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Protein profiles of CCL5, HPGDS and NPSR1 in plasma reveal association to childhood asthma

C. Hamsten^{1,2}, A. Häggmark³, J. Grundström¹, M. Mikus³, C. Lindskog⁴, J. R. Konradsen^{5,6}, A. Eklund⁷, G. Pershagen⁸, M. Wickman^{8,9}, J. Grunewald⁷, E. Melén^{8,9}, G. Hedlin^{5,6}, P. Nilsson³ & M. van Hage¹

¹Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

²Center for Inflammatory Diseases, Karolinska Institutet, Stockholm, Sweden

³Affinity Proteomics, SciLifeLab, School of Biotechnology, KTH-Royal Institute of Technology, Stockholm, Sweden

⁴SciLifeLab, Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden

⁵Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden

⁶Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

⁷Respiratory Medicine Unit, Department of Medicine Solna and CMM, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

⁸Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

⁹Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden

Correspondence

Marianne van Hage, MD, PhD

Karolinska Institutet

Department of Medicine Solna, Immunology and Allergy Unit

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Karolinska University Hospital, L2:04

SE - 171 76 Stockholm, Sweden

Tel +46-8-5177 6701, Fax +46-8-33 57 24

E-mail: marianne.van.hage@ki.se

Short title: Plasma levels of CCL5, HPGDS and NPSR1 in asthmatics

Abstract

Asthma is a common chronic childhood disease with many different phenotypes that need to be identified. We analyzed a broad range of plasma proteins in children with well-characterized asthma phenotypes to identify potential markers of childhood asthma. Using an affinity proteomics approach, plasma levels of 362 proteins covered by antibodies from the Human Protein Atlas were investigated in a total of 154 children with persistent or intermittent asthma and controls. After screening, chemokine ligand 5 (CCL5) hematopoietic prostaglandin D synthase (HPGDS) and neuropeptide S receptor 1 (NPSR1), were selected for further investigation. Significantly lower levels of both CCL5 and HPGDS were found in children with persistent asthma, while NPSR1 was found at higher levels in children with mild intermittent asthma compared to healthy controls. In addition, the protein levels were investigated in another respiratory disease, sarcoidosis, showing significantly higher NPSR1 levels in sera from sarcoidosis patients compared to healthy controls. Immunohistochemical staining of healthy tissues revealed high cytoplasmic expression of HPGDS in mast cells, present in stroma of both airway epithelia, lung as well as in other organs. High expression of NPSR1 was observed in neuroendocrine tissues, while no expression was observed in airway epithelia or lung. In conclusion, we have utilized a broad-scaled affinity proteomics approach to identify three proteins with altered plasma levels in asthmatic children, representing one of the first evaluations of HPGDS and NPSR1 protein levels in plasma.

Keywords:

Asthma, biomarker, childhood, plasma, protein

Abbreviations:

CCL5: chemokine (C-C motif) ligand 5

EBC: exhaled breath condensate

FeNO: exhaled nitric oxide

HPA: Human Protein Atlas

HPGDS: hematopoietic prostaglandin D synthase

NPSR1: neuropeptide S receptor 1

PGD₂: prostaglandin D₂

Asthma affects approximately 300 million people worldwide and is characterized by bronchial hyperresponsiveness and reversible expiratory airflow limitation. Genome-wide association studies have revealed many candidate risk genes involved in different functions and pathways. Childhood asthma is a common chronic disease often characterized by eosinophilic activity and allergic inflammation (1, 2). The disease may lead to airway remodeling even in young children (1). Several phenotypes of asthma have been described, based upon the time of presentation of first wheeze, the presence of allergic sensitization, eosinophilic or non-eosinophilic inflammation, response to treatment, asthma severity and allergic comorbidities (1). Markers of T_H2 inflammation include allergen-specific IgE, high levels of exhaled nitric oxide (FeNO), sputum eosinophilia and increased airway periostin levels (3). However, as these markers might not be as discriminatory in early-onset asthma (3), new biomarkers are needed to allow early identification of childhood asthma and proper treatment (2).

The aim of this study was to analyze plasma profiles of a broad range of selected proteins in well-characterized children with persistent or intermittent asthma, as well as matched healthy controls, in order to identify potential novel protein biomarkers of childhood asthma. This was performed by an affinity proteomics approach using antibody suspension bead arrays developed within the Human Protein Atlas (HPA) (4, 5). A set of 91 protein targets covered by 131 HPA antibodies was selected among genes and proteins with postulated associations to asthma and inflammation. These were supplemented with a set of 380 HPA antibodies covering 271 proteins assembled without any disease bias. In total 511 antibodies (362 proteins) were used to analyse a cohort of 70 children with a paediatric allergist's diagnosis of persistent asthma (including daily administration of inhaled corticosteroids) together with 20 healthy controls from the Swedish Search study (6) (Table 1, for details see Supporting information).

After an initial analysis of plasma protein levels, the three proteins displaying the strongest association with asthma were further investigated; chemokine (C-C motif) ligand 5 (CCL5, HPA010552), hematopoietic prostaglandin D synthase (HPGDS, HPA024035) and neuropeptide S receptor 1 (NPSR1, HPA007489). To verify the findings, 36 children with mild intermittent asthma (diagnosed asthma according to GA₂LEN criteria at 16 years of age with no inhaled corticosteroid treatment during the last year prior to sampling) and 48 age-matched healthy controls were included from the BAMSE birth cohort (Table 1) (7, 8). Furthermore, the number of HPA antibodies used for analysis in the two independent cohorts was expanded to at least two per target protein. For further technical validation, CCL5 levels in selected samples were analyzed using an external antibody (AF278, R&D Systems, Minneapolis, MN) by suspension bead array as well as by ELISA (DRN00B, R&D Systems). Both assays correlated to previous results ($\rho=0.70$ and $\rho=0.92$, respectively, see Fig S1 in Supporting information).

We found significantly lower levels of both CCL5 and HPGDS in children with persistent asthma compared to healthy controls ($p=0.004$ and $p=0.002$ respectively, Fig 1A), while differences were not as pronounced in children with mild intermittent asthma. Interestingly, plasma levels of these two proteins were positively correlated in both sample materials ($\rho=0.72$ and $\rho=0.53$, $p<0.001$, Fig S2 in Supporting information). In addition, NPSR1 was found at higher levels in asthmatic children compared to healthy controls with the largest difference in children with mild intermittent asthma compared to matched healthy controls ($p=0.03$, Fig 1A). CCL5 is a chemotactic cytokine for T cells, eosinophils and basophils that has been shown to induce eosinophilic airway inflammation and airway hyperresponsiveness as well as to contribute to airway recruitment of fibrocytes in severe asthma (9). Increased levels of CCL5 have been reported in broncho-alveolar lavage (BAL) (10) and exhaled breath condensate (EBC) (11) from adult asthmatic patients and in BAL of inhaled corticosteroid treated asthmatic children (12). In contrast to our results, increased levels of CCL5 in serum from adult asthmatics compared to controls have also been described (13). However, the lower CCL5 levels we noted in the children with persistent asthma could be explained by the use of inhaled corticosteroids, which has been shown to reduce CCL5 production (14). Furthermore, asthma exacerbations following glucocorticoid withdrawal have been linked to increased CCL5 mRNA expression in the airway epithelium (15). We also found a non-significant trend for decreased CCL5 levels in the mild intermittent asthma group. This could indicate a systemic reduction of the cytokine levels, which possibly results from the established airway-specific increases observed in EBC, BAL, and lung tissue of asthmatics (10-12).

The prostaglandin pathway, where HPGDS enables the conversion of prostaglandin H_2 to prostaglandin D_2 (PGD₂), is known to be upregulated in severe asthmatics (16). Increased levels of PGD₂ have been shown in BAL from patients with severe asthma compared to healthy controls (16). In addition, increased HPGDS mRNA levels have been found in airway epithelium of severe asthmatics and the levels correlated with tryptase mRNA in lung epithelial cells, suggesting that mast cells could be the source of HPGDS in asthmatic patients (16). HPGDS can also be expressed by macrophages, dendritic cells and Th2 lymphocytes (16). Consequently, the lower levels of HPGDS in plasma that we observed among patients with persistent asthma could be due to HPGDS expressing cells homing to lung tissue. Furthermore, the lower levels of HPGDS protein in plasma may also reflect a lower expression of this protein in persistent asthma. Antibody-based protein profiling using immunohistochemistry was performed for *in situ* localization of HPGDS and NPSR1 expression in 44 different organs, including airway epithelia of nasopharynx and bronchus, as well as lung parenchyma from non-asthmatic subjects (Fig 1B). High cytoplasmic expression of HPGDS was observed in mast cells present in stroma of both airway epithelia, lung and other organs, while no or only low expression was observed in other normal cell types. It has been shown that the expression of HPGDS mRNA in synovial fluid dendritic cells is inversely correlated with C-reactive protein levels in patients with inflammatory arthritis (17), suggesting that HPGDS expression decreases when inflammation is present. Interestingly, all the patients with persistent asthma in this study are treated with inhaled corticosteroids to dampen the inflammation in the lung. Taken together, this indicates that the reduction of CCL5 and HPGDS in plasma of asthmatic children could reflect altered expression patterns due to corticosteroid use and/or disease-specific recruitment of cells to the lung.

In contrast to CCL5 and HPGDS, the NPSR1 levels were increased in plasma from both children with persistent and children with intermittent asthma compared to healthy controls, even though the differences did not reach significance in the group of persistent asthmatics. In concordance with our data, NPSR1 expression was also found to be higher in peripheral blood eosinophils from patients

with persistent asthma compared to patients with mild asthma and healthy controls, suggesting that the NPSR1 signalling pathway is of importance to asthma, in particular in more severe disease (18). The results are also in line with our previous report on the association of the NPSR1 locus with childhood asthma (19). Immunohistochemical analysis revealed that NPSR1 is highly expressed in neuroendocrine tissues, such as adrenal medullar cells, parathyroid gland, and neuroendocrine cells of the gastrointestinal tract (Fig 1B). However, in contrast to earlier observations by Vendelin et al (20), no NPSR1 expression was observed in airway epithelium or lung. This may reflect that the antibody we used for staining recognizes the A-isoform of NPSR1, which has lower expression in bronchus compared to the B-isoform (20).

To assess whether the obtained results should be seen as general markers of inflammation in the respiratory system, sera from 40 newly diagnosed and untreated sarcoidosis patients with active disease as well as 30 controls were included in the study (See Table S1 in Supporting Information). Significantly higher protein levels of NPSR1 were observed in sarcoidosis patients compared to controls ($p=0.05$, Fig S3 in Supporting Information). No significant differences were observed for CCL5 or HPGDS, but the trend for CCL5 was similar to the results observed among the asthma patients and controls. A role for CCL5 has been implicated in many inflammatory conditions, including sarcoidosis (21), so a broader link to inflammatory airway disease is not unexpected. The NPSR1 gene locus has also been associated to inflammatory diseases other than asthma, such as inflammatory bowel disease and rheumatoid arthritis (22, 23). In this light, our results thus further indicate that NPSR1 expression is relevant to inflammatory conditions of the airways, which in turn can be detected as altered levels in peripheral blood. However, whether these protein levels are causative or indicative of airway inflammation remains to be deduced.

The strength of the study is the large screening effort compared to other proteomic methods, both in number of proteins as well as samples included. By using an affinity proteomics approach, plasma levels of 362 proteins in a total of 154 asthmatic children and controls were investigated. With respect to limitations, individual antibody affinities, low abundant plasma proteins as well as short temporal effects on protein levels may influence the results towards increasing false negatives. Nonetheless, these shortcomings do not influence the results concerning altered plasma levels of these three proteins in asthmatic children but rather open up for future screening efforts.

In conclusion, we have utilized a broad-scaled affinity proteomics approach to identify three proteins (CCL5, HPGDS and NPSR1) with altered plasma levels in asthmatic children compared to healthy controls, representing the first evaluation of HPGDS and NPSR1 in plasma. The role of these proteins in the inflammatory process, and as easily accessible markers of disease development in paediatric asthma, needs to be further investigated.

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Author contributions

The study was conceived by CH, MvH, GH, AH and PN. CH and AH participated in all stages of the project and performed the experiments. CL provided immunohistochemistry data. JK, GH, EM, JGd and AE provided the patient material. CH, JGm and MvH wrote the manuscript together with AH, MM and PN. All authors contributed to interpretation of the data and provided critical review of the manuscript.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Supporting information

Data S1. Methods.

Table S1. Sample characteristics of sarcoidosis patients and controls.

Figure S1. Comparison of CCL5 results using HPA and commercial antibodies in the suspension bead array format as well as compared to ELISA format.

Figure S2. Correlation of CCL5 and HPDGS plasma levels in both asthmatic cohorts.

Figure S3. Results for CCL5, HPGDS and NPSR1 in a cohort of 40 sarcoidosis patients compared to 30 controls.

REFERENCES

1. Pijnenburg MW, Baraldi E, Brand PL, Carlsen KH, Eber E, Frischer T, et al. Monitoring asthma in children. *Eur Respir J* 2015;**45**(4):906-925.
2. Moschino L, Zanconato S, Bozzetto S, Baraldi E, Carraro S. Childhood asthma biomarkers: present knowledge and future steps. *Paediatr Respir Rev* 2015.
3. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;**18**(5):716-725.
4. Schwenk JM, Gry M, Rimini R, Uhlén M, Nilsson P. Antibody suspension bead arrays within serum proteomics. *J Proteome Res* 2008;**7**(8):3168-3179.

5. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. *Science* 2015;**347**(6220):1260419.
6. Konradsen JR, Nordlund B, Lidegran M, Pedroletti C, Grönlund H, van Hage M, et al. Problematic severe asthma: a proposed approach to identifying children who are severely resistant to therapy. *Pediatr Allergy Immunol* 2011;**22**(1 Pt 1):9-18.
7. Kull I, Melén E, Alm J, Hallberg J, Svartengren M, van Hage M, et al. Breast-feeding in relation to asthma, lung function, and sensitization in young schoolchildren. *J Allergy Clin Immunol* 2010;**125**(5):1013-1019.
8. Wickman M, Kull I, Pershagen G, Nordvall SL. The BAMSE project: presentation of a prospective longitudinal birth cohort study. *Pediatr Allergy Immunol* 2002;**13 Suppl 15**:11-13.
9. Isgro M, Bianchetti L, Marini MA, Bellini A, Schmidt M, Mattoli S. The C-C motif chemokine ligands CCL5, CCL11, and CCL24 induce the migration of circulating fibrocytes from patients with severe asthma. *Mucosal Immunol* 2013;**6**(4):718-727.
10. Alam R, York J, Boyars M, Stafford S, Grant JA, Lee J, et al. Increased MCP-1, RANTES, and MIP-1α in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am J Respir Crit Care Med* 1996;**153**(4 Pt 1):1398-1404.
11. Zietkowski Z, Tomasiak MM, Skiepkó R, Bodzenta-Lukaszyk A. RANTES in exhaled breath condensate of stable and unstable asthma patients. *Respir Med* 2008;**102**(8):1198-1202.
12. Fitzpatrick AM, Higgins M, Holguin F, Brown LA, Teague WG, National Institutes of Health/National Heart L, et al. The molecular phenotype of severe asthma in children. *J Allergy Clin Immunol* 2010;**125**(4):851-857 e818.
13. Saad-El-Din Bessa S, Abo El-Magd GH, Mabrouk MM. Serum chemokines RANTES and monocyte chemoattractant protein-1 in Egyptian patients with atopic asthma: relationship to disease severity. *Arch Med Res* 2012;**43**(1):36-41.
14. John M, Oltmanns U, Binder C, Meiners S, Gellert K, Chung KF, et al. Inhibition of chemokine production from human airway smooth muscle cells by fluticasone, budesonide and beclomethasone. *Pulm Pharmacol Ther* 2004;**17**(1):41-47.
15. Castro M, Bloch SR, Jenkerson MV, DeMartino S, Hamilos DL, Cochran RB, et al. Asthma exacerbations after glucocorticoid withdrawal reflects T cell recruitment to the airway. *Am J Respir Crit Care Med* 2004;**169**(7):842-849.
16. Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, et al. Prostaglandin D(2) pathway upregulation: relation to asthma severity, control, and TH2 inflammation. *J Allergy Clin Immunol* 2013;**131**(6):1504-1512.
17. Moghaddami M, Ranieri E, James M, Fletcher J, Cleland LG. Prostaglandin D(2) in inflammatory arthritis and its relation with synovial fluid dendritic cells. *Mediators Inflamm* 2013;**2013**:329494.
18. Ilmarinen P, James A, Moilanen E, Pulkkinen V, Daham K, Saarelainen S, et al. Enhanced expression of neuropeptide S (NPS) receptor in eosinophils from severe asthmatics and subjects with total IgE above 100IU/ml. *Peptides* 2014;**51**:100-109.

19. Melen E, Bruce S, Doekes G, Kabesch M, Laitinen T, Lauener R, et al. Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma. *Am J Respir Crit Care Med* 2005;**171**(10):1089-1095.
20. Vendelin J, Pulkkinen V, Rehn M, Pirskanen A, Raisanen-Sokolowski A, Laitinen A, et al. Characterization of GPRA, a novel G protein-coupled receptor related to asthma. *Am J Respir Cell Mol Biol* 2005;**33**(3):262-270.
21. Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in inflammation. *Expert Opin Ther Targets* 2013;**17**(12):1439-1460.
22. D'Amato M, Bruce S, Bresso F, Zucchelli M, Ezer S, Pulkkinen V, et al. Neuropeptide s receptor 1 gene polymorphism is associated with susceptibility to inflammatory bowel disease. *Gastroenterology* 2007;**133**(3):808-817.
23. D'Amato M, Zucchelli M, Seddighzadeh M, Anedda F, Lindblad S, Kere J, et al. Analysis of neuropeptide S receptor gene (NPSR1) polymorphism in rheumatoid arthritis. *PLoS One* 2010;**5**(2):e9315.

Table 1. Sample characteristics of studied subjects in two independent cohorts: Search and BAMSE.

	Search		BAMSE	
	Persistent asthma	Control	Intermittent asthma	Control
No. Subjects	70	20	36	48
Age [yr]	14 (6-18)	8 (4-16)	16 (15-17)	16 (16-18)
Gender [M/F]	40/30	17/3	13/23	28/20
ICS dose [μ g]	400 (100-3200)	0	0	0
FEV ₁ [% predicted]	88 (37-117)	NA	83 (51-114)	90 (72-115)
FEV ₁ /FVC ratio	0.83 (0.47-0.98)	NA	0.86 (0.65-0.97)	0.87 (0.71-0.97)
FE _{NO} [ppb]	18 (4-118)	NA	15 (5-57)	13 (6-79)
Total IgE	300 (6.3-2400)	NA	NA	NA
Blood neutrophils [$\times 10^9$ /l]	2.7 (0.7-6.8)	3.0 (1.8-5.5)	3.5 (1.1-7.3)	3.6 (1.2-5.9)
Blood eosinophils [$\times 10^9$ /l]	0.3 (0-2.1)	0.2 (0-1.3)	0.1 (0.09-0.5)	0.1 (0.09-0.5)

All values presented as median (range). M, male; F, female; ICS, inhaled corticosteroids; FEV₁, forced expiratory volume during 1 second; FVC, forced vital capacity; FE_{NO}, fraction of nitric oxide in exhaled air.

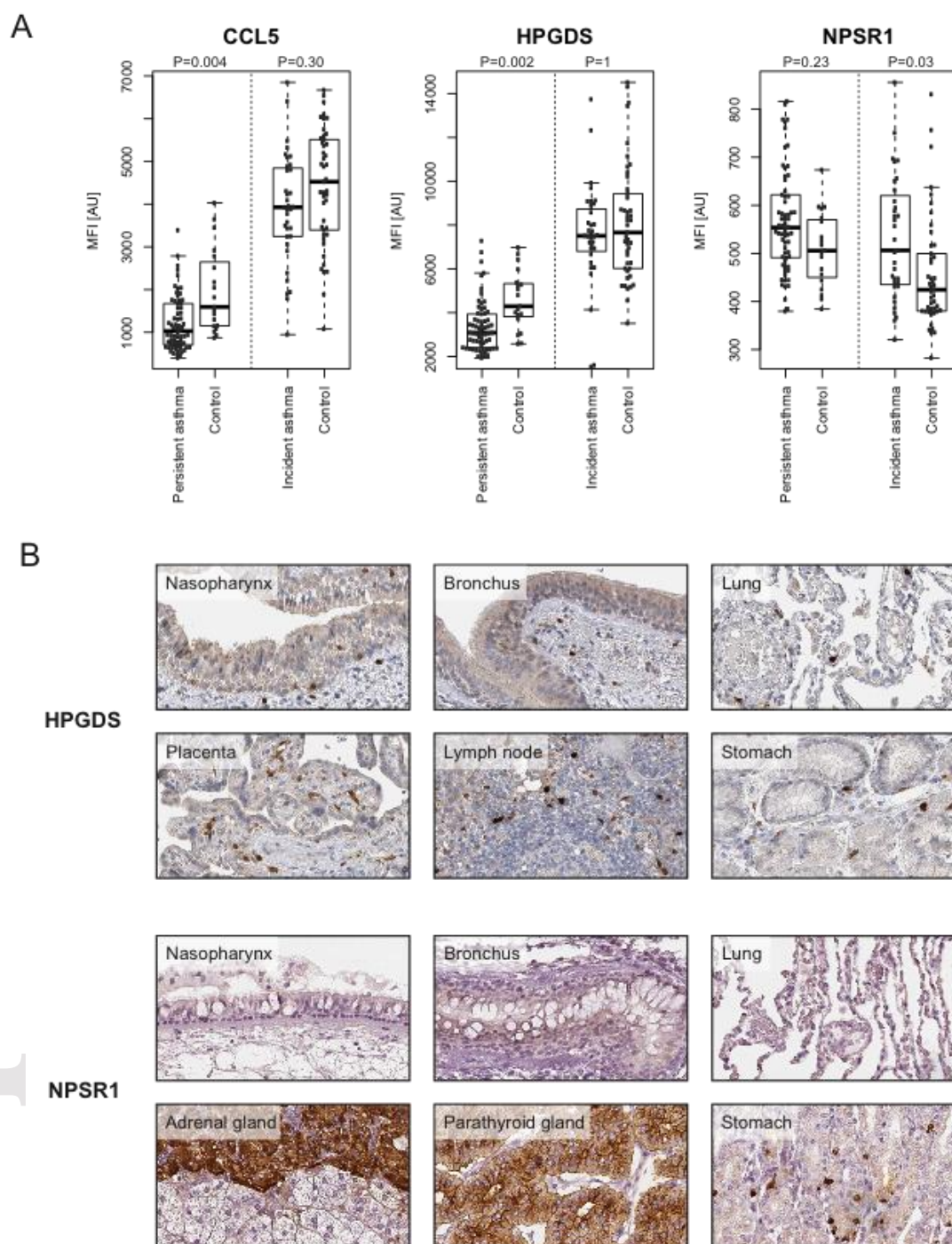


Figure 1. A Protein levels of CCL5, HPGDS and NPSR1 in plasma show significant differences among children with both persistent and intermittent asthma compared to healthy controls (FDR-adjusted P-values are displayed in the plots). MFI, median fluorescent intensity expressed in arbitrary units (AU). **B** Immunohistochemical staining in selected healthy tissues displaying high cytoplasmic expression of HPGDS in mast cells present in stroma of both airway epithelia, lung and other organs while no or low expression was observed in other normal cell types. NPSR1 was highly expressed in neuroendocrine tissues, such as adrenal medullar cells, parathyroid gland, and neuroendocrine cells of the gastrointestinal tract. No expression was observed in airway epithelia or lung.

SUPPORTING INFORMATION

Protein profiles of HPGDS and NPSR1 in plasma reveal association to childhood asthma

C. Hamsten^{1,2}, A. Häggmark³, J. Grundström¹, M. Mikus³, C. Lindskog⁴, J. R. Konradsen^{5,6},
A. Eklund⁷, G. Pershagen⁸, M. Wickman^{8,9}, J. Grunewald⁷, E. Melén^{8,9}, G. Hedlin^{5,6}, P.
Nilsson³ & M. van Hage¹

¹Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

²Center for Inflammatory Diseases, Karolinska Institutet, Stockholm, Sweden

³Affinity Proteomics, SciLifeLab, School of Biotechnology, KTH-Royal Institute of Technology, Stockholm, Sweden

⁴SciLifeLab, Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden

⁵Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden

⁶Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

⁷Respiratory Medicine Unit, Department of Medicine Solna and CMM, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

⁸Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

⁹Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden

Correspondence

Marianne van Hage, MD, PhD

Karolinska Institutet

Department of Medicine Solna, Immunology and Allergy Unit

Karolinska University Hospital, L2:04

SE - 171 76 Stockholm, Sweden

Tel +46-8-5177 6701, Fax +46-8-33 57 24

E-mail: marianne.van.hage@ki.se

METHODS

Samples

Plasma samples from children with persistent asthma, further classified as either problematic severe or controlled persistent asthma, were selected from the Swedish Search study described elsewhere (1). Matching controls were recruited from children admitted for elective surgical procedures unrelated to asthma at Astrid Lindgren's Hospital, Stockholm, Sweden. Plasma samples from 16-year-old children with incident asthma and matching healthy controls were selected from the Swedish birth cohort BAMSE (2) using the criteria of diagnosed asthma at 16 according to GA₂LEN criteria but with no inhaled corticosteroid treatment during the last year prior to sampling. Matched controls were selected from non-asthmatic and non-atopic 16-year-olds.

Sera from 40 sarcoidosis patients diagnosed according to the WASOG criteria (3) were included along with 30 control sera from non-smoking healthy subjects reporting no respiratory infections within 6 weeks prior to sampling.

Suspension bead array

Selected antibodies available through the Human Protein Atlas (HPA), anti-CCL5 (AF278, R&D Systems) as well as anti-human IgG (Jackson ImmunoResearch, 309-005-082), anti-albumin (Dako, A0001), rabbit IgG from non-immunized rabbits (Bethyl) and protein-free buffer as technical controls were immobilized onto color-coded magnetic beads (Magplex, Luminex corp) as previously described (4). In brief, antibodies were diluted to 1.6 µg/ml in MES (2[N-Morpholino]-ethanesulponic acid) and covalently linked to NHS/EDC activated

carboxylated beads, each antibody to a unique bead ID. After removal of unbound antibodies and blocking of the bead surface all identities were combined into an array in suspension.

Suspension bead array assays were performed as previously reported (4). In short, plasma or serum samples were distributed in 96-well microtiter plates, diluted 1:10 in Phosphate buffered saline (PBS) and labelled with a ten fold molar excess biotin over total protein amount. Prior to analysis, labelled samples were further diluted 1:50 in assay buffer (0.5% (w/v) polyvinylalcohol, 0.8% (w/v) polyvinylpyrrolidone and 0.1% (w/v) casein (all SigmaAldrich) in 1xPBS supplemented with 10% (v/v) rabbit IgG) and heat treated in a thermocycler (Techne TC-PLUS) at 56°C for 30 min followed by cooling to 20°C for 15 min. Over night incubation of 45 µl sample and 5 µl bead array was performed at RT on a plate rotator. The beads were washed in 3x100 µl PBST (0.05% Tween-20) to remove unbound proteins, using a wash station (BioTek EL406). Bound proteins were then cross-linked to the antibodies through 10 min incubation with 0.4% paraformaldehyde in PBS. The wash cycle was repeated followed by incubation for 20 min with a streptavidin conjugated fluorophore (R-phycoerythrin, Invitrogen, SA10044 diluted 1:600 in PBST) as detection reagent. After a final wash cycle, 100 µl PBST was added to the beads and the relative abundance of proteins measured in an LX200 or FlexMap3D instrument (Luminex Corp.). At least 32 beads per identity were required for statistical reliability and the median fluorescence intensity (MFI) per identity was used for data processing.

Immunohistochemistry

Paraffin blocks corresponding to 44 different tissue types were used for production of tissue microarrays (TMAs) as previously described(5), and each tissue type was represented by 1 mm cores from three individuals. Four-micrometer sections of the TMA blocks were

deparaffinised in Neo-Clear® (Merck Millipore, Darmstadt, Germany), hydrated in graded alcohols and blocked for endogenous peroxidase in 0.3% hydrogen peroxide. Antigen retrieval was performed by immersing and boiling the slides in Citrate buffer®, pH6 (Lab Vision, Freemont, CA) for 4 min at 125°C followed by cooling of the slides to 90°C. Automated immunohistochemistry was performed using an Autostainer 480 instrument® (Lab Vision) as previously reported (5). Primary antibodies anti-HPGDS (HPA024035, Atlas Antibodies) and anti-NPSR1 (HPA007489, Atlas Antibodies), and the secondary reagent (UltraVision LP HRP polymer®, Lab Vision) were applied for 30 min each at RT and the slides were developed for 10 min using Diaminobenzidine (Lab Vision) as chromogen. Incubations were followed by rinse in Wash buffer® (Lab Vision) for 5 min and slides were counterstained in Mayers hematoxylin (Histolab) and cover slipped using Pertex® (Histolab) as mounting medium. Incubation with PBS instead of primary antibody served as negative control. The dilution of the primary antibody was optimized by testing the antibody on a selection of normal tissues and comparison with previously published literature, gene characterization data and RNA expression data. The AperioScanScope XT Slide Scanner (Aperio Technologies, Vista, CA) system was used to capture digital whole slide high-resolution images.

Statistical analysis

Statistical analysis and visualization was performed in R (6) and Prism 6 (GraphPad software, La Jolla, CA). For data generated by suspension bead array assay, MFI based on >32 events per bead ID were used and after removal of sample-free wells, outlying samples were identified by robust principal component analysis (7) and removed from further analysis. Suspension bead array data were then normalized using Probabilistic Quotient Normalization (8) while ELISA results were analysed as raw data. Group comparisons were performed using

Wilcoxon rank sum or Kruskal-Wallis tests where P values less than 0.05 following false discovery rate corrections (9) were regarded as significant. Correlation coefficients were calculated using Spearman correlation.

REFERENCES

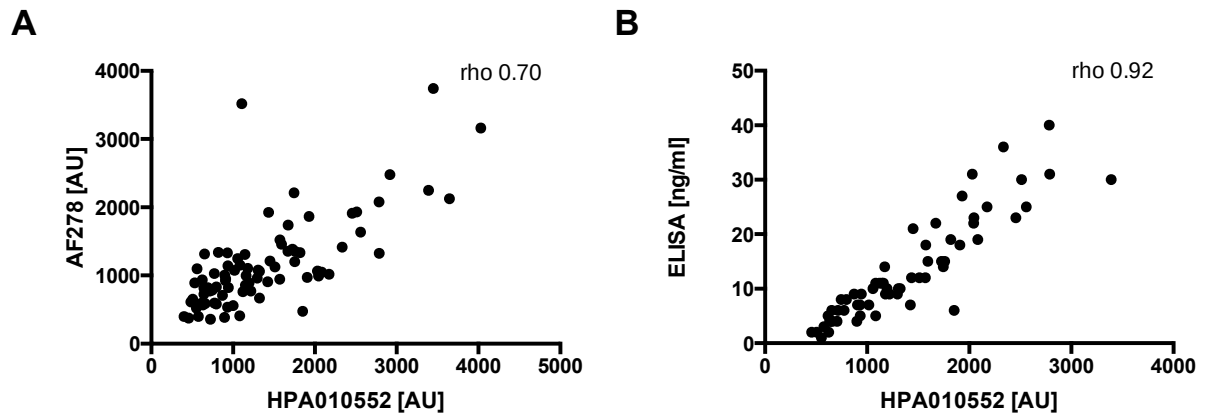
1. Konradsen JR, Nordlund B, Lidegran M, Pedroletti C, Grönlund H, van Hage M, et al. Problematic severe asthma: a proposed approach to identifying children who are severely resistant to therapy. *Pediatr Allergy Immunol* 2011;**22**(1 Pt 1):9-18.
2. Wickman M, Kull I, Pershagen G, Nordvall SL. The BAMSE project: presentation of a prospective longitudinal birth cohort study. *Pediatr Allergy Immunol* 2002;**13 Suppl 15**:11-13.
3. Hunninghake GW, Costabel U, Ando M, Baughman R, Cordier JF, du Bois R, et al. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;**16**(2):149-173.
4. Schwenk JM, Gry M, Rimini R, Uhlén M, Nilsson P. Antibody suspension bead arrays within serum proteomics. *J Proteome Res* 2008;**7**(8):3168-3179.
5. Kampf C, Olsson I, Ryberg U, Sjöstedt E, Pontén F. Production of tissue microarrays, immunohistochemistry staining and digitalization within the human protein atlas. *J Vis Exp* 2012(63).
6. Ihaka R, Gentleman R. R: a language for data analysis and graphics. *Journal of computational and Graphical Statistics* 1996:299-314.
7. Hubert M, Rousseeuw PJ, Vanden Branden K. ROBPCA: A new approach to robust principal component analysis. *Technometrics* 2005;**47**(1):64-79.
8. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. *Anal Chem* 2006;**78**(13):4281-4290.
9. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 1995;**57**(1):289-300.

136 **Table S1.** Sample characteristics of sarcoidosis patients and controls

	Sarcoidosis	Control
No. Subjects	40	30
Age [yr]	44 (26-67)	24 (20-35)
Gender [M/F]	(24/16)	(9/21)
Smoking [N/E/C]	(19/13/8)	30/0/0

137 Age presented as median (range). M, male; F, female; N, never; E, ever; C, current.

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140 **Figure S1.** Comparison of the suspension bead array results for the CCL5-binding antibody
 141 HPA010552 to **A** an external antibody (AF278, R&D Systems) using suspension bead array
 142 assay and **B** compared to ELISA data (DRN00B, R&D Systems) showing high correlations
 143 (Spearman's rho 0.70 and 0.92)

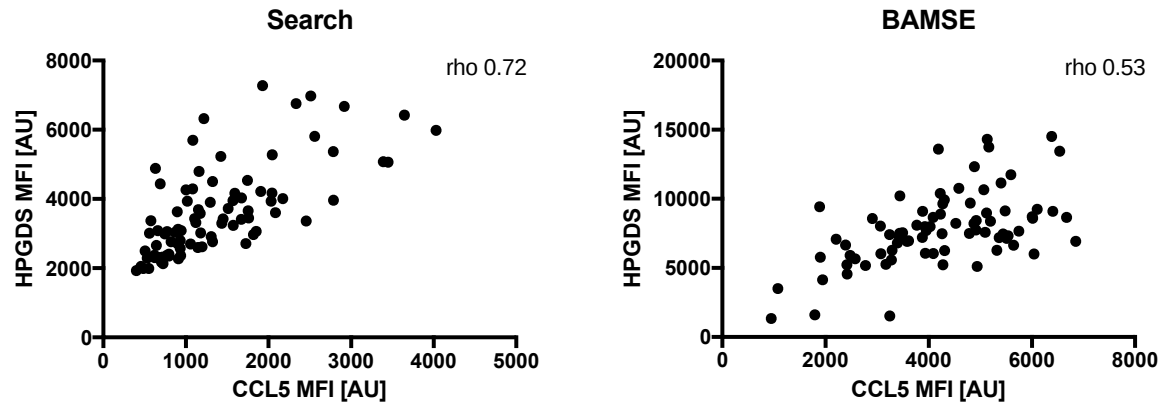


Figure S2. Plasma levels of CCL5 and HPGDS were found to correlate among all samples from both the Search cohort (persistent asthma and healthy controls) as well as the BAMSE cohort (incident asthma and healthy controls).

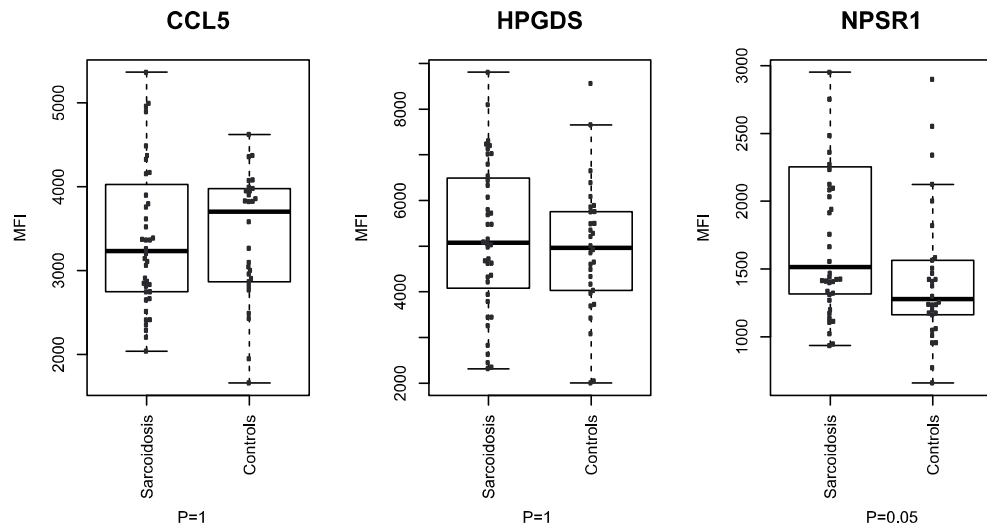


Figure S3. Plasma levels of NPSR1 but not CCL5 or HPGDS were significantly different in 40 sarcoidosis patients compared to 30 controls, although a similar trend was observed for CCL5 as compared to asthma.