In Vivo Confocal Microscopy of Conjunctiva-Associated Lymphoid Tissue in Healthy Humans

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Purpose. To investigate modifications with aging of the presence, distribution and morphologic features of conjunctiva-associated lymphoid tissue (CALT) in healthy human subjects using laser scanning in vivo confocal microscopy (IVCM).

Methods. A total of 108 (age range, 17–75 years) subjects were enrolled. In vivo confocal microscopy of the tarsal and bulbar conjunctiva, and impression cytology (IC) with CD3 (intra-epithelial T-lymphocytes) and CD20 (intra-epithelial B-lymphocytes) antibody immunofluorescence staining were performed. The main outcomes were subepithelial lymphocyte density (LyD), follicular density (FD), and follicular area (FA). The secondary outcomes were follicular reflectivity (FR), and lymphocyte density (FlyD), and CD3 and CD20 positivity.

Results. Conjunctiva-associated lymphoid tissue was observed in all subjects (97% only superior and 5% in both superior and inferior tarsus). Lymphocyte density ranged from 7.8 to 165.8 cells/mm2 (46.42 [18.37]; mean [SD]), FD from 0.5 to 19.4 follicles/mm2 (5.3 [3.6]), and FA from 1110 to 96,280 mm2 (26,440 [26,280]). All three parameters showed a highly significant inverse cubic relationship with age (P < 0.001); that is, in the first and last parameters a steep decline up to 35 years and above 65 years of age, with a plateau phase between these ages, whereas FA had a gradually decreasing rate of loss over the studied age range. CD3 and CD20 IC were consistent with these results.

Conclusions. In vivo confocal microscopy was effective in revealing CALT and modifications these structures undergo with aging. Aging correlated with an involution of all parameters defining lymphoid structures. These modifications may account for the decrease of mucosal immune response and increase of ocular surface diseases in the elderly.

Keywords: conjunctiva-associated lymphoid tissue (CALT), mucosa-associated lymphoid tissue (MALT), conjunctiva, in vivo confocal microscopy, ocular aging

Eye-associated lymphoid tissue, which comprehensively forms the immune system of the eye, is recognized as a component of the mucosa-associated lymphoid tissue (MALT) found in different organs of the body.1,2 Mucosa-associated lymphoid tissue is composed of lymphoid cells situated in and closely underneath the epithelium. These cells detect antigens and induce an immune response.

Eye-associated lymphoid tissue includes the lacrimal gland, the conjunctiva-associated lymphoid tissue (CALT) and the lacrimal drainage-associated lymphoid tissue (LDALT).2–4

Conjunctiva-associated lymphoid tissue is located in the lamina propria and consists of a diffuse layer of a specialized secretory lympho-epithelium with a variable number of lymphoid follicles and crypt-associated lymphoid structures.5–7 Lymphoid tissue contains T-lymphocytes and IgA-secreting plasma cells, which are responsible for the efferent arm of the conjunctival immune system. Scattered lymphocytes are also present within the overlying epithelium (intra-epithelial lymphocytes [IEL]). Lymphoid follicles are composed of B- and T-lymphocytes, macrophages, and dendritic cells. These follicles sample antigens and function as the afferent arm. In postmortem human eyes, follicles were mainly observed in the tarsal and orbital conjunctiva of the upper and lower lids, but also were present in the fornix and rarely in the bulbar conjunctiva.7 The crypt-associated lymphoid tissue consists of lymphoid cell accumulations aggregated along the crypt wall creating a dense lining, or disposed as dense roundish spots of lymphoid tissue similar to solitary follicles. The presence of highly specialized vessels with tall endothelia (high endothelial venules [HEVs]) that are important for lymphocyte homing to the conjunctiva indicates the integration of CALT as an outpost of the central immune system.8

Evidence from histological studies have led to the hypothesis that CALT is an active immunological interface responsible for detection of ocular surface antigens and is involved in initiating and regulating local ocular surface-related immune responses.2,3,6,9–11 In humans and in numerous studies conducted on rabbits, dogs, mice, and guinea pigs, CALT presented morphologic and functional features analogous to MALT described in other regions.10–14
As reported by Osterlind,\textsuperscript{12} CALT undergoes a developmentally regulated reorganization that results in shifts in the cell population profile and altered responses to novel antigens during the course of life.

In vivo confocal microscopy (IVCM) can noninvasively characterize the entire ocular surface in physiological conditions and several diseases.\textsuperscript{15,16} Recently, IVCM was used to evaluate alterations induced by toxic stimuli and antitumoral medications in the rabbit CALT.\textsuperscript{17,18} In humans, the presence of CALT is extremely difficult to observe in vivo for both anatomical and technical reasons. Recently, IVCM was used to explore human CALT follicles in two young patients with vernal keratoconjunctivitis and were reported to appear similar, although smaller, to those observed in rabbits,\textsuperscript{17} and in a patient with CALT lymphoma.\textsuperscript{19} However, to date, the modification of the characteristics of CALT with age has not been methodically investigated in vivo. The aim of this study was to describe the IVCM features and distribution of CALT, and modifications with aging in a population of healthy human subjects with a wide range of age.

**Materials and Methods**

**Design of the Study**

Our institutional review board (Department of Medicine and Ageing Science, G. d’Annunzio University of Chieti-Pescara, Chieti, Italy) approved this cross-sectional study. After receiving an explanation of the nature and possible consequences of the study, written informed consent was obtained from all subjects before enrollment. The research adhered to the tenets of the Declaration of Helsinki.

**Subjects’ Enrollment**

One hundred eight eyes of 108 consecutive healthy Caucasian subjects (50 males and 58 females) referred for a routine visit at the Ophthalmology Clinic of the G. d’Annunzio University of Chieti-Pescara (Chieti, Italy) were enrolled from January to December 2013, and examined. On completion of their routine visit and if they met the inclusion/exclusion criteria, 241 subjects (8.7% of the patients visited yearly) agreed to participate in the study, only 44.8% of whom returned for subsequent examinations.

Before enrollment, a detailed medical history was obtained. Each subject underwent a complete ophthalmic examination, including best-corrected visual acuity (BCVA), central corneal thickness (CCT), Goldmann applanation tonometry, anterior segment biomicroscopy, and funduscopy.

Inclusion criteria were as follows: age older than 16 years, BCVA ≥ 8/10, IOP lower than 18 mm Hg, CCT ranging from 530 to 570 μm, and absence of abnormalities of the anterior and posterior segment structures, ocular adnexa, and periorcular skin at slit lamp examination.

Exclusion criteria included the following: history of ocular trauma or surgery, meibomian gland dysfunction (MGD) (according to criteria for diagnosing MGD),\textsuperscript{20,21} dry eye (tear film breakup time < 10 seconds, and Schirmer Test I with topical anesthesia < 10 mm), ocular allergy, or any other ocular or systemic disease in the past 24 months that could affect the ocular surface tissues, current or long-term topical ocular or systemic medications in the past 24 months that could have modified CALT status, or contact lens wear. Pregnant women also were excluded. Both eyes were evaluated, but one eye per subject was randomly chosen (using a computer-generated random-number list) for statistical analysis.

**In Vivo Confocal Microscopy**

Laser-scanning confocal microscopy of the conjunctiva was performed using HRT III Rostock Cornea Module (RCM; Heidelberg Engineering GmbH, Dossenheim, Germany) to study CALT structures. Briefly, after topical anesthesia (oxybuprocaine hydrochloride 0.4%), the objective of the laser-scanning microscope was gently placed in contact with the conjunctiva separated by a polymethyl methacrylate contact cap. A drop of 0.2% polyacrylic gel (Viscotirs Gel; CIBA Vision Ophthalmics, Marcon, Venezia, Italy) served as a coupling medium to minimize compression during examination.

In vivo confocal microscopy examinations were performed to identify and analyze the main component of the CALT (the diffuse lymphoid layer and IEL follicles, and crypt-associated lymphoid structures) throughout the tarsal and bulbar conjunctiva. Figure 1 shows the areas of interest and whole conjunctival surface explored, in which confocal images were recorded.

Before performing IVCM, the superior eyelid was everted and a caliper was used to divide the lid into three equal lengths. Two parallel lines, perpendicular to the tarsal margin, were drawn with methylene blue from the tarsal to the lid margin, thus obtaining three areas (nasal, central, and temporal) that were approximately 7 × 7 mm.

Subsequently, confocal scanning was initiated at the upper tarsal conjunctiva from the most superficial to the deepest layers, which could be visualized with a satisfactory resolution. The procedure was repeated by moving the lens either along the entire lid length (from nasal to temporal margin) with...
minute vertical and horizontal movements or from eyelid margin toward the fornix.

Afterward, the four quadrants of the bulbar conjunctiva (temporal superior and inferior, nasal superior and inferior) were evaluated, asking the subject to gaze in the opposite direction of the analyzed quadrant. To evaluate the lower tarsal conjunctiva, the inferior eyelid was completely evverted with the subject gazing up. Because of technical difficulties, the RCM cannot reach the inferior and superior fornix conjunctiva and, therefore, these sectors were not examined.

The depth of CALT was checked manually using the x-y-z position of the optical section; HRT III/RCM automatically calculated the focus position (μm). Confocal microscopy cannot analyze conjunctiva more than 80 to 90 μm of depth due to the high hyperreflectivity of underlying tissues such as sclera or tarsum.17 Thus, the effective depth at which CALT structures could be clearly visualized was under 70 to 80 μm.

A total of 144 images were acquired to analyze tarsal conjunctiva (24 each for nasal, central, and temporal tarsal conjunctiva of the upper and lower lid) and 48 for bulbar conjunctiva (12 for each quadrant) at approximately every 10 μm of depth. Typical sessions for LSCM examination lasted between 5 and 8 minutes. A single operator (VF) performed confocal examinations and selected the images, which were evaluated by a second operator (LA). Both operators were masked for subject age. Ninety-six randomly selected images of the layers from 20 to 50 μm were defined as the intrafollicular layers, at the largest diameter point of the follicle. To precisely characterize IEL, all subjects underwent IC of the superior tarsum and immunofluorescence staining with antibodies directed against lymphocyte CD3 and CD20 antigens.

The CD3 complex serves as a T-cell coreceptor that associates noncovalently with the T-cell receptor (TCR). Both TCR and the CD3 protein complex are defining features of lymphocytes belonging to the T-cell lineage and, therefore, can be used as T-cell markers (for both CD4 and CD8 lymphocytes).22 CD20 is an activated-glycosylated phosphoprotein expressed on the surface of all B-lymphocytes beginning at the

The presence of these structures was also evaluated.

**Impression Cytology (IC)**

To precisely characterize IEL, all subjects underwent IC of the superior tarsum and immunofluorescence staining with antibodies directed against lymphocyte CD3 and CD20 antigens.

The Crypt-Associated Lymphoid Tissue and HEVS.

The Diffuse Lymphoid Layer. Cellular elements located beneath and within the conjunctival epithelium, morphologically different from epithelial cells, were considered. The following parameter was evaluated: the density of presumed lymphocytes (LyD). Presumed lymphocytes were counted calculating nonepithelial cells in the whole scanning image (400 × 400 μm), distinguishing cell populations on the basis of their size, morphology, and reflectivity. The Cell Count software (Heidelberg Engineering) was used, and the final counts of cells were the averages of 36 randomly selected high-quality images (where conjunctival subepithelium was regularly represented) taken from the upper and lower tarsal conjunctiva (six for each sector) and eight images taken from the bulbar parts.

**Follicles.** Subepithelial roundish/oval-shaped structures resembling lymphoid follicular spots were considered. To standardize the analysis of the organized CALT, the zone from 0 to 20 μm was defined as the superficial epithelial dome, and the layers from 20 to 50 μm were defined as the intrafollicular layers. The following follicular parameters were considered: follicular density (FD), follicular area (FA) (area of each follicle calculated using ImageJ [http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA]), perifollicular lymphocytes density (FLyD), and follicular reflectivity (FR) (this parameter depended on the complexity of the inner connective reticular architecture and was quantified with an arbitrary grading scale, which ranged from 1 to 4, where 1 indicated low reflectivity, 2 medium reflectivity, 3 high reflectivity, and 4 very high reflectivity; Figs. 2D–G). As for superficial lymphocytes, these cells were evaluated and counted using the Cell Count software (the final counts of cells were the averages of five randomly selected images). The final counts of follicular parameters were the averages of 36 randomly selected high-quality images (400 × 400 μm) taken from the upper and lower tarsal conjunctiva (six for each sector) and eight images taken from the bulbar parts. FA, FR, and FLyD were measured at the intra-follicular layers, at the largest diameter point of the follicle.

**Crypt-Associated Lymphoid Tissue and HEVS.** The presence of these structures was also evaluated.

**Comparison with other methods**

To compare the results obtained with the LSCM and IC, we compared the results obtained with LSCM and IC of 14 human subjects (6 men and 8 women, mean age 50 ± 8 years) on whom a complete ophthalmic examination had been performed.

**Classification of the lymphoid follicle reflectivity at the point of the largest diameter, according to the complexity of the inner connective reticular architecture: 1, low reflectivity (D); 2, medium reflectivity (E); 3, high reflectivity (F); and 4, very high reflectivity (G). Scale bar: 100 μm.
pro-B phase (CD45R- , CD117+) and progressively increasing in concentration until maturity. Impression cytology was performed from 28 to 32 hours after confocal microscopy to avoid misinterpretation due to the mechanical pressure during execution of IVCM. The central part of the superior tarsum, which was the largest and still marked with the methylene blue, was chosen for the examination and centered with the IC stripping membrane.

Briefly, after topical anesthesia with oxybuprocaine hydrochloride 0.4% (Novesin; Novartis, Basel, Switzerland), a strip was applied to the central part of the superior tarsal conjunctiva, within the borders of the marked site, and then pressed gently by a glass rod. Impression cytology samples were collected using Millicell-CM 0.4 mL (12 mm of diameter) (Millipore, Bedford, MA, USA); cells were fixed with cytology fixative (Biox; Bio Optica, Milan, Italy).

For CD3 and CD20 immunofluorescence staining, the Millicell membranes were hydrated with distilled water and placed in 80% alcohol for 2 minutes. The membranes were washed in distilled water and PBS was added for 2 minutes, followed by two washes with Wash Buffer (Dako, Glostrup, Denmark) of 2 minutes each. Then the filters were incubated with ribonuclease A (Sigma-Aldrich Corp., St. Louis, MO, USA) diluted 1:1500 in PBS for 20 minutes at room temperature. The samples were washed and anti-rabbit Alexa fluor 488 (Invitrogen, San Diego, CA, USA) 1:50 or CD20 (Dako) 1:25, both diluted in antibody diluent (Dako), were incubated overnight at 4°C. Samples were washed and anti-rabbit Alexa fluor 488 (Invitrogen, San Giuliano Milanese, Italy) for CD3 or anti-mouse Alexa Fluor 488 (Invitrogen) for CD20, diluted 1:200, and propidium iodide at 1:150 (both in antibody diluent) (Dako), were added and incubated for 1 hour at room temperature. Membranes were mounted with a drop of Fluorescent Mounting Medium (Dako) and the cells visualized with a Zeiss confocal laser-scanning microscope (510; Carl Zeiss Microlmaging, GmbH, Vertrieb, Germany). Five different fields for each sample were evaluated and two masked expert observers (AM and LB) in concordance counted the positive-staining cells.

The LyD, FD, and FA, were the primary outcomes. Follicular reflectivity and FlyD with IVCM, and CD3 and CD20 positivity at IC were secondary outcomes. The modification of all these parameters with aging was also evaluated.

Statistical Analysis

The sample size calculation indicated that at least 100 patients were necessary to have a z of 0.05 and a β of 0.80. The overall relationships between primary and secondary outcome measures, aging and sex, were evaluated with Curve Estimation Analysis for linear, quadratic, and cubic regression models. The maximum/minimum and inflection points that represent the ages at which the rate of decrease changed or inverted trend were estimated from plotted data. For post hoc analysis, subjects were divided into three groups based on age: early (<40 years), middle (40–60 years), and late (>60 years) adulthood and differences between groups were tested using Pearson χ² test. Statistical analysis was conducted using IBM SPSS Statistics for Mac, Version 20.0 (IBM Corp., Armonk, NY). Significance was set at P = 0.05.

RESULTS

Demographic and Clinical Data

The age ranged for all 108 subjects from 17 to 75 years. Mean IOP and CCT (SD) were 13.66 (2.35) mm Hg and 551.53 (13.94) μm, respectively. No macroscopic abnormalities were found in the bulbar, tarsal, and fornix conjunctiva, after complete slit-lamp examination and photographic analysis.

Presence, Structure, and Topographic Distribution of CALT

In vivo confocal microscopy documented the presence of CALT in all subjects, and three different forms of lymphoid tissue were seen: a diffuse subepithelial layer of presumed lymphocytes, subepithelial roundish structures probably representing follicles, and conjunctival crypts with aggregations of presumed lymphocytes. Follicles and the diffuse lymphoid layer were found in all subjects; the crypt-associated lymphoid tissue was found in the 32% of subjects and presented as interfollicular crypts, but was not found in the isolated form. Lymphoid structures were almost exclusively observed throughout the tarsal regions of the conjunctiva: only in the upper tarsum in 97% of subjects, and very infrequently in the lower (5% of cases in both superior and inferior tarsum).

Conversely, confocal microscopy was not able to find lymphoid tissue within the bulbar conjunctiva. Thus, the anatomic description of CALT, and statistical analysis of considered parameters, derived from images taken at the superior tarsal conjunctiva.

Diffuse Lymphoid Layer

The diffuse lymphoid layer was located within the lamina propria of the subepithelium (20–30 μm of depth), with features of an inhomogeneous carpet-like layer of hyperreflective spots (Figs. 3A–D). This layer presented several cellular elements (presumably lymphocytes or plasma cells), which were very hyperreflective, smaller in size, and somewhat irregularly shaped (void or multilobed) when compared with superficial epithelial cells. Scattered or isolated presumed lymphocytes (IEL), with the same characteristics, were detected also within the carpet of superficial epithelial cells (10–20 μm of depth) (Figs. 4A, 4D). The cellular features of presumed lymphocytes appear similar to those of tarsal conjunctiva inflammatory cells described in vivo in healthy and inflamed eyes. Lymphocyte density ranged from 7.8 to 165.8 cells/mm² (46.42±18.37%; mean [SD]) and showed a significant reduction with age (P < 0.001) (Fig. 5A), with a more marked and steep decrease in the first 35 years of life and after 65 years; in middle age (40–60 years) the density presented a plateau-like phase. The positivity for CD3 and CD20 at IC, confirmed that presumed IELs were immune cells. Although statistical analysis was not performed on IELs, this lymphocyte population also presented an evident reduction with age at IVCM (Figs. 4A, 4D). This was confirmed with immunofluorescence, which showed intra-epithelial CD20- and CD3-positive cells in 65% and 80% of subjects younger than 40 years (Figs. 4B, 4C, 6), in 70% and 60% of subjects aged from 40 to 60 years, and in 30% and 20% of subjects older than 60 years, respectively (Figs. 4E, 4F, 6) (P < 0.05).

Lymphoid Follicles

Most of the lymphoid follicles presented an oval/lenticular shape, both the dome and deep pole were distinguishable (Figs. 2A–C), and were located beneath the subepithelial lymphocyte layer (20–80 μm of depth). Each follicle presented well-defined borders, were slightly more reflective than surrounding tissue, and showed a loosely arranged and low-reflective network-like feature probably representing the inner connective framework. Because follicles are three-dimensional structures, the reflectivity was not homogeneous and was
higher at the intermediate layers, and lower at the dome and deep pole (Figs. 2A–C). Follicles were frequently surrounded by a lympho-epithelium corresponding to inter-follicular crypts, which hosted scattered hyperreflective spots of cells probably representing lymphocytes (Figs. 3E–H).

Follicular density (follicles/mm²), FA (μm²), FLyD (cells/mm²), and FR ranged from 0.5 to 19.4 (5.3 [3.6]; mean [SD]), 1110 to 96,280 (26,440 [26,280]; mean [SD]), 1.1 to 107.9 (17.5 [21.3]; mean [SD]), and 1 to 4 (2.8 [1.0]; mean [SD]), respectively, and showed a significant reduction with age (P < 0.001, R² = 0.597, 0.887, 0.840, and 0.914, respectively) (Figs. 5B–E). Subepithelial lymphocytes and follicles were rare in subjects older than 75 years. Although FD and FLyD decreased similarly to LyD, with a plateau-like phase between 40 and 60 years, FA showed a less evident plateau-like phase. Conversely, FR progressively and significantly (P < 0.001) increased with age.

The comparison between men and women for all parameters for the entire population, and for the three groups...
considered for the IC, did not show statistically significant differences ($P > 0.05$).

**Crypt-Associated Lymphoid Tissue and Presumed HEVs**

Crypt-associated lymphoid tissue presented features of inter-follicular grooves hosting scattered or clustered hyperreflective cells at bottom (probably lymphocytes) (Figs. 3E–H, 7A). In vivo confocal microscopy was not able to identify the isolated form of crypts in any of the subjects. Even though no parameter was defined to statistically analyze this structure, intracryptal lymphocytes evidently reduced with aging. Presumed HEVs were difficult to identify in vivo and were sometimes (6% of subjects) observed within the follicles, rather than within the lymphoid layer. Compared with blood vessels (low endothelial venules) (Fig. 7B), presumed HEVs presented a thicker and moderately hyperreflective wall with larger and hyperreflective cellular elements inside (Fig. 7C). Because of the difficulty in identifying presumed HEVs, modifications with aging were not analyzed.

**DISCUSSION**

Laser scanning IVCM documented the presence of CALT in all healthy subjects, with an evident involution associated with aging. These observations were consistent with previous ex vivo studies, which reported lymphoid follicles in most human conjunctival tissues (from 50% to 100% of cases),7,25,26 with a decrease in number with age.12 Moreover, our findings were in accordance with histology also concerning the structural organization because CALT comprised a diffuse lymphoid layer, follicles, and the interfollicular crypt-associated lymphoid tissue, with the lymphoid layer and follicles being the structures more represent-
findings seem to be in line with previous studies. Thus, our study also supported the hypothesis that lymphoid structures represent a normal component of the conjunctival immune system and not an inflammatory response to stimuli.

To date, IVCM has been used to study CALT in rabbits, to evaluate the toxicity of antiglaucoma drugs (prostaglandin analogs and their preservative [benzalkonium chloride]), and cell reactions occurring during conjunctival inflammation models. In humans, Zhivov et al. first described the characteristics of CALT in vivo in healthy subjects, reporting features in line with histology. In our study, follicles and interfollicular crypts presented at IVCM the same characteristics described by Zhivov and coworkers. Later, Liang et al., in a study that evaluated CALT in rabbits under inflammatory stimuli, documented lymphoid follicles also in two young human patients with vernal keratoconjunctivitis, with features similar to, although smaller, than those observed in rabbits. Even though Liang et al. analyzed eyes with an ocular inflammatory condition, our findings were in accordance with their findings because follicles presented as well-defined roundish structures, with internal collagen architecture, located beneath the subepithelial lymphoid layer.

Postmortem and in vivo studies reported that follicular diameter ranged from 200 to more than 300 μm. In this study, we considered the follicular area rather than the diameter, because a two-dimensional parameter better expresses the global dimension of an irregular structure. Therefore, a direct comparison with previous studies that reported results using only the diameter was not possible. Nonetheless, because in young to middle-aged subjects the follicles almost filled the entire confocal image, which was 400 × 400 μm, our findings seem to be in line with previous studies.

In the present study, we did not find significant differences in follicular parameters between males and females. These results seem to be in agreement with those reported in rabbits, where from adolescence, the number of follicles in males and females is similar (females presented 1% fewer follicles than males). Previous histological studies in humans were not reported.

Besides follicles, and in line with histological evidence, IVCM identified a carpet of subepithelial hyperreflective elements probably representing lymphocytes, and scattered IEL dispersed within the superficial layers in every normal human conjunctiva. Immunohistochemistry confirmed that these intraepithelial cells were CD3-positive T-lymphocytes and CD20-positive B-lymphocytes. Nevertheless, we cannot confirm the cellular composition of the diffuse layer because IC acquires a sample of only the more superficial layers of the epithelium.

In our study, the crypt-associated lymphoid tissue was less frequently identifiable with respect to the other lymphoid components, probably because the anatomical organization of the conjunctiva relegates this part of the CALT to less accessible locations in vivo. Similarly, the identification of HEVs was difficult because, in vivo, it is challenging to differentiate between blood and lymphatic vessels. Nonetheless, presumed HEVs presented different features compared with blood vessels that are thick-walled structures. This was consistent with electron microscopy findings where HEVs were described as venules with an increased height of the endothelial cells surrounded by a basement membrane and a pericyte layer.

Also, the topographic distribution of CALT was consistent with that reported in histological studies, with lymphoid structures mainly representing within the superior tarsal conjunctiva.

As previously hypothesized, this particular distribution permits CALT, when projected onto the ocular surface during eye closure, to overlap the cornea and occupy a suitable position to promote corneal immune protection during blinking and overnight.

The present study confirmed the results of a previous study that reported that CALT features and distribution depended on the age, because significant age-related modifications of all lymphoid structures were found. The main modifications were an evident decrease of the cellular density in the superficial lymphoid layer, along with a reduction of the density and area of follicles.

These results were in line with the well-documented decrease of the mucosal immunity during aging. In the intestine, where mucosal lymphoid structures are well represented, Peyer's patches are present at birth, and increase their number and size during the first 10 years of life; they rapidly decline by age 20, followed by a slower decline for the remainder of life, but are never fully lost. The same changes have been described in the bronchus- and larynx-associated lymphoid tissues.

In the eye, similar age-dependent variations were reported in the tear-duct-associated lymphoid tissue (TALT), LDALT, and CALT. Concerning human CALT, follicles were not found in the newborn, then became most numerous just before puberty, and slowly decreased in number until old age. Similar modifications were reported in rabbits, where the number of follicles increased until adolescence, stabilized through early adulthood, and dramatically declined in elderly rabbits. In our study, information from the pre-pubertal period, when lymphoid structures present a florid developing phase, lack. For the rest of life, our results were in line with literature concerning follicle density, which showed an evident decline with age, but appeared opposite concerning follicular size, because area decreased. In fact, in rabbits, diameter was reported to increase with age. Because studies that evaluated the follicular size modifications with age in humans are not available, comparison cannot be done. We hypothesize that the longer life of humans may differently modify the lymphoid structures during aging and, thus, follicular size may present a different behavior with respect to rabbits. Aged follicles presented a more densely arranged network of hyperreflective fibrils compared with young follicles, with rare cells: this aspect was probably due to involution phenomena, which consisted of connective fiber deposition and lymphocyte loss. These features were in line with signs of aging described in postmortem lymph nodes, which presented an age-dependent tendency toward the replacement of areas populated with immune cells by connective and lipomatous tissue.
Also, the diffuse lymphoid layer and IEL presented an evident involution with age, with a progressive reduction of the subepithelial cellular density, and the CD3 and CD20 positivity.

In summary, all these atrophic modifications of CALT may be intended as a part of the age-related involution that similarly affects most ocular surface structures, such as meibomian glands and conjunctival components.\textsuperscript{36,37}

The present study has some limitations. First, due to technical difficulties in performing confocal microscopy in children, there is a lack of information of CALT features during childhood, when the mucosal immunity is flourishing. Thus, we cannot confirm, in vivo, previous results that reported that follicles were not present in the newborn and increased just before puberty.\textsuperscript{5,6} Further in vivo studies are required to explore in vivo CALT in this interval. Second, due to technical difficulties, we did not study orbital and fornical parts of the conjunctiva. Nonetheless, as reported by Knop and Knop,\textsuperscript{6} the fornix did not represent the preferred location of CALT. Third, even though we were able to discriminate different cellular subtypes on the basis of size, shape, and reflectivity, confocal microscopy cannot certainly characterize the single immune cells present within the subepithelial CALT structures. Similarly, in vivo we can only presume that particular vessels, morphologically different with respect to the most part of observed blood vessels, were really HEVs.

Moreover, the calculation of the volume (even though the compression during examination may significantly affect this variable) would have defined more accurately the whole follicular structure. Therefore, further studies aimed at describing the three-dimensional feature of follicles and modifications with aging, are warranted. Finally, we cannot confirm that observed CD3 and CD20 positivity corresponded to real values, as the mechanical stress applied during IVCM could have led to lympho-cellular immigration within the epithelium and increased the density of presumed lymphocytes. To minimize this likelihood, minimal pressure during examination was applied.

**CONCLUSIONS**

There is growing evidence that conjunctival lymphoid tissue is involved in pathogenesis of several ocular surface diseases, such as inflammatory and autoimmune disorders, dry eye, infections, and toxic reactions to medications.\textsuperscript{18,38–40} Laser scanning confocal microscopy was successfully used to assess CALT and could be a pertinent tool in the future for the comprehension of ocular surface defense mechanisms in normal and pathological conditions. Further studies evaluating the in vivo modifications of CALT in ocular surface disease could define the role of the mucosal immune system in their development and evolution.

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