Diurnal variation on tear stability and correlation with tear cytokine concentration

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A B S T R A C T

Purpose: To investigate the effect of time of day on tear evaporation rate (TER) and tear break-up time, and its possible relationship with the concentration of inflammatory tear molecules (cytokines) in healthy subjects.
Methods: Participants with healthy ocular surfaces attended 3 visits, including the screening visit (V0), the 2nd visit (V1) and the 3rd visit (V2). There were 7-day intervals between visits. Participants with Dry Eye Disease (DED) were excluded by using appropriate clinical tests during V0. Clinical evaluation (TER and Non-Invasive Tear Break-Up Time (NITBUT)) and tear collection were performed during V1 and V2, between 9 and 10AM and 3-4PM. The relative humidity and temperature of the examination room were also measured. The tear fluid concentrations of 15 cytokines were measured by multiplex bead analysis.
Results: Seven men and 10 women (mean age $\pm$ S.D; 25.1 $\pm$ 6.63 years old) participated in the study. There were no differences in neither the TER and NITBUT outcomes, nor humidity and temperature among times or visits. Eleven out of the 15 cytokines measured were detectable in tear fluids in > 50% of the participants. In the tear levels, no significant (p > 0.05) inter- and/or intra-day differences were detected for EGF, fractalkine, IL-1RA, IL-1β and IP-10. However, significant inter-day differences were found in the tear levels of IL-10 (p = 0.027), IFN-γ (p = 0.035) and TNF-α(p = 0.04) and intra-day differences in the tear levels of IL-8/CXCL8 (p = 0.034) and MCP-1 (p = 0.002). A significant correlation between TER and IL1-β, IL-2, and Fractalkine (p = 0.03, p = 0.03 and p = 0.046, respectively) was found at V1.
Conclusions: NITBUT and TER values had no significant variability over the course of a day (AM versus PM), or on different days in healthy participants when humidity and temperature were constant. However, some tear molecule levels did show inter- and intra-day variability, having an inconsistent and moderate correlation with TER diurnal variation.

1. Introduction

The tear film is a thin fluid layer, which covers and lubricates the ocular surface. It plays an important role in the maintenance of ocular health, comfort and the optical quality of the eye [1,2]. Tear volume is important for a healthy ocular surface, and thus, a reduced volume increases the likelihood of developing signs and symptoms of ocular dryness [2–4]. The lipid layer is an essential component for the stabilization of the tear film, as it helps to retard tear evaporation and thus maintains tear volume [5]. A higher tear evaporation rate (TER) has been

associated with increased tear film thinning, tear break-up [3,6] and tear osmolarity [7], which can also trigger symptoms of dryness and discomfort [5]. Moreover, a relationship between increased evaporation and decreased tear stability has been reported [8,9]. Intra- and inter-day variation of TER has already been examined [10]. These authors showed that after controlling humidity (whose higher values resulted in reduced TER), temperature, diurnal variation or different days had no influence on TER in healthy participants. Moreover, these authors also showed that TER measurements are most repeatable during the evening [2,10].

It has been reported that there is a positive correlation between tear

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film instability, tear hyperosmolarity and the potential activation of inflammatory mediators [11,12]. There are some molecule levels in tears that vary depending on the time of day, i.e. mid-day or evening [13,14]. For example, in the study by Benito et al [13], Epidermal Growth Factor (EGF), CX3CL1/fractalkine, CXCL10/IP-10, and Vascular Endothelial Growth Factor (VEGF) tear levels in healthy participants were found to be consistently higher in the evening, compared to the mid-day measurements. In addition, these authors also observed that the frequency of detection of some tear molecules, and their repeatability, was higher in the evening than in the mid-day period. Thus, it was concluded that tear samples should be obtained in the evening to find more reproducible inter-day levels and therefore improve accuracy and reliability of tear levels data. The concentrations of most tear molecules have been shown to be reproducible over time, having low inter-day variability [15,16]. However, some cytokines, such as interleukin (IL)-10 and IL-1β, have shown higher inter-day variability [13]. This information is important when studying tear molecule levels in common ocular surface conditions like dry eye disease (DED). It has been reported that there is inter and intra-day variation in both tear stability and cytokine levels, then there might be some relationship between both variables. It would be of great interest to clinicians and researchers to understand if, besides the effect of time of day, different values of tear stability may lead to different levels in tear molecules. This study aims to understand better if there is a correlation between clinical tests (like NITBUT and TER) and the presence of some tear molecules. Thus, the objective of this pilot study was to investigate the effect of time of day on TER and tear break-up values, and its possible relationship with tear molecule levels in healthy subjects.

2. Materials and Methods

2.1. Participants and study visits

This pilot study was approved by the Glasgow Caledonian University (GCU), School of Health and Life Sciences Ethics committee (HLS/LS/A17/059) and was conducted in accordance with the Declaration of Helsinki guidelines. Written consent was obtained from all participants after explanation of the study protocol.

The inclusion criteria were: age between 18 and 40 years old, no current contact lens use, no active ocular allergies, no use of any ophthalmic drops within the previous week of the screening visit and commencement of the study, no use of any systemic medications known to affect tear production (including antihistamines, antidepressants, diuretics and corticosteroids) within 30 days of any study visit, no previous history of ophthalmic surgery and no active ocular disease, specifically DED. This condition was defined as having ocular symptoms determined with the Ocular Surface Disease Index questionnaire (OSDI $\geq$ 13 points [17]), and at least 2 of following tests altered (in at least one eye): 1) fluorescein tear break-up time (FTBUT, of $\leq$ 7 sec; 2), Corneal Fluorescein Staining (CFS) $\geq$ grade 2, in any of the corneal areas and 3) Schirmer 1 test of $\leq$ 5 mm in 5 min.

Participants were evaluated during a screening visit (V0) for recruitment, and during two follow-up visits scheduled within a 7-day interval. During these two visits, participants were evaluated at two different time points in the day, one in the morning between 9:00 and 10:00 AM (AM moment), and another during the afternoon, between 3:00 and 4:00 PM (PM moment).

2.2. Clinical evaluation and data collection

The OSDI questionnaire [17] was first administered to measure symptoms of ocular discomfort and dryness. Then, FTBUT was measured by instillation of sodium fluorescein and using a slit-lamp microscope with a cobalt blue filter and the Wratten #12 yellow filters (https://www.kodak.de/ek/DE/de/corp/default.htm). Three measurements were taken from each eye and the mean value was calculated and

recorded. Following this, corneal integrity was examined by CFS, and using the Efron Clinical Grading Scale (0-Normal, 1-Trace, 2-Mild, 3-Moderate, 4-Severe) [18]. Lastly, the Schirmer strip was inserted into the external canthus of the eyelid margin, without topical anaesthesia [19]. The length in mm of the moistened strip was measured five minutes later.

The diagnostic tests performed in the screening visit were taken from both eyes, then one eye was randomly selected and evaluated for the rest of the study. During both visits, the relative humidity and temperature of the examination room was measured using the external room sensor of the Delfin Eye-Vapometer (Delfin Technologies Ltd, Kuopio, Finland).

The following clinical procedures were performed during the morning and afternoon measurements in both visits (V1 and V2): 1) Tear evaporation rate (TER; g/m^{2}h) assessment; 2) Non-Invasive tear break-up time (NITBUT; sec); and 3) tear sample collection. The TER assessment was measured using the Delfin Eye-Vapometer, which was placed on the test eye of the participant and TER was measured with both eyes opened and after blinking normally. Then, the NITBUT value was measured using a Keeler Tearscope® (Keeler Ltd, UK). Three measurements of the NITBUT were performed and the mean value was calculated.

For the tear sample collection, 2 µl of tears were collected from each subject at both visits. Tears were collected from the tear meniscus in the temporal canthus of the test eye, using 1 µl glass capillary micropipettes (Drummond Scientific Co., Broomall, PA, USA), and avoiding reflex tearing as much as possible. Tear samples were maintained separately, without pooling. Each sample of collected tears was diluted 1:10 in ice-cold cytokine assay buffer (Merck Millipore, UK) in a lo-bind sterile eppendorf tube (Sigma, UK). This low volume sample (1 µl) has been previously shown to be sufficient for tear molecule analysis using a low sample volume protocol [20–22]. The collected (basal) tears were centrifuged using the SciSpin Micro 24R (SciQuip, UK) at 8000 rpm for 30 s (4 °C), then the tear samples were transferred to a −80 °C freezer until analysis.

2.3. Analysis of tear cytokines

Levels of tear molecule samples were determined by a commercial multiplex bead analysis (Milliplex MAP human cytokine/chemokine magnetic bead panel, Millipore, Watford, UK). Levels of the following 15 molecules were measured: EGF, fractalkine/CX3CL1, interferon (IFN)-γ, IL-10, IL-17A, IL-1 receptor antagonist (IL-1RA), IL-1β, IL-2, IL-6, IL-8/CXCL8, interferon inducible protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, and VEGF. A low sample volume protocol was used as previously described [20–22]. Each diluted tear sample (10 µl) was incubated with antibody-coated capture beads overnight under agitation at 4 °C. After washing, the beads were further incubated with biotin labelled anti-human cytokine and chemokine antibodies, followed by streptavidin phycoerythrin incubation. Finally, the beads were washed and analysed in a Luminex IS-200 (Luminex Corp., Austin, TX, USA). Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units to cytokine concentration units (pg/ml) using BioRad analysis software. Some cytokine concentrations were detected

Table 1
Average humidity and temperature registered in the examination room during visits 1 and 2, and during the morning (AM) and afternoon (PM).

	Visit 1		Visit 2	
	AM	**PM**	**AM**	**PM**
Relative humidity (%)	43.35 ± 6.71	41.57 ± 9.72	48.8 ± 9.68	45.03 ± 7.11
Temperature (°C)	19.61 ± 1.03	20.31 ± 1.10	19.34 ± 1.44	20.64 ± 1.29

Variables are presented as mean ± standard deviation (SD).

Table 2

Outcomes of the TER and NITBUT values obtained during visits 1 and 2, and during the morning (AM) and afternoon (PM).

	Visit 1		Visit 2	
	AM	**PM**	**AM**	**PM**
TER (g/m^2/h)	73.31 ± 35.02	58.30 ± 24.09	63.11 ± 33.83	56.05 ± 25.61
NITBUT (s)	19.90 ± 11.21	13.26 ± 5.46	15.43 ± 7.02	15.52 ± 8.99

Variables are presented as mean ± standard deviation (SD). TER: tear evaporation rate; NITBUT: non-invasive tear break-up time.

Table 3

Detection rates and concentrations of tear cytokines for each visit and collection time.

		AM			PM		
Cytokine	Visit	N out of 17	%	Molecule concentration (pg/mL)	N out of 10	%	Molecule concentration (pg/mL)
IL-1 β	1	17	100	12 [7–28]	10	100	12 [6–18]
	2	17	100	11 [5–22]	10	100	12 [8–16]
IL-1RA	1	17	100	901 [116–33593]	10	100	2991 [112–19414]
	2	17	100	2041 [19–18884]	10	100	2739 [109–20681]
IL-2	1	17	100	5 [2–25]	8	80	5 [2–10]
	2	13	76.5	3 [0–17]	10	100	5 [2–11]
IL-4	1	6	35.3	NA	2	20	NA
	2	4	23.5	NA	1	10	NA
IL-6	1	9	52.9	NA	3	30	NA
	2	4	23.5	NA	2	20	NA
IL-8/CXCL8	1	17	100	96 [26–992]	10	100	77 [21–140]
	2	17	100	78 [4–390]	10	100	79 [33–253]
IL-10	1	17	100	22 [4–104]	9	90	12 [4–69]
	2	13	76.5	12 [2–69]	9	90	12 [3–54]
IP-10/	1	17	100	19753 [7040–30767]	10	100	15783 [7271–24666]
CXCL10	2	16	94.1	14804 [5345–31404]	10	100	20935 [9301–26794]
IL-17A	1	1	5.9	NA	0	0	NA
	2	0	0	NA	0	0	NA
EGF	1	17	100	828 [239–2504]	10	100	585 [270–3160]
	2	16	94.1	792 [109–3664]	10	100	1170 [85–4077]
Fractalkine/CX3CL1	1	17	100	797 [90–2330]	10	100	546 [99–1510]
	2	15	88.2	669 [124–2070]	10	100	552 [420–906]
IFN- γ	1	15	88.2	19 [1–77]	7	70	10 [1–32]
	2	16	94.1	7 [0–49]	8	80	6 [0–19]
MCP-1/	1	17	100	129 [38–895]	10	100	433 [33–2163]
CCL2	2	17	100	115 [22–899]	10	100	453 [61–3006]
TNF- α	1	14	82.4	8 [0–41]	6	60	3 [1–17]
	2	11	64.7	1 [0–20]	9	90	5 [2–14]
VEGF	1	8	47.1	NA	2	20	NA
	2	4	23.5	NA	3	30	NA

Concentration is presented as median [range: min–max]. N: number of participants in which the molecule was detected; AM/PM: morning/afternoon; CI: Confidence interval; NA: not applicable (due to a low percentage of detection).

as "Out of Range" (OOR, meaning that the value was less than the minimum detectable concentration: MinDC), or were extrapolated beyond the standard range (meaning that the values are outside the standard curve range). To avoid biased results, the statistical analysis was restricted to molecules with percentage of detection values higher than 50% (i.e., with < 50% of sample falling below the OOR).

2.4. Intrasession repeatability of tear evaporation rate

A study to assess the intrasession repeatability of the TER measurements using the Delfin Eye-Vapometer was also performed in healthy participants. The inclusion criteria were the abovementioned for the main study (section 2.1). Clinical tests for screening and study purposes were carried out during the same visit, thus non-invasive tests were performed during the screening. Consequently, to detect DED during the screening, volunteers underwent OSDI (cut-off value ≥ 13) and NITBUT (cut-off value < 10 s, mean value of 3 measures, EASYTEARview; EasyTear, Trento, Italy) tests as recommended by the TFOS DEWS II diagnostic methodology report [3].

To perform TER measurements, participants were instructed to stare a distant target while being seated on a chair. Then, three consecutive TER measurements were obtained by a single examiner with the eyes

open. The selection of the eye was performed following a computer-generated random table.

2.5. Statistical analysis

The Shapiro-Wilk test was used to test for normality of the data. Then, analysis of variance (ANOVA) for normally distributed data and the Friedman test for non-normally distributed data were performed to assess changes in the conditions of the evaluation room, or in clinical variables between moments and visits.

For the analysis of the concentrations of tear molecules, first, a Regression on Order Statistics (ROS) was used to impute the values fall below the limit of detection (LOW values); ROS method is based on a simple linear regression model using ordered detected values and distributional log normal quantiles to estimate the concentration of the low values. Molecules that were detected in<50% of the samples were not further analyzed. The R package, NADA (Non-detects and data analysis), was used for this analysis [23].

Then, to analyse the tear levels of the molecules evaluated, the allocated values and a base 2 logarithmic transformation were used. The logarithmic transformation was used to reduce or remove the skewness of the original data. To evaluate the relationship between the levels of

Table 4
Significant effects of diurnal variation or different days on the tear molecules.

Effect	Comparisons	IL-2				IL-8/CXCL8				IL-10			
		Est	Dif. \vskip5 \hfill\hbox \rot90{(I)-(II)}	95% CI dif.	Test; \vskip5 \hfill\hbox \rot90{p-value}	Est	Dif. \vskip5 \hfill\hbox \rot90{(I)-(II)}	95% CI dif.	Test; \vskip5 \hfill\hbox \rot90{p-value}	Est	Dif. \vskip5 \hfill\hbox \rot90{(I)-(II)}	95% CI dif.	Test; \vskip5 \hfill\hbox \rot90{p-value}
Visit	(I) V1	2.46	0.5	−0.024/ 1.017	t = 1.938; p = 0.06	6.27	0.1	−0.369/ 0.563	t = 0.423; p = 0.675	4.25	0.7	0.083/ 1.322	t = 2.305; **p = 0.027**
	(II) V2	1.96				6.17				3.55			
Moment	(I) AM	2	−0.41	−0.982/ 0.157	t = -1.465; p = 0.15	6.5	0.55	0.042/ 1.064	t = 2.193; **p = 0.034**	3.9	0.02	−0.659/ 0.689	t = 0.046; p = 0.964
	(II) PM	2.42				5.94				3.89			
Visit* Moment	(I) V1 AM vs (II) V2 AM	2.58 vs 1.42	1.16	1.159/ 0.312	t = 3.719; **p = 0.003**	6.73 vs 6.26	0.47	0.469/ 0.279	t = 1.678; p = 0.350	4.53 vs 3.28	1.25	1.247/ 0.371	t = 3.362; **p = 0.009**
	(I) V1 PM vs (II) V2 PM	2.33 vs 2.5	−0.17	− 0.166/ 0.406	t = -0.410; p = 0.977	5.8 vs 6.08	−0.27	−0.275/ 0.364	t = -0.755; p = 0.874	3.97 vs 3.81	0.16	0.158/ 0.484	t = 0.326; p = 0.988
	(I) V1 AM vs (II) V1 PM	2.58 vs 2.33	0.25	0.251/ 0.38	t = 0.659; p = 0.912	6.73 vs 5.8	0.93	0.925/ 0.341	t = 2.713; **p = 0.047**	4.53 vs 3.97	0.56	0.56/ 0.452	t = 1.240; p = 0.606
	(I) V2 AM vs (II) V2 PM	1.42 vs 2.5	−1.08	−1.075/ 0.38	t = -2.826; **p = 0.036**	6.26 vs 6.08	0.18	0.181/ 0.341	t = 0.531; p = 0.951	3.28 vs 3.81	−0.53	−0.529/ 0.452	t = -1.172; p = 0.648

V1/V2: visit 1 and 2; AM/PM: morning/afternoon; Est: Molecule concentration estimation (expressed as logarithmic transformation base 2); Dif: difference; CI: confidence interval. Negative values mean an increase in the molecule concentration, and positive values mean a decrease in the molecule concentration.

the tear molecules and the day (V1-V2), as well as the diurnal variation (AM-PM), and their interaction (visit & time of the day), a linear mixed model was performed [24]. To fit the model, the likelihood ratio was used. To measure the effects, the Least Squares Means and its confidence intervals and p-values were used. If there was more than one comparison, the model was fitted using the Tukey method for multiple comparisons. Residual analysis was used to check the required assumption and to assess the appropriateness of the fitted models. Models were fitted with the R package lme4 [25]. Marginal means were estimated with the R package emmeans [26]. The model was fitted for each molecule, and p-value correction for each comparison was done. The Westfall and Young method (free step-down resampling approach) [27] was used to control the probability of false positives (Family-wise Error Rate).

For the analysis of the correlation between the clinical variables and the tear molecules levels, the relative change during the day was calculated for all the variables for both visits, V1 and V2. The relative change of TER and NITBUT was calculated using the following formula:

$$Relative\ change = \frac{(X\ value\ AM\ moment - X\ value\ PM\ moment)}{X\ value\ AM\ moment}$$

The relative change of the level of cytokines was calculated using the difference of the base 2 logarithmic transformation of the concentration value between morning and afternoon (PM moment – AM moment).

The normality of the relative change was analyzed using the Shapiro-Wilk test and correlation analysis between the relative change of TER, NITBUT and cytokine level was performed using Pearson test. Correlation was classified as follows: 0.00–0.20, poor; 0.21–0.50, fair; 0.51–0.70, moderate; 0.71–0.90, very strong, and > 0.90, almost perfect correlation [28].

To estimate the intrasession repeatability of TER measurements, the within-subject coefficient of variation (CV_w) and the intraclass correlation coefficient (ICC) were calculated [29,30].

3. Results

3.1. Participants and study visits

Seventeen participants (7 men and 10 women) with a mean age of 25.1 ± 6.6 years old (range: 18–38 years) were recruited. The results of diagnostic tests performed at the screening visit were as follows: OSDI, 4.5 ± 4.7; FTBUT (OD and OS), 9.23 ± 3.29 s and 12.44 ± 3.30 s, respectively; Schirmer test (OD and OS), 27.3 ± 8.6 mm and 23.9 ± 10.4 mm, respectively. Differences (p = 0.009) in the mean age were found between men and women (30.43 ± 6.6 vs. 21.40 ± 3.4, respectively), however, there were no differences (p > 0.05) in the results of the rest of the tests performed.

The average relative humidity and temperature of the examination room during visits is presented in Table 1. There were no differences either in the humidity nor the temperature values registered among moments or visits (ANOVA, p > 0.05).

3.2. Clinical evaluation

The data of TER and NITBUT obtained on both days and during the morning and afternoon visits are presented in Table 2. There were no differences in the TER and NITBUT outcomes among moments or visits (Friedman, p = 0.06 and p = 0.11, respectively).

3.3. Analysis of tear cytokines

Out of the 15 tear molecules analysed, 11 showed a detection of > 60%. Four tear molecules, IL-17A, IL-4, IL-6 and VEGF, were not considered for further analysis due to their low detection values (<50%. Table 3).

According to the linear mixed model, no significant (p > 0.05) inter- and/or intra-day differences in the tear levels were detected for EGF, fractalkine, IL-1RA, IL-1 β and IP-10 (data not shown). On the other hand, significant inter-day (effect of the visit) differences were found in the tear levels of IL-2, IL-10, IFN- γ and TNF- α and intra-day (effect of the moment) differences in the tear levels of IL-2, IL-8/CXCL8 and MCP-1 (Table 4).

Particularly, IL-2 was 1.4 times higher at V1 than at V2, although this difference was significant only in the morning. Moreover, at V2 the IL-2 molecule level during the morning was significantly 2 times lower than the afternoon. In addition, the IL-8/CXCL8 tear level was 1.2 times higher in the morning than in the afternoon, with this difference higher

IFN- γ				MCP-1				TNF- α			
Est	Dif. \vskip5\hfill \hbox\rot90 {(I)-(II)}	95% CI dif.	Test; \vskip5\hfill \hbox\rot90{p-value}	Est	Dif. \vskip5\hfill \hbox\rot90 {(I)-(II)}	95% CI dif.	Test; \vskip5\hfill \hbox\rot90{p-value}	Est	Dif. \vskip5\hfill \hbox\rot90 {(I)-(II)}	95% CI dif.	Test; \vskip5\hfill \hbox\rot90{p-value}
3.36 2.33	1.02	0.076/ 1.978	t = 2.196; **p = 0.035**	7.73 7.79	−0.06	−0.365/ 0.24	t = -0.424; p = 0.675	2.45 1.72	0.72	0.034/ 1.413	t = 2.132; **p = 0.040**
3.05 2.64	0.41	−0.616/ 1.432	t = 0.806; p = 0.425	7.05 8.48	−1.43	−2.204/ −0.661	t = -4.2; p = **0.002**	1.86 2.32	−0.46	−1.212/ 0.291	t = -1.240; p = 0.222
3.56 vs 2.54	1.02	1.025/ 0.567	t = 1.806; p = 0.288	7.1 vs 6.99	0.11	0.109/ 0.179	t = 0.613; p = 0.927	2.69 vs 1.02	1.67	1.668/ 0.413	t = 4.037; **p = 0.001**
3.15 vs 2.13	1.02	1.022/ 0.74	t = 1.382; p = 0.529	8.36 vs 8.6	−0.23	−0.234/ 0.233	t = -1.004; p = 0.748	2.21 vs 2.43	−0.22	−0.22/ 0.539	t = -0.409; p = 0.977
3.56 vs 3.15	0.41	0.409/ 0.688	t = 0.595; p = 0.933	7.1 vs 8.36	−1.26	−1.261/ 0.371	t = -3.396; **p = 0.022**	2.69 vs 2.21	0.48	0.484/ 0.503	t = 0.961; p = 0.772
2.54 vs 2.13	0.41	0.407/ 0.688	t = 0.591; p = 0.934	6.99 vs 8.6	−1.6	−1.604/ 0.371	t = -4.320; **p = 0.004**	1.02 vs 2.43	−1.4	−1.404/ 0.503	t = -2.791; **p = 0.039**

at V1. Similarly, the concentration of IL-10 was 1.4 times higher during the morning of V1 than during the morning of V2. In addition, MCP-1 was 2.7 times higher during the morning than the afternoon, with this difference observed at both visits. The level of TNF- α was also higher in the morning of V1 than in V2, and in V2 the TNF- α tear levels were 2.6 times higher in the morning than in the afternoon.

3.4. Correlation between clinical variables and concentration of tear cytokines

There were no significant (p > 0.05) correlations between the relative changes observed in clinical variables (TER and NITBUT) and changes in the concentration of tear molecules between the AM and PM values, except for the ones found at V1 for TER and IL1- β, IL-2 and Fractalkine (Table 5).

3.5. Intrasession repeatability of tear evaporation rate

Twenty-three participants (8 men and 15 women) with a mean age of 23.4 ± 3.9 years old (range: 18–37 years) were recruited. During the screening, participants showed a mean OSDI score of 5.1 ± 4.1 and a mean NIBUT of 18.2 ± 7.3 s. The CV_w for TER measurements was 13.9% (95% CI: 11.2%; 18.3%) and the ICC value was 0.94 (95% CI: 0.88; 0.97).

4. Discussion

The time of day may have an impact on TER and tear break-up values, as well as on tear molecule levels. Increasing the knowledge in this field is important, as the variation of pro- or anti-inflammatory molecules depending on the evaporation of the tear film could influence the pro-inflammatory status of the ocular surface in some adverse environments. A better understanding of how molecules and tear stability behave and interact or how they vary throughout the day, can help to study other eye conditions, such as dry eye (specially evaporative-type). Thus, this pilot study was focused on the analysis of these interactions in healthy subjects. The results of this study showed that tear stability, as measured with NITBUT and evaporimetry, did not change significantly intra-day or inter-day in healthy participants, however, changes in some tear molecule levels were observed.

Changes in the quality of the tears or in the tear chemistry could result in a change in the stability of the tear film [31]. Tear quality and tear stability can be assessed using different clinical tests, such as the Schirmer test or FTBUT. However, both of these tests are invasive and, consequently, their application can affect the properties of the tear film. Thus, in this study, TER measurements and the NITBUT test were used to evaluate the tear stability. Evaporimetry is used to indirectly measure the TER of the aqueous component of the tear film. Several devices have been used to measure TER [10,32,33]. Recently, it has been reported

Table 5
Relationship between the relative change in clinical variables (tear evaporation and break-up) and levels of tear molecules during V1 and V2.

	Relative change		IL-1 β	IL-1RA	IL-2	IL-8/CXCL8	IL-10	IP-10	EGF	Fractalkine	IFN- γ	MCP-1	TNF- α
V1	TER	Correlation (r)	−0.70	−0.42	−0.69	−0.45	−0.63	−0.02	0.14	−0.64	−0.31	−0.44	−0.50
		p-value	**0.03**	0.23	**0.03**	0.20	0.053	0.95	0.70	**0.046**	0.39	0.20	0.15
	NITBUT	Correlation (r)	−0.15	−0.03	0.04	−0.34	0.17	−0.24	−0.24	0.20	−0.40	0.07	−0.41
		p-value	0.68	0.93	0.91	0.34	0.64	0.50	0.51	0.57	0.26	0.86	0.23
V2	TER	Correlation (r)	0.05	−0.11	−0.15	−0.18	−0.26	−0.09	−0.15	−0.05	−0.11	−0.46	0.20
		p-value	0.90	0.75	0.68	0.62	0.46	0.80	0.68	0.90	0.77	0.19	0.58
	NITBUT	Correlation (r)	−0.03	0.06	−0.003	−0.38	0.08	−0.45	−0.01	−0.28	−0.43	−0.05	−0.50
		p-value	0.93	0.87	0.99	0.28	0.83	0.20	0.97	0.43	0.22	0.88	0.14

V1/V2: visit 1/2; TER: tear evaporation rate; NITBUT: non-invasive tear break-up time.

that a non-invasive commercial and validated instrument (Eye-Vapometer) can be also be used to measure TER [10]. Thus, it was decided to use it in the present study. Moreover, when measuring the rate of evaporation of tears *in vivo*, environmental factors such as relative humidity and temperature, have important effects on the evaporation rate, so that it is better to control them in experimental settings [34]. In the present study, the relative humidity and temperature of the examination room were monitored in a normal indoor environment (i.e. relative humidity was close to 40% and temperature ranged from 19 to 22 °C), as recommended by the Workplace Health Committee [35]. In addition, no differences in humidity or temperature were found between the evaluation days of the study.

Regarding the TER, although it has been measured only with the eyes open, this study examined change with repeated measures. Thus, any adjustment was deemed unnecessary. However, the area of the exposed ocular surface and the volume under the eye google can influence the rate of evaporation [36], therefore the evaporation value relates to the total evaporation and not only the tear film evaporation. The TER outcomes in the present study showed no association with day or diurnal variations, when relative humidity and temperature were constant. The average TER values ranged between 56.05 and 73.31 g/m^2/h, which are within the normal ranges for healthy subjects [37]. These findings are in line with other studies that reported similar evaporation rate values on multiple days, with the same range of relative humidity [2,38]. However, those values are considerably less than the mean thinning rate (in free air) of 193.2 g/m^2/h reported in the study of Kimbal et al [39]. Nevertheless, this discrepancy could be due to the fact that evaporation rate measurements in these studies were performed using preocular chambers, which restrict air flow over the tear film surface, permitting a thick layer of humid air to build up, which retards evaporation. In addition, the standard deviation of the evaporation measurements was large, which may have contributed to the discrepancy with other studies. However, this variance observed in TER measurements cannot be assigned to a large measurement error from Delfin Eye-Vapometer, because the intrasession repeatability of the instrument was adequate (CV$_w$ = 13.9% and ICC = 0.94). Previous authors have assessed the repeatability of the same commercially available Eye-Vapometer and they also reported good ICC values (ICC = 0.84) [40]. However, clinicians and researchers should bear in mind that several factors, as mentioned previously, can influence the rate of evaporation [36]. Thus, the statistical analysis in this study was designed to compare the same subject throughout the visits and these factors have been constant through all the measurements. Regarding the diurnal variation, it has been reported that evaporation is lowest on waking, and rises within 2 h to a constant value for the rest of the day [41]. In contrast, Wojtowicz et al found a diurnal variation of TER between the morning (8–9 AM) and afternoon (4–5 PM) [2].

Likewise, in this study the NITBUT outcomes showed no significant inter- or intra-day changes. This finding is consistent with the results of other studies also performed in healthy subjects, where no diurnal variation was found either for FTBUT or NITBUT values [42,43]. However, other authors have found a decrease in the tear stability during the afternoon [44,45]. Nevertheless, these discrepancies could be caused by the inherent variability of the FTBUT and NITBUT variables.

Regarding the tear molecule levels, the results confirmed that EGF, fractalkine, IL-1RA, IL-1 β and IP-10 tear levels did not change either intra- or inter-day. Similar results were found in the study of Benito et al. [13], where EGF, fractalkine, IL-1RA and IP-10 tear levels showed a high inter-day reproducibility in healthy participants, both in the morning and afternoon. Conversely, the results of this study also showed that tear levels of some molecules were significantly different between days (IL-10, IFN- γ and TNF- α) and time of day (morning vs afternoon; IL-8/CXCL8 and MCP-1). It has been previously reported that there are inter-day and intra-day variations in the concentrations of IL-10 and TNF- α [13]. In contrast, IL-8/CXCL8 showed relatively constant values throughout the day [14]. Variation of IL-2, IFN- γ and MCP-1 has not

been reported in these studies because these molecules were either not detected or studied [13,14]. Besides, in this study the changes in concentration for the tear molecules assessed did not seem to simply reflect the effects of tear evaporation, as TER values did not significantly change during the day. Although participants had no signs or symptoms of dry eye according to the Schirmer test and OSDI scores, the increases found in the concentrations of some pro-inflammatory cytokines during the afternoon visit could indicate that there is a predisposition of these molecules to show raised levels towards the end of the day. As has been previously studied, diurnal variation of pro-inflammatory cytokines could be caused due to the diurnal rhythm of cortisol [46]. However, this increase of pro-inflammatory cytokines does not necessarily indicate an acute inflammation of the ocular surface, but rather, a regulation of both pro- and anti-inflammatory cytokines to control the inflammatory status [14]. Altogether, the interaction and regulation of the circadian and immune systems seems to be focused at optimizing immune responses around the clock [47].

In this study there were some molecules (IL-17A, IL-4, IL-6 and VEGF) that had a very low percentage of detection (<50% in all cases) and they were not considered for further statistical analysis, because of the unreliability of the data that could bias the outcomes. Some of these molecules also showed low detection rates in healthy subjects in previous studies, or were more related to ocular anomalies, such as DED (including severe DED related to Sjögren's syndrome), or uveitis [20,48–51]. It is possible that the low detection rate of these molecules in these samples could be related to a very low concentration in tears, below the detection level of this multiplex assay (Milliplex MAP human cytokine/chemokine magnetic bead panel).

The differences observed in the tear molecules levels in the present study in comparison with those reported in other studies, could be due to the specific hours of sample collection, since it has been distinguished two ranges of time, morning (9:00–10:00 AM) and afternoon (3:00 PM to 4:00 PM). These two time periods were chosen in this study as the optimal collection times that coincided with office working hours. In addition, the environmental conditions associated to the climate of Glasgow (more humid and northerly than other research centres) could be causing this difference.

Regarding the correlation between the relative change in clinical variables (TER and NITBUT) and tear molecule levels, a significant moderate and inverse correlation was found between IL and 1 β, IL-2 and fractalkine levels and TER variation in V1. A previous study also showed an inverse relationship between Fractalkine levels and tear production (Schirmer's test), suggesting its relationship with tear parameters [20]. However, this finding was not detected in V2; so this might be the result of the variability of the clinical tests used for assessing the tear film and the healthy status of the ocular surface of this cohort [52,53]. This lack of a consistent correlation could be also caused by the fact that relating the biochemical properties of tears, *in vitro*, to the stability of the tear film, *in vivo*, is affected by many unknown factors [31]. Moreover, the lack of differences between some of the parameters measured could be due to the fact that the difference was smaller than the experimental deviation or deviation between subjects.

There were some limitations in this study. The first one is the small sample size of the study, as well as the differences in the mean age of the women and men recruited, although all participants were young adults. The present work was a pilot study designed for exploratory purposes, therefore further studies, including larger sample sizes and other age groups, are warranted. Another limitation is that TER was measured only with the eyes open, thus it was not possible to isolate the tear evaporation from the skin one. Transepidermal water loss can be affected by environmental and intrinsic factors, however, environmental factors were monitored during the study and they were kept constant through the study. Thus, it was assumed that tear and skin evaporation was changing similarly in different days or moments. Also, due to the requirements of the research, the number of tear samples analysed were different in the morning than in the afternoon, only tear samples from 10

participants collected during the afternoon were analysed. However, the linear mixed model analysis used in this study allowed us to analyse repeated measurements made on the same participant and incorporating random effects and fixed effects. In addition, to avoid biased results due to the low detection levels of some tear molecules, a Regression on Order Statistics was performed. According to the simulation study of Lubin et al [54], the reproducibility of imputed data has limited bias when less than half of the measurements are below the limit of detection. Therefore, the statistical analysis was restricted to molecules with percentages of detection greater than 50%.

In summary, this study showed that the NITBUT and TER values had no significant variability over a day (AM versus PM), or on different days in healthy participants, when humidity and temperature were constant. However, the levels of some tear-borne molecules did show inter- and intra-day variability, though without any consistent correlation with TER diurnal variation. Finally, intrasession reliability of TER measurements using Delfin Eye-Vapometer is adequate.

5. Disclosure

The authors report no conflicts of interest and have no proprietary interest in any of the materials mentioned in this article.

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