

# Age-Related Loss of Human Vitreal Viscoelasticity

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**Purpose:** To determine the viscoelasticity of human vitreous bodies and its changes with age in order to benefit the understanding and therapy of vitreoretinal diseases.

**Methods:** In a postmortem study, 190 human vitreous bodies were extracted from 33- to 92-year-old donors, analyzed with regard to their viscoelastic properties via dynamic mechanical analyses, and compared with bovine and porcine vitreous. Postmortem intervals and donor-related parameters were examined as potential parameters influencing vitreous viscoelasticity. Dynamic moduli of different hyaluronic acid (HA) solutions as well as human vitreous treated with HA injections were determined by frequency sweep tests.

**Results:** With age the viscoelasticity of human vitreous bodies decreased significantly and independently of postmortem intervals, diabetes, and the donor's sex. The storage modulus  $G'$  and loss modulus  $G''$  correlated strongly with the donor's age with  $r = -0.789$  and  $r = -0.764$ , respectively. Bovine and porcine vitreous bodies exhibited dynamic moduli comparable only to the viscoelastic properties of aged human vitreous and are thus limited models for the simulation of the human vitreous. The viscoelasticity of aged human vitreous bodies was found to be increased after intravitreal injections of highly concentrated HA.

**Conclusions:** The present postmortem study is the first to show a significant age-related reduction in the viscoelasticity of entire human vitreous bodies. Highly concentrated HA injections may serve as a possible therapeutic approach for restoring the viscoelasticity of aged vitreous bodies.

**Translational Relevance:** These findings improve the understanding and therapy of the vitreous liquefaction with age and the associated vitreoretinal diseases.

## Introduction

The exact function of the human vitreous body is not yet fully understood. However, the vitreous body is given immense importance with regard to the embryogenesis of the eye, the tamponing properties in the first decades of life, the maintenance of homeostasis in the eye and its manifold interactions with neighboring structures. With increasing age, the human vitreous body liquefies and consequently changes its properties.<sup>1–5</sup> This age-related vitreous liquefaction is associated with various vitreoretinal diseases such as posterior vitreous detachment, retinal detachment, and diseases of the vitreoretinal interface.<sup>6–8</sup>

In recent years, remarkable work has been done to characterize vitreal changes in terms of size, biochemistry, and structure. Postmortem studies of fresh human eyes observed an age-related linear decrease in gel volume and weight with a simultaneous increase in the liquid portion.<sup>1,2</sup> Within the reduced gelatinous vitreous body, substantial structural changes appear as thickened and collapsed fibers,<sup>3</sup> which are associated with the increased collagen content within the gel.<sup>2</sup> Moreover, levels of hyaluronic acid (HA) as a major and stabilizing component of the vitreous body were found to be reduced within the aged gelatinous vitreous.<sup>2,4,5</sup> However, information about the viscoelastic properties of human vitreous humors with aging, which are closely related to the biochemical

and structural composition and thus to the vitreous liquefaction, have been limited.

By definition, viscoelasticity describes the behavior of materials to respond to deformation in a partially elastic, reversible manner (introduced energy is available after deformation) and a partially viscous, irreversible manner (energy got lost). These rheological properties are determined as storage modulus ( $G'$ ) and loss modulus ( $G''$ ) via dynamic mechanical analyses/frequency sweep tests. To date, viscoelastic properties has been described for different species such as rabbit,<sup>9</sup> ovine,<sup>10,11</sup> porcine,<sup>10,12–16</sup> bovine,<sup>10,12,15–19</sup> and human vitreous bodies.<sup>10,20,21</sup> Age-related changes in vitreal viscoelasticities have been reported by Colter et al.<sup>11</sup> for ovine vitreous. The dynamic moduli of adult ovine vitreous were found to be lower than infant samples.<sup>11</sup> Moreover, a recent study by Tram et al.<sup>21</sup> reported the localized stiffening of the human vitreous body with age using shear rheology to analyze the dynamic moduli of sectioned gel portions. However, age-related changes of the entire human vitreous body remained unexplored and are of particular clinical interest for the understanding and therapy of vitreoretinal diseases, since liquid pockets are known to increasingly arise within the gelatinous vitreous body due to liquefaction,<sup>3,22</sup> which potentially decrease the human vitreal viscoelasticity macroscopically. In addition, good knowledge about vitreous viscoelasticity is also essential for the development of artificial vitreous body replacement as a future therapeutic approach for vitreoretinal diseases. An ideal vitreous substitute should be based on the natural, juvenile, and healthy vitreous body with regard to its viscoelastic properties.

The present study therefore aims at examining the viscoelasticity of the entire human vitreous body with age and to assess the possible therapeutic potential of intravitreal HA injections with respect to the improvement of the vitreal, viscoelastic properties.

## Materials and Methods

### Vitreous Body Dissection

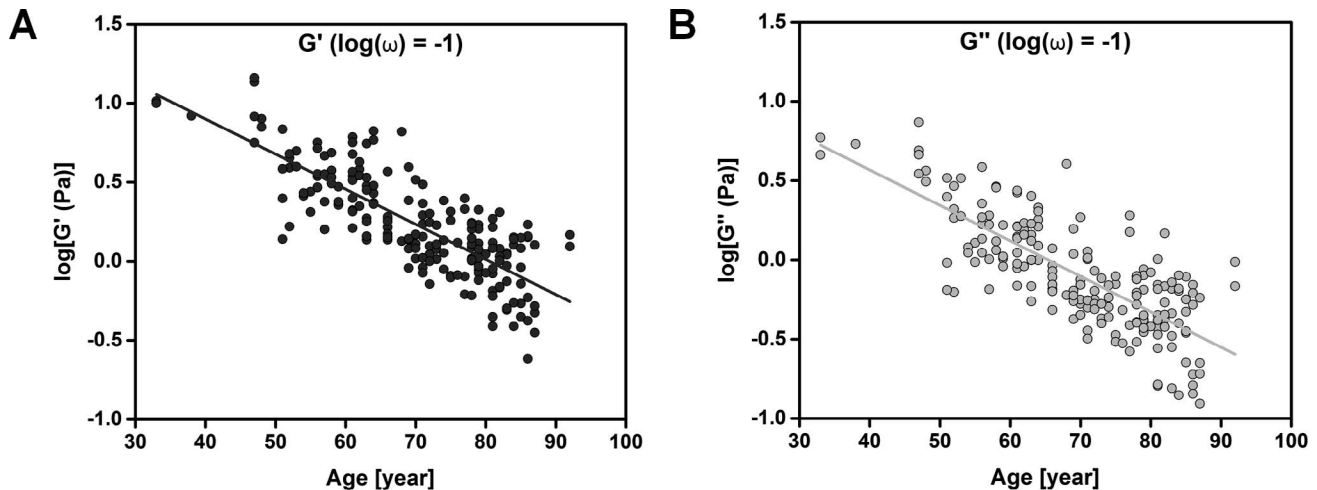
All procedures including the acquisition and dissection of the donor eyes has been carried out in accordance with the tenets of the Declaration of Helsinki for the use of human tissue as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the local ethical committee (application number:

97/17). One hundred ninety human research eyes (4 unpaired eyes, 93 paired eyes, from 33- to 92-year-old donors, aged  $70 \pm 12$  years) were obtained from The German Society for Tissue Transplantation (DGFG, Hannover, Germany) with a written consent from the donor's next of kin. Bovine and porcine eyes (animals aged 1–2 years and 8–10 months, respectively) were obtained for research purpose freshly as abattoir by-products by Emil Faerber GmbH (Zweibruecken, Germany).

Vitreous body dissection was performed under a sterile bench. The globes were treated for 5 minutes with 10% povidone iodine (Mundipharma GmbH, Limburg an der Lahn, Germany) and washed twice with phosphate-buffered saline ((-,-), Sigma-Aldrich, Taufkirchen, Germany). An initial incision into the sclera was made with a surgical blade (B. Braun, Melsungen, Germany) 3.5 mm posterior to the limbus. Then, a circular 360° incision was performed using a surgical scissor (Geuder AG, Heidelberg, Germany) to remove the anterior segment of the eye. Afterward, the eye was slightly flipped to gently luxate the vitreous with forceps (Geuder AG). The exposed vitreous was transferred to a six-well plate (Greiner Bio-One, Frickenhausen, Germany) and put into closed interim storage at 4°C. The vitreous extraction procedure was the same for human, bovine, and porcine samples. The duration from the time of death to the final extraction of human vitreous bodies was captured as the postmortem interval for further calculations. The postmortem intervals for bovine and porcine samples were  $\leq 2$  hours.

### Dynamic Mechanical Analysis

Entire, gelatinous vitreous samples were transferred with forceps and subjected to a frequency sweep between 0.1 and 100  $s^{-1}$   $\leq 2$  hours after vitreous extraction. The shear measurements were carried out using the rotational rheometer Physica MCR 101 (Anton Paar, Graz, Austria) with a parallel plate geometry and a Peltier element. 500 grit sandpaper was used to reduce the wall slip.<sup>14,21</sup> Depending on the size of the vitreous, the parallel plate was lowered in such a way that the test geometry was fully occupied by the vitreous sample and the normal force was minimized, as described previously.<sup>12</sup> Since the storage and loss moduli were found to be independent of temperature in the range of 20°C to 37°C<sup>9,16–18,20</sup> (see [Supplementary Fig. S3](#)), the dynamic moduli were determined at  $T = 20^\circ C$ , a temperature usually applied for material characterization and comparison.



**Figure 1.** Dynamic mechanical analyses of extracted human vitreous bodies ( $n = 190$ ) revealed an age-related reduction of the storage modulus  $G'$  (A) and loss modulus  $G''$  (B).

Human, bovine, and porcine vitreous bodies were compared in the linear viscoelastic region at a frequency of  $0.1 \text{ s}^{-1}$  as artificially increased dynamic moduli may occur at high frequencies due to inertial effects of the geometry.<sup>11,14</sup>

In addition, high molecular weight HA (Healon GV, Johnson & Johnson Vision Care, Jacksonville, FL) was dissolved in balanced salt solution (Thermo Fisher Scientific, Dreieich, Germany) to concentrations of 0, 3, 4, 5, 6, 7, and 14 mg HA/mL and characterized in terms of its viscoelastic properties. Then, the dynamic moduli of human vitreous humors (five paired eyes, from 66- to 85-year-old donors, aged  $75 \pm 7$  years) were measured. Afterward, 0.3 mL Healon GV (14 mg HA/mL) was injected through a 27G cannula into the center of the left eye's extracted vitreous body. The extracted vitreous bodies of the right eyes remained untreated (control). Storage and loss moduli of untreated and treated vitreous samples were analyzed immediately.

### Statistical Evaluation

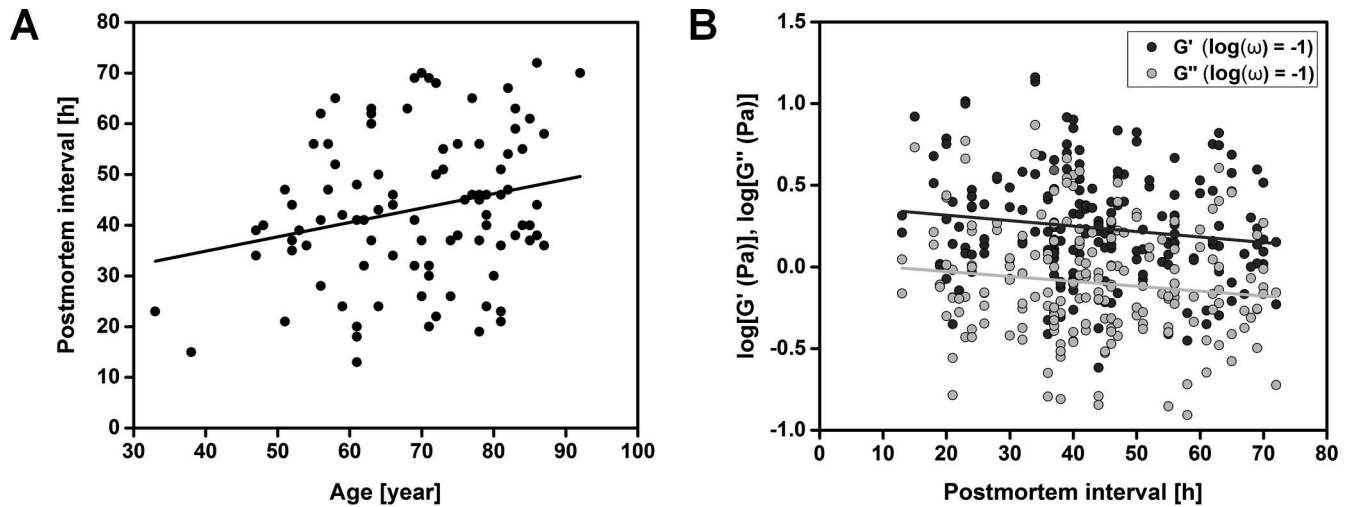
Graphical illustration of data and statistical analyses were performed using OriginPro and IBM SPSS Statistics. Differences between groups were considered significant by  $P < 0.05$  and were evaluated with bivariate and partial correlations. Two-tailed Student's  $t$ -test were used to compare the dynamic moduli of young and older human vitreous bodies as well as of bovine and porcine vitreous samples. As defined by the World Health Organization, donors of an age  $\leq 65$  years were considered as “young” and donors  $> 65$  years as “old.”

## Results

### Reduced Viscoelasticity of Human Vitreous Bodies With Aging

The dynamic moduli  $G'$  and  $G''$  of 190 human vitreous bodies (4 unpaired eyes, 93 paired eyes) extracted from 33- to 92-year-old donors (aged  $70 \pm 12$  years) were analyzed (Fig. 1). All human vitreous exhibited a gel-like behavior with  $G' > G''$ . With increasing age  $G'$  and  $G''$  were found to be reduced ( $r = -0.802$ ,  $P < 0.001$  and  $r = -0.770$ ,  $P < 0.001$ , respectively). Storage and loss moduli of younger donors (33–65 years old, 69 eyes) ranged from  $G' = 1.4$  to  $14.5 \text{ Pa}$  ( $\log(G') = 0.14$ – $1.16 \text{ Pa}$ ) and  $G'' = 0.55$  to  $7.39 \text{ Pa}$  ( $\log(G'') = -0.26$ – $0.87 \text{ Pa}$ ), respectively. In contrast, the dynamic moduli of older donors (66–92 years old, 121 eyes) decreased to  $G' = 0.24$  to  $6.60 \text{ Pa}$  ( $\log(G') = -0.62$ – $0.82 \text{ Pa}$ ) and  $G'' = 0.12$  to  $4.04 \text{ Pa}$  ( $\log(G'') = -0.91$ – $0.61 \text{ Pa}$ ) (Fig. 1). Vitreous bodies of older donors exhibited significantly lower dynamic moduli than these of younger donors ( $P < 0.001$ ). The difference between the highest and the lowest storage modulus measured in this study (14.5 and 0.24 Pa, respectively) was 14.26 Pa. Hence, the elastic portion of the aged vitreous body appeared to be reduced up to a factor of 60.

Interestingly, postmortem intervals were found to be increased with the donor's age ( $r = 0.236$ ,  $P < 0.001$ , Fig. 2A; Table). To examine the effect of postmortem intervals on the dynamic moduli of human vitreous bodies, both parameters were plotted against each other (Fig. 2B; Table).  $G'$  and  $G''$



**Figure 2.** A positive correlation was found between postmortem intervals and the donor's age (A). However, postmortem intervals did not affect the viscoelastic characteristics  $G'$  and  $G''$  significantly (B).

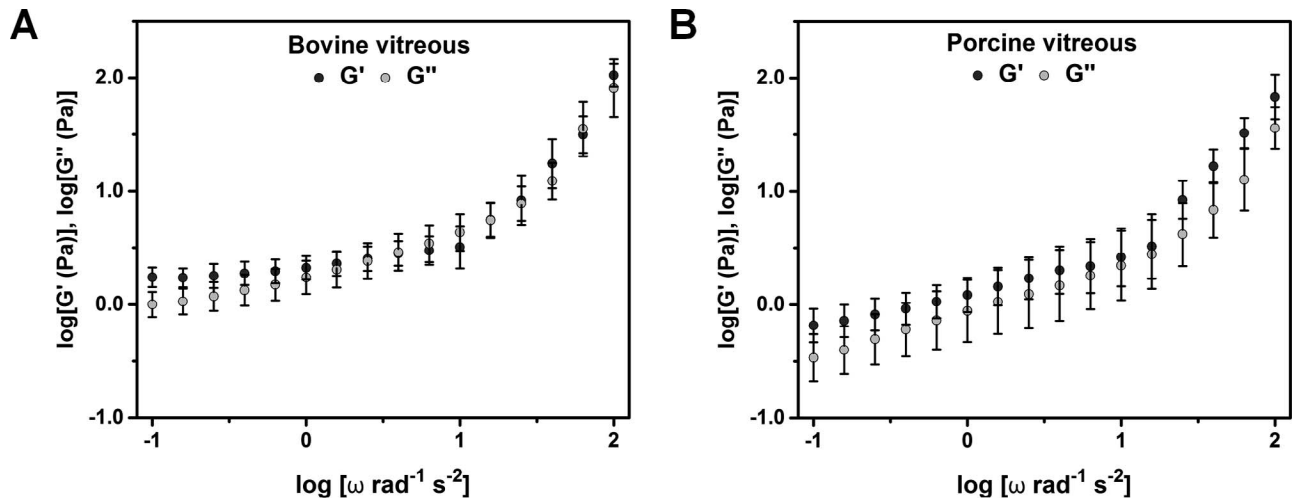
negatively correlated with postmortem intervals ( $r = -0.144$ ,  $P = 0.047$  and  $r = -0.124$ ,  $P = 0.089$ , respectively). However, both effects were found to be not significant after partial correlation against the

donor's age. To exclude the impact of the period from time of death to final vitreous extraction, the relation between dynamic moduli and age was partially correlated against the respective postmortem intervals

**Table.** Statistical Evaluation of the Age-Related Decrease in Vitreal Viscoelasticity

Bivariate Correlation		Partial Correlation ( <i>Control Variables</i> )		Pearson Coefficient	<i>P</i> Value	
$G'$	Age			-0.802	<0.001	
$G''$	Age			-0.770	<0.001	
Age	Postmortem			0.236	0.001	
$G'$	Postmortem			-0.144	0.047	
$G''$	Postmortem			-0.124	0.089	
$G'$	Postmortem	Age		0.077	0.295	
$G''$	Postmortem	Age		0.093	0.202	
$G'$	Age	Postmortem		-0.798	<0.001	
$G''$	Age	Postmortem		-0.768	<0.001	
$G'$	Sex			-0.126	0.084	
$G''$	Sex			-0.141	0.052	
$G'$	Age	Sex		-0.805	<0.001	
$G''$	Age	Sex		-0.774	<0.001	
Age	Diabetes			0.275	<0.001	
$G'$	Diabetes			-0.199	0.006	
$G''$	Diabetes			-0.154	0.034	
$G'$	Diabetes	Age		0.038	0.603	
$G''$	Diabetes	Age		0.094	0.198	
$G'$	Age	Diabetes		-0.793	<0.001	
$G''$	Age	Diabetes		-0.766	<0.001	
$G'$	Age	Postmortem	Sex	Diabetes	-0.789	<0.001
$G''$	Age	Postmortem	Sex	Diabetes	-0.764	<0.001

Differences between groups were considered significant by  $P < 0.05$ .



**Figure 3.** Frequency sweep tests of extracted bovine ( $n = 15$ ) (A) and porcine ( $n = 15$ ) (B) vitreous bodies. The *error bars* display the standard deviations. Storage moduli  $G'$  were found to be higher than loss moduli  $G''$  at low frequencies indicating a gel-like behavior for both animal sources (with bovine  $>$  porcine,  $P < 0.001$ ).

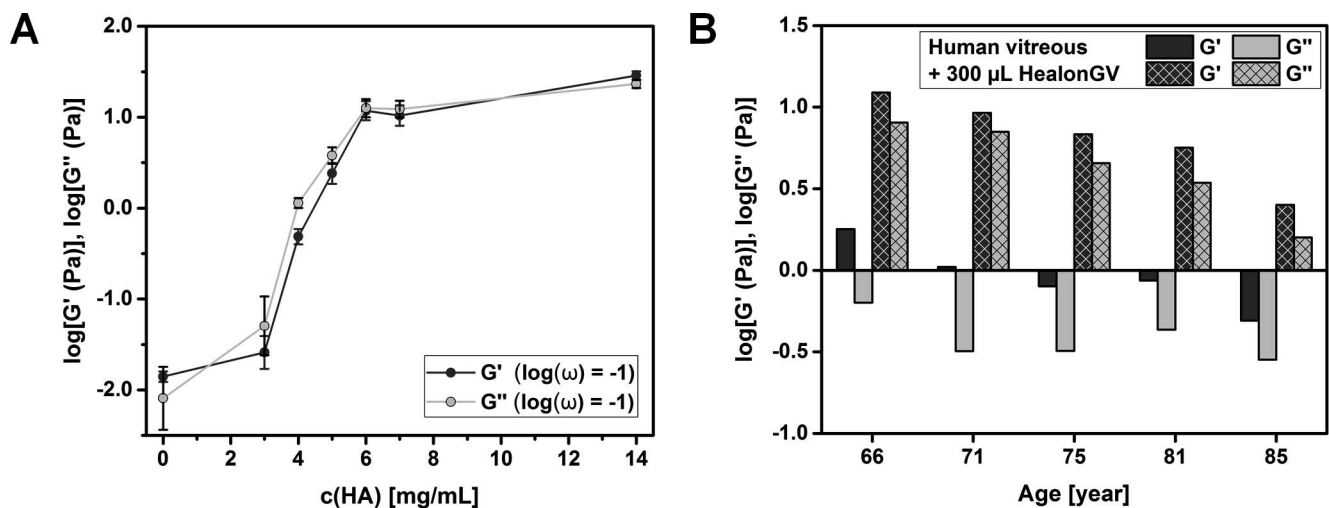
(Table). In addition, the viscoelasticity of human vitreous bodies and its age-related reduction were found to be independent from the donor's sex and diabetes (Table).

Human vitreous bodies were also compared with bovine and porcine samples (Fig. 3). Bovine vitreous humors resulted in higher dynamic moduli than porcine vitreous ( $P < 0.001$ ) with  $G' > G''$  ( $\log(G') = 0.24 \pm 0.09$  Pa,  $\log(G'') = 0.00 \pm 0.11$  Pa and  $\log(G') = -0.18 \pm 0.15$  Pa,  $\log(G'') = -0.47 \pm 0.21$  Pa, respectively). Both bovine and porcine samples exhibited comparable viscoelastic properties with

human vitreous bodies at high age. However, dynamic moduli of young human vitreous bodies were found to be higher than both animal sources.

### Increased Vitreal Viscoelasticity Due to HA Injections

To assess the possible therapeutic potential of intravitreal HA injections with respect to the improvement of the vitreal viscoelasticity, differently concentrated HA solutions were analyzed in terms of their dynamic moduli (Fig. 4A). The solution's



**Figure 4.** (A) Increased dynamic moduli of HA solutions with increasing HA concentration and gel-like behavior (storage modulus  $G' >$  loss modulus  $G''$ ) at 14 mg HA/mL (Healon GV). The *error bars* display the standard deviations of three independent experiments. (B) Low viscoelastic properties of human vitreous bodies extracted from old donors were enhanced due to the intravitreal injection of 300  $\mu\text{L}$  Healon GV.

viscoelasticity was found to be elevated with increasing HA concentrations. HA solutions ranging from 0 to 7 mg HA/mL behaved like a sol with  $G' < G''$ . In contrast, higher concentrations of HA (14 mg HA/mL) resulted in a gel-like dynamic behavior with  $G' > G''$  ( $\log(G') = 1.46 \pm 0.05$  Pa and  $\log(G'') = 1.37 \pm 0.04$  Pa) comparable with the moduli of human vitreous bodies of younger donors.

In order to improve the viscoelastic properties of aged vitreous bodies, human vitreous samples from 66- to 85-year-old donors with low viscoelastic characteristics were treated post mortem with 300  $\mu$ L Healon GV injections through a 27G cannula and subsequently characterized in terms of their dynamic moduli (Fig. 4B). Human vitreous bodies without intravitreal injections (untreated) served as controls to exclude methodological errors and were found to be unaffected (data not shown). In contrast, the vitreal viscoelasticity increased due to the injection of 300  $\mu$ L Healon GV. On average, the storage modulus was found to be increased by  $7.4 \pm 1.2$  Pa after the HA treatment.

## Discussion

The present postmortem study aimed to determine the human vitreal viscoelasticity with age and is the first to reveal a significant age-related reduction in the viscoelasticity of entire human vitreous bodies. The decrease in viscoelastic properties with age is largely exponential (see also Supplementary Fig. S2) meaning that the greatest proportion of viscoelasticity gets lost during the first years of vitreous liquefaction. As the present study only had access to vitreous bodies of donors older than 32 years and the vitreous liquefaction is known to begin as early as age 4,<sup>23</sup> future studies should examine human vitreous bodies from youthful donors to approach the age that represents the starting point of the age-related loss of vitreal viscoelasticity. Nevertheless, the present study provides one of the largest sample sizes applied for rheological tests of the human vitreous to date.<sup>10,20,21,24</sup>

The reduced viscoelastic properties of the entire human vitreous with age are related with the vitreous composition. Due to liquefaction, the vitreous alters in terms of size, biochemistry, and structure.<sup>1,2,4,5,7</sup> The vitreous network that is mainly composed of water (approximately 99%), collagens and HA undergoes a tremendous phase separation with age. The depolymerization of the network has been attributed to enzymatic digestions<sup>12,25</sup> and results in the increasing release of HAs.<sup>2,4,5</sup> As HAs are known to

retain water molecules, their release is accompanied by the loss of water and vitreous weight/volume.<sup>1,2</sup> The age-related decrease in vitreous weight and volume has been also demonstrated in the present study while only small differences occurred between paired eyes indicating an appropriate dissection procedure (Supplementary Fig. 1). Moreover, the vitreous network degradation is associated with increased concentrations of collagens, which aggregate to thickened and collapsed fibers.<sup>3</sup> A recent study reported an age-related localized stiffening of sectioned, gelatinous vitreous bodies using shear rheology and attributed the elevated storage moduli with the vitreous dehydration and increased collagen concentration.<sup>21</sup> However, the viscoelastic properties of entire human vitreous bodies with aging remained unexplored and are of particular interest for the understanding and therapy of vitreoretinal diseases since the vitreal viscoelasticity functions rather macroscopically. Here, the dynamic mechanical properties of the vitreous were previously attributed with the maintenance of its function such as keeping an intact visual pathway and stabilizing the surrounding tissues.<sup>19,26</sup> In addition, it has been also discussed recently that the viscoelasticity of the vitreous humor is protecting the eye against physical impacts spanning from internal low frequency mechanical stress and vibrations to external mechanical traumata.<sup>6</sup> The present study, therefore, aimed to determine the viscoelastic properties of the entire vitreous humor with age. Even though the vitreous liquefaction results in the localized stiffening of vitreous gel portions,<sup>21</sup> the viscoelasticity of entire human vitreous bodies were found to be reduced macroscopically with aging. As a consequence, the human eye may become less protected against motions and deformations with age. This age-related loss of human vitreal viscoelasticity supports the findings of Colter et al.<sup>11</sup> who reported lower dynamic moduli of the entire adult than the infant vitreous, using ovine eyes. Due to the progressive vitreous phase separation, pockets of liquid arise increasingly inside the vitreous,<sup>3,22</sup> which may result in macroscopically less viscoelastic vitreous bodies with age. One could also speculate that the mechanical characteristics were reduced to a certain extent because of the network degradation. Although thickened and collapsed collagen fibers should increase the storage modulus in theory based on an increased network density,<sup>21</sup> the length and rigidity of collapsed collagen fibers, however, may be decreased due to depolymerization processes. For instance, previous studies showed a decreased stiffness of

bovine vitreous bodies as a result of enzymatic degradation.<sup>12,19</sup> Future studies might, therefore, further investigate the relationship between the vitreous network degradation and the mechanic properties of the entire vitreous including the degree of liquid pocket formation within the vitreous gel.

Besides the donor's age, further variables were considered as potential influences on the vitreal viscoelasticity. The period from time of death to final vitreous extraction (postmortem interval) was proven as positive correlative with age and is in accordance with previous studies that attributed prolonged postmortem intervals until finding of elderly individuals with certain household situations (e.g., living alone).<sup>27</sup> After death, the characteristic of human vitreous bodies are changing with time.<sup>28</sup> Therefore, postmortem intervals were assumed as an influencing factor in respect of the age-related loss of vitreal viscoelasticity. However, neither the postmortem intervals nor donor-related parameters such as sex and diabetes have been shown to have a significant effect on the decrease in human vitreal viscoelasticity with age and could be excluded as potential methodological errors. Nevertheless, the found effects have been eliminated using partial correlations resulting in a strong relation between the dynamic moduli and age with  $r = -0.789$  for  $G'$  and  $r = -0.764$  for  $G''$ .

The age-related changes in the human vitreal viscoelasticity shown were also compared with bovine and porcine sources as established model systems in practice. Our results confirm the reported findings<sup>10,15</sup> of higher dynamic moduli of bovine than porcine vitreous bodies. Tested bovine and porcine samples (aged 1–2 years and 8–10 months, respectively) in theory correspond to the human age of approximately 18 years. However, bovine and porcine vitreous bodies prove to be only limited usable animal models for the simulation of the human vitreous humor, since the dynamic moduli of the bovine and porcine samples exclusively represent the viscoelastic properties of older humans, but not the ideal condition of a young healthy human vitreous humor.

As a potential therapeutic approach for restoring the viscoelasticity of aged vitreous bodies, HA injections were investigated. With increasing concentration, HA solutions became more viscous up to a gel-like behavior ( $G' > G''$  at 14 mg/mL) comparable with the moduli of human vitreous bodies of younger donors and thus favorable for the enhancement of liquefied vitreous. Here, an injection of highly concentrated and viscoelastic HA (300  $\mu$ L Healon GV) provides a threefold quantity of HA compared

with a juvenile vitreous body.<sup>5</sup> Postmortem studies of human vitreous bodies extracted from 66- to 85-year-old donors with weak viscoelastic characteristics revealed increased vitreal viscoelasticities after the injection of a highly concentrated HA dose. Consequently, lost viscoelastic properties of 20 to 30 years could be restored. Although HAs were approached previously to treat retinal detachment,<sup>29–34</sup> the present study is the first to reveal improved viscoelastic properties of the human vitreous after intravitreal injections of highly concentrated HA. The injected HA, however, may be degraded enzymatically in vivo by hyaluronidases and thus may serve only as a short-term approach, as described previously.<sup>29–34</sup> Moreover, this approach may be also limited by the increase of the intraocular pressure due to volume expansion.<sup>35</sup> However, intravitreal HA injections was found to be beneficial to treat hypotonic eyes<sup>36</sup> and retinal detachment.<sup>30</sup> Hence, future studies involve in vivo analyses concerning the stability of injected HA on site as extracted vitreous bodies liquefy in vitro with time. Here, diffusion and biodegradation studies of various volumes of injected HA are of particular interest beside the overall acceptance such as the preservation of the intraocular pressure.

Taken together, the present postmortem study is the first to show a significant age-related reduction in the viscoelasticity of entire human vitreous bodies independent of postmortem intervals and donor-related parameters such as sex and diabetes. These findings provide new insights into the alteration of human vitreous bodies with age and will benefit the understanding and therapy of vitreoretinal diseases such as the design of vitreous substitutes.

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