Exploration of Early-Life Candidate Biomarkers for Childhood Asthma Using Antibody Arrays

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Electronic Repository: E Table 1. List of Proteins Assessed in the Antibody Array.

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Abstract

Background: Proteomic approaches identifying biomarkers have been applied to asthma to only a very limited extent.

Methods: With an antibody array (RayBiotech, Norcross, GA) the relative intensity and rank differences of 444 proteins were compared in 24 plasma samples obtained at age 3, 11 from children with and 12 without asthma diagnoses at ages 5 and 9. Protein candidates identified by antibody array were quantitated by ELISA in an enlarged sample. Proteins found to differentiate children with and without asthma were also examined for association with known Year 1 asthma risk factors, eczema and wheeze.

Results: In the antibody array, four proteins had rank differences between asthma and non-asthma groups (FDR <0.1). By ELISA, mean log ($\pm$ SEM) erythropoietin (EPO) level (IU/L) was lower (0.750 ± 0.048 vs. 0.898 ± 0.035 ; $p=0.006$) and mean ($\pm$ SEM) soluble GP130 (sGP130) level (ng/mL) was higher in the asthma vs. the non-asthma group (302 ± 13 vs. 270 ± 8 ; $p=0.041$). The other 2 array proteins (galactin-3, eotaxin-3) did not differ by ELISA by asthma. EPO related to the asthma risk factor, first year eczema, whereas sGP130 related to first year wheeze.

Conclusions: Through two independent assessments, age 3 plasma levels of EPO and sGP130 were found related to childhood asthma.

Key words: asthma; plasma; biomarker; erythropoietin; soluble GP130

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INTRODUCTION

Asthma is a lung disease which features chronic inflammation, episodic bronchospasm, airway hyper responsiveness, and airway remodeling [reviewed in Elias *et al.* (1)], and is a serious public health problem worldwide. The pathobiology remains relatively obscure. Studies have identified associations of asthma with variants in various genes [reviewed in Vercelli (2)], but these account for only a small portion of the familial inheritance patterns. The emerging area of plasma proteomics holds promise for biomarker discovery in complex diseases. Thus far plasma proteomic methodologies have been applied to the study the biology of asthma to only a very limited extent (3, 4)

Antibody array is a technique of profiling multiple protein analytes simultaneously in samples or tissues (5). Different from quantitative methods, antibody arrays generate signal intensities that allow assessment of relative concentrations of analytes among samples. Findings of significant relative differences can be assessed for quantitative validation by other methods.

We hypothesize that asthma development in childhood is accompanied by alterations in plasma proteins. Potential biomarkers for childhood asthma were sought through application of antibody array for 444 proteins in plasma obtained at age 3 from a subset of children enrolled in a longitudinal study and followed through age 9 for development of asthma. Top candidates were tested for quantitative differences with ELISA assays. The results point to potential biomarkers for childhood asthma that open the possibility of distinct pathways, and that if found to be generalizable, will allow for new directions of possible asthma treatment and/or prevention.

METHODS

Birth Cohort Subgroups and Samples

Cryopreserved plasma samples were from subjects in the Tucson Infant Immune Study (IIS), a birth cohort enrolled without selection and followed longitudinally through age 9 (6). Samples obtained at age 3 were from either children whose parents had reported the child had a physician diagnosis of asthma at ages 5 and 9 and active wheezing in the past year at both ages (asthma group) or children whose parents reported the child did not wheeze or have a diagnosis of asthma at age 3, 5, or 9 (non-asthma group).

Sample sizes, Assays, and Analyses

Samples (n=12 each from asthma and non-asthma groups) were sent to RayBiotech (Norcross, GA) where they were assayed by biotin-label-based antibody array (L-series 507) for 507 proteins. Group size was determined by availability of asthma group subjects meeting all criteria at point samples were submitted to Raybiotech and (for nonasthma group) availability of funds. The intensity values of signals from plate for each subject were evaluated by Axon GenePix laser scanner. To adjust for plate-to-plate variation, normalization by RayBiotech was done by multiplying the intensity values on each plate by the ratio of the positive controls on that plate to the mean of the positive controls on one randomly selected plate. Background was removed by subtracting each plate's mean negative control from each value as described (5). One sample from the asthma group had background intensity values much greater than the other 23 and was excluded from further analysis. Proteins with mean intensities (mean of all samples) near background levels (less than 100, n=63) were excluded from further analysis. This value of 100 was selected as 3 times the mean background noise. Intensity values for remaining proteins (ranging from 100 to 19250) were ranked within each subject.. Group mean ranks for each protein were compared using Student's t-test and were judged noteworthy if the false discovery rate (FDR) adjusted p value was less than 0.1 (7). Noteworthy proteins were further considered for ELISA evaluation.

Quantitation of the candidate proteins identified by antibody array screening was performed using Quantikine ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Sample size of asthma group (our study being longitudinal) could be expanded to 18 samples and included all of the subjects in the IIS population who both met the criteria for persistent childhood asthma (n=33) and had sufficient samples at age 3 for analysis. The non-asthma group was increased to 54 (3 times larger than the asthma group) randomly selected from among 146 in the IIS population who met the criteria and had sufficient samples at age 3. Samples were run as 2-4 replicates and values below the lowest standard were assigned one-half the lowest standard. For proteins with less than 15% undetectable values, distributions of values were assessed for normality and if skewed to the right were log transformed. Groups were compared by Student's two-sample t test. For proteins with greater than 15% undetectable values, groups were compared using Mann-Whitney comparison of medians. Odds ratios with mutual adjustment were calculated to assess independence of the associations between two protein biomarkers and asthma. Proteins differing by asthma status were further assessed by dividing the asthma group into those diagnosed by or after age 3. These groups were compared by ANOVA and post-hoc Fisher's protected LSD test. A p-value <0.05 was considered significant.

Blood samples were assayed for hemoglobin by Sonora Quest Laboratories (Tucson, AZ). Total IgE values at 3y were available from assays previously run by Immulite 2000 as previously described (8).

RESULTS

Identification of Plasma Protein Candidate Biomarkers for Childhood Asthma through Antibody Arrays

In comparing the asthma (n=11) and non-asthma (n=12) groups based on the ranks of intensity values from the antibody array assays, 4 proteins were identified as differentiating the two groups (FDR adjusted p-value <0.1; Figure 1). The mean ranks of erythropoietin (EPO) and galectin-3 were lower and the mean ranks of eotaxin-3 and soluble GP130 (sGP130) were higher in the asthma compared to the non-asthma group. The 444 proteins assessed are listed in E Table 1 or <http://www.raybiotech.com/l-series-507-label-based-human-array-1-membrane-2.html>.

Quantitation of Candidate Biomarkers for Childhood Asthma

In the enlarged sample set, we quantified by ELISA each of the four plasma proteins that were identified by rank analysis in the antibody arrays. EPO had a lower mean log concentration in the asthma compared to the non-asthma group (Figure 2A). The direction of change agreed with that of the rank differences in the antibody array. Also in accord with rank difference direction, mean concentrations of sGP130 were increased in the asthma group (Figure 2B). Mean galectin-3 concentrations did not differ significantly between the two groups (Figure 2C). For eotaxin-3, for which 55% had at least one undetectable value, a nonparametric comparison indicated there was no difference in median values between the asthma and non-asthma groups (data not shown). Limiting the analysis to those samples also assayed in the antibody array revealed a similar significant difference in the two groups for EPO although the difference for sGP130 was not significant.

When modeled together, logistic regression analysis revealed the independence of the associations of EPO and sGP130 plasma levels for asthma risk (Table 1). Levels for these two plasma biomarkers did not correlate with each other ($r=0.12$, $p=0.33$). Total IgE, well known to be increased in asthma, was increased in the plasma samples of the children with asthma

155 compared to those without asthma (mean log $\pm$ SEM: 1.775 $\pm$ 0.141 vs. 1.104 $\pm$ 0.089 IU/ml,
156 respectively, $p < 0.001$). Total IgE correlated inversely with EPO ($r = -0.36$; $p = 0.002$, $n = 70$) but not
157 with sGP130 ($r = -0.19$; $p = 0.14$, $n = 66$). Hemoglobin levels did not differ by asthma group,
158 although levels in the combined groups tend toward their known inverse relation with EPO ($r =$
159 -0.20 , $p = 0.07$).

161 In a post-hoc analysis, the asthma group was subdivided into those who had a diagnosis of
162 asthma already by the age of 3 and those who gained their diagnosis after age 3. A
163 concentration response was evident with lower levels of EPO in those already diagnosed at age
164 3 (Table 2). Similar stepwise increases were observed for total IgE. In contrast, sGP130 levels
165 did not differ by age of diagnosis in the asthmatic children.

167 **Relation of plasma protein biomarkers to early life asthma risk factors**

168 Eczema and wheezing in the first year of life are well-established, independent risk factors for
169 childhood asthma (9) and in the subset of IIS children show odds of more than 5 and 9
170 respectively (Table 3). Interestingly, the mean log plasma EPO was lower in children with
171 eczema in the first year of life but was unrelated to first year wheezing (Table 4). In contrast,
172 mean sGP130 showed no relation to eczema but was higher in the children with first year
173 wheezing (Table 4). Maternal asthma, also a significant risk factor for asthma in this subset,
174 was unrelated to either plasma protein biomarker (data not shown).

176 **DISCUSSION**

178 Our study identified four candidate protein biomarkers of asthma development using rank
179 analysis of 444 proteins by antibody array in plasma samples obtained at age 3 from a subset
180 children enrolled in the Tucson Infant Immune Study, a birth cohort. Of these four proteins, we

181 identified quantitative differences by ELISA for two: EPO had lower mean levels in the asthma
182 compared to the non-asthma group and sGP130 had higher levels in the asthma group. Neither
183 eotaxin-3 nor galactin-3 yielded quantitative differences between the two groups.

184
185 Plasma proteomics offers the opportunity to follow hypothesis generating approaches to study
186 of the biology of a complex disease like asthma. Differences in plasma proteins so identified
187 suggest that the disease may have systemic involvement (in contrast to being completely organ
188 constrained). Given that plasma contains several thousand proteins (10), antibody arrays do
189 not assess the entire protein spectrum and carry some bias in regard to limitations based on
190 availability of antibody pairs for protein detection. In contrast, though, they are much less limited
191 by relative concentrations than are mass spectrometry-based methods.

192
193 Only a few studies have yet pursued examining plasma or serum for asthma-related differences
194 by either of these approaches. In one study utilizing two-dimensional gel electrophoresis and
195 matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.
196 Verrills *et al.* (3) identified 14 proteins that showed relative differences in serum of adult
197 asthmatics vs. controls (without correcting for multiple comparisons). Validation of a few of the
198 14 proteins was attempted by ELISA: one yielded concentrations that validated the direction of
199 change identified in the proteomic analysis (haptoglobin), one was not significant in the
200 proteomic analysis but was increased in ELISA (ceruloplasmin), and one showed a difference
201 opposite in direction (anti-thrombin III). None of the 14 proteins was among the proteins on the
202 antibody arrays employed in our study. In a recent report, Reubsaet *et al.* (4) performed a
203 limited multiplex antibody-based assay in plasma from a group of children all of whom exhibited
204 wheezing in the first 3 years of life. Among 16 cytokines and chemokines assessed, they
205 reported 3 analytes (CXCL10, CCL17, CCL22) that differed between children who subsequently
206 gained a diagnosis of asthma by age 6 and those who did not. Although these analytes were

among the 444 we assessed in the antibody array employed herein, they did not show a difference related to asthma in our study, possibly related to differences in study design. Reubsaet *et al.* studied only children with a history of early life wheezing, required evidence of allergic sensitization for a diagnosis of asthma, and included no consideration for multiple comparisons. Thus, it is possible that their findings may relate to an allergy susceptible group within asthma.

ELISA for plasma EPO levels provided quantitative validation of the rank differences and agreed in direction with data generated by antibody array. Thus, decreased concentration of EPO in plasma at age 3 appears to serve as a biomarker differentiating subjects with asthma from those without. The quantitative differences were greatest in children who had already acquired a diagnosis of asthma by age 3.

Although EPO has long been known for its actions in increasing production of red blood cells, other effects noted more recently involve protective effects on various cell types and include anti-inflammatory (11-13), anti-apoptotic (14, 15), and antioxidant effects (16, 17).

EPO functions through two classes of receptor made up of combinations of monomeric EPO receptor (EpoR) and CD131, the common beta chain (18, 19). While EpoR homodimerizes upon binding of its ligand, common beta chain has been shown to heterodimerize with monomeric EpoR (19, 20). It has been suggested that the protective effects on various tissues act through the heterodimerized receptor (21).

To our knowledge, altered EPO levels have not been implicated in human asthma. However, a recent study suggested that EPO is decreased in plasma of subjects experiencing exacerbations of COPD (22). A few studies in mice have suggested the potential therapeutic

value of EPO. . Following EPO administration, Tyagi *et al.* (23) found significant inhibition of bronchoconstriction induced by carbachol and In a model of chronic asthma, Karaman *et al.* (24) reported improvement in histological assessments of ovalbumin-induced airway hyper responsiveness and airway inflammation . Such findings support the possibility that the reduced levels of EPO associated with asthma shown in our study may increase risk for cell damage and also show a need for future studies to examine the local airway milieu for differences in EPO.

Soluble GP130 has also not been clearly associated with asthma or early life wheeze, but such roles are not without plausibility as sGP130 participates in a complex network with a membrane-bound form of GP130 together with IL-6 and both soluble and membrane-bound IL-6R (25). sGP130 is suggested to serve as a natural inhibitor of IL-6 (26) and its inflammatory activity. Soluble IL6R was shown (27) increased in airways of asthmatics.

We confirm that eczema and wheezing are associated independently with risk for asthma, suggesting that they may identify distinct paths to asthma. In this regard, our two biomarkers align differentially with these two risk factors: low plasma EPO levels associate with eczema in the first year of life whereas high sGP130 associate with wheeze. Further studies are needed to explore the possibility that the two biomarkers may mark separate paths involved in asthma development.

Previous reports, differ as to whether eotaxin-3 is increased in asthma (28, 29). Our results could not corroborate an increased plasma level associated with asthma. Galectin-3, has been reported to function as a soluble receptor for IgE (30), but has not been clearly associated with asthma and we were unable to identify differences in plasma levels between our asthma and non-asthma groups.

Our study has several advantages: the use of samples from a non-selected birth cohort that has longitudinal detailed information on respiratory symptoms and age of acquiring asthma diagnoses; a broad, hypothesis-generating, screening approach to identifying candidate plasma biomarkers; and a step validating the screening results through quantitation. A limitation of our study is a relatively small sample size and thus other markers in the antibody array may be present differentially in asthma but such differences were not detected because of insufficient power.

In summary, our study demonstrates the utility of antibody array as a screening tool for proteomic identification of candidate biomarkers in human plasma for childhood asthma and the importance of quantitation for follow-up of candidates so identified. We suggest that children with asthma have decreased levels of circulating EPO and increased levels of sGP130. In addition, EPO relates to the early life risk factor, eczema, and sGP130 relates to early life wheezing. Further studies are warranted to assess generalizability and to establish the biological actions and possible therapeutic effects of treatment with EPO and/or approaches to decrease sGP130 in childhood asthma.

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376

377 Table 1. Biomarker Odds Ratios for Asthma Before and After Mutual Adjustment.

Asthma Biomarker	Marginal Odds Ratio	95% CI	p	Mutually Adjusted Odds Ratio	95% CI	p
EPO	0.01	0.00-0.36	0.011	0.01	0.00-0.50	0.022
SGP130	1.01	1.00-1.02	0.047	1.02	1.00-1.03	0.018

378

379 Table 2. Mean levels of Plasma Biomarkers at Age 3 in Relation to Age of Asthma Diagnosis

	n	No asthma	n	Asthma Diagnosed After Age 3	n	Asthma Diagnosed by Age 3	ANOVA P
Mean (SEM) log EPO IU/ml	54	0.898 (0.026)	7	0.830 (0.070)	10	0.706 (0.068)	0.018*
Mean (SEM) sGP130 ng/ml	50	260 (8)	7	304 (23)	8	305 (14)	0.10
Mean (SEM) log IgE IU/ml	54	1.14 (0.09)	7	1.43 (0.19)	10	2.00 (0.19)	0.001*

380 *Asthma diagnosed by age 3 and no asthma groups differ significantly by Fisher's protected
381 LSD post hoc test. P=0.006 for EPO and <0.001 for IgE.

382 Table 3. Year 1 Risk Factor Odds Ratios for Asthma Before and After Mutual Adjustment

Risk Factor	Marginal Odds Ratio	95% CI	p	Mutually Adjusted Odds Ratio	95%CI	p
Eczema Yr 1	5.0	1.6-15.9	0.007	5.9	1.5-22.7	0.009
Wheeze Yr 1	8.3	2.5-27.7	0.001	9.4	2.5-36.2	0.001

383

384 Table 4. Relation of Mean Levels of the Candidate Biomarkers to Eczema and Wheezing in the
 385 First Year of Life

Risk Factor		n	mean (SEM) log EPO IU/ml	n	Mean (SEM) sGP130 ng/ml
Eczema Year 1	No	49	0.900 (0.028)	45	279 (9)
	Yes	21	0.770 (0.043)	19	276 (11)
ANOVA p			0.014		0.85
Wheeze Year 1	No	51	0.871 (0.028)	49	266 (8)
	Yes	19	0.830 (0.053)	15	320 (9)
ANOVA p			0.44		0.001

386

387 **Figure Legends**

388 Figure 1: Rank of intensity signals for four proteins with significant rank differences between
389 non-asthma and asthma groups from antibody array analysis (FDR adjusted p-value < 0.1).

390

391 Figure 2: Comparisons of non-asthma and asthma groups for plasma levels of analytes assayed
392 by ELISA for (A) erythropoietin (EPO; 0.898 ± 0.035 vs. 0.750 ± 0.048); (B) soluble GP130
393 (sGP130; 270 ± 8 vs. 302 ± 13); and (C) galectin-3 (1.56 ± 0.11 vs. 1.39 ± 0.25). Groups are
394 compared by Student's t-test.