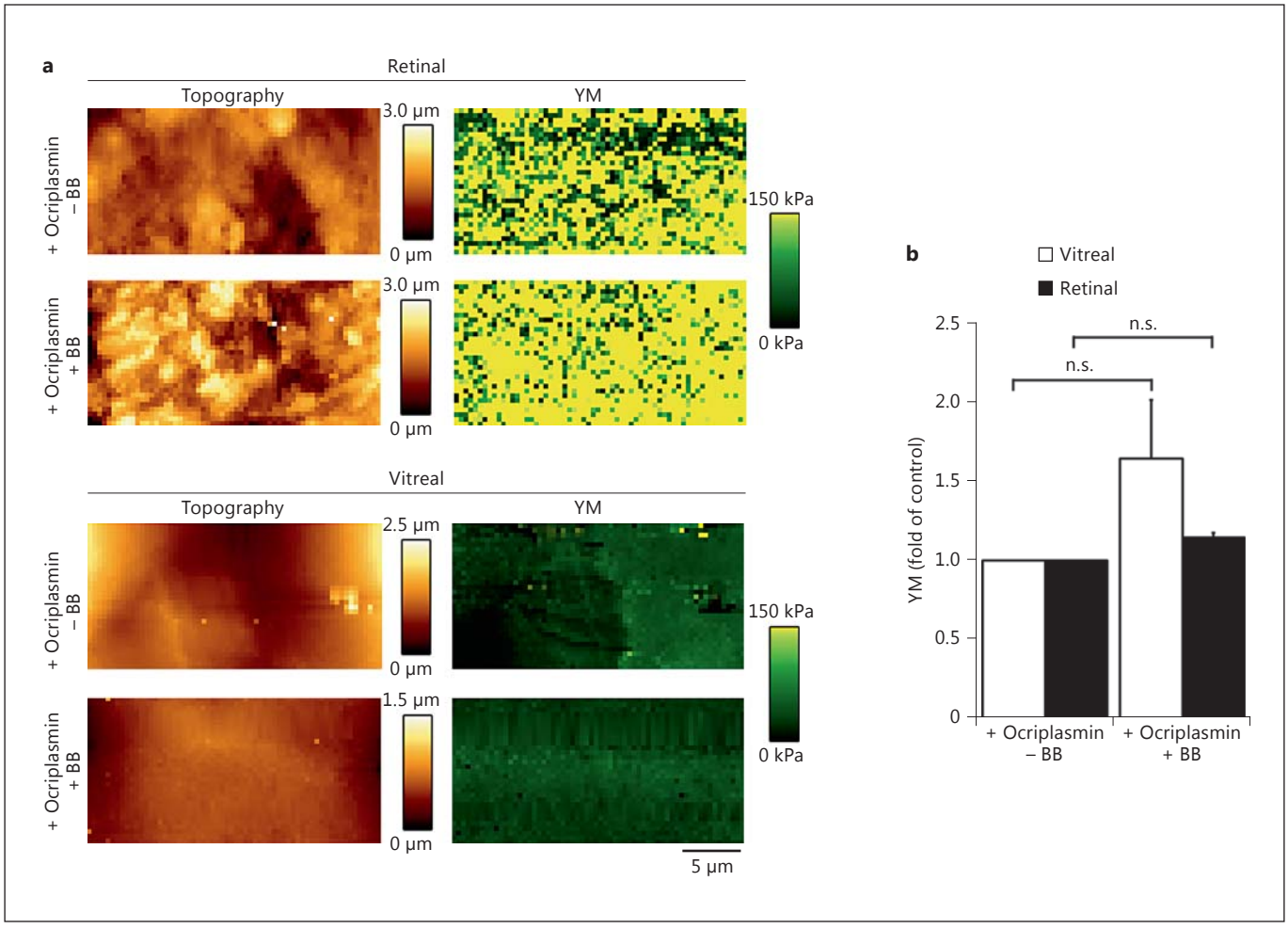

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Fig. 3. a Topography and elasticity maps of 3 patients treated with ocriplasmin before and after additionally receiving BB. **b** Average of YM maps of both ILM sides. YM before BB application was set to 1 and compared to the value after BB application. BB did not significantly alter YM patients treated with ocriplasmin.

could not be seen in unstained ILM specimens. In addition, we observed a more pronounced stiffening effect of the ILM following the use of indocyanine green compared to BBG, especially after illumination of the stained specimen. This may be related to the fact that indocyanine green has photosensitizing properties which may increase this effect. Furthermore, we observed differences in tissue elasticity when analyzing the retinal and vitreal surface of the ILM both in unstained and stained specimens, with the retinal surface of the ILM being stiffer than the vitreal side. The confirmation of our previous results assured that the AFM is a reliable technique with reproducible results. Based on our findings, we are confident that a potential enzymatic effect of ocriplasmin on the ILM interfering with the stiffness of the tissue would have been detectable by AFM, if present.

Ocriplasmin is currently used and approved for the treatment of small macular holes and VMT smaller than 1,500 µm by intravitreal injection [2]. Applied intravitreally, the enzyme has been shown to induce a posterior detachment and liquefaction of the vitreous. Clinical reports and small case series raised concern on potential side effects of the enzyme. These studies reported on clinical symptoms and findings including varying degrees of retinal dysfunction, such as visual acuity loss, dyschromatopsia, nyctalopia, visual field constriction, outer retinal signal abnormalities or defects on spectral domain optical coherence tomography, macular hole enlarge-

ment, macular detachment with subretinal fluid accumulation, and electroretinography changes [6, 7, 12–14, 19–25].

The ILM of the retina is a multi-laminar structure mainly composed of type IV collagen, laminin and fibronectin and essentially represents the basement membrane of Müller cells. Under higher magnification, it typically reveals a smooth vitreal surface and a rough surface which is oriented towards the retina. This distinct morphological appearance helps to identify the respective surface during microscopy studies as performed herein. Although it was experimentally shown that ocriplasmin seems not to affect the structural aspect of the vitreal ILM surface using electron microscopy [1], we do not have any information on potential alterations of the biomechanical properties of the ILM following the contact of the retinal surface with ocriplasmin, which may very well not be detectable using microscopy. Such alterations may be the result of the known enzymatic properties of ocriplasmin, with laminin and fibronectin being important enzymatic targets. Both laminin and fibronectin are also relevant components of the ILM. The proteolysis of these structures may allow the drug to even penetrate the ILM and reach the outer layers of the neurosensory retina, thereby cleaving laminin throughout the retinal layers [12–14]. Of note, laminin was not only reported to be a component of the ILM as mentioned, but also of the outer plexiform layer, the external limiting membrane, and the interphotoreceptor matrix [26–28]. Although cleavage of more than one protein may be responsible for the various manifestations of ocriplasmin-induced retinal dysfunction, it was hypothesized that degradation of intraretinal laminin plays an important role in ocriplasmin-associated side effects [12–14].

However, the present investigation has some limitations. Of note, AFM provides measurements of the tissue surface elasticity, which does only indirectly provide information about the deeper aspects of the ILM. Nevertheless, we did not see any changes of the stiffness of the ILM following ocriplasmin exposure and it seems very reasonable to assume that an affection of deeper ILM layers is unlikely in the presence of this result. More importantly, AFM measurements as performed herein do not represent direct measurements of the changes of the tensile strength of the ILM. Nevertheless, as the ILM represents an irregularly structured meshwork of various fibers, one may transfer our findings and conclude that a change of the tensile properties is very unlikely to occur.

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Disclosure Statement

The authors indicate no financial conflict of interest.

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