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Genetic variations in the *TLR3* locus are associated with eosinophilic esophagitis

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Abstract

Background: Eosinophilic esophagitis (EoE) is an antigen-driven disease mediated by an abnormal immune Th2 response.
Objective: The objective of this article is to investigate genes associated with regulating immune responses leading to disease susceptibility.
Methods: Twenty-seven tag single nucleotide polymorphisms (tSNPs) selected in five candidate genes (*TLR3*, *TLR4*, *FOXP3*, *FLG* and *TSLP*) were genotyped in 218 EoE patients and 376 controls. Skin prick tests were carried out in EoE patients with a panel of 17 aeroallergens and 22 plant- and animal-derived foods.
Results: Five tSNPs located in the *TSLP* locus and one tSNP located in the *TLR3* locus were significantly associated with EoE. The interactions between *TLR3* and *TSLP* loci were analyzed. *TLR3*+/*TSLP*− and *TLR3*−/*TSLP*+ individuals showed a significantly reduced susceptibility to EoE compared to *TLR3*−/*TSLP*− individuals (OR = 0.66, $p = 0.036$ and OR = 0.23, $p = 0.00014$, respectively). Likewise, *TLR3*+/*TSLP*+ individuals showed the most decreased susceptibility of developing EoE (OR = 0.16, $p = 0.0001$). However, the interaction gain attributed to the combination of both genes was negative (IG = −4.52%), which indicated redundancy or independent effect. Additionally, *TLR3* locus was found to be associated with aeroallergen and food sensitization in EoE patients (OR = 9.67, $p_c = 0.025$ and OR = 0.53, $p_c = 0.048$, respectively).
Conclusion: *TLR3* constitutes a novel genetic susceptibility locus for developing EoE, and the effects would be independent of *TSLP*.

Keywords

Eosinophilic esophagitis, genetic susceptibility, thymic stromal lymphopoietin, Toll-like receptor, single nucleotide polymorphism

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Key summary

Summary of established knowledge on this subject

1. Eosinophilic esophagitis (EoE) results from an abnormal immune response to food and airborne allergens, which are characterized by a predominantly Th2-type inflammatory response.
2. Thymic stromal lymphopoietin (*TSLP*) has been identified as a major candidate gene involved in the pathogenesis of the disease.
3. Primary esophageal epithelial cells express *TSLP* in response to Toll-like receptor 3 (*TLR3*) signaling.

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What are the significant and/or new findings of this study?

1. We have replicated the association of the *TSLP* gene with EoE.
2. This study describes the *TLR3* gene as a novel genetic susceptibility locus for developing EoE for the first time.
3. The *TLR3* gene was found to be associated with aeroallergen and food sensitization in EoE patients.
4. Our results suggest that there is a non-synergic effect between *TSLP* and *TLR3* genes, which suggests that the interaction of both genes have redundancy or independent effects.

Introduction

Eosinophilic esophagitis (EoE) is a chronic inflammatory condition that is clinically characterized by symptoms of esophageal dysfunction and histologically characterized by eosinophil-predominant inflammation.[1] The current annual incidence of EoE is estimated to be 7.2 new cases per 100,000 people, and the prevalence in males is approximately 2.5-fold higher than in females.[2] EoE results from an abnormal immune response to food and airborne allergens, and most patients with EoE also exhibit several atopic diatheses, including allergic rhinitis, bronchial asthma and eczema, which are the most common.[3] EoE patients also have a high rate of immunoglobulin (Ig)E-mediated food allergy, which is estimated to be 10 times higher compared to the general population, which suggests that the pathogenesis of EoE and atopy involve common processes[4,5] characterized by a predominantly T helper 2 (Th2)-type inflammatory response.[6] EoE today represents one of the most relevant topics in gastroenterology and allergology, but after 20 years of research on the causes of this disorder, a full explanation of the pathophysiology of EoE has yet to be determined. The familiar associations of cases reveal common genetic predispositions that underlie the increased concordance of EoE in monozygotic (58%) and dizygotic twins (36%) compared to siblings (2.4%).[7] Research on candidate-gene identification and genome-wide association studies (GWAS) have identified multiple genes that are likely contributing to the development of EoE,[8–10] among which thymic stromal lymphopoietin (*TSLP*) has a predominant position.[11] TSLP is a cytokine produced by esophageal epithelial cells, which acts by driving dendritic cells (DCs) toward a Th2 response and constitutes an essential link between epithelial cell activation and allergic inflammation.[12] The eosinophilic infiltration of EoE can therefore be considered the consequence of Th2-type inflammation driven by TSLP secreted by esophageal epithelial cells, which appears under the influence of a genetic predisposition.[10,11] In addition to *TSLP*, calpain 14 (*CAPN14*), *EMSY, LRRC32, STAT6, ANKRD27* and *CCL26* are known to play relevant roles in the origin of EoE.[13]

However, the same studies of a familiar association and others assessing the effects of early life exposures on the risk of EoE also demonstrate the substantial influence that the environment (including exposure to dust mites and microorganisms, foods consumed, antibiotic use and even the esophageal microbiota)[14] has through epigenetic mechanisms. Changes in the esophageal microbiota linked to EoE are arising as a novel but potentially essential causative factor in triggering and maintaining EoE[15] with Toll-like receptors (TLRs) as the interaction point between bacteria and mucosal immunity. In fact, the activation and maturation of antigen-presenting cells (APCs) and regulatory T cells (Tregs) depend on TLR-mediated signaling. TLRs are transmembrane pattern recognition receptors located in intestinal epithelial cells and basal lamina that respond to microbial signals and distinguish different types of pathogens from commensal bacteria.[16] Increasing evidence points toward a relevant role for genetic polymorphisms affecting TLR function in inappropriate inflammatory responses.[16] TLR2, 4, 6, 7, 8 and 9 are significantly associated with allergic asthma and atopy,[17,18] and TLR4-dependent signals provided by the intestinal commensal flora inhibit the development of allergic responses to food antigens;[18,19] TLR3 can signal inflammatory responses in human epithelial cells.[20,21] Preliminary results have documented changes in the expression levels of several TLRs that reverse after effective dietary therapy.[22]

An impairment of immune homeostasis maintained by CD4+CD25+FOXP3+Tregs, which is a subtype of T cell that expresses the interleukin (IL)-2 receptor alpha chain (CD25) and the transcriptional regulator forkhead box P3 (FOXP3) protein, also arises as potentially involved in EoE. Tregs are important components in the immunoregulatory suppression of T cell proliferation and function, which directly or indirectly suppresses effector cells in allergic inflammation, including eosinophils.[23,24] Tregs cells are significantly increased in the esophageal tissue of EoE patients, which suggests that a negative feedback mechanism exists to regulate an inflammatory response triggered by external stimuli or allergen exposure.[25] In this sense, TLR4 is associated with the amplification of the suppressive function of Tregs and may influence allergic responses to food antigens.[18]

On the other hand, polymorphisms in epithelium-specific genes have also been associated with EoE

susceptibility. A loss of function caused by a single nucleotide polymorphism (SNP) in the filaggrin gene (*FLG*) (2282del14) is associated with EoE independent of the atopic status of the patient.[26] IL-13, which is a Th2-type cytokine that is over-expressed in esophageal epithelial cells from EoE patients, strongly decreases *FLG* expression.[26,27]

With this background, our research aims to analyze the role of genes associated in regulating immune responses of patients with EoE. First, we will evaluate whether the *TLSP* loci are associated with a risk for developing EoE in a Spanish Caucasian population. Second, possible associations between *TLR3, TLR4, FOXP3*, and *FLG* and susceptibility of presenting EoE will be studied.

Patients and methods

Patients

A total of 218 adult patients with EoE (170 males; mean age at diagnosis 35.4 years) and 376 healthy ethnically matched bone marrow donors were selected for this study. To avoid the confounding effects of the genetic associations investigated with other conditions, EoE patients who had other autoimmune conditions (i.e. celiac disease, diabetes mellitus and thyroid disorders) were excluded. All patients and controls were unrelated Spanish Caucasian individuals who were recruited at two Spanish hospitals: Hospital Universitario Virgen del Rocío (Sevilla) and Hospital General de Tomelloso (Ciudad Real). This study was approved by the Comité de Ética de la Investigación del Hospital Universitario Virgen del Rocío, Sevilla, on April 9, 2012 (Code 2012PI/079). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institutions' human research committee. Informed consent for the procedures was obtained from all patients included in the study.

Participants were diagnosed as having EoE if they met the following accepted criteria:[28] (i) symptoms related to esophageal dysfunction, (ii) one or more mucosal biopsies that demonstrated at least 15 eosinophils per high-powered field (hpf), (iii) persistence of eosinophilic infiltration after an eight-week trial with a proton-pump inhibitor drug at double doses, and (iv) exclusion of other potential causes of esophageal eosinophilia.

Allergic sensitization tests

To evaluate allergic comorbidities, skin prick tests (SPTs) were carried out in every EoE patient by using standard methods[29] with a panel of 17 commonly distributed aeroallergens (including mites, molds, pollen and animal dander) and 22 plant- and animal-derived food antigens (milk, egg, shrimp, squid, clam, cod fish, chicken meat, walnut, peanut, pepper, tomato, potato, onion, soy, lentil, carrot, peach, kiwi, melon, banana, wheat and rice). SPTs were performed at the flexor surface of the forearm with reactions read out after 15 to 20 minutes. SPTs were positive if the mean wheal diameter was $\geq$3 mm over the negative control. Saline solution and histamine solution were respectively used as negative and positive controls. Sensitization to airborne or food allergens was considered as a dichotomous variable and was exclusively based on the positivity or negativity of skin allergy testing.

SNP selection and genotyping

Genomic DNA was extracted from blood leukocytes using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and stored at –20ºC.

Five candidate genes previously identified as relevant for EoE pathophysiology were selected based on their function in innate host defense: *TLR3, TLR4, FOXP3, FLG* and *TSLP*. Tag single nucleotide polymorphisms (tSNPs) were selected across each locus from the designated set of common SNPs genotyped in the Central European-like Utahns (CEU) population (HapMap Project, Release 28, Phase II+III, National Center for Biotechnology Information (NCBI) build 36 assembly, dbSNP b126; http://www.hapmap.org). The tSNPs selection was performed with pairwise $r^2 \geq 0.80$ and minor allele frequency (MAF) ≥ 0.05 using the Haploview v4.0 software (http://www.broad.mit.edu/mpg/haploview/download.php).[30] According to the above rules, 27 tSNPs that permitted us to capture 52 SNPs were selected (Table 1). Genotyping was performed using TaqMan® SNP Genotyping Assays (Applied Biosystems, Barcelona, Spain) in a LightCycler 480 (Roche, Barcelona, Spain). To verify inter-experimental reproducibility and accuracy, 8% of the samples were duplicated. A 90% sample quality control rate and 90% SNP genotyping success rate was imposed on the analysis.

Statistical analyses

Allele frequency distributions were compared using the χ^2 test, and a corrected p value (p_c) was calculated from 10,000 permutations (Haploview program). Genotypes of each SNP were assessed according to dominant (AA vs. AB+BB (A, major allele; B, minor allele)) or recessive (AA+AB vs. BB) models. The odds ratios (ORs) with their corresponding 95% confidence intervals (95% CIs) and logistic regression were calculated

Table 1. Tag SNPs included in this study.

Gene	CHR	Tag SNPs	SNPs captured
TLR3	4	rs5743303	rs5743303, rs5743312, rs3775296
		rs5743305	rs5743305
		rs11721827	rs11721827, rs11730143, rs11732384
		rs7657186	rs7657186, rs13108688
		rs13126816	rs13126816, rs6552950
		rs7668666	rs7668666
		rs3775292	rs3775292
		rs3775291	rs3775291
TLR4	9	rs1927914	rs1927914, rs2149356, rs1927911
		rs10759932	rs1927907, rs10759932, rs5030717
		rs5030728	rs5030728, rs2770146
		rs12377632	rs12377632
		rs11536889	rs11536889
		rs7873784	rs7873784
FLG	1	rs2065956	rs7522925, rs2065956, rs12407748
		rs2065958	rs2065958
		rs11582620	rs11582620
		rs11204981	rs2184953, rs11204981, rs3126066, rs11584340, rs3120659, rs11204980, rs6587666
		rs11586114	rs11586114, rs11204978, rs2065955
FOXP3	X	rs2280883	rs3761548, rs2280883
		rs3761549	rs3761549, rs3761547
TSLP	5	rs2289276	rs11466741, rs2289276
		rs1898671	rs1898671
		rs10062929	rs11466750, rs10062929
		rs2289277	rs2289277
		rs2289278	rs2289278
		rs11466749	rs11466749

CHR: chromosome; SNP: single-nucleotide polymorphism; *TLR*: Toll-like receptor; *FLG*: filaggrin gene; *FOXP3*: forkhead box P3; *TSLP*: thymic stromal lymphopoietin.

using OpenEpi v3.01 software online (http://www.openepi.com). *P* values < 0.05 were considered statistically significant.

Gene-gene interactions were evaluated using the non-parametric multifactor dimensionality reduction (MDR) method.[31,32] To interpret combination effects, an entropy-based analysis was used. Interaction entropy uses information gain to gauge whether interactions between two (or more) variables that were considered independent exist.[33] Entropy is estimated for each individual attribute (i.e. main effects) and each pair-wise combination of attributes (i.e. interaction effects). Therefore, the main effects of each factor can be compared to the interaction effect to determine whether interactions are additive or non-additive. There is evidence for a synergistic interaction when a positive value is obtained combining two (or more) variables. Conversely, there are redundancy or independent effects when a negative value is obtained by loss of information.[33]

Results

Aeroallergen and food sensitization, as estimated by a positive SPT result, were found in 72.22% and 61.46% of the whole series of 218 patients with EoE, respectively. The successful rate of genotyping was >98% for all SNPs included, and genotypes were unequivocally assigned for all the cases except in 11 controls. The study population was found to be in the Hardy-Weinberg equilibrium for all the polymorphisms that were analyzed ($p > 0.05$).

No statistically significant differences were found between EoE patients and control individuals in the distribution of the allelic frequencies of any of the SNPs of *TLR4, FOXP3* and *FLG* loci (data not shown). However, five tSNPs located in the *TSLP* locus and one tSNP located in the *TLR3* locus were significantly associated with EoE (Table 2). Since the prevalence of EoE in males is higher than in females, a gender-stratified analysis of EoE patients and controls was carried out to determine whether *TSLP* and *TLR3* variants contribute to this gender bias (Table 3). Significant differences remained unchanged between patients and controls in the male group, and only the rs2289276 polymorphism in the *TSLP* gene was not associated after *p* correction ($p = 0.013$ and $p_c = 0.065$). Regarding female patients, no significant differences in the distribution of alleles were found, and the trend in the distribution was similar to the one observed in the male group. The *TSLP* gene polymorphism rs10062929 was found to be associated before *p* correction ($p = 0.016$).

To further clarify whether the contribution of *TSLP* and *TLR3* loci to EoE susceptibility detected in univariate analyses might have synergistic effects, a logistic regression test was performed to analyze interactions between the two polymorphisms with the highest statistical significance (rs3775292 of the *TLR3* locus and rs10062929 of the *TSLP* gene). In both polymorphisms, statistically significant differences were observed between patients and controls in a dominant model (Table 4). Therefore, patients and controls carrying a C allele (CG or CC) in rs3775292 were classified as TLR3+. Similarly, individuals carrying the A allele

Table 2. Minor allele frequencies of the SNPs studied in the *TSLP* and *TLR3* genes in EoE patients and healthy controls.

Gene SNP	MA	Controls ($n = 365$)	Patients ($n = 218$)	p	p_c	OR (95% CI)
TSLP						
rs2289276	T	0.309	0.233	0.0078	0.0429	0.68 (0.51–0.90)
rs1898671	T	0.389	0.484	0.0023	0.0142	1.47 (1.14–1.89)
rs10062929	A	0.123	0.036	6,18E-06	1.0E-05	0.68 (0.51–0.90)
rs2289277	G	0.432	0.298	1,71E-05	1.0E-04	0.67 (0.51–0.90)
rs2289278	G	0.046	0.045	0.941	NS	
rs11466749	G	0.135	0.071	0.0018	0.0106	0.68 (0.51–0.90)
TLR3						
rs5743303	T	0.192	0.210	0.5072	NS	
rs5743305	A	0.415	0.344	0.0172	0.0584	
rs11721827	C	0.082	0.077	0.7728	NS	
rs7657186	A	0.251	0.194	0.0247	NS	
rs13126816	A	0.246	0.305	0.0298	NS	
rs7668666	A	0.276	0.273	0.9166	NS	
rs3775292	C	0.271	0.196	0.0039	0.0288	0.65 (0.49–0.87)
rs3775291	T	0.309	0.334	0.3732	NS	

SNP: single-nucleotide polymorphism; MA: minor allele; EoE: eosinophilic esophagitis; *TSLP*: thymic stromal lymphopoietin; *TLR3*: Toll-like receptor 3; OR (95% CI): odds ratio with 95% confidence interval; NS: not significant $p > 0.05$. p_c: corrected p value, 10,000-fold permutation testing.

Table 3. Distribution of allelic frequencies of the SNPs studied in the *TSLP* and *TLR3* genes after stratification by gender.

		Males					Females				
Gene SNP	MA	Controls ($n = 174$)	Patients ($n = 170$)	p	p_c	OR (95% CI)	Controls ($n = 191$)	Patients ($n = 48$)	p	p_c	OR (95% CI)
TSLP											
rs2289276	T	0.318	0.229	0.013	0.0654		0.299	0.244	0.3079	NS	
rs1898671	T	0.366	0.493	0.0013	0.0063	1.68 (1.22–2.31)	0.397	0.453	0.3395	NS	
rs10062929	A	0.132	0.038	7.66E-05	0.0003	0.26 (0.12–0.53)	0.113	0.027	0.0167	0.0807	0.20 (0.04–0.85)
rs2289277	G	0.443	0.287	6.13E-05	0.0003	0.26 (0.12–0.53)	0.425	0.333	0.1219	NS	
rs2289278	G	0.042	0.054	0.4625	NS		0.053	0.013	0.1287	NS	
rs11466749	G	0.145	0.065	0.0016	0.0084	0.26 (0.12–0.53)	0.128	0.090	0.3429	NS	
TLR3											
rs5743303	T	0.200	0.216	0.6372	NS		0.175	0.188	0.7914	NS	
rs5743305	A	0.426	0.348	0.0353	NS		0.410	0.330	0.1562	NS	
rs11721827	C	0.066	0.078	0.5152	NS		0.084	0.073	0.5176	NS	
rs7657186	A	0.253	0.189	0.0456	NS		0.250	0.208	0.3951	NS	
rs13126816	A	0.233	0.291	0.0908	NS		0.264	0.356	0.0870	NS	
rs7668666	A	0.280	0.288	0.8200	NS		0.263	0.219	0.3700	NS	
rs3775292	C	0.284	0.190	0.0036	0.0282	0.59 (0.41–0.84)	0.258	0.218	0.4277	NS	
rs3775291	T	0.286	0.323	0.2899	NS		0.333	0.375	0.4429	NS	

SNP: single-nucleotide polymorphism; MA: minor allele; *TSLP*: thymic stromal lymphopoietin; *TLR3*: Toll-like receptor 3; OR (95% CI): odds ratio with 95% confidence interval; NS: not significant $p > 0.05$.
p_c: corrected p value, 10,000-fold permutation testing.

Table 4. Distribution of genotypes of the SNP rs3775292 (*TLR3*) and rs10062929 (*TSLP*) and dominant and recessive models.

			Dominant model		Recessive model	
	Controls	EoE patients	*p*	OR (95% CI)	*p*	OR (95% CI)
*TLR3*_rs3775292						
CC	28	9	0.0042	0.60 (0.42–0.85)	0.09	0.52 (0.22–1.10)
CG	144	67				
GG	193	142				
*TSLP*_rs10062929						
AA	6	2	1.1E-6	0.23 (0.11–0.44)	0.36	0.36 (0.01–2.46)
AC	78	13				
CC	276	203				

Model dominant: CC+CG vs GG (*TLR3*) and AA+AC vs CC (*TSLP*).
Model recessive: CC vs CG+GG (*TRL3*) and AA *vs* AC+CC (*TSLP*).
SNP: single-nucleotide polymorphism; EoE: eosinophilic esophagitis; *TLR3*: Toll-like receptor 3; *TSLP*: thymic stromal lymphopoietin; OR (95% CI): odds ratio with 95% confidence interval.

Table 5. Frequencies of combinations of rs3775292 (*TLR3*) and rs10062929 (*TSLP*) in EoE patients and controls.

	TLR3-/TSLP-	TLR3+/TSLP-	TLR3-/TSLP+	TLR3+/TSLP+
Patients ($n = 218$)	127 (58.25%)	77 (35.33%)	9 (4.13%)	5 (2.29%)
Controls ($n = 360$)	145 (40.27%)	132 (36.65%)	46 (12.77%)	37 (10.27%)
OR (95% CI)	1	0.66 (0.44–0.96)	0.23 (0.10–0.53)	0.16 (0.05–0.47)
p	<0.0001	0.036	0.00014	0.00010

OR (95% CI) and p values were calculated using logistic regression.
EoE: eosinophilic esophagitis; *TLR3*: Toll-like receptor 3; *TSLP*: thymic stromal lymphopoietin; OR (95% CI): odds ratio with 95% confidence interval.

(AA or AC) in rs10062929 were classified as TSLP+. Individuals with 0 copies of both SNPs alleles (TLR3–/TSLP–) were used as a reference group. A statistically significant lower susceptibility to EoE was found in individuals carrying TLR3+/TSLP– and TLR3–/TSLP+ compared to individuals carrying the TLR3–/TSLP– genotype with OR = 0.66 ($p = 0.036$) and 0.23 ($p = 0.00014$), respectively. Furthermore, the susceptibility to EoE was reduced substantially among individuals carrying TLR3+/TSLP+ with an OR = 0.16 ($p = 0.0001$) (Table 5). To complement logistic regression analyses, MDR software was used to further evaluate gene-gene interactions. For analyzing whether the observed interaction between both attributes, *TLR3* and *TSLP*, had or did not have a synergistic effect, we found a negative interaction gain value (IG = –4.52%) (Table 6). This negative value occurred because the percentage of entropy removed by the interaction of both *TLR3* and *TSLP* was 3.36% less than the sum of the percentage of entropy removed by each attribute (0.78% and 7.09%, respectively). This result suggests that the interaction of both genes had redundancy or independent effects.

Finally, the possible associations between *TSLP* and *TLR3* gene polymorphisms and the allergic phenotype of EoE patients was investigated. Case-only phenotype analyses of patients with EoE revealed no association between *TSLP* and sensitization to food or inhalant allergens. In contrast, two SNPs in the *TRL3* locus, rs11721827 and rs5743303, were found to be associated with sensitization to aeroallergens and food, respectively. The frequency of the rs11721827C allele was significantly higher among EoE patients sensitized to aeroallergens (9.4% vs. 1.1% in non-sensitized patients, $p_c = 0.025$, OR = 9.67). In contrast, the frequency of the rs5743303T allele was lower among patients with EoE who were sensitized to food (17.5 vs. 28.5%; $p_c = 0.048$) with an OR of 0.53 (Table 7).

Discussion

This study describes the *TLR3* gene as a novel genetic susceptibility locus for developing EoE for the first time. Additionally, we have replicated the association of the *TSLP* gene with EoE described in an American

Table 6. Information gain estimated for each individual locus and combination of both loci.

	Information gain by each locus						Information gain by combination of both loci			Interaction gain
Attributes	H (A)	H (AC)	H (B)	H (BC)	I (A; C)	I (B; C)	H (AB)	H (ABC)	I (AB;C)	IG (A; B; C)
TLR3	1.2493	1.2414			0.0078 (0.78%)					
TSLP			0.9629	0.892		0.0709 (7.09%)				
TLR3/TSLP							0.9987	0.9651	0.0336 (3.36%)	**−0.0452 (−4.52%)**

H (A), H (B), H (AB): entropy attributed to *TLR3* and *TSLP* individually and the combination of both loci.
H (AC), H (BC), H (ABC): conditional entropy attributed to *TLR3* and *TSLP* individually and the combination of both loci.
I (A; C) = H (A) − H (AC): information gain (percentage of entropy removed) by *TLR3* attribute (main effect).
I (B; C) = H (B) − H (BC): information gain (percentage of entropy removed) by *TSLP* attribute (main effect).
I (AB; C) = H (AB) − H (ABC): information gain (percentage of entropy removed) by the combination of both attributes (interaction effect).
IG (A; B; C) = I (A; C) + I (B; C)−I (AB; C): interaction gain by the combination of both attributes.
TLR3: Toll-like receptor 3; *TSLP*: thymic stromal lymphopoietin.

Table 7. Minor allele frequencies of the SNPs studied in the *TLR3* gene in EoE patients.

		Aeroallergens					Food allergy				
TLR3 SNP	MA	Negatives ($n = 54$)	Positives ($n = 164$)	p	p_c	OR (95% CI)	Negatives ($n = 84$)	Positives ($n = 134$)	p	p_c	OR (95% CI)
rs5743303	T	0.255	0.199	0.2521	NS		0.285	0.175	0.014	0.048	0.53 (0.32–0.88)
rs5743305	A	0.362	0.351	0.8575	NS		0.331	0.367	0.4906	NS	
rs11721827	C	0.011	0.094	0.0071	0.0255	9.67 (1.29–72.28)	0.038	0.092	0.0603	NS	
rs7657186	A	0.196	0.204	0.8569	NS		0.190	0.208	0.6861	NS	
rs13126816	A	0.315	0.303	0.8297	NS		0.294	0.314	0.698	NS	
rs7668666	A	0.277	0.268	0.873	NS		0.308	0.250	0.2329	NS	
rs3775292	C	0.234	0.196	0.4262	NS		0.223	0.196	0.5358	NS	
rs3775291	T	0.394	0.326	0.2337	NS		0.346	0.342	0.9308	NS	

SNPs: single-nucleotide polymorphisms; *TLR3*: Toll-like receptor 3; EoE: eosinophilic esophagitis; MA: minor allele; OR (95%CI): odds ratio with 95% confidence interval; NS: not significant $p > 0.05$.
p_c: corrected p value, 10,000-fold permutation testing.

cohort of European origin in a Spanish population.[9–11] This result confirms the role played by SNPs in the *TSLP* gene in the pathogenesis of EoE. However, the association between *TSLP* and EoE susceptibility was statistically significant for male patients only, which probably occurred because of the low number of female patients with EoE included in our study. Thus, an equivalent role for *TSLP* in both genders cannot be ruled out since a non-significant trend in SNP frequencies was present in the female group. It is notable that the direction of the disease risk (OR) was similar in this study and previous studies. Therefore, rs2289276, rs10062929, rs2289277 and rs11466749 had an OR < 1 and, in contrast, rs1898671 had an OR > 1.

Since the *TSLP* gene has been identified as a well-established genetic marker that confers risk for developing EoE,[9–11] we analyzed the contribution of *TLR3* in relation to *TLSP* using a logistic regression method. This model suggests that certain alleles of the *TLR3* gene contribute to EoE susceptibility when associated alleles of the *TSLP* gene are absent, whereas *TLR3* could also act as an additional susceptibility factor in individuals with associated *TSLP* alleles. In addition, we used the MDR method[31,32] to better study the interaction between *TLR3* and *TSLP* genes. MDR is a non-parametric and model-free method alternative to logistic regression that is effective for relatively small sample sizes. The interaction entropy analysis revealed no

statistical epistasis between *TLR3* and *TSLP* genes (IG = –4.52%). Biological epistasis is the result of physical interactions among biomolecules within gene regulatory networks and biochemical pathways.[34] In this manner, *TLR3* has been revealed as a key regulator of *TSLP* expression and function.[20,21] *TLR3* recognizes double-stranded RNA (dsRNA), which is found in some viruses, and the stimulation of primary esophageal epithelial cells with poly I:C (a dsRNA mimetic) induces the expression of *TSLP* messenger RNA (mRNA).[19] Although the absence of statistical epistasis does not necessarily imply absence of biological epistasis, our results suggest a non-synergic effect between both loci, which suggests that the interactions of both genes have redundancy or independent effects.

The present study also examined the effects of *TLR3* SNP polymorphisms when EoE patients expressed aeroallergen or food sensitization because *TLR3* has been shown to be involved in the development of these allergic conditions.[17,19] We found that different tSNPs were independently associated with aeroallergen and food sensitization (rs11721827 and rs5743303, respectively), which suggests that the two variants could act as markers of the etiological variants. The influence of the hypothetical etiological variants could be related to differences in the 3′UTR region, the post-transcriptional control, mRNA expression levels or alternative forms of the transduced protein, which would affect its function.

Taken together, we highlight a critical role for the *TLR3* locus in the pathogenesis of EoE. We propose that activation of the innate immune system in the esophageal epithelium involving *TLR3* signaling and expression of *TLSP* mRNA is likely to interact with adaptive allergic responses ultimately triggered by allergens. This finding presents *TSLP* and *TLR3* as new potential molecular targets for therapeutic intervention in EoE, which highlights a key role for innate immunity in the development of specific allergic diseases.

The strengths of our research included the analysis of a large series of patients with EoE collected at two referral centers in Spain as well as the selection of a higher number of control individuals, the development of molecular studies to evaluate SNP of the major genetic markers identified as conferring risk to EoE and key factors involved in regulating mucosal immune responses in allergies. The selection of the control group from healthy bone marrow donors is recognized as the most convenient strategy, but it has led to some controversy in the literature since the donors could not completely represent the whole population of the place that they come from. Volunteers who act as donors for organs and tissues are usually healthy people in whom several illnesses have been excluded. For this reason, donors could easily present a better health status than standard populations. Despite those issues, our control group represents the same populations from where the patients with EoE came from, so we are confident that it does not constitute a drawback for our study.

Finally, since no replication cohort was considered in our study, further studies to reproduce our findings in broader populations and to validate the findings of *TLR3* as a novel genetic susceptibility locus for EoE are warranted.

In conclusion, our research reinforces the role played by *TSLP* polymorphisms in conferring susceptibility to EoE and identifies for the first time that *TLR3* acts as a novel genetic susceptibility locus for developing EoE. This effect would be independent of the previously established association of EoE with *TSLP*.

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The author contributions are as follows: RAC, JRGL and JQ contributed to the conception and design of the research and performed the experiments, analysis and interpretation of the data; SC, AJL and JMB contributed samples, patient information and analysis and interpretation of the data. RAC, JRGL, AJL and JQ drafted the manuscript. All authors read and approved the final version.

Declaration of conflicting interests

None declared.

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Ethics approval

This study was approved by the Comité de Ética de la Investigación del Hospital Universitario Virgen del Rocío, Sevilla (Code 2012PI/079).

Informed consent

Informed consent was obtained from all individual participants included in the study. All participants included in the study consented to the publication of the data extracted from the statistical study. No individual patient data are reported.

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