Research Article

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Rheological analysis of saliva samples in the context of phonation in ectodermal dysplasia

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Abstract: Ectodermal dysplasia (ED) is a rare inherited disorder affecting embryonic ectoderm structures, leading to reduced development of skin appendages and certain eccrine glands. Displaying reduced salivation and impaired acoustic quality in males, ED offers a unique chance to study the role of laryngeal mucus in the phonation process. This study analyzed saliva rheology as a non-invasive substitute for laryngeal mucus to investigate potential causal relationships. Saliva samples from 36 ED patients and 99 controls were collected for 5 min following a 15 min abstention from eating, drinking, or smoking. The viscoelastic properties have been measured by a rheometer with a parallel plate system. Storage (G') and loss (G'') moduli were recorded and compared between ED and controls. ED subjects exhibited significantly lower G' and G'' at lower frequencies and strains, yet slightly higher values at increased frequencies and strains among males. These findings suggest reduced resistance of saliva to external forces for ED. Transferred to the laryngeal level, this behavior might impair the mucus' retention rate on the vocal folds. The results also hint at altered hyaluronic acid content in ED, guiding further correlation studies investigating voice conspicuities.

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Abbreviations

ED	Ectodermal dysplasia
$ED_{m/f/b}$	Ectodermal dysplasia in males/females/boys
C	Controls
$C_{m/f/b}$	Controls in males/females/boys
XLHED	X-linked hypohidrotic ectodermal dysplasia

1 Introduction

In spite of the major role of the human voice in social and professional aspects of today's society, the phonation process is not yet fully understood [1–4]. Recently, the impact of the laryngeal mucus as a hydrating boundary layer in the larynx and vocal tract on phonation and the role of the structural compositions and tissue properties of the vocal folds have come into focus of research [5–11]. *Ex vivo* experiments allow direct access and complete control of the boundary conditions, but typically lack, to some degree, in realism of the acoustic modulation in the vocal tract [10]. Various diseases with altered mucus production that also affect the voice, such as ectodermal dysplasia (ED), cystic fibrosis, and Sjögren's disease [12–15], provide a suitable model for *in vivo* studies, although direct access to the larynx is profoundly limited.

Among other symptoms, the most common hypohidrotic form of ED is characterized by a reduced number and activity of all glands of ectodermal origin, including the salivary and mucosal glands in the whole respiratory tract [16,17]. Although the reduced glandular function is an obviously contributing factor, the striking voice of patients with ED cannot yet be fully explained. The symptoms of ED result from a hereditary disorder in the development of the ectoderm [18], and the most frequent form of ED is inherited X-linked recessively, so that males are more strongly affected by ED than women [16,19].

Previous studies by Semmler et al. [12] reported a significantly reduced salivary function in the experimental group of ED subjects compared to the control group by measuring the generated saliva weight over a certain duration. Furthermore, this study demonstrated a reduced voice quality objectively and systematically by means of the multimodal measurement protocol established by the European Laryngological Society for a functional assessment of voice pathologies [12,20]. A recent study by Pelka et al. [21] presented statistically significant differences between male ED and control subjects concerning the mechanical parameters representing tissue characteristics of the vocal folds derived from high-speed videoendoscopy. Despite these findings, no clear correlation with voice quality in ED subjects has yet been revealed [12,20,21].

Another important factor could be the laryngeal mucus with its rheological characteristics, which acts as a boundary layer in the fluid-structure-acoustic interaction, generating the primary voice signal in the larynx [10,22]. The long-term goal is to further investigate whether there is an explanation for the striking vocal characteristics of ED subjects on the laryngeal level. Due to the invasive extraction procedure required for laryngeal mucus samples [11], the investigation is instead performed on resting saliva, which is believed to be a suitable substitute since the salivary gland function is severely affected in subjects with ED. Therefore, the aim of the present study is an initial exploration of differences in the rheology of saliva samples between ED subjects and controls to expand our knowledge about this issue and guide future research approaches.

So far, the viscoelastic properties of human saliva have barely been studied in patients with reduced salivary function [23,24], and it is unclear which factors in saliva contribute to its rheological properties [25]. Rereddy et al. found that post-radiation and anticholinergic-induced xerostomia patients, despite similar shear viscosities, exhibited significantly increased extensional viscosity compared to healthy controls, indicating altered saliva rheology associated with dry mouth conditions [23]. The study by Chaudhury et al. suggests that qualitative changes in mucin glycosylation – particularly reduced sialylation of MUC7 – contribute to altered extensional rheology of saliva in Sjögren's syndrome. These changes may underlie the sensation of oral dryness, independent of the saliva volume [24].

In line with other rheological studies on human and simulated/artificial saliva [26–29], in our approach, we employ a plate-plate rheometer to analyze the viscoelastic properties at variable frequency and shear stress.

We hypothesize that the different viscoelastic characteristics of saliva in ED subjects could emerge from divergent compositions of hyaluronic acids at specific molecular masses [12,30]. Once the transferability of our findings between saliva and mucus is established, substitute solutions would be conceivable. We are confident that our work will eventually contribute to improving patient care for the ED as well as increase our understanding of the phonation process in general.

2 Methods

2.1 Study participants

Initially, the experimental group examined in this study included 46 test persons with a clinically diagnosed ED. These participants were recruited via the German ED patient organization (Ektodermale Dysplasie e.V.) in 2022. For further analysis, only patients with genetically confirmed X-linked hypohidrotic ectodermal dysplasia (XLHED) were taken into account [31]. Some samples had to be excluded because of insufficient saliva volume or unsuccessful measurements.

Overall, the experimental group (with XLHED) included 14 male subjects (ED_m), 17 female subjects (ED_f) aged between 13 and 71 years, and 5 boys up to 12 years of age (ED_b), which were investigated separately for statistical purposes. The frequency sweep included 36 people with XLHED. In the strain sweep, 31 people with XLHED were considered.

The healthy control group, without current or previous voice pathologies, comprised 44 male test subjects (C_m) and 50 female test subjects (C_f) aged between 13 and 64 years and additionally 5 boys (C_b). In the control group, the frequency sweep included 99 healthy subjects and 85 healthy subjects in the strain sweep.

The numbers and mean values of the age in all six subgroups are shown in Table 1. Please note that the group classification (adult/children) was differentiated on the basis of

Table 1: Included number of subjects, mean values, and standard deviations of the investigated parameter age averaged for all test groups

			Frequency sweep			
	ED_m $N = 14$	**C_m $N = 44$**	**ED_f $N = 17$**	**C_f $N = 50$**	**ED_b $N = 5$**	**C_b $N = 5$**
Age (years)	37.8 ± 16.3	32.2 ± 14.0	45.2 ± 12.2	35.0 ± 13.8	10.0 ± 1.1	10.2 ± 1.3
			Strain sweep			
	ED_m $N = 13$	**C_m $N = 35$**	**ED_f $N = 14$**	**C_f $N = 45$**	**ED_b $N = 4$**	**C_b $N = 5$**
Age (years)	36.6 ± 16.4	34.7 ± 14.6	45.5 ± 13.0	36.1 ± 14.3	10.3 ± 1.1	10.2 ± 1.3

voice status. In this study, all subjects up to the age of 12 years had not yet undergone voice change according to perceptual assessment. The study is covered by a positive vote of the local ethics committee (ref. No 21-437-B) of the medical faculty at the Friedrich-Alexander-Universität Erlangen-Nürnberg.

2.2 Data acquisition and measurement protocol

Samples of resting saliva were obtained for a period of 5 min in a collection cup. The subjects had been instructed not to eat, drink, smoke, use chewing gum, etc., for at least 15 min beforehand. The samples were then transferred and aliquoted to Eppendorf tubes and deep-frozen on dry ice for transport and long-term storage at −20°C before collective analysis. The saliva samples were slowly thawed at 4°C over the duration of 1 h and centrifuged for 5 min at 3,000 × g prior to the rheological measurement to separate any potential solid components. The samples were stored at 4°C until the measurement to avoid any degeneration.

The samples were measured with a plate-plate rheometer of type HAAKE™ MARS™ 60 Rheometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a plate diameter of 25 mm and a gap of 120 µm at 25°C. For our available sample volumes (<200 µl), we chose the largest possible plate diameter. It has been shown before that thin-gap parallel-disk configurations provide reliable results for low viscosities down to a gap of 20 µm [32].

All measurements were conducted following a pre-shear for homogenizing the dispensed saliva sample at 0.5 Hz. In the stress-controlled frequency sweep, the oscillation frequency was varied between f = 1–10 Hz in 15 steps at a regulated stress of τ = 0.1 Pa. Subsequently, in the stress-controlled strain sweep, the applied shear stress was systematically varied between τ = 0.01–20 Pa at 0.5 Hz in 30 steps. Each set of measurements took 9.4 min per sample, and on average, 15 samples were measured in each batch. The measurements were carried out blinded and in no particular order. Storage modulus G' in Pa and loss modulus G'' in Pa were determined in both frequency and strain sweeps.

2.3 Data processing and analysis

To enable meaningful comparisons over all measurements, the data points of the strain sweep were interpolated in 30 logarithmically distributed steps by a piecewise cubic Hermite interpolation in the largest shared range, where all measurements provide interpolated values [33,34]. This resulted in a strain range of γ = 0.1–70%. The interpolation was performed with Matlab R2021b.

The statistical analysis was conducted with regard to differences between ED subjects and controls, but separately for the male and female subgroups due to the X-linked genetic inheritance of ED, which yields more pronounced and consistent symptoms in males. The male children were evaluated separately to reveal age-related differences.

For every investigated frequency in the frequency sweep and every investigated shear strain in the interpolated strain sweep, an individual mean value comparison over the absolute values of storage and loss moduli is performed in each group (male/female/boys). Additionally, a mean value comparison is performed on the shear strain values of the cross-over points of the loss and storage moduli curves in the strain sweep.

A Shapiro–Wilk test with a significance level of p = 0.05 was used to test the subgroups for normal distribution. The nonparametric Mann–Whitney U test for comparison of two groups was used with a significance level of p = 0.05 to look for statistically significant differences between the subgroups (ED_m <> C_m, ED_f <> C_f, ED_b <> C_b). All statistical tests were conducted utilizing SPSS version 29.

3 Results

3.1 Mean value comparison of storage and loss moduli

The numbers of subjects included in each subgroup for the frequency sweep and the strain sweep are listed in Table 1. In Tables 2–5, the mean values and standard deviations for each group are illustrated. Tables 2 and 3 show the rheological measurements at the different frequencies. Tables 4 and 5 present the rheological measurements in relation to different shear strains. The results of the statistical analysis are presented in Tables A1–A4: Tables A1 and A2 in relation to the different analyzed frequencies and Tables A3 and A4 with regard to the different amplitude swipes. The subgroups in the columns were ordered for separate group comparison of males (left: ED_m <> C_m), females (middle: ED_f <> C_f), and boys (right: ED_b <> C_b). Statistically significant differences (p < 0.05 in the Mann–Whitney U test) between ED subjects and controls are highlighted in bold letters in both tables.

Focusing on the frequency sweeps of the adult males, which are visualized in Figure 1, the male ED group (ED_m)

Table 2: Mean values and standard deviations of all investigated frequencies for storage modulus *G′* averaged for all test groups

Storage modulus *G′* from frequency sweep on resting saliva						
Frequency *f* (Hz)	***G′*_ED$_m$** ***N* = 14** **Mean ± std**	***G′*_C$_m$** ***N* = 44** **Mean ± std**	***G′*_ED$_f$** ***N* = 17** **Mean ± std**	***G′*_C$_f$** ***N* = 50** **Mean ± std**	***G′*_ED$_b$** ***N* = 5** **Mean ± std**	***G′*_C$_b$** ***N* = 5** **Mean ± std**
1.00	**0.30 ± 0.26**	**0.61 ± 0.58**	0.29 ± 0.18	0.46 ± 0.42	**0.22 ± 0.15**	**1.51 ± 0.82**
1.18	**0.31 ± 0.25**	**0.65 ± 0.62**	0.33 ± 0.45	0.45 ± 0.42	**0.17 ± 0.13**	**1.47 ± 0.80**
1.39	**0.29 ± 0.29**	**0.67 ± 0.66**	0.28 ± 0.21	0.47 ± 0.44	**0.22 ± 0.16**	**1.54 ± 0.80**
1.64	**0.33 ± 0.28**	**0.80 ± 0.81**	0.41 ± 0.45	0.52 ± 0.44	**0.28 ± 0.14**	**1.66 ± 0.85**
1.93	0.57 ± 0.60	0.69 ± 0.65	0.72 ± 1.30	0.52 ± 0.45	**0.32 ± 0.23**	**1.63 ± 0.99**
2.28	0.36 ± 0.40	0.64 ± 0.66	0.79 ± 2.07	0.65 ± 0.78	0.95 ± 1.82	1.68 ± 1.02
2.68	1.28 ± 2.29	0.99 ± 1.29	1.41 ± 1.99	1.01 ± 1.58	0.84 ± 0.59	1.79 ± 0.99
3.16	1.20 ± 2.01	2.55 ± 6.89	1.83 ± 4.42	2.06 ± 4.97	0.51 ± 0.47	1.70 ± 1.11
3.73	3.80 ± 6.70	2.37 ± 5.45	3.69 ± 6.58	2.06 ± 2.51	6.56 ± 11.89	1.00 ± 0.77
4.39	4.99 ± 7.84	2.49 ± 4.15	3.25 ± 5.96	1.96 ± 3.13	3.24 ± 4.53	0.92 ± 0.75
5.18	8.27 ± 9.94	8.65 ± 14.4	8.30 ± 8.85	5.71 ± 7.39	5.21 ± 6.47	2.68 ± 3.25
6.11	14.6 ± 12.5	8.85 ± 11.2	16.7 ± 17.6	12.8 ± 19.2	17.3 ± 21.9	7.45 ± 4.06
7.20	22.4 ± 23.0	12.3 ± 14.7	22.5 ± 21.3	17.1 ± 18.8	27.4 ± 25.5	6.31 ± 2.97
8.48	56.7 ± 49.4	35.1 ± 45.3	53.8 ± 50.7	43.4 ± 54.1	**31.3 ± 9.91**	**10.8 ± 7.60**
10.0	**106.1 ± 80.5**	**61.9 ± 71.7**	88.3 ± 71.8	86.9 ± 100.9	50.3 ± 37.7	34.6 ± 20.9

Statistically significant differences ($p < 0.05$ in Mann–Whitney *U* test) between ED subjects and controls are highlighted in bold style.

shows statistically significantly lower values for storage modulus *G′* and loss modulus *G″* concerning the lower frequencies below 2 Hz (Tables A1 and A2). Within this range, the mean values of the storage modulus *G′* for ED_m (blue in Figure 1) are only 41–49% of the mean values of C_m (green in Figure 1), while the mean values of the loss modulus *G″* are 59–68% for ED_m compared to C_m. For higher frequencies over 3.7 Hz, the mean values of storage and loss moduli *G′* and *G″* are mostly higher for ED_m compared to C_m. However, only for few investigated frequencies, the differences displayed statistical significance. At 10 Hz, the *G′* mean value for ED_m is 171% of the C_m mean value and the *G″*

Table 3: Mean values and standard deviations of all investigated frequencies for loss modulus *G″* averaged for all test groups

Loss modulus *G″* from frequency sweep on resting saliva						
Frequency *f* (Hz)	***G″*_ED$_m$** ***N* = 14** **Mean ± std**	***G″*_C$_m$** ***N* = 44** **Mean ± std**	***G″*_ED$_f$** ***N* = 17** **Mean ± std**	***G″*_C$_f$** ***N* = 50** **Mean ± std**	***G″*_ED$_b$** ***N* = 5** **Mean ± std**	***G″*_C$_b$** ***N* = 5** **Mean ± std**
1.00	**0.19 ± 0.09**	**0.28 ± 0.20**	0.18 ± 0.08	0.24 ± 0.15	**0.16 ± 0.05**	**0.53 ± 0.27**
1.18	**0.19 ± 0.11**	**0.31 ± 0.19**	0.26 ± 0.31	0.25 ± 0.16	**0.12 ± 0.08**	**0.54 ± 0.27**
1.39	**0.21 ± 0.15**	**0.32 ± 0.21**	0.23 ± 0.13	0.29 ± 0.13	**0.22 ± 0.10**	**0.55 ± 0.26**
1.64	**0.26 ± 0.14**	**0.44 ± 0.26**	0.32 ± 0.20	0.34 ± 0.21	0.28 ± 0.02	0.55 ± 0.28
1.93	**0.25 ± 0.16**	**0.41 ± 0.32**	0.48 ± 0.71	0.32 ± 0.17	**0.22 ± 0.11**	**0.62 ± 0.27**
2.28	0.36 ± 0.26	0.58 ± 1.10	0.70 ± 0.79	0.53 ± 0.41	0.53 ± 0.52	0.59 ± 0.34
2.68	1.05 ± 1.98	0.57 ± 0.63	0.85 ± 1.21	0.87 ± 2.80	0.65 ± 0.32	0.71 ± 0.15
3.16	2.15 ± 4.32	1.29 ± 3.32	0.70 ± 1.03	0.83 ± 1.40	0.62 ± 0.80	0.66 ± 0.36
3.73	6.18 ± 11.86	2.39 ± 6.24	4.34 ± 9.25	4.03 ± 11.98	2.16 ± 2.18	1.07 ± 0.75
4.39	**5.16 ± 6.36**	**2.54 ± 5.46**	3.67 ± 5.62	1.71 ± 2.15	6.20 ± 11.3	1.14 ± 0.27
5.18	7.97 ± 12.27	5.71 ± 11.17	8.80 ± 15.62	4.12 ± 7.22	4.09 ± 5.22	1.09 ± 0.21
6.11	12.0 ± 25.1	8.97 ± 16.4	13.2 ± 18.3	10.3 ± 19.3	6.22 ± 7.53	8.21 ± 10.7
7.20	**20.3 ± 14.3**	**9.73 ± 10.1**	15.3 ± 12.8	12.5 ± 11.0	**17.1 ± 10.3**	**5.76 ± 3.57**
8.48	47.3 ± 44.5	23.7 ± 33.8	25.1 ± 37.6	23.7 ± 35.92	14.8 ± 16.3	31.5 ± 41.4
10.0	47.9 ± 55.7	50.6 ± 98.4	20.7 ± 24.0	52.2 ± 65.2	26.9 ± 53.4	86.9 ± 98.7

Statistically significant differences ($p < 0.05$ in the Mann–Whitney *U* test) between ED subjects and controls are highlighted in bold style.

Table 4: Mean values and standard deviations of all investigated shear stress amplitudes for storage modulus G' averaged for all test groups

Storage modulus G' from strain sweep on resting saliva						
Shear strain γ (%)	**G'_ED$_m$ N = 13 Mean ± std**	**G'_C$_m$ n = 35 Mean ± std**	**G'_ED$_f$ N = 14 Mean ± std**	**G'_C$_f$ N = 45 Mean ± std**	**G'_ED$_b$ N = 4 Mean ± std**	**G'_C$_b$ N = 5 Mean ± std**
0.10	**0.40 ± 0.28**	**0.77 ± 0.62**	0.38 ± 0.18	1.10 ± 3.31	**0.24 ± 0.09**	**1.54 ± 0.71**
0.13	**0.39 ± 0.24**	**0.75 ± 0.58**	0.35 ± 0.19	1.03 ± 2.91	**0.23 ± 0.09**	**1.51 ± 0.67**
0.16	**0.37 ± 0.21**	**0.72 ± 0.55**	0.34 ± 0.18	0.95 ± 2.55	**0.24 ± 0.08**	**1.43 ± 0.61**
0.20	**0.36 ± 0.19**	**0.69 ± 0.50**	0.34 ± 0.16	0.87 ± 2.14	**0.23 ± 0.08**	**1.34 ± 0.55**
0.25	**0.33 ± 0.16**	**0.65 ± 0.46**	0.34 ± 0.14	0.77 ± 1.70	**0.23 ± 0.07**	**1.24 ± 0.49**
0.31	**0.31 ± 0.14**	**0.61 ± 0.43**	**0.30 ± 0.15**	**0.67 ± 1.24**	**0.23 ± 0.07**	**1.13 ± 0.43**
0.39	**0.29 ± 0.11**	**0.56 ± 0.38**	0.28 ± 0.14	0.57 ± 0.82	**0.23 ± 0.06**	**1.01 ± 0.38**
0.49	**0.26 ± 0.09**	**0.51 ± 0.34**	0.26 ± 0.13	0.48 ± 0.48	**0.22 ± 0.05**	**0.90 ± 0.33**
0.61	**0.24 ± 0.09**	**0.46 ± 0.29**	0.25 ± 0.12	0.41 ± 0.30	**0.21 ± 0.04**	**0.77 ± 0.28**
0.76	**0.22 ± 0.09**	**0.40 ± 0.25**	0.23 ± 0.11	0.36 ± 0.25	**0.20 ± 0.03**	**0.65 ± 0.23**
0.96	**0.20 ± 0.08**	**0.35 ± 0.20**	0.21 ± 0.10	0.31 ± 0.20	**0.19 ± 0.03**	**0.54 ± 0.19**
1.20	**0.18 ± 0.07**	**0.30 ± 0.17**	0.19 ± 0.09	0.27 ± 0.16	**0.17 ± 0.03**	**0.45 ± 0.15**
1.50	**0.16 ± 0.06**	**0.26 ± 0.14**	0.16 ± 0.08	0.23 ± 0.12	**0.15 ± 0.03**	**0.36 ± 0.12**
1.89	**0.13 ± 0.05**	**0.22 ± 0.11**	0.14 ± 0.07	0.19 ± 0.08	**0.12 ± 0.02**	**0.29 ± 0.09**
2.36	**0.11 ± 0.04**	**0.18 ± 0.09**	0.13 ± 0.06	0.16 ± 0.06	**0.10 ± 0.01**	**0.23 ± 0.06**
2.96	**0.09 ± 0.04**	**0.15 ± 0.07**	0.11 ± 0.05	0.13 ± 0.05	**0.08 ± 0.01**	**0.19 ± 0.05**
3.71	**0.07 ± 0.02**	**0.13 ± 0.06**	0.09 ± 0.04	0.11 ± 0.05	**0.07 ± 0.02**	**0.15 ± 0.04**
4.65	**0.05 ± 0.02**	**0.10 ± 0.05**	0.08 ± 0.03	0.09 ± 0.04	**0.07 ± 0.01**	**0.12 ± 0.04**
5.83	**0.05 ± 0.02**	**0.08 ± 0.04**	0.07 ± 0.02	0.07 ± 0.03	0.06 ± 0.02	0.10 ± 0.03
7.31	**0.04 ± 0.02**	**0.07 ± 0.04**	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.02	0.08 ± 0.03
9.17	0.04 ± 0.02	0.05 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.06 ± 0.03
11.5	0.04 ± 0.02	0.04 ± 0.03	0.05 ± 0.03	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02
14.4	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.04	0.03 ± 0.02	0.04 ± 0.01	0.02 ± 0.02
18.1	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.05	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
22.6	0.05 ± 0.03	0.04 ± 0.03	0.06 ± 0.06	0.04 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
28.4	0.06 ± 0.04	0.05 ± 0.04	0.07 ± 0.07	0.06 ± 0.04	0.05 ± 0.02	0.03 ± 0.04
35.5	0.09 ± 0.05	0.06 ± 0.04	0.10 ± 0.06	0.08 ± 0.05	0.07 ± 0.02	0.05 ± 0.05
44.6	0.12 ± 0.06	0.09 ± 0.05	0.13 ± 0.05	0.11 ± 0.05	0.10 ± 0.04	0.08 ± 0.05
55.9	0.15 ± 0.05	0.12 ± 0.05	0.16 ± 0.04	0.14 ± 0.05	0.14 ± 0.05	0.11 ± 0.04
70.0	**0.18 ± 0.03**	**0.15 ± 0.05**	**0.19 ± 0.03**	**0.16 ± 0.04**	0.18 ± 0.03	0.14 ± 0.04

Statistically significant differences ($p < 0.05$ in the Mann–Whitney U test) between ED patients and controls are highlighted in bold type.

mean values for ED$_m$ are at 203% for 4.39 Hz and 208% for 7.2 Hz of the C$_m$ mean values (Tables A1 and A2).

Throughout all frequencies for both G' and G'', the ED$_f$ values behaved mostly similar to the ED$_m$ group, lower storage and loss moduli than C$_f$ for lower frequencies and higher or equal values than C$_f$ for higher frequencies (Figure 2). In contrast to the male subgroups, none of the differences in G' and G'' between the female subgroups were statistically significant, as illustrated in Tables A1 and A2.

Concerning the boys, the statistical analysis yields a similar trend to the male adults, with significant differences of G' and G'' at lower frequencies. Between 1 and 2 Hz, the mean values of the storage modulus G' for ED$_b$ are only 12–20% of the mean values of C$_b$, while the mean values of the loss modulus G'' are 22–40% for ED$_b$ compared to C$_b$.

Considering the strain sweeps in Tables 4 and 5, it can be observed that the ED$_m$ group displays statistically significantly lower G' and G'' values, particularly at lower strains (Figure 3). Between 0.1 and 7.3%, the mean values of the storage modulus G' for ED$_m$ are only 52–61% of the mean values of C$_m$, while the mean values of the loss modulus G'' are 58–71% for ED$_m$ compared to C$_m$. Conversely, at all higher strains between 7.3 and 70%, the ED$_m$ group tends to have equal or slightly higher G' values compared to group C$_m$, with only one significant case at 70% with G' mean value for ED$_m$ being 120% of the C$_m$ mean value. Similarly, for G'', the ED$_m$ group tends to have slightly higher or equal values compared to group C$_m$. Apart from that, these differences are not statistically significant according to Tables A3 and A4.

Similar to the frequency sweeps, over all investigated strain levels for both G' and G'', the ED$_f$ values displayed

Table 5: Mean values and standard deviations of all investigated shear stress for loss modulus *G″* averaged for all test groups

Loss modulus *G″* from strain sweep on resting saliva						
Shear strain *γ* (%)	**G''_ED$_m$** **N = 13** **Mean ± std**	**G''_C$_m$** **N = 35** **Mean ± std**	**G''_ED$_f$** **N = 14** **Mean ± std**	**G''_C$_f$** **N = 45** **Mean ± std**	**G''_ED$_b$** **N = 4** **Mean ± std**	**G''_C$_b$** **N = 5** **Mean ± std**
0.10	**0.23 ± 0.12**	**0.35 ± 0.22**	0.21 ± 0.11	0.78 ± 3.08	0.89 ± 1.53	0.56 ± 0.23
0.13	**0.23 ± 0.12**	**0.35 ± 0.22**	**0.20 ± 0.11**	**0.75 ± 2.91**	0.82 ± 1.39	0.55 ± 0.22
0.16	**0.22 ± 0.12**	**0.35 ± 0.21**	0.21 ± 0.10	0.72 ± 2.71	0.74 ± 1.22	0.55 ± 0.22
0.20	**0.22 ± 0.12**	**0.35 ± 0.21**	0.21 ± 0.10	0.68 ± 2.47	0.54 ± 0.82	0.54 ± 0.21
0.25	**0.21 ± 0.12**	**0.34 ± 0.20**	0.20 ± 0.11	0.64 ± 2.19	0.54 ± 0.82	0.54 ± 0.21
0.31	**0.20 ± 0.11**	**0.33 ± 0.20**	**0.19 ± 0.10**	**0.58 ± 1.87**	0.43 ± 0.61	0.53 ± 0.19
0.39	**0.19 ± 0.10**	**0.33 ± 0.19**	**0.19 ± 0.10**	**0.52 ± 1.53**	0.32 ± 0.39	0.51 ± 0.18
0.49	**0.18 ± 0.09**	**0.32 ± 0.18**	**0.19 ± 0.09**	**0.46 ± 1.20**	0.22 ± 0.20	0.50 ± 0.17
0.61	**0.18 ± 0.08**	**0.30 ± 0.17**	**0.18 ± 0.09**	**0.41 ± 0.94**	**0.15 ± 0.07**	**0.48 ± 0.15**
0.76	**0.17 ± 0.07**	**0.29 ± 0.16**	**0.17 ± 0.08**	**0.38 ± 0.83**	**0.12 ± 0.04**	**0.45 ± 0.14**
0.96	**0.16 ± 0.06**	**0.27 ± 0.15**	**0.16 ± 0.07**	**0.35 ± 0.73**	**0.12 ± 0.05**	**0.43 ± 0.13**
1.20	**0.15 ± 0.05**	**0.26 ± 0.13**	**0.15 ± 0.07**	**0.32 ± 0.62**	**0.12 ± 0.05**	**0.39 ± 0.11**
1.50	**0.14 ± 0.04**	**0.23 ± 0.12**	**0.14 ± 0.06**	**0.28 ± 0.51**	**0.11 ± 0.04**	**0.35 ± 0.10**
1.89	**0.13 ± 0.04**	**0.21 ± 0.11**	**0.13 ± 0.05**	**0.25 ± 0.40**	**0.11 ± 0.04**	**0.31 ± 0.09**
2.36	**0.12 ± 0.03**	**0.19 ± 0.09**	**0.12 ± 0.04**	**0.21 ± 0.31**	**0.11 ± 0.03**	**0.27 ± 0.08**
2.96	**0.11 ± 0.02**	**0.17 ± 0.08**	**0.11 ± 0.04**	**0.19 ± 0.24**	**0.11 ± 0.03**	**0.24 ± 0.06**
3.71	**0.10 ± 0.02**	**0.15 ± 0.07**	0.11 ± 0.03	0.17 ± 0.21	**0.10 ± 0.02**	**0.21 ± 0.05**
4.65	**0.09 ± 0.03**	**0.14 ± 0.06**	0.10 ± 0.03	0.15 ± 0.18	**0.10 ± 0.11**	**0.18 ± 0.04**
5.83	**0.07 ± 0.03**	**0.12 ± 0.05**	0.10 ± 0.02	0.13 ± 0.15	**0.09 ± 0.01**	**0.16 ± 0.04**
7.31	**0.05 ± 0.03**	**0.10 ± 0.05**	0.09 ± 0.03	0.11 ± 0.12	**0.07 ± 0.01**	**0.14 ± 0.04**
9.17	**0.05 ± 0.03**	**0.07 ± 0.05**	0.08 ± 0.05	0.09 ± 0.10	0.05 ± 0.03	0.11 ± 0.03
11.5	**0.05 ± 0.04**	**0.07 ± 0.04**	0.07 ± 0.06	0.07 ± 0.08	0.03 ± 0.02	0.09 ± 0.04
14.4	0.06 ± 0.05	0.07 ± 0.04	0.06 ± 0.06	0.07 ± 0.07	0.02 ± 0.01	0.04 ± 0.04
18.1	0.07 ± 0.06	0.08 ± 0.05	0.08 ± 0.06	0.08 ± 0.07	0.03 ± 0.03	0.04 ± 0.03
22.6	0.10 ± 0.06	0.09 ± 0.06	0.10 ± 0.07	0.10 ± 0.07	0.08 ± 0.02	0.07 ± 0.04
28.4	0.13 ± 0.06	0.11 ± 0.06	0.13 ± 0.06	0.13 ± 0.06	0.12 ± 0.03	0.09 ± 0.06
35.5	0.16 ± 0.05	0.13 ± 0.06	0.17 ± 0.04	0.16 ± 0.05	0.15 ± 0.03	0.13 ± 0.05
44.6	0.19 ± 0.03	0.16 ± 0.05	0.20 ± 0.02	0.18 ± 0.04	0.18 ± 0.03	0.17 ± 0.03
55.9	0.20 ± 0.02	0.19 ± 0.04	**0.21 ± 0.01**	**0.20 ± 0.03**	0.20 ± 0.02	0.20 ± 0.02
70.0	0.21 ± 0.05	0.20 ± 0.03	0.21 ± 0.01	0.20 ± 0.05	0.21 ± 0.01	0.20 ± 0.01

Statistically significant differences ($p < 0.05$ in the Mann–Whitney U test) between ED patients and controls are highlighted in bold style.

similar trends to the ED$_m$ group, lower storage and loss moduli than C$_f$ for lower strains and slightly higher or equal values than C$_f$ at higher strains (Figure 4). Only two of the differences in G' between the female subgroups were statistically significant at 0.31% and 70%. However, the analysis on the storage modulus G'' showed statistically significant differences in the continuous range of 0.3–2.96% with mean values of 33–58% for ED$_f$ compared to C$_f$ and at two further strain levels of 0.13% and 55.9%.

In the subgroup of boys, the statistical analysis of G' reflects the tendency of the male adults with significant differences of G' at lower strain levels (Table 4). Between strains of 1–4.7%, the mean values of the storage modulus G' for ED$_b$ are only 16–58% of the mean values of C$_b$. G'' displayed no statistical differences in the strain sweeps for the lowest and highest strain levels, but for strain levels 0.6–7.3%, where the mean values of the loss modulus G'' are 30–56% for ED$_b$ compared to C$_b$ (Table 5).

3.2 Cross-over points in amplitude/strain sweeps

Overall strain measurements, a common behavior can be observed: the storage modulus G' is larger than the loss modulus G'' for lower strain levels until a characteristic cross-over point is reached. For higher strain levels, the rheological characteristic is then generally inverted, yielding a larger loss modulus G'' than storage modulus G'. For all available strain sweeps, an additional analysis was conducted on the original measurement data (before interpolation) in order to determine and compare the position

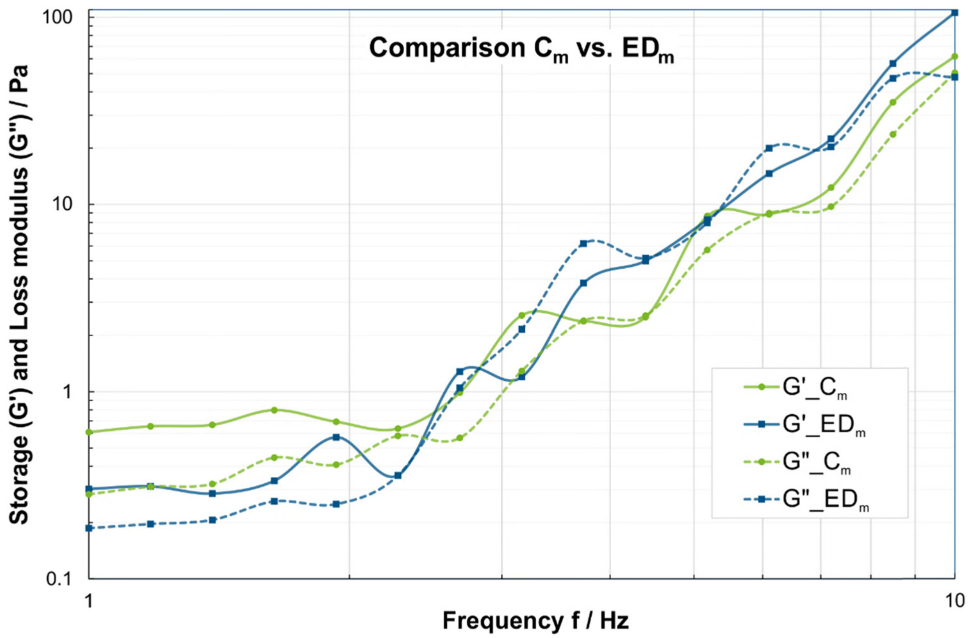

Figure 1: Comparison of storage modulus (solid lines) and loss modulus (dashed lines) in frequency sweeps at τ = 0.01 Pa between saliva samples from male ED subjects (ED_m) in blue and male controls (C_m) in green. Please note that the dashed/solid lines between the data points are for visualization of group affiliation and do not indicate an interpolation or dependency.

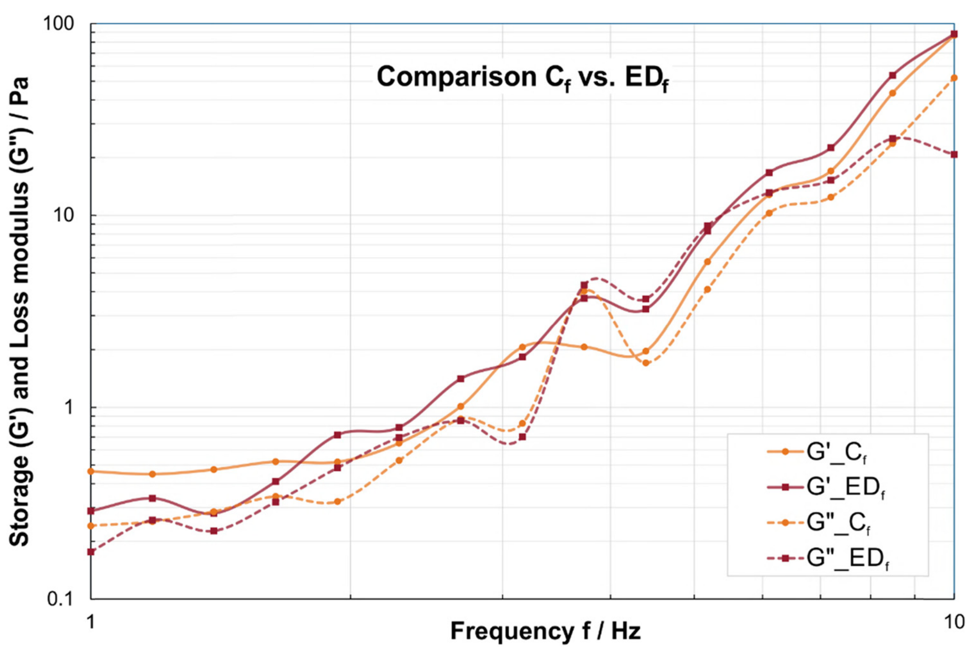

Figure 2: Comparison of storage modulus (solid lines) and loss modulus (dashed lines) in frequency sweeps at τ = 0.01 Pa between saliva samples by female ED subjects (ED_f) in red and female controls (C_f) in orange. Please note that the dashed/solid lines between the data points are for visualization of group affiliation and do not indicate an interpolation or dependency.

of the lowest cross-over points of G' and G'' on the shear strain axis.

Even though the averaged curves in Figure 4 indicated a shift of the cross-over points between ED_f and C_f toward higher strain levels for female ED subjects compared to their corresponding controls, the statistical analysis in Table 6 as well as the boxplot diagram in Figure 5 demonstrated that the cross-over points of G' and G'' in

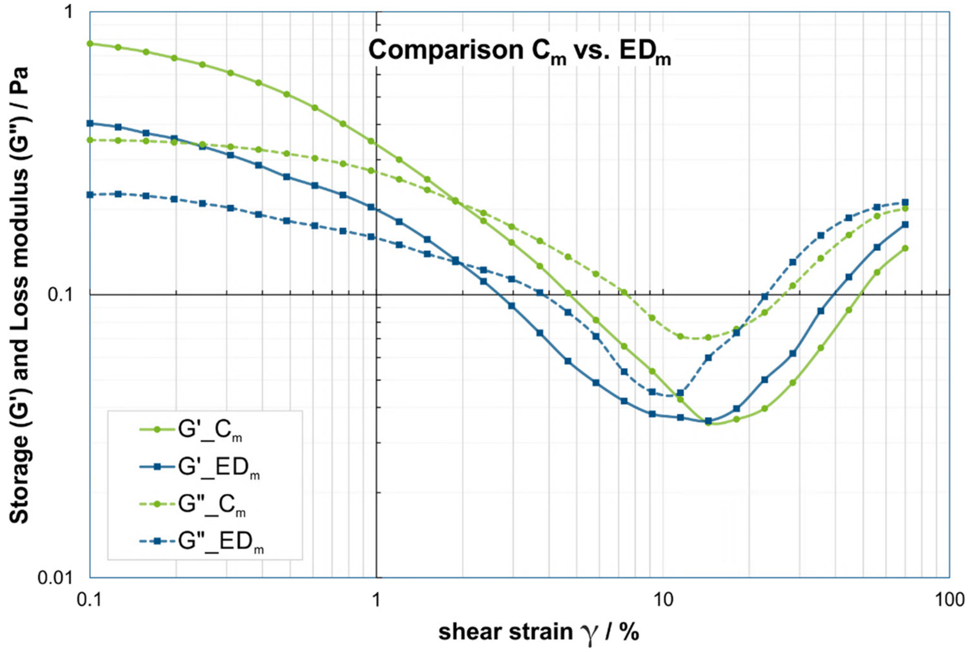

Figure 3: Comparison of storage modulus (solid lines) and loss modulus (dashed lines) in strain sweep at f = 0.5 Hz between saliva samples from male ED subjects (ED_m) in blue and male controls (C_m) in green. Please note that the dashed/solid lines between the data points are for visualization of group affiliation and do not indicate an interpolation or dependency.

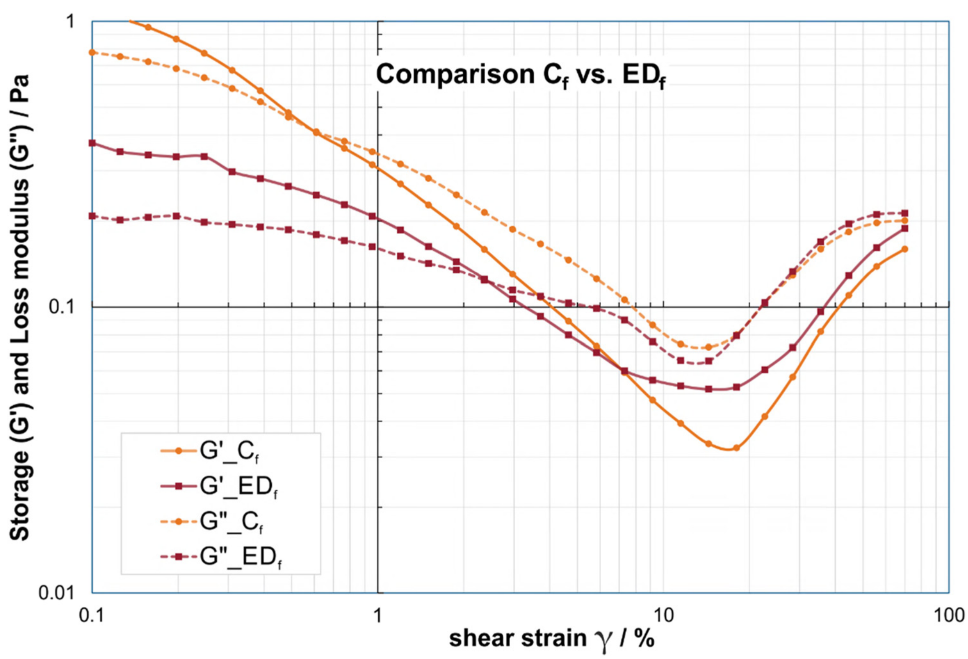

Figure 4: Comparison of storage modulus (solid lines) and loss modulus (dashed lines) in strain sweep at f = 0.5 Hz between saliva samples from female ED subjects (ED_f) in red and female controls (C_f) in orange. Please note that the dashed/solid lines between the data points are for visualization of group affiliation and do not indicate an interpolation or dependency.

the strain sweeps were not significantly displaced for male and female adults. Only for the boys, a statistically significant difference could be detected. The cross-over points of C_b are shifted toward lower strain levels compared to the ED_b group and all other groups, respectively.

Table 6: (a) Mean values and standard deviations of all investigated cross-over points in the shear stress analysis averaged for all test groups and (b) detailed results of Mann–Whitney *U* test for comparison of two groups calculated separately for male (ED_m vs C_m), female (ED_f vs C_f), and boys (ED_b vs C_b) test subjects

(a) Shear strain of cross-over points between *G′* and *G″* in strain sweeps

ED_m $N = 13$	C_m $N = 35$	ED_f $N = 13$	C_f $N = 45$	ED_b $N = 5$	C_b $N = 5$
Mean ± std	Mean ± std	Mean ± std	Mean ± std	Mean ± std	Mean ± std
2.03 ± 1.01	2.21 ± 1.25	2.57 ± 1.37	2.14 ± 1.24	**2.17 ± 0.77**	**1.58 ± 0.24**

(b) Statistical results: Mann–Whitney *U* ($p < 0.05$)

ED_m <> C_m		ED_f <> C_f		ED_b <> C_b	
Z	*p*-value	*Z*	*p*-value	*Z*	*p*-value
−0.104	0.917	−1.388	0.165	**−1.984**	**0.047**

Statistically significant differences ($p < 0.05$ in Mann–Whitney *U* test) between ED patients and controls are highlighted in bold type.

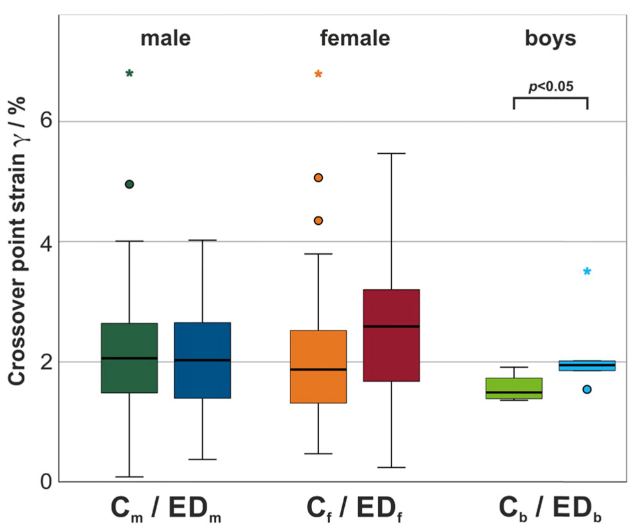

Figure 5: Boxplot diagram with median and interquartile ranges of the lowest cross-over points in strain sweeps for all test groups. Statistically significant difference within each gender group is indicated by brackets ($p < 0.05$).

4 Discussion

The rheology of resting saliva as a substitute for laryngeal mucus was analyzed in order to identify the underlying causality of the conspicuous voice in patients with ED. The statistical analysis of the rheological characteristics determined by frequency and amplitude sweeps yielded statistically significantly lower values for storage modulus *G′* and loss modulus *G″* for the male ED group (ED_m/ED_b) compared to their corresponding male control group (C_m/C_b) at the lower frequencies and lower strain levels. It has to be noted that the data obtained from the frequency sweeps display high standard deviations, as shown in Tables 2 and 3, and therefore should be interpreted carefully. The significant differences in the viscoelastic characteristics for the quasi-static range (low frequency/ low strain) indicate a reduced resistance against external forces on the saliva in male ED patients compared to healthy control subjects. This rheological divergence carries implications for the retention of saliva on saliva-coated tissue as well as for omnipresent tribomechanical loading cases, for instance, during swallowing. It is possible that the typical swallowing problems in ED are not only due to the reduced saliva production, but also to the altered rheological properties [35].

Even though a statistically significant difference was found in the cross-over points of storage and loss moduli in the strain sweep for the boys, this result must be considered with caution in view of the small number of participants. The cross-over point represents a measure for describing the gradual transition from the domination of elastic effects to the domination of viscous effects with increasing strain, comparable with the transition from a viscoelastic solid state to a viscoelastic liquid state [36]. Significant variations of this metric may further affect the adherence of saliva to surrounding tissue under mechanical stress, hence promoting the viscous-dominated behavior of saliva, with ED boys displaying the cross-over point at elevated strains compared to their controls.

To our knowledge, this rheological study demonstrates for the first time that male subjects with ED not only exhibit reduced saliva production compared to the control groups [12], but that the viscoelastic properties of the saliva are also significantly different. Since the glandular function is systematically affected in the most common hypohidrotic ED [16], these findings can most likely also be extended to the laryngeal mucus [10,12]. When transferring the findings on ED saliva to the laryngeal level, we hypothesize that this viscoelastic behavior would certainly influence the retention rate of the laryngeal mucus on the vocal folds. We assume that an increased effluent into the airways could contribute to the increased susceptibility to respiratory tract infections observed in ED patients [37,38]. Microaspiration, in which secretion of the upper respiratory tract, along with particulate material and microorganisms, reaches the respiratory tract by passing the vocal folds, is known as an important pathogenic mechanism in pneumonia [39].

The deviating viscoelasticity of the saliva from ED males raises the question of its origin. A study by Rebenda et al. could illustrate a strong dependency between the molecular weight of hyaluronic acids and the viscoelastic properties of the corresponding solutions by means of rheological measurements [30,40]. In general, the hyaluronic acid solutions with lower molecular weights displayed lower viscosity. For smaller molecular weights, the progression of storage and loss moduli was found to start at considerably lower values at low frequencies but display a greater increase toward higher frequencies than large molecular weights. Referring to the present study on ED, the progression of the G' and G'' of the male ED groups (ED_m/ED_b) compared to their corresponding male control groups (C_m/C_b) mirrors the behavior of hyaluronic acid solutions with smaller molecular weight, which suggests that the content of hyaluronic acid in saliva is systematically altered in male ED subjects [30]. We hypothesize that the significant differences found in the viscoelastic characteristics may be, at least in part, resulting from a decreased molecular weight of hyaluronic acid in ED.

Overall performed measurements, the comparison between ED subjects and controls in the female subgroups displayed similar trends to the corresponding male subgroups, but yielded no statistical significance in the frequency sweeps. However, in the strain sweeps, the loss modulus was statistically significantly lower for ED_f than C_f. This is in line with former studies on ED and the fact that the most frequent ED is inherited X-linked, which means that women are typically less affected [16,19]. An in-depth analysis regarding the differences between male and female ED subjects with thorough consideration of the genetic background would be highly desirable [31].

5 Limitations/outlook

A natural limitation of studies on rare diseases such as ED is the limited number of participants, which limits the power of the statistical analyses and therefore valid conclusions.

Another justifiable limitation is the use of easily accessible saliva samples instead of laryngeal mucus, which must be collected in an invasive procedure. Nevertheless, a thorough investigation of the saliva in the ED may provide helpful hints toward systemically altered parameters in the glandular function. In this regard, future studies are planned to analyze the composition of saliva samples in more detail and to determine the content of enzymes, proteins, and hyaluronic acid, also taking into account the distribution over different molecular masses.

Furthermore, it could provide important insights to take both saliva and mucus samples from people without ED who are placed under sedation/anesthesia for other reasons in order to analyze the correlation of these two secretions and thus better assess the transferability of our findings.

So far, the various influences of eating, drinking, and smoking habits, as well as medication and also the exact genetic variants, have not yet been taken into account due to the small group sizes, which would be highly desirable in subsequent studies. Despite difficulties in practical realization, it would be further favorable if the exact time of measurement in the circadian rhythm were also taken into account [41].

6 Conclusions

The present study showed statistically significantly lower values for storage modulus G' and loss modulus G'' concerning the lower frequencies and lower strains for the male ED group ED_m compared to the control group C_m. The same effect was only observed in the loss module G'' of the strain sweeps of female ED subjects ED_f. The significant differences found in the viscoelastic properties indicated a reduced resistance of the resting saliva against external forces in ED patients compared to healthy control subjects. Transferred to the laryngeal level, these findings would have an influence on the retention rate of the laryngeal mucus on the vocal folds. The altered progression of storage and loss moduli in ED subjects could be explained by deviating distributions in the molecular mass of hyaluronic acid.

Even though further studies are needed, the presented data provide a promising indication toward the underlying causes on the laryngeal level leading to the voice conspicuities in ED subjects. Once we understand this relationship, new therapeutic approaches similar to saliva substitutes could emerge, from which ED and other diseases with a reduced glandular function, like Sjögren, etc., would benefit [42–44]. By prospectively allowing for correlations between rheological characteristics of mucus, saliva, and the genetic expression, targeted treatments could emerge. Although ED is a rare disease, we can gain knowledge of the fundamental principles of phonation and the role of mucus by investigating the voice of ED patients in comparison to healthy control subjects.

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Author contributions: ME performed the statistical analysis, contributed to the interpretation of data, and drafted the manuscript. BT contributed to the measurements, data curation, interpretation of data, and revision of the manuscript. AH performed the rheological analysis. SW contributed to the data acquisition and the rheological analysis. SS contributed to data analysis, interpretation of the rheological data, and editing of the manuscript. DD contributed to the interpretation of the data and the review of the manuscript. OW supervised data analysis, contributed to the interpretation of the data, and edited the manuscript. HS provided his expertise in genetic analysis and contributed to the review of the manuscript. MS designed the study, supervised the data acquisition, data analysis, and revision of the manuscript. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that they have no competing interests.

Ethical approval: The present study is covered by a positive vote of the local ethics commission (ref. No 21-437-B) at the medical faculty at the Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Data availability statement: The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

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Appendix A

Table A1: Detailed results of Mann–Whitney *U* test for comparison of two groups calculated separately for male (ED_m <> C_m), female (ED_f <> C_f), and boys (ED_b <> C_b) test subjects

Resting saliva	**Storage modulus *G′***					
Frequency *f* (Hz)	**Mann–Whitney *U* (*p* < 0.05)**					
	ED_m <> C_m		**ED_f <> C_f**		**ED_b <> C_b**	
	Z	***p*-value**	***Z***	***p*-value**	***Z***	***p*-value**
1.00	**−2.126**	**0.034**	−1.398	0.162	**−2.611**	**0.009**
1.18	**−2.235**	**0.025**	−1.607	0.108	**−2.611**	**0.009**
1.39	**−2.726**	**0.006**	−1.621	0.105	**−2.611**	**0.009**
1.64	**−2.907**	**0.004**	−1.484	0.138	**−2.611**	**0.009**
1.93	−1.127	0.260	−0.749	0.454	**−2.193**	**0.028**
2.28	−1.799	0.072	−1.715	0.086	−1.567	0.117
2.68	−0.109	0.913	−0.836	0.403	−1.567	0.117
3.16	−0.218	0.827	−0.173	0.863	−1.567	0.117
3.73	−0.709	0.479	−0.562	0.574	−0.313	0.754
4.39	−1.663	0.096	−0.648	0.517	−1.358	0.175
5.18	−0.999	0.318	−1.016	0.310	−0.940	0.347
6.11	−1.790	0.073	−0.807	0.420	−0.104	0.917
7.20	−1.726	0.084	−1.383	0.167	−1.776	0.076
8.48	−1.908	0.056	−0.879	0.379	**−2.402**	**0.016**
10.0	**−2.071**	**0.038**	−0.908	0.364	−0.940	0.347

Significant *p*-values ($p < 0.05$) are highlighted in bold type.

Table A2: Detailed results of Mann–Whitney *U* test for comparison of two groups calculated separately for male (ED_m <> C_m), female (ED_f <> C_f), and boys (ED_b <> C_b) test subjects

Resting saliva	**Loss modulus *G″***					
Frequency *f* (Hz)	**Mann–Whitney *U* (*p* < 0.05)**					
	ED_m <> C_m		**ED_f <> C_f**		**ED_b <> C_b**	
	Z	***p*-value**	***Z***	***p*-value**	***Z***	***p*-value**
1.00	**−2.199**	**0.028**	−1.643	0.100	**−2.611**	**0.009**
1.18	**−2.853**	**0.004**	−1.023	0.306	**−2.611**	**0.009**
1.39	**−2.344**	**0.019**	−1.441	0.150	**−2.193**	**0.028**
1.64	**−2.871**	**0.004**	−0.598	0.550	−1.776	0.076
1.93	**−2.262**	**0.024**	−0.584	0.560	**−2.611**	**0.009**
2.28	−0.945	0.345	−0.216	0.829	−0.940	0.347
2.68	−0.491	0.624	−0.072	0.943	−0.104	0.917
3.16	−0.236	0.813	−0.375	0.708	−0.940	0.347
3.73	−0.236	0.813	−0.620	0.536	−0.940	0.347
4.39	**−3.271**	**0.001**	−1.470	0.142	−0.104	0.917
5.18	−1.108	0.268	−0.461	0.645	−1.149	0.251
6.11	−1.127	0.260	−0.562	0.574	−0.104	0.917
7.20	**−2.435**	**0.015**	−0.821	0.411	**−2.193**	**0.028**
8.48	−1.690	0.091	−0.636	0.526	−0.522	0.602
10.0	−0.763	0.445	−1.081	0.280	−1.358	0.175

Significant *p*-values ($p < 0.05$) are highlighted in bold type.

Table A3: Detailed results of Mann–Whitney *U* test for comparison of two groups calculated separately for male ($\mathrm{ED_m}$ <> $\mathrm{C_m}$), female ($\mathrm{ED_f}$ <> $\mathrm{C_f}$), and boys ($\mathrm{ED_b}$ <> $\mathrm{C_b}$) test subjects

Resting saliva Shear strain *γ* (%)	**Storage modulus *G′***					
	Mann–Whitney *U* (*p* < 0.05)					
	$\mathrm{ED_m}$ <> $\mathrm{C_m}$		**$\mathrm{ED_f}$ <> $\mathrm{C_f}$**		**$\mathrm{ED_b}$ <> $\mathrm{C_b}$**	
	Z	***p*-value**	***Z***	***p*-value**	***Z***	***p*-value**
0.10	**−2.424**	**0.015**	−1.693	0.091	**−2.449**	**0.014**
0.13	**−2.378**	**0.017**	−1.835	0.066	**−2.449**	**0.014**
0.16	**−2.517**	**0.012**	−1.853	0.064	**−2.449**	**0.014**
0.20	**−2.424**	**0.015**	−1.871	0.061	**−2.449**	**0.014**
0.25	**−2.633**	**0.008**	−1.746	0.081	**−2.449**	**0.014**
0.31	**−2.703**	**0.007**	**−2.049**	**0.040**	**−2.449**	**0.014**
0.39	**−2.819**	**0.005**	−1.960	0.050	**−2.449**	**0.014**
0.49	**−3.097**	**0.002**	−1.960	0.050	**−2.449**	**0.014**
0.61	**−2.958**	**0.003**	−1.800	0.072	**−2.449**	**0.014**
0.76	**−2.865**	**0.004**	−1.693	0.091	**−2.449**	**0.014**
0.96	**−2.888**	**0.004**	−1.800	0.072	**−2.449**	**0.014**
1.20	**−2.819**	**0.005**	−1.710	0.087	**−2.449**	**0.014**
1.50	**−2.912**	**0.004**	−1.568	0.117	**−2.449**	**0.014**
1.89	**−2.912**	**0.004**	−1.479	0.139	**−2.449**	**0.014**
2.36	**−2.865**	**0.004**	*−1.497*	*0.134*	**−2.449**	**0.014**
2.96	**−2.842**	**0.004**	*−1.318*	*0.187*	**−2.449**	**0.014**
3.71	**−2.888**	**0.004**	−1.140	0.254	**−2.449**	**0.014**
4.65	**−2.842**	**0.004**	−0.481	0.630	**−2.205**	**0.027**
5.83	**−2.772**	**0.006**	−0.036	0.972	−1.960	0.050
7.31	**−2.424**	**0.015**	−0.463	0.643	−0.980	0.327
9.17	−1.844	0.065	−1.318	0.187	−0.245	0.806
11.5	−0.476	0.634	−1.497	0.134	−0.490	0.624
14.4	−0.313	0.754	−1.479	0.139	−1.715	0.086
18.1	−0.313	0.754	−1.514	0.130	−1.715	0.086
22.6	−0.893	0.372	−1.051	0.293	−1.470	0.142
28.4	−1.148	0.251	−0.445	0.656	−1.470	0.142
35.5	−1.334	0.182	−0.641	0.521	−1.470	0.142
44.6	−1.380	0.167	−1.176	0.240	−0.735	0.462
55.9	−1.404	0.160	−1.675	0.094	−0.735	0.462
70.0	**−2.053**	**0.040**	**−2.227**	**0.026**	−1.470	0.142

Significant *p*-values ($p < 0.05$) are highlighted in bold type.

Table A4: Detailed results of Mann–Whitney *U* test for comparison of two groups calculated separately for male ($\mathrm{ED_m}$ <> $\mathrm{C_m}$), female ($\mathrm{ED_f}$ <> $\mathrm{C_f}$), and boys ($\mathrm{ED_b}$ <> $\mathrm{C_b}$) test subjects

Resting saliva Shear strain *γ* (%)	**Loss modulus *G″***					
	Mann–Whitney *U* (*p* < 0.05)					
	$\mathrm{ED_m}$ <> $\mathrm{C_m}$		**$\mathrm{ED_f}$ <> $\mathrm{C_f}$**		**$\mathrm{ED_b}$ <> $\mathrm{C_b}$**	
	Z	***p*-value**	***Z***	***p*-value**	***Z***	***p*-value**
0.10	**−2.501**	**0.012**	−1.942	0.052	−1.225	0.221
0.13	**−2.346**	**0.019**	**−1.996**	**0.046**	−1.225	0.221
0.16	**−2.434**	**0.015**	−1.924	0.054	−1.225	0.221
0.20	**−2.412**	**0.016**	−1.782	0.075	−1.225	0.221
0.25	**−2.589**	**0.010**	−1.942	0.052	−1.225	0.221
0.31	**−2.678**	**0.007**	**−2.031**	**0.042**	−1.225	0.221
0.39	**−2.811**	**0.005**	**−2.067**	**0.039**	−1.225	0.221
0.49	**−2.921**	**0.003**	**−2.067**	**0.039**	−1.960	0.050
0.61	**−2.921**	**0.003**	**−2.102**	**0.036**	**−2.449**	**0.014**
0.76	**−3.032**	**0.002**	**−2.120**	**0.034**	**−2.449**	**0.014**
0.96	**−3.054**	**0.002**	**−2.298**	**0.022**	**−2.449**	**0.014**
1.20	**−3.054**	**0.002**	**−2.316**	**0.021**	**−2.449**	**0.014**
1.50	**−3.076**	**0.002**	**−2.370**	**0.018**	**−2.449**	**0.014**
1.89	**−3.054**	**0.002**	**−2.227**	**0.026**	**−2.449**	**0.014**
2.36	**−3.121**	**0.002**	**−2.298**	**0.022**	**−2.449**	**0.014**
2.96	**−3.121**	**0.002**	**−2.049**	**0.040**	**−2.449**	**0.014**
3.71	**−3.298**	**<0.001**	−1.835	0.066	**−2.449**	**0.014**
4.65	**−3.364**	**<0.001**	−1.461	0.144	**−2.449**	**0.014**
5.83	**−3.231**	**0.001**	−0.748	0.454	**−2.449**	**0.014**
7.31	**−3.143**	**0.002**	−0.125	0.901	**−2.205**	**0.027**
9.17	**−2.235**	**0.025**	−0.036	0.972	−1.960	0.050
11.5	**−2.014**	**0.044**	−0.624	0.533	−1.960	0.050
14.4	−1.173	0.241	−0.891	0.373	−0.245	0.806
18.1	−0.443	0.658	−0.053	0.957	−0.980	0.327
22.6	−0.664	0.507	−0.089	0.929	−1.225	0.221
28.4	−1.195	0.232	−0.321	0.748	−1.470	0.142
35.5	−1.505	0.132	−0.249	0.803	−0.980	0.327
44.6	−1.682	0.093	−0.927	0.354	−0.980	0.327
55.9	−1.306	0.192	**−1.996**	**0.046**	−0.735	0.462
70.0	−1.549	0.121	−1.764	0.078	−1.470	0.142

Significant *p*-values ($p < 0.05$) are highlighted in bold type.