

RESEARCH LETTER

Gender-specific differences in the molecular signatures of adult Eosinophilic Oesophagitis

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The influence of gender on the prevalence, presentation and pathogenesis of allergic diseases is being increasingly recognized.¹ Eosinophilic oesophagitis (EoE) is a chronic inflammatory disorder of the oesophagus, seen in both children and adults and is three to four times more common in males than females.² Diagnosis of EoE has relied on quantification of oesophageal eosinophils by biopsy and patient-reported symptoms, but recent studies have supported the use of tissue-based molecular biomarker assessment, based on gene expression signatures,³ aiding patient diagnosis.⁴ Strong correlations between gene expression profiles and symptoms were found, particularly dysphagia.⁴ Early work looking at the EoE-associated transcriptional profile concluded that there were no differences between males and females,⁵ but this was performed in childhood EoE and recent studies have demonstrated that gender significantly influences the clinical presentation of adult EoE, particularly dysphagia.⁶⁻⁸ We investigated whether the EoE-associated transcriptional profile of biopsies from adult EoE patients displays differences related to gender.

We assessed the molecular expression patterns of 40 genes associated with specific aspects of EoE pathology, including epithelial barrier, eosinophils, mast cells and cellular proliferation, in pretreatment biopsies from male and female control or EoE patients. Based on a power calculation of sample size required to detect a 30% difference between EoE patients with an alpha probability of .05 and power of .80 for the gene *MS4A2*, which encodes the β chain of the high affinity IgE receptor that we have previously described as elevated in adult EoE,⁹ we investigated the gene expression profiles of 22 adult EoE patients (11 male, 11 female) vs 15 control patients (4 males, 11 females). All patients were undergoing routine assessment for EoE at gastroenterology clinics at Northwestern Memorial Hospital and consented to biopsies for research under IRB Approval number STU00021171. Control patients were undergoing assessment for

oesophageal complaints but determined to not have EoE. Patients were selected randomly by the study coordinator and biopsies identified before being sent to the laboratory for gene expression analysis. Oesophageal biopsies were taken prior to introduction of any specific therapy, but all patients were on proton pump inhibitor treatment, as per standard clinical practice, and reported dysphagia symptoms. As shown in Table S1, no significant differences were found between the ages of Control vs EoE or in male vs female for any group. Eosinophils were significantly elevated in EoE vs control but not different between male and female within the EoE patients ($P=.44$). Endoscopic features typical of EoE were also elevated in EoE but not significantly different between male and female EoE patients.

Real-time RT-PCR (Taqman primer and probe sets, Applied Biosystems, Foster City, CA, USA) was performed on collected biopsies that were immediately placed into RNeasy lysis solution (Qiagen, Germantown, MD, USA) before gene expression assessed relative to the housekeeping gene *GAPDH*, for which all groups were within one cycle threshold of each other. 18/40 genes were statistically different between control and EoE patients using Nonparametric rank two-way ANOVA (full table of ranks and P values shown in Table S2); these included eosinophil-associated genes (*IL5*, *CCL24*, *CCL26*), mast cell-associated genes (*MS4A2*, *TPSAB1*, *HDC*, *KITLG*) and epithelial barrier genes (*SFRP1*, *KRT14*, *DSG1*, *CRISP3*). In considering gender as a variable within each group, significant differences were observed between male and female EoE patients in 8 of these 18 genes, as well as one (*FOS*) that had not achieved significance between the combined populations. As shown in Figure 1A, expression of *CCL24*, *CCL26* and *ALOX15*, key eosinophil-associated transcriptional signatures in EoE, as well as mast cell-associated genes *TPSAB1*, *HDC* and *KITLG* were significantly higher in male over female EoE patients. In contrast, barrier-related genes were

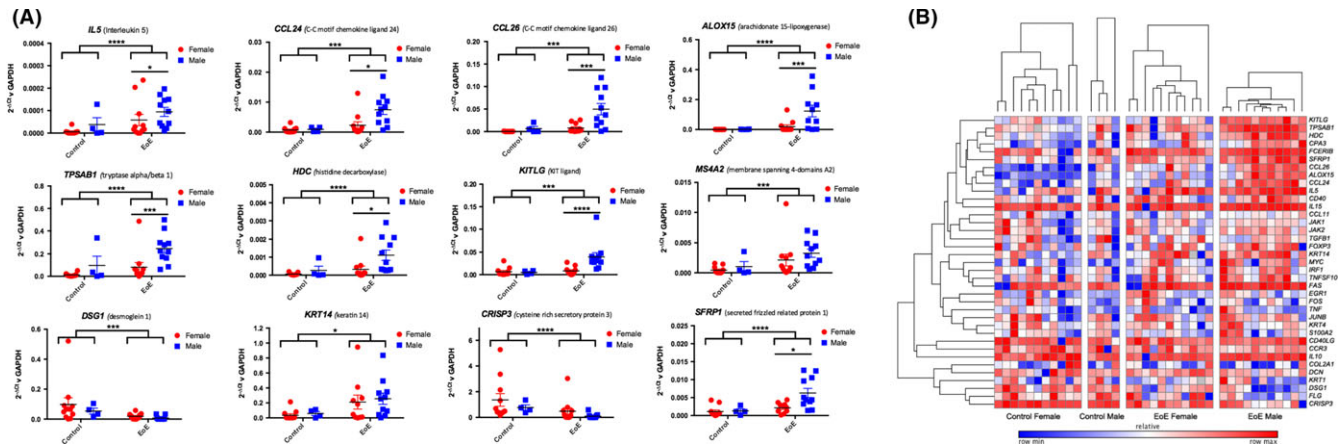


FIGURE 1 Gender-specific differences in the gene expression of adult EoE patients. (A) Gene expression levels relative to a housekeeping gene, GAPDH, was determined by real-time RT-PCR in oesophageal biopsy samples from control or EoE patients. * $P<.05$, ** $P<.005$, *** $P<.001$ by nonparametric rank ANOVA between either all controls and EoE patients or between male and female within each group. (B) Heatmap representation of normalized and log-transformed expression for all genes tested

significantly altered between control and EoE patients but were similar between genders, with only *SFRP1* being higher in male than female. In order to assess global patterns of expression, the relative expression for each individual compared to the average of the control expression level was calculated and log₂-transformed. Figure 1B shows a distinct pattern of expression in EoE male patients that is less evident in EoE female, particularly the high expression of mast cell and eosinophil-associated genes and the lower relative expression of some epithelial or barrier-associated signatures.

We next assessed whether the molecular signatures present in our patient cohort correlated with the pathology reported frequency of eosinophils in the biopsies. No specific gene was found to correlate with peak eosinophil counts (data not shown) and so we utilized principal component analysis (PCA), a method of orthogonal transformation on matrix-type data, to examine the variances between our entire data set (PAST3.12 software). One patient in the EoE group was removed due to nonconformity of the matrix because of the absence of expression data for more than three genes. Principal component 1 (PC1) accounted for 37.7% of the overall variance within the data matrix (Table S3) and showed clustering of all male

EoE patients on the positive side of the axis (Figure 2A). The individual values for PC1 correlated significantly with peak eosinophils in male EoE patients ($P<.05$) but not in female EoE patients (Figure 2B).

Taken together, our findings demonstrate a clear influence of gender on the molecular signatures of biomarkers for EoE. The influence of gender is most prominent within the eosinophil and mast cell-associated markers, perhaps implying a difference in inflammatory processes. Mast cells have been shown to express significant levels of oestrogen, progesterone and androgen receptors,¹⁰ but the biological functions remain poorly defined. The lack of gender influences on barrier-related differences also suggests that mucosal integrity, such as has been described to be associated with the epithelial differentiation cluster (EDC) genes in EoE,¹¹ might be a primary aspect of EoE pathogenesis but that gender-related influences then alter the inflammation-associated signature. This hypothesis might also align with the absence of gender-specific differences in the paediatric EoE patients,⁵ in who sex hormone effects are less likely to participate than occurs in adults. Importantly, the associations between molecular signatures and eosinophils in male EoE patients would suggest that the pathology in these patients is driven

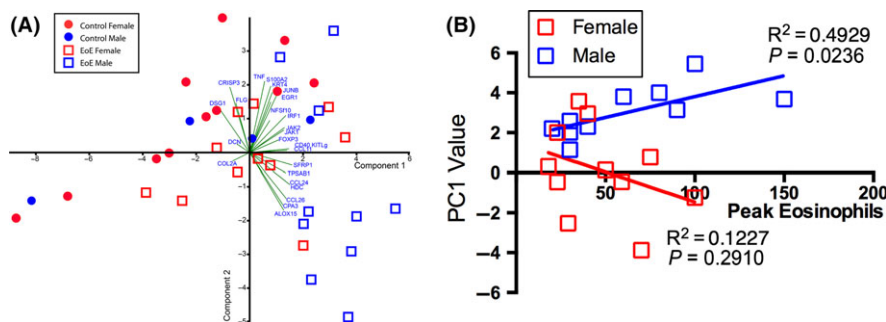


FIGURE 2 Multivariate molecular signatures associate with eosinophils in male but not female EoE. (A) Principal component analysis was utilized to determine principal components most contributing to overall variance with PC1 vs PC2 plotted. Loading influences of individual genes are represented by the linear lines. (B) PC1 score values correlate with peak eosinophils in male but not female EoE. Linear regression for goodness of fit and P value for slope being nonzero were performed using GraphPad Prism

by some synergistic influence of the signatures we examined. In contrast, the lack of association between these in females could suggest that alternative, yet to be determined, mechanisms regulate eosinophils in female EoE. Supporting the concept that gender differences might modify disease pathogenesis, a recent study described an “EoE-like” disease within families where EoE was prevalent and that this phenotype was female dominant¹²; how this new phenotype relates to our patients remains to be determined. One limitation of our study is that we focused on self-reported gender, potentially increasing the risks of variability if there are influences of biological sex status. A second limitation relates to the potential for confounder influences that we are unable to ascertain in these patients, including atopic status, comorbid diseases and ethnicity. We are also unable to conclude whether these differences in molecular signatures between genders are specific to EoE or reflect a broader alteration in oesophageal biology between men and women; gender-related differences in transcriptional signatures have been found in other oesophageal diseases, including Barrett's oesophagus and/or oesophageal adenocarcinoma.¹³ While such mechanistic research is vital to understanding differences between paediatric and adult EoE, our findings presented here offer the initial evidence that gender-related differences in the molecular biomarker signatures of EoE require consideration in understanding adult EoE.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Online Repository

Title: Gender-specific differences in the molecular signatures of adult Eosinophilic Esophagitis

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	Control				EoE				Control v EoE (p values)		
	Total (n=15)	Female (n=11)	Male (n=4)	F v M p value	Total (n=22)	Female (n=11)	Male (n=11)	F v M p value	Total	Female	Male
Mean Age ±SEM (range)	48.5±10.5 (24—60)	51.0±6.8 (35—59)	41.7±15.1 (24—60)	0.15	42.8±15.5 (23—82)	44.9±14.4 (24—78)	40.7±16.2 (23—82)	0.55	0.23	0.24	0.92
Mean Peak Eos ±SEM (range)	0.13±0.5 (0—2)	0±0 (0—0)	0.5±0.9 (0—2)	0.1	53.3±33.6 (18—150)	47.4±24.8 (18—100)	59.1±39.6 (20—150)	0.44	<0.00001	<0.00001	0.016
Endoscopic Features (n)											
Exudate	0	0	0	1.0	10	6	4	0.67	0.002	0.01	0.51
Rings	4	2	2	0.52	20	9	11	0.48	<0.0001	0.009	0.057
Edema	0	0	0	1.0	4	3	1	0.59	0.13	0.21	1.0
Furrows	0	0	0	1.0	17	8	9	1.0	<0.0001	0.001	0.01
Strictures	0	0	0	1.0	14	8	6	0.66	<0.0001	<0.0001	0.1

Online Repository Table 1: Patient demographics and endoscopic results. Eosinophil counts were assessed on biopsy by pathological examination. Endoscopic features were as described in the endoscopic report by a trained gastroenterologist and assessed at the same time as the biopsy taken for pathology. Statistical differences were determined by Fisher's exact test using GraphPad Prism.

		CONTROL RANK		EoE RANK				
Gene (Chromosome)	Protein	FEMALE	MALE	FEMALE	MALE	CONTROL v EOE	FEMALE v MALE	Interaction
<i>SFRP1</i> (8)	Secreted frizzled related protein 1	10.0 ± 2.4	11.8 ± 4.0	17.7 ± 2.4	28.5 ± 2.4	0.0002	0.0366	0.1254
<i>CCL24</i> (7)	C-C motif chemokine ligand 24	10.5 ± 2.5	14.3 ± 4.2	16.6 ± 2.5	28.4 ± 2.5	0.0020	0.0150	0.1987
<i>FAS</i> (10)	Fas cell surface death receptor	11.6 ± 2.8	12.8 ± 4.6	17.3 ± 2.8	27.0 ± 2.8	0.0050	0.1109	0.2021
<i>IL5*</i> (5)	interleukin 5	9.4 ± 2.6	14.5 ± 4.3	19.1 ± 2.6	26.8 ± 2.6	0.0013	0.0481	0.6820
<i>CCL26*</i> (7)	C-C motif chemokine ligand 26	6.7 ± 2.0	15.5 ± 3.4	19.7 ± 2.0	28.5 ± 2.0	<.0001	0.0010	0.9926
<i>MS4A2</i> (11)	membrane spanning 4-domains A2	9.7 ± 2.6	13.5 ± 4.4	19.2 ± 2.6	26.6 ± 2.6	0.0012	0.0862	0.5658
<i>DSG1*</i> (18)	desmoglein 1	25.7 ± 2.6	25.8 ± 4.3	16.3 ± 2.6	9.3 ± 2.6	0.0002	0.2705	0.2675
<i>CRISP3*</i> (6)	cysteine rich secretory protein 3	25.6 ± 2.5	26.5 ± 4.1	16.7 ± 2.5	8.5 ± 2.5	<.0001	0.2234	0.1355
<i>HDC</i> (15)	histidine decarboxylase	10.5 ± 2.5	13.0 ± 4.1	16.5 ± 2.5	28.8 ± 2.5	0.0008	0.0170	0.1022
<i>MYC</i> (8)	MYC proto-oncogene, bHLH transcription factor	14.2 ± 3.0	8.8 ± 5.0	19.9 ± 3.0	23.4 ± 3.0	0.0087	0.7879	0.2314
<i>KITLG</i> (12)	KIT ligand	12.2 ± 2.3	12.8 ± 3.8	13.6 ± 2.3	29.9 ± 2.3	0.0019	0.0044	0.0075
<i>CPA3*</i> (3)	carboxypeptidase A3	10.1 ± 2.6	11.5 ± 4.3	19.5 ± 2.6	26.9 ± 2.6	0.0004	0.1640	0.3387
<i>CCR3*</i> (3)	C-C motif chemokine receptor 3	21.5 ± 3.2	22.0 ± 5.4	17.5 ± 3.2	13.5 ± 3.2	0.1151	0.6588	0.5773
<i>CD40</i> (20)	CD40 molecule	13.8 ± 3.2	15.0 ± 5.4	19.2 ± 3.2	22.2 ± 3.2	0.1145	0.5926	0.8157
<i>IL15</i> (4)	interleukin 15	14.0 ± 2.9	12.0 ± 4.8	15.7 ± 2.9	26.5 ± 2.9	0.0263	0.2184	0.0764
<i>TNFSF10</i> (3)	TNF superfamily member 10	17.2 ± 3.1	9.8 ± 5.2	16.3 ± 3.1	23.5 ± 3.1	0.0959	0.9832	0.0590
<i>COL2A1</i> (12)	collagen type II alpha 1 chain	20.6 ± 3.3	17.8 ± 5.5	19.5 ± 3.3	14.0 ± 3.3	0.5368	0.2988	0.7472
<i>IRF1</i> (5)	interferon regulatory factor 1	15.1 ± 3.3	16.0 ± 5.5	17.6 ± 3.3	22.0 ± 3.3	0.2867	0.5087	0.6644
<i>FLG*</i> (1)	filaggrin	21.2 ± 3.0	27.8 ± 4.9	18.3 ± 3.0	11.0 ± 3.0	0.0092	0.9216	0.0599
<i>FOXP3</i> (X)	forkhead box P3	17.1 ± 3.2	10.0 ± 5.4	18.2 ± 3.2	21.5 ± 3.2	0.1123	0.6335	0.1863
<i>CCL11</i> (17)	C-C motif chemokine ligand 11	16.1 ± 3.2	14.3 ± 5.4	16.4 ± 3.2	22.8 ± 3.2	0.2644	0.5576	0.2945
<i>DCN</i> (12)	decorin	19.9 ± 3.2	17.5 ± 5.3	21.4 ± 3.2	12.9 ± 3.2	0.6874	0.1690	0.4395
<i>IL10</i> (1)	interleukin 10	19.9 ± 3.1	13.0 ± 5.1	12.6 ± 3.1	23.2 ± 3.1	0.6962	0.6257	0.0241
<i>TNF</i> (6)	tumor necrosis factor	20.2 ± 3.3	12.5 ± 5.5	16.5 ± 3.3	19.4 ± 3.3	0.6960	0.5528	0.1924
<i>EGR1</i> (5)	early growth response 1	14.9 ± 3.2	10.3 ± 5.3	21.9 ± 3.2	19.9 ± 3.2	0.0358	0.3878	0.7290
<i>JAK1</i> (1)	Janus kinase 1	16.7 ± 3.4	16.3 ± 5.6	18.6 ± 3.4	19.3 ± 3.4	0.5479	0.9845	0.8918
<i>NFKB1</i> (4)	nuclear factor kappa B subunit 1	13.3 ± 3.2	23.8 ± 5.4	18.6 ± 3.2	19.9 ± 3.2	0.8454	0.1389	0.2434
<i>FOS</i> (14)	Fos proto-oncogene, AP-1 transcription factor subunit	21.7 ± 3.1	7.0 ± 5.2	18.9 ± 3.1	17.2 ± 3.1	0.3321	0.0349	0.0916
<i>S100A2</i> (1)	S100 calcium binding protein A2	18.5 ± 3.3	21.0 ± 5.5	14.4 ± 3.3	20.0 ± 3.3	0.5190	0.3162	0.6915
<i>TGFB1</i> (19)	transforming growth factor beta 1	14.7 ± 3.3	18.8 ± 5.5	19.9 ± 3.3	19.2 ± 3.3	0.4868	0.6824	0.5558
<i>CD40LG</i> (X)	CD40 ligand	21.2 ± 3.3	14.0 ± 5.5	17.5 ± 3.3	16.9 ± 3.3	0.9189	0.3396	0.4113
<i>JAK2</i> (9)	Janus kinase 2	17.7 ± 3.1	10.5 ± 5.2	15.2 ± 3.1	23.8 ± 3.1	0.1592	0.8517	0.0415
<i>KRT4</i> (12)	keratin 4	18.0 ± 3.4	17.5 ± 5.6	16.2 ± 3.4	20.0 ± 3.4	0.9334	0.6845	0.5973
<i>JUNB</i> (19)	jun B proto-oncogene	16.1 ± 3.4	17.5 ± 5.6	19.0 ± 3.4	19.3 ± 3.4	0.5657	0.8362	0.8889
<i>KRT14</i> (17)	keratin 14	11.5 ± 2.8	15.5 ± 4.7	20.3 ± 3.0	25.0 ± 2.8	0.0114	0.2095	0.9242
<i>TPSAB1*</i> (16)	tryptase alpha/beta 1	9.1 ± 2.3	15.3 ± 3.7	17.8 ± 2.2	28.9 ± 2.2	0.0002	0.0032	0.3681
<i>ALOX15*</i> (17)	arachidonate 15-lipoxygenase	7.7 ± 2.0	15.5 ± 3.2	20.5 ± 2.0	29.4 ± 2.0	<.0001	0.0013	0.8055
<i>IL4R</i> (16)	interleukin 4 receptor	13.6 ± 3.0	14.0 ± 5.0	20.1 ± 3.3	21.9 ± 3.0	0.0604	0.7716	0.8472
<i>FN1</i> (2)	fibronectin 1	13.3 ± 3.0	15.3 ± 5.0	19.6 ± 3.3	22.4 ± 3.0	0.0772	0.5185	0.9105
<i>KRT1</i> (12)	keratin 1	19.9 ± 3.3	19.8 ± 5.5	17.6 ± 3.5	17.5 ± 3.3	0.5682	0.9698	0.9986

Online Repository Table 2: Table of significance values for each gene assessed.

Significant differences between the $2^{-\Delta CT}$ values for indicated groups were determined using Nonparametric rank ANCOVAs by ranking each expression variable observation from 0 to 36 and performing normal parametric analysis on the ranked values. Category means > 17.5 indicate more observations in that category were above the median while means < 17.5 indicate more observations were below the median. P values less than 0.05 were considered significant and are highlighted in bold. * indicates genes also present in the EoE Diagnostic Panel ¹

PC	EIGENVALUE	% VARIANCE
1	11.3102	37.701
2	4.27807	14.26
3	2.49066	8.3022
4	1.81845	6.0615
5	1.67195	5.5732
6	1.1886	3.962
7	1.13371	3.779
8	0.985094	3.2836
9	0.865756	2.8859
10	0.702993	2.3433
11	0.566319	1.8877
12	0.475369	1.5846
13	0.393044	1.3101
14	0.377688	1.259
15	0.330794	1.1026
16	0.271296	0.90432
17	0.238649	0.7955
18	0.206534	0.68845
19	0.178438	0.59479
20	0.131724	0.43908
21	0.0960762	0.32025
22	0.0861421	0.28714
23	0.0591991	0.19733
24	0.0444733	0.14824
25	0.0391065	0.13036
26	0.0291442	0.097147
27	0.0117358	0.039119
28	0.0100289	0.03343
29	0.00604558	0.020152
30	0.00272912	0.0090971

Online Repository Table 3: Principle Component Contributions to Variance.

Principle Components Analysis was performed on the matrix-formed dataset and the eigenvalue and relative contribution to variance within the data was determined using PAST software.

Additional Methods:

Bioinformatic Analysis

Data generated by Real-time RT-PCR ($2^{-\Delta CT}$ for each gene versus GAPDH) was normalized to the mean value of the control patient group and Log2 transformed. Heatmap visualization was generated using GENE-E software developed at The Broad Institute and available at <http://www.broadinstitute.org/cancer/software/GENE-E/> Principle Components Analysis was performed using PAST3 software developed at The University of Oslo and available at <http://folk.uio.no/ohammer/past/>

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