Online Supplement

Quantile (median) linear regression on the association of AEC eotaxin-2 protein levels and age of asthma onset, adjusted for confounders.

	Coefficient	p	95% C.I.
Age of asthma onset (years)	2.10	< 0.001	1.2 – 2.9
Age at enrollment (years)	.12	0.8	-1.14 – 1.4
Gender	-3.94	0.8	-36 – 28
Severity	3.61	0.7	-17.7 – 24.8
Body Mass Index	.93	0.3	-.9 – 2.9
Model R^2=0.22			

METHODS

SARP protocol [Details as previously described (1)] Mild asthma subjects had an FEV1 of >80% predicted, used no inhaled corticosteroids (CS), had symptoms < 2 days per week, and had rare exacerbations. Mild/Moderate asthma + ICS (Mild/Mod+ICS) used inhaled CS (<1000 mcg fluticasone or equivalent/day) on a daily basis, had an FEV1 of >60% predicted and were without a history of ED/urgent care visits or systemic CS use in the past year. Severe asthma was defined by the ATS 2000 workshop definition (2). Severe asthmatics were on treatment with high dose inhaled CS ($\geq$1000 mcg fluticasone/day) or systemic CS (SCS) and met at least 2 of 7 minor criteria. Normal controls had no history of respiratory symptoms or

disease, normal pulmonary function and a negative methacholine challenge. These subjects were recruited through local advertisements, in participating SARP cities. Although atopic NCs were allowed, no NC was treated for rhinitic symptoms. No subject smoked within the previous year or had a greater than 10 pack year history. All subjects answered questions on their asthma and underwent allergy skin testing. Pre- and maximum post-bronchodilator spirometry testing was performed. Frequent and/or severe asthma exacerbations were identified by one or more ED visits for asthma or 3 or more oral CS bursts in the previous year. Sputum induction was done at least 1-2 weeks prior to bronchoscopy and only on subjects with an FEV1$\geq$60% predicted. If the FEV1 was <60% predicted, spontaneous sputum was utilized if available. The study protocol was approved by the Institutional Review Boards at the University of Pittsburgh and National Jewish Health and written informed consent was obtained from all subjects.

Sputum induction and processing. Sputum was processed according to the method of Fahy (3). Sputum eosinophils were counted using a Diff-Quick stain Protocol Hema 3 (Fisher Scientific, Pittsburgh, PA) on cytospins and expressed as percentage of 300 inflammatory cells.

Bronchoscopy with airway brushing and lavage. Bronchoscopy was performed as previously described (4, 5). Six to 8 airway brushings (containing a mix of 85-95% epithelial cells and 5-15% inflammatory cells) were obtained from different subsegments of the right or the left lower lobes and immediately placed into TriZOL for mRNA or Mammalian Cell-PE Lysis Buffer for protein analysis (G-Biosciences, St. Louis, MO). BAL was obtained from either the

lingula or right middle lobe and processed as previously described (6). Eosinophils percentages in BAL were determined from the percent of total cells after counting 300 cells.

Real time quantitative PCR analysis. Primers and probes were labeled with 5'-reported dye 6-carboxy fluorescein (FAM) and 3'-quencher dye 6-carboxy N, N, N', N' tertramethyl-rhodamine (TAMRA) obtained from Applied Biosystems (Assays on Demand, Foster City, CA, USA). Catalogue: eotaxin-1 Hs00237013_m1; eotaxin-2 Hs00171082_m1; eotaxin-3 Hs00171146_m1). VIC labeled glyceraldehyde 3 phosphate dehydrogenase (GAPDH) primer and probes were obtained from Applied Biosystems (Genbank accession number NM-002046, Catalogue: 4310884E). qRT-PCR was performed on the ABI Prism 7900 sequence detection system. All mRNA measurements were indexed to GAPDH values ($1/2^{\Delta Ct} \times 1000$). Due to limited sample availability, low Ct values or non-significant results, mRNA for all eotaxin isoforms was not obtained on all subjects. A Ct value of 40 was considered undetectable. Samples were excluded if the GAPDH exceeded 27.

Enzyme linked immunosorbent assays. Eotaxin protein levels in AEC and BAL cell lysates and BAL fluid were measured by ELISA with antibodies from R&D Systems (Minneapolis, MN) by ELISATech (Aurora, CO), as described in online reference. The lower limit of detection of the assays was 16 pg/ml. All eotaxin protein levels from cell lysates were corrected for cell number and reported as pg/10^6 cells. Samples with undetectable protein were assigned a value

of 5 pg/10^6 cells for statistical calculations. Eotaxin levels in BAL fluid were reported per ml of BAL fluid. Due to limited sample availability and low or no detection for some isoforms, protein levels of all the eotaxin isoforms were not able to be obtained on all subjects.

References

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