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SUPPORTING INFORMATION

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Respiratory allergies with no associated food allergy disrupt oral mucosa integrity

To the Editor,

Respiratory allergy is the most common allergy worldwide. Studies in Europe have shown that up to 30% of the population suffers from allergic rhinoconjunctivitis, while up to 20% suffer from asthma. This group of allergic diseases is characterized by an aberrant immune response to aeroallergens. The prevalence of aeroallergens varies in different regions depending on the climate.[1] Patients overexposed to grass pollen are frequently sensitized to the pan-allergen profilin. In fact, we have recently demonstrated that a fraction of these patients develops severe profilin-mediated food reactions that are associated with intense oral mucosa remodeling,[2] and with unique metabolic and transcriptomic signatures.[3]

In the present study, we aim to investigate whether oral mucosa is compromised in the absence of food allergy. We formulated the hypothesis that an intense systemic inflammation could induce structural changes in the oral mucosa after respiratory sensitization.

To test this hypothesis, we took advantage of respiratory phenotypes associated with olive pollen (seasonal allergy) and mites (perennial allergy), previously described.[4,5] We recruited patients with respiratory allergy to olive pollen from Cordoba, Spain (n = 5), defined by clinical history and with positive skin prick test (SPT) to olive allergens Ole e1 and/or Ole e7, and patients with allergy to house dust mites (HDM) from Gran Canaria, Spain (n = 6), who experienced urticaria, respiratory distress or pruritus after ingestion of mite-contaminated flours. Clinical and demographic data of allergic participants are summarized in Table 1. Oral mucosa biopsies were obtained from the cheek lining of the subjects by using a scalpel or punch and used for histological and immunohistochemical analyses (AppendixS1). We analyzed structural components and cell infiltrate in oral mucosal samples from these patients compared with a control group of nonallergic subjects (n = 5).

First, we studied whether the oral mucosal barrier integrity was compromised. For this purpose, we determined tight junction (TJ) and adherens junction (AJ) protein expression. We observed a significant and similar decrease in occludin and E-cadherin expression in both groups of allergic patients in accordance with previous results[2] (Figure 1A-D). However, we could not find significant differences in claudin-1 expression (Figure 1E,F) as previously reported.[2] Next, we studied fibrosis in the oral connective tissue of these patients. Augmented fibrosis has been previously associated with epithelial disruption.[2,6.] We observed increased collagen deposition in olive pollen and HDM-allergic patients (Figure 1G,H). An affected epithelial barrier together with increased fibrosis of the oral mucosa suggest that extensive structural changes are taking place in respiratory phenotypes associated with olive pollen and mites.

Increased angiogenesis has been previously described in the oral mucosa of patients with inflammatory pathologies.[7] No significant differences were found in this study for the angiogenic factor VEGFa (data not shown). However, when measuring capillary density, an increased number of blood vessels was observed in HDM-allergic patients (Figure 1I). Regarding immune recruitment to the oral mucosal tissue, we could not find major differences in immune cell counts when comparing allergic groups with nonallergic controls. We focused on phagocytes, mast cells, innate lymphoid cells and lymphocytes. For this purpose, the expression of langerin, MCT, FcεRI, CRTH2, CD3, CD4 and FoxP3 was determined. Numbers of langerin+ cells among the experimental groups were similar; however, FcεRI expression in these cells tended to be higher in the oral epithelium of allergic patients (data not shown). Regarding the connective tissue, MCT+ cells represented almost 100% of the FcεRI-expressing cells in nonallergic subjects. In contrast, FcεRI/MCT ratio revealed that other cell types were also FcεRI+ in the allergic populations (Figure 1J). CD3+ and CD4+ counts were higher in the connective tissue of nonallergic subjects (data not shown). The majority of CD3+ cells were also CD4+ (Figure 1K). Among the CD3+ cells, HDM patients showed a significantly increased proportion of regulatory T cells (Tregs). Although not significant, this trend was also found for olive pollen allergic patients (Figure 1L). We also stained for eosinophils (Figure 1M) and neutrophils (Figure 1N) but found hardly any positively stained cell.

Based on these results, we conclude that oral mucosal integrity is compromised in respiratory phenotypes in the absence of food allergy. Moreover, oral mucosa structural changes take place with no associated local recruitment of inflammatory infiltrate, which is presumably directed to the airway mucosa, but higher numbers of

Abbreviations: AJ, adherens junction; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; FFPE, formalin-fixed paraffin embedded; HDM, house dust mites; ILC, innate lymphoid cell; MCT, mast cell tryptase; S1P, sphingosine-1-phosphate; SPT, skin prick test; TJ, tight junction; Treg, regulatory T cell; VEGFa, vascular endothelial growth factor A.

TABLE 1 Detailed information on study allergic patients

Age	Sex	Specific IgE (kU/L)							Positive SPT	FEV1/FVC	FVC	FEV1	BT	Self-reported reactions[A]	NSAID intolerance
		Total IgE (kU/L)	*Ole e1*	*Ole e7*	*Ole e9*	*D Pte*	*D Farinae*	*L Destructor*							
Olive															
25	M	193	0	79.40	0	Nd	Nd	Nd	Ole	90	134	118	NEG	NC, RHI, RESP	No
18	M	193	0	85.50	0	Nd	Nd	Nd	Ole, lol, cyp	102	99	98	NEG	NC, RHI, RESP, PRU	No
40	F	Nd	8.05	72.20	0	0.82	Nd	0	Ole, pte	98	125	123	NEG	NC, RHI, RESP	No
18	F	3301	241	696	181	Nd	Nd	Nd	Ole, lol, sal, cyp, alt, cat, dog	95	115	111	NEG	NC, RHI, RESP	No
31	M	240	7.47	19.60	0	Nd	Nd	0	Ole, lol, cyp, cat, lepi	94	81	75	POS	NC, RHI, RESP	No
HDM															
39	F	1113	Nd	Nd	Nd	22	14	5	Pte, far, lepi, tyro, blo	107	95	99	POS	UR, EE, RESP, DIZZ	Yes
39	F	47	Nd	Nd	Nd	1.57	1.42	0.70	Pte, far, lepi, tyro, blo	92	85	74	POS	RHI, RESP, PRU, EE, UR	Yes
36	F	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Pte, far, lepi, tyro, blo, a siro	106	79	86	NEG	RHI, RESP, PRU, CG	Yes
44	F	180	Nd	Nd	Nd	4	4	0.80	Pte, far, lepi, blo, a siro	105	88	91	POS	RHI, RESP, PRU, AE, CG	Yes
40	F	116	Nd	Nd	Nd	20	13	1.60	Pte, far, lepi, tyro, blo, a siro	93	97	87	POS	NC, ABP, DI, VO, DIZZ, PRU	Yes
37	F	67	Nd	Nd	Nd	8.50	Nd	1.30	Pte, far, lepi, tyro, blo	96	111	102	NEG	CG, TO, AE, PRU, HY, F	Yes

Abbreviations: M/F, male/female; Nd, nondetermined parameter; NSAID, nonsteroidal anti-inflammatory drugs. Test: FEV1/FVC: Tiffeneau-Pinelli index. FVC:forced vital capacity. FEV1: first second of forced expiration volume. B.T: bronchodilation test. SPT: ole: *Olea europea*, lol: *Lolium perenne*, sal: *Salsola kali*, cyp: *Cupressus arizonica*, alt: *Alternaria alternata*; *pte*: *Dermatophagoides pteronyssinus*, *far: Dermatophagoides farinae*, *lepi: Lepidoglyphus destructor*, *tyro: Tyrophagus putrescentiae*, *blo: Blomia tropicalis*, *a siro: Acarus siro*. Reactions[A] Olive pollen allergy: reactions reported during pollen-season/HDM allergy: reported during last anaphylactic episode. ABP: abdominal pain, AE: angioedema, CG: cough, DI: diarrhea, DIZZ: dizziness, EE: eyelid edema, F: fainting, HY: hypotension, NC: nasal congestion, PRU: pruritus, RESP: respiratory difficulties, RHI: rhinitis, TO: thorax oppression, UR: urticaria, VO: vomit.

Tregs. In this sense, Morita et al reported that IL-33-stimulated mast cells promoted expansion of Tregs in a mouse model of papain-induced allergic inflammation.[8] In this model, IL-2 produced by IL-33-stimulated mast cells promoted Tregs expansion, which suppressed papain-induced airway inflammation. Tregs have been described to contribute to tissue homeostasis by promoting wound healing and repair processes. Thus, we hypothesize that the increased numbers of Tregs we observed in allergic patients are recruited to protect against tissue damage and maintain barrier integrity.

Since we could not find increased recruitment of inflammatory cells in the oral mucosa of respiratory allergic patients, we hypothesize that systemic changes could account for the altered oral mucosal features observed in these phenotypes. Under inflammatory conditions, epithelial cells can release IL-33. This alarmin disrupts epithelial barrier and activates type 2 innate lymphoid cells (ILC2s). ILC2s and

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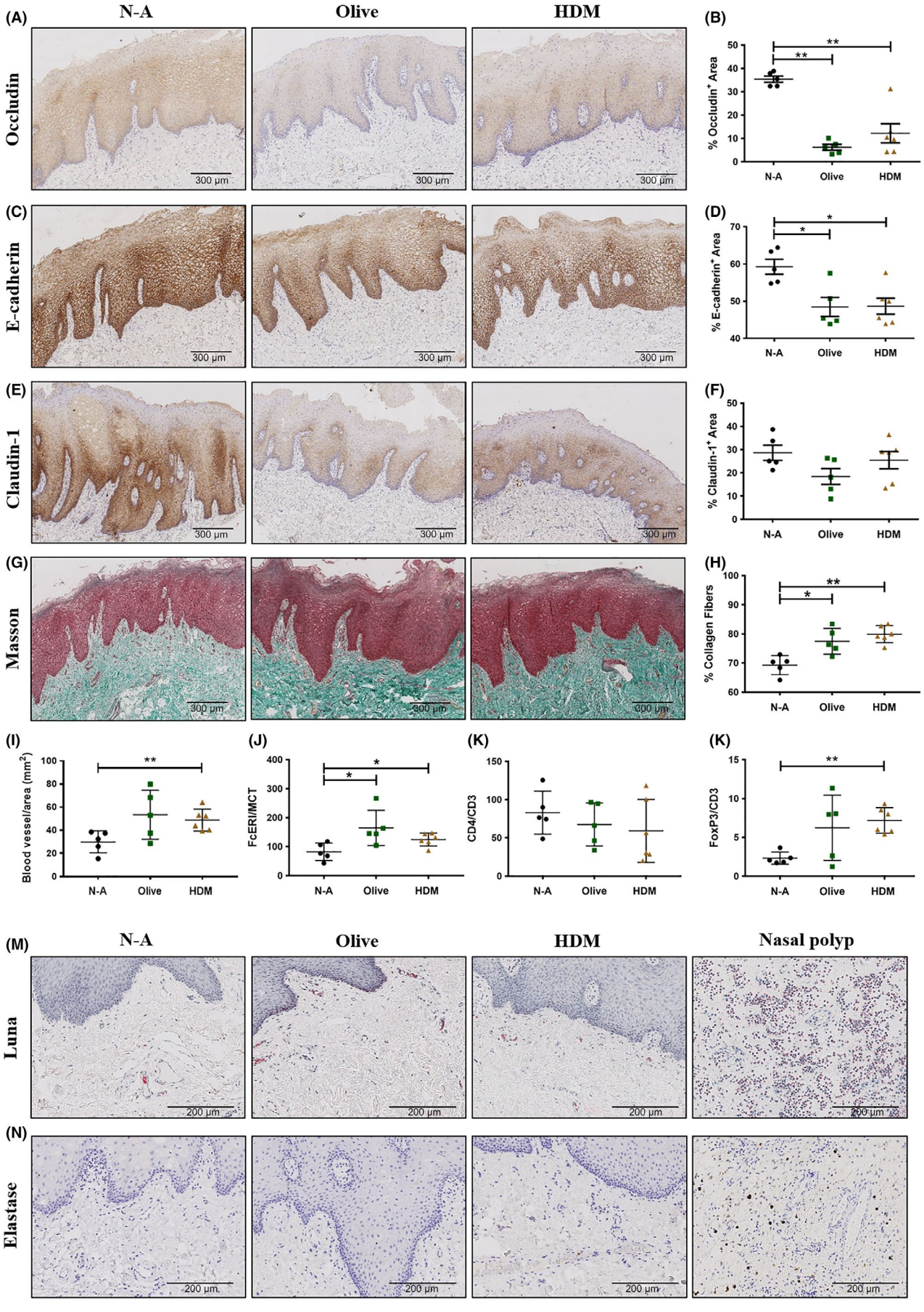

FIGURE 1 Histological and immunohistochemical analyses of FFPE oral mucosal sections from nonallergic subjects (N-A) and patients allergic to olive pollen (Olive) or HDM (HDM). Staining and quantification for occludin (A-B), E-cadherin (C-D), claudin-1 (E-F), and collagen fiber deposition (Masson trichrome) (G-H) in all groups. Quantification is the percentage-stained area (mm^2) of epithelial (B, D, and F), or connective tissue area (H). Capillary density (blood vessel/mm^2) in the connective tissue (I). FcεRI + cells in the connective tissue expressed as the percentage of total MCT+ cells (J). Frequency of CD4+ (K) and FoxP3+ (L) cells expressed as the percentage of total CD3+ cells in the whole tissue. Luna (M) and elastase (N) representative images for each experimental group. Nasal polyp was used as a positive control. Images were captured at 8X (A, C, E, and G), and 20X (M, N) magnification. Scatter plots show mean ± SD $^*P < 0.05$, $^{**}P < 0.01$

Th2 cells, and their associated cytokines, play major roles in type 2 inflammation. It has been reported that Th2 cytokines, such as IL-4 and IL-13, induced impaired barrier function in bronchial epithelial cells. IL-13 appeared to be a key effector cytokine in ILC2-induced bronchial epithelial leakiness in the study by Sugita et al[9] This cytokine has TJ disruptive effects and induces IgE production. Although we could not find differences in Th2- or ILC2-associated markers locally, in the multi-omics study of severe profilin-mediated food allergy, Obeso et al described increased sphingosine-1-phosphate (S1P) and lysophospholipids synthesis along with altered platelet functionality systemically.[3] We believe that systemic changes may also reflect the leaky oral mucosal barrier found in the patients of the current study.

To our knowledge, this is the first study describing an affected oral mucosa in the absence of food allergy and opens new ways of understanding barrier alterations associated with respiratory allergy. A disrupted barrier could be responsible for the progression to food allergy when the affected oral mucosa gets in direct contact with a trigger.[2] In fact, many food allergies are associated with previous respiratory sensitizations (PR10, lipid transfer proteins, profilins).[10]

These reports highlight the need to understand how systemic inflammatory factors affect the integrity of TJs and to further investigate common metabolic and transcriptomic fingerprints that might be useful for the identification of new therapeutic targets to prevent allergic progression.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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The potential role of CD16^{high}CD62L^{dim} neutrophils in the allergic asthma

To the Editor:

Asthma is a chronic disease, characterized by chronic inflammation, airway obstruction and airway hyperresponsiveness. Neutrophils and eosinophils both contribute to disease development and aggravation, as they are found in the airways and lungs of patients with asthma.

Previous research has emphasized the importance of eosinophils in allergic asthma, whereas neutrophils have not been extensively studied. This is likely because the neutrophils are present within the lung at all times, even in asymptomatic patients. Nevertheless, blood neutrophils have been shown to increase during the first hours after allergen bronchial challenge test[1] as well as during the late asthmatic airway response (LAR).[2] The role and perception of neutrophils changed when Pillay et al[3] identified three subsets of neutrophils. In this study, neutrophils were subdivided based on their expression of CD16 and CD62L, each subset representing a different stage of maturity and activity. Neutrophils released from the bone marrow express low levels of CD16 and high levels of CD62L; this CD16^{dim}CD62L^{high} subset is considered to represent the immature neutrophil. These neutrophils are absent in the circulation in healthy donors, but appear rapidly after an lipopolysaccharide (LPS) challenge, and are even more pronounced in severely injured patients. Neutrophils expressing high levels of CD16 and CD62L (CD16^{high}CD62L^{high}) are mature, but nonactivated, whereas the CD16^{high}CD62L^{dim}-expressing neutrophils are believed to be mature and activated and play an important role in systemic inflammation.[3] We have recently shown a role for these subsets in allergic rhinitis[4] and an in vitro model of asthma.[5] In the latter, the CD16^{high}CD62L^{dim} subset (produced by LPS in vitro stimulation) was found to enhance the response to bradykinin in both human bronchioles and murine tracheae. No such effects were obtained for the other neutrophil subsets. The response was due to an upregulation of bradykinin receptor 2, through release of TNFα by the neutrophils.[5]

Using flow cytometry, we examined the presence and the distribution of the CD16^{high}CD62L^{high} and the CD16^{high}CD62L^{dim} subsets in peripheral blood from patients with allergic asthma during an exacerbation induced by an inhaled allergen bronchoprovocation test (Figure 1A). The bronchial challenge test presently used is an exacerbation model of allergic asthma common in research settings.[6] Regional Ethical Review Board (2012/800) approved the study, and all participants provided written informed consent.

Nine nonsmoking patients (aged 18-50 years; 6 males and 3 females) with a physician-confirmed diagnosis of allergic asthma (according to GINA[7]) were included. All of them exhibited less than 40% of activated neutrophils in peripheral blood before the bronchial challenge test. All patients had a positive skin prick test and elevated levels of specific IgE to the allergen used for their respective challenge (3 cat, 2 birch, 2 grass pollen and 2 horse allergen (ALK-Abello)). Seven of the patients could be classified as eosinophilic phenotype based on their blood eosinophils (>150 cells/mcL). One patient had a slightly lower number of eosinophils, and one patient cannot be defined due to lack of data. Five patients had only an early asthmatic response following allergen, while four patients presented with both an early and a late repose (Table 1). Blood samples were collected in sodium heparin tubes before and 24 hours after completion of the allergen bronchial challenge test. Cells were stained and analysed using flow cytometry. Neutrophils were gated on CD45, CD16 and CD62L (Figure 1B,C). Statistical analyses were performed using GraphPad Prism software (version 6.0, GraphPad