

Age-Related Changes to Human Tear Composition

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PURPOSE. We characterize age-associated alterations in the expression of inflammatory mediators and tissue remodeling factors in human tears.

METHODS. A total of 75 consecutive volunteers (32 male/44 female; 19–93 years) underwent clinical assessment of ocular surface status, ocular surface disease index (OSDI) grading and tear sampling. The volunteers were categorized into three groups: young (18–40 years), middle-aged (41–60 years), and old (>60 years). Total protein profiles and chip-based protein array evaluations were conducted to investigate the expression of 60 potential candidates, including pro-/anti-inflammatory mediators and tissue remodeling factors. Appropriate validations were performed using conventional assays. Multiple comparisons for regression between potential candidates and age were performed, as well as statistical analyses among the three age groups. Nonpooled samples were used for quantifications.

RESULTS. Pearson analysis of chip-arrays identified 9 of 60 potential candidates. Specifically, IL-8, IL-6, and regulated on activation, normal T cell expressed and secreted (RANTES; $P < 0.0083$) protein as well as matrix metalloproteinase (MMP)-1, IL-3, and TNF- α ($P < 0.05$) correlated positively with aging. MIP-3 β showed an opposite tendency. Western blot and ELISA analysis corroborated the array data. OSDI grading did not correlate with aging.

CONCLUSIONS. Dynamic changes to tear protein profiles occur with aging. Our study identifies the expression of IL-8, IL-6, RANTES, MMP-1, and MIP-3 β as increasing with age. These select inflammatory and matrix remodeling factors may be relevant to the development of novel diagnostic tools and therapeutics in the context of age-related ocular surface disease.

Keywords: aging, protein tear-print, inflammation, ocular surface, discomfort, para-inflammation

As the irreversible impairment of physiological tissue and organ function.^{1,2} At the ocular surface, the wide array of changes that occur with aging may significantly impair an individual's quality of life.³ Anatomical and physiological changes that occur with aging include progressive atrophy of the lacrimal glands, keratinization of the Meibomian gland ducts, alterations in tear quality and quantity, as well as formation of conjunctival folds.^{4,5} Furthermore, aging has been associated with decreased corneal nerve fiber density, diminished frequencies of goblet cells, and alterations to hormone signaling and immune homeostasis (resulting in a higher incidence of autoimmune diseases in the elderly).^{6–8} In light of the plethora of anatomical and physiological changes that occur with aging, as well as the fact that age-related disorders (e.g., dry eye disease) are associated with increased inflammatory proteins in the tears,^{8,9} it perhaps is unsurprising that aging itself is associated with altered tear composition.¹⁰

Given that tear proteins are critically involved in preserving metabolic and immune homeostasis of the ocular surface, these proteins have attracted attention as potential diagnostic indicators.^{11–13} Gradual changes in cytokines, chemokines, as well as growth and tissue remodeling factors have been observed with normal aging, and it has been proposed that

alterations in these components may explain the age-related anatomical and physiological changes observed at the ocular surface.^{10,14,15} In the setting of chronic ocular inflammatory disease, the overexpression of cytokines and growth factors, as well as altered expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), have been implicated in driving inflammation and fibrotic remodeling of the ocular surface tissues.¹⁶ These observations suggest that tear protein composition data might be used to identify pathological molecules that drive disease, and accordingly, may represent the first steps toward the development of targeted therapeutic strategies.^{17,18}

We further characterized the alterations in human tear composition that occur with aging. In particular, we evaluated/selected pro- and anti-inflammatory molecules, as well as tissue remodeling factors, that have been identified as key mediators of ocular surface pathology.

METHODS

Study Population

A total of 76 consecutive volunteers older than 18 years were enrolled from December 2014 to June 2015. Subjects were



TABLE. Study Population

Groups	Subjects	Age, Range	Mean Age*	Sex, F/M	OSDI†
Population	76	19-93		44/32	–
Young	35	19-40	27.60 ± 6.39	21/14	11.01 ± 6.95
Middle-aged	23	41-60	50.17 ± 5.49	15/8	11.94 ± 13.47
Elderly/old	18	61-93	62.62 ± 3.70	8/10	16.12 ± 7.43

* Data are mean ± SD.

† OSDI score (0-100 points) was calculated according to the Allergan® suggestions and clustered as follows: normal, score 0-12; mild, score 13-22; moderate, score 22-32; severe, score 33-100.³¹

divided into three age groups (young, 18-40; middle-aged, 41-60; and old, >60). Participants with a history and/or signs of ocular surface disease, contact lens wear, systemic disease (including malignancies, metabolic diseases, psychiatric diseases), or pregnancy were excluded from the study. Furthermore, patients taking medications (either systemic or topical) at examination were excluded. All experimental procedures were performed in accordance with guidelines established by the Association for Research in Vision and Ophthalmology and adhered to the tenets of the Declaration of Helsinki concerning human subjects. The procedures for patient recruitment, tear sampling, and biochemical analysis were reviewed and approved by the intramural ethical committee (University Campus Bio-Medico, Rome, Italy). All participants provided written informed consent to proceed to conjunctival imprint and tear sampling.

Ocular Surface Disease Index (OSDI) Questionnaire

An OSDI questionnaire (Allergan, Inc., Irvine, CA, USA) was completed by each subject to provide a brief screening of ocular discomfort.¹⁹ OSDI grading was as follows: normal (0-12 points), mild (13-22 points), moderate (23-32 points), or severe (33-100 points).

Tear Sampling and Handling

Subjects underwent late morning or early afternoon non-anesthetized tear collection via the eye-flush procedure, as described previously.²⁰ Briefly, the sampling procedure involved topical ocular administration of 30 µL sterile balanced salt solution (BSS; Alcon Laboratories, Inc., Fort Worth, TX, USA) and prompt tear collection by means of a sterile individually-wrapped plastic micropipette (PBI Intl., Milan, Italy). Tear samples were mixed with a cocktail of protease inhibitors (Pierce Biotechnology, Rockford, IL, USA) and stored at -20°C pending biochemical analysis. Sample delivery to the laboratory or analysis was conducted using an isothermal cage (CryoCooler; Starlab Int GmbH, Ahrensburg, Germany).

Total Protein Analysis

All samples were subjected to total protein analysis. Tear volume was recalculated according to the guidelines of the procedure, adjusting for a dilution factor, as described previously.²⁰ Following centrifugation at 16,000g for 10 minutes, samples were diluted at a ratio of 1:2 in cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM, NaF, and 1 mM PMSF; pH 7.5). Following this, the samples were quickly sonicated (VibraCell; Sonics, Newtown, CT, USA) and clarified by centrifugation (12500g/7 min). The total protein quantification was done (3 µL/tear

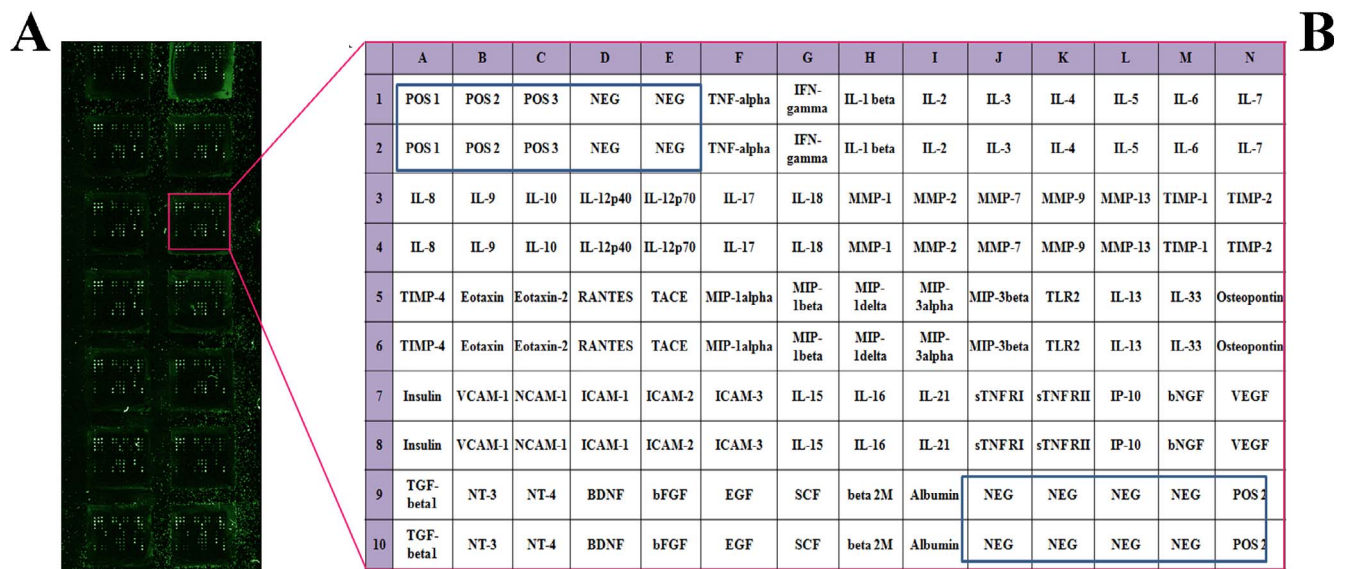


FIGURE 1. Array and subarray schematizations. (A) Representative glass-slide containing 14 subarrays. For each subarray, the antibody-based structure was designed according to the map in (B), framed area/subarray), displaying the position of all candidate factors investigated. Each spot contains specific capture antibodies (targets): appropriate checks and confirmation of specificity were carried out by manufacture. Bordered, positive and negative internal controls.

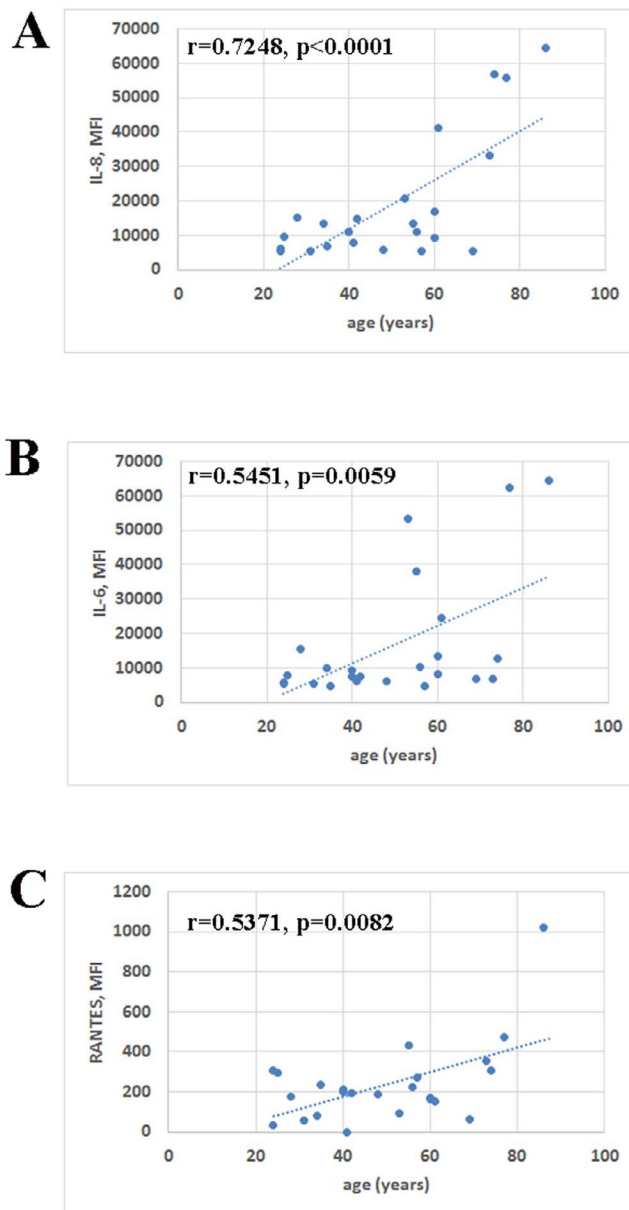


FIGURE 2. Biochemical variables and aging (D). Pearson correlation coefficients between age factor and IL-8 (A), IL-6 (B), and RANTES (C) fold-changes. Coefficient and *P* values from correlation analysis are detailed inside panels and indicate the accuracy of correlation. Pearson rank's correlation analysis. A tendency toward a positive correlation between age factor and each biochemical factor also was observed for IL-2 and IL-21.

sample) using a digital A1000 Nanodrop spectrophotometer equipped with the A280 program (Celbio, Milan, Italy). Protein electrophoresis (30 μ g total proteins heated in loading buffer at 75°C/5 min) was conducted under reducing conditions (4%–12% precast resolving SDS-PAGE mini gels; 130 V/frontline; MiniProtean3 apparatus; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Electrophoresed proteins were transferred to Hybond-membranes (0.22 μ m; GE Healthcare, Buckinghamshire, UK), under semi-dry conditions (13 V/45 min; Trans-Blotting apparatus; Bio-Rad), and stained with a Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL, USA). Images were digitally captured using 1D Kodak Image Software

(Kodak, Tokyo, Japan), quantified, and exported as 8-tiff-based files for figure assembly in Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA). Samples that overexpressed albumin, IgG or fibronectin bands were pretreated with a bead-based depletion kit (GE Healthcare).²¹

Chip-Based Array

For analysis, 24 samples were tested, including young ($n = 10$), middle-aged ($n = 8$), and old ($n = 6$) protein extracts. The samples were selected randomly from patients with normal OSDI values. Each glass slide included 14 identical subarrays containing 60 preselected protein candidates assembled in customized G-series arrays (Ray-Biotech, Norcross, CA, USA). Candidates were selected retrospectively based on previous investigations by our laboratory^{20,22–25} and others,^{26–28} and were grouped as follows: pro/anti-inflammatory (cytokines, chemokines, and adhesion molecules and neurotrophins), tissue remodeling factors (MMPs), tissue-specific inhibitors (TIMPs), fibrogenic and angiogenic factors, and other soluble receptors/proteins. Briefly, equal quantities of protein extracts (350 ng/mL) were hybridized into array-wells and the antigen-antibody immunoreaction, including incubation and washing conditions, was conducted according to a standard procedure (Ray-Biotech). Spin-dried glass-slides were scanned in a GenePix 4400 Microarray platform (Molecular Devices LLC, Sunnyvale, Silicon-Valley, CA, USA). The specific acquisition area (array/spot) was first selected manually and then automatically adjusted for size, brightness, and contrast by the software. Normalized Fluorescent Intensity (FI) data were calculated by the GenePix Pro 6.0 software (Axon; Molecular Devices). Inter-assay normalizations were guaranteed by the presence of multiple internal controls for each subarray. The minimum sensitivity ranged between 3.8 and 56 pg/mL.

Western Blotting and ELISA

All samples were subjected to Western blotting analysis for the appropriate validations. Briefly, SDS-PAGE was blocked in 5% BSA-TBS (TBS, 20 mM Tris-HCl and 150 mM NaCl, pH 7.5) and immunoblotted with anti-human monoclonal or polyclonal antibodies specific for IL-8, IL-6, and MIP3 β (respectively, MAB208, MAB206, and AF361; all from R&D Systems, Minneapolis, MN, USA), and regulated on activation, normal T cell expressed and secreted protein (RANTES; ab-9679; Abcam, Cambridge, MA, USA). Antibodies were diluted in 0.5% TX-TBS at 0.1–1 μ g/mL final concentration. The labeling step with species-specific POD-conjugated antibodies (at least 1:10000; raised in donkey; Jackson ImmunoResearch Europe Ltd, Suffolk, UK) and the developing steps with ECL-based chemiluminescence kit (West Femto Sensitivity Substrate; Pierce, Rockford, IL) were performed according to standard procedures. An immunoblot-specific signal was acquired with the 1D Image Station Analyser (Kodak, Tokyo, Japan). Data were saved as 8-bit TIFF files and exported to be shown after Adobe Photoshop CS3 assembly (Adobe Systems, Inc.). For IL-8, ELISA also was performed according to manufacturer's instructions (DY208 duo-set ELISA kit; R&D Systems).

Impression Cytology and Goblet Cell Staining

In representative volunteers ($n = 9$; $n = 3$ /group), conjunctival imprint at the bulbar temporal conjunctiva was performed (Millicell; 0.22 μ m membranes; 13 mm diameter; Millipore, Burlington, MA, USA) in the presence of topical anesthesia (Novesina; Novartis Farma, Origgio, VA, USA), according to a standard procedure.²⁹ Imprints were fixed (Bio-Fix spray; Bio-Optica, Milan, Italy) and processed for PAS staining, to visualize

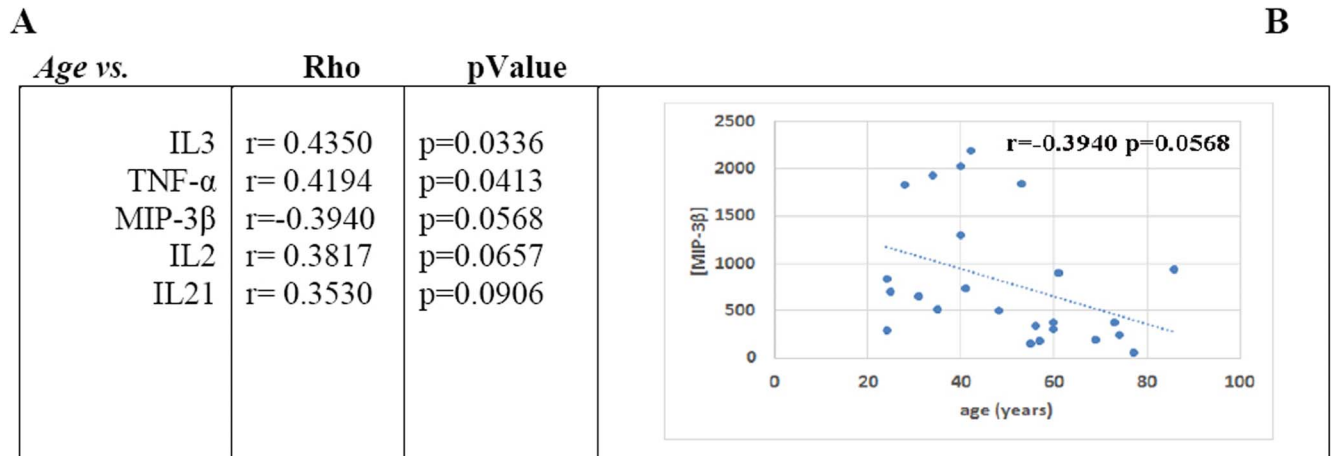


FIGURE 3. Biochemical variables and aging (II). Correlation coefficients between age and respectively IL-3 ($P < 0.05$), TNF- α ($P < 0.05$), IL-2, and IL-21 fold-changes. (A) An opposite tendency was detected for MIP-3 β (B).

goblet cells (Bio-Optica). Stained membranes were removed from the plastic support (Millicell) and mounted on glass slides. Images were acquired at the Eclipse Ci microscope equipped with the NIS Elements Image software for optic field light transmission acquisition (Nikon, Tokyo, Japan).

Statistical Analysis

Statistical analyses were conducted using the SPSS 15.0 statistic package (SPSS, Inc., Chicago, IL, USA) and scatter/histogram

panels were produced by Prism software (GraphPad, San Diego, CA, USA). The nonparametric Kolmogorov-Smirnov and Shapiro-Wilk tests assessed the Gaussian distribution of data. For chip array analysis, the specific FI averages (mean \pm SD) were calculated from replicates (2 spots) of nonpooled tear samples. FI values were normalized by subtracting the background signal estimated by the software (Axon; Molecular Device). A P value ≤ 0.00083 with fold-changes >2 for 60 targets was considered significant in protein-array analysis with a Bonferroni's correction ($0.05/60$), while a P value ≤ 0.05 was

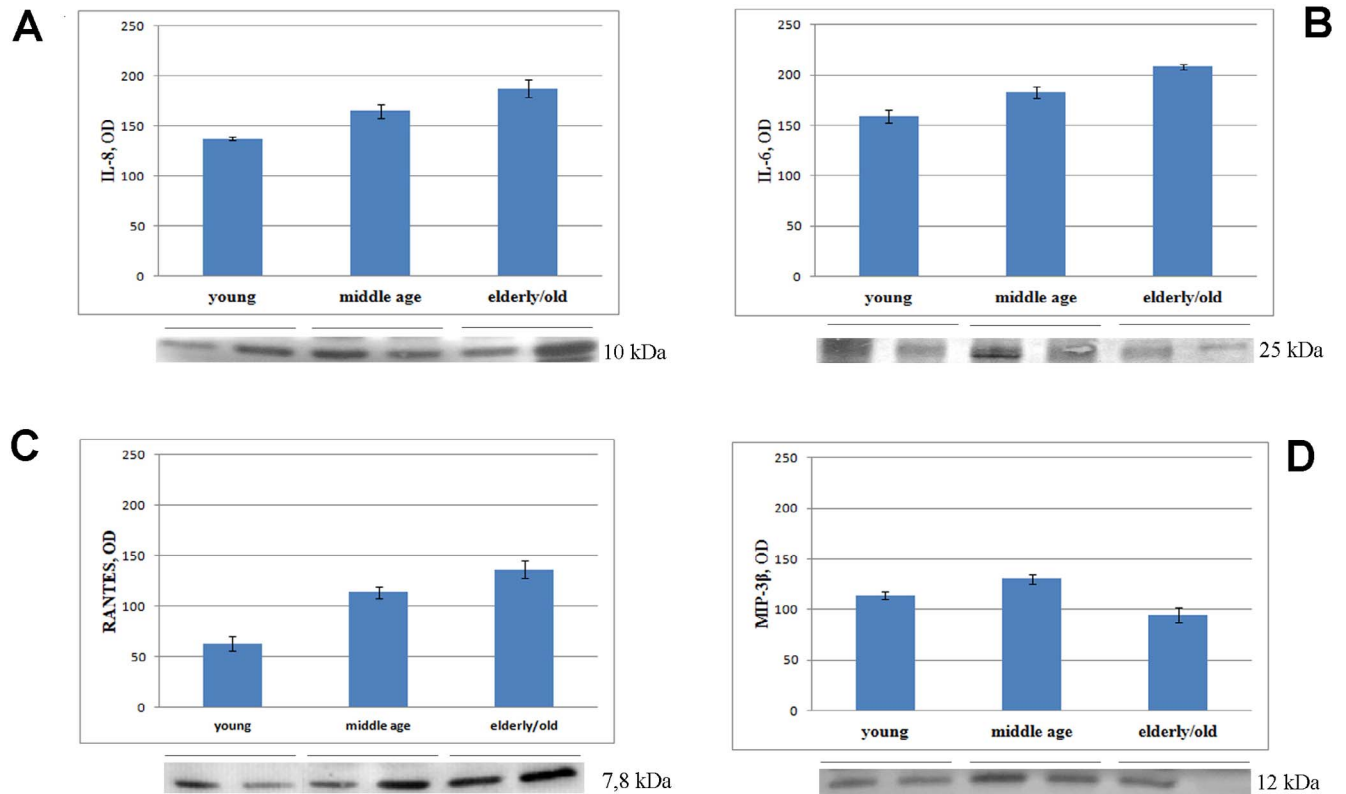


FIGURE 4. Array validation analysis. Biomarkers having significant changes (multiparametric analysis corrected for Bonferroni test) were subjected to Western blotting. The IL-8 (A), IL-6 (C), RANTES (D), and MIP-3 β (E) protein expressions in tears. Below each histogram (OD), the related SDS-PAGE (two per group). Expected protein size is shown adjacent to each immunoblot.

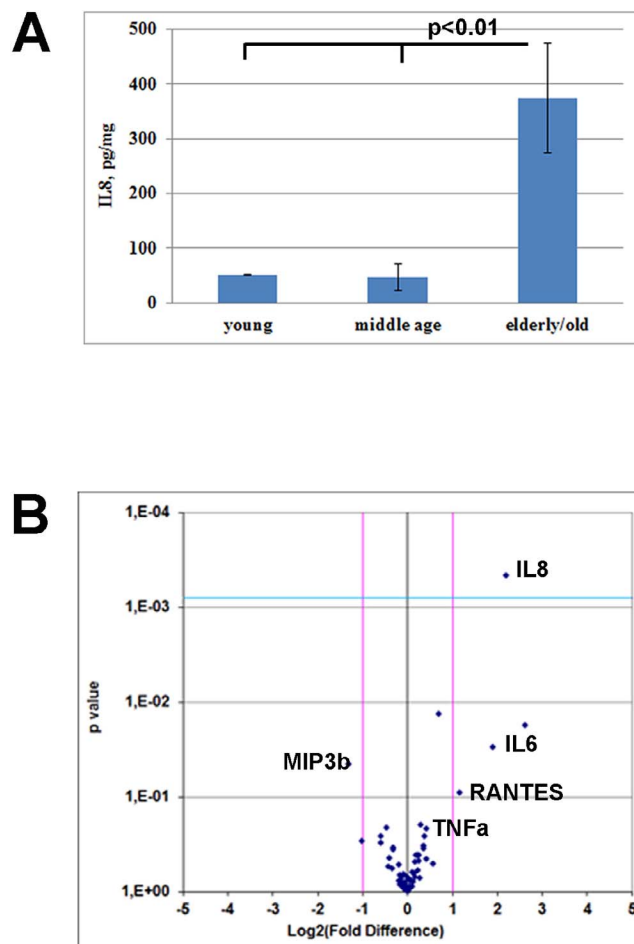


FIGURE 5. Summarizing results. (A) Bar graph illustrating IL-8 levels in tears from young, middle-aged, and old groups. Note the higher increase of IL-8 in tear samples from the old group, with respect to middle-age and young groups. (B) A Volcano plot specific for old versus young groups, with the expression of IL-8, IL-6, RANTES, and MIP-3 β highlighted. Fold changes (Log₂ [FC]; x axis) are ranked according to the statistical significance (*P* values, as negative Log₁₀; y axis). For each marker, the fold changes (from mean of FI values) were provided by 2-sided unpaired *t*-test comparisons. Both ± 2 FC and $P \leq 0.05$ were used as initial cutoffs. The pink lines indicate differences of ± 1 FC (log₂) while the blue one shows the high significance.

used in comparisons (optical density [OD] or ratios). Correlations between candidate biomarkers and age were calculated. Aging groups were compared using ANOVA analysis coupled to Bonferroni post hoc.

RESULTS

The characteristics of the study population are summarized in the Table. As depicted in Figure 1, the protein arrays contained 60 potential candidates clustered as follows: pro/anti-inflammatory factors (cytokines, chemokines, adhesion molecules, neurotrophins) and tissue remodeling mediators (MMPs and TIMPs; fibrogenic and angiogenic factors).^{30–32} Analysis of Pearson correlation coefficients identified 8 of 60 protein candidates that were observed to vary significantly with aging. As shown in Figure 2, the expression of IL-8 ($r_{bo} = 0.7248$; $P < 0.0001$; Fig. 2A), IL-6 ($r_{bo} = 0.5451$; $P = 0.0059$; Fig. 2B), and RANTES ($r_{bo} = 0.5371$; $P = 0.0082$; Fig. 2C) were correlated

with age. Furthermore, the positive correlations between age and IL-3, TNF α , MIP-3 β , IL-2, and IL-21 are detailed in Figure 3A. An increasing trend also was observed for IL-2 and IL-21 with age. In contrast, a decreasing trend was found for MIP-3 β expression (the scatter plot is shown in Fig. 3B).

To further delineate the effects of aging on tear composition, the tear print samples were categorized according to the following age groups: young (18–40 years, 10 subjects), middle-aged (41–60 years; eight subjects), and old (>60 years; six subjects; Table). ANOVA analysis coupled to Bonferroni post hoc was used for multiple comparisons ($F = 6.11 - Df_n = 21 - Df_d = 1239$; $P < 0.0001$) and confirmed: (1) increased IL-8 ($P < 0.001$), IL-6 ($P < 0.001$), and MMP-1 ($P < 0.01$) protein levels in tears from the old group relative to the young group; (2) increased IL-8 ($P < 0.001$), IL-6 ($P < 0.01$), and MMP-1 ($P < 0.05$) protein levels in tears from the old group compared to the middle-aged group; and finally (3) elevated IL-7 ($P < 0.01$) protein levels in tears from the middle-aged group compared to the young group. Western blot analysis corroborated the IL-8 (Fig. 4A), IL-6 (Fig. 4B), RANTES (Fig. 4C), and MIP-3 β (Fig. 4D) array-outcomes. IL-8 was further quantified by ELISA analysis, thereby confirming a strong association between IL-8 and aging, as shown in Figure 5A. The Volcano plot indicating the cytokines of interest is shown in Figure 5B.

ANOVA analysis also identified an increase in IL-7 expression in the middle-aged group with respect to old ($P < 0.001$) and groups ($P < 0.05$) young. No significant correlation was observed between IL-7 and age factor ($P = 0.7410$). Finally, an increased protein tear-print MI of array outputs demonstrated inverse correlation with decreased goblet cell number at conjunctival impression cytology (respectively in young, Figs. 6A, 6B; middle-age, Figs. 6C, 6D; and old, Figs. 6E, 6F subgroups).

DISCUSSION

Tear composition changes with age.¹⁰ This study reports the protein array data for tears isolated from a population of volunteers, grouped according to age. Our results analysis suggested candidate biomarkers with diagnostic and therapeutic potential in the setting of age-related ocular surface disease.

Significant changes in tear protein synthesis (including pro/anti-inflammatory and tissue remodeling factors) have been reported in ocular surface diseases.^{11,12} Moreover, it has been proposed that some mediators released into the tear film by ocular surface tissues in response to inflammation, might themselves drive tissue inflammation, thereby establishing a positive feedback loop.^{33–35} Tear film proteins are potential biological markers of subclinical tissue damage at the ocular surface, and may permit early clinical intervention with the arrest of positive feedback loops. The scarcity of data available in the published literature on the biochemical analysis of tears from normal human controls (i.e., without ocular surface disease) highlights the need for systematic studies on these subjects.^{33–35}

The candidate factors that were selected retrospectively have been previously implicated (by our laboratory^{20,22–25} and others^{26–28}) in the promotion or regulation of inflammation, or alternatively in low-grade tissue remodeling, at the ocular surface. Importantly, several of these soluble signaling factors (e.g., IL-6) also have been reported as important components of the secretory patterns of senescent cells.^{35–37} Our analysis of the correlation between tear proteins and age unveils a dynamic age-related change in the tear protein profiles of volunteers without ocular surface disease. Specifically, our data showed a positive correlation of IL-8, IL-6, and

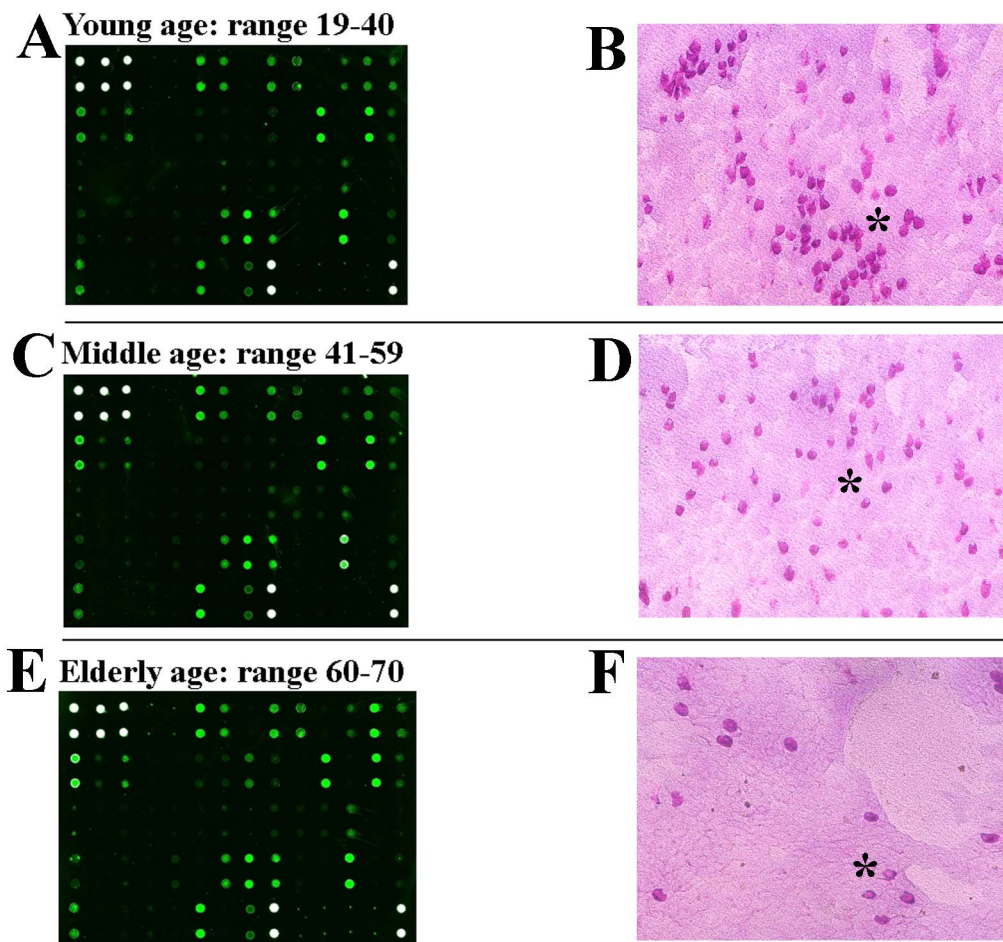


FIGURE 6. Chip-array versus conjunctival imprint illustrations. A comparison between tear subarrays (A, C, E) and conjunctival imprints (B, D, F), randomly selected for representative illustration. (A, C, E) Representative images following hybridization and acquisition by the GenePix platform. (B, D, F) From *left to right*, $\times 20$ /optic as produced by Nikon acquisition.

RANTES with age, which is suggestive of low-grade inflammation (IL-8, IL-6, and RANTES) and dynamic tissue remodeling (IL-6) being more common among the elderly population.³⁷

Aging is associated with cellular senescence and widespread tissue dysfunction.¹⁻³ Our data are consistent with the increased prevalence of chronic, low-grade inflammation of the ocular surface among the elderly population. Biochemical factors may function to trigger or perpetuate inflammation, as observed in dry eye disease and other autoimmune disorders.^{35,38} Interestingly, our data showed a correlation between age and TNF α /IL-3 as well as IL-2/IL-21, cytokines that are involved in sustaining T helper-mediated immune responses.^{39,40} Specifically, IL-3 (from activated T cells) contributes to immune homeostasis and host defense by modulating the activity of various immune effectors (dendritic cells, granulocytes, monocytes, and mast cells), either alone or in collaboration with other cytokines.⁴¹ IL-2 is known primarily for driving the proliferation of activated T cells, and IL-21 is renowned for its role in Th17 immunity.^{42,43} Yet, both of these cytokines exert broad pleiotropic functions that modulate the activities of immune cells (including T, B, and NK cells).^{42,43} It is possible that IL-2 and IL-21 contribute toward age-associated immune dysregulation, which is observed as increased autoimmunity among the elderly, but also to impaired host defense against

infectious insults.^{26,32} MMP-1 has been shown previously to be elevated during ocular surface inflammation.^{32,44-46} Our data demonstrated a positive correlation between age and MMP-1 expression in the tears of subjects, further suggesting that a state of chronic, low-grade ocular surface inflammation may be more prevalent among the elderly population. Interestingly, the negative correlation that we observed between aging and MIP-3 β does not seem to be consistent with published data indicating that the recruitment of leucocytes is increased in aged tissues.⁴⁰

Categorizing the volunteers by age (46% young, 30% middle-age, and 23% old) allowed us to confirm the previously observed trends in IL-8, IL-6, and RANTES expression. Moreover, comparisons between the age categories unveiled a selective increase in IL-7 expression in the middle-age relative to the young groups. The increased IL-7 expression in middle-age may have additional implications for immune homeostasis and tissue remodeling, due to the capacity of IL-7 to inhibit TGF- β production and upregulate SMAD7.^{47,48} The absence of a significant difference in VEGF expression between the old and young groups is notable, in light of the persistence of corneal angiogenic privilege with age.^{39,49} Interestingly, given the association of conjunctival goblet cell loss and ocular surface inflammation,⁵⁰ our data (Fig. 6) suggest an increased protein tear-print MI of array outputs in aged subjects, that

correlated inversely with goblet cell number at conjunctival impression cytology.

A limitation of this study is that neuromediators (such as VIP, NPY, CGRP, and SP) and BDNF, NT-4, and NGF were not evaluated by our protein-array platform. Given the importance of these factors in the context of age-related nerve degeneration and neuroinflammation, further investigation of their expression is required.^{22,23,25}

Life expectancy is increasing worldwide, and age-related ocular surface disorders can markedly reduce human quality of life.^{3,51} A high-quality tear film is a major determinant of ocular surface well-being.³³ Physiological cell senescence, together with environmental stressors and epigenetic factors (sunlight, VDT, natural/artificial climate, topical drugs, surgery, infections, diseases) may contribute toward dysregulation of ocular surface immunity and predispose to tissue damage.^{52,53} Understanding the changes in pro-/anti-inflammatory factors and tissue remodeling factors that occur with aging will enable the development of novel diagnostic tests based on biomarkers. Moreover, defining these critical proteins may permit the design of targeted, personalized artificial tear substitutes.

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