Association of Serum Surfactant Protein D and SFTPD gene variants with asthma in Danish children, adolescents and young adults

Running title: Serum Surfactant Protein D and Asthma

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Abstract

Background: Surfactant Protein D (SP-D) is a pattern recognition molecule belonging to the collectin family expressed in multiple human organ systems including the lungs. Previous studies have shown that SP-D concentrations in bronchoalveolar lavage samples decreases and serum concentrations increases in patients with asthma possibly attributable to a combination of induced SP-D synthesis and decreased air-blood barrier integrity. The aims of this study were to investigate if the serum level of SP-D and common variations in the SP-D gene were associated to asthma in adolescents and young adults.

Methods: Prospective observational study including 449 adolescents and young adults (age 11-27 years) previously diagnosed with asthma during a two-year period from 2003 to 2005 (0-16 years). At follow-up from 2016 to 2017 314 healthy controls with no medical history of asthma were recruited. Serum SP-D was analyzed on samples obtained at baseline as well as samples obtained at follow-up. SP-D genotyping was performed for rs721917, rs2243639 and rs3088308.

Results: No differences were found in mean levels of sSP-D and SFTPD genotype among subjects with *current asthma*, *no current asthma* and *controls*. Serum SP-D and SFTPD genotype were not associated to any clinical parameters of asthma. Furthermore, baseline sSP-D was not associated to asthma at follow-up.

Conclusion: Serum surfactant protein D and common SP-D gene variants were not associated with asthma in Danish adolescents and young adults with mild to moderate asthma. Serum surfactant protein D did not demonstrate any value as a clinical biomarker of asthma.

Keywords: adolescent, asthma, biomarker, child, surfactant protein d

Introduction

Asthma is a common chronic airway disease characterized by intermittent obstruction of the airways caused by chronic inflammation[1]. The clinical assessment of asthma is based on airway symptoms, lung function and measures of airway hyperresponsiveness that is not always readily available in primary care nor feasible in preschool children. There is a need for objective and readily available markers of airway inflammation in patients with asthma.

The pathophysiology of asthma is complex and influenced by many factors including genetic susceptibility, exposure to ubiquitous allergens, irritants, respiratory tract infections during early childhood and altered airway microbiome[2,3]. Surfactant Protein D (SP-D) is a pattern recognition molecule belonging to the collectin family expressed in multiple human organ systems including alveolar type II cells and Clara cells[4,5]. SP-D plays a role in the innate immune system by binding bacteria, viruses, fungi and parasites for clearance via opsonization in phagocytes, and aids in the removal of allergens[6-8]. Several experimental studies have indicated that SP-D concentrations in broncho-alveolar lavage samples decreases and serum concentrations increases in patients with asthma possibly attributable to a combination of induced SP-D synthesis and decreased air-blood barrier integrity[9-12]. Recent clinical studies have shown a correlation of serum SP-D (sSP-D) with asthma severity and a decreased BAL/serum SP-D ratio supporting the hypothesis of leakage of degraded SP-D to the circulation[13,14], whereas other studies have failed to demonstrate an association[15,16].

The surfactant protein D-encoding gene (SFTPD) is located at the genomic position 10q22.2-23.1. Three structural single nucleotide polymorphisms (SNP), rs721917, rs2243639 and rs3088308 have previously been linked to circulating levels of SP-D and pulmonary disease[17]. In particular single nucleotide polymorphism in rs721917 has been shown to be associated with several chronic and acute respiratory diseases, although the data on the association to asthma have been conflicting[18-20]

We intended to elucidate the clinical significance of SP-D in childhood asthma in a real-life setting. The objectives of this study were to investigate if the serum level of SP-D and SP-D gene variants were associated with asthma in children, adolescents and young adults, and whether serum SP-D measured at the time of asthma diagnosis was associated with persistent asthma. We hypothesized that serum SP-D was increased in subjects with persistent asthma and associated with asthma severity.

Method

Study design

The study is a prospective follow-up including 449 adolescents and young adults (11-27 years) from a closed cohort of children diagnosed with asthma during 2003-2005 (0-16 years) at all four paediatric outpatient clinics in the Region of Southern Denmark. At follow-up during 2016-2017, an additional 314 healthy

controls with no medical history of asthma and within the same age range were recruited. Controls were recruited through notice on the digital learning platforms from the same schools as the origin of cases.

Baseline data collection at time of diagnosis

Baseline data was extracted from a structured database and biobank established prospectively during the baseline examination. The data was collected prospectively and consecutively by the treating staff and included a structured questionnaire-based interview (regarding atopic heredity, medical history, use of medication, and environmental factors), clinical examination by a pediatrician, blood sampling, skin prick test, spirometry with reversibility test and exercise test where appropriate.

Follow-up data collection

The follow-up examination was performed by the research team which included experienced medical doctors and pediatric nurses with specialty within the field of pediatric asthma and allergology. Each participant completed a structured questionnaire-based interview (regarding atopic heredity, medical history, use of medication, and environmental factors), clinical examination, blood sampling, measurement of fractional exhaled nitric oxide (FeNO), spirometry with reversibility test and mannitol provocation test.

The subjects completed questionnaires regarding asthma control (Asthma Control Test) and quality of life (Asthma Quality of Life Questionnaire)[21,22]. Controls completed the same examination program as the cases except for mannitol provocation test and asthma questionnaires.

Serum Surfactant Protein D

Serum levels of Surfactant Protein D were measured by immunoassay as previously described[23(p)]. Serum SP-D was analyzed on samples obtained at baseline as well as samples obtained at follow-up.

SFTPD genotyping

DNA purification and genotyping were carried out by PentaBase Aps, Odense, Denmark using Maxwell® 16 Blood DNA Purification Kit (Promega, AS1010) and nuclease resistant probes, EasyBeacons™ , developed by PentaBase[24], in a 2-step Real-Time PCR, followed by melt analysis. SP-D genotyping was performed for 3 single-nucleotide variations conferring amino acid substitutions in the mature protein (rs721917, rs2243639 and rs3088308). Information on the SFTPD specific primers and probes (*Table E1*) and a detailed description of the method is provided in the online repository.

Definition of asthma

Current asthma at follow-up was defined by recurrence or persistence of at least two of three symptoms: cough, wheeze, and shortness of breath (not triggered only by infection) within the last 12 months *and at*

least one of the following: positive bronchodilator reversibility test (salbutamol/terbutaline) and/or positive mannitol test. A baseline exercise test was performed whereas mannitol test was used at follow-up. Subjects were defined as having asthma if they had a history of symptoms and a daily use of inhaled corticosteroids (ICS), fixed combination of ICS and long-acting beta-2-adrenoreceptor agonists (LABA) or classical exercise-induced asthma symptoms and clinical effect of inhaled beta-2-adrenoreceptor agonists. *Allergic asthma* was defined as having current asthma and concurrent allergic sensitization to ≥ 1 inhalant allergens. *Severe asthma* was defined as poor control despite maximal maintenance therapy (GINA step 4)[25].

Allergic sensitization

Specific immunoglobulin E (s-IgE) at follow-up was measured quantitatively using Single ImmunoCAP™ after an initial qualitative screening with ImmunoCap™ Phadiatop™ (Thermo Fisher Diagnostics Aps, Allerød, Denmark). The samples were tested for the presence of s-IgE for the following five food allergens (milk, egg, peanut, wheat and soy) and 10 inhalant allergens (birch, timothy grass, mugwort, horse, dog, cat, dermatophagoides pteronyssinus, dermatophagoides farinae, alternaria and cladossporium). Sensitization was defined as s-IgE ≥ 0.35 kU/l.

Clinical measures

Lung function was measured by spirometry (SpiroUSB, Carefusion Ltd, United Kingdom), reversibility test was performed with salbutamol (Buventol Easyhaler™), and mannitol provocation testing was performed using a commercially available test kit (Osmohale™, Pharmaxis Ltd, Australia). All measurements were performed according to generally accepted methods and criteria[26–28]. Parameters obtained for statistical analysis were forced expiratory flow rate at 1 second (FEV1), forced vital capacity (FVC), FEV1/FVC and forced expiratory flow 25-75% (FEF25-75). We used the multi-ethnic reference values for spirometry developed by the European Respiratory Society Task Force[29].

Classification of the response to mannitol was performed in subjects with a positive test according to the cumulative dose of mannitol required to provoke a 15% reduction in FEV1[30]. Fractional exhaled nitric oxide (FeNO) was measured before spirometry using NIOX VERO™.

Statistical Analysis

The distribution of data was assessed visually using histograms and normal quantile-quantile (QQ) plots. Due to the non-normal distribution of observations of sSP-D, approximation to normal distribution was achieved by logarithmic transformation (the natural logarithm, ln) before further statistical analysis. All reported values of sSP-D and 95% confidence intervals were ln-transformed.

The subjects were classified according to the clinical assessment at follow-up: *current asthma*, *no current asthma and controls* and subgroups of *allergic phenotype* and *response to mannitol*.

A regression-based comparison using linear regression was used to test differences between ln sSP-D as outcome and classification as exposure with an initial F-test to test overall difference between groups.

The relationships between ln(sSP-D) and continuous clinical measures were assessed by estimating kernel-weighted local polynomial regression ("lpoly" command in Stata). The associations between sSP-D as outcome and relevant exposure variables were estimated by linear regression models and presented by back-transformed coefficients ($e^{\ln(\beta)}$) and confidence intervals (95%) with associated p-values. The coefficients are interpreted as the fold increase per unit change in a continuous variable or the fold difference between levels of a binary explanatory variable[31]. The longitudinal association of baseline sSP-D with current asthma at follow-up were estimated using logistic regression models.

All models were estimated using both a univariate model and a multivariate model to accommodate the uncertainty of potential confounders. In the multivariate models we included age, sex, BMI (numeric), smoking status and ethnicity, all factors that previously have been reported to affect the constitutional levels of SP-D[32,33].

The multivariate models assessing the association between sSP-D and clinical measures of asthma were estimated on all study subjects and included asthma classification at follow-up as an interaction term. Furthermore, all models were re-estimated including smoking status, gender and genotype in separate models as potential effect modifiers. Smoking status at follow-up was defined as current active smoking whereas smoking status at baseline was defined as current parental smoking.

Analysis of genotype association were performed by initial testing of Hardy-Weinberg equilibrium of each SNP among the groups ("genhw" command in stata)[34]. Logistic regression models were estimated to assess the relationship between asthma classification and genotypes.

To evaluate the importance of missing values on sSP-D at follow-up, subjects with a missing sSP-D were assigned both the highest and the lowest measured value in the study population, and the main analysis was re-estimated to determine if conclusions changed.

All statistical analysis were performed using Stata 15 (StataCorp). Statistical testing was 2-sided and $P < 0.05$ was considered statistically significant. Due to the risk of type one error caused by multiple testing in the complete manuscript, findings should be interpreted as exploratory.

Ethics

The study was conducted in accordance with the Declaration of Helsinki approved by the Regional Scientific Ethical Committee of Southern Denmark (S-20120093) and the Danish Data Protection Agency (95-50819). Before enrolment, informed consent was obtained from each participant and from the parents of participants below 18 years of age.

Results

Study population

The asthma diagnosis was confirmed in a total of 1014 children at baseline during 2003-2005 of whom 449/1014 subjects participated in the follow-up examination 2016-2017 (follow-up rate, 0.443). Of the 449 subjects included, 196 were classified as having *current asthma* and 253 as having *no current asthma*. A descriptive comparison of included subjects and dropouts is available in the online supplemental information (*Table E2*). 314 *healthy controls* with no medical history of asthma were recruited at follow-up.

The basic and clinical characteristics of the study population stratified by classification at follow-up are presented in *Table 1*. The groups had significant differences according to age, sex, BMI, ethnicity and current smoking.

Blood samples were obtained in 98.7% (753/763) and spirometry with reversibility test performed in 99.6% (760/763) of the participants. Mannitol test was performed in subjects with prior history of asthma in which 94.4% (424/449) were positive. Serum samples were available from 89.8% (403/449) and spirometry in 46% (207/449) of the subjects at baseline [age 5-16 years].

Association of sSP-D with potential confounders

Significant relationships were found between sSP-D and age (expβ, 0.97 [95% CI, 0.96 to 0.98]), female sex (expβ, 0.76 [95% CI, 0.70 to 0.82]), BMI (expβ, 0.97 [95% CI, 0.96 to 0.98]) and ethnicity (expβ, 1.58 [95% CI, 1.26 to 1.97]) but no significant association to current smoking (expβ, 0.95 [95% CI, 0.83 to 1.10]).

Association of sSP-D with asthma at follow-up

All observations stratified by classification at follow-up are presented in *Figure 1A*. No difference was found in mean levels of sSP-D among subjects with *current asthma* 6.72 (95% CI, 6.64 to 6.80), *no current asthma* 6.79 (95% CI, 6.71 to 6.86) and *controls* 6.70 (95% CI, 6.65 to 6.76). Based on the findings regarding potential confounders a multivariate model was estimated which did not change the conclusion.

When comparing mean levels of sSP-D in subgroups no differences were found according to allergic phenotype, *allergic asthma* 6.75 (95% CI, 6.66 to 6.84), *non-allergic asthma* 6.61 (95% CI, 6.42 to 6.80) (*Figure 1B*) and severity, *mild asthma* 6.76 (95% CI, 6.62 to 6.90), *moderate asthma* 6.70 (95% CI, 6.60 to 6.81) and *severe asthma* 6.68 (95% CI, 6.35 to 7.02) (*Figure 1C*).

No associations were found between sSP-D and FEV1, FVC, FEF 25-75% and response to SABA (*Figure 2*), nor to self-reported atopic symptoms other than asthma, ACT-score, FeNO, and allergic sensitisation (data not shown). We found no association of sSP-D with the outcome of mannitol test nor to severity of the response to mannitol (*Figure 3*). There was a weak association of sSP-D with FVC in the unadjusted model that diminished when age was included in the model.

No significant effect modification by gender, smoking status, genotype nor asthma classification at follow-up was found in any model.

Association of baseline sSP-D with current asthma at follow-up

To investigate the longitudinal relationship of sSP-D obtained at baseline with asthma status at follow-up multivariate logistic regression models were estimated with asthma classification at follow-up as response variable: *current asthma* vs *no current asthma*. The models included age, sex, BMI, ethnicity and active parental smoking as covariates. No association of baseline sSP-D was found with *current asthma* at follow-up nor to FEV1, FVC, FEF 25-75% and response to mannitol.

Association of baseline sSP-D with clinical measures obtained at baseline

The associations of baseline sSP-D with clinical measures obtained at baseline were evaluated and a weak association of baseline sSP-D with FEV1 at baseline (age 5-16 years) was found in the adjusted model (expβ, 0.99 [95% CI, 0.99-1.00], p = 0.011). No association of baseline sSP-D with FVC, FMEF, peak flow, atopic comorbidity, allergic sensitisation and blood eosinophils obtained at baseline was found.

SFTPD SNP associations with sSP-D, asthma and lung function

The frequencies of SNPs in the total number of genotyped subjects (n=749) are presented in *Table E2* in the supporting information. All SNPs were in Hardy-Weinberg equilibrium A univariate model and a multivariate model including all three SNPs were estimated to evaluate the association between genotypes and asthma. No association of STFPD variants, rs721917, rs3088308 and rs2243639 with current asthma (*Table 2*) nor to allergic asthma, FEV1, FVC and FEF 25-75% was found (data not shown). We found a strong association of rs721917 genotype with constitutional levels of sSP-D both at baseline and follow-up (*Table 3*) and a high degree of tracking comparing the baseline levels of sSP-D with sSP-D at follow-up (*Figure E3*).

Missing values

To investigate the importance of missing values of sSP-D at follow-up (missings, n=11) on the association with asthma at follow-up, subjects with a missing sSP-D were assigned both the highest and the lowest measured value in the study population and the main analysis was re-estimated which did not change the conclusion at follow-up. When the subjects where assigned the lowest measured value of sSP-D, the mean levels of sSP-D in subjects with current asthma were 6.72 (95% CI, 6.64 to 6.80), no current asthma 6.72 (95% CI, 6.64 to 6.81) and controls 6.68 (95% CI, 6.62 to 6.74). When the subjects where assigned the highest measured value of sSP-D, the mean levels of sSP-D in subjects with current asthma were 6.72 (95% CI, 6.64 to 6.80), no current asthma 6.83 (95% CI, 6.75 to 6.91) and controls 6.72 (95% CI, 6.67 to 6.78).

Discussion

Main findings

In the present study, we found no association of serum SP-D with asthma in adolescents and young adults with predominantly mild to moderate asthma. We found no association of sSP-D with lung function, FeNO, allergic sensitization, response to SABA and mannitol. Baseline sSP-D at the time of asthma diagnosis during childhood was not associated with persistent asthma into adolescence and young adulthood. Furthermore, we evaluated three single nucleotide polymorphisms, rs721917, rs3088308 and rs2243639, in the SP-D gene and found no association with asthma. To our knowledge no previous data on the association of sSP-D with asthma in children, adolescents and young adults has been published.

Interpretation of the findings

The results are in agreement with previous clinical studies reporting data on sSP-D in patients with mild to moderate asthma[15] but deviated in contrast to two recent studies that found that sSP-D is associated to asthma severity and the degree of small airway dysfunction[14] and that SP-D concentrations in BAL samples were reduced and serum concentrations elevated in patients with severe treatment-resistant asthma[13]. The reduced level of BAL SP-D and a concurrent increase in sSP-D has also been observed in patients with COPD and in subjects exposed to tobacco smoke[35,36] and has been suggested to reflect non-specific inflammation within the distal airways and alveoli suggesting an impaired endothelial barrier and altered permeability which allows leakage of both full-size and degraded SP-D into serum[13]. This hypothesis is supported by the study by Sin et al who found an increased level of airway SP-D messenger RNA, indicating that the reduced level of BAL SP-D is not because of reduced synthesis but rather leakage into serum[35]. On the contrary, a study recently reported a decreased level of sSP-D in subjects with aspirin-exacerbated respiratory disease[37].

In the present study we found no indications of leakage of SP-D into serum in a population of adolescents and young adults who was diagnosed with asthma at four pediatric outpatient clinics in the Region of Southern Denmark during 2003-2005. The majority of the population was receiving medication according to GINA step 1-3 with varying symptom control assessed by Asthma Control Test whereas only 6 patients with *current asthma* had severe disease defined as poor asthma control despite maximal maintenance therapy (GINA step 4) and acceptable adherence to treatment. It can be speculated that the differences in the local environment in the airways of our cases compared to controls are minimal and without any influence on the endothelial barrier of the airways.

It is well-known that severe asthma represents a distinct phenotype characterized by an altered airway microbiome associated with neutrophilic airway inflammation, a more pronounced expression of Th2 signature molecules and reduced lung function[38-41].

In experimental studies it has been shown that asthma is associated with an increased expression of SP-D in the airways and that SP-D seems to provide negative regulatory control of IL-13 that promotes key features in asthma such as eosinophil infiltration, production of IgE and airway hyperresponsiveness[42]. SP-D gene deficiency induces hyper-eosinophilia, increased levels of IL-5 and IL-13 and a lowered IFN-gamma to IL-4 ratio, a response that is reversible by treatment with SP-D[43,44]. The nature of the present study does not allow us to conclude on the underlying cellular mechanisms in the airway tissue although we note that we did not find any indirect evidence of these pathways on the clinical parameters in our study.

A previous study investigating the heritability of sSP-D found that the genetic variant, rs721917, accounts for 39% of the variation in sSP-D[33]. Furthermore, is has been shown that the rs721917 genotype varies significantly according to ethnicity[32]. Several previous studies on SP-D in relation to pulmonary disease do not include SFTPD variations in the analysis. This gives rise to some uncertainty if associations previously reported are explained by a causal relationship or just a result of differences in genotypes among the groups. This implies the importance of evaluating the genotype in studies reporting on sSP-D.

Strength and limitations

The major strengths of this study are the large sample size including comprehensive characterized subjects in a real-life clinical setting and the ability to include relevant confounders in the analysis. An important draw-back of the study is the lack of airway samples, such as bronchoalveolar lavage fluid and bronchial biopsies, which limits our ability to investigate the molecular mechanisms in the target tissue related to changes in the airway microbiome and immunological responses. Another weakness of the study is the fact that only 6 subjects with *current asthma* had severe disease defined as poor asthma control despite maximal maintenance therapy (GINA step 4) and acceptable adherence to treatment. It is therefore only possible to note that we did not find any association of sSP-D with increased asthma severity but not to draw any conclusion about patients with severe asthma.

Conclusion

Serum surfactant protein D and three common single nucleotide polymorphisms in the Surfactant Protein D gene, rs721917, rs3088308 and rs2243639, were not associated with asthma in Danish adolescents and young adults with mild to moderate asthma. Serum surfactant protein D did not demonstrate any value as a clinical biomarker of asthma in adolescents and young adults with mild to moderate asthma.

Author contributions

SH and AH were responsible for the baseline study as a whole from design to conduction of the study and data collection. LA, BH, SH and AH designed and initiated the follow-up study. The clinical examinations

were performed by BH, RA, JJP and THP. BH was responsible for uniformity and consistency of the study across the hospitals and supervised all examinations. GL was responsible for laboratory analysis of SP-D. CBA facilitated the genetic analysis and interpretation. BH was responsible for data collection, data analysis and writing the manuscript. All co-authors have contributed substantially to the interpretation of the data, provided crucial intellectual input and approval of the final draft of the manuscript.

Data Availability Statement:

The standard terms for research projects and the Danish Act on Processing of Personal Data define the rules of data sharing and will be followed. Data used for the manuscript may be obtained in anonymous form after application for permission to the regional Danish Data Protection agency (https://www.datatilsynet.dk/english/).

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Table 1. Characteristics of the study population according to classification at follow-up: *controls*, *no current asthma* and *current asthma*.

	Controls (n=314) % (cases/total)	No current asthma (n=253) % (cases/total)	Current asthma (n=196) % (cases/total)	p-value
Basic characteristics				
Gender - female	61.8 (194/314)	39.9 (101/253)	45.9 (90/196)	<0.001
Age - mean (sd)	18.4 (4.5)	17.6 (4.4)	18.7 (4.1)	0.030
BMI - mean (sd)	22.2 (3.8)	22.9 (4.7)	23.2 (5.2)	0.031
Ethnicity - caucasian	98.1 (308/314)	96.4 (244/253)	95.9 (188/196)	0.312
Parental asthma †	10.5 (33/314)	29.6 (75/253)	33.2 (65/196)	<0.001
Siblings - median (iqr)	1 (1-2)	1 (1-2)	1 (1-2)	0.480
Active smokers	5.4 (17/314)	8.7 (22/253)	10.7 (21/196)	0.080
Symptomscore (ACT) ‡				
Well-controlled (score>19)	-	94.0 (236/251)	77.4 (151/195)	
Poor controlled (score≤19)	-	6.0 (15/251)	22.6 (44/195)	
Patient-reported symptoms				
Hayfever	2.9 (9/314)	44.7 (113/253)	74.5 (146/196)	<0.001
Eczema	2.2 (7/314)	11.1 (28/253)	27.7 (54/195)	<0.001
Food Allergy	1.0 (3/314)	5.1 (13/253)	10.2 (20/196)	<0.001
Urticaria	0.6 (2/314)	4.3 (11/253)	13.8 (27/196)	<0.001
Current medication §				
No treatment	-	87.0 (220/253)	11.7 (23/196)	
SABA only	-	13.0 (33/253)	19.9 (39/196)	
ICS low dose	-	-	29.6 (58/196)	
ICS low dose + LABA	-	-	29.6 (58/196)	
ICS high dose + LABA	-	-	9.2 (18/196)	
Add on (thiotropium/biologicals)	-	-	-	
Allergic sensitisation				
Inhalant allergens	18.8 (58/309)	54.5 (134/246)	78.1 (153/196)	<0.001
Food allergens	2.6 (8/309)	10.6 (26/246)	16.3 (32/196)	<0.001

Notes: †, medical history of asthma in ≥ 1 parent; ‡, not filled in by controls; §, self-reported symptoms
Abbreviations: sd, standard deviation; ACT, Asthma Control Test; SABA, short-acting beta2 agonists; ICS, inhalant corticosteroids; LTRA, leukotriene receptor antagonists

Table 2. Association of SFTPD polymorphisms and asthma at follow-up (2016-2017). Unadjusted logistic regression models are presented with odds ratios, 95% confidence intervals and related p-values.
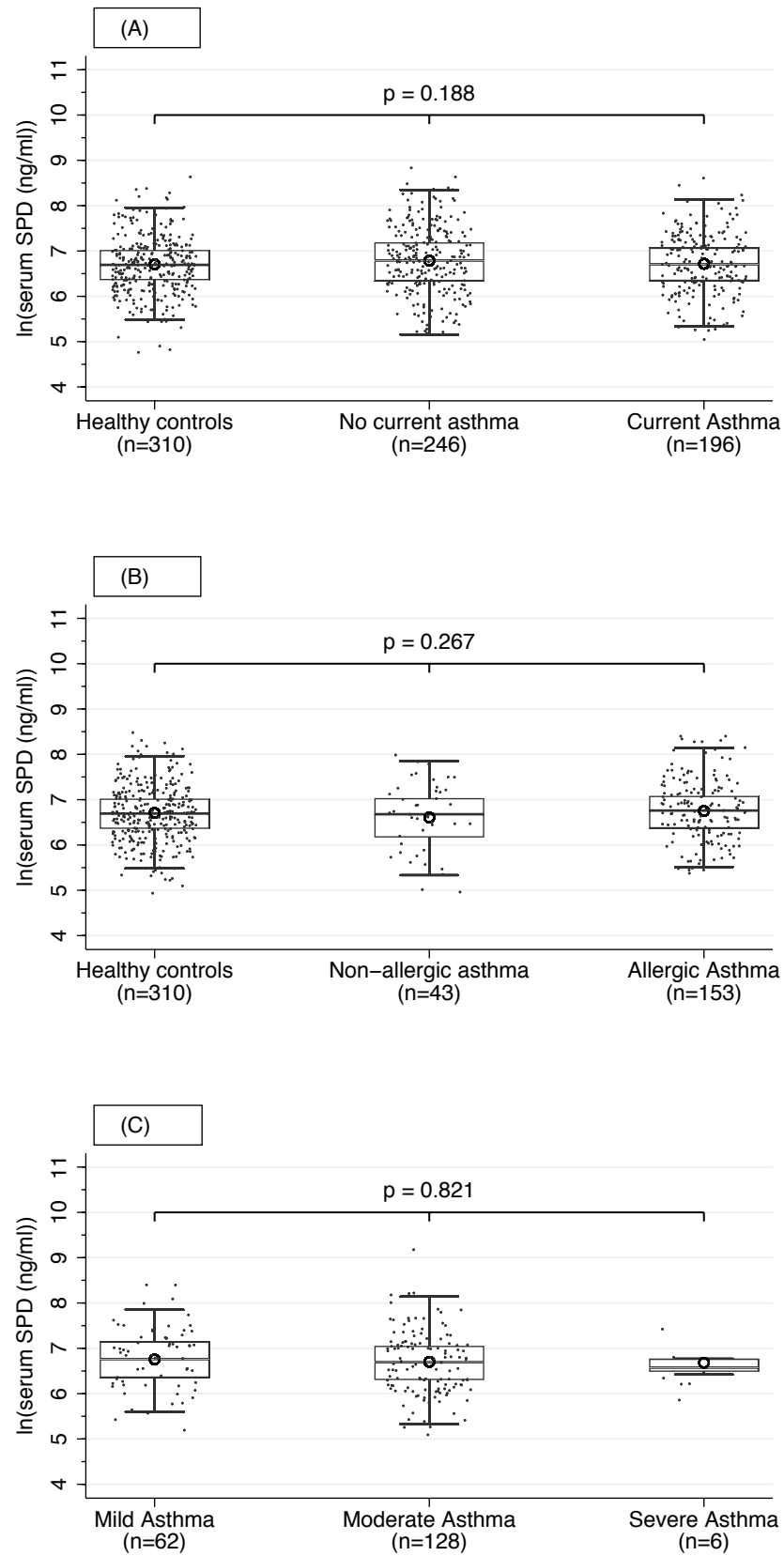
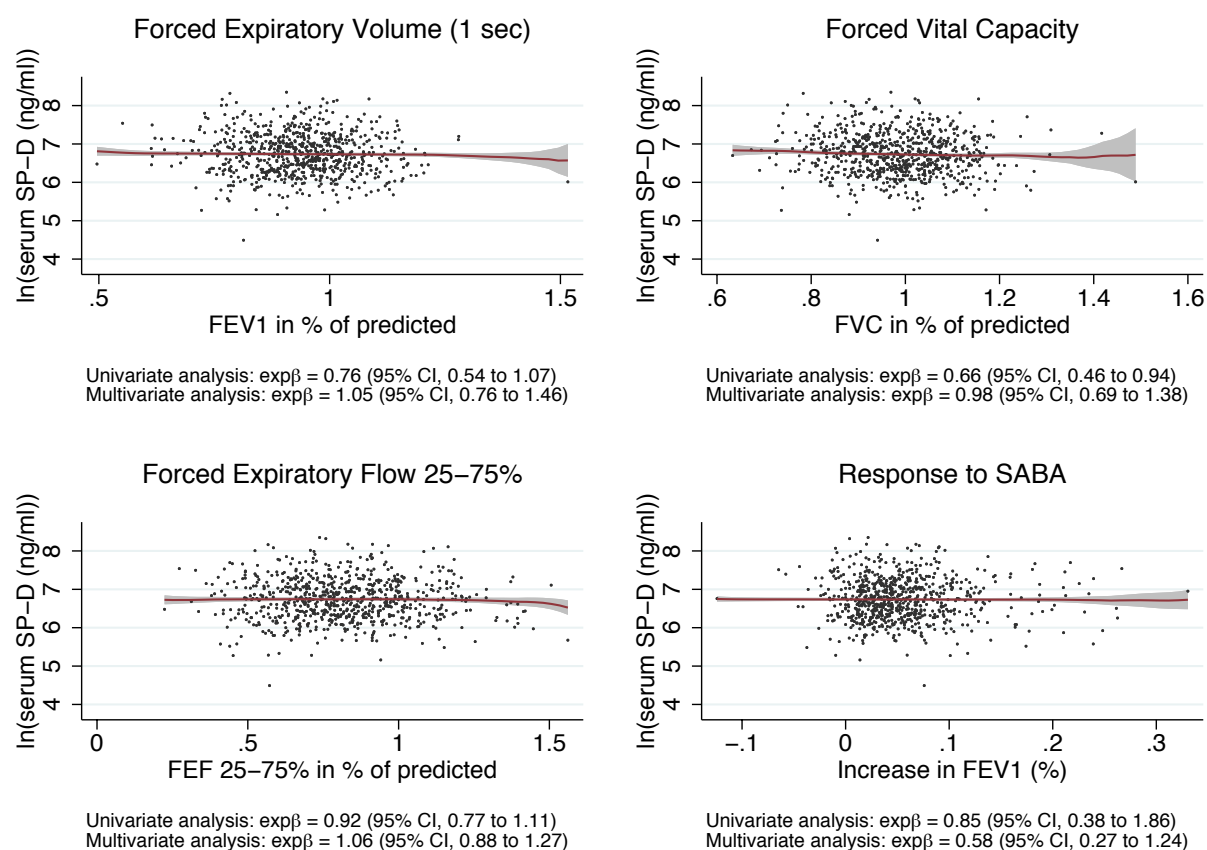| SNP	Genotype	Current asthma' vs 'Controls' (n=502)		'Current asthma' vs 'No current asthma' (n=442)	
		OR (95% CI)	p-value	OR (95% CI)	p-value
rs2243639	CC	Ref.	-	Ref.	-
	CT	1.06 (0.71; 1.57)	0.787	0.90 (0.60; 1.37)	0.633
	TT	0.77 (0.45; 1.33)	0.343	0.83 (0.46; 1.49)	0.537
rs3088308	AA	Ref.	-	Ref.	-
	AT	0.83 (0.48; 1.45)	0.518	0.93 (0.52; 1.67)	0.801
	TT	6.21 (0.69; 56.05)	0.104	5.06 (0.56; 45.68)	0.149
rs721917	AA	Ref.	-	Ref.	-
	AG	1.33 (0.89; 2.00)	0.164	1.11 (0.73; 1.69)	0.627
	GG	1.11 (0.66; 1.87)	0.687	1.23 (0.71; 2.15)	0.459

Table 3. Associations of sSPD with SFTPD variants *rs2243639*, *rs3088308* and *rs721917*. The models are estimated for sSPD both measured at follow-up and baseline. The linear slope coefficients are presented as back-transformed coefficients $e^{\ln(\beta)}$ with 95% confidence intervals. The multivariate models include all three SFTPD variants.

Variant	Genotype	Univariate model expβ (95% CI)	p-value	Multivariate model expβ (95% CI)	p-value
Follow-up					
rs2243639	CC	Ref.	-	Ref.	-
	CT	1.04 (0.95; 1.13)	0.404	0.86 (0.77; 0.95)	0.003
	TT	1.13 (1.01; 1.28)	0.035	0.76 (0.65; 0.88)	<0.001
rs3088308	AA	Ref.	-	Ref.	-
	AT	0.75 (0.66; 0.84)	<0.001	0.83 (0.74; 0.94)	0.003
	TT	0.65 (0.42; 1.00)	0.048	0.77 (0.50; 1.18)	0.222
rs721917	AA	Ref.	-	Ref.	-
	AG	0.79 (0.73; 0.86)	<0.001	0.74 (0.67; 0.82)	<0.001
	GG	0.68 (0.61; 0.76)	<0.001	0.60 (0.52; 0.70)	<0.001
Baseline					
rs2243639	CC	Ref.	-	Ref.	-
	CT	1.01 (0.90; 1.13)	0.848	0.89 (0.78; 1.02)	0.091
	TT	1.08 (0.93; 1.27)	0.309	0.83 (0.68; 1.00)	0.056
rs3088308	AA	Ref.	-	Ref.	-
	AT	0.75 (0.64; 0.88)	<0.001	0.79 (0.67; 0.94)	0.007
	TT	0.63 (0.40; 1.00)	0.050	0.69 (0.43; 1.09)	0.113
rs721917	AA	Ref.	-	Ref.	-
	AG	0.85 (0.76; 0.95)	0.004	0.82 (0.72; 0.94)	0.005
	GG	0.76 (0.66; 0.89)	<0.001	0.74 (0.60; 0.90)	0.003

Figure 1. Boxplot presenting log-transformed serum levels of SP-D (unadjusted) measured at follow-up stratified by (A) classification at follow-up, (B) allergic phenotype and (C) asthma severity.

Figure 2 Association of serum SP-D with parameters obtained from spirometry. Local kernel-weighted mean of ln(SP-D) on FEV1, FVC, FEF 25-75% and response to SABA (% increase in FEV1). Shaded areas indicate 95% confidence bands. The linear slope coefficients are presented as back-transformed coefficients $e^{\ln(\beta)}$ with 95% confidence intervals from a univariate and multivariate model. The multivariate model includes adjustment for age, sex, BMI, active smoking, ethnicity and asthma classification at follow-up.

Univariate analysis: expβ = 0.76 (95% CI, 0.54 to 1.07)
Multivariate analysis: expβ = 1.05 (95% CI, 0.76 to 1.46)

Univariate analysis: expβ = 0.66 (95% CI, 0.46 to 0.94)
Multivariate analysis: expβ = 0.98 (95% CI, 0.69 to 1.38)

Univariate analysis: expβ = 0.92 (95% CI, 0.77 to 1.11)
Multivariate analysis: expβ = 1.06 (95% CI, 0.88 to 1.27)

Univariate analysis: expβ = 0.85 (95% CI, 0.38 to 1.86)
Multivariate analysis: expβ = 0.58 (95% CI, 0.27 to 1.24)

Figure 3. Boxplot presenting log-transformed serum levels of SP-D (unadjusted) measured at follow-up and stratified by response to mannitol. Mannitol test was only performed in subjects previously diagnosed with asthma: *current asthma* and *no current asthma*.