Title: Analysis of the differences in ocular surface damage and inflammatory signs between healthy and evaporative dry eye participants.

Running short title: Comparison of MGLA and OS alterations between healthy and EDE.

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ABSTRACT

Objective: To distinguish between EDE severity levels by analysing the MGLA, conjunctival hyperemia and corneal staining in healthy and EDE participants.

Methods: 100 participants were recruited based on OSDI, TO, TFBUT, TMH, and LLP to be categorised as healthy (Group 1) or EDE (Group 2). Group 2 was divided into Group 2A (mild symptoms), 2B (moderate symptoms), and 2C (severe symptoms). In a second session, MGLA, conjunctival hyperemia, and corneal staining were measured.

Results: Positive correlation between MGLA, conjunctival hyperemia, and corneal staining were found (all $r \geq 0.221, p \leq 0.027$). Significant differences were found: MGLA between Group 1 vs. 2C and 2C vs. 2A or 2B; conjunctival hyperemia between Group 1 vs. 2A, 2B or 2C; corneal staining between Group 1 vs. 2B or 2C and 2A vs. 2B or 2C (all $p \leq 0.049$).

Conclusion: Severe EDE participants have higher MGLA, conjunctival hyperemia, and corneal staining values than healthy, mild, or moderate EDE participants.

Keywords: Evaporative dry eye, Meibography, Meibomian gland loss area, Conjunctival hyperemia.

Since the mid-90s the Dry Eye Disease (DED) has been in the spotlight for many clinicians and remains poorly understood. DED is a global issue that affects between 5 to 50% of the population, with a prevalence of 75% in studies that only involve signs and between 8.7% to 30.1% in studies that combine signs and symptoms.[1-3] The Tear Film & Ocular Surface Society in the Dry Eye Workshop II (TFOS DEWS-II)[4] has defined the DED as *"a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles".* According to the classification of the DEWS-II report, there are two main DED types: aqueous deficient dry eye (ADDE), often related to the Sjögren Syndrome, and evaporative dry eye (EDE).[4] It is important to differentiate between both DED types due to the subsequent management that will be different for each. EDE by meibomian gland dysfunction (MGD) is the most prevalent DED type,[2] being defined by TFOS as "*a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. It may result in alteration of the tear film, symptoms of the eye irritation, clinically apparent inflammation, and ocular surface disease*".[5] Many tests have been carried out and proposed to examine the ocular surface as a part of the test battery to diagnose or grade the severity of EDE,[6] such as the evaluation of the physiology of the tear film, meibomian glands (MG) anatomy or corneal and conjunctival integrity. Non-contact infra-red meibography is a non-invasive technique that provides the observer with an in-vivo image of the MG structure, roughly localized in the dimensions of the tarsal plate of both eyelids;[6] conjunctival hyperemia is a non-specific sign that usually indicates inflammation which can occur with tear film disorders, like excessive evaporation due to an altered lipid

layer produced by the MG;[6] and finally, corneal staining is an important marker of disease severity in DED patients and is observed with instillation of sodium fluorescein dye, which stains cells that have their integrity compromised.[6-8] Abnormalities in the MG function could lead to a tear film instability, causing corneal staining and conjunctival hyperemia.[6,8] Despite their habitual clinical use, it remains unclear whether one or more of these procedures can distinguish between the severity levels of either dry eye subtype.[6] To provide insight into this problem, the current work focused on EDE and aimed to investigate differences in meibomian gland loss area (MGLA), conjunctival hyperemia and corneal staining in a sample containing healthy as well as various levels of disease severity participants.

METHODS

Study and sample design

This cross-sectional study was formed by volunteers who attended the Optometry Service for an ocular surface exploration or visual examination. The present study was approved by the Bioethics Committee of the institution (approval number: USC-40/2020), the study protocol had adhered to the tenets of the Declaration of Helsinki and every participant has signed an informed consent to be included. Participants were excluded if they had a history of a conjunctival, scleral, or corneal disease, active ocular disease or ocular allergy, prior eye surgery (including refractive surgery or eyelid tattooing), glaucoma, diabetes mellitus, thyroid disorders, were pregnant or breast-feeding, wore contact lenses, had a systemic inflammatory/autoimmune disease or were following any pharmacological treatment/systemic drug that can disturb the normal function of the ocular surface.

The sample was determined whether participants presented a DED compatibility diagnostic based on DEWS-II criteria.[6,9] On a first screening session, the following

parameters were measured: OSDI questionnaire, tear osmolarity (TO), tear meniscus height (TMH), lipid layer patterns (LLP) and Tear film break-up time (TFBUT). Based on the DEWS-II diagnostic guidelines,[6,10,11] participants were categorized as dry eye if they accomplish the following criteria: OSDI score > 12, TO > 308 mOsm/L and/or a TFBUT < 10 s. To be ensured of the inclusion of only EDE and the exclusion of possible ADDE participants in the study, only those that had a LLP < Wave category in a Guillon scale and a TMH ≥ 0.2 mm were included in the study.[6,9,12] A LLP lower than Wave category indicate a thinner lipid layer usually related with EDE type.[6,12] The quantitative assessment of the tear menisci is the most direct approach to studying the tear film volume; this test was proposed as the main method to diagnose the presence of ADDE subtype in DED participants.[6,9]

A total of 208 participants were primarily screened but only 100 (mean ± SD of 43.4 ±16.5 years old) met the exhaustive inclusion and exclusion criteria. The sample was recruited into two main groups: Group 1 (Healthy) and Group 2 (EDE). Group 2 was then divided into 3 subgroups according to their OSDI score: Group 2A – EDE with mild symptoms [12, 22), Group 2B – EDE with moderate symptoms [22, 32), and Group 2C – EDE with severe symptoms [32, 100). The four groups were conformed by 25 participants each. Participants were scheduled for a second session, where study parameters were evaluated: MGLA, nasal and temporal conjunctival hyperemia and corneal staining. The sample and study design were summarized in Figure 1.

Procedures

All procedures were performed by the same observer and only the right eye of each participant was examined to avoid overstating the precision of statistical estimates.[13] To mask the data, measurements were associated with an alphanumeric code for the later randomised analysis by a second observer. All the videos recorded, both in the

screening session and the study session, were captured by a DC-4 camera attached to a Topcon SL-D2 slit-lamp. Throughout the study, laboratory conditions of temperature, light, and humidity were within the following range: temperature 20-23°C, humidity 50-60%.

The present study includes many day-to-day clinical procedures; therefore, all tests are summarised in Table 1. MGLA, conjunctival hyperemia and corneal staining procedures have been described in detail to facilitate the study reproducibility.

Topcon CA-800 meibography and meibomian gland loss area evaluation

Meibography images were captured by the Topcon® CA-800 topographer (TOPCON Corporation, Tokyo, Japan) by using an infra-red camera which allows to observe the MG in-vivo.[14-16] Participants were requested to look up, lower eyelid was everted, and several images of the total tarsus were taken.

All the meibography images were evaluated with the open-source software ImageJ to calculate the MGLA.[17] First, the images were exported from the CA-800 topographer to a computer. Then, ImageJ software was used to enhance contrast and to measure the following areas (Figure 2A). For calculating the MGLA, two areas were measured: the total tarsus area (Figure 2B) and the MG area (Figure 2C). After defining the area and clicking on *Analyse* > *Measure* in the ImageJ, the number of pixels of each area measured was provided. These values were exported to an excel sheet and the difference between the pixels of both areas was calculated. The result obtained was the MGLA expressed as a percentage. The MGLA data were categorised into 4 subgroups depending on the severity of the loss according to the Meiboscale proposed by Pult et al.:[17] MGLA 1 (<25%), MGLA 2 (25 – 50%), MGLA 3 (50 – 75%) and MGLA 4 (>75%).

Conjunctival redness

Ocular surface and conjunctival images were captured under 16x magnification with diffuse white light. Participants were instructed to look to the right and to the left for capturing images of the nasal and temporal bulbar conjunctiva. Images were categorised following the Brien Holden Vision Institute (BHVI) grading scale (4 severity subgroups): 1 (very slight hyperemia), 2 (slight hyperemia), 3 (moderate hyperemia), and 4 (severe hyperemia).[7]

Corneal staining

Corneal staining was performed by instilling non-preserved 2% sodium fluorescein dye onto inferotemporal bulbar conjunctiva. Videos of the ocular surface were captured with 16x magnification, Wratten 12 yellow filter and cobalt blue light.[18] A video of the cornea was recorded in all the gaze positions. Corneal staining images were extracted from the recorded videos and analysed following the Oxford grading scale.[19]

Statistical analysis

IBM SPSS Statistics v.23 software (SPSS Inc., IL) was used for the data analysis. A significance value of $p < 0.05$ was used for all statistical tests. The normality of the data was checked using the Kolmogorov–Smirnov test; all parameters were not normally distributed (Kolmogorov–Smirnov, all $p \leq 0.001$).[20,21]

Due to the non-continuous distribution of the data, correlations between values were assessed by performing the Spearman Rho Correlation test.[20,21] Since data showed a non-continuous distribution, differences in the value among groups were obtained by a Kruskal-Wallis test, while differences by pairs were assessed using the Mann-Whitney U test.[20]

RESULTS

Descriptive statistics of the studied parameters expressed as median (IQR) for the whole sample were the following values: the MGLA was 2 (2-3) (range between 1 to 4), the

nasal and temporal conjunctival hyperemia was 2 (2-2) and 2 (2-3) (range between 1 to 4) respectively, and 1(1-2) (range between 0 to 4) for the corneal staining values. The frequency distribution of the data is shown in Figure 3.

Correlations between MGLA, conjunctival hyperemia and corneal staining

Spearman Rho test showed a positive correlation between the MGLA and the nasal conjunctival hyperemia ($r = 0.368$; $p < 0.001$), temporal conjunctival hyperemia ($r = 0.221$; $p = 0.027$), and corneal staining ($r = 0.276$; $p = 0.005$). No correlations between the corneal staining and the nasal or temporal conjunctival hyperemia were found ($p \geq 0.070$).

Differences in MGLA, conjunctival hyperemia and corneal staining between groups

Descriptive statistics according to the groups established on the sample design are shown in Table 2. Participant distribution of the studied parameters expressed as a percentage for the whole sample was the following: for the MGLA parameter 12% in Garde 1, 51% in Grade 2, 32% in Grade 3, and 5% in Grade 4; for the nasal conjunctival hyperemia parameter 5% in Garde 1, 48% in Grade 2, 37% in Grade 3, and 10% in Grade 4; for the temporal conjunctival hyperemia parameter 1% in Garde 1, 57% in Grade 2, 32% in Grade 3, and 10% in Grade 4; for the corneal staining parameter 31% in Garde 0, 44 % in Grade 1, 16% in Grade 2, 8 % in Grade 3 and 1 % in Grade 4.

Age and Gender differences among groups

Significant differences in both age and gender among groups were obtained (both, $p \leq 0.038$). Paired analysis showed significant differences in age category when Group 1 was compared versus Group 2B or Group 2C (both, $p \leq 0.011$). Regarding gender, significant differences were found between Group 1 versus Group 2C ($p = 0.025$) and between Grupo 2A versus 2C ($p = 0.024$).

MGLA differences among groups

Significant differences in MGLA values among groups were obtained ($p = 0.007$). Paired analysis showed significant differences in the MGLA category when Group 1 was compared versus Group 2C ($p = 0.001$); also, significant differences were obtained when Group 2C was confronted with Group 2A ($p = 0.028$), or Group 2B ($p = 0.049$). No significant differences in MGLA were found when Group 1 was compared to Group 2A or Group 2B (both $p \geq 0.073$).

Conjunctival hyperemia differences among groups

Significant differences in conjunctival hyperemia both, nasal ($p = 0.047$) and temporal ($p = 0.024$), among groups were found. Nevertheless, the paired analysis only showed statistically significant differences in conjunctival hyperemia for both areas (nasal and temporal) when comparing Group 1 versus Group 2A, Group 2B and Group 2C (all $p \leq 0.044$). No statistically significant differences were found when the pairing analysis was performed between Group 2A with Group 2B and Group 2C, or Group 2B versus Group 2C (all $p \geq 0.077$).

Corneal staining differences among groups

Significant differences in corneal staining values among groups were found ($p = 0.047$). However, pairwise comparison only showed significant differences when Group 1 was confronted with Group 2B or Group 2C (both $p \leq 0.013$). Also, significant differences were found when comparing Group 2A versus Group 2B and Group 2C (both $p \leq 0.041$). No significant differences were found when Group 1 was compared to Group 2A, and between Group 2B versus Group 2C (both $p \geq 0.339$).

DISCUSSION

Technology improvements made on the MG observation techniques have boosted investigations in the DED field. Many researchers have theorised about symptoms and

signs and their relationship with different ocular surface assessment tests. Nowadays, there is no consensus on whether comparing participants severity status grouped according to their DED symptomatology severity shows different values in MGLA, conjunctival hyperemia or corneal staining.[22,23]

To the author's knowledge, there are not many publications about how ocular surface parameters vary in healthy participants or participants with EDE. Age and gender are parameters that could influence ocular surface aspects so their implication must be considered. The present study found differences in age between the studied groups (Table 2). These findings corroborate the hypothesis that age is associated with the severity of the disease, as Pult et al.[24] have postulated. Regarding gender, the present study observed more women than men in Group 2C (EDE severe symptomatology group), similar to Borrelli et al.[25] findings where women reported highly dry eye symptomatology than men.

Recent research performed by Crespo-Treviño et al.[26] showed similar findings that the observed in the present study, where EDE participants showed more MG morphological alterations than healthy ones. In the present study, a special emphasis was made to differentiate ADDE from EDE. Measurement of the LLP and TMH was considered as inclusion criteria according to DEWS-II, due to a LLP lower than Wave category could be potentially originated by MG alterations (therefore, EDE type) or a lower and irregular meniscus potentially could be considered indicative of tear film aqueous deficiency.[6,9,12]

In tear film physiology there can be many triggers related to initiate symptomatology compatible with DED. It is important to find out whether the MG loss could be one of those triggers, or simply a factor that increases symptomatology severity. There is some controversy on how MG alterations contribute to increasing ocular surface

symptomatology. Pult et al.[24] found a positive correlation between symptomatology and MGLA values. The relationship between the alteration of the MG and ocular discomfort was set earlier to the meibography implementation and corroborated by investigators years later.[24,27,28] Nevertheless, researchers found no relationship between symptomatology and MG alterations.[29-32] There is something in common between these publications, all of them request a further investigation to clarify how this relationship works.[24,27-32] The present study found that healthy participants had less MG loss than EDE participants with severe DED symptomatology. The presence of symptoms does not follow a linear distribution with signs of DED.[6] Nonetheless, in the case of the EDE subtype, severe DED symptomatology is associated with higher MG loss. It is known that the destruction of the MG habitually happens by the obstruction of the MG orifices and the impossibility to excrete the meibum. MGLA has been proposed as one of the main tests for MGD diagnosis, which is the principal cause of the EDE subtype.[5] With the present findings, it could be hypothesised that MG loss is not a trigger that initiates the DED symptomatology, but it plays an important role in the enhancement of it. MG loss must be considered in the daily clinic for EDE management.

At the time of assessing the multiple signs that can be potential triggers of DED symptomatology, many procedures can be performed.[6] Conjunctival redness is a non-specific sign that usually indicates inflammation of the ocular surface, not a sign of specific damage as it occurs with corneal staining.[8] Conjunctival hyperemia can be a consequence of an unstable tear film that could be originated by an inefficient MG secretion or deficient tear film production. To the author's knowledge, there are no investigations that have assessed whether differences in conjunctival hyperemia exist between healthy and EDE participants grouped by their symptomatology severity. The present study found that EDE participants presented higher values of conjunctival

redness than healthy participants, so conjunctival hyperemia could play a possible role in EDE cases. Further investigation is needed to clarify the relationship between the DED symptomatology severity and conjunctival redness in EDE participants.

The principal parameter that indicates ocular surface damage is the corneal staining, being considered as an important parameter to consider in the DED diagnosis.[6] Many researchers have tried to prove if a relationship between DED symptomatology severity and corneal staining in MGD participants exists: some studies have found weak positive correlations between symptomatology severity and corneal staining were obtained,[27,28,31,33] while other authors' showed that there is no relationship between both parameters.[29,34,35] In a recent research, Llorens-Quintana et al.[35] studied how MG irregularity could be associated with dry eye symptomatology and various ocular surface parameters like corneal staining. They found an inverse correlation between MG irregularity and dry eye symptomatology, but they observed that MG irregularity only could be studied in patients with less than 32% of MG loss because shortened glands are rarely tortuous. Also, they have found no relationship between corneal staining and MG irregularity or MG loss. Xiao et al.[33] studied a sample of the two main delivery MGD categories (high-delivery and low-delivery) segregated into the four MGD states (Hypersecretory MGD, undefined MGD, hyposecretory MGD, and obstructive MGD); they found that low-delivery groups (Hyposecretory and obstructive MGD) showed worse dry eye symptoms and ocular surface staining scores than the high-delivery MGD groups. The present study found that healthy and EDE participants with lower values of symptomatology showed lower values of corneal staining. However corneal staining could be originated from an unstable tear film due to the poor quality of the MG secretion.[22,36]

The principal study limitation of the present study was that only the MGLA was measured and no other MG parameters (MG expressibility, free eyelid border and MG orifices alteration) could more likely be indicatives of being the cause of higher symptomatology values.[37] Anyway, not a consistent conclusion could be formed about how the MG function is altered by only analysing its anatomy. Nevertheless, the MGLA measurement is an easier and faster procedure that supplies useful information about the MG status than analysing other MG parameters in dry eye routine assessments. In fact, understanding how MGLA influences or is related to other ocular surface parameters could clarify and simplify the EDE subtype diagnosis. Further investigation with a complete battery of tests focused on all MG aspects will be interesting, orientated to find the MG parameter that originates the DED symptomatology in EDE participants.

In conclusion, the present study shows that EDE participants with severe symptomatology have higher values of MGLA, conjunctival hyperemia, and corneal staining than healthy participants or EDE participants with mild or moderate symptomatology.

DECLARATION OF INTEREST

The authors report there are no competing interests to declare.

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411 Table 1 – Test procedures, method and categorization performed. MGLA: Meibomian

412 gland loss area; OSDI: Ocular surface disease index; TFBUT: Tear film break-up time;

413 BHVI: Brien Holden vision institute. ADDE: Aqueous deficient dry eye.

Procedures performed on the screening session			
Parameter	**Material**	**Method**	**Categorization**
Dry eye symptomatology	OSDI questionnaire	Self-administered by an informatized OSDI questionnaire.	Categorized into 4 groups: [11,38] • Normal [0, 12) • Mild [12, 22) • Moderate [22, 32) • Severe [32, 100)
Osmolarity	TearLab (TearLab, San Diego, CA, USA)	Following manufacturer's protocol.	Values > 308 mOsm/l use to be related to an altered ocular surface.[6,39]
Tear film break-up time	SL-D2 and DC-4 camera (TOPCON Corporation, Tokyo Japan)	Fluorescein instillation onto inferotemporal bulbar conjunctiva. Performed under slit-lamp with 16x magnification, Wratten 12 yellow filter and cobalt blue light during the TBUT video recording.[18,40] Videos were analysed by counting frames with the VirtualDub software; these frames were converted into seconds (15 frames per second).[41]	Values < 10 s use to be related to an altered ocular surface.
Lipid layer pattern	Tearscope (Keeler, Windsor, UK) SL-D2 and DC-4 camera (TOPCON Corporation, Tokyo Japan)	Instrument attached to a slit-lamp with the video recording system.	Cut-off value < Wave to exclude possible ADDE participants. Categorized following the Guillon categories:[12,42] • Open Meshwork • Close Meshwork • Wave • Amorphous Colour Fringe
Tear meniscus height	Tearscope (Keeler,	Instrument attached to a slit-lamp with the video recording system	Cut-off value ≥ 0.2 mm to exclude possible ADDE participants.

	Windsor, UK) SL-D2 and DC-4 camera (TOPCON Corporation, Tokyo Japan)	Measured the lower meniscus height with ImageJ software.[9]	
Procedures performed on the study session			
Parameter	**Material**	**Method**	**Categorization**
Meibography	Topcon CA-800 topographer (TOPCON Corporation, Tokyo Japan)	The lower eyelid was everted, and several images of the total tarsus were taken. Images were evaluated with ImageJ software, and two areas were measured: the total tarsus area and the meibomian gland area. The difference between both areas (expressed as a percentage) is the MGLA.	Categorized following the Pult et al.[17] Meiboscale: • MGLA 1 (<25%) • MGLA 2 (25-50%) • MGLA 3 (50-75%) • MGLA 4 (>75%)
Conjunctival redness	SL-D2 and DC-4 camera (TOPCON Corporation, Tokyo Japan)	Nasal and temporal conjunctiva images were captured under 16x magnification with diffuse white light.	Categorized following the BHVI grading scale:[7] • 1 (very slight hyperemia) • 2 (slight hyperemia) • 3 (moderate hyperemia) • 4 (severe hyperemia)
Corneal staining	SL-D2 and DC-4 camera (TOPCON Corporation, Tokyo Japan)	Fluorescein instillation onto inferotemporal bulbar conjunctiva. Performed under slit-lamp with 16x magnification, Wratten 12 yellow filter and cobalt blue light. A video of the cornea was recorded in all the gaze positions.	Graded according to the Oxford staining score.[19] • 0 (Absent) • 1 (Minimal) • 2 (Mild) • 3 (Moderate) • 4 (Severe)

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Table 2 – Descriptive statistics and analysis of differences (Kruskal-Wallis test) between age, gender and the studied parameter divided by group severity categories. Data are expressed as mean ± SD (Range) (parametric variables) or median (IQR) (non-parametric variables). OSDI: Ocular surface disease index; MGLA: Meibomian gland loss area; TFBUT: Tear film break-up time; IQR: Interquartile range; BHVI: Brien Holden vision institute; SD: Standard deviation.

Study Group		**Age (Years)**	**Gender (Male 1 - Female 2)**	**Studied parameters**			
Number	**Characteristics**			**MGLA (Meiboscale)**	**Nasal Conjunctival Hyperemia (BHVI)**	**Temporal Conjunctival Hyperemia (BHVI)**	**Corneal Staining (Oxford Grade)**
Group 1	OSDI 1 Normal (0 – 12), + Osmolarity < 308 mOsm/l and TFBUT ≥ 10 s	36.6 ± 17.3 (20 – 63)	2 (1-2)	2 (1-2)	2 (2-3)	2 (2-2.5)	0 (0-1)
Group 2A	OSDI 2 Mild (13 – 22) + Osmolarity ≥ 308 mOsm/l and/or TFBUT < 10 s	45.0 ± 15.7 (21 – 68)	2 (1-2)	2 (2-2)	3 (2-3)	3 (2-3)	0 (0-1)
Group 2B	OSDI 3 Moderate (23 – 32) + Osmolarity ≥ 308 mOsm/l and/or TFBUT < 10 s	47.7 ± 16.6 (20 – 70)	2 (1-2)	2 (2-2)	3 (2-3)	3 (2-3)	1 (1-2)
Group 2C	OSDI 4 Severe (> 33) + Osmolarity ≥ 308 mOsm/l and/or TFBUT < 10 s	49.8 ± 14.9 (20 – 70)	2 (2-2)	3 (2-3)	3 (2-3)	2 (2-3)	1 (1-2)
Analysis of differences		p = 0.038	p = 0.035	p = 0.007	p = 0.047	p = 0.024	p = 0.010

Figure 1 – Summary of the study design, sample size and clinical procedure.

Figure 2 – The procedure of the contrast enhancement on tarsus images, measurement of the total tarsus area and measurement of meibomian glands area for the MGLA calculation. A: Inferior tarsus image before contrast enhancement; B: Total tarsus area after contrast enhancement. C: Meibomian glands area after contrast enhancement.

Figure 3 – Frequency distribution of the data. The p value reported in the figure is from Kruskal-Wallis analysis. A: MGLA (Meiboscale); B: Nasal Hyperemia (BHVI); C: Temporal Hyperemia (BHVI); D: Corneal Staining (Oxford grade). *$p < 0.05$ versus Group 1 (Mann-Whitney U test), **$p < 0.05$ versus Grupo 2A 1 (Mann-Whitney U test), ***$p < 0.05$ versus Group 2B 1 (Mann-Whitney U test).