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Activation of the Complement and Coagulation Systems in the Small Airways in Asthma

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Asthma · Complement system · Coagulation · Small airways · Exhaled particles · Asthma control

Abstract

Background: Several studies have shown the importance of the complement and coagulation systems in the pathogenesis of asthma. ***Objectives:*** We explored whether we could detect differentially abundant complement and coagulation proteins in the samples obtained from the small airway lining fluid by collection of exhaled particles in patients with asthma and whether these proteins are associated with small airway dysfunction and asthma control. ***Method:*** Exhaled particles were obtained from 20 subjects with asthma and 10 healthy controls (HC) with the PExA method and analysed with the SOMAscan proteomics platform. Lung function was assessed by nitrogen multiple breath washout test and spirometry. ***Results:*** 53 proteins associated with the complement and coagulation systems were included in the analysis. Nine of those proteins were differentially abundant in subjects with asthma as compared to HC, and C3 was significantly higher in inadequately controlled asthma as compared to well-controlled asthma. Several proteins were associated with physiological tests assessing small airways. ***Conclusions:*** The study highlights the role of the local activation of the complement and coagulation systems in the small airway lining fluid in asthma and their association with both asthma control and small airway dysfunction. The findings highlight the potential of complement factors as biomarkers to identify different sub-groups among patients with asthma that could potentially benefit from a therapeutic approach targeting the complement system.

Introduction

Asthma is a heterogeneous disease and is considered an umbrella diagnosis for multiple phenotypes driven by different immunological mechanisms, so-called endotypes,

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and this diverseness of asthma results in varying responses to different therapies [1]. Therefore, there has been increased interest in understanding the molecular pathways driving the inflammation in asthma in the recent years in order to identify new therapeutic targets and develop more targeted therapy.

For many years, asthma was considered to predominately be mediated by the adaptive immune response. However, there is increasing recognition that innate immune system plays an important role in asthma pathophysiology as well [2]. The complement system, which plays a central role in the innate immune response and represents a functional bridge between the innate and adaptive immunities, may act as a key regulator of the type 2 inflammation in asthma and has therefore been recognized as a potential therapeutic target for asthma treatment [3–5]. Several complement factors are synthesized locally in the lungs by the alveolar type 2 cells [6], providing a basis for local complement system activation and initiation of inflammation.

The complement cascade can be activated through three main pathways: classical, lectin, and alternative pathways [7]. Upon activation, the cleavage of complement components C3 and C5 generates the anaphylatoxins C3a and C5a [7], which have been long recognized to play an important role in the pathogenesis of asthma [8]. Studies have shown that the levels of complement factors C3a and C5a in bronchoalveolar lavage fluid increase after allergen challenge in asthma [9]. C3a is a potent pro-inflammatory mediator involved in eosinophil and mast cell activation [10, 11], smooth muscle contraction, and regulation of vascular permeability [12]. Furthermore, complement C3, the central component of the complement system, has been linked to asthma severity and it has been recently reported that high concentrations of plasma complement C3 are associated with high risk of asthma hospitalizations and exacerbations [12].

The complement system also links the immune system with the coagulation system [13]. The crosstalk between the complement and coagulation system has been illustrated by the ability of the complement system to enhance coagulation as well as the ability of certain coagulation enzymes to activate the complement components. Therefore, it seems there is simultaneous activation of both cascades in most pathophysiological situations [13], and several studies have highlighted the role of coagulation system in the asthma pathophysiology as well [14]. Asthma is associated with a procoagulant state in the bronchoalveolar space, and it has been shown that the abnormalities in coagulation and the fibrinolytic pathways in the distal airways contribute to airway hyperresponsiveness and airway closure in asthma [15].

Asthma was originally considered to predominantly involve the central airways. However, more recent evidence revealed that inflammation extends beyond the central airways and involves the entire lung, including the small distal airways [16]. Dysfunction of the small airways (airways with a diameter <2 mm) has been related to asthma control, severity, and risk of exacerbation [17]. Several physiological tests, all reflecting different aspects of small airways dysfunction, can be used to assess small airways. However, due to its complexity, no single variable defines small airway disease, but impulse oscillometry, nitrogen multiple breath washout, and spirometry can all contribute [18]. Therefore, there has lately been increased interest in exploring for potential biomarkers for small airway dysfunction [19], and especially methods to characterize and monitor pathological processes and possibly identify novel drug targets.

Results from the present study focussing on differences between subjects with asthma with and without small airway dysfunction and healthy controls (HC), respectively, have been presented earlier [20]. During the analyses of protein profiles in exhaled particles, it has been evident that proteins belonging to the complement and coagulation systems are overrepresented and seem of particular interest, both in the present study and also in another study population including smokers [21]. In the present study, we have therefore performed a post hoc with the aim to explore if clinical characteristics in asthma are associated with activation of the complement and coagulation systems. Furthermore, due to the previously reported association of plasma C3 and asthma severity, and C3 being the central component of the complement system, we explored whether different asthma sub-groups could be identified based on the abundance of C3 in the small airway lining fluid.

Methods

Study Design and Participants

Twenty subjects with respiratory physician-diagnosed asthma and ten HC were included in the study. Subjects were recruited from our previous studies or by an advertisement in a daily paper. Some results from the study have been presented earlier but with a different general approach [20].

In order to include subjects with asthma with different degrees of peripheral airway involvement, all were screened with the nitrogen multiple breath washout test and lung clearance index (LCI) was assessed. LCI is a robust measure of global ventilation inhomogeneity and is recognized to reflect changes in the small airways [22–25], even though it cannot discriminate between structural and inflammatory alterations in airway structure. Ten subjects with asthma and LCI z-score <2 (normal range) and ten

subjects with asthma and LCI z-score ≥2.9, representing the subjects with the highest LCI z-score in our population where subjects were recruited from, were included in the study. The control group consisted of ten healthy non-asthmatic subjects without respiratory symptoms or a history of respiratory diseases and an LCI z-score <2.

Exclusion criteria were current smoking, smoking within the last 10 years, smoking history of >10 pack-years, and diagnosis of systemic inflammatory disease, cardiovascular disease, or pregnancy. Participants provided a written informed consent prior to the measurements and the Regional Ethics Committee at the University of Gothenburg approved the study (390-06).

Study Assessments

All subjects filled out a questionnaire on medical history, symptoms, use of medication, and smoking history. Additionally, all subjects with asthma filled out the Asthma Control Questionnaire (ACQ-6) [26, 27] and were divided into two groups based on the ACQ score; those with a score ≤1.0 were classified as having well-controlled asthma (WCA) and those with a score >1.0 as inadequately controlled asthma (ICA) [28].

Blood samples were obtained and analysed for hsCRP and white blood cell differential count. All subjects were instructed to withdraw from inhaled corticosteroids, long-acting bronchodilators, and short-acting bronchodilators at least 3 days, 24 h, and 6 h prior to the examination, respectively.

Fractional Exhaled Nitric Oxide

Fractional exhaled nitric oxide (FENO) is a non-invasive method of measuring airway inflammation in asthma [29]. A single measurement at an expiratory flow of 50 mL/s (FENO50) was performed using the NIOX MINO device (Aerocrine AB, Stockholm, Sweden).

Spirometry

Spirometry was performed using a Spirare spirometer (Spirare, Stockholm, Sweden) before and after bronchodilation with 400 μg of salbutamol in accordance with ATS/ERS criteria [30]. Forced vital capacity (FVC), forced expired volume in 1 s (FEV_1), forced expiratory flow at 25–75% of FVC (FEF25–75), and flow when 75% of FVC has been exhaled (FEF75) were expressed as z-scores according to the ERS Global Lung Function Initiative [31].

Nitrogen Multiple Breath Washout Test

Nitrogen multiple breath washout test was performed using the Exhalyzer® D device (Eco Medics AG, Duernten, Switzerland) and software (Spiroware 3.1) in accordance with the current guidelines [32]. Outcomes calculated were S_{cond} and S_{acin} indices (both corrected for expired tidal volume), which are indices of ventilation inhomogeneity in the conductive and acinar zones of the lungs, respectively, as well as LCI to assess global ventilation inhomogeneity. Z-scores were calculated as described by Kjellberg et al. [23].

Skin Prick Tests

Skin prick tests to common allergens in Sweden were performed. A positive histamine control and negative control were used. A positive result was defined as a wheal diameter ≥3 mm.

Exhaled Particles

Exhaled particles (PEx) were collected using the PExA 1.0 instrument (PExA AB, Gothenburg, Sweden), as previously described [33, 34]. Study subjects breathed via a mouthpiece and a two-way, non-re-breathing valve into the instrument which consists of a thermostated box (36°C) containing an optical particle counter (Grimm Aerosol Technik GmbH & Co, Ainring, Germany) and an impactor (Dekati Ltd, Tampere, Finland). The measured particle sizes cover diameters between 0.41 and 2.98 μm. Subjects inhaled HEPA-filtered air for a minimum of three breaths before the sampling in order to remove particles from ambient air. All subjects wore a nose clip throughout the procedure. A standardized breathing manoeuvre was used [35, 36], starting with an exhalation at normal flow rate to residual volume, breath holding for 5 s, followed by a maximal inhalation to total lung capacity, immediately followed by a normal exhalation to functional residual capacity. Exhalation flow was measured by an ultrasonic flow metre (OEM flow sensor; Spiroson-AS, Medical Technologies, Zürich, Switzerland), enabling live visualization of the expiratory flow and derived volume [34]. Between breathing manoeuvres, the subject breathed particle-free air tidally for 30–60 s. The sampling consisted of two consecutive sampling sessions with a short break in between, and a total of 240 ng of exhaled particles was collected from each subject. After collection, the sample holder was transferred to a clean air room and the substrate was cut out with a scalpel from the sample holder area and placed in Millipore Ultrafree-MC LH Centrifugal Filter insert (FC30LH25) and stored at −80°C for subsequent extraction and SOMAscan analysis, as detailed below. True blank samples were generated by applying the same sample-handling procedure as for real samples but without collecting the exhaled particles from the study subjects.

SOMAscan Analysis and Processing of Data

SOMAscan (SomaLogic Inc, Boulder, USA) is an aptamer-based proteomics platform that uses slow off-rate-modified DNA aptamers (SOMAmers) as high-affinity protein capture reagents to quantify proteins. PEx sample preparation and SOMAscan analysis has been previously described in detail [20, 21]. Prior to SOMAscan analysis, the volume of sample buffer was adjusted to reach the same concentration of PEx in all samples in order to normalize the samples for the differences in the collected amount of PEx. Intra-plate and inter-plate normalization were performed by SomaLogic according to their SOMAscan assay good laboratory practice data quality control procedures, as described previously in details [37], and the data were reported in relative fluorescent units.

Limit of detection (LOD) was calculated as the mean plus 3 standard deviations based on three blank samples. 53 proteins that take part in the activation and regulation of the complement system and coagulation with relative fluorescent unit values >LOD in more than 80% of the samples were selected and included in further analysis (online suppl. Table S1; for all online suppl. material, see https://doi.org/10.1159/000531374, Additional file 1). To account for any remaining systematic differences, the set of detected proteins was subjected to group median based normalization.

Statistical Analysis

Statistical analyses of the protein data were performed using general linear model-based statistics (Qlucore Omics Explorer 3.7 software, Qlucore AB, Lund, Sweden). SOMAscan data were

Table 1. General characteristics and clinical data of the subjects included in the study

	HC	WCA	ICA	*p* value			
				HC versus asthma	HC versus WCA	HC versus ICA	WCA versus ICA
Group size, *N* (F/M)	10 (7/3)	12 (4/8)	8 (3/5)	0.070	0.087	0.168	0.848
ACQ score	NA	0.33 (0.04; 0.67)	1.67 (1.50; 2.08)	NA	NA	NA	**<0.001**
Age, years	52 (32; 62)	42 (31; 59)	50 (41; 58)	0.619	1.000	1.000	1.000
BMI, kg/m^2	24.4 (21.8; 25.8)	22.6 (21.8; 27.8)	25.7 (25.0; 27.3)	0.350	1.000	0.209	0.229
Former smokers, *n* (%)	3 (30)	6 (50)	5 (62.5)	0.196	0.342	0.168	0.582
Age of asthma onset, years	NA	7.5 (5; 21.8)	28 (8.3; 44.8)	NA	NA	NA	0.135
N of positive SPT	0 (0; 2)	5 (3; 6)	3 (1; 5)	**0.001**	**0.002**	0.180	0.655
Allergic rhinitis, *n* (%)	1 (10)	10 (83)	3 (38)	**0.004**	**<0.001**	0.163	**0.035**
SABA, *n* (%)	0	6 (50)	6 (75)	NA	NA	NA	0.264
ICS-LABA, *n* (%)	0	5 (42)	6 (75)	NA	NA	NA	0.142
ICS + SABA, *n* (%)	0	4 (33)	1 (13)	NA	NA	NA	0.292
OCS, *n* (%)	0	1 (8)	3 (38)	NA	NA	NA	0.110
Montelukast, *n* (%)	0	2 (17)	4 (50)	NA	NA	NA	0.111
hsCRP, mg/L	0.48 (0.33; 0.58)	0.60 (0.21; 1.08)	0.88 (0.41; 4.08)	0.317	1.000	0.675	1.000
B-neutrophils; 1.8–7.5 × 10^9/L	2.9 (2.2; 4.0)	3.5 (2.9; 5.2)	4.0 (3.0; 6.0)	0.067	0.452	0.201	1.000
B-eosinophils; 0.04–0.4 × 10^9/L	0.10 (0.10; 0.15)	0.20 (0.13; 0.38)	0.20 (0.20; 0.53)	**0.008**	0.069	0.058	1.000
B-lymphocytes; 0.8–4.5 × 10^9/L	2.0 (1.6; 2.2)	2.1 (1.4; 2.5)	1.8 (1.5; 2.1)	0.846	1.000	1.000	1.000
FENO50, ppb	20.5 (11.5; 23.0)	30.0 (15.8; 65.3)	31.0 (19.3; 76.5)	0.061	0.379	0.218	1.000
FEV1 z-score, post	0.12 (−0.97; 0.58)	−0.83 (−1.80; 0.20)	−1.24 (−1.53; −0.70)	**0.019**	0.204	0.067	1.000
FVC z-score, post	−0.15 (−1.10; 0.54)	−0.12 (−0.94; 0.36)	−0.64 (−0.78; 0.14)	0.948	1.000	1.000	1.000
FEV1/FVC, post	0.80 (0.78; 0.86)	0.75 (0.70; 0.82)	0.69 (0.64; 0.72)	**0.002**	0.084	**0.007**	0.815
FEF75 z-score, post	0.53 (0.18; 0.83)	−0.93 (−1.44; 0.57)	−1.16 (−1.74; −0.55)	**0.001**	**0.040**	**0.010**	1.000
FEF25–75 z-score, post	0.24 (−0.13; 0.45)	−1.26 (−1.71; 0.31)	−1.51 (−2.06; −1.10)	**0.006**	0.112	**0.024**	1.000
Reversibility, %	2.0 (0.8; 4.5)	6.0 (3.3; 9.0)	15.0 (6.5; 23.3)	**0.001**	0.112	**0.002**	0.314
LCI z-score	0.80 (0.60; 1.03)	1.50 (1.05; 5.00)	3.90 (1.43; 5.18)	**0.005**	0.074	**0.030**	1.000
S_{acin} z-score	0.50 (0; 1.13)	0.65 (0; 1.48)	1.55 (0.25; 2.93)	0.267	1.000	0.327	0.768
S_{cond} z-score	−0.50 (−1.33; 2.23)	1.35 (−0.75; 3.18)	3.30 (−0.73; 4.90)	**0.013**	0.142	0.064	1.000
PEx concentration, kN/L	25.2 (11.8; 33.0)	11.2 (6.5; 26.6)	14.9 (6.4; 16.4)	0.127	0.575	0.497	1.000

Median values and IQR are presented, unless specified otherwise. Kruskal-Wallis test was used for multi-group comparisons, and *p* values were adjusted by the Bonferroni correction for multiple tests. Mann-Whitney test was used for two group comparisons of continuous data and χ^2 test for categorical data. ACQ, Asthma Control Questionnaire; SABA, short-acting beta$_2$-agonist; ICS, inhaled corticosteroid; LABA, long-acting beta$_2$-agonist; OCS, oral corticosteroid.

log$_2$ transformed before the analysis to achieve normal distribution. General linear model, with each variable normalized to mean 0 and variance of 1, was used to determine differences in protein abundance between different groups, and all the analyses were adjusted for the subjects' age, due to significant correlations between the abundance of proteins and age. Correction for multiple testing (*q* value) has been performed using the Benjamini-Hochberg method. Proteins with *p* value <0.05 and *q* value <0.30 based on the group comparisons were considered to be of interest in this explorative study. Linear regression was used to assess correlations between mean ACQ score, physiological measurements of lung function, and protein levels. Statistical analysis of clinical and demographic data was performed using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA) with the significance level set to $p < 0.05$. Subjects with asthma were divided into tertiles based on the abundance of C3 (T1, lowest protein abundance; T2, median protein abundance; T3, highest abundance), and the differences in clinical data between the tertiles were assessed using the non-parametric Kruskal-Wallis test.

Results

Demographic and Clinical Characteristics

Demographic and clinical characteristics of the subjects included in the study are shown in Table 1. Subjects with asthma had significantly higher levels of blood eosinophils compared to subjects without asthma. However, no significant difference was observed between subjects with well-controlled asthma and ICA. There was no significant difference in FENO50 values between these groups. FEV1 z-score, FEV1/FVC, FEF75 z-score, and FEF25–75 z-score were significantly lower in subjects with asthma as compared to HC, and LCI and S_{cond} z-score were significantly higher (Table 1).

Asthma versus Healthy Controls

Two-group comparison adjusted for age revealed 9 proteins (C1q receptor (CD93), complement C4, C7, protein kinase C alpha type (PRKCA), coagulation factor

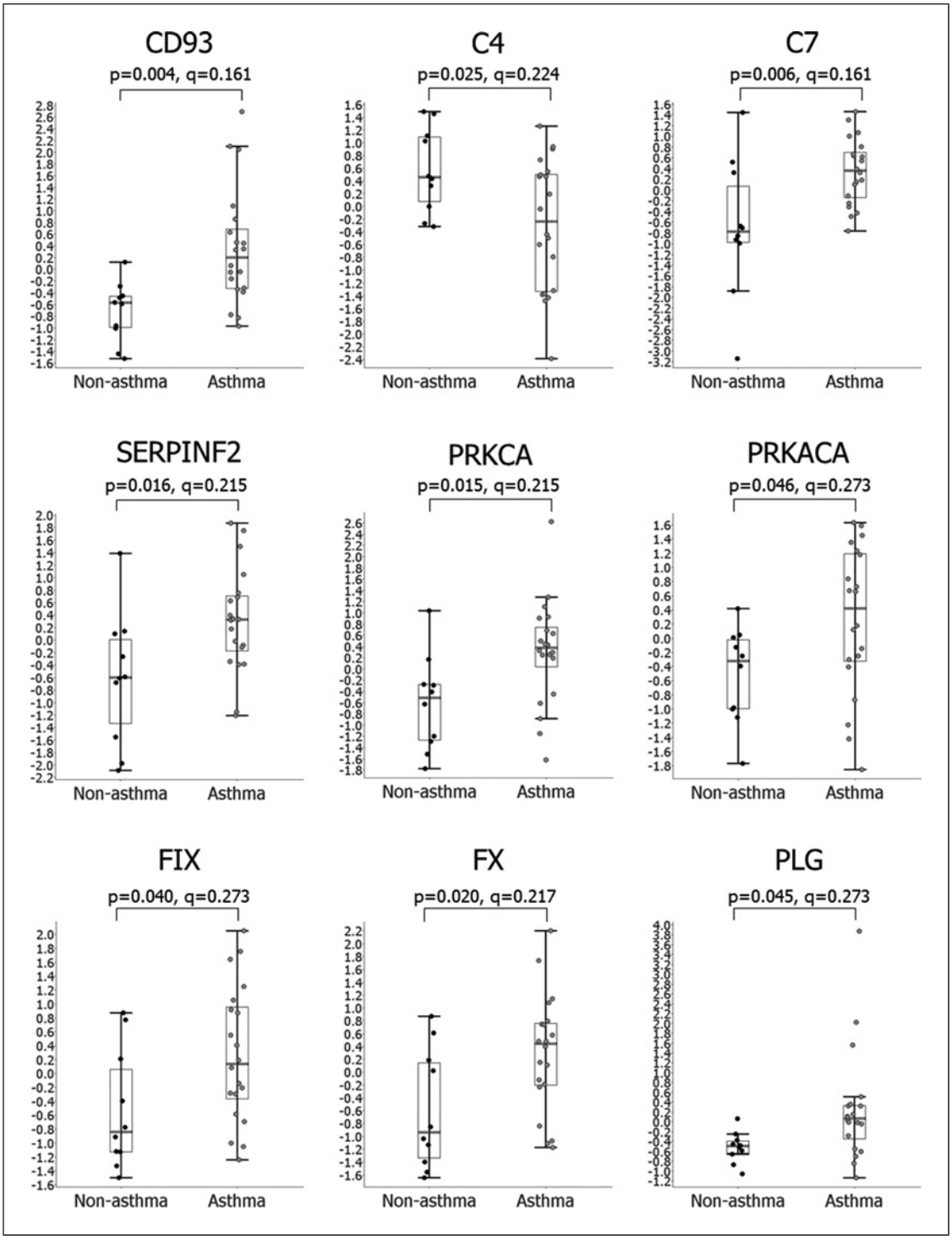

Fig. 1. Proteins identified as differentially abundant in subjects with asthma as compared to healthy controls (non-asthma). *y*-axis shows normalized abundance of protein levels ($\log_2$ transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile, and median value is marked with dotted line. *p* values and *q* values are shown over each box plot. Protein abundance data were adjusted for age.

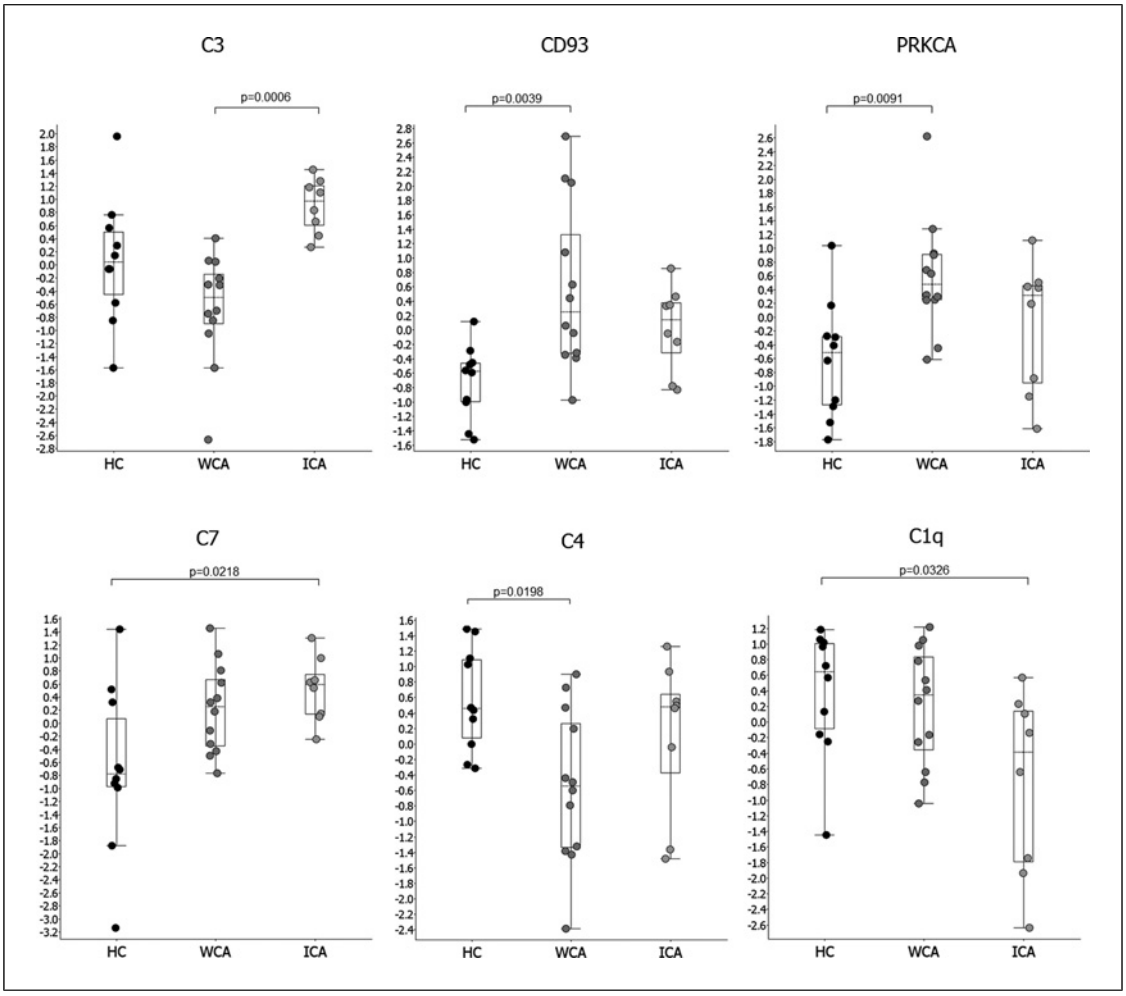

Fig. 2. Proteins identified as differentially abundant between inadequately controlled asthma (ICA), well-controlled asthma (WCA), and healthy controls (HC). *y*-axis shows normalized abundance of protein levels (log_2 transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile and median value is marked with dotted line. Tukey's post-hoc test was used for pairwise comparisons and only statistically significant differences ($p < 0.05$) are displayed. Protein abundance data were adjusted for age.

X and IX (FX and FIX), angiostatin (PLG), alpha-2-antiplasmin (SERPINF2), and cAMP-dependent protein kinase catalytic subunit alpha (PRKACA)) to be differentially abundant ($p < 0.05$) in subjects with asthma as compared to HC (Fig. 1).

Asthma Control

When comparing subjects with ICA, WCA, and HC, we found significant differences in the abundance of C3 (p = 8.93E-4, q = 0.047), CD93 (p = 0.006, q = 0.152), PRKCA (p = 0.012, q = 0.201), C7 (p = 0.020, q = 0.266), C4 (p = 0.025, q = 0.266), and C1q (p = 0.032, q = 0.284) between the groups. After correcting for multiple testing, C3 was significantly higher in ICA as compared to WCA, but not HC (Fig. 2). CD93 and PRKCA were significantly increased and C4 decreased in WCA, but not ICA. The abundance of C7 was higher and of C1q lower in ICA as compared to HC.

Furthermore, significant correlation was observed between ACQ score in subjects with asthma and levels of C3 (R = 0.64, p = 0.003, q = 0.156), C3b (R = 0.55, p = 0.013, q = 0.185), C4b (R = 0.50, p = 0.030, q = 0.262), PROS1 (R = 0.61, p = 0.006, q = 0.156), APOE (R = 0.57, p = 0.011, q = 0.185), and VWF (R = −0.53, p = 0.020, q = 0.201).

Correlation with the Clinical Data

The abundance of 5 proteins showed significant correlation ($p < 0.05$) with LCI, S_{cond}, and S_{acin} z-scores and the abundance of 9 proteins with FEF25–75 z-score and FEF75 z-score (Fig. 3; online suppl. Table S2, Additional file 1).

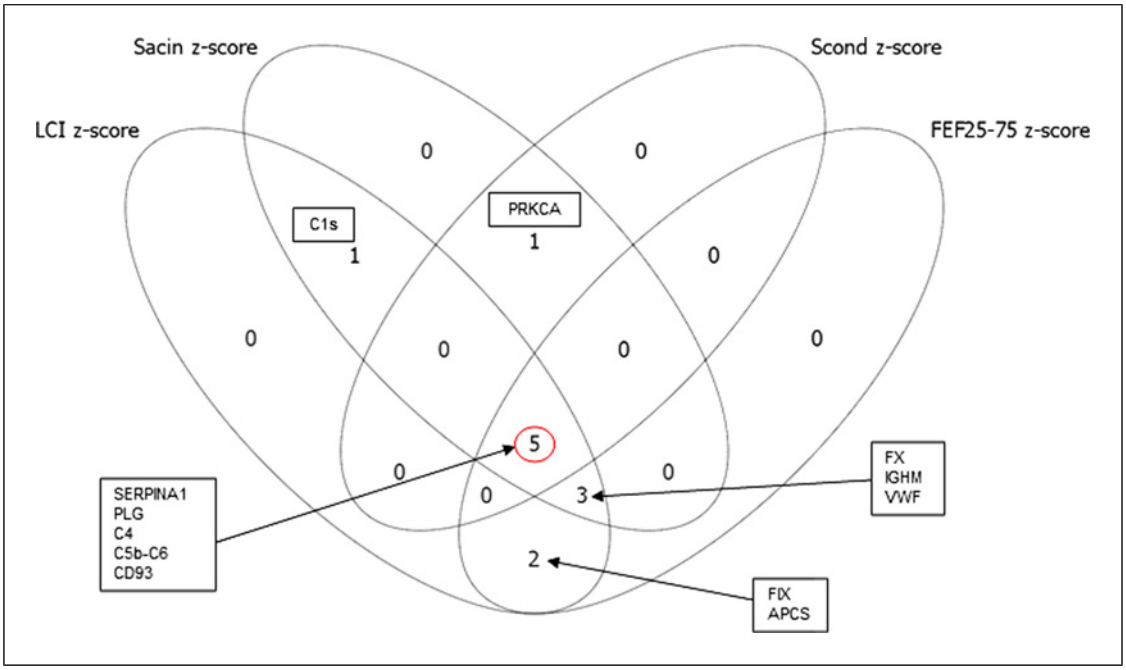

Fig. 3. Venn diagram showing the number of proteins significantly correlated ($p < 0.05$) with different lung function variables. The names of the proteins significantly correlated to all four lung function variables are displayed.

C3 Tertiles

By comparing clinical data between the C3 tertiles, significant differences were observed in CRP levels, LCI z-score, FEF25–75 z-score, FEF75 z-score, and ACQ score between the tertiles (Table 2). Pairwise comparisons of the tertiles and adjustment of p values for multiple comparisons using the Bonferroni correction revealed significant higher ACQ score in T3 as compared to T1 and T2 (Fig. 4). Subjects in T3 and T1 also appeared to have more small airway dysfunction according to the LCI z-score and FEF25–75 z-score as compared to T2, and plasma CRP levels appeared to be higher in T3; however, after Bonferroni correction for multiple testing, p values did not reach statistical significance (Fig. 4). No significant differences were observed in the number of blood neutrophils and eosinophils, age of asthma onset, FENO50, and the use of medication between the tertiles (Table 2).

Discussion

Results from this explorative study highlight the role of the complement and coagulation system in the pathogenesis of asthma, as several complement and coagulation proteins were identified to be differentially abundant in subjects with asthma. Additionally, the abundance of several of these proteins was correlated to the severity of small airway dysfunction as well as asthma control.

When comparing subjects with asthma and HC, we have observed several complement and coagulation proteins to be altered in subjects with asthma (CD93, C4, C7, PRKCA, FX, FIX, PLG, SERPINF2, and PRKACA). Several of these proteins have been previously described to be altered in patients with asthma. CD93, also called complement component C1q receptor or C1QR is considered to be associated with various inflammatory diseases, including asthma. Studies have shown increased serum CD93 levels in patients with stable asthma [38], as well as increased serum CD93 levels during asthma exacerbations that decrease after treatment [39]. In agreement with these studies, we have also observed increased abundance of CD93 in the small airway lining fluid in patients with asthma, suggesting an increased leucocyte migration, complement activation, and clearance of apoptotic cells in the small airways of these subjects [39]. Furthermore, the abundance of CD93 was correlated with several physiological measurements of small airway function, suggesting that CD93 plays a role in the small airway dysfunction. PRKCA or protein kinase C

Table 2. General characteristics and clinical data of the subjects with asthma divided into C3 tertiles

	Subjects with asthma – by complement C3 tertiles			*p* value
	T1	T2	T3	
N	7	6	7	
Age	38 (27; 60)	41 (32; 57)	52 (46; 58)	0.395
BMI, kg/m^2	25.0 (22.3; 28.7)	22.3 (21.6; 27.8)	25.3 (25.0; 26.3)	0.333
Sex (F/M)	2/5	3/3	2/5	0.655
HsCRP, mg/L	0.65 (0.56; 2.00)	0.27 (0.14; 0.65)	1.20 (0.45; 4.20)	**0.050**
B-neutrophils; 1.8–7.5 x 10^9/L	3.3 (2.5; 5.0)	3.5 (2.8; 5.3)	4.3 (3.1; 6.2)	0.377
B-eosinophils; 0.04–0.4 x 10^9/L	0.20 (0.10; 0.60)	0.25 (0.20; 0.33)	0.20 (0.20; 0.60)	0.609
FENO50, ppb	45 (21; 73)	20 (13; 50)	25 (19; 48)	0.327
LCI z-score	2.90 (1.20; 6.70)	1.10 (0.65; 1.45)	4.0 (3.0; 5.2)	**0.047**
S_{acin} z-score	1.10 (0.00; 4.70)	0.15 (0.00; 1.03)	1.60 (1.00; 3.30)	0.098
S_{cond} z-score	2.20 (−0.90; 4.40)	0.50 (−1.43; 1.40)	3.70 (0.70; 5.20)	0.124
FEF25–75 z-score, post	−1.41 (−3.05; −1.17)	0.58 (−1.19; 0.89)	−1.53 (−2.22; −1.39)	**0.030**
FEF75 z-score, post	−0.99 (−2.66; −0.90)	0.71 (−0.77; 0.81)	−1.17 (−1.90; −0.68)	**0.047**
Age of asthma onset, years	6 (3; 8)	14 (7; 32)	34 (7; 48)	0.164
ACQ score	0.33 (0; 0.83)	0.42 (0.17; 0.92)	1.67 (1.50; 2.17)	**0.003**
SABA, *n* (%)	3 (43)	4 (67)	5 (71)	0.509
ICS-LABA, *n* (%)	4 (57)	2 (33)	5 (71)	0.351
ICS + SABA, *n* (%)	1 (14)	3 (50)	1 (14)	0.240
OCS, *n* (%)	1 (14)	0 (0)	3 (43)	0.140
Montelukast, *n* (%)	2 (29)	0 (0)	4 (57)	0.081

Median values and IQR are presented, unless specified otherwise. *p* values are based on a non-parametric Kruskal-Wallis test for continuous data and χ^2 test for categorical data. SABA, short-acting beta2-agonist; ICS, inhaled corticosteroid; LABA, long-acting beta2-agonist; OCS, oral corticosteroid.

alpha type plays an important role in platelet activation [40] and inflammation [41] and is suggested to contribute to the pathogenesis of asthma [42]. Increased PRKCA activity in the lymphocytes of asthmatic patients has been reported previously [42], and this is in line with our findings, exhibiting increased abundance of PRKCA in subjects with asthma. Also, coagulation factors IX and X were more abundant in the small airway lining fluid in patients with asthma. Factor X transcript levels have been previously reported to be increased in the lungs of asthmatic mice treated with ovalbumin, and the role of factor X in airway remodelling by stimulating mucin production has been suggested [43]. In the present study, subjects with asthma also had an increased abundance of alpha-2-antiplasmin (SERPINF2), and this has been previously observed in a rat model of asthma [44]. We have also found increased abundance of angiostatin (PLG) in patients with asthma as well as significant correlations with LCI, S_{cond}, S_{acin}, FEF75, and FEF25–75. Angiostatin is an endogenous fragment of plasminogen and can be produced in high concentrations at sites of inflammation. It can inhibit monocyte and neutrophil chemotaxis, and studies suggest it might inhibit inflammation [45].

A significant correlation was observed between various measurements of small airway dysfunction and levels of complement C4, as well as levels of C5b–C6 complex. Decrease in the levels of C4 and an increase in the levels of C5b–C6 were associated with a higher degree of small airway dysfunction according to the multiple breath washout and spirometry measurements. These findings suggest there is local activation of the complement system in the small airway, resulting in consumption of C4 and production of C5b–C6, and that the complement activation plays an important role in the pathogenesis of small airway dysfunction in asthma.

In addition to the role of the complement activation in the small airway dysfunction, there appears to be an association between the complement activation and asthma control. We have observed differences in levels C3 between ICA and WCA, and several complement and coagulation proteins correlated significantly with the ACQ score.

Due to the previously reported association between plasma C3 and asthma severity [12] as well as the increased abundance of C3 in subjects with ICA seen in our study, we have decided to explore whether different asthma sub-groups could be identified based on the levels

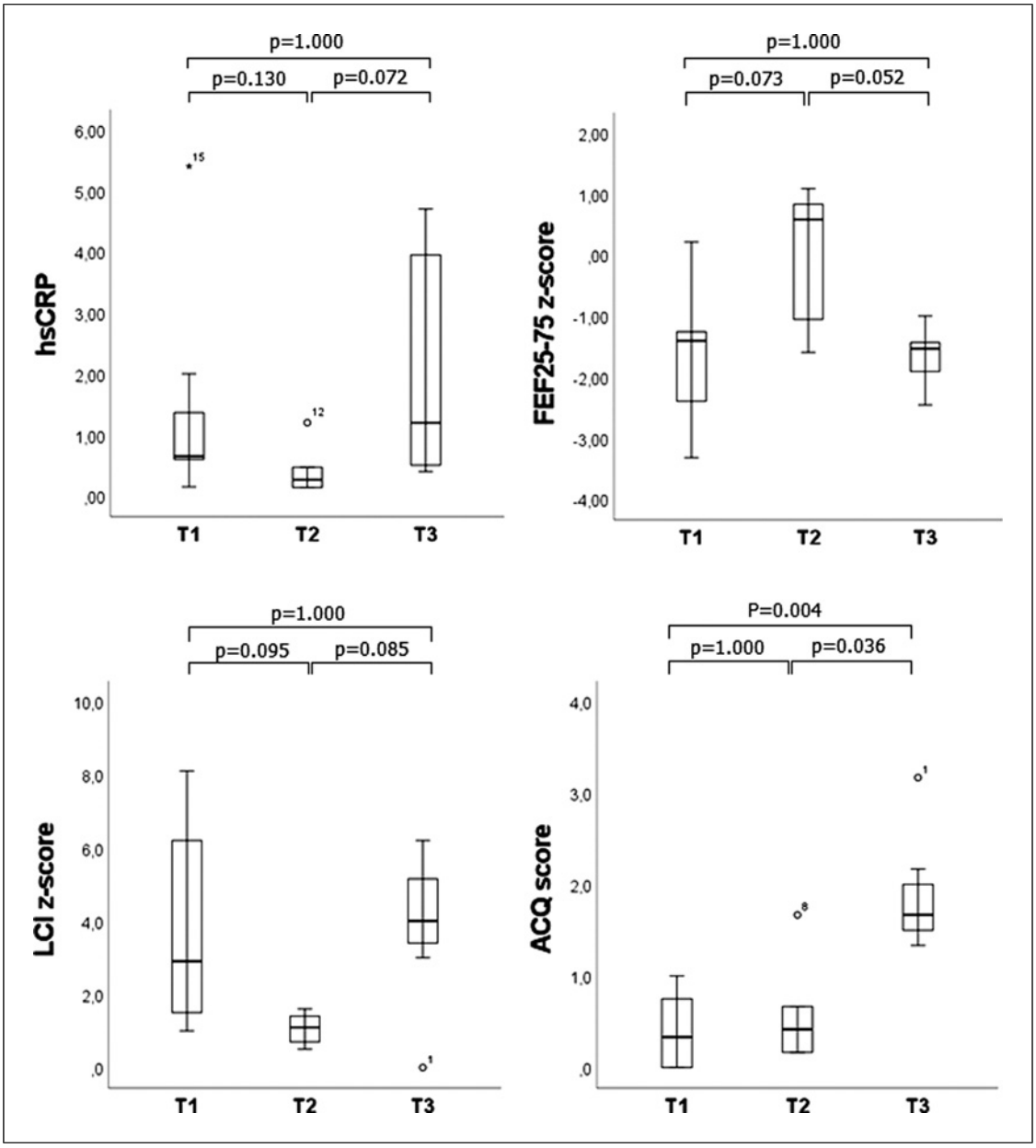

Fig. 4. Boxplots of hsCRP (mg/L), FEF25–75 z-score (post BD), LCI z-score, ACQ score in subjects with asthma divided into tertiles based on the complement C3 abundance in the small airway lining fluid (T1, lowest abundance; T2 median abundance; T3, highest abundance). Horizontal lines represent the median, boxes represent the interquartile range (IQR), and whiskers represent the range (excluding outliers). Circles represent high potential outliers (>1.5 IQR and ≤IQR above quartile 3), and the stars represent high extreme values (>3 IQR above quartile 3). *p* values have been adjusted by the Bonferroni correction for multiple tests.

of C3. Interestingly, we observed that subjects with asthma with both lowest and highest levels of C3 appeared to have signs of small airway dysfunction. These groups, however, differed in the degree of systemic inflammation and asthma control as subjects with high C3 levels in small airways appeared to have more systemic inflammation and poor asthma control, but not those with low levels of C3. Although the results did not reach statistical significance after the correction for multiple testing and the analyses would have to be repeated in a larger cohort, the trend was still seen as interesting in this explorative study. Decrease in complement C3 levels can

occur when C3 is consumed due to over-activation of the complement system, resulting in increased inflammation. In addition, C3 is an acute-phase reactant and can be increased in chronic inflammation. Given that the activation of C3 leads to production of C3a, which is a potent pro-inflammatory mediator, increased levels of C3 may contribute to the inflammatory process by fuelling the complement activation [46].

There are several limitations that need to be considered. First, the sample size is small and an independent validation cohort is needed to confirm our findings. Second, due to the small sample size and the exploratory nature of the study, a more inclusive approach was taken and the differences were identified as significant at p value <0.05 and q < 0.30. Third, in order to be able to balance groups, an ACQ cut-off of 1 was chosen for ICA, instead of the more optimal cut-off of 1.50 [28]. Forth, due to the unspecificity of the SOMAscan aptamers for C3a and C3a des Arginine, and because C5a did not fulfil the data pre-processing criteria (not detected >LOD in more than 80% of the samples), these proteins were excluded from the final analysis.

Conclusion

The study highlights the role of the local activation of the complement and coagulation systems in the small airway lining fluid in asthma and their association with both asthma control and small airway dysfunction. The findings highlight the potential of complement factors as biomarkers to identify different sub-groups among patients with asthma that could potentially benefit from a therapeutic approach targeting the complement system.

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Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki, in accordance with the relevant guidelines and regulations. The Regional Ethics Committee at the University of Gothenburg approved the study (approval number 390-06) and participants provided written informed consent prior to the measurements.

Conflict of Interest Statement

A.C.O. is a chair-holder and a board member of PExA AB (www.PEXA.se). J.Ö. reports personal fees from PExA AB during the conduct of the study and was employed by PExA AB while writing the manuscript but not during the planning and completion of the study. H.K.O. is a full-time employee of AstraZeneca.

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Author Contributions

A.C.O. and J.Ö. designed the study; S.K., A.C.O., and J.Ö. conceptualized the analyses plan; S.K. and J.Ö. performed data analyses; S.K., J.Ö., A.C.O., K.N.E., B.N., K.F., H.K.O., and L.V. interpreted the data; S.K. drafted the manuscript; S.K., J.Ö., K.N.E., K.F., L.V., H.K.O., and A.C.O. have substantively revised the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The datasets generated and/or analysed during the current study are not publicly available due to explorative nature of the study and complexity of the dataset but are available from the corresponding author on reasonable request.

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