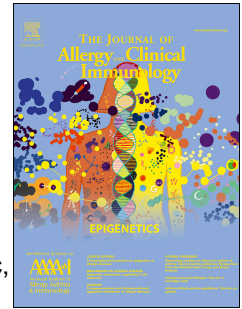


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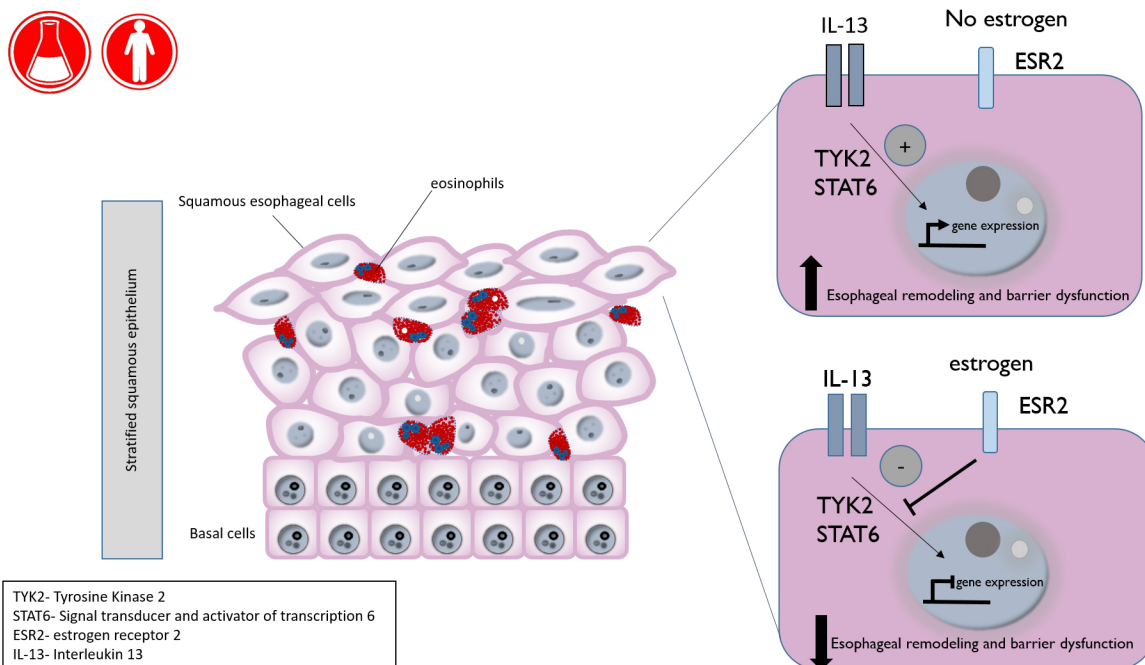
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Estrogen protects esophageal epithelium from IL-13-induced barrier dysfunction and remodeling



17-beta estradiol protects esophageal epithelium from IL-13-induced barrier dysfunction and remodeling

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Key Words: Eosinophilic Esophagitis, interleukin-13, estrogen, hormone, barrier dysfunction

29

30 **Abbreviations:**

31 ALI- Air liquid interface

32 DHT- Dihydrotestosterone

33 DIS- Dilated intercellular spaces

34 DSG1- Desmoglein 1

35 E2 - 17-beta estradiol

36 EoE- Eosinophilic esophagitis

37 EDP- Eosinophilic diagnostic panel

38 EPC2- Primary human esophageal keratinocyte cells

39 ERE- Estrogen Response Element

40 ESR1- Estrogen receptor 1

41 ESR2- Estrogen receptor 2

42 IL4r- Interleukin 4 receptor

43 IL-13- Interleukin 13

44 IL-13 α - Interleukin 13 receptor alpha

45 KSFM- Keratinocyte serum-free media

46 RPKM- Reads per kilobase per million

47 SOCS1- Suppressor of cytokine signaling 1

48 STAT6- Signal transducer and activator of transcription 6

49 TER- Transepithelial resistance

50 TYK2- Tyrosine kinase 2

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Abstract

Background: The incidence of Eosinophilic esophagitis (EoE) is higher in males than females and the underlying molecular basis for this gender bias remains unclear.

Objective: To delineate the contribution of the sex hormone estrogen to the EoE phenotype and esophageal epithelial barrier function and remodeling.

Methods: We performed demographic and incidence analyses of EoE in males and females from a single center pediatric cohort. Estrogen responsive gene expression analyses and estrogen receptor immunofluorescence staining of esophageal biopsies from EoE patients and controls were performed. The effect of E2 on IL-13-induced signaling pathways, gene expression, and esophageal epithelial architecture and barrier function in a primary human esophageal keratinocyte culture system (EPC2-ALI) was examined.

Results: We observed a male predominance in EoE. Analyses of RNA-seq datasets revealed a significant dysregulation of the estrogen responsive gene network and expression of the estrogen receptors ESR1 and ESR2 in esophageal biopsies from EoE patients compared with controls. IL-13 stimulation of EPC2-ALI cells led to altered cellular architecture with induced dilation of intercellular spaces, and barrier dysfunction. Pretreatment of EPC2 cells with E2 prior to IL-13 exposure abrogated IL-13-induced architectural changes and esophageal barrier dysfunction. Mechanistically, E2 protective effects were dependent on ESR2 and associated with diminishing of IL-13-induced TYK2 and STAT6 phosphorylation and EoE dysregulated gene expression.

Conclusions: Estrogen responsive genes are modified in EoE patients compared to controls. E2 attenuated IL-13 induced architectural changes and esophageal epithelial barrier dysfunction via ESR2-dependent process via inhibition of the IL-13/TYK2/STAT6 pathway. Estrogen hormone signaling may protect against development of EoE in females.

Capsule Summary

Eosinophilic esophagitis (EoE) is more common in males than females for reasons that are not yet fully understood. We demonstrate significant dysregulation of the expression of the estrogen receptors and estrogen responsive gene network in EoE. We show that the sex hormone estrogen abrogates IL-13-induced esophageal architectural changes and barrier dysfunction via the downregulation of IL-13-induced Tyk2-STAT6 signaling. These studies suggest that differential estrogen signaling may explain gender-bias in EoE.

Key Messages:

- Eosinophilic esophagitis (EoE) is 3:1 more common in males than females and the underlying molecular basis for the increased male incidence in EoE is unclear.
- Demographic, incidence and RNAseq analyses of EoE in males and females from a single center pediatric cohort we revealed significant dysregulation of the expression of the estrogen receptors and estrogen responsive gene network in EoE.
- Mechanistic analyses on a differentiated primary human esophageal keratinocyte culture system (EPC2-ALI) revealed that the sex hormone estrogen protected the esophageal epithelium from IL-13-induced esophageal architectural changes and barrier dysfunction.
- Estrogen hormone signaling may help protect against development of EoE in females and targeting this pathway maybe an approach for therapeutic intervention for suppression of esophageal inflammation and development of the EoE phenotype.

Introduction:

Eosinophilic esophagitis (EoE) is a chronic, immune mediated disease characterized by eosinophil infiltration of the esophageal epithelium, resulting in impairment of esophageal function and development of feeding intolerance, dysphagia, food impaction, or strictures^{1, 2}. Epidemiological studies in North America, Europe and Asia, have identified that EoE is two to four times more frequent in males than in females³⁻⁶. The EoE male predominance appears to persist across demographic groups, regardless of age, geographical region, socioeconomic status, or race^{4, 7, 8}. Clinical studies indicate that while there is no difference in disease severity between males and females, the clinical and histologic presentation of EoE can differ between the sexes⁴. Moreover, adult male EoE patients are more likely to have stricturing disease and a longer duration of symptoms prior to presentation⁴. Furthermore, male pediatric patients with EoE are more likely to present with food impaction and feeding refusal, while female children with EoE report more abdominal pain⁴. Intriguingly, males are also at greater risk than females of developing other esophageal diseases including GERD, Barrett's esophagus and esophageal adenocarcinoma (1.7:1, 2:1 and 8-9:1, respectively)^{9, 10}. The underlying cause of increased risk of esophageal-related disorders such as EoE in males and whether dissimilarities in EoE between males and females represent differences in disease endotypes or reflective of differential underlying disease-driving mechanisms remains unknown.

Clinical and experimental evidence suggests that the manifestations of EoE are a consequence of chronic esophageal allergic inflammation and impaired epithelial barrier function which drives esophageal remodeling and disease onset¹¹. Dysregulation of expression of a number of key epithelial barrier regulatory genes including desmosomal cadherin desmoglein-1 (DSG1)¹², leucine-rich repeat-containing protein 31 (LRRC31)¹³, kallikrein (KLK) serine proteases¹³, and calpain-14 (CAPN14)¹⁴, have been linked with EoE and shown to alter esophageal epithelial barrier function. Furthermore, Interleukin 13 (IL-13), a known pro-allergic cytokine has been linked to dysregulation of expression of esophageal epithelial barrier genes in EoE^{15, 16}. Indeed, administration of IL-13 to esophageal epithelial cells is sufficient to dysregulate epithelial barrier regulatory gene expression and esophageal barrier function¹². IL-13 binds to either the Type II IL-4 receptor (IL4R α and IL-13R α 1 subunits) or Type II IL-13 receptor (IL-13R α 2)¹⁷ which leads to phosphorylation of receptor associated Janus kinases, including TYK2 and JAK2^{18, 19}. Activation of TYK2 or JAK2 promotes phosphorylation of the cytosolic transcription factor STAT6²⁰⁻²² and recruitment of STAT6 into the nucleus and induction and suppression of STAT6-dependent gene expression including inflammation related genes such as Eotaxin-3 (*CCL26*) and genes related barrier function such as the desmosome related protein desmoglein 1 (*DSG1*)^{12, 23}.

There is emerging evidence that the underlying basis for sex-related susceptibility to various inflammatory conditions are sex hormones²⁴. Sex hormones influence the onset and severity of immune-mediated pathologic conditions by modulating innate and adaptive immunity during prenatal, prepubertal, and postpubertal stages of life²⁵⁻²⁷. Endogenous estrogen hormones of which 17 β -estradiol (E2) is the predominant form, can impact physiologic processes beyond reproductive function, including modulation of inflammation and tissue differentiation, via genomic and non-genomic pathways²⁸⁻³¹. Indeed, many hematopoietic cells including B and T lymphocytes, granulocytes, myeloid cells and NK cell populations express estrogen receptors³². Estrogens are bound in the blood to hormone binding proteins²⁸ and can dissociate from these binding proteins, cross plasma membranes, and stimulate gene expression via an estrogen receptor-dependent and -independent pathways^{29, 30}. Estrogen canonical signaling occurs via binding of estrogens to the cytosolic estrogen receptors ESR1 and ESR2 that can form homodimers or heterodimers²⁹. Ligand binding and dimerization of the ESRs leads to translocation of the ESR to the nucleus, and stimulation of gene expression through binding DNA Estrogen Response Elements (ERE), or indirectly via interactions with other transcription factors in a concentration and tissue specific manner.^{28, 30} Estrogen can also activate rapid signal transduction cascades, such as MAP kinases, upon binding to cytoplasmic ER or membrane bound G-protein coupled receptors, resulting in cytoplasmic alterations or gene regulation.^{29, 30, 33} Herein, we examine the male to female ratio of EoE and association with age of diagnosis. We explore the involvement of the sex hormone estrogen signaling in the EoE transcriptome and define the impact of estrogen on IL-13-induced esophageal barrier function. We observed differences in estrogen responsive gene expression in patients with EoE compared to controls, and show that estrogen receptor expression was increased in esophageal tissue of patients with EoE compared to controls. Employing an *in vitro* EoE model system we show that pretreatment of primary esophageal cells with the estrogen hormone 17- β estradiol (E2) abrogated IL-13-induced barrier dysfunction and associated architectural changes. The suppression of IL-13-induced barrier function was dependent on ESR2-signaling and associated with decreased activation of IL-13-induced TYK2 and STAT6 signaling and downstream IL-13-mediated transcriptional events. Our data suggests that E2 may exert protective effects on the esophageal epithelium through downregulation of inflammation-induced esophageal barrier dysfunction. Collectively, these studies identify potential pathways that may explain the reduced incidence of EoE in females.

Methods:

CCED Cohort Analysis: A retrospective review was performed of the electronic medical record database at the Cincinnati Center for Eosinophilic Disease (CCED). The CCED is part of a pediatric tertiary care referral center, and is affiliated with an adult tertiary care academic medical center (<https://www.cincinnatichildrens.org/cced>). The medical record database includes demographic and histologic data of all patients with a biopsy confirmed diagnosis of EoE treated in the CCED. Inclusion criteria for the query included patients of any age with a diagnosis of EoE and a clinical visit between January 2000 and January 2017. Patients were further divided by sex and age at initial diagnosis.

RNA sequencing (RNA-seq) and bioinformatical analysis: RNA-seq results of human esophageal biopsies were obtained from previous analysis³⁵. Samples were from patients ages 1-34 years at time of biopsy, with all but three patients being <18 years. For RNA-seq of EPC2 ALI cells, RNA was isolated from cells using the RNEasy Kit (QIAGEN Incorporated, Germantown, MD, USA) according to the manufacturer protocol. RNA quality was assessed using the Agilent 2100 Expert bioanalyzer (Agilent Technologies Incorporated, Clara, CA, USA) and only the samples with a RNA Integrity Number (RIN) higher than 8 were processed for sequencing. RNA samples were subjected to RNA sequencing at the CCHMC Gene Discovery and Genetic Variation Core as previously described³⁶. The sequencing reads were aligned against GRCh37 genome model using TopHat2.04 with Bowtie 2.03^{37, 38}, while the separated alignments were unified with Cuffmerge³⁹ using UCSC model as reference. Raw data was then uploaded on Biowardrobe⁴⁰ (<http://biowardrobe.com>) and analyzed using Differential Sequencing (DeSeq) tool.

RNA sequencing and gene expression quantification: Transcriptomic analyses were performed in StrandNGS. After removal of barcodes and primers, raw reads were aligned to the mm10 genome using annotations provided by UCSC and the following parameters: (1) minimum percent identify = 90; (2) maximum percent gaps = 5; (3) minimum aligned read length = 25; (4) number of matched to output per read = 1; and (5) ignore reads with more than 5 matches. The aligner (COBWeb) was based on the Burrow Wheeler Transform method. Reads per kilobase per million (RPKM) were computed using aligned reads and the Expectation-Maximization algorithm for the maximum likelihood estimation of expression. Further, RPKM minimal threshold was defined as 1 and normalized using the DESeq algorithm, which computes a normalization factor (NF) for each sample. Within each sample, each transcript is divided by that transcript's geometric mean across samples. The within-sample median of these values is that sample's NF. To obtain normalized counts, a sample's raw RPKM are divided by that sample's NF. Finally, normalized per-transcript RPKM were baselined to the median of all samples (N=40448 transcripts). Reasonably expressed transcripts (raw RPKM >3 in 100% of samples in at least one condition) were included for differential analysis (N=21096 transcripts). Differential expression was

determined through t-tests ANOVAs with a p-value cutoff of 0.05 and a fold change requirement of >1.5 (M v F in Ctrl N = 63, M v F in EoE N = 191, EoE v Ctrl N = 6647). Data can be accessed through NCBI's GEO repository at GSE58640. Pathway analysis was performed using topptgene.cchmc.org, which collects ontological data from more than 30 individual repositories. The heatmaps were generated using R's heatmap.2 defaults, where the Euclidean measure is used to obtain the distance matrix and complete agglomeration method for clustering.

Immunofluorescent Staining: Formalin-fixed, paraffin-embedded, de-identified human esophageal biopsy samples of post-pubertal (ages 15.2-17.9 years at time of biopsy) male and female healthy controls and patients with EoE were obtained from the CCED slide repository. Slides were de-paraffinized, then underwent antigen retrieval with Tris-EDTA pH 9. The samples were rinsed with PBS, then underwent heat-induced epitope retrieval using a pressure cooker. The tissue was blocked with 4% donkey serum for one hour, then exposed to primary antibodies diluted in PBS. Primary antibodies included ESR1 (rabbit anti-human, 1:1,000 dilution, Invitrogen) and ESR2 (rabbit anti-human, 1:200 dilution, Invitrogen). Biopsy samples were treated with primary antibodies at 4° C overnight, rinsed with PBS, then exposed to secondary antibody for one hour at room temperature (goat anti-rabbit Alexa Fluor 488 nm, 1:1,000 dilution, Invitrogen). The samples were exposed to chromosome counterstain 4',6-diamidino-2-phenylindole (DAPI), and digital fluorescence images were recorded (Zeiss Axiovision).

Air Liquid Interface Cell Culture System: Primary human keratinocyte cells (EPC2), a human telomerase reverse transcriptase-immortalized esophageal epithelial cell line that has been characterized in multiple studies, was used for all experiments^{41, 42}. Cells were seeded at a concentration of 1×10^4 cells per well on a 0.4-mm pore-size permeable membrane support system (Corning Incorporated, Corning, NY), then grown in low-calcium (0.09 mM $[Ca^{+2}]$) keratinocyte serum-free media (KSFM) to confluence. After cells reached confluence, typically Day 3 of culture, media was changed to high-calcium KSFM (1.8 mM $[Ca^{+2}]$) for three additional days. On Day 7, the media was removed from the inner chamber of the ALI system to expose cells to air and subsequently inducing epithelial stratification and differentiation. Treatment was started on Day 10 or Day 11 of culture, with 24–48 hours of exposure to IL13 (100 ng/mL) and/or E2 (1-100 nM). The esophageal epithelial equivalent was analyzed on Day 12, or five days after air exposure.

Hematoxylin and Eosin Staining: Cells were fixed by adding 4% paraformaldehyde directly to top and bottom of transwells. The cell plates were placed on ice, and the support membranes were removed

from the transwells. The cells on the support membranes were dehydrated with 70% ethanol, then paraffin embedded. The cells and support membranes were cut, placed on slides, de-paraffinized, and stained with hematoxylin and eosin by the CCHMC tissue processing core.

Quantification of Dilated Intercellular Spaces (DIS): DIS values represent automated space measurement quantification of a compilation of 40x images from H&E stained samples from each treatment group using Image-Pro Plus software (Media Cybermetrics, Rockville, MD, USA). DIS percentages represent a ratio of DIS area to total tissue area.

Western Blot: Cells were rinsed with cold PBS, and the culture plate was placed on ice. The cell culture membrane support system was cut from the plastic transwells and placed in lysis buffer with protease inhibitor. Cells were scraped from the membranes and the suspension was centrifuged at 4° C at 10,000 x g for 10 minutes. The supernatant was removed and protein quantification was performed using serial bicinchoninic acid (BCA) assay. The samples were boiled for 10 minutes with SDS sample buffer. The samples were separated using SDS-PAGE gradient gels (Novex Life Technologies, Invitrogen), followed by Western blot analysis. The membranes were blocked with 5% milk, then incubated with primary antibody overnight at 4° C. Primary antibodies included STAT6 (rabbit anti-human, 1:1,000 dilution, Santa Cruz Biotechnology), phosphorylated STAT6 (rabbit anti-human, 1:1,000 dilution, Santa Cruz Biotechnology), TYK2 (rabbit anti-human, 1:1,000 dilution, Thermo Fisher Scientific), and phosphorylated TYK2 (rabbit anti-human, 1:500 dilution, Thermo Fisher Scientific). Following serial rinsing with TBS with 20% Tween, the membranes were exposed to secondary antibody (LI-COR Biosciences) for 1 hour at room temperature. Quantitative expression analysis using two color infrared imaging was used to compare protein expression (Odyssey Imager, LI-COR Biosciences).

RNA sequencing and Quantitative PCR: After completion of treatment course, cells were treated with Trizol, scraped from the support membrane, then frozen in Trizol suspension at -80° C. RNA was extracted using RNA isolation kit (Thermo Fischer Scientific) then analyzed for purity and quantified using Nanodrop 2000c (Thermo Scientific). Messenger RNA was either submitted to Cincinnati Children's Hospital Medical Center Bioprocessing CORE for RNA sequencing or amplified to complementary DNA. RNA sequencing is expressed in reads per kilobase per million (RPKM). Quantitative PCR was performed using SYBR Green. *SOCS1* forward primers (5'- TCC CCT TCC AGA TTT GAC CG -3') and reverse primers (5'- CCA CAT GGT TCC AGG CAA GT -3') were designed from Integrated DNA Technologies. Relative

expression of *SOCS1* was compared to baseline *HPRT* expression using real time PCR (CFX96R, Bio-Rad Laboratories).

Transepithelial Resistance (TER) Analysis: Cells were grown in EPC2 -ALI transwells as described⁴³. After completion of treatment course, the top of the transwells were removed. The bottom portion of the transwells were placed in chamber sliders (P2320T slider, 6.5 mm diameter, sized for 0.33 cm² growth area). The chamber sliders were inserted into the dual chambers of an Ussing apparatus (P2300 dual Ussing chamber, Physiologic Instruments, San Diego, CA) containing Ringers buffer (pH 7.4). The Ussing chambers were mounted on 8-chamber Ussing system (EM-CSYS-8 EasyMount Ussing Chamber System, Physiologic Instruments, San Diego, CA). The transepithelial potential difference was measured using two paired Ag and AgCl electrode tips made from 4% agar dissolved in 3 M KCl. The electrodes were connected to a voltage clamp amplifier (VCC-MC8 multichannel voltage clamp, Physiologic Instruments, San Diego, CA). The potential difference of the electrodes and the fluid resistance compensation were calibrated prior to insertion of the transwells into the chamber. Potential difference was monitored continuously with an open circuit for 15-30 minutes prior to establish equilibrium. Rapid-pulse voltage pulses of 620 ms cycles were delivered every 1 second to yield a current response for calculation of resistance across the membrane using Ohm's law. Output was collected using Acquire & Analyze Rev II software. In some experiments mature EPC2-ALI cells (> 1500 $\Omega \cdot \text{cm}^2$) were treated with either vehicle (0.1% DMSO) or highly selective ESR1 antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP dihydrochloride) MPP (100pM) or 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) (10 μ M) 30 minutes prior to exposure to E2 (100 nM). Twenty-four hours later EPC2-ALI cells were stimulated with IL-13 (100 ng) in the presence of vehicle, MPP or PHTPP and E2 for twenty-four hours and TER measurements were performed using an EVOM (World Precision Instruments, Inc., Sarasota, FL, USA).

Statistical Analysis: Categorical data was expressed as percentages or ratios, and continuous data as mean $\pm$ standard deviation, unless otherwise stated. All analyses were performed using Prism 7.0 software (Prism Software Corp., Irvine, CA). Comparisons of categorical data such as sex or age groupings were performed using chi-square or Fisher exact test. Statistical significance for continuous data was determined using Student's t-test, or Mann-Whitney for nonparametric data such as age at diagnosis. In experiments comparing multiple experimental groups, analysis was performed using one-

way analysis of variance with Bonferroni or Holm-Sidak post-test. Significance was considered for $p \leq 0.05$.

RESULTS:

Increased incidence of EoE in males

To determine the prevalence of EoE in males and females in our local cohort, we examined the number of patients with a diagnosis of EoE cared for at the Cincinnati Center for Eosinophilic Disease (CCED). A retrospective review of the electronic medical record database from January 2000 through January 2017 found that 2,013 patients were given a diagnosis of EoE ($n = 2,013$, males $n = 1,485$, females $n = 528$) (Supplemental Figure 1A). The male to female ratio for all patients of all ages was 2.85 (Supplemental Figure S1B). To compare the effect of age at diagnosis between genders in pediatric patients, the cohort was stratified by age at initial diagnosis to groups < 5 years, 5-9 years, 10-14 years and 15-19 years (Supplemental Figure S1C and D). Examination of the male to female ratio revealed that the male predominance was most pronounced in patients diagnosed at < 5 years of age, with a male to female ratio of 3.8 (37% of cohort, $n = 666$, male $n = 527$, female $n = 139$) (Supplemental Figure S1C and D). The male sex predominance decreased as age of initial diagnosis increased, with a male to female ratio of 2.95 in patients age 5-10 years (23.5 % of cohort, $n = 423$, male $n = 316$, female $n = 107$), 2.93 for patients age 10-14 years (21.0 % of cohort, $n = 377$, male $n = 281$, female $n = 96$) and 2.31 for patients age 15-19 years (11.2 % of cohort, $n = 167$, males $n = 90$, females $n = 77$) (Supplemental Figure S1C and D). The male to female ratio of patients diagnosed at < 5 years was statistically different than the male to female ratio of patients ages 15-19 years ($p < 0.01$). We observed no statistically significant differences between other age divisions. When stratified by sex, the average age of diagnosis for males was significantly lower than that of females (8.7 ± 7.6 years vs 11.9 ± 10.8 years; males and females respectively, mean $\pm$ SD; $p < 0.001$). These analyses demonstrate the increased incidence of EoE in male patients in our cohort (male to female ratio 3 : 1 in patients younger than 20 years) and that the male gender predominance was more pronounced in children < 5 years.

Altered expression of estrogen responsive genes in EoE

To begin to determine the contribution of sex hormones to the increased male predominance in EoE, we examined expression of the sex hormone responsive genes in esophageal biopsy samples from four female and six male EoE patients and three of both healthy female and male controls using RNA sequencing data from a previously described cohort of patients (Supplemental Table S1)³⁵. The estrogen responsive genes were derived from the estrogen responsive gene reference database

(<http://datam.i2r.a-star.edu.sg/ergdbV2/>)⁴⁴ which consists of 1,069 validated estrogen responsive human genes. While this gene list does not contain all estrogen responsive genes^{45, 46}, it serves as an initial screen to identify the potential involvement of estrogen responsive gene expression in the pathogenesis of EoE. Of the 1,069 estrogen responsive genes in the published reference database, 1,057 (98.8%) were present in the UCSC gene model based RNA sequencing data set. Analysis showed that 29.9% (317 of 1057 genes, $p < 0.05$, $FC > 1.5$) of estrogen responsive genes were significantly dysregulated (up or down) in male or female EoE patients compared to controls (Figure 1). This is significantly different than the 5% of genes ($n = 53$ genes) anticipated to be dysregulated by chance ($p < 0.01$, 10^4 permutations of 30,000 genes). Of the 317 dysregulated genes, 58.7% ($n = 186$ genes) were up regulated in patients with EoE compared to controls, while 41.3% of genes ($n = 131$) were down regulated in patients with EoE (Figure 1A). 58 of these genes were altered specifically in females with EoE ($n = 43$ upregulated, $n=15$ down regulated) and 44 were altered in males with EoE ($n = 13$ upregulated, $n = 31$ down regulated) (Table 1). When examining the entire dysregulated transcriptome of EoE patients compared to controls, 8% of all dysregulated genes were predicted to be estrogen responsive (172 of 2199 genes, $p < 0.01$, $FC > 2$) (Supplemental Table S2). String protein-protein interaction network analysis⁴⁷ of the 172 differentially expressed estrogen response genes in EoE revealed one major cluster / Node for the interactions of genes associated with the regulation of DNA synthesis (CDC45, RRM1 and RRM2) cell cycle checkpoints (CCNB1, CCNG2) and mitosis progression (TPX2, AURKA, MAD2L1, BUB1B, CDC20, KIF2C) (Figure 1B). This major cell cycle regulatory network was enjoined to a cluster of innate immune genes (Cluster Node 2) involved in chemoattraction (CXCL1, CCR1, ITGAE and ICAM1), innate immune recognition (TLR2, IRF9) and cell activation (CD44, CD69) (Figure 1B). A confirmatory gene expression cohort of 10 EoE and 10 control patients showed approximately 8% of dysregulated genes in EoE were estrogen responsive (GEO deposition: GSE113341).

Gene ontology analysis of the estrogen responsive genes altered in male and female EoE patients (Table 1) demonstrated numerous altered pathways in several broad categories including cell cycle regulation (*PKMYT1*, *PMP22*, *FOXM1*, *NUSAP1*, *CKS2*, *PTTG1*, *THBS1*, *SFRP1*, *PTPRK*, *ANLN*, *BE2C*, *TPX2*, *BAK1*, *BIRC5*, *TACC3*, *CDC20*, *TOP2A*, *KIF11*, *KIFC1*, *CCNB1*, *AURKB*), kinase binding (*TOP2A*, *ITGAV*, *PRC1*, *PKP2*, *CD44*, *TPX2*, *KIF20A*, *PTPRK*, *TRIB2*, *FOXM1*), and inflammatory processes (*CYBB*, *SAA1*, *THBS1*, *SOC3*, *CD44*, *ICAM1*, *CCL5*, *IFIH6*, *PBK*, *PTGES*, *ITGAV*, *STAB1*, *CXCL1*, *BCL6*). Both male and female EoE patients compared to controls had decreased expression of estrogen responsive genes related to regulation of membrane permeability (*BCL2*, *BCL2L1*, *PPIF*, *IER3*, *PMAIP1*), response to hormones (*CDKN1A*, *NCOA2*, *ANXA2*, *FOSL2*, *NUCB2*, *BCL2*, *TRIM24*, *ME1*, *RXRA*, *AREG*, *CITED2*, *GJA1*, *TIMP3*,

PIK3R1, DHCR24, PRKCE, BAIAP2L2, MARCKS) and response to wounding (*KRT6B, ACHE, ANXA2, ACTG1, BCL2, IL1RL2, RXRA, ZFP36, S100A9, GJA1, TIMP3, IER3, PIK3R1, PRKCE, PARD3, MVK*) ($p < 0.05$) (Table 1). Female EoE patients had increased expression of DNA binding and repair genes (*RAD51, CDC6, RFC3, BRCA1, POLA2, RFC5, RPA3, FEN1*) and decreased expression of genes related to cellular adherence and cell-cell junctions (*PTPN12, PGM5, EFNB2, NDRG1*) and NFkB signaling (*BLNK, IL1R1, CXCL2, NFKBIA*) compared to males ($p < 0.05$). Male EoE patients had increased expression of genes related to steroid hormone receptor activity (*RARA, NR2C1*) and signal transduction (*LGALS1, SLA, DUSP6, IGF1R, RARA, NR2C1, CD8A*) and decreased expression of a set of genes related to epithelial cell proliferation (*SOX9, ZNF703, CCND1, B4GALT1, TGFA*) compared to females (Table 1, $p < 0.05$). These findings suggest numerous genes altered in active EoE are estrogen responsive, including gene groups related to membrane permeability and cell-cell junctions, and that there is differential gene dysregulation between males and females that could contribute to the observed male predominance of EoE.

EDP Gene Panel and estrogen responsive genes in EoE

Recently the CCED group developed an eosinophilic diagnostic panel (EDP) consisting of 94 dysregulated genes in the EoE transcriptome and two housekeeping genes (*GAPDH* and *18S*)⁴⁸. The EDP has shown utility as a diagnostic and disease activity surveillance test, and could serve as an indicator of how changes in estrogen responsive genes might correlate with disease activity^{48, 49}. We found that 10 of the 94 EDP genes (10.6%) were part of the estrogen responsive genes database (*CA2, CFB, CITED2, CRISP3, CXCL1, EML1, FKBP5, KRT23, PHLDB2, and SLC16A6*). We reviewed the RNAseq analysis of biopsy samples from the male and female EoE vs control cohort described above to identify overlap with the EDP gene list, and found that 7 of the 10 EDP genes (*CA2, CFB, CITED2, CRISP3, CXCL1, EML1* and *SLC16A6*) were significantly dysregulated (up or down) between the EoE and control populations (Supplemental Table S3 and Figure 1C). Interestingly, two of these estrogen responsive genes (*CXCL1* and *CRISP3*) were also some of the most altered genes in the EoE patients, with FC of 56.9 ($p < 0.001$) and FC of -621.5 ($p < 0.001$) respectively.

Esophageal gene expression in Healthy Females vs Males:

Male and female patients with EoE had differences in esophageal biopsy gene expression profile (Table 1). To evaluate what differences may be sex specific, we compared esophageal biopsy gene expression between healthy female and male controls from the same cohort described above (Females $n = 3$, Males $n = 3$, not age controlled). We found 128 genes with FC > 3 (Up $n = 66$, Down $n = 62$, 10^4

permutations) that differed between female and male controls, but none reached statistical significance. The most altered genes were sex-chromosome specific (*XIST*, FC +113, F v M and *RPS4Y1*, FC -162 F v M). Gene ontology analysis identified Molecular Function genes related to Glutathione transferase activity (*GSTT2B*, *GSTM1*, *GSTM3*, *GSTT2*). Two of the differently expressed genes were part of the EDP (*GLDC*, -5.84 FC FvM, and *UPK1A*, +3.36 FC FvM). *GLDC* is glycine decarboxylase, part of the mitochondrial glycine degradation system. *UPK1A* is a transmembrane protein that modulates membrane permeability, and is also one of the most down-regulated genes included in the EDP. Although the +3 FC difference in expression did not reach statistical significance ($p = 0.1$) the degree of dysregulation of *UPK1A* in EoE patients merits further investigation.

EoE patients have increased expression of estrogen receptors compared to healthy controls

Given these differences in expression of estrogen responsive genes in EoE, we next examined expression of the hormone receptors ESR1, and ESR2 in esophageal tissue from post-pubertal (ages 15.2 to 17.9 years) male and female EoE and control patients (Figure 2). RNA expression analyses revealed that both *ESR1* and *ESR2* were expressed at low levels in both male and female control patients and that levels were not significantly different between the sexes (Figure 2 A and B). The level of expression of both ESR1 and ESR2 were increased in both males and females with EoE compared to controls, however, gene expression levels were low with RPKM values < 1 for *ESR1* and *ESR2* in esophageal tissue (Figure 2 A and B).

Examination of the cellular distribution and localization of the estrogen receptors in primary esophageal biopsy samples from male and female control and EoE patients by immunofluorescence revealed expression of ESR1 and ESR2 at baseline (Figure 2 E and F). Notably, expression of ESR1 and ESR2 appeared to be restricted to the basal layer of the esophageal epithelium (Figure 2E and F, a and c; white arrows). We did not observe differences in the level of expression of ESR1 and ESR2 between males and female controls (Figure 2E and F; Upper and lower left panels and 2F Upper and lower right panels). In EoE, we observed an altered expression pattern of ESR1 and ESR2 in both male and female patients with EoE compared to controls (Figure 2 E and F). Notably, ESR1 and ESR2 expression was preserved within the basal layer of the esophageal epithelium, however positive staining was also observed within the basal proliferative zone of the esophageal epithelium (Figure 2E and F; yellow arrows). We did not observe differences in the level of expression of ESR1 and ESR2 between males and female EoE within the basal layer and basal proliferative zone (Figure 2E and F; Upper and lower left panels and 2F Upper and lower right panels).

E2 pretreatment protects against IL-13–induced changes in esophageal barrier dysfunction and remodeling

Given the observed alteration in estrogen responsive genes in individuals with EoE, coupled with differential expression of estrogen receptors in esophageal tissues of EoE patients we examined the effects of E2 on IL-13-induced esophageal epithelial dysfunction in an *in vitro* model developed from esophageal epithelial cells (EPC2) grown in air liquid interface (ALI). We have previously reported that IL-13 stimulation of EPC2-ALI cells induces a gene expression profile resembling EoE, with a 22% overlap of dysregulated genes¹⁶. IL-13 also induces esophageal epithelial dysfunction (barrier impairment, proliferation) similar to what is observed in EoE¹⁶. We have previously demonstrated that IL-13 stimulation of EPC2-ALI cells leads to changes in the esophageal architecture including dilated intercellular spaces, epithelial proliferation and barrier dysfunction¹². IL-13 stimulation of EPC2 cells significantly decreased transepithelial resistance (TER) compared to control (Figure 3A), which is indicative of barrier dysfunction ($\Omega \cdot \text{cm}^2$; 2021 ± 516 vs 1258 ± 334 ; Vehicle vs IL-13; mean $\pm$ SD; $n=10$; $p < 0.001$). Pretreatment of EPC2 cells with E2 (100 nM) for 24 hours prior to IL-13 exposure (Figure 3A) attenuated the IL-13-induced decrease in TER (1291 ± 190 vs 1597 ± 363 ; Vehicle + IL-13 vs E2 + IL-13; mean $\pm$ SD; $n = 10$; $p < 0.05$). Treatment of EPC2 cells with E2 alone had no effect on esophageal epithelial barrier function (Figure 3A). These findings indicate that estrogen mitigates IL-13 induced esophageal epithelial barrier dysfunction.

The observed reduction in IL-13 induced esophageal epithelial barrier dysfunction by estrogen led us to examine the impact of estrogen on IL-13-induced esophageal architectural changes. We show that IL-13 exposure led to an overall disruption of cellular architecture, with increased dilation of intercellular spaces and irregularities of apical cellular layers in EPC2-ALI cells compared to vehicle treated cells (Figure 3B and C). Notably, these morphological changes were ameliorated with pretreatment of cells with 100 nM E2 for 24 hours prior to IL-13 exposure. In fact, the esophageal architecture of the EPC2-ALI cells stimulated with IL-13 in the presence of E2 resembled that of vehicle treatment alone group (Figure 3B and C). Quantification of the dilated intercellular spaces (DIS) (Figure 3B) showed IL-13 induced an increased percentage of DIS compared to vehicle (8.2 ± 4.1 vs 3.9 ± 2.7 ; Veh+IL13 vs Veh; mean $\pm$ SD; $n=10$; $p<0.05$), while E2 pretreatment prior to IL-13 exposure attenuated IL-13 induced DIS (8.2 ± 4.1 vs 2.4 ± 2.2 , Veh+IL13 vs E2+IL13; mean $\pm$ SD; $n=10$; $p<0.05$) (Figure 3B). We concluded that E2 promotes a protective effect against IL-13 induced esophageal barrier dysfunction and architectural changes in EPC2-ALI cells.

17- β estradiol pretreatment abrogates IL-13-induced activation of TYK-2/STAT6 signaling pathway

To begin to unravel the molecular basis of E2 suppression of IL-13-induced esophageal epithelial architectural remodeling and barrier dysfunction, we examined the gene expression profile of IL-13-stimulated EPC2-ALI cells in the presence and absence of E2 using RNA sequencing analysis. Cells exposed to IL-13 for twenty-four hours had alteration of 246 genes (Up $n = 105$, Down $n = 141$, $FC > 3$), including genes related to epithelial barrier function (*CA2*), inflammatory response (*TNFAIP6*, *SOCS1*), chemokines (*CCL26*), protease activity (*CAPN14*) and ion transport (*ANO1*)^{14, 50} (Figure 3D and Table 2). Gene ontology demonstrated gene expression related to Cytokine Signaling, Regulation of Cell Adhesion and Endothelial Cell Proliferation (Table 3). Pretreatment of EPC2-ALI cells with E2 prior to IL-13 exposure led to an alteration in the IL-13-induced gene expression profile. Moreover, we observed abrogation of some IL-13-induced genes including decreased expression of *ANO1* and *SOCS1*. There were similar levels of expression of other IL-13 induced genes despite E2 pretreatment, including *CCL26* (Table 2). E2 pretreatment increased expression of *CA2*, *TNFAIP6*, and *CAPN14* (Table 2). This indicates that E2 treatment prior to IL-13 exposure diminishes the induction of some, but not all, IL-13 related genes. Interestingly, the cells pretreated with E2 and then exposed to IL-13 had alteration in nearly 10 times more genes compared to Vehicle than IL-13 treatment alone compared to Vehicle, (Total $n = 1077$, Up $n = 519$, Down $n = 588$, $FC > 3$), with gene ontology being related to Cell Proliferation and Keratinocyte Differentiation (Table 3). E2 treatment alone altered expression of 125 genes (Up $n = 42$, Down $n = 83$, $FC > 3$), as well as enhancement of expression of the desmosome associated protein *DSG1* ($FC 1.73$). Gene ontology (Table 3) showed E2 treatment alone induced genes related to Cellular Oxidant Detoxification and Gene Silencing ($p < 0.05$). Pretreatment with E2 for 24 hours prior to IL-13 exposure resulted in differential expression of 100 genes compared to IL-13 treatment alone (Up $n = 45$, Down $n = 55$, $FC > 3$, $p < 0.05$), and gene ontology demonstrated genes related to Responses to Oxidative Stress (Table 3).

We next examined changes in genes found in the EDP in EPC2-ALI cells treated with IL-13, as many of these genes have been shown to correlate with clinical EoE disease activity⁴⁹, and may provide additional insight into the effects of E2 on genes related to the EoE transcriptome. We found 17 of the 94 EDP related genes were altered, including *ALOX15*, *ANO1*, *ARG1*, *CA2*, *CCL26*, *CDA*, *CDH26*, *CTSC*, *FLG*, *GCNT3*, *H19*, *IGFL1*, *MSRB3*, *PMCH*, *PNLIPRP3*, *SLC16A6*, and *TNFAIP6* ($FC > 2$, $p < 0.05$). Of the 17 genes, two were E2 responsive genes (*CA2*, *SLC16A6*). The effect of E2 pretreatment prior to IL-13 exposure varied, with some genes having decreased expression, while no effect was observed in others (Table 2).

The IL-13-induced EoE gene transcriptome is predominantly STAT6-dependent^{51, 52}. Given the observed effect of E2 on IL-13-induced genes we hypothesized that E2 protective effects were through alteration of IL-13-induced STAT6 signaling. To test this, we examined IL-13-induced STAT6 phosphorylation in EPC2-ALI cells by western blot analyses. We show that IL-13-stimulation of EPC2-ALI cells induced STAT6 phosphorylation (Figure 4A). Notably, IL-13 stimulation led to increased levels of pSTAT6 within 5 minutes and maximal pSTAT6 was observed within 30 minutes (Figure 4A). Pretreatment with E2 decreased IL-13-induced phosphorylation of STAT6 in EPC2 cells compared to IL-13 treatment alone at 30 minutes (-0.495 FC; $n = 3$; $p < 0.05$) (Figure 4A). Importantly, we observed no effect of E2 on total STAT6 levels suggesting that E2 was suppressing STAT6 phosphorylation and activation (Figure 4A and Supplemental Figure S2). Consistent with this observation, we observed a reduced expression of IL-13-induced genes in EPC2-ALI cells following E2 exposure including *CCL26* (Eotaxin-3) (IL-13 induction of *CCL26* +4.0 FC; E2 + IL-13 -1.0 FC) and *SOCS1*⁵³ (IL-13 induction of *SOCS1* +7.0 FC; E2 + IL-13 -2.0 FC). The reduction in IL-13 signaling was not attributed to reduced receptor expression as our EPC2-ALI cell RNAseq analyses did not reveal any significant effect of E2 on the mRNA expression of the IL-13 receptor sub units (IL4R α , IL-13R α 1 or IL-13R α 2 receptor) (Supplemental Figure S3).

IL-13 induction of STAT6-dependent pathways requires activation of receptor associated tyrosine kinases, including JAK2 and TYK2^{23,19}. TYK2 specifically has been implicated with IL-13 induced activation of STAT6¹⁹. To determine whether E2 is inhibiting the IL-13-STAT6 pathway via Tyk2, we examined the level of Tyk2 phosphorylation in EPC2-ALI cells following E2 exposure. IL-13 treatment of EPC2-ALI cells induced a 7.55-fold increase in the actin-corrected ratio of phosphorylated TYK2 (pTYK2) to total TYK2 at 5 minutes after exposure to IL-13 (0.017 ± 0.015 vs 0.128 ± 0.05 , mean $\pm$ standard deviation, Vehicle vs Vehicle + IL-13 at 5 minutes, $n = 3$, $p < 0.05$) (Figure 4B). Pretreatment with E2 prior to IL-13 exposure resulted in only 1.4-fold change in pTYK2/TYK2 at 5 minutes after IL-13 exposure (0.017 ± 0.015 vs 0.024 ± 0.02 , Vehicle vs E2+IL-13 at 5 minutes, $n = 3$, not significant). Levels of pTYK2 in the E2 pretreated group was decreased after 5 minutes of exposure to IL-13 compared to vehicle pretreatment (0.128 ± 0.05 vs 0.024 ± 0.02 , mean $\pm$ STD, $n = 3$, $p < 0.05$). This decreased pTYK2 compared to total TYK2 in E2 pretreated cells indicates E2-mediated suppression of TYK2 phosphorylation and activation.

17- β estradiol mediated abrogation of IL-13-induced changes in esophageal barrier dysfunction is ESR2-dependent.

To determine the requirement of ESR1 or ESR2 in 17- β estradiol mediated abrogation of IL-13-induced changes in esophageal barrier dysfunction, we employed the highly selective ESR1 and ESR2 receptor antagonists, MMP and PHTPP⁵⁴⁻⁵⁸. Recent studies have demonstrated that antagonism of ESR-pathway inhibits esophageal epithelial proliferation⁵⁴, we therefore generated mature EPC2-ALI monolayers and then pretreated the monolayers with MMP or PHTPP for twenty-four hours and subsequently examined the effect of E2 on IL-13-induced esophageal epithelial barrier dysfunction. As anticipated, IL-13 stimulation of EPC2 cells significantly decreased TER compared to vehicle control (Figure 5) and pretreatment of EPC2-ALI cells with E2 inhibited the IL-13-mediated response (Figure 5). Pretreatment of EPC2 cells with ESR1 antagonist MMP had no impact on E2-mediated inhibition, however exposure of EPC2 cells with PHTPP released the E2-mediated inhibition of IL-13-induced esophageal epithelial barrier dysfunction (Figure 5). These findings indicate that estrogen mitigates IL-13 induced esophageal epithelial barrier dysfunction through ESR2.

Discussion:

EoE is more frequently diagnosed in males than in females and the underlying cause of increased risk in males has not been fully delineated. Herein, we confirmed the male predominance in EoE in a single center pediatric cohort and that the male predominance is most pronounced in patients diagnosed at < 5 years of age. We reveal that that esophageal epithelium expresses both *ESR1* and *ESR2* and that 29.9% of the genes dysregulated in EoE are estrogen responsive genes involved in multiple pathways, including inflammation, membrane permeability, and cell-cell junctions. Mechanistic studies revealed that 17- β estradiol attenuated IL-13 induced esophageal epithelial barrier dysfunction and architectural remodeling and that suppression of IL-13-induced esophageal dysfunction by E2 was associated with decreased IL-13-induced TYK2/STAT6 phosphorylation and activation. Based on our findings, we propose that estrogen hormone signaling confers a protective effect against the development of EoE.

The approximate 3:1 male predominance in EoE observed in our CCED cohort is consistent with previous epidemiologic studies from around the world⁷⁻⁹. Analyses of clinical registries from five tertiary care institutions in the United States has revealed a 72% male EoE predominance in the US⁴. Consistent with this, van Rhijn et al reported a 74% male predominance in an adult and pediatric European cohort³, and a systematic review of EoE studies performed in Asian countries reported a 73% male predominance among EoE patients of all ages⁵. Interestingly, we show that the increased male to female ratio in EoE was most pronounced in children diagnosed at < 5 years of age, with 3.8 males for every female. Consistent with this, previous studies have reported that males are more likely than females to be diagnosed as children in an EoE⁷ (48% of males verses 36% of females were diagnosed before age 18 years old) and that males were diagnosed at an average younger age than females (25 $\pm$ 19 vs 29 $\pm$ 20 years).

There is anecdotal clinical evidence supporting a role for sex hormones in the development of EoE or atopic diseases. For example, during infancy and childhood, asthma, atopic dermatitis (AD) and allergic rhinitis (AR) are more common in males than females⁵⁹, however a female predominance develops in asthma and atopic dermatitis around the age of puberty and continues throughout adulthood until menopause⁶⁰. Furthermore, males with AD had lower levels of circulating estradiol than healthy controls⁶¹. We propose that females may be protected from developing EoE or atopic disease through circulating estrogens. Estrogen levels are higher on average in females than males during all Tanner stages of development, including infants and pre-pubescent children⁶². However, a direct correlation between total circulating estrogen levels and risk of developing EoE seems unlikely to

completely explain the sex bias in EoE, as our cohort showed a decrease in the male predominance for age of initial diagnosis during teenage years, and clinical experience does not show a corresponding change in symptoms and disease activity in females with EoE as estrogen levels rise during puberty. The observations that females have an alleviation of asthma symptoms during life phases associated with higher levels of circulating sex hormones such as pregnancy whereas others can have an exacerbation of symptoms⁶³ further suggests a more complex role for sex hormones possibly related to dose and tissue specificity to confer protective effects in onset and development of allergic diseases⁶³.

Our molecular studies revealed expression of estrogen receptors ESR1 and ESR2 in the esophageal epithelium in healthy controls and that receptor expression is increased in patients with active EoE. Previous studies have demonstrated moderate positive staining for ESR1, and weak positive staining for ESR2 in normal esophageal tissue⁶⁴. We observed comparable levels of expression of both ESR1 and ESR2 in healthy individuals, with increased expression of both in male and female patients with active EoE. Our demonstration of induction of E2 genes in our EPC2-ALI model following E2 exposure indicates presence of ESR receptors and active estrogen signaling pathways in esophageal cells supports the concept that estrogens exert physiologic effects in esophageal tissue via estrogen receptor-related signaling. Consistent with this argument, we show that pharmacological antagonism of ESR2 released the E2-mediated protective response against IL-13-mediated barrier dysfunction. ESR2 has previously been reported to play a pivotal role in E2-mediated enhancement of intestinal epithelial barrier function. While the molecular basis of ESR2-mediated protection of intestinal epithelial barrier properties is not fully elucidated, the E2-ESR2-axis has been shown to stimulate the upregulation of tight and adherence junctional proteins occludin and JAM-A in intestinal epithelial cells to reinforce the epithelial barrier³³. Interestingly, experimental analyses have identified the existence of ESR1 and ESR2 isoforms⁶⁵. The biological properties of the ESR1 and ESR2 isoforms are still unclear, however they have been shown to have differential expression in various tissues and cell lines^{65, 66}. RNAseq analyses of esophageal biopsy samples revealed increased mRNA expression of ESR2 isoform 4 in EoE compared with controls (Supplemental Table S4). Previous studies have reported that ESR2 isoform 1 is the full functional isoform and that ESR2 isoforms (2-5) do not possess intrinsic activity. However these isoforms can interact with ESR2 isoform 1 and form functional heterodimers that possess altered tissue distribution and ligand binding affinity and functionality⁶⁷. Future analyses of ESR2 isoform 1 and isoform 4 interactions in esophageal epithelial barrier function are currently under further investigation.

The molecular basis of E2 suppression of IL-13-induced barrier dysfunction appears to relate to dampening of IL-13-induced TYK2/STAT6 signaling axis. Moreover, we showed that E2 pretreatment

suppressed IL-13-induced phosphorylation of TYK2 and STAT6 and subsequent activation of some IL-13 induced genes. RNAseq analyses revealed that the effects of E2 occurred independently of any changes in IL-13 receptor subunits (*IL4R α* , *IL13R α 1*, *IL13R α 2*) mRNA which suggests the observed loss of IL-13-mediated effects are not a consequence of alterations in receptor expression. Furthermore, the observed comparable levels of total TYK2 between vehicle and E2 exposure suggests that suppression is not related to alteration in total TYK2 protein levels. TYK2 being a non-receptor tyrosine-protein kinase phosphorylates downstream STATs including STAT6 triggering STAT dimerization, nuclear translocation leading to DNA binding and activation of gene expression⁶⁸. TYK2 undergoes post translational modification to induce positive and negative regulatory effects including phosphorylation, dephosphorylation and ubiquitination⁶⁹. Suppressors of cytokine signaling (SOCS) proteins are a family of molecules known to be induced by JAK-STAT signaling act as a negative feedback loop⁷⁰. However we observed no effect of E2 on expression of the SOCS inhibitors of TYK2 such as SOCS1 and SOCS3 in EPC2-ALI cells, suggesting that the E2-mediated suppression is not through upregulation of this counter-regulatory mechanism^{71, 72}. TYK2 can also be negatively regulated by phosphatases such as Src homology 2 domain containing protein tyrosine phosphatase (SHP) 1 and protein tyrosine phosphatase (PTP) 1B which can directly interact with TYK2 and modulate TYK2 signaling functions⁷³. Analyses of the EPC2-ALI + E2 gene expression studies did not reveal any obvious alterations in phosphatase pathways and we are currently further examining the molecular basis of E2 suppression of TYK-2 phosphorylation. Given the experimental evidence indicating intricate cross talk between JAKs and estrogen receptors the molecular basis of suppression may be through multiple different signaling mechanisms.

We have previously reported that IL-13 stimulation of primary esophageal and EPC-ALI cells induces esophageal barrier dysfunction¹², which is thought to be in part due to decreased expression of intercellular adhesion molecules such as desmoglein-1 (DSG1). Consistent with this, DSG1 has been shown to be downregulated in EoE patients compared to controls¹². RNA sequencing data from our EPC2-ALI model revealed that E2 alone was sufficient to induce esophageal *DSG1* expression. Estrogens have been implicated in protection against esophageal epithelial barrier dysfunction through the direct induction of major component of cellular tight junctions including occludin^{74 75 76}. Bioinformatic analyses of the dysregulated estrogen responsive genes in EoE and E2 stimulated EPC-ALI cells identified other estrogen responsive genes involved in preservation and maintenance of the esophageal barrier. One such gene was *UPK1A* which encodes for a transmembrane protein that modulates membrane permeability. *UPK1A* is one of the most down-regulated genes in EoE, with a 38-fold reduction ($p < 0.01$) in esophageal expression in EOE compared to controls⁴⁸. Although *UPK1A* was not included in the

Estrogen Gene Database used in this study, *UPK1A* has been shown to be an estrogen responsive gene in the genitourinary system, with a -71 fold decrease in expression in *ESR1* knockout mice⁷⁷. We observed a +3.36 FC increased expression on *UPK1A* in esophageal tissue of healthy females compared to healthy male controls. *UPK1A* is active in esophageal tissue as a tumor suppressor of esophageal squamous cell carcinoma, which interestingly is 2:1 more common in males than females⁷⁸, and is an antagonist to matrix metalloproteinases.^{78, 79} Increased expression of matrix metalloproteinases MMP-2 and MMP-14 have been implicated in EoE⁸⁰. Although we did not observe an effect on *UPK1A* mRNA expression following E2 exposure in our EPC2-ALI model, the difference in expression of this gene between healthy males and females, coupled with the marked dysregulation in EoE patients compared to controls indicative of variable *UPK1A* expression, and the known connection to estrogen may contribute to male predominance in EoE. Collectively, these data suggest that while E2 may dampen IL-13-induced TYK-2/STAT-6 signaling axis and reduce IL-13 induced barrier dysfunction, some of the protective effects of estrogen may extend beyond just suppression of IL-13/STAT6 signaling to include direct regulation of barrier enhancer gene expression.

In conclusion, we demonstrate increased incidence of EoE in males and that this was associated with dysregulation of estrogen-responsive gene expression including genes related to inflammation, cell-cell junctions, and the EDP genes *CA2*, *CFB*, *CITED2*, *CRISP3*, *CXCL1*, *EML1* and *SLC16A6*. Using an EPC2-ALI model, we showed that E2 exposure abrogated IL-13-induced architectural changes of dilated intercellular spaces and barrier dysfunction in esophageal epithelial cells. Mechanistically we show that E2 suppression of IL-13-effects were associated with decreased phosphorylation of TYK2 and STAT6 and dampened IL-13-response. These studies suggest that estrogen hormone signaling may play a role in protecting against the development of EoE in females.

Figure legends:

Figure 1: Estrogen responsive genes in EoE: RNA sequencing expression analysis of genes included in the estrogen responsive gene database (n=317 of 1057 genes, $p < 0.05$, $FC > 1.5$, 10^4 permutations of 30,000 gene data set) from esophageal biopsies of 10 patients with EoE (numbers 1-10, female n = 4, male n =6) and 6 Control patients (numbers 11-16, female n = 3, male n = 3). Upregulated genes are represented in yellow and down regulated genes in blue. Magnitude of expression is represented by darkness of the color. (B) String protein-protein interaction network analysis of the 172 differentially expressed estrogen response genes in EoE. The network nodes represent the proteins encoded by the genes. Functional partner predictions are based on available experimental data, databases, text-mining, and homology. Cluster nodes 1-3 are defined by identified Gene pathway analyses of the associated genes. (C) Heat map analysis of the expression of 10 estrogen responsive genes (determined by estrogen responsive gene database) included in the EoE Diagnostic Panel (EDP, 94 genes) isolated from esophageal biopsies of n = 6 control patients (numbers 1-3 and 8 – 11, female n = 3, male n = 4) and 10 patients with EoE (numbers 4-7 and 12-17, female n = 4, male n =6) patients. The EDP expression heat maps were generated using R's heatmap.2 defaults as described in Material and Methods. The seven genes are significantly expressed ($\log FC > 1.5$, $p < 0.05$) in EoE patients compared to control. The difference in expression ($\log FC$) of male EoE and control patients and female EoE and control patients are also shown. Upregulated genes are represented in yellow and down regulated genes in blue. Magnitude of expression is represented by darkness of the color.

Figure 2: *ESR1* and *ESR2* gene expression: RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of (A) *ESR1* and (B) *ESR2* in esophageal biopsies from healthy control (female n =3, male n =3) and EoE patients (female n= 4, male n=6). *ESR1* and *ESR2* expression was elevated in EoE patients compared to controls. There was no difference in expression between males and females in EoE or control patient groups (C-D). RPKM was < 1 for *ESR1* and *ESR2*. (E-F) *ESR1* and *ESR2* Immunofluorescence staining of esophageal biopsies from male and female patients with EoE and healthy controls: Immunofluorescence analyses for *ESR1* and *ESR2* in control (Ctrl, no eosinophils present in biopsy) and representative active EoE biopsy (EoE, maximum of 62 (male) and 90 (female) eosinophils per high-power field [HPF]) is shown. The bar is 20 μm for X 20 images (left panels) and 50 μm for X 40 images (right panels). A total of 2 control and 4 active EoE biopsies were stained. White line indicates single cell basal layer. White arrow indicates positive staining cell within the single cell basal layer. Yellow arrow indicates positive staining within basal proliferative zone.

Figure 3: Transepithelial resistance (TER) and cellular architecture of esophageal epithelial cells exposed to IL-13 $\pm$ 17 β -estradiol. (A): Transepithelial Resistance (TER) of Vehicle (Veh) or 17 β -estradiol (E2)-pretreated EPC2-ALI monolayer following IL-13 exposure. (B): Quantification of dilated intercellular spaces (DIS) and (C) representative H&E staining of EPC2-ALI multicell layer cultures of Vehicle (Veh) or E2-pretreated EPC2-ALI monolayer following IL-13 exposure. (D) RNAseq expression analysis represented by reads per kilobase of transcript per million mapped reads (RPKM) of esophageal epithelial cells (EPC2-ALI) exposed to Vehicle (Veh), IL-13 $\pm$ 17 β -estradiol. Upregulated genes are represented in yellow and down regulated genes in blue. Magnitude of expression is represented by darkness of the color. EPC2-ALI monolayers were pretreated with Vehicle (0.01% EtOH) or E2 (100 nM) for 24 hrs and subsequently stimulated with Vehicle (PBS) or IL-13 (100 ng/mL) in the presence of E2 for 24 hrs and TER measurements were performed using an 8-chamber EasyMount Ussing Chamber System as described in Material and Methods. C, DIS formation (percentage of total area) was quantitated by

using morphometric analyses and expressed as the mean $\pm$ SEM ($n = 3$ independent experiments). * $P < 0.05$. Magnification: (C) $\times 100$.

Figure 4: TYK2 and STAT6 phosphorylation after exposure to IL-13 $\pm$ 17 β -estradiol in esophageal epithelial cells. (A) Representative Western Blot of EPC2 cell lysate for P-STAT6 and STAT6 at 0, 5, 30, and 60 minutes after IL-13 (100 ng/mL) exposure with or without 24 hrs pretreatment with E2 (100 nM) or Vehicle (0.01% EtOH) analyzed by means of immunoblotting with anti-STAT6, anti-p-STAT6, and anti-GAPDH. Quantitative expression of p-STAT6 levels corrected to housekeeper gene (GAPDH), with $n=3$, * $p < 0.05$, student t-test. (B) TYK2 phosphorylation after exposure to IL-13 $\pm$ 17 β -estradiol in esophageal epithelial cells. Representative Western Blot of EPC2-ALI cell lysate for phosphorylated TYK2 (PTYK2) and TYK2 at 0, 5, 30, and 60 minutes after IL-13 (100 ng/mL) exposure with or without 24 hrs pretreatment with E2 (100 nM) or Vehicle (0.01% EtOH). Analyzed by means of immunoblotting with anti-TYK2, anti-PTYK2, and anti-actin. Quantitative expression of p-TYK2 levels corrected to total TYK2, with $n=3$, * $p < 0.05$, student t-test.

Figure 5. 17- β estradiol mediated abrogation of IL-13-induced changes in esophageal barrier dysfunction is ESR2-dependent. Mature EPC2-ALI cells ($> 1500 \Omega \cdot \text{cm}^2$) were treated with either vehicle (0.1% DMSO) or MPP (100pM) or PHTPP (10 μ M) 30 minutes prior to exposure to E2 (100 nM). Twenty-four hrs later EPC2-ALI cells were stimulated with IL-13 (100 ng/ml) in the presence of vehicle, MPP or PHTPP and E2 for 24 hrs and TER measurements were performed as described in the Material and Methods. Data represented as average $\pm$ SEM of $n = 6 - 13$ individual cultures per group. * $p < 0.05$ compared with Veh + IL-13 + E2, multi-group ANOVA analyses and Holm-Sidak Post-test.

Table 1: Gene ontology of estrogen responsive genes in EoE: Gene ontology of up and down regulated gene expression from RNA sequencing analysis of genes included in the estrogen responsive gene database (total $n = 317$, $\text{FC} > 1.5$, $p < 0.05$) in EoE patients (female $n = 4$, male $n = 6$) compared to controls (female $n = 3$, male $n = 3$).

Table 2: Gene expression of EoE-related genes in esophageal epithelial cells exposed to IL-13 $\pm$ 17 β -estradiol: Gene expression of EoE-related IL-13 induced genes (column 1) and gene function (column 2) from RNA sequencing of EPC2-ALI treated with E2 (100 nM) or Vehicle (0.01% EtOH) for 48 hours, with exposure to IL-13 (100 ng/ μ L) at 24 hours. Included are genes with FC expression > 2 in either Vehicle compared to Vehicle + IL-13 (column 3) or E2 pretreatment + IL-13@24 hours compared to E2 alone (column 4) or Vehicle + IL-13@24 hours (column 5). The genes included in the EoE Diagnostic Panel (*) and estrogen responsive gene database (**) are indicated.

Table 3: Gene Ontology Profile of esophageal epithelial cells exposed to IL-13 $\pm$ 17 β -estradiol: Gene ontology profile of EPC2-ALI after IL-13 $\pm$ E2. Column one is comparison between treatment groups Vehicle (0.01% EtOH) or E2 (100 nM 17- β estradiol) for 48 hours with or without addition of IL-13 (100 ng/mL) at 24 hours. Column two and three represent Gene Ontology grouping and associated biological or cellular activity. Column four is the number of genes dysregulated per ontology grouping. Column five is p value of ontology grouping association using hypergeometric probability mass function. Column six are individual genes from the ontology grouping.

Supplemental Figure S1: Sex Predominance and Age at Diagnosis of EoE at CCED: (A) All male and female patients diagnosed with EoE in the Cincinnati Center for Eosinophilic disease (CCED) from 1/2000 through 12/2016 (Total n = 2013, males n = 1458, females n = 528). (B) Male predominance in EoE, with a male to female ratio of 2.81 (73.8% male, 26.2% female) (C) Male and female patients with EoE separated by age of initial diagnosis into 5 year increments. Patients <5 years (total n = 666, male n = 527, female n = 139), age 5-10 years (total n = 423, male n = 316, female n = 107), age 10-14 years (n = 377, male n = 281, female n = 96) and age 15-19 years (n = 167, males n = 90, females n = 77). (D) Chi square analysis demonstrated a significant difference in M:F ratios of patients < 5 years compared to those age 15-19 years (3.8 vs 2.31, p<0.01). (D) We observed no statistically significant differences between other age divisions.

Supplemental Figure S2: Ratio of P-STAT6 / STAT6 in EPC2-ALI esophageal epithelial cells following exposure to IL-13 ± 17β-estradiol in esophageal epithelial cells. Quantitative expression of p-STAT6 / STAT6 relative levels normalized to control (0 minutes Veh), with n = 3, *p < 0.05, student t-test. Western Blot of EPC2-ALI cell lysate for phosphorylated STAT6 (P-STAT6) at 0, 5, 30, and 60 minutes after IL-13 (100 ng/mL) exposure with or without 24 hrs pretreatment with E2 (100 nM) or Vehicle (0.01% EtOH). Analyzed by means of immunoblotting with anti-P-STAT6 and anti-STAT6.

Supplemental Figure S3. RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of (A) *IL4RA*, (B) *IL13RA1* and (C) *IL13RA2* in EPC2-ALI multicellular layer treated with E2 (100 nM) or Vehicle (0.01% EtOH) for 24 hours, with exposure to IL-13 (100 ng/μL) at 24 hours.

Supplemental Table S1. Patient characteristics of EoE and control patients. Description of age of biopsy collected, gender percentage and maximum eosinophils per high power field of patient in cohort.

Supplemental Table S2. Dysregulated Estrogen Responsive Genes within the EoE Transcriptome. Significantly dysregulated genes identified by RNA sequencing expression analysis of esophageal biopsies of n = 6 control patients (numbers 1-3 and 8 – 11, female n = 3, male n = 4) and 10 patients with EoE (numbers 4-7 and 12-17, female n = 4, male n = 6) patients were cross referenced against estrogen responsive gene reference database (<http://datam.i2r.a-star.edu.sg/ergdbV2/>)⁴⁴ which consists of 1,069 validated estrogen responsive human genes. Column one indicates Gene ID, Column 2, Gene symbol and Column 3 fold change (FC) between EoE and control.

Supplemental Table S3: Estrogen Responsive Genes in EoE Diagnostic Panel (EDP): RNA sequencing analysis (Log FC) and gene function of the 10 genes that are included in both the EoE Diagnostic Panel (EDP, 94 genes) and the estrogen responsive gene database (1,069 genes). Transcripts isolated from esophageal biopsies of EoE (female n = 4, male n = 6) and control patients (female n = 3, male n = 3). The 7 genes that are significantly expressed (Log FC > 1.5, p < 0.05) in EoE patients compared to control are shown in bold. The difference in expression (Log FC) of male EoE and control patients and female EoE and control patients are also shown.

Supplemental Table S4. RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of *ESR1* and *ESR2* isoforms in esophageal biopsies from healthy control (female n = 3, male n = 3) and EoE patients (female n = 4, male n = 6). *ESR1* and *ESR2* expression was elevated in EoE patients compared to controls.

ACCEPTED MANUSCRIPT

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Table 1. Gene ontology analysis of the significantly dysregulated estrogen responsive genes in esophageal biopsy samples from male and female control and EoE

Group	Ontology	Activity	Number of Genes	p value	Specific Genes
Up Regulated [M&F EoE] (n=130)	Biological Process	Inflammatory Response	14 of 714	1.75E-07	<i>CYBB, SAA1, THBS1, SOCS3, CD44, ICAM1, CCL5, IFI16, PBK, PTGES, ITGAV, STAB1, CXCL1, BCL6</i>
	Biological Process	Regulation of Cell Cycle	21 of 1012	2.26E-11	<i>PKMYT1, PMP22, FOXM1, NUSAP1, CKS2, PTTG1, THBS1, SFRP1, PTPRK, ANLN, BE2C, TPX2, BAK1, BIRC5, TACC3, CDC20, TOP2A, KIF11, KIFC1, CCNB1, AURKB</i>
	Molecular Function	Kinase Binding	12 of 693	5.29E-06	<i>TOP2A, ITGAV, PRC1, PKP2, CD44, TPX2, KIF20A, PTPRK, TRIB2, FOXM1</i>
Down Regulated [M&F EoE] (n=85)	Biological Process	Response to Hormone	18 of 1035	9.15E-06	<i>CDKN1A, NCOA2, ANXA2, FOSL2, NUCB2, BCL2, TRIM24, ME1, RXRA, AREG, CITED2, GJA1, TIMP3, PIK3R1, DHCR24, PRKCE, BAIAP2L2, MARCKS</i>
	Biological Process	Regulation of Membrane Permeability	5 of 76	5.55E-05	<i>BCL2, BCL2L1, PPIF, IER3, PMAIP1</i>
	Biological Process	Responses to Wounding	16 of 973	6.00E-05	<i>KRT6B, ACHE, ANXA2, ACTG1, BCL2, IL1RL2, RXRA, ZFP36, S100A9, GJA1, TIMP3, IER3, PIK3R1, PRKCE, PARD3, MVK</i>
Up Regulated [M EoE] (n=13)	Biological Process	Steroid Hormone Receptor Activity	2 of 59	3.43E-04	<i>RARA, NR2C1</i>
	Molecular Function	Positive Regulation of Signal Transduction	7 of 1488	6.03E-07	<i>LGALS1, SLA, DUSP6, IGF1R, RARA, NR2C1, CD8A</i>
Down Regulated [M EoE] (n=31)	Biological Process	Gland Development	5 of 153	1.56E-06	<i>SOX9, ZNF703, CCND1, B4GALT1, TGFA</i>
	Biological Process	Epithelial Cell Proliferation	5 of 394	1.49E-04	<i>SOX9, ZNF703, CCND1, B4GALT1, TGFA</i>
Up Regulated [F EoE] (n=43)	Biological Process	DNA Replication	8 of 346	1.46E-07	<i>RAD51, CDC6, RFC3, BRCA1, POLA2, RFC5, RPA3, FEN1</i>
	Biological Process	Serine/threonine/tyrosine Kinase Activity	2 of 49	3.53E-03	<i>MAP2K6, RPS6KA1</i>
Down Regulated [F EoE] (n=15)	Cellular Component	Adherens Junctions	4 of 484	1.69E-04	<i>PTPN12, PGM5, EFNB2, NDRG1</i>
	Pathways	NFKB Signaling Pathway	4 of 205	2.13E-06	<i>BLNK, IL1R1, CXCL2, NFKBIA</i>

patients

Table 2

Table 2. Gene Ontology Analyses of differentially expressed genes from RNASeq analyses of IL-13-stimulated EPC2-ALI cells in the presence and absence of E2.

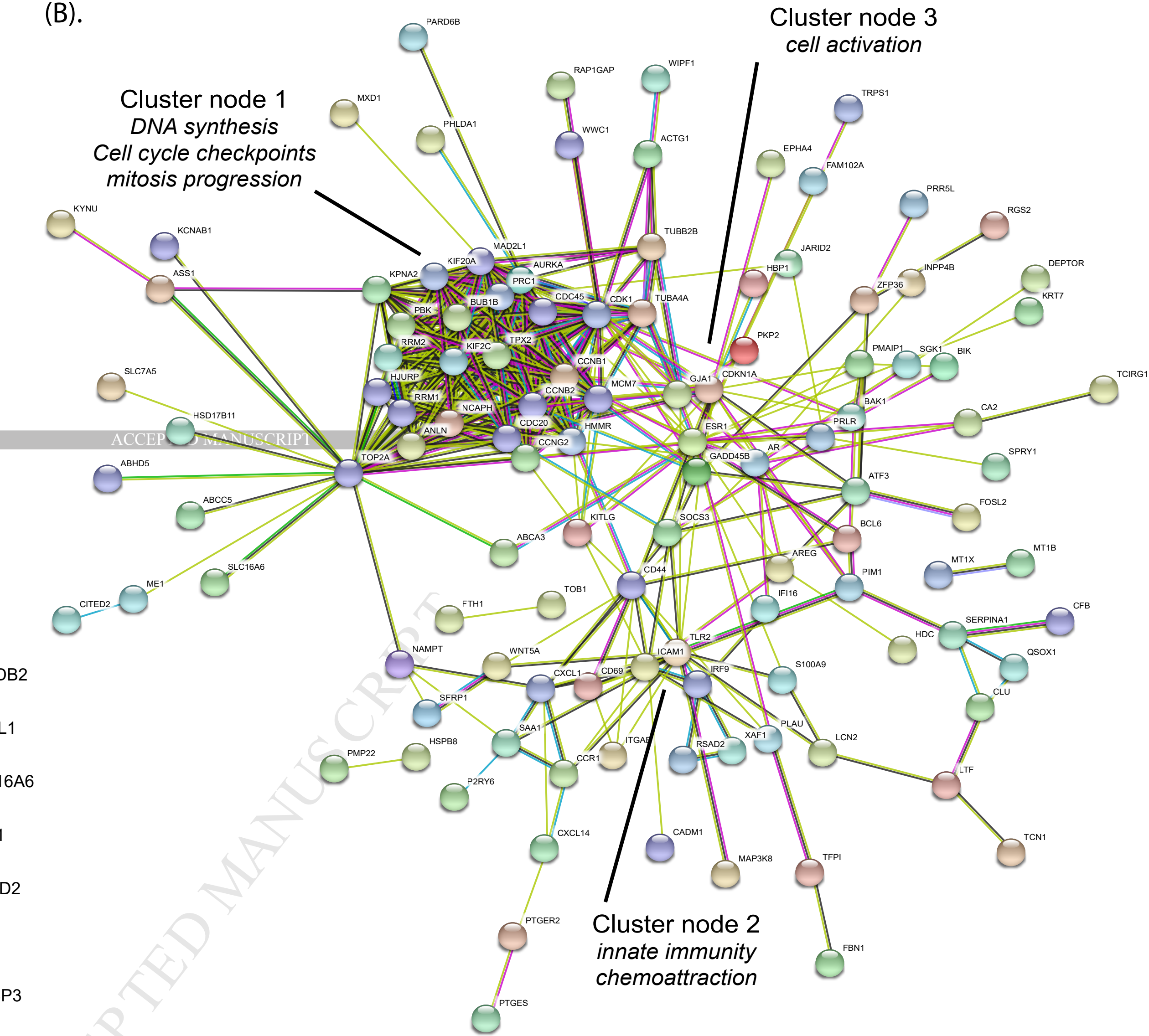
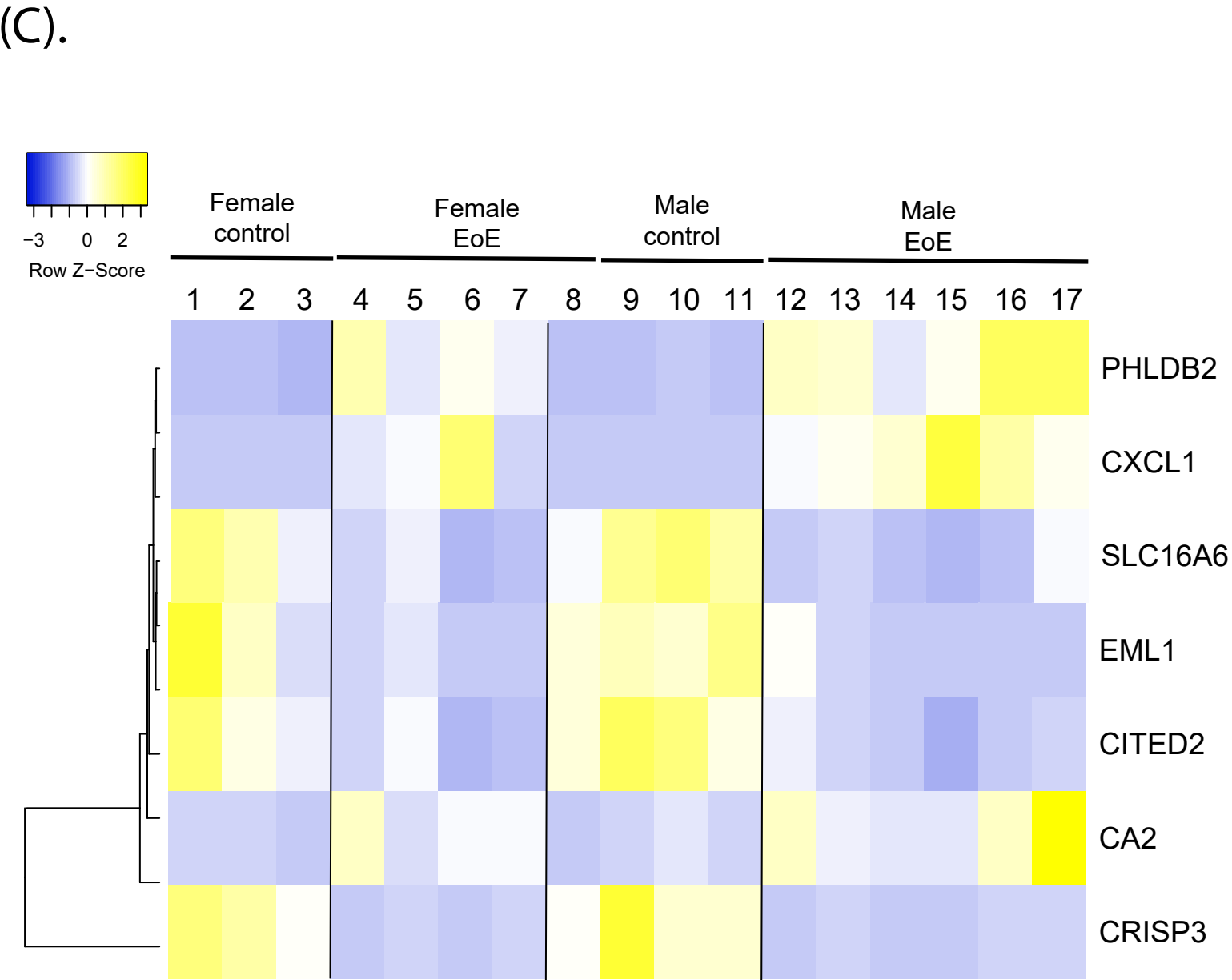
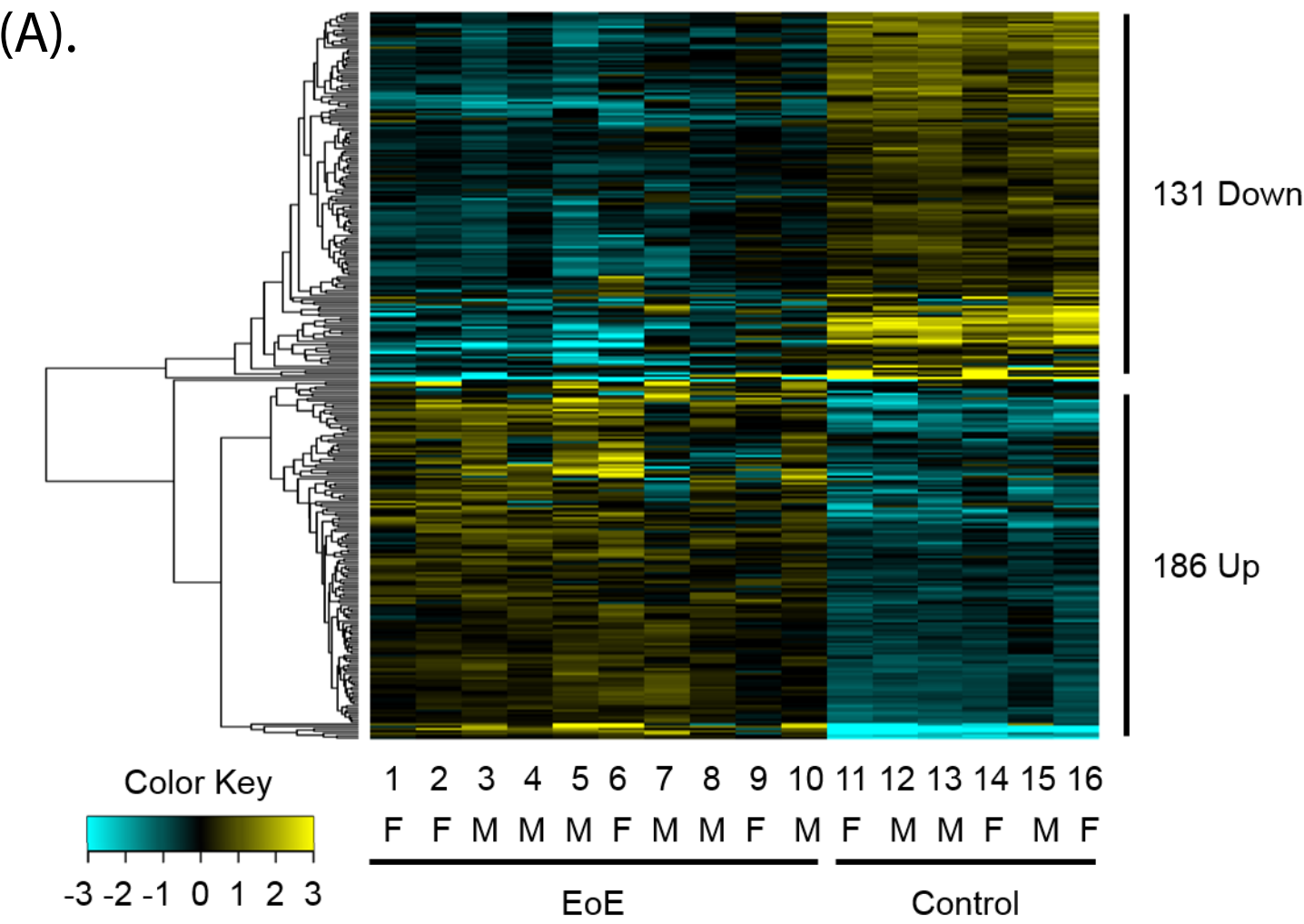
Gene	Function	V + IL13@24h vs Veh Fold Change	E2 + IL-13@24h vs E2 Fold Change	E2 + IL-13@24h vs V + IL-13 @24h Fold Change
ALOX15	Inflammation Process	2.6	2.9	1.1
ANO1	Ion Channel	5.5	8.4	-1.6
ARG1	Inflammation Process	-6.7	-6.7	1.9
CA2**	Epithelial Related	3.1	3.1	1.6
CCL26	Chemokine	3.8	4.8	-1.2
CDA	Pyrimidine Salvage	-2.9	-2.7	-1.2
CDH26	Cell Adhesion	2.2	2.2	1.2
CTSC	Tissue Remodeling	3.9	3.2	1.3
FLG	Epithelial Related	-3.3	-2.9	-1.2
GCNT3	Epithelial Related	5.3	6.0	1.0
H19	Steroid Responding Element	-2.2	-2.1	-1.8
IGFL1	Proliferation/Growth	-3.5	-4.0	1.2
PMCH	Inflammation Process	2.5	3.8	1.5
PNLIPRP3	Lipoprotein Metabolism	-2.3	-2.4	1.8
SLC16A6**	Ion Channel	-2.8	-3.2	1.4
SPINK7	Epithelial related	1.3	1.6	2.0
TNFAIP6	Inflammation Process	36.6	59.1	1.6
CAPN14*	Endopeptidase Activity	120.0	128.1	1.1
SLC9A3*	Ion Channel	1.1	1.2	-2.2
SOCS1*	Inflammation Response	6.9	9.9	-2.0

* Known IL-13 induced gene not included in EDP

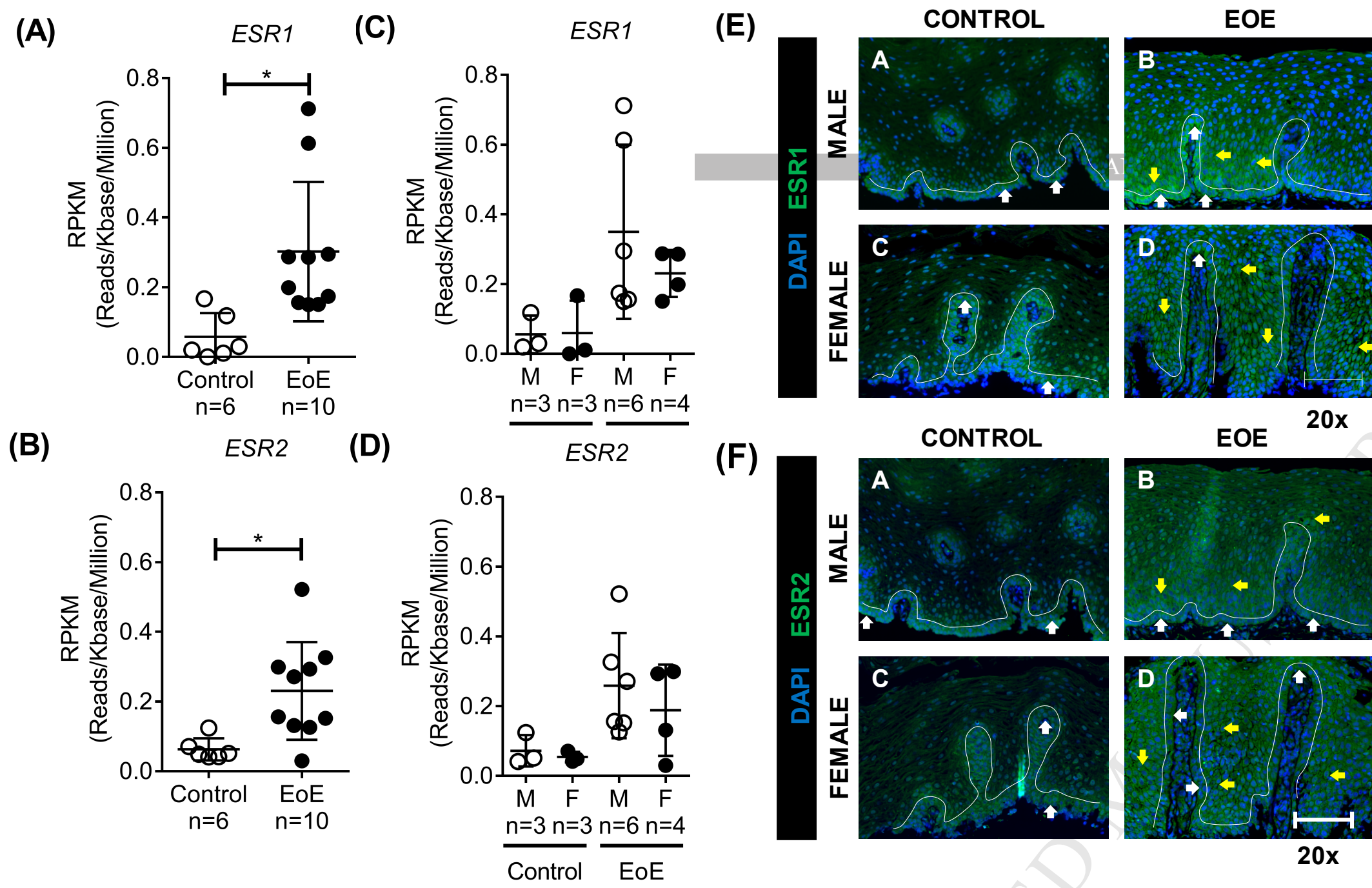
** EDP gene also included in estrogen responsive gene database

Table 3. Gene ontology analysis of IL-13 and β 17-estradiol dysregulated genes in ECP2-ALI cells identified by RNAseq analyses.

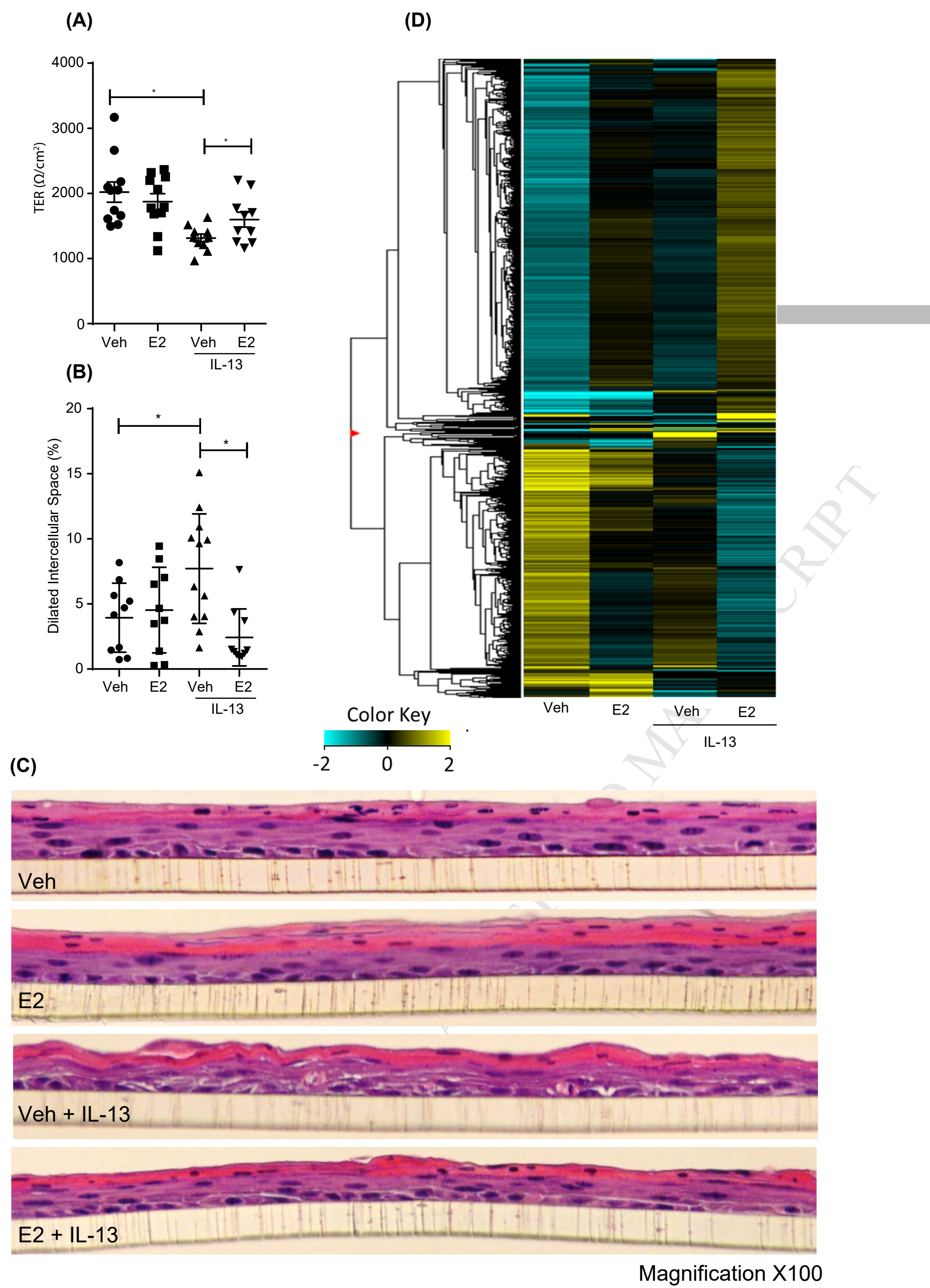
Group	Ontology	Activity	Number of Genes	p value	Specific Genes
Vehicle vs Vehicle+IL13 (n=246)	Biological Process	Regulation of Cell Adhesion	17 of 709	9.38E-05	<i>CYP1B1, C1QTNF1, COL1A1, DPP4, SFRP1, TNFSF18, TNC, C13orf15, MAP3K14, ST6GAL1, APOD, TGM2, RASGRP1, THBS1, EGR3, CD59, B4GALNT2</i>
	Biological Process	Regulation of Endothelial Cell Proliferation	6 of 103	2.27E-04	<i>CCL26, CCL24, C13orf15, ARG1, THBS1, EGR3</i>
	Biological Process	Response to Cytokine	18 of 825	1.89E-04	<i>CHI3L1, CCL26, IL22RA1, CISH, MME, CCL24, PID1, CARD14, COL1A1, SFRP1, TNFSF18, MAP3K14, BIRC3, IFI35, ARG1, ENTPD2, OASL, SOCS1</i>
Vehicle vs E2 (n=125)	Biological Process	Cellular Oxidant Detoxification	4 of 87	1.61E-04	<i>SZT2, HBA1, HBA2, HBB</i>
	Biological Process	Gene Silencing	6 of 257	1.52E-04	<i>KMT2B, POLR2A, TERT, KMT2D, HIST1H4H, MSL3P1</i>
Vehicle+IL13 vs E2+IL13 (n=100)	Biological Process	Response to Oxidative Stress	4 of 440	4.32E-04	<i>KLF2, HBA1, HBA2, HBB</i>
	Cellular Component	Innate Immune Response in Mucosa	2 of 24	1.86E-04	<i>HIST1H2BJ, HIST1H2BC</i>
Vehicle vs E2+IL13 (n=1107)	Biological Process	Mitotic Cell Cycle Process	96 of 932	3.39E-11	<i>NUSAP1, KIF18A, MIS18BP1, SPC25, MCM6, CDC7, CDC45, FZR1, ANAPC10, NDC80, ANXA1, RGCC, BIRC3, STIL, SMC2, NUP35, NDC1, HAUS1, MARK4, REEP4, PLK4, TJP3, PBK, NEDD1, BID, NCAPG, DBF4, BUB1, BUB1B, NPM1, CALM2, NUMA1, TERT, ZWINT, CCNB1, ITGB3BP, ORC4, ORC5, CCNE2, BRD4, MASTL, CDK1, CDC6, CDC34, TOP2A, PCNA, CDK7, CDKN3, PTTG1, CENPC, CENPE, ORC3, CETN2, CETN3, CHEK1, HMMR, TTK, CKS2, ANLN, PIM1, NUP37, ASPM, DSCC1, HSPA2, PLK3, HSP90AA1, TOM1L2, VRK1, KIF23, POLE2, NABP1, EPGN, KIF20B, PRIM1, TNKS1BP1, TCF7L1, KNTC1, ERCC6L, PSMA2, PSMA3, PSMA4, DLGAP5, PSMC6, NUF2, SPDL1, KNL1, ZWILCH, NUP107, RBBP8, RPS27L, CEP55, FANCI, CKAP2, SKA3, RPA3, MAD2L1</i>
	Biological Process	Keratinocyte Differentiation	19 of 140	9.55E-05	<i>EVPL, ALOX15B, FLG, FOXN1, ANXA1, TMEM79, ADAM9, CASP3, LCE1A, TGM1, C1orf68, CDSN, CASP14, OVOL2, PPL, CSTA, DNASE1L2, KRT10, LOR</i>
	Biological Process	Skin Development	33 of 280	7.14E-06	<i>EVPL, ALOX12B, ALOX15B, FLG, FOXN1, ANXA1, TMEM79, GRHL3, KRT27, ADAM9, SLC27A4, CASP3, LCE1A, TGM1, C1orf68, CDSN, CASP14, OVOL2, COL1A1, WNT7A, COL5A3, CLDN4, PPARD, PPL, IGFBP5, CSTA, TCF7L1, PRSS8, JUP, DNASE1L2, KRT10, LOR, ALOXE3</i>



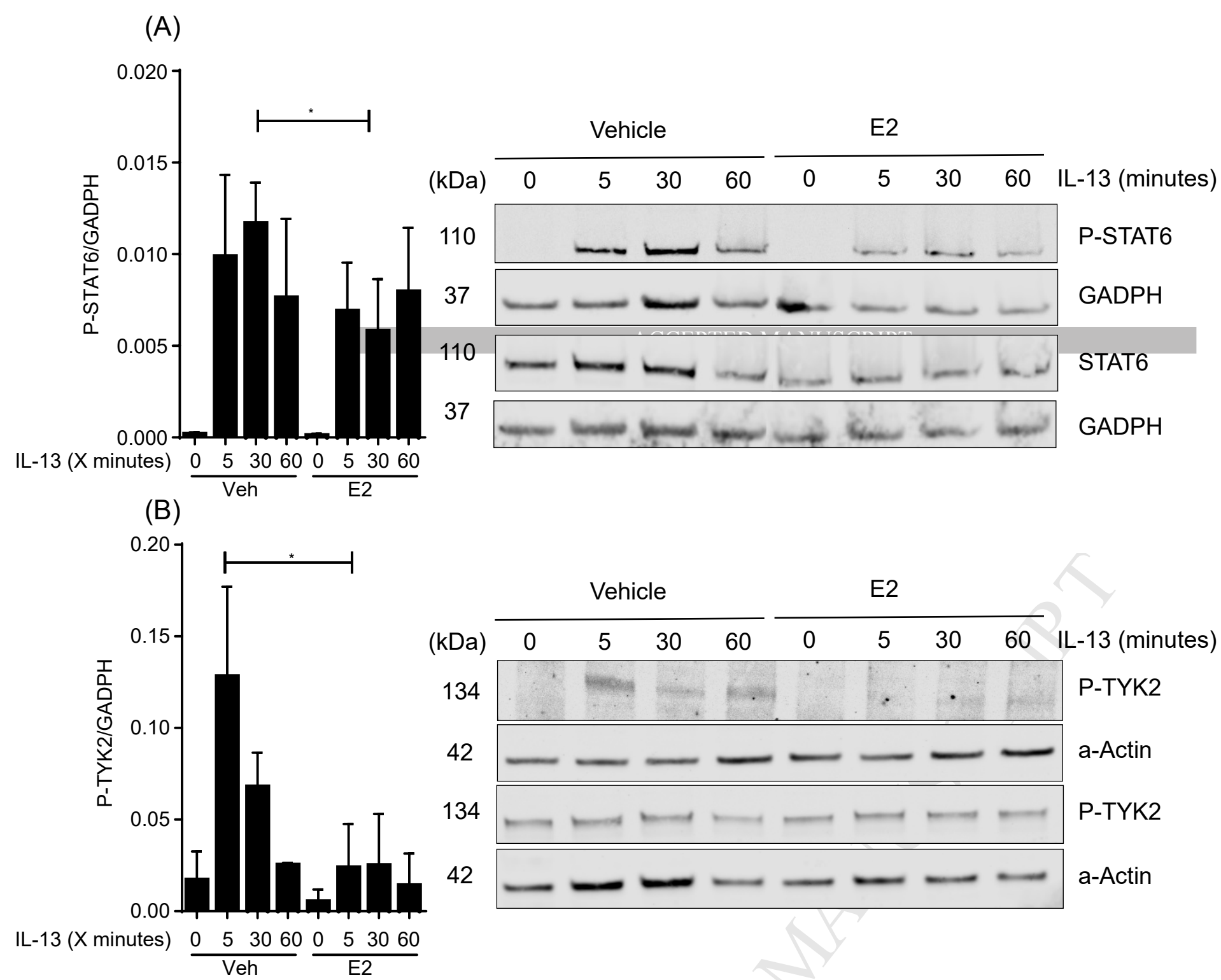
Wheeler et al., Figure 1



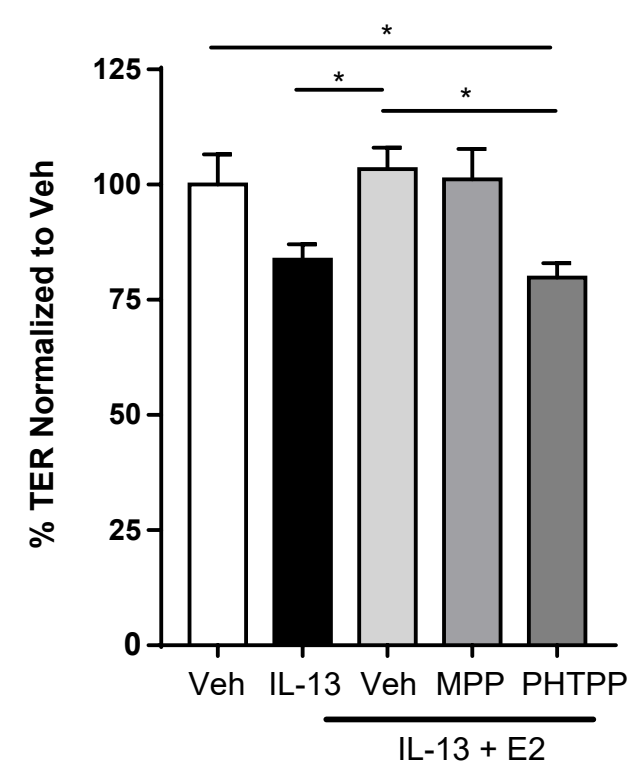
Wheeler et al., Figure 2



Wheeler et al., Figure 3



Wheeler et al., Figure 4



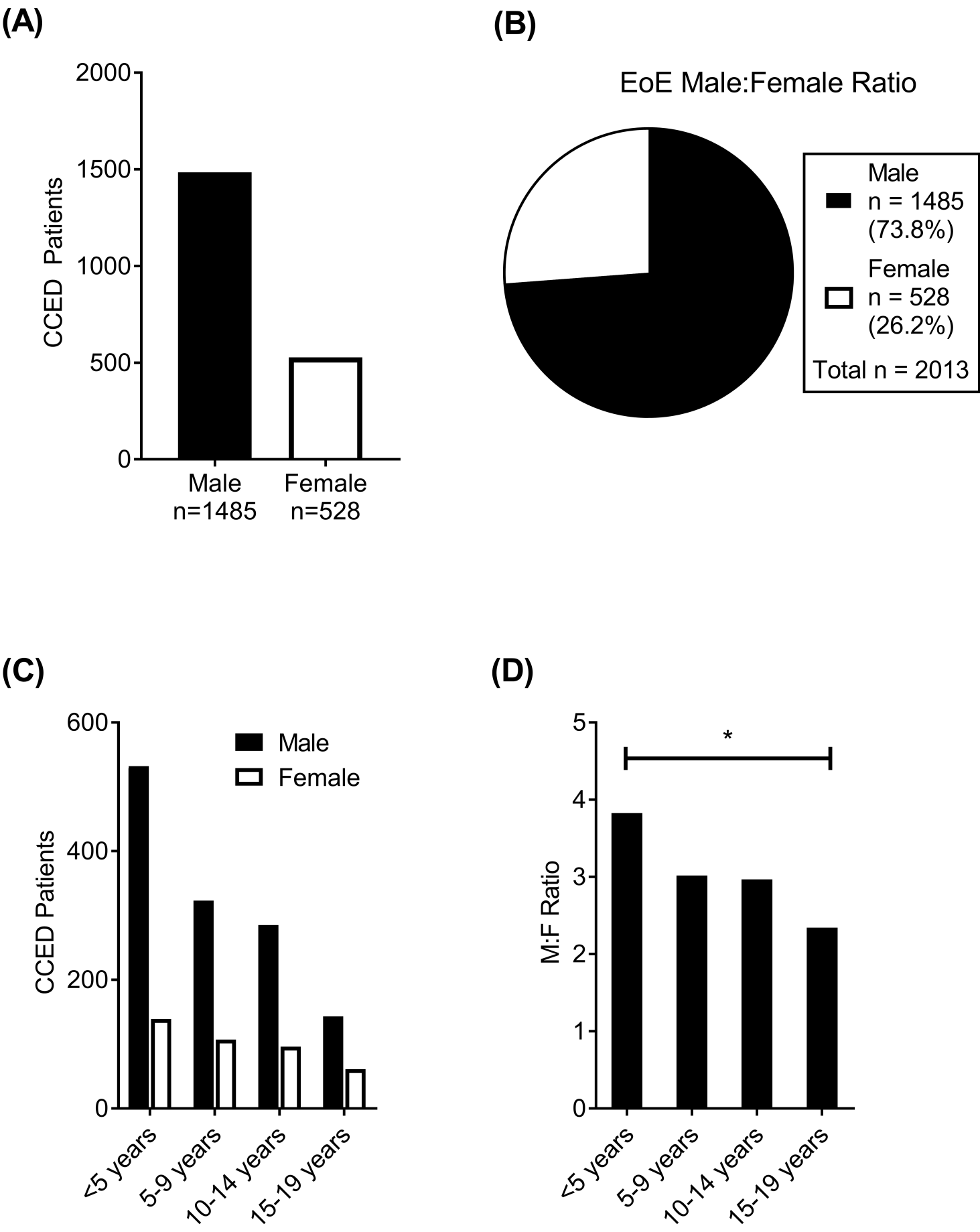
Gene ID	Gene Symbol	EoE v Ctrl
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79570	NKAIN1	-3.6938274
11004	KIF2C	2.090421
23648	SSBP3	-2.1834307
1718	DHCR24	-2.0940933
4808	NHLH2	-7.9068274
6281	S100A10	-2.1445158
6280	S100A9	-4.878775
3428	IFI16	2.0866728
5768	QSOX1	-3.2194555
5997	RGS2	2.665855
467	ATF3	2.9852965
3776	KCNK2	-2.38742
91543	RSAD2	3.5567057
6241	RRM2	2.0773802
151354	FAM84A	-2.2526572
2355	FOSL2	-2.282333
4084	MXD1	-4.736478
23397	NCAPH	2.0073497
8808	IL1RL2	-2.0251696
8942	KYNU	-2.7693355
7456	WIPF1	2.0911493
7035	TFPI	4.2318144
8828	NRP2	3.3540428
7277	TUBA4A	-2.4781528
2043	EPHA4	2.4812098
92737	DNER	-2.8724897
55355	HJURP	2.15619
9467	SH3BP5	-2.7454515
51099	ABHD5	-2.5065684
1230	CCR1	2.8903162
4057	LTF	10.861932
7869	SEMA3B	2.1535842
23166	STAB1	3.7305193
7474	WNT5A	-2.138783
23024	PDZRN3	-3.8128614
90102	PHLDB2	15.272408
7881	KCNAB1	-2.7669294
10057	ABCC5	3.204606
604	BCL6	2.0432694
9076	CLDN1	2.1800225

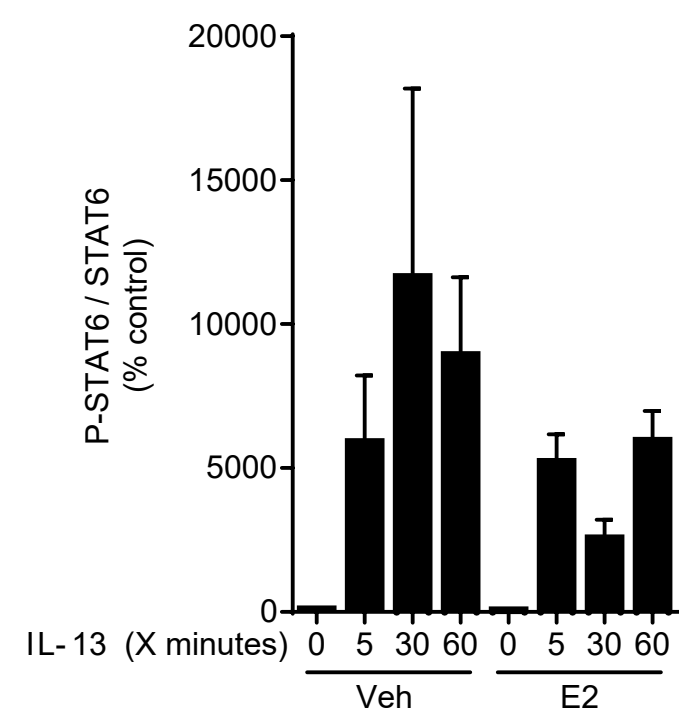
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374	AREG	-2.3094673
901	CCNG2	-3.9330554
51170	HSD17B11	2.3237138
64116	SLC39A8	2.0273166
10252	SPRY1	-2.9855664
8821	INPP4B	-3.0631502
7097	TLR2	2.207184
5618	PRLR	-5.397557
891	CCNB1	2.0676951
8507	ENC1	-3.2233987
22936	ELL2	-2.5925186
28966	SNX24	-2.7428026
5307	PITX1	-2.479032
9547	CXCL14	-4.473342
10112	KIF20A	2.1949399
3161	HMMR	2.422021
23286	WWC1	-2.4100356
2568	GABRP	2.3744614
23138	N4BP3	-2.054488
347733	TUBB2B	-4.456426
3720	JARID2	-2.0282407
8870	IER3	-2.7523484
629	CFB	7.014401
578	BAK1	2.2390006
1026	CDKN1A	-2.3305557
5292	PIM1	-3.3060715
10321	CRISP3	-422.97067
60481	ELOVL5	2.3214505
4199	ME1	-4.9494467
4082	MARCKS	-2.1363783
2697	GJA1	-4.3366895
5570	PKIB	-3.0308394
6446	SGK1	7.824201
10370	CITED2	-3.0182765
2099	ESR1	3.0552385
154043	CNKSRR3	-2.5714743
27075	TSPAN13	-2.537181
1124	CHN2	2.140382
54443	ANLN	2.2491665
23089	PEG10	-4.953466
43	ACHE	-5.82186
10135	NAMPT	-2.7723346

26959	HBP1	-2.2921069
1191	CLU	2.1993902
55872	PBK	2.1030056
1846	DUSP4	-3.552755
6422	SFRP1	4.6600194
760	CA2	7.6309357
7227	TRPS1	-2.4738355
64798	DEPTOR	-3.7762537
23237	ARC	-2.70144
286343	LURAP1L	34.32622
6812	STXBP1	-2.2465713
399665	FAM102A	-2.3775804
3934	LCN2	-3.3471174
9536	PTGES	5.5027094
445	ASS1	2.4493604
83641	FAM107B	-6.6630917
25805	BAMBI	-2.0078208
1326	MAP3K8	2.031808
983	CDK1	2.10686
55222	LRRC20	-10.495367
5328	PLAU	2.0051374
26509	MYOF	2.39125
4925	NUCB2	-4.158169
6288	SAA1	7.083697
960	CD44	2.9550786
79899	PRR5L	2.5568686
6947	TCN1	-5.5606546
2495	FTH1	-11.136274
10312	TCIRG1	2.3476403
5031	P2RY6	3.182197
23705	CADM1	2.9595108
969	CD69	4.993349
5318	PKP2	5.0620847
51474	LIMA1	2.2021432
3855	KRT7	-5.2352414
3854	KRT6B	-3.1959434
22822	PHLDA1	-4.090617
4254	KITLG	5.5764656
8693	GALNT4	4.4703536
26353	HSPB8	-2.2381966
10379	IRF9	2.1404727
5732	PTGER2	3.5021675
5265	SERPINA1	2.8276248

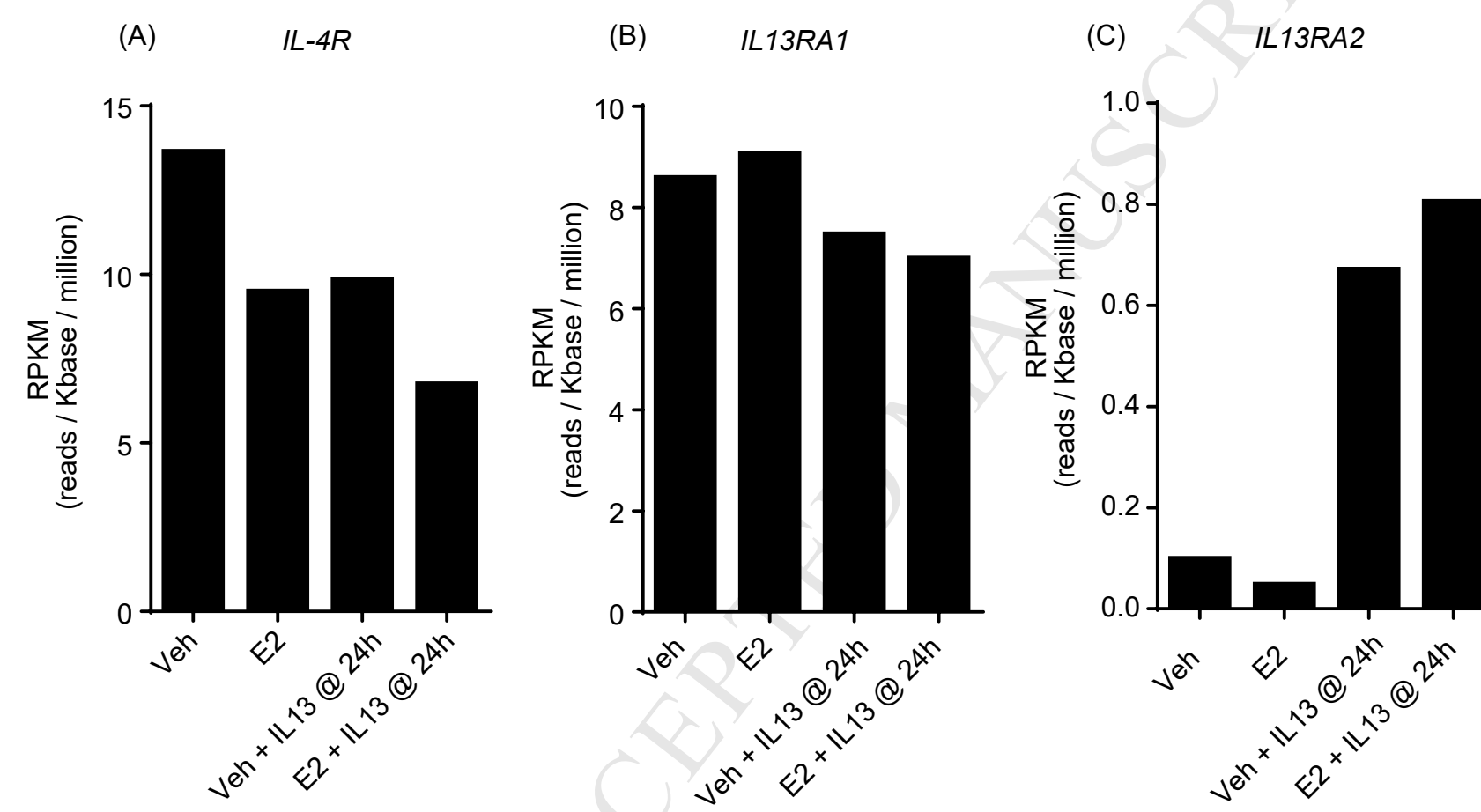
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701	BUB1B	2.0855703
2200	FBN1	2.052866
3067	HDC	33.882904
771	CA12	-3.3110154
10260	DENND4A	2.4901874
79875	THSD4	-2.0536993
9055	PRC1	2.0548787
21	ABCA3	-2.6582265
9516	LITAF	3.4651341
83719	YPEL3	-2.0160918
81831	NETO2	-2.27692
267	AMFR	-4.0804906
4500	MT1L	-3.78365
4490	MT1B	-4.607791
4501	MT1X	-3.2173896
8140	SLC7A5	-4.241863
3682	ITGAE	2.1115844
489	ATP2A3	2.4045455
54739	XAF1	2.4633784
5376	PMP22	3.613479
7153	TOP2A	2.3354392
10140	TOB1	-3.0223722
3838	KPNA2	2.1269157
9120	SLC16A6	-4.122575
54549	SDK2	-2.5351462
146722	CD300LF	4.5996666
9021	SOCS3	4.1641707
71	ACTG1	-2.6543965
5366	PMAIP1	-3.2186942
4616	GADD45B	-7.566502
3383	ICAM1	2.9324749
23770	FKBP8	-2.1683078
7538	ZFP36	-2.8289816
645	BLVRB	-2.4865913
22974	TPX2	2.0029418
26013	L3MBTL1	2.199974
84612	PARD6B	-2.0653572
8537	BCAS1	-2.7189233
6790	AURKA	2.3851943
8318	CDC45	2.2055566
80115	BAIAP2L2	-12.881658
638	BIK	2.3142154

367	AR	-3.504336
51566	ARMCX3	2.2777658





Wheeler et al., Supplemental Figure S2



Supplemental Table S1. Demographics of patient cohorts examined in RNAseq analysis.

RNAseq cohort (NL=6, EoE=10)

	Age	Male	Female	Max Eosinophils per high powered field (hpf)
<i>NL</i>	12.7 ± 6.0	50%	50%	0 ± 0
<i>EoE</i>	15.1 ± 12.4	60%	40%	163.6 ± 92.4

Supplemental Table S3. Eosinophil Diagnostic panel (EDP) expression analyses of estrogen responsive genes in esophageal biopsy samples from control and EoE cohorts.

Estrogen Responsive EDP Genes	Function	Log FC All [EoE vs Control]	<i>p</i> value	Log FC Male [EoE vs Control]	Log FC Female [EoE vs Control]
CA2*	Epithelial Related	2.65	8.0E-04	0.72	0.89
CFB*	Inflammation	2.50	1.6E-03	1.10	1.15
CITED2	Inflammation	-1.42	3.1E-03	-0.52	-0.47
CRISP3*	Epithelial Related	-7.99	3.0E-04	-2.41	-2.28
CXCL1*	Chemokine	-2.14	4.0E-03	1.30	0.87
EML1*	Cytotubule Assembly	-2.86	7.0E-04	-0.64	-0.63
FKBP5	Steroid-Responding Element		ns		
KRT23	Remodeling		ns		
PHLDB2	Epithelial Related		ns		
SLC16A6*	Ion Channel	-1.89	5.0E-04	-0.75	-0.56

*Significantly expressed in EoE compared to Control

Supplemental Table S4. ESR1 and ESR2 Isoform mRNA expression in NL and EoE biopsy samples.

	NL	EoE	Significance
ESR Isoforms	RPKM (Reads/Kbase/million)	RPKM (Reads/Kbase/million)	
ESR1			
Isoform 1	0.02 ± 0.00	0.06 ± 0.02	n.s.
Isoform 2	0.02 ± 0.02	0.06 ± 0.02	n.s.
Isoform 3	0.02 ± 0.01	0.09 ± 0.03	n.s.
Isoform 4	0.0 ± 0.0	0.03 ± 0.02	n.s.
ESR2			
Isoform A	0.0 ± 0.0	0.01 ± 0.01	n.s.
Isoform B	0.0 ± 0.0	0.06 ± 0.03	n.s.
Isoform C	0.04 ± 0.01	0.05 ± 0.03	n.s.
Isoform D	0.02 ± 0.01	0.01 ± 0.01	n.s.
Isoform E	0.01 ± 0.00	0.08 ± 0.03	p < 0.05

*RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of ESR1 and ESR2 isoforms in esophageal biopsies from healthy control (female n =3, male n =3) and EoE patients (female n= 4, male n=6). ESR1 and ESR2 expression was elevated in EoE patients compared to controls.

Supplemental Figure S1: Sex Predominance and Age at Diagnosis of EoE at CCED: (A) All male and female patients diagnosed with EoE in the Cincinnati Center for Eosinophilic disease (CCED) from 1/2000 through 12/2016 (Total n = 2013, males n = 1458, females n = 528). (B) Male predominance in EoE, with a male to female ratio of 2.81 (73.8% male, 26.2% female) (C) Male and female patients with EoE separated by age of initial diagnosis into 5 year increments. Patients <5 years (total n = 666, male n = 527, female n = 139), age 5-10 years (total n = 423, male n = 316, female n = 107), age 10-14 years (n = 377, male n = 281, female n = 96) and age 15-19 years (n = 167, males n = 90, females n = 77). (D) Chi square analysis demonstrated a significant difference in M:F ratios of patients < 5 years compared to those age 15-19 years (3.8 vs 2.31, p<0.01). (D) We observed no statistically significant differences between other age divisions.

Supplemental Figure S2: Ratio of P-STAT6 / STAT6 in EPC2-ALI esophageal epithelial cells following exposure to IL-13 ± 17β-estradiol in esophageal epithelial cells. Quantitative expression of p-STAT6 / STAT6 relative levels normalized to control (0 minutes Veh), with n = 3, *p < 0.05, student t-test. Western Blot of EPC2-ALI cell lysate for phosphorylated STAT6 (P-STAT6) at 0, 5, 30, and 60 minutes after IL-13 (100 ng/mL) exposure with or without 24 hrs pretreatment with E2 (100 nM) or Vehicle (0.01% EtOH). Analyzed by means of immunoblotting with anti-P-STAT6 and anti-STAT6.

Supplemental Figure S3. RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of (A) *IL4RA*, (B) *IL13RA1* and (C) *IL13RA2* in EPC2-ALI multicellular layer treated with E2 (100 nM) or Vehicle (0.01% EtOH) for 24 hours, with exposure to IL-13 (100 ng/μL) at 24 hours.

Supplemental Table S1. Patient characteristics of EoE and control patients. Description of age of biopsy collected, gender percentage and maximum eosinophils per high power field of patient in cohort.

Supplemental Table S2. Dysregulated Estrogen Responsive Genes within the EoE Transcriptome. Significantly dysregulated genes identified by RNA sequencing expression analysis of esophageal biopsies of n = 6 control patients (numbers 1-3 and 8 – 11, female n = 3, male n = 4) and 10 patients with EoE (numbers 4-7 and 12-17, female n = 4, male n = 6) patients were cross referenced against estrogen responsive gene reference database (<http://datam.i2r.a-star.edu.sg/ergdbV2/>)⁴⁴ which consists of 1,069 validated estrogen responsive human genes. Column one indicates Gene ID, Column 2, Gene symbol and Column 3 fold change (FC) between EoE and control.

Supplemental Table S3: Estrogen Responsive Genes in EoE Diagnostic Panel (EDP): RNA sequencing analysis (Log FC) and gene function of the 10 genes that are included in both the EoE Diagnostic Panel (EDP, 94 genes) and the estrogen responsive gene database (1,069 genes). Transcripts isolated from esophageal biopsies of EoE (female n = 4, male n = 6) and control patients (female n = 3, male n = 3). The 7 genes that are significantly expressed (Log FC > 1.5, p < 0.05) in EoE patients compared to control are shown in bold. The difference in expression (Log FC) of male EoE and control patients and female EoE and control patients are also shown.

Supplemental Table S4. RNA sequencing gene expression represented by reads per kilobase of transcript per million mapped reads (RPKM) of *ESR1* and *ESR2* isoforms in esophageal biopsies from healthy control (female n = 3, male n = 3) and EoE patients (female n = 4, male n = 6). *ESR1* and *ESR2* expression was elevated in EoE patients compared to controls.