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## Original Research

# Topical fluorometholone treatment and desiccating stress change inflammatory protein expression in tears



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## ABSTRACT

**Purpose:** It was hypothesized that tear protein biomarkers could predict the effects of topical steroid treatment and desiccating stress in patients with dry eye disease (DED). To test this concept, a randomized, double-masked, controlled clinical trial with 41 patients was conducted.

**Methods:** The patients were treated topically with either 0.1% fluorometholone (FML) or polyvinyl alcohol (PA). Tear samples were collected using 1 µl glass capillaries at recruitment into the study and after a 3-week treatment period, both before and after 2 h exposure to desiccating stress, in a controlled environment chamber. Relative quantification of tear proteins was conducted by NanoLC-MSTOF using sequential window acquisition of all theoretical mass spectra (SWATH). Ocular surface integrity (corneal and conjunctival staining and conjunctival hyperemia) was selected as the key DED-related sign and analyzed with proteomic data. Analysis of covariance (ANCOVA) and linear models were used to analyze the data with R.

**Results:** 758 proteins were identified and relatively quantified from each tear sample. Analysis revealed 9 differentially expressed proteins between FML and PA treatments after 3 weeks and 7 after desiccating stress ( $P < 0.05$ ). We also identified several differentially expressed proteins at the initial collection, which could be used to predict changes of conjunctival and corneal staining and conjunctival hyperemia after FML treatment and after desiccating stress. These proteins include complement C3 (C3) and calmodulin like 5 (CALML5), which could also differentiate the severity of DED at baseline.

**Conclusions:** The identified proteins could be further used as biomarkers to identify patients most benefiting from FML treatment.

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## 1. Introduction

Tear fluid is a complex, extra-cellular fluid from various secretory sources, including the lacrimal glands, cornea, conjunctival cells and blood ultra-filtrates [1]. Tears bathe the ocular surface as

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they deliver and remove nutrients and metabolic products from the anterior surface of the eye, improve the retinal image, and contribute to the mechanical, antimicrobial and anti-inflammatory defense of the ocular surface [2,3]. Any dysfunction in tear flow, production or composition can cause adverse effects to the health of the ocular surface and the visual process.

Dry eye disease (DED) is an immune-based multifactorial disease of the ocular surface resulting from a dysfunction of the lacrimal functional unit, which maintains a homeostatic environment of the ocular surface [4–6]. Management of DED usually begins with artificial tears, which mainly increase the aqueous layer of the tear film and may dilute inflammatory cytokines, which are upregulated in DED [6,7]. In more severe DED, topical steroids or other anti-inflammatory medications such as topical cyclosporine are commonly used [6,8]. Several studies have shown that corticosteroids suppress the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF- $\kappa$ B) pathways [9–11]. They have been demonstrated to be effective, in particular during and after desiccating stress, but are used with caution in long-term treatment due to adverse side effects [8,12].

The present study continues from a publication by Pinto-Fraga et al. (2016) revealing the positive action of topical 0.1% fluorometholone (FML) in DED patients in adverse environments in comparison to polyvinyl alcohol vehicle (PA) treatment [13]. Conjunctival hyperemia as well as corneal and conjunctival staining were significantly decreased in patients with FML treatment in comparison to vehicle, PA. In our study, tear samples collected from these patients were used for proteomics analysis. The hypothesis of the current study was that topical FML treatment would benefit the patients, ameliorating the desiccating stress effects, and that this would be reflected in changes of the tear proteome. Additionally, another aim was to identify proteins specifically indicating and predicting the treatment effects combined with desiccating stress.

## 2. Methods

### 2.1. Study outline

The study was a single randomized, double-masked, vehicle-controlled, parallel-group, phase 3 clinical trial (clinicaltrials.gov: identifier NCT0205102313) with 41 patients who had been previously diagnosed with either moderate or severe DED. This clinical trial was fully approved by an Ethics Committee and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

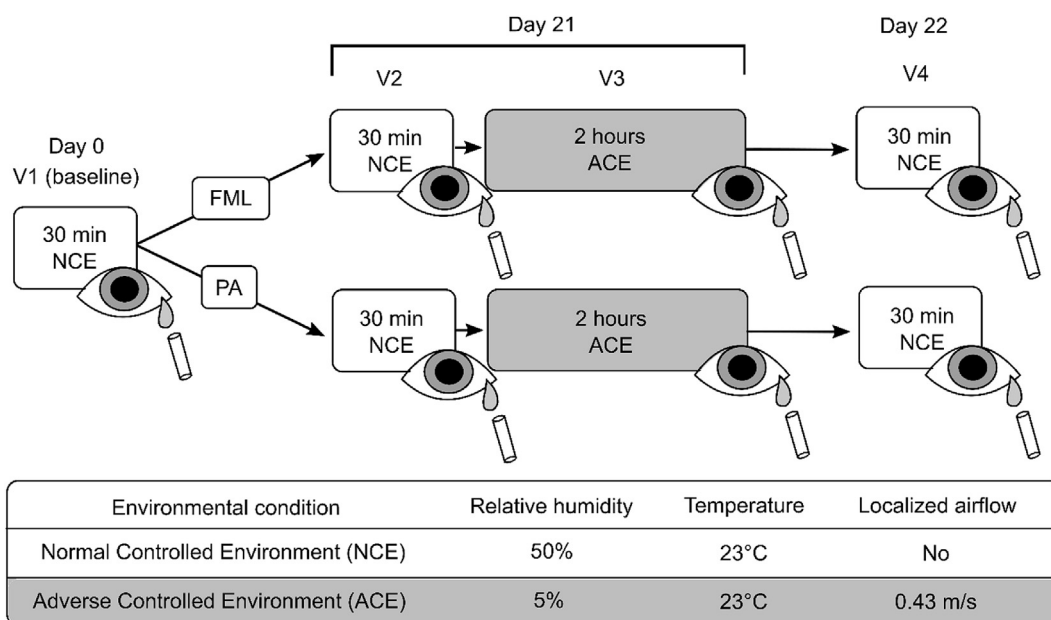
As shown in Fig. 1, the clinical trial was conducted during a 22-day period during which DED patients underwent 4 visits in the environmental chamber within the Controlled Environmental Research Laboratory (CERLab, Instituto Universitario de Oftalmobiología Aplicada, University of Valladolid) [14,15]. Clinical information and tear samples were collected from the patients during each visit [13]. Clinical examinations included fluorescein corneal staining, lissamine green conjunctival staining, and slit-lamp biomicroscopy, among others. Clinical examinations were conducted on both eyes and a randomly chosen eye was used in this study, along with tear samples from the same eye.

### 2.2. Tear collection

Unstimulated tear samples were collected from one eye of all 41 DED patients using 1  $\mu$ l Microcap<sup>®</sup> tubes (Drummond, Broomall, PA, USA) from the open eye [14,15], avoiding tissue contact to prevent reflex tearing. Tear collection was the first test performed in order to avoid any influence from other tests. Samples were immediately transferred to storage tubes at  $-80^{\circ}\text{C}$  until processed.

### 2.3. Chemicals and materials

Acetonitrile (ACN), formic acid (FA), water (UHPLC-MS grade),



**Fig. 1. Flowchart of the study workflow.** The 22-day study period included 4 visits (V) to the environmental chamber with either NCE or ACE, where relative humidity, temperature and localized airflow were controlled. DED patients were recruited in baseline (V1) and randomized to use either 0.1% fluorometholone (FML) or its vehicle, polyvinyl alcohol (PA), four times per day for 21 days, i.e. 3 weeks (V2). Immediately after V2 patients were exposed to desiccating stress for 2 h (V3). After one day (24 h) recovery, during which the patients still took their assigned topical medication, the study was concluded (V4). Tear capillary samples were collected from patients during each visit (V1–V4) after the exposures to either NCE or ACE.

triethylammonium bicarbonate buffer 1 M (TEAB), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), trifluoroacetic acid (TFA), ammonium bicarbonate (ABC) and urea were all purchased from Sigma Aldrich (St. Louis, MO, USA). Halt™ Protease Inhibitor Cocktail and sample clean up tips (C18) were from Thermo Fisher Scientific (San Jose, CA, USA). Bio-Rad DC™ kit and bovine serum albumin standard were purchased from Bio-Rad (Hercules, CA, USA) and 30 kDa molecular weight cut off (MWCO) centrifugal devices from PALL (Port Washington, NY, USA). Retention time calibration peptides (Hyper reaction monitoring (HRM) Calibration Kit) were purchased from Biognosys AG (Zurich, Switzerland).

#### 2.4. Sample preparation and analysis

Tear samples were dissolved from capillary tubes in 0.5% SDS, 50 mM ABC, HALT™ protease inhibitor, incubated on ice for 60 min and centrifuged with low rpm to avoid the breaking of the capillaries (3000 rpm, 2 min). Protein concentration was measured using Bio-Rad DC protein quantification kit. Average amount of protein recovered per sample was  $20 \pm 9 \mu\text{g}$  (SD). The smallest amount taken to tryptic digestion was  $6 \mu\text{g}$  of total protein. Seven patients had less than  $6 \mu\text{g}$  of protein in the tear samples and were therefore not analyzed further. Two additional patients were excluded from the study for other reasons.

Samples were then subjected to reduction, alkylation, and tryptic digestion. These steps were performed according to the description in the supplementary method materials. For mass spectrometry, analysis samples were eluted to the same concentration and  $2.6 \mu\text{g}$  of sample was injected into NanoLC-TripleTOF (Sciex 5600). Two technical replicates were produced from each sample. Analysis of the samples was done by NanoLC-TripleTOF mass spectrometry using SWATH acquisition as described in the supplementary method material.

#### 2.5. Protein identification and quantification

As part of the SWATH analysis method, a relative protein quantification library, consisting of >870 tear proteins, was created using tear samples from this study and from another clinical study consisting of glaucoma patients and their controls. Overall library consisted of 37 different patients/samples and 64 data-dependent analysis (DDA) runs with same LC gradient and instrument settings, which were used for SWATH analyses. The library was created using Protein Pilot® 4.5 (Sciex, Redwood City, USA) and all DDA runs spectra were identified against UniprotKB/SwissProt. Quantification was done by Peak Viewer® and Marker viewer® (Sciex, Redwood City, USA). FDR 1% was used in the library creation and only distinctive peptides were used in the quantification. Retention time calibration was done for all samples using HMR retention time calibration peptides. Five transitions per peptide and 1–15 peptides were used for peak area calculations. All proteins with significant or interesting findings in the data analysis were subjected to manual inspection of peptides. This consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), sufficient signal to noise ratio inspection (>7) and chromatogram inspection in relation to library chromatogram. Also, variation of technical replicate results were calculated as means to all samples/protein. Peptides were eliminated from result processing if manual inspection requirements were not fulfilled. Proteins with missing values were excluded from consideration. Results are presented as combination of protein-specific peptides peak areas from SWATH mass spectrometry measurement and referred to as protein expression.

#### 2.6. Data analysis

Data processing included  $\log_2$ -transformation and percentile normalization in which the sample distributions were normalized based on the global median. The quality of the technical replicates was analyzed by analysing the intraclass correlation (ICC) and Pearson correlation was used to generate p-values (P) in permutation tests ( $n = 1000$  permutations/technical replicate). Means of the dependent technical replicates were used for further analysis.

The treatment effect was analyzed using analysis of covariance (ANCOVA) in order to evaluate the differences in protein levels at each visit to control for protein expression in the previous visit. The normality of residuals was tested using the Shapiro-Wilk normality test and the homogeneity of variance was tested using the Bartlett test of homogeneity of variances. Additional assumptions of ANCOVA, i.e., the assumption of parallel slopes and independence of covariate (previous visit) and independent variable (treatment effect) were in addition tested accordingly. Post hoc analysis in ANCOVA was conducted using pairwise Tukey tests on adjusted means, using the multcomp package in R (R Core Team. Foundation for Statistical Computing, Vienna, Austria). Protein associations for given biological functions (inflammation) were identified in R based on their UniProt (UniProt.ws) term and its offspring terms as well as by using the associated terms provided by Ingenuity® Pathway Analysis (IPA, QIAGEN Redwood City, USA).

Multiple linear regression was used to evaluate which proteins were related to percentage changes in specific clinical signs. The protein expression level of the previous visit and treatment effect were accounted for as independent variables. The interaction terms of treatment effect and the baseline levels of protein expression were included in the model and were of main interest. The clinical signs were evaluated in percentage changes as in the previously published work as qualitative variables (1). The severity comparisons at baseline were conducted using Wilcoxon rank sum test.

$$\text{if } \begin{cases} Y_{\text{post}} > Y_{\text{pre}} \rightarrow \Delta Y = \frac{Y_{\text{post}} - Y_{\text{pre}}}{Y_{\text{max}} - Y_{\text{pre}}} * 100 \\ Y_{\text{post}} = Y_{\text{pre}} \rightarrow \Delta Y = 0 \\ Y_{\text{post}} < Y_{\text{pre}} \rightarrow \Delta Y = \frac{Y_{\text{post}} - Y_{\text{pre}}}{Y_{\text{pre}} - Y_{\text{min}}} * 100 \end{cases} \quad (1)$$

where  $Y_{\text{max}}$  = maximum value,  $Y_{\text{min}}$  = minimum value,  $Y_{\text{pre}}$  = initial value,  $Y_{\text{post}}$  = final value,  $\Delta Y$  = percentage change.

Benjamini-Hochberg adjustment was applied to all initial p-values (P) where applicable to account for the multiple testing issues. The significance threshold was chosen as  $\alpha = 0.05$ . R software version 3.2.3 was used to analyze data. IPA was used to conduct pathway analysis and identify proteins connected to inflammatory pathways.

### 3. Results

Altogether, 758 proteins were identified and relatively quantified from each 1  $\mu\text{l}$  sample (32 patients, 128 samples). The SWATH data consisted of reproducible results with a mean intraclass correlation (ICC) coefficient of 0.97 between technical replicates. Permutation tests using Pearson correlation with the technical replicates showed that 80% of the technical replicates had a  $P < 0.05$ , which suggests that the technical replicates are of relatively good quality. Two approaches were adopted to analyze the proteomic data; the first was to evaluate the effects of FML on the tear proteome as such and after a desiccating stress at the planned visits, and the second was the incorporation of the clinical signs and their relationship to the proteomic data. The patient characteristics

of different treatment and severity groups can be found from Appendix A (Tables A.1 and A.2).

### 3.1. FML treatment and desiccating stress effects on tear proteins

When the protein expression levels were examined, 9 proteins were found to differ between FML and vehicle (PA) groups after 3-week treatment period (adjusted model  $P < 0.05$  and treatment effect  $P < 0.05$ ), and 7 proteins were differentially expressed in these groups after desiccating stress (Table 1). Most of these proteins were associated to inflammation according to the linked gene ontology (GO) terms. In addition, similar tests were conducted for the follow-up (V4) data but only one protein showed statistically significant changes between the groups. This was more specifically Ig heavy chain V-I region V35 (P23083) (Baseline ( $P < 0.001$ ; Treatment ( $P = 0.019$ ;  $\log_2$  ratio (post hoc) =  $-0.52$ ). The significant proteins were analyzed further to test ANCOVA assumptions, and the results can be found in Appendix B (Table B.1).

### 3.2. Proinflammatory proteins and their connections to clinical signs

The ability of the expressed proteins to predict subsequent clinical sign changes was evaluated next. Ocular surface integrity (corneal and conjunctival staining) and conjunctival hyperemia were selected as the key DED-related signs since they showed statistically significant clinical changes in this patient group (Appendix C, Table C.1).

Individual protein expression levels and treatment, FML or PA, were used to predict the DED-related sign changes between initial baseline and after the 3-week treatment period (V1-V2). Sixty-eight potentially predictive proteins (model  $P$  (adj.)  $< 0.05$  and interaction term  $P < 0.05$ ) were identified for conjunctival staining change and 38 potentially predictive proteins for corneal staining change. In addition, there were 28 proteins with potential connections to the conjunctival hyperemia change between V1 and V2. Appendix D (Tables D.1–4) shows the statistically significant results. Corneal and conjunctival staining change results had 7 proteins in common: complement C3 (C3), Ig mu chain C region (IGHM) and 14-3-3 protein sigma (SFN), calmodulin like 5 (CALML5), LIM and SH3 domain protein 1 (LASP1), quinone oxidoreductase (CRYZ) and

proline-rich protein 4 (PRR4). CALML5 was also associated with conjunctival hyperemia. C3, IGHM, SFN and LASP1 displayed similar regression patterns in comparison to each other and similarly CALML5, CRYZ and PRR4 behave similarly according to the data.

All statistically significant proteins were analyzed further by pathway analysis (IPA). The top results are listed in Appendix E (Table E.1), indicating that many identified proteins are connected to apoptosis-, migration- and inflammation-related pathways. Three inflammation-associated proteins common for corneal and conjunctival staining (C3, IGHM and SFN) and CALML5 were examined in more detail (Fig. 2). These proteins were further found to be connected to proinflammatory cytokines and NF- $\kappa$ B (Appendix E, Fig. E.1). The analysis showed that FML-treated patients with low initial baseline expression levels in C3, IGHM and SFN and high baseline expression of CALML5 were more likely to exhibit greater improvement in staining scores compared to patients with higher initial expression levels. Alternatively, FML-treated patients with low CALML5 expression and/or high C3, IGHM and SFN expression in the baseline appear to be less likely to have a great improvement in their clinical signs (Fig. 2). PA treatment of DED patients had little effect on corneal or conjunctival staining and the protein baseline expression levels but higher CALML5 expression in the baseline predicted worsening of conjunctival hyperemia.

### 3.3. DED severity differences on baseline protein expression

The relationship of CALML5, C3, IGHM and SFN baseline expression levels and the DED severity status, i.e., moderate or severe, was examined next. Expression differences of CALML5 and C3 were significantly different in moderate and severe patients ( $P = 0.002$  and  $P = 0.009$  respectively) (Fig. 3). In severe DED patients CALML5 was downregulated ( $\log_2$  fold change of  $-0.79$ ) and C3 was upregulated ( $\log_2$  fold change 0.93). The correlation (Spearman's rank correlation) between C3 and CALML5 baseline expression levels was negative and statistically significant ( $\rho = -0.5$ ,  $P = 0.004$ ), which indicates that these proteins are connected suggesting that they could be used as predictive markers for DED severity at baseline.

**Table 1**

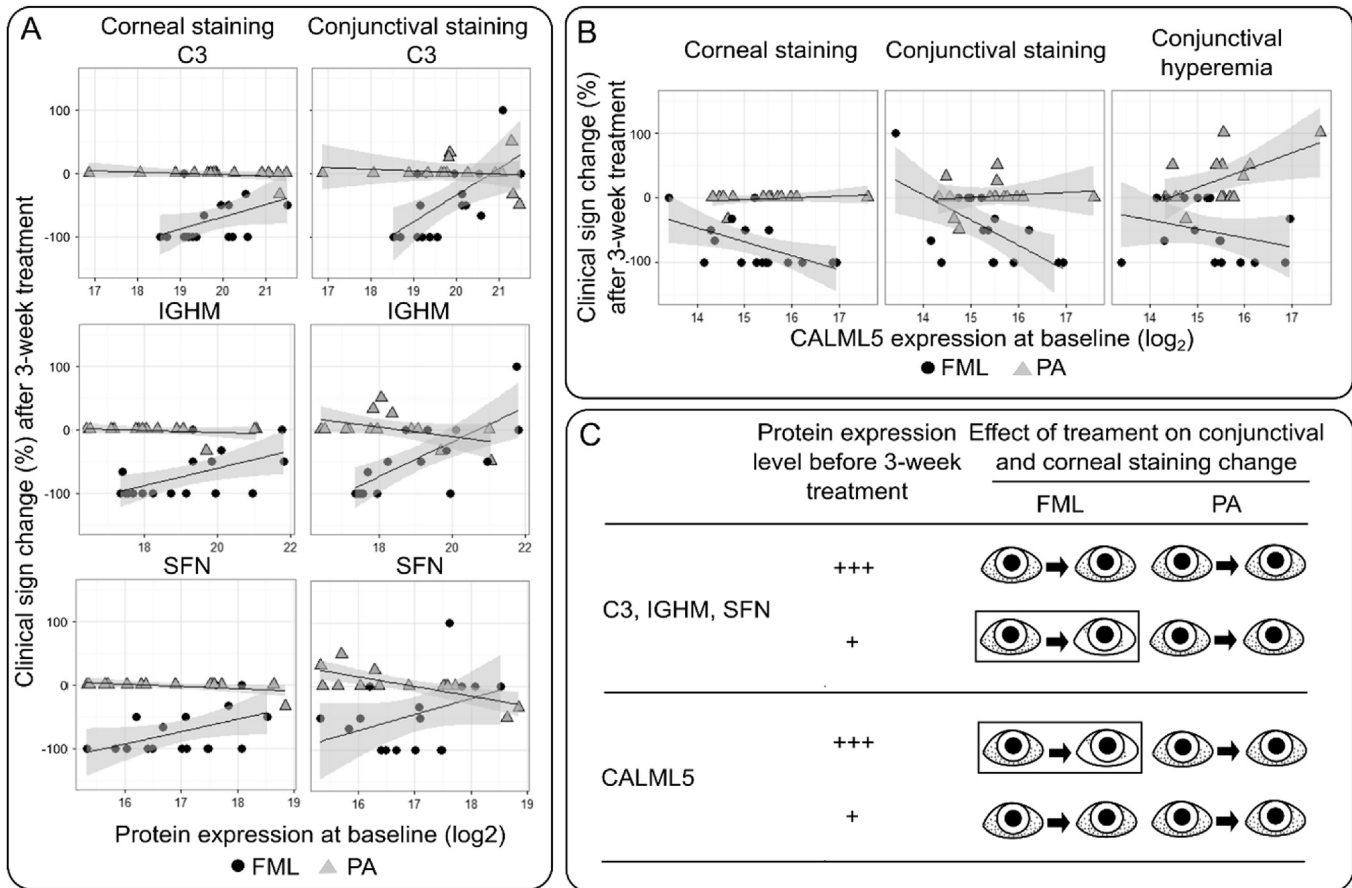
Protein abundance difference between treatments (0.1% fluorometholone (FML), polyvinyl alcohol (PA)) after 3-week treatment period and after desiccating stress.

Time point	UniProt	Full name	Gene name	Baseline (P)	Treatment (P)	Model (P)	Model (Adj. P)	$\log_2$ ratio	
After 3-week treatment period	<b>P63261<sup>b</sup></b>	<b>Actin, cytoplasmic 2</b>	<b>ACTG1</b>	<b>&lt;0.001</b>	<b>0.029</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.552</b>	
	<b>P08758<sup>b</sup></b>	<b>Annexin A5</b>	<b>ANXA5</b>	<b>&lt;0.001</b>	<b>0.009</b>	<b>&lt;0.001</b>	<b>0.001</b>	<b>0.567</b>	
	P00751 <sup>a,b</sup>	Complement factor B	CFB	<0.001	0.046	<0.001	<0.001	-0.485	
	P01042 <sup>a,b</sup>	Kininogen-1	KNG1	<0.001	0.005	<0.001	<0.001	-0.891	
	P01743	Ig heavy chain V-I region HG3		0.001	0.040	0.002	0.013	-0.452	
	<b>P02511<sup>b</sup></b>	<b>Alpha-crystallin B chain</b>	<b>CRYAB</b>	<b>0.019</b>	<b>0.040</b>	<b>0.013</b>	<b>0.050</b>	<b>1.206</b>	
	P11766 <sup>b</sup>	Alcohol dehydrogenase class-3	ADH5	0.230	0.028	0.010	0.043	-0.773	
	P47755	F-actin-capping protein subunit alpha-2	CAPZA2	0.008	0.048	0.009	0.040	-0.595	
	P18827 <sup>a,b</sup>	Syndecan-1	SDC1	0.114	0.007	0.012	0.049	-1.322	
	After desiccating stress	P98160 <sup>b</sup>	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	<0.001	0.050	<0.001	<0.001	-0.281
		<b>P00558<sup>b</sup></b>	<b>Phosphoglycerate kinase 1</b>	<b>PGK1</b>	<b>0.01</b>	<b>0.046</b>	<b>0.007</b>	<b>0.035</b>	<b>0.496</b>
<b>P06744<sup>b</sup></b>		<b>Glucose-6-phosphate isomerase</b>	<b>GPI</b>	<b>&lt;0.001</b>	<b>0.039</b>	<b>&lt;0.001</b>	<b>0.002</b>	<b>0.578</b>	
P20061 <sup>b</sup>		Transcobalamin-1	TCN1	<0.001	0.049	<0.001	<0.001	-0.426	
<b>P10599<sup>b</sup></b>		<b>Thioredoxin</b>	<b>TXN</b>	<b>&lt;0.001</b>	<b>0.010</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.767</b>	
<b>Q13510</b>		<b>Acid ceramidase</b>	<b>ASAH1</b>	<b>&lt;0.001</b>	<b>0.011</b>	<b>&lt;0.001</b>	<b>0.002</b>	<b>0.767</b>	
<b>P04746<sup>b</sup></b>		<b>Pancreatic alpha-amylase</b>	<b>AMY2A</b>	<b>&lt;0.001</b>	<b>0.030</b>	<b>&lt;0.001</b>	<b>0.001</b>	<b>0.565</b>	

Positive  $\log_2$  ratio values (bold) indicate higher protein expression in FML than PA and negative values lower expression in FML.

<sup>a</sup> Proteins connected to inflammation based on UniProt accession.

<sup>b</sup> Connections to inflammation based on Ingenuity® Pathway Analysis (IPA).



**Fig. 2.** Summary of the clinical sign changes during 3-week treatment and their connections to protein baseline expressions. A: Complement C3 (C3), ig mu chain C region (IGHM) and 14-3-3 protein sigma (SFN) expression levels in the baseline (x-axis) can be used to predict the corneal and conjunctival staining change (%) (y-axis) in patients treated with 0.1% fluorometholone (FML). Treatments are fitted by separate lines and each dot represents a patient. B: Calmodulin like 5 (CALML5) expression level in the baseline (x-axis) could be used to predict corneal and conjunctival staining and conjunctival hyperemia change (%). C: The results for these four proteins for different treatments are summarized.

### 3.4. Desiccating stress effects in conjunctival staining differ based on the treatment and protein expression

The clinical signs before and after the 2 h controlled adverse environment exposure (V2-V3) were also analyzed in connection to the proteomics data and 68 statistically significant proteins were associated with conjunctival staining change (%). Significant proteins found in V1-V2 and V2-V3 for conjunctival staining change had 12 proteins in common and these included proteins related to lacrimal gland (prolactin-inducible protein (PIP), lysozyme C (LYZ), Proline-rich protein 4 (PRR4)), cystatins (cystatin-S (CST4), cystatin-SN (CST1), cystatin-B (CSTB)), as well as alcohol dehydrogenase class 4 mu/sigma chain (ADH7), 60S acidic ribosomal protein P2 (RPLP2), nucleobindin-2 (NUCB2), histone H1.4 (HIST1H1E), secreted frizzled-related protein 1 (SFRP1). Fig. 4 shows some of these results along with the severity of staining. Proteins unique to only these results included alpha-enolase (ENO1), plasma serine protease inhibitor (SERPINA5) and phospholipid transfer protein (PLTP) which had similar patterns as CSTB but are not visualised further here.

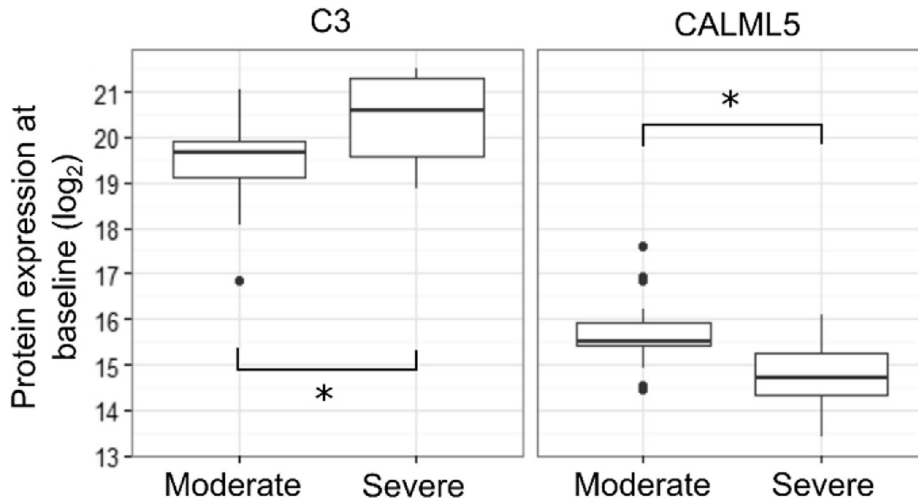
Patients receiving FML were not greatly affected by the protein expression levels prior to the desiccating stress, but patients receiving PA were (Fig. 4A). For most proteins, excluding only CSTB, lower expression levels prior to desiccating stress indicated greater conjunctival staining. DED severity and protein expression levels were again compared. At both visits, CST4 decreased with patients with severe DED in comparison to moderate DED ( $P = 0.025$  and

$P = 0.005$  respectively) in baseline ( $\log_2$  fold change =  $-1.019$ ) and after 3-week treatment ( $\log_2$  fold change =  $-1.341$ ) (Fig. 4B). In addition, CST1 ( $\log_2$  fold change =  $-1.58$ ,  $P = 0.004$ ), ADH7 ( $\log_2$  fold change =  $0.573$ ,  $P = 0.033$ ) and NUCB2 ( $\log_2$  fold change =  $-0.832$ ,  $P = 0.024$ ) were statistically significant in patients after the 3-week treatment but were omitted from the visualisation for simplicity. There were no statistically significant proteins related to the desiccating stress effect (V2-V3) in corneal staining or conjunctival hyperemia and no proteins were related to clinical changes after the desiccating stress effect and follow-up, i.e., V3-V4.

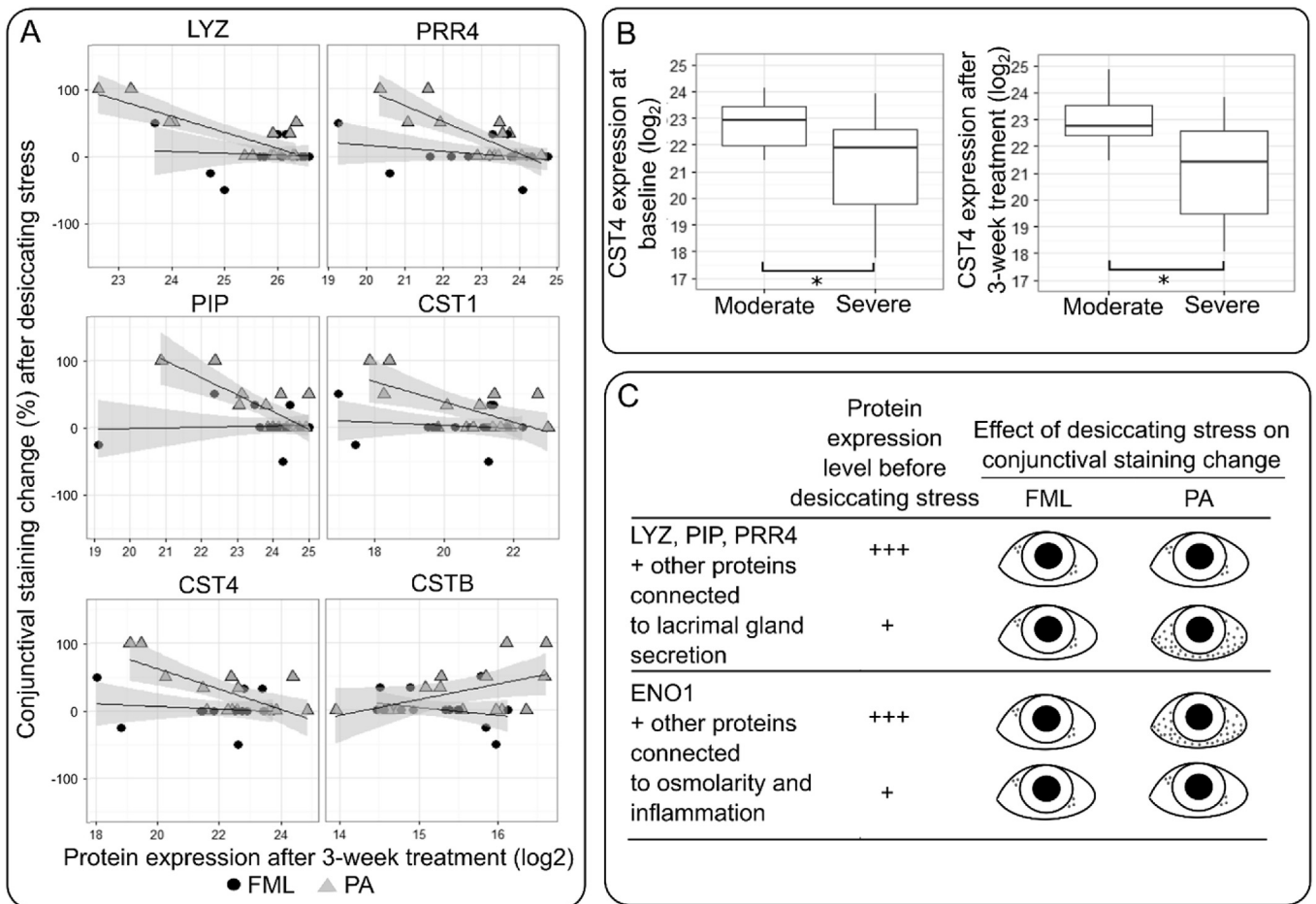
## 4. Discussion

The results of this study show that the tear proteome reflects the biological status of the ocular surface and that DED and desiccating stress change the proteome to a more inflammatory status, which is remediated by steroid treatment. Two of the proteins upregulated in FML-treated patients, ANXA5 and CRYAB, have been associated with epithelial wound healing [16] and anti-inflammatory functions via e.g. suppressing of NF- $\kappa$ B activation [17–21] while another protein also upregulated in FML-treated patients, ACTG1, has been associated with keratoconus [22]. In addition, FML treatment downregulated CFB, KNG1, ADH5 and SDC1, which are all proinflammatory proteins [23,24], acting most likely via NF- $\kappa$ B [25], as well as through TNF- $\alpha$  and IFN- $\gamma$  [26]. These differentially expressed proteins associated with FML treatment suggest that





**Fig. 3. Complement C3 (C3) and calmodulin like 5 (CALML5) baseline expression levels between different dry eye (DED) severity levels in baseline.** Upregulation of C3 and downregulation of CALML5 at baseline (y-axis) are associated with severe DED (x-axis). In the boxplots, the bottom and the top of the box are the first and third quartiles respectively and the horizontal line in the middle is the median. The vertical lines show the lowest and highest values still within 1.5 interquartile range (IQR) of the lower or upper quartile and the points represent outliers. \*P < 0.05.



**Fig. 4. Summary of the clinical sign changes after desiccating stress and their connections to protein expressions prior to stress.** A: Lysozyme C (LYZ), proline-rich protein 4 (PRR4), cystatin-S (CST4), prolactin-inducible protein (PIP), cystatin-SN (CST1) and cystatin-B (CSTB) expression levels after 3-week treatment (x-axis) can be used to predict the conjunctival staining change (%) after the desiccating stress (y-axis) in patients treated with polyvinyl alcohol (PA). Treatments are fitted by separate lines and each dot represents a patient. B: CST4 separated patients based on severity at baseline and after the 3-week treatment period. C: The results from the analysis are summarized.

proinflammatory activity is reduced in DED patients as a result of FML, possibly via NF- $\kappa$ B pathway. This is also supported by the preceding clinical study [13]. Thus, the results of these studies confirm the anti-inflammatory properties of FML, a synthetic glucocorticoid. FML is weaker than many other glucocorticoids e.g., prednisolone and dexamethasone, but, in particular with DED patients, it tends to lead to less pressure elevations than other steroids commonly used to reduce inflammation [27,28]. These identified proteins are potential biomarkers for FML-treatment and should be examined further in future.

In desiccating stress, PGK1, GPI, and TXN, were significantly upregulated in FML-treated patients. These proteins have been associated with proinflammatory functions in patients with rheumatoid arthritis [29–31], but the connections to the eye have not been extensively studied. An FML-downregulated protein, HSPG2 has been connected to the barrier functions of the eye and cell adhesion and it is potentially upregulated when IOP increases [32], while another FML-downregulated protein, TCN1, has also been shown to be downregulated in DED [33]. Based on these results, cellular stress, likely due to the desiccating stress, appears to be worse for FML-treated patients. Alternatively, the effects of the stress may occur later for PA-treated patients and the process is simply accelerated for FML-treated patients. In any case, no differences at the final 24 h post-stress visit, were observed. These desiccating stress effects with FML-treated patients do require further work, potentially with more time points, in order to better understand the occurring changes.

Pinto-Fraga et al. (2016) [13], as well as our own analysis, revealed that corneal and conjunctival staining and conjunctival hyperemia improved during the 3-week FML treatment period, and the effects of desiccating stress were reduced among moderate to severe DED patients receiving FML treatment. Integrating proteomics data with the clinical signs suggested that initial baseline protein expression levels could be used to predict the treatment effect. Four proteins with predictive relationships were highlighted in this study: CALML5, C3, IGHM and SFN.

CALML5 regulates barrier function proteins and terminal epidermal differentiation genes, and it has been reported that a knockout of CALML5 disrupts both of these biological functions [34]. In our results, this protein was downregulated in patients with a more severe DED condition, which could be explained by disruption of barrier functions and epidermal differentiation. One recently discovered interaction partner of CALML5 is SFN [34], which was also identified as a protein of interest. SFN is an epithelial cell specific, secreted 14-3-3 family protein and there is evidence of ocular surface expression in the corneal and conjunctival epithelium [35]. It has been shown that as CALML5 is depleted in differentiated keratinocytes, many SFN interactors have altered abundance, which suggests that CALML5 is in some way modulating SFN. Therefore, our results, which suggest that both of these proteins are significant to FML-treatment effect prediction, seem feasible based on previous studies.

C3 is well-known for its important role in the activation of complement system, which has been previously connected to DED [36]. Furthermore, C3 is increased in severe inflammatory diseases [37] and primary Sjögren's syndrome onset was reduced in C3-gene knockout mice [38]. IGHM, similar to C3, has been shown to be a secreted tear protein [3] connected to the immune system. Both of these proteins have a similar relationship to changes in clinical signs as SFN, suggesting that the immune system and complement cascade related proteins, such as C3 could be further used to identify which patients would benefit most from the FML treatment. In addition, C3, similar to CALML5, does separate clinically assigned severe and moderate patients from each other, and these

two proteins had a statistically significant (negative) correlation, which suggests that they could be connected to each other and changed expression of either of the two, could indicate the severity of DED of a given patient.

IPA pathway analysis showed that the four proteins associated with FML-treatment prediction were connected to proinflammatory cytokines and P38 MAPK, as well as phosphoinositide 3-kinase (PI3K) and NF- $\kappa$ B complexes, either directly or via, e.g., immunoglobulins and caspases. Proinflammatory cytokines are some of the main targets of corticosteroids, and they are known to also inhibit NF- $\kappa$ B [39,40], which is a transcription factor regulating the synthesis of several proinflammatory proteins, and there is also evidence that NF- $\kappa$ B-mediated pathways are activated in DED related chronic graft-versus-host disease [41]. In addition, topical modulation of NF- $\kappa$ B activation leads to improvement of clinical markers of DED in mice exposed to desiccating stress [42]. The effects of FML could therefore explain why these proteins, and more specifically their initial baseline expression levels, would provide us more information as to how patients will react to FML treatment.

Similarly to the treatment effects, the severity and individual protein expression levels mattered during the desiccating stress, but only for patients who were not receiving FML. Patients who continued to have high proinflammatory protein expressions while also suffering from lowered lacrimal gland production (based on LYZ, PIP and PRR4) after the 3-week treatment, were likely to experience more severe desiccating stress effects in the conjunctiva if they were not treated with FML. Hence, in the desiccating stress, the initial severity of the patient no longer matters as long as they have been treated with FML prior to the desiccating stress. From these results CST4 was most notable, since it could separate the severe and moderate patients in both baseline and after the 3-week treatment period. Hence, in addition to providing information of the severity of desiccating stress reaction, it again could tell us of the severity of the dry eye, even after corticosteroid treatment. CST4 has previously been connected to DED and meibomian gland dysfunction, where it was notably reduced in comparison to control samples [43].

These results were only applicable with conjunctival staining, while corneal staining and conjunctival hyperemia did not display a similar relationship with the proteomics. This could be due to the V3 sampling/visit time point taking place immediately after the desiccating stress, resulting in inconsistency between the clinical and proteomic data. Following the development of clinical signs and proteomics more closely, i.e., including more time points, during the 24-h window after the desiccating stress could have provided us with better understanding of the speed and timing of stress effects in the ocular surface.

Small sample amount (1  $\mu$ l/patient/time point) could be considered as a limiting factor of this study. Due to the small quantities of samples, it was not possible to carry out further validation of the results, and the reproducibility of sample recovery on 1  $\mu$ l tear samples could not be fully confirmed. Furthermore, due to limitations in proteomic sample preparation, sensitivity, and technological features of LC-MSTOF, we were unable to quantify small proteins such as cytokines as well as some of the well-known markers for dry eye, such as matrix metalloproteinase-9 (MMP9), in this study. Improved results for MSTOF could potentially be acquired by optimizing the digestion protocol to, e.g., MMP9, but this would be likely to have negative effects on other proteins of analysis. The results presented in this study should be complemented and validated by independent clinical studies using either immunoassays or targeted MS/MS approach.

The reasons why there were no statistically significant results in proteomic comparisons immediately after the desiccating stress (V3) and after 24 h recovery time (V4) can only be speculated. The

24 h may not have been a sufficient time for the effects of recovery to occur. When C57BL/6 mice were exposed to desiccating stress, tear volume and ocular surface parameters recovered within 2 weeks, while non-obese diabetic mice, which are known develop spontaneous dry eye and other related conditions, did not show signs of recovery during the study (1 month after the desiccating stress was removed) [44]. Hence, considering the individual proteomic profiles of severe-to-moderate dry eye patients and their individual initial recovery patterns, it can be expected that more uniform changes in proteomics can be seen only several days or even weeks later. Our results further highlight the value of tear fluid proteomics in DED and for the development of therapeutic options.

## 5. Conclusions

Our study identified several differentially expressed proteins between the FML and PA treatments. Our results suggest that the FML treatment reduces the inflammation more efficiently than PA treatment during the 3-week period. In addition, the clinical signs were examined together with the proteomic data, and we identified several proteins that could be considered potential biomarkers. These proteins could not only indicate the changes in clinical signs, but in some cases, could also differentiate moderate and severe DED patients. Further validation is needed to verify these biomarkers.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jtos.2017.09.003>.

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