InflammAging at Ocular Surface: Clinical and Biomolecular Analyses in Healthy Volunteers

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PURPOSE. To assess the ocular surface in volunteers who consider themselves as healthy, in order to evaluate how para-inflammatory mechanisms fail with age, and thus investigate the phenomenon of "InflammAging."

METHODS. In this observational prospective cohort study, volunteers were categorized into three groups according to age: young (19–40 years), middle-aged (41–60 years), and older adults (61–93 years). Clinical assessments included tear breakup time (T-BUT) and Schirmer test type I. Dry eye symptoms were evaluated by the Ocular Surface Disease Index (OSDI) questionnaire. Conjunctival mRNA and protein expression of intercellular adhesion molecule-1 (ICAM-1), MUC5AC, and IL-8 were measured by real-time PCR and immunofluorescence.

RESULTS. A total of 82 volunteers (38 males and 44 females) were enrolled. T-BUT decreased significantly with increasing age (young: 11.13 ± 0.18 seconds; middle-aged: 10.83 ± 0.56 seconds; older: 9.00 ± 1.00 seconds, $P < 0.05$). Schirmer test values decreased significantly with age (young: 20.6 ± 1.0 mm; middle-aged: 19.2 ± 1.2 mm; older: 16.0 ± 1.1 mm, $P < 0.05$). OSDI scores increased with age in both groups, but they were substantially higher in women. Conjunctival expression of inflammatory markers ICAM-1, IL-8, and MUC5AC increased with age.

CONCLUSIONS. Clinical signs, symptoms, and biomarkers of chronic inflammation increased with age in a cohort of volunteers who considered themselves healthy, indicating an age-related progressive impairment of ocular surface system function.

Keywords: health volunteers, aging, ocular surface, dry eye, para-inflammation

The ocular surface is a complex morpho-functional unit in which multiple anatomical and cellular components (cornea, conjunctivae, lacrimal glands, Meibomian glands, tear film, immune cells, and nervous fibers) cooperate to preserve its homeostasis.[1] This equilibrium promotes the repair of the ocular surface following daily repetitive insults.[2,3] In other organs (such as the cardiovascular, endocrine, and nervous systems), the self-limiting innate immune mechanisms that maintain and restore homeostasis have been termed "para-inflammation."[2] According to this paradigm, para-inflammation is a physiologic process that permits tissues to adapt to injuries and that restores their normal functions.[2] However, if dysregulated, para-inflammation may trigger overt inflammation, resulting in the clinical signs observed in several chronic disorders, such as type 2 diabetes, neurodegenerative and cardiovascular diseases.[4] There is evidence that cumulative age-associated low-grade oxidative insults associated to dysregulation of para-inflammation contribute to age-related macular degeneration.[5] Although an association between aging and ocular surface damage has been presumed, there is no agreement on what constitutes "healthy" aging of ocular surface system. We hypothesize that a persistent dysregulation of para-inflammation: (1) occurs with aging in the ocular surface, (2) compromises homeostasis, and (3) fosters an often asymptomatic inflammatory condition "InflammAging."

InflammAging is a chronic, subclinical form of dysregulated para-inflammation that arises with aging.[6-12] In this prospective cohort study, we assessed clinical and biomolecular changes of the ocular surface in volunteers of different ages who consider themselves healthy, thus investigating InflammAging at ocular surface.

MATERIALS AND METHODS

Eighty-two consecutive volunteers (38 males and 44 females) were enrolled for our open observational prospective cohort study between December 2014 and June 2015. Volunteers were categorized into three groups according to age: 35 subjects in the young group (19–40 years), 23 subjects in the middle-aged group (41–60 years), and 24 subjects in the older group (61–93 years) (Table 1).[13] Eligible subjects were at least 18 years of age and described themselves as "healthy." Exclusion criteria included history and/or signs/symptoms of ocular surface disease as well as contact lens use. Further exclusion criteria included systemic conditions (including cardiovascular, metabolic, neoplastic, and psychiatric diseases) and pregnancy. In addition, all patients using any topical and/or systemic medications within the 3-month period preceding the examination were excluded.

TABLE 1. Study Population

Groups	Subjects	Age Range	Mean Age	Sex, F/M
Population	82	19–93	50.00 ± 18	44/38
Young	35	19–40	27.60 ± 6.39	21/14
Middle-aged	23	41–60	50.17 ± 5.49	15/8
Old	24	61–93	62.62 ± 3.70	4/4

All experimental procedures were performed in accordance with guidelines established by the ARVO and adhered to the tenets of the Declaration of Helsinki concerning human subjects. The procedures performed were reviewed and approved by the Intramural Ethical Committee (University Campus Bio-Medico, Rome, Italy). All participants provided written informed consent to proceed for clinical and laboratory analysis.

Clinical Assessment

The clinical examination included an assessment of best-corrected visual acuity, slit-lamp biomicroscopy, tear breakup time (T-BUT), and Schirmer test type I. Schirmer test was performed without topical anesthesia by placing a narrow filter paper strip (5 × 35 mm strip of Whatman #41 filter paper) in the inferior cul-de-sac. Standard T-BUT measurement was performed by instilling a fluorescein drop into the inferior fornix.[14] The time lapse between the last blink and the appearance of the first randomly distributed dark discontinuity in the fluorescein-stained tear film was measured three times, and the mean value of the measurements was calculated. We also evaluated corneal fluorescein staining in all enrolled subjects, although we did not perform any analysis of these data in light of previous studies reporting that approximately 20% of "healthy subjects" show positive staining.[15,16] Moreover, our patients were excluded if a diagnosis of ocular surface disease had previously been given. The Ocular Surface Disease Index (OSDI) questionnaire was also administered. OSDI is a 12-item validated questionnaire designed to provide a rapid assessment of symptoms related to ocular surface irritation and their impact on vision-related functioning in patients with ocular surface disease (13–22 mild, 23–32 moderate, and >33 severe).[13]

Immunofluorescence, Epifluorescence Acquisition and Integrated Density Analysis

Two imprints (impression cytology; ICs) from each bulbar conjunctiva, specifically the inferior-nasal and temporal conjunctiva, were sampled (0.22-μm membranes; Millipore, Milan, Italy). ICs were quickly fixed by using spray citofix (Bio-Fix spray; Bio-Optica, Milan, Italy), according to a standardized procedure.[17] Cytofixed conjunctival ICs were probed with primary antibodies specific for mouse anti-human MUC5AC (M7316, Clone CLH2, 4 μg/mL; Dako, Kidlington, UK) and mouse anti-human intercellular adhesion molecule-1 (ICAM-1, F7143, Clone 6.5B5, 4 μg/mL) antibodies. Specific binding of the first antibody was detected by using cy2 or cy3-conjugated species specific antibodies (1:300–500; Jackson ImmunoResearch, West Grove, PA, USA). Nuclear staining was performed with 4′,6-Diamidino-2-phenylindole (DAPI) (5 μg/mL; Molecular Probe, Eugene, OR, USA) in PBS supplemented with RNAse (20 μg/mL), to reduce nonspecific signal due to cytoplasm/nucleoli RNA background and improve the quality of nuclear acquisition, according to a standard protocol.[18] Images were acquired using direct Ni Eclipse microscope equipped with the DS-Ri1 digital camera and the NIS Elements Imaging Software

(×20/0.50 plan fluor, ×40/0.75 plan fluor, and ×60/1.40 plan Apo; Nikon, Tokyo, Japan). Isotype-matched IgG antibodies (Vector Laboratories Ltd., Burlingame, CA, USA) were incubated in parallel and used as internal controls.

RNA Isolation, cDNA Synthesis, and Relative Real-Time PCR Amplification

Specimens for each group were processed for RNA isolation by 1:1 extraction in Trifast reagent (EuroClone, Milan, Italy). RNAs were subjected for cDNA synthesis and specific amplification. The reverse transcription (cDNA) was carried out in a programmable thermal cycler (LifePRO/BIOER; EuroClone) according to the IMPROM protocol (Promega, Madison, WI, USA).

Specific amplifications with human primer pairs were carried out for: MUC5AC (NM_001304359; For: tcc acc ata tac cgc cac aga; 215 bp); ICAM-1 (J03132; For: atg agt gcc cag gga ata tg; 107 bp); IL-8 (BC013615; For: aag ctg gac tcg gtc ttt ga; 142 bp); H3 (NM_005324; For: gtc tgc agg ctg gca tag aag; 113 bp). Primers (possibly one intron-spanning) were designed according to a standard procedure by using the Primer3 software (freely available) and produced by MWG Biotech (10 pM; Biotech, Ebersberg, Germany). Real-time PCR amplifications were carried out in 48-well microplates (Eco Illumina, San Diego, CA, USA). Samples were run in triplicate and single cycle threshold (Ct) values were produced at the end of amplification. Ct averages were calculated from replicates and expressed as real cycle threshold ($\Delta Ct = \Delta Ct_{target} - \Delta Ct_{reference}$) and reported as normalized ΔCts ± standard deviation (SD) in the text. To note, Ct values are inversely proportional to transcript expression. Furthermore, Ct values were analyzed by the Relative Expression Software Tool (REST) 384–2006,[19] in order to ascertain the increase/decrease difference in target gene expression, with respect to reference genes (H3) and compared to control group, herein represented by the young. Fold changes, according to the $2^{-(\Delta\Delta Ct)}$ formula where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$ in log2-scale, were employed in the preparation of bar graphs (Supplementary Fig. S2).[20,21] Finally, PCR products were confirmed for their specific amplification by the single melting curves obtained during amplification.

Statistical Analyses

The differences between mean values between groups were assessed with 1-way ANOVA nonparametric tests, while the differences in mean values between the groups were assessed with paired sample t-tests or ANOVA followed by a Tukey-Kramer post hoc analysis.[15] In subgroup analysis among sex, <10% age variability among subgroups was considered. $P \leq 0.05$ was selected as the limit of statistical significance. Data values are expressed as the mean ± SD (in the text) and mean ± SEM (in the graphics). The statistical package used was StatView II for PC (Abacus Concepts, Inc., Berkley, CA, USA). We anticipated that power of 80% would be sufficient to detect statistically significant differences at an α level of 0.05 with a large standardized difference between the three groups (balanced 1-way analysis of variance power calculation: $k = 3, f = 0.4$, significance level = 0.05 , power = 0.8): $k = 3; n = 21.10364, f = 0.4$, significance level = 0.05, power = 0.8.[22]

RESULTS

Age-Associated Changes to Tear Film Stability, Tear Production, and OSDI Scores

In a population of asymptomatic volunteers who consider themselves "healthy" tear film stability, as assessed by T-BUT,
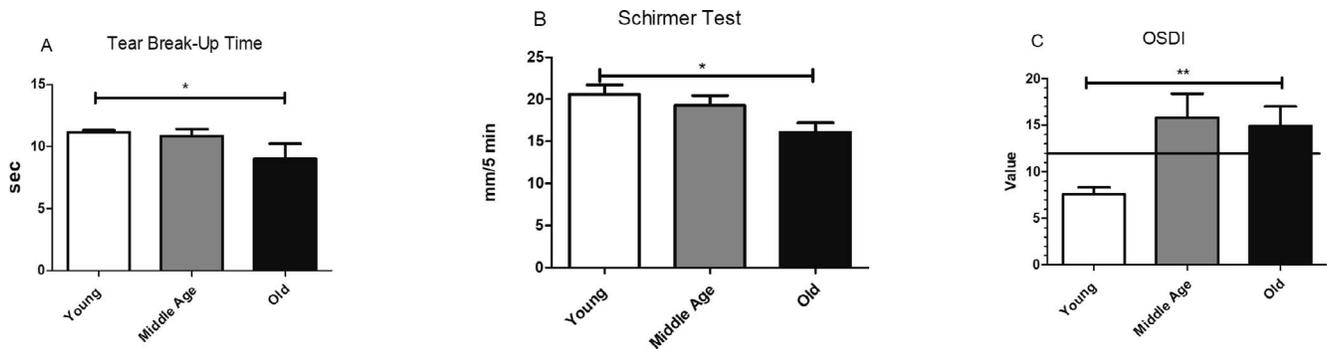
FIGURE 1. Clinical assessments of ocular surface system function. From left to right: T-BUT (**A**), Schirmer test (**B**), and OSDI (**C**) analysis as categorized. A tendency for decreased T-BUT and a significant decrease for Schirmer test were observed in old groups with respect to both middle age and young ones. Increased OSDI scores were recorded for old and middle-aged groups, with respect to the young. ANOVA analysis followed by Tukey-Kramer post hoc: *P* values are reported in the panels. Data are mean values ± SD. Study population is represented by volunteers considering themselves as healthy subjects. In panel **C**, the *black line* defines the normal OSDI limit at score 13.[13]

decreased significantly with increased age (young [19–40 years]: 11.13 ± 0.18 seconds; middle-aged [41–60 years]: 10.83 ± 0.56 seconds; older [61–93 years]: 9.00 ± 1.00 seconds; $P < 0.05$; Fig. 1A). Notably, T-BUT reached borderline unstable values (i.e., <10 seconds)[16] in the older group. Tear production was also observed to decrease with age, as determined by the Schirmer test (young [19–40 years]: 20.6 ± 1.0 mm; middle-aged [41–60 years]: 19.2 ± 1.2 mm; older [61–93 years]: 16.0 ± 1.1 mm, $P < 0.05$; Fig. 1B). Analysis of OSDI data demonstrated elevated scores in the middle-aged and older groups relative to the young, consistent with a mild form of ocular surface disease[16] (young [19–40 years]: 7.60 ± 0.76; middle-aged [41–60 years]: 16.00 ± 2.60; older [61–93 years]: 15.00 ± 2.10; $P < 0.01$, Fig. 1C).

In age-matched sex subgroup analyses (male: young [26.2 ± 4.1 years], middle-aged [50.4 ± 6 years], old [68.8 ± 6 years]; female: young [28 ± 7 years], middle-aged [51 ± 5.8 years], old [82.6 ± 8.6 years]), similar decreases in T-BUT (Figs. 2A, 2B) and Schirmer values (Figs. 2C, 2D) with increased age were observed. In the female study population, significantly elevated OSDI scores were demonstrated in the middle-aged (41–60 years) group relative to the young (19–40 years) group, yet in the male population, OSDI scores between the young (19–40 years) and middle-aged (41–60 years) were similar (Table 2). No significant change in T-BUT values was observed in the male compared to the female overall populations (Fig. 3A). However, both male and female T-BUT values demonstrated a tendency to decrease with increased age, and only in the female group did the difference between young and old reach statistical significance. Females reported significantly higher OSDI scores compared to males (Fig. 3B; Table 2).

Immunofluorescence and Molecular Analyses

Immunofluorescence analysis was used for investigating ICAM-1 and MUC5AC immunoreactivity in conjunctival imprints. As shown in Figure 4, ICAM-1 positive cells were increased in middle-aged (41–60 years) and older (61–93 years) imprints, as compared to young (19–40 years). A trend to an increase of MUC5AC positivity was observed in older (61–93 years) with respect to young (19–40 years) and middle-aged (41–60 years) groups (Fig. 5). Both figures show the merged staining (Fig. 5, left panels) and the single ICAM-1 or MUC5AC (Fig. 5, green, middle) specific immunofluorescence (channels) over a DAPI (Fig. 5, blue, right) channel. The nuclear monolayer distribution (DAPI) is clearly visible. Molecular analyses conducted on conjunctival imprints demonstrated increased MUC5AC expression in the older group (61–93 years) compared to the young (19–40 years). IL-8 transcript was significantly increased in middle age conjunctival imprints as compared to young ones (10.86-fold change as 2log [$2-\Delta\Delta Ct$]; nCt 10.09 ± 0.95 middle age versus nCt 20.95 ± 2.91 young; $P < 0.001$, 1-way ANOVA [Tukey-Kramer post hoc]-REST coupled analysis), while a slight increase was detected in old imprints as compared to young ones (1.92-fold change as 2log [$2-\Delta\Delta Ct$]; Ct 19.77 ± 0.15 old versus 20.95 ± 2.91 young; $P < 0.001$, 1-way ANOVA [Tukey-Kramer post hoc]-REST coupled analysis) (Fig. 6).

DISCUSSION

Our data demonstrate clinical and biomolecular changes of ocular surface in asymptomatic volunteers who considered themselves healthy. With increased age: (1) tear film stability decreases, (2) tear production decreases, (3) OSDI scores increase (with higher scores in middle-aged women), and (4) inflammatory markers rose (i.e., ICAM-1, MUC5AC, and IL-8). ICAM-1 has been reported as an overall mediator of inflammation, MUC5AC is a marker of reduced goblet cell density, and IL-8 expression has been associated with aging.[13,17,23,24] The changes in ICAM-1, MUC5AC, and IL-8 expression reported herein support the concept of age-related ocular surface system impairment, and the instigation of a persistent state of InflammAging.

In clinical practice, several degenerative changes of the ocular surface have been observed with age.[16] T-BUT values

**TABLE 2.** Study Population Clinical Features

	Age		T-BUT		Schirmer Test		OSDI	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Young	26.2 ± 4.1	28.0 ± 7.0	10.9 ± 0.2	11.4 ± 0.2	20.7 ± 0.2	20.5 ± 1.3	6.2 ± 1.3	8.2 ± 1.1
Middle-aged	50.4 ± 6.0	51.0 ± 5.8	10.0 ± 0.1	11.0 ± 0.6	20.3 ± 1.2	18.7 ± 1.2	5.9 ± 1.2	20.2 ± 3.4
Old	68.8 ± 6.0	82.6 ± 8.6	8.6 ± 1.6	9.6 ± 0.3	15.6 ± 1.6	16.6 ± 1.4	12.5 ± 2.4	17.6 ± 3.5

Tear Break-up Time

Schirmer Test

OSDI
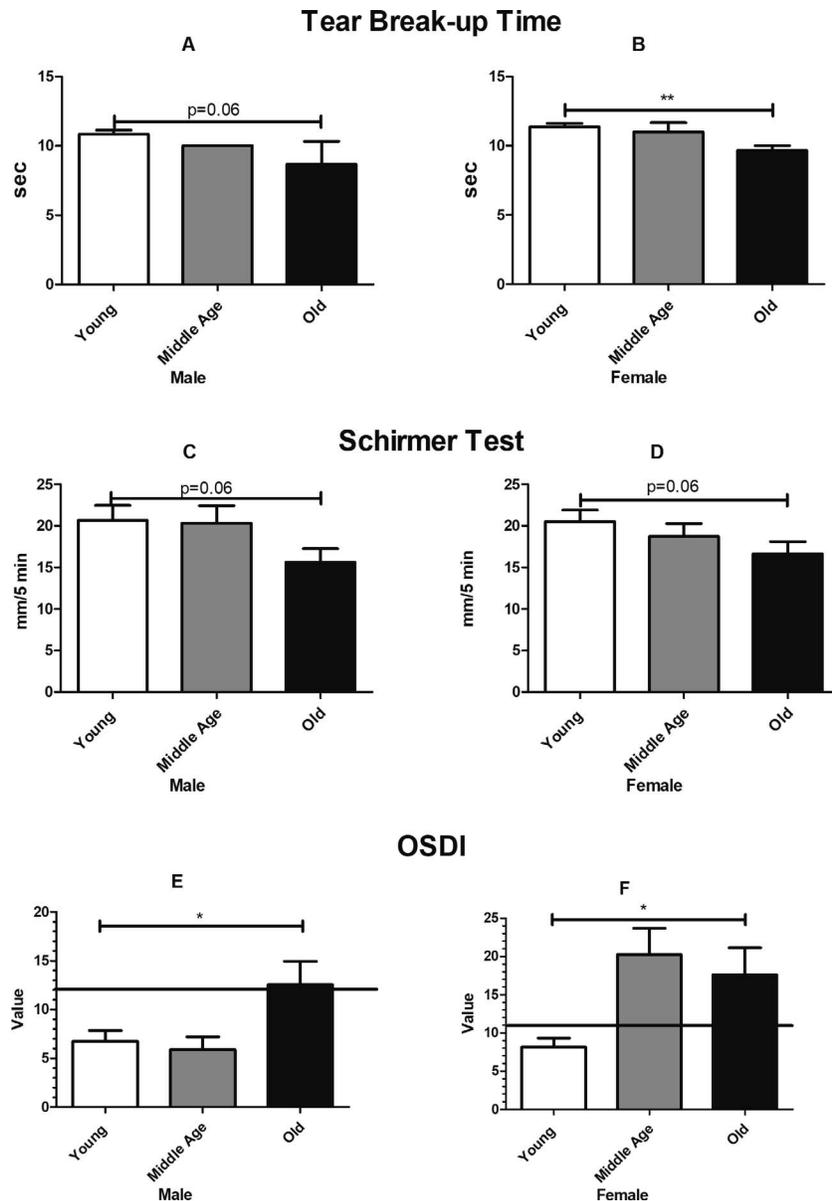
FIGURE 2. Clinical assessments of ocular surface system function according to male (*left side*) and female (*right side*) subgroups. (A) A tendency for decreased T-BUT values was observed in male old subjects with respect to their middle-age and young counterparts. (B) A decrease in T-BUT values was observed in female old group as compared to middle-age and young ones. (C, D) A significant decrease of Schirmer values was observed in old subjects with respect to middle age and young counterparts, with no differences between sex categorization. (E, F) Analysis of OSDI questionnaires showed increased symptoms in old male subjects with respect to both middle aged and young male groups. In female subjects, the younger population showed decreased symptoms with respect to both middle age and old groups. *Black lines* in the graphics point at normal OSDI limit (score 13). ANOVA analysis followed by Tukey-Kramer post hoc: *P* values are reported in the panels. Data are mean values ± SD. Study population is represented by volunteers considering themselves as healthy subjects. The normal OSDI limit was set at score 13.[13]

decrease[14] in association with abnormal lid margin anatomy, erythema, keratinization, and telangiectasia, as well as with alterations to the lipid profiles of Meibomian gland secondsretions.[17] These observations of age-related ocular surface degeneration, which contribute to tear film instability,[18] are consistent with our data. We report an association between increased age and decreased tear volume, as measured by the Schirmer test. The well-known decrease in corneal sensitivity with increased age[19] may partially account for reduced Schirmer test scores in the older adults. Cumulative oxidative stress, such as in chronic inflammation,[20,21] may also cause lacrimal gland dysfunction resulting in decreased tear production.

Despite the volunteers in our study were referring to themselves as "healthy," it is remarkable that OSDI questionnaires completed by the middle-aged and older volunteers documented symptoms of "mild dry eye disease (DED)" (i.e., OSDI >13; Fig. 1C). Subgroup analysis taking into account the sex of the volunteers revealed that OSDI scores for men were <13 in each age group, thus they did not qualify as "mild DED" (Fig. 2E). OSDI scores in women were elevated in middle-aged and older participants compared to young (Fig. 2F). Female participants reported higher OSDI scores at earlier age than men. Although sex-based differences in OSDI scores have been reported previously in ocular surface diseases,[22,23] our results highlighted a sex-specific suscepti-
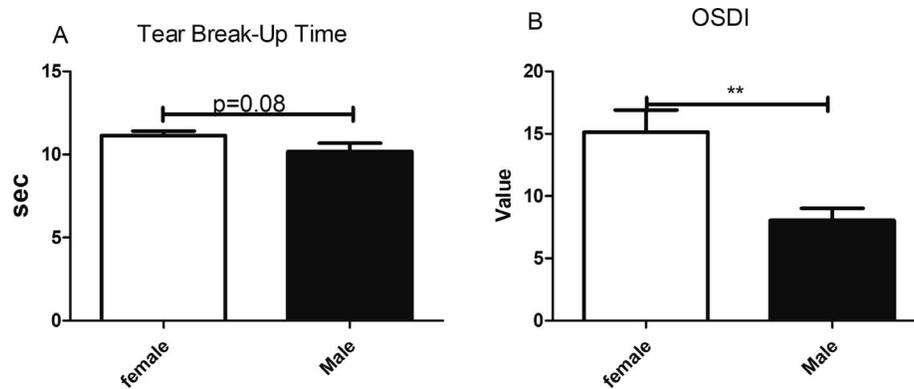
FIGURE 3. Sex intragroup comparison for T-BUT and OSDI questionnaire analysis. (**A**) T-BUT analysis with respect to female and male distribution showed no significant difference ($P = 0.08$). (**B**) A significant decrease of OSDI symptom score was observed in male with respect to female subjects ($P = 0.001$). ANOVA analysis followed by Tukey-Kramer post hoc: P values are reported in the panels. Data are mean values ± SD. Study population is represented by volunteers considering themselves as healthy subjects. The normal OSDI limit was set at score 13.[13]

bility to age-related ocular surface discomfort in the self-referring "asymptomatic" population. It may be speculated that sex-specific hormonal changes influence T-BUT values during aging. According to the questionnaire scores, some subjects who consider themselves "healthy" would in fact be classified as having ocular surface disease at the OSDI. This underlines the sensitivity of the OSDI score to ocular surface symptoms. It also suggests a relative lack of awareness of those subjects to their subjectively and objectively quantifiable age-related ocular surface dysfunction (InflammAging).

In addition to these signs and symptoms of ocular surface dysfunction, we observed age-related increased expression of the inflammatory marker ICAM-1,[24] which was significantly associated with increased IL-8 and MUC5AC target gene expression. Upregulated ICAM-1 has previously been report-ed to predispose to immune-based inflammation,[25] indicative of an age-associated state of chronic inflammation of ocular surface in our subjects. Moreover, the relative increase in IL-8 is likely to be associated with the recruitment of innate immune cells, such as neutrophils and macrophages. In aged humans, infiltrates of lymphocytes are detected in approximately 63% of lacrimal glands, and the incidence of lymphocytic infiltration has been reported to be higher in older individuals.[26] The goblet cell-derived mucin, MUC5AC, plays an important role in epithelial protection by clearing debris from the ocular surface.[27] It is likely that the increase in MUC5AC expression observed in our cohorts represented a compensatory mechanism for the decreased tear film stability and volume observed in our subjects. It is well known that subclinical persistence of low grade inflamma-
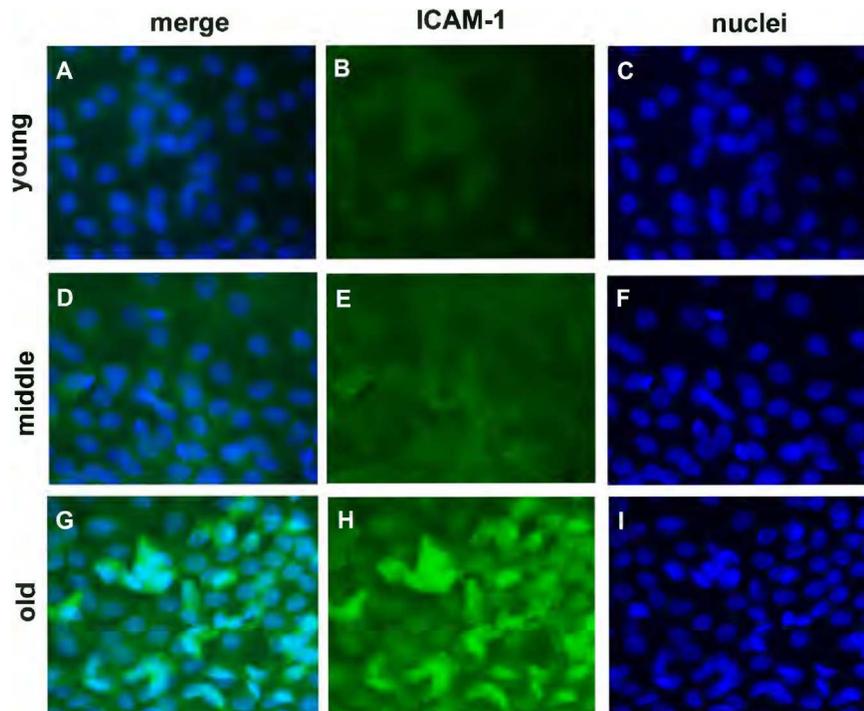

FIGURE 4. ICAM-1 immunoreactivity at aging. Confocal analysis of ICAM-1 immunostained (*green*) imprints. Representative merge panels (**A**, **D**, **G**) showing the specific ICAM-1 immunoreactivity (**B**, **E**, **H**, *green*) in respectively young (**A–C**), middle age (**D–F**), and old (**G–I**) groups over a nuclear DAPI counterstaining (**C**, **F**, **I**, *blue*). Magnification: ×400.
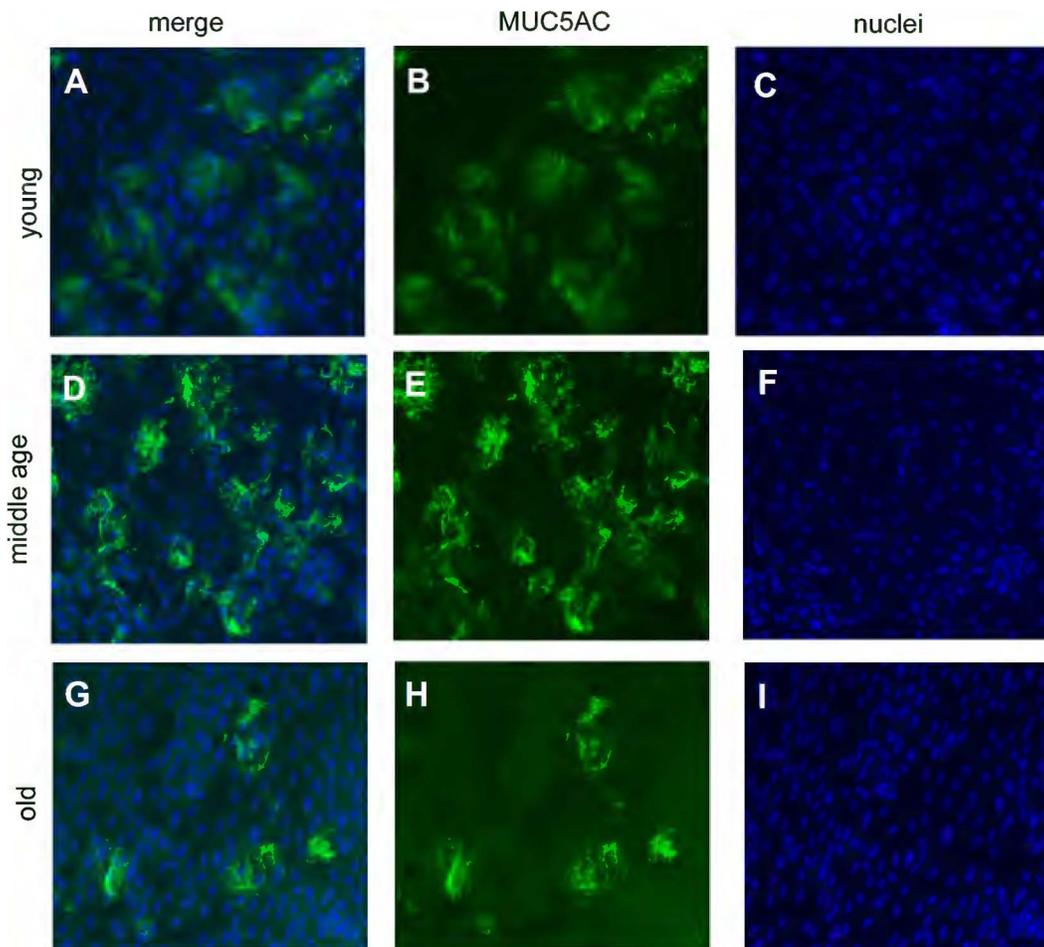
FIGURE 5. MUC5AC immunoreactivity at aging. Confocal analysis of MUC5AC immunostained (*green*) imprints. Representative merge panels (**A**, **D**, **G**) showing the specific MUC5AC immunoreactivity (**B**, **E**, **H**, *green*) in respectively young (**A–C**), middle age (**D–F**), and old (**G–I**) groups over a nuclear DAPI counterstaining (**C**, **F**, **I**, *blue*). Magnification: ×400.

tion contributes critically to many human diseases that were previously not considered to be inflammatory disorders, including obesity,[28] atherosclerosis,[29] and various neurodegenerative disorders.[30]
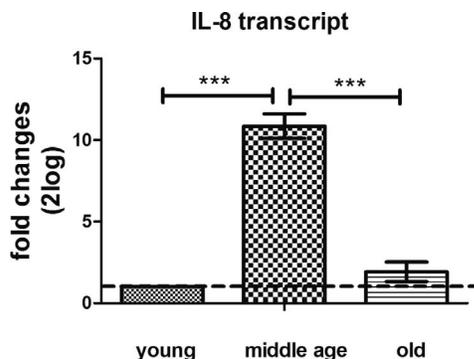
FIGURE 6. Fold changes in IL-8 transcript expression. Relative real-time PCR amplification was carried out, and REST Analysis was performed to obtain fold changes (± SD) expression with respect to young imprints (used as control; referred as 1, as indicated by *dashed line*). A significant increase was observed for middle age versus young ($P < 0.001$), while a slight increase was detected in old versus young ($P > 0.05$). A difference in IL-8 transcript expression was also observed between middle age and old ($P < 0.001$) 1-way ANOVA Tukey-Kramer post hoc.

In our study, self-considered "healthy" volunteers demonstrated subclinical persistent low-grade ocular surface inflammation with aging, suggestive of an ineffective attempt of parainflammation to restore ocular surface homeostasis. The capacity of parainflammation to restore ocular surface homeostasis necessarily deteriorates in InflammAging.[5] Dysfunction or dysregulation of the ocular surface immune response in this para-inflamed setting might lead to various immune-related diseases, such as infection and autoimmunity.[5] In an experimental murine model, increased activity of IFN-γ+ effector T cells,[31] and decreased suppressive function by CD4+CD25+ Foxp3+ T regulatory cells (Tregs) has been observed with aging.[11] Several groups have reported that Tregs become dysfunctional with age, losing their suppressive ability, producing significant amounts of inflammatory cytokines, such as IL-17, and becoming "pathogenic exTregs."[10,32] In older populations, the dysfunctional homeostatic system and the increased inflammatory environment, accompanied by increased numbers of dysfunctional regulatory T cells may actively participate in the initiation and development of immune-mediated ocular surface diseases such as keratoconjunctivitis sicca.[33]

This study has several limitations: (1) corneal esthesiometry was not performed despite corneal sensitivity being a potential confounder, (2) only selected Dry Eye Workshop (DEWS) II parameters and inflammatory biomarkers were assessed, and (3) age-sex matching in older group was not achieved. Our

findings should be confirmed by further studies, investigating wider range of clinical analysis and biomarkers, in order to better evaluate the complexity of the parainflammatory homeostatic mechanisms in the ocular surface.

These data unveil a different point of view about aging and the ocular surface unit. We propose that aging is not only a risk factor for ocular surface disease, but in fact represents a preclinical and/or subclinical condition. We believe that, with aging, para-inflammatory compensatory mechanisms fail to adequately restore ocular surface homeostasis, leading to a persistent asymptomatic inflammatory state, termed InflammAging.

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