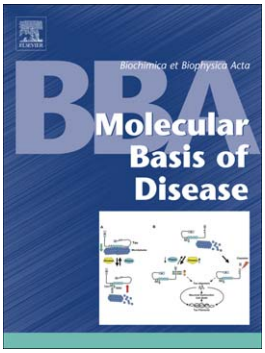

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FUNCTIONAL HETEROGENEITY OF PULMONARY SURFACTANT PROTEIN-D IN CYSTIC FIBROSIS .

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Running Title: SP-D activity in CF.

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ABSTRACT:

Pulmonary surfactant protein-D (SP-D) is a soluble collagenous C-type lectin with important anti-microbial and anti-inflammatory properties. Although it is subject to functionally relevant modification by common polymorphisms and unregulated inflammation, the functional status of SP-D in cystic fibrosis (CF) remains unclear. Given the importance of infection and inflammation in CF lung pathology we have undertaken the first systematic analysis of SP-D lectin activity in this population.

By ELISA, we found that airway lavage fluid SP-D expression was greater in CF compared to control patients but was reduced in CF patients with infection and correlated negatively with markers of neutrophilic inflammation. In a functional assay, the percentage of SP-D capable of binding zymosan rarely exceeded 60% in CF or control patients and similarly restricted binding activity was observed towards maltose-agarose. SP-D lectin activity also correlated negatively with infection and neutrophilic inflammation but there was little evidence of major proteolytic degradation among the non-bound material. SP-D which failed to bind zymosan exhibited features of lower oligomeric form compared to bound material when tested by native gel electrophoresis. Furthermore, when separated by gel chromatography, high and low oligomeric populations of SP-D were observed in CF lavage fluid but only high oligomeric forms exhibited substantial lectin activity towards yeast derived mannan.

Our data demonstrate that oligomeric heterogeneity underlies functional diversity amongst SP-D in health and disease and that dynamic regulation of oligomerisation is an important feature of SP-D biology.

Keywords: Cystic Fibrosis, Innate Immunity, Lung, Surfactant Protein-D, Lectin, Inflammation

1. INTRODUCTION:

Surfactant protein-D (SP-D) is a soluble multimeric collagenous C-type lectin. Although initially and most widely described as a lung specific protein, SP-D is also expressed at a number of other mucosal sites [1]. In common with other collectins including mannose binding lectin (MBL) and surfactant protein-A (SP-A), SP-D recognises carbohydrate structures on a wide range of pathogens - including viruses, bacteria and fungal species - in a calcium dependent fashion [2]. The consequences of such recognition vary among the collectins and in the case of SP-D, pathogen agglutination, inhibition of replication and enhanced phagocytosis are primary outcomes. Clearly important, but less well understood, is the ability of SP-D to regulate inflammatory activity. This aspect of SP-D biology is most apparent in the knockout mouse which develops a spontaneous and progressive emphysematous inflammatory lung disease [3].

SP-D expression has been examined in a number of lung diseases, including cystic fibrosis (CF). Exacerbations characterised by protracted and ineffective neutrophil dominated inflammatory responses to pulmonary infection are a primary cause of progressive lung damage in CF and for this reason there has been much interest in the pulmonary innate immune system in this population. Although a number of studies have been undertaken, a clear consensus on the status of SP-D in CF has not emerged. Earlier studies demonstrated a near absence of SP-D in CF lung secretions [4], but more recently μg/ml quantities have been detected in CF bronchoalveolar lavage fluid (BALF) [5]. Furthermore, reports of a complete absence [5], or significant reduction [4, 6] of SP-D expression in CF patients with infection contrast with others demonstrating relatively abundant immunoreactive SP-D in patients infected with *Pseudomonas aeruginosa* [7]. Whilst the amount of detectable SP-D in CF BALF correlates negatively with markers of neutrophil activity and SP-D is susceptible to neutrophil mediated inactivation in vitro [5-10], the functional status of endogenous SP-D in CF lung secretions has not been systematically addressed. This is important as physiological concentrations of calcium are

known to protect SP-D from neutrophil mediated protease inactivation [6, 9-11] and in some studies little or none of the SP-D found in CF exhibits features of proteolytic degradation [7, 12].

For this reason we wished to examine whether the widely reported inactivation of SP-D by neutrophils in vitro is reflected in the functional status of this molecule in CF lung secretions. Here we examine the expression of SP-D in airway secretions from two independent CF cohorts and control patients. We also report the first systematic assessment of the SP-D lectin activity in CF using a novel zymosan binding assay.

2. MATERIALS AND METHODS:

2.1. Patient recruitment and sample processing:

Samples were included from two independently collected cohorts of CF patients and a cohort of control patients (see Table 1) as follows:

Cohort A (25 samples) was recruited from the Children's CF unit at the University Hospital of Wales as previously described [13]. Lavage was collected by flexible bronchoscopy in 10/25 cases with the remainder collected by non-bronchoscopic lavage. In all cases lavage was conducted for diagnostic reasons by instillation of 1ml/kg normal saline (up to a maximum of 20ml) which was immediately suctioned back. A portion of this lavage, representing the bronchial airway, was available for this study. Processing and storage of lavage fluid at -80°C occurred within 45 minutes of collection as previously described [13]. Briefly, lavage fluid was centrifuged at 500g/5 min at 4°C, cell free lavage was aliquoted at 25µl and stored at -80°C for later analysis. Among CF cohort A, clinically significant microbes were cultured from 10 samples (*Pseudomonas aeruginosa* (n=5), *Burkholderia cepacia* (n=1), *Stenotrophomonas maltophilia* (n=2; of which n=1 coincident with *P. aerugionosa*), *Aspergillus fumigatus* (n=1), *Moraxella catarrhalis* (n=1), *Haemophillus influenzae* (n=2; of which n=1 coincident with *M.*

catarrhalis)). 15 samples either yielded no culturable microbes or yielded microbes suggestive of normal microflora (*Klebsiella pneumonia, alpha haemolytic streptococci)* or microbes not considered to be of clinical significance in the absence of other symptoms *(Candida, coliforms)*.

Control lavage was collected by non-bronchoscopic lavage from nine non-CF subjects who were undergoing tonsillectomy and/or adenoidectomy at the University Hospital of Wales. These patients were well at the time of surgery and were not in receipt of medication for respiratory symptoms as previously reported [13]. Samples were processed and stored as described for CF cohort A. For this cohort and CF cohort A, ethical approval was obtained from the South East Wales Local Research Ethics Committee and written informed consent was obtained.

Cohort B (19 samples) was recruited as previously described [11] from Southampton General Hospital from children undergoing fiberoptic bronchoscopy for diagnostic reasons. For cohort B, 3 x 10ml portions of normal saline were instilled and aspirated in children younger than 3 years of age. This was increased to 3 x 20ml portions in children > 3 years of age. This sampling represents bronchoalveolar lavage (BAL). The study was approved by the Southampton and Wessex Local Research Ethics Committees and written informed consent was obtained from the parents. BAL from cohort B was initially filtered through a 100μm nylon cell strainer (BD Biosciences) prior to centrifugation at 500g/10 minutes at 4°C. Aspirated cell free BALF was immediately stored at -80°C. For this study, 1ml aliquots from cohort B were available. Consequently, one freeze/thaw cycle was undertaken on these samples to prepare 25μl aliquots subsequently used throughout the study. A single freeze thaw step was found to have no discernible impact on SP-D binding activity and recovery when determined for three lavage fluid samples (data not shown). Limited clinical data and no microbiology data were available for cohort B and this cohort was primarily used to independently replicate observations made with the main cohort A. Owing to some differences in collection and processing methodologies, no direct comparison was made between cohort B and either cohort A or the control cohort.

Immediately prior to analysis, samples from both cohorts were thawed on ice and subjected to micro-centrifugation at 10,000g for 1 minute. Not all samples from the original studies were available for analysis here.

2.2. Reagents:

Unless otherwise stated, all reagents were from Fisher Scientific (Loughborough, UK). Affinity purified goat anti-human SP-D raised against recombinant human SP-D was from R&D systems (Abingdon, UK). Minimally cross reactive HRPO-conjugated donkey anti-goat IgG was from Jackson ImmunoResearch (Suffolk, UK). Native SP-D was purified from pulmonary alveolar proteinosis bulk lavage as previously described [14], and stored at -80°C in the presence of 1mM EDTA.

2.3. ELISA:

SP-D was quantified in lavage fluid using a two monoclonal antibody sandwich ELISA from Hycult Biotech (Uden, Netherlands) according to the manufacturer's instructions (measurable range: 6.3-400ng/ml). Lavage samples were diluted between 1:2 and 1:100 and measured in duplicate. Values for CXCL8 have previously been reported in cohort A [13] but not in the context of SP-D expression; this novel analysis is reported here.

2.4. Elastase activity assays:

Values for elastase activity in cohorts A and B have been previously reported [11, 13] but not in the context of SP-D expression; this novel analysis is reported here.

2.5. Zymosan and maltose-agarose binding assays:

10µl of a Zymosan-A suspension (1% w/v in 154mM NaCl) was added to a 0.5ml microcentrifuge tube and washed twice with 10 volumes of TBS-Ca (20mM Tris-HCl, 154mM NaCl, 20mM $CaCl_2$; pH 7.6) with microcentrifugation at 10,000g (13,000rpm at r=5.3cm) for 1 minute between washes. The pellet was resuspended with 10µl of TBS-Ca and 10µl of either

freshly thawed BALF or, as a positive control, 10µl of native SP-D diluted to 2 or 20µg/ml in 154mM NaCl with 1.5mg/ml protease free BSA (Sigma, Dorset, UK) to give a final concentration of 10mM $CaCl_2$ in all binding assays. Where Ca^{2+} or carbohydrate dependence of the binding assay was tested, native SP-D or lavage was diluted to give a final concentration of 10mM EDTA in TBS or 100mM D-Maltose plus 10mM $CaCl_2$ in TBS in the binding assay. In some cases, 1mM PMSF was included to inhibit serine protease activity in assays where EDTA was used. Where the capacity of lavage to influence native SP-D binding to zymosan was tested, 10µl of native SP-D (2µg/ml) in TBS-Ca was pre-incubated with 10µl of lavage prior to incubation with zymosan. For all assay formats, samples were incubated for 30 minutes at 37°C with occasional gentle mixing to maintain zymosan in suspension over the course of the assay. Post assay, samples were microcentrifuged at 10,000g for 1 minute and the supernatant was carefully aspirated to a fresh tube, avoiding disturbance of the zymosan pellet. Following aspiration of the supernatant, the zymosan pellet was washed once with 100µl TBS-Ca as described above and the washed pellet was resuspended in 20µl TBS with 20mM EDTA. Where samples were subsequently subjected to SDS-PAGE both pellet and supernatant fractions were immediately treated with SDS sample buffer with β-mercaptoethanol as a reducing agent and boiled at 100°C for 2 minutes prior to electrophoretic separation. For native-PAGE, samples were treated with cold sample buffer without SDS but supplemented with 0.002% Coomassie Blue G250 (Sigma, UK). Samples were not boiled but immediately separated as described below. The reproducibility of the zymosan binding assay was confirmed by assaying at least seven lavage samples from this and a previous study [15] at different time points up to 10 months apart (data not shown).

Maltose-agarose pull down assays were similar to the zymosan binding assays with the following differences. 10µl of maltose agarose (Sigma, Dorset, UK) was washed three times in 1ml TBS-Ca prior to commencement of the binding assay. Binding steps for both parallel zymosan and maltose-agarose assays were carried out on a flat bed roller to avoid settling of the maltose-agarose during the assay. Assays were carried out in a final volume of 30µl, with only 20µl of the supernatant fraction aspirated for SDS-PAGE to avoid disturbing the agarose pellet;

once washed, the pellet was resuspended in 30µl of TBS-EDTA, 20µl of which was prepared for for SDS-PAGE as described above.

2.6. Native- and SDS-PAGE, Western Blot and densitometry:

Native PAGE was performed using NuPage 3-8% Tris-Acetate gels (Life Technologies, UK) and a tris-glycine buffer system where the cathode buffer was supplemented with 0.002% Coomassie Blue G250. Electrophoresis proceeded for 90-120 min at a constant voltage of 150V. Proteins were transferred to nitrocellulose using NuPage Transfer buffer (Life Technologies, UK) supplemented with 10% methanol at a constant voltage of 100V for 1 hour. For proteins separated under denatured and reduced conditions SDS-PAGE was performed using 10% gels with a 4% stacking gel. Gels were separated at a constant current of 45mA per gel for 45-60min. Proteins were transferred to nitrocellulose in a tris-glycine buffer supplemented with 20% methanol at a constant voltage of 100V for 1 hour. In all cases, following transfer to nitrocellulose, blots were blocked for 30 min at room temperature with PBS 5% w/v skimmed milk powder (PBS-milk). Blots were incubated overnight at 4°C with 0.1µg/ml goat anti-human SP-D in PBS-milk. Blots were washed three times with PBS-0.05% v/v Tween and once with PBS. Blots were incubated with minimally cross-reactive HRPO-conjugated donkey anti-goat IgG for 45 min at room temperature, washed three times with PBS-Tween, three times with PBS and developed using ECL prime substrate (GE Lifesciences, UK) prior to exposure to X-Ray film (Fuji, UK). The limit of sensitivity of SP-D detection by western blot was approximately 5ng/lane (equivalent to 500ng/ml under the conditions used here) and lavage samples with ELISA determined SP-D below this level were not detectable by western blot in this study.

Developed blots were scanned and the entire blot area was uniformly adjusted for brightness and contrast. Presented images represent all bands visible in lanes from the original blot. Densitometry was performed on the blots using ImageJ v1.42 as previously described [13] and percentage zymosan bound SP-D for any given data point refers to the percentage of the total band area present in the pellet fraction. As SP-D exists in a number of oligomeric forms of

differing molecular weights, densitometry was only undertaken on samples reduced with β-mercaptoethanol immediately prior to SDS-PAGE. Reducing all SP-D oligomeric forms to single subunits ensured equivalent transfer efficiency and antibody reactivity of bands in pellet and supernatant fractions during western blot which is essential for accurate comparison of SP-D content in each fraction.

2.7. **Analytical gel filtration and mannan binding assays**:

Lavage fluid (200µl) from six CF patients was applied to a Superose 6 10/300GL (GE Lifesciences, UK) column and was eluted in TBS-1mM EDTA at a flow rate of 24ml/h on an AKTAprime (GE Lifesciences, UK), collecting 0.8ml fractions which were immediately transferred to ice. Fractions were assayed for SP-D by a sandwich ELISA as described (Leth-Larsen 2003), except the capture antibody was replaced with a polyclonal rabbit antibody raised against a recombinant fragment of human SP-D, as previously reported [16]. Plates were developed with 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma, UK) and arrested with H_2SO_4 before absorbance was measured at 450nm on a SpectraMax 340PC spectrophotometer (Molecular Devices, UK).

Fractions were also assayed for specific SP-D lectin activity in a solid phase mannan binding assay as follows: Maxisorp 96-well plates (Nunc, Denmark) were coated overnight at 37°C with 50µg/ml mannan from *S. cerevisiae* (Sigma, UK) and blocked with TBS 0.05% Tween 20 (TBST). Gel chromatography fractions were made 0.05% (v/v) with respect to Tween 20 and 10mM with respect to either $CaCl_2$ or EDTA. Fractions were applied to mannan coated plates for 90 minutes at room temperature. Plates were washed three times with TBST-10mM calcium (TBST-C) or TBST-10mM EDTA (TBST-E) and goat anti-SP-D (1µg/ml) was applied in TBST-C for 60 minutes at room temperature. Plates were washed and HRPO-conjugated minimally cross reactive donkey anti-goat IgG was applied for 30 minutes at room temperature. Plates were washed, developed with TMB and arrested with H_2SO_4 before absorbance was measured at 450nm on a SpectraMax 340PC spectrophotometer (Molecular Devices, UK).

2.8. Statistics

Unless otherwise indicated, all data presented in the text refer to (median; interquartile range). Two group comparisons of non-parametric data were undertaken using the Mann-Whitney test. Spearman's r-values are used to describe correlations. In all cases statistical significance was achieved at $p<0.05$. Data remained statistically significant after the application of Holm's sequential Bonferroni correction where appropriate [17]. All data were analysed using GraphPad Prism v5.01 (GraphPad Software Inc).

3. RESULTS:

Table 1: Description of cohorts under study.

	Control	CF Cohort A	CF Cohort B
N	9	25	19
Age (decimal years)	8.28 (6.53-10.93)	6.41 (0.07-15.74)	9.67 (0.39-17.92)
Sex (M:F)	5:4	15:10	NK[a]
Weight (kg)	NK	22 (4.8-81.2)	NK
BMISDS	NK	0.09 (-7.7-3)	NK
FEV_1 (% predicted)	NK	80 (37-110); (N=12)[b]	82.2 (68-145); (N=12)[b]
FVC (% predicted)	NK	88.5 (51-106); (N=12)[b]	88.45 (73-161); (N=12)[b]
Lavage culture verified infection (N)	0	10[c]	NK
Lavage neutrophils (10^6/ml)	0.007 (0-0.0157)	0.54 (0.03-4); (N=18)[d]	NK
Lavage mononuclear cells (10^6/ml)	0.096 (0-0047-0.38)	0.4 (0.04-2.5); (N=18)[d]	NK
Lavage elastase positive:negative (N)	0:9	14:11	11:8
Lavage free elastase activity (nM)[e]	0	74 (8-1250)	40 (2-1657)

Data are expressed as median (range) or as number of samples (N). [a]NK=not known. [b]Lung function data were available for a proportion of cohort A and B and were not available for children <4 years of age. [c]Please see materials and methods for additional information. [d]Differential cell counts were available for a proportion of the cohort as previously described [13]. [e]Elastase activity values refer to elastase positive samples only.

3.1. SP-D expression in CF airway lavage fluid is inversely related to infection, markers of neutrophilic inflammation and age: SP-D was detected by ELISA in CF lavage fluid from cohort A (5332ng/ml; 1957-9595) at levels 2.4 fold higher than control BALF (2214ng/ml; 1545-2902) (figure 1a). Within CF cohort A SP-D expression was 3.6 fold lower in those with clinically important infections (1892ng/ml; 359-6376) compared to those without (6879ng/ml; 4683-12200) (figure 1b). SP-D expression negatively correlated with elastase activity (data not shown: Spearman r=-0.71; p=0.004) and CXCL8 expression (data not shown: Spearman r=-0.45; p=0.02) in CF cohort A. SP-D expression also negatively correlated with age in CF cohort A (figure 2a: Spearman r=-0.4; p=0.04). Patients in whom elastase activity was detected were significantly older than elastase negative counterparts (9.2 years; 3.6-13.2 vs. 2 years; 0.7-6.4: p=0.02) and infected patients were also substantially older than their non-infected counterparts (8.7 years; 3.6-13.5 vs. 2.8 years; 0.4-8.8) although this did not reach statistical significance (p=0.08). Data for microbiology or CXCL8 expression were not available for cohort B (median BALF SP-D: 1073ng/ml; 239-2414), however we confirmed that SP-D expression correlated negatively both with elastase activity (data not shown: Spearman r=-0.8; p=0.002) and age (data not shown: Spearman r=-0.47; p=0.04).

3.2. Measuring SP-D lectin activity with a functional assay: SP-D lectin activity was measured in a zymosan binding assay. Native SP-D interactions with zymosan were maltose and EDTA sensitive (figure 3a) as previously described [15]. When tested in the same assay, lavage fluid from CF cohorts A and B as well as the control cohort contained substantial amounts of SP-D which failed to bind zymosan. Within control and CF lavage, a minor band migrating 3-4kDa and occasionally 6-8kDa (figure 3b&c; closed arrows) higher than the main SP-D monomer (figure 3b&c; asterix) were sometimes identified by Western blot as part of the non-binding SP-D pool. Similar minor forms of higher molecular weight SP-D have previously been described [7, 18]. Occasionally, a minor band migrating below the main SP-D monomer was observed in CF lavage which contained active neutrophil elastase (figure 3b; open arrow).

This likely corresponds to partially proteolysed SP-D as previously described in CF [6, 7]. Lavage SP-D interactions with zymosan were also predominantly calcium and maltose dependent (Figure 3c). As reported in previous studies [9-11], calcium chelation with EDTA in CF lavage sometimes resulted in a loss of detectable SP-D due to proteolysis (data not shown). Protease inhibition with 1mM PMSF preserved SP-D under these conditions (figure 3c; right hand panel). The pH of airway secretions in normal and pathological conditions has been reported to be slightly acidic. The pH dependence of SP-D zymosan interactions were therefore also tested and were found to be stable when tested between pH 5.6 and 7.6 (figure 3d).

When maltose-agarose was used as a substrate for lavage fluid SP-D binding, the pattern of binding was largely similar to that obtained with zymosan (figure 4). However in some cases (figure 4; samples 2b & 3b) SP-D demonstrated weaker binding activity towards maltose-agarose.

3.3. Restricted lavage SP-D lectin activity is related to infection and markers of neutrophil inflammation: Following a zymosan binding assay and western blot of bound and non-bound fractions, the percentage of zymosan bound SP-D was quantified by densitometry for all available patient samples. In CF cohort A, 3/25 samples with the lowest SP-D concentration contained insufficient antigen to be quantified in the zymosan binding assay and are not included in subsequent analyses. In cohort B, 6/19 BALF samples were similarly below the limit of detection for the assay. Those samples below the limit of detection for western blot were also observed to have SP-D <500ng/ml by ELISA. Further concentration of these samples was not undertaken as this has been reported to alter the molecular structure of SP-D in a functionally relevant manner [19]. A substantial proportion of SP-D in lavage from CF cohort A failed to bind zymosan (55%) and similar findings were observed in the control cohort (46%) however there was no difference between the groups, (p=0.56), (figure 5a). Similar findings were observed in CF cohort B where 61% of SP-D failed to bind zymosan (data not shown). The proportion of SP-D with zymosan binding activity was significantly lower in infected compared

to non-infected CF patients (p=0.02; figure 5b). The majority of native purified SP-D included as a positive control in all assays for cohorts A and B exhibited binding to zymosan (95%; 90-98). Total zymosan binding SP-D (calculated using ELISA and zymosan binding assay data) was higher in CF cohort A than controls (p=0.01; figure 6a) but was significantly lower in CF samples with culture confirmed infection compared to those without (p=0.04; figure 6b).

Samples containing the lowest detectable SP-D zymosan binding activity were tested for the ability to inhibit exogenously added native SP-D binding to zymosan. Substantial inhibition of native SP-D binding to zymosan was not observed by lavage samples yielding a positive culture for *Aspergillus fumigatus* (figure 7), *Burkholderia cepacia* or *Stenotrophomonas maltophilia* (data not shown) suggesting that competitive inhibition by microbial ligands within these samples was not responsible for the restricted lectin activity. Furthermore, addition of the serine protease inhibitor PMSF had little impact on the assay results in the presence of calcium (figure 7).

In samples where active elastase was present, the majority of SP-D in the non-bound fraction was of identical molecular weight to the bound material and SP-D degradation products were only occasionally observed in a small minority of samples by western blot (figure 3b). Despite this, both the percentage and total concentration of SP-D with lectin activity was significantly lower in elastase positive samples from cohort A compared to those in which elastase was not detected (figure 8a & b). The percentage and total concentration of SP-D binding to zymosan also negatively correlated with expression of the neutrophilic chemokine, CXCL8, in this cohort, (figure 8c & d). Similar relationships between elastase activity and SP-D lectin activity were not observed in cohort B (data not shown), however those analyses may have been compromised by the smaller sample size and the fact that almost half of the elastase positive samples in that cohort were not detectable in the zymosan binding assay and are not therefore represented in the analysis. Furthermore, CXCL8 expression data was not available in cohort B.

3.4. Oligomeric variants of SP-D in CF airway lavage fluid exhibit distinct lectin activity:

As the oligomeric status of SP-D is known to have a profound impact on its ligand binding activity [20-22], native gel electrophoresis was used to assess the oligomeric status of SP-D in lavage fluid. A range of oligomeric forms were evident in both control and CF samples (Figure 9a) including very large oligomers barely capable of entering the gel. Analysis of bound and non-bound fractions from a zymosan binding assay, revealed that high oligomeric forms of SP-D predominantly migrated with zymosan whilst low oligomeric forms remained in the supernatant (Figure 9b; upper panel). Intermediate oligomeric forms evident in the non-fractionated blot (arrow; figure 9a), were less prevalent following the binding assay. Partial assembly of SP-D trimers to multimers in response to ligand binding has previously been reported [19] and we suggest that these intermediate forms may have formed larger oligomers in response to zymosan binding.

The size distribution of SP-D was also examined by gel chromatography of lung lavage fluid from six CF patients. Two distinct populations of SP-D separating at different molecular weights were observed in all samples tested (Figure 10a). Although present in different proportions, both populations eluted at the same position in all lavage samples. The mobility of both populations relative to globular standards was consistent with 'high' (dodecameric and multimeric) and 'low' (trimeric) forms of SP-D previously described by Leth-Larsen [22]. Fractions from five of these samples were available for parallel assays of lectin activity. This showed substantial calcium dependent SP-D mannan binding activity in fractions corresponding to high oligomeric forms whilst SP-D in fractions of lower oligomeric form exhibited only weak or absent mannan binding activity in any of the samples tested (figure 10b & c).

4. DISCUSSSION:

We present a comprehensive analysis of SP-D expression and function in lung secretions from paediatric CF patients and controls. To our knowledge, this is the first systematic analysis of SP-D lectin activity in the CF population.

In keeping with previous studies, SP-D expression was reduced in lavage fluid from CF patients with infection or evidence of inflammation compared to non-infected CF patients. Importantly however, SP-D was present in all but a few CF samples representing both bronchial (Cohort A) and bronchoalveolar (Cohort B) airway when tested by specific dual monoclonal antibody ELISA. There has been considerable variability in reported values for BALF SP-D in CF, ranging from a near absence [4, 23] to expression in the μg/ml range [5]. Several factors may explain this variability: In addition to differences in BAL sampling and processing methodology, techniques used to measure SP-D in CF range from semi-quantitative western blot [6, 7] to ELISA, some of which use antibodies reactive with human SP-D but raised against a different species [5, 23]. Also, some previous studies applied specific microbial density thresholds to define infection [5, 6], whereas we define infection on the basis of reported positive culture in line with other reports [12, 24-26]. This may explain why we observe immunoreactive SP-D both by Western blot and ELISA even in culture positive CF samples. Also, in some previous studies, the proportion of patients with culture verified infection was higher [4, 7] and the age range of CF patients substantially older [7, 23] than those studied by us. This may be relevant in light of the negative correlation between age, infection and SP-D concentration reported here. Previous population based studies have described increased circulating SP-D with age [27]. The age related decline in lung rather than circulating SP-D observed here probably reflects advancing lung disease in this population, an interpretation supported by the increased prevalence of infection and active elastase in older patients. In summary, our observation of substantial SP-D expression in CF confirms some reports [5, 7] but not others [4]. However the observed negative association between SP-D, infection and inflammation agrees with the

findings of a number of previous studies [4-6] and suggests that detectable SP-D is subject to dynamic modulation in the face of ongoing inflammatory disease.

The primary objective of this study was to examine the carbohydrate binding activity of SP-D in CF patients. We observed considerable heterogeneity in the capacity of both CF and control lavage SP-D to bind the yeast derived mannan rich particle, zymosan. Such heterogeneity was observed in lavage fluid collected from two distinct cohorts of CF patients. Lavage collected from cohort A was mainly of bronchial origin whilst that from cohort B was reflective of bronchoalveolar lavage according to the lavage procedure used. Nevertheless, inflammatory activity (reflected in free elastase activity) was relatively similar between both cohorts which may reflect the largely bronchial nature of airway disease in CF. Although SP-D inactivation by CF relevant proteases is well described, the functional consequences of this remain unclear as physiological concentrations of calcium render SP-D resistant to proteolysis [6, 9, 11]. As previously reported, SP-D expression was lower in elastase positive CF samples here, however in the majority of cases substrate bound and non-bound SP-D migrated at an identical molecular weight on SDS-PAGE under reducing conditions. The absence of a major population of proteolytically fragmented SP-D in this study is consistent with two previous reports [7, 12] and indicates that limited proteolysis is not responsible for the observed restriction of lectin activity. Minor forms of SP-D migrating 3-8kDa higher than the main monomer were observed in many samples but did not associate with zymosan or maltose-agarose and were exclusively found in the supernatant fraction. These resemble forms of SP-D previously observed in CF BALF [7] and are thought to represent a hyper-glycosylated form of SP-D speculated by Mason et al. to limit oligomerisation to trimeric species [18]. The inability of these forms to bind saccharide ligands in our assays is consistent with the well documented reduction of opsonic and anti-viral activity of low oligomeric forms of SP-D [20-22]. In light of this, we used both native gel electrophoresis and size exclusion chromatography to further investigate the molecular distribution and lectin activity of SP-D in airway lavage. SP-D capable of binding zymosan exhibited features of high molecular weight when assessed by western blot under

native conditions, whilst non-bound material was of lower molecular weight. This observation was confirmed by size exclusion chromatography which revealed two populations of SP-D in CF airway lavage fluid. Consistent with data from the zymosan binding assay, high molecular weight SP-D bound to yeast derived mannan in a calcium dependent fashion whilst lower molecular weight material exhibited limited if any capacity to recognise such polysaccharides. The distribution of these two populations of SP-D is consistent with reports of a common SP-D polymorphism influencing the structure and lectin activity of SP-D. The presence of Thr at position 11 limits SP-D oligomerisation to trimers with restricted lectin activity whilst Met at that position gives rise to dodecameric oligomers with unrestricted lectin activity towards complex carbohydrates and a range of bacteria and Influenza virus in addition to functionally restricted trimeric oligomers [22]. Although the frequency of that polymorphism was not determined here, its distribution among Danish CF patients is not significantly different from the normal population [28]. In addition to these common polymorphisms, post-translational modification by nitric oxide derivatives [29] and elements of the neutrophil respiratory burst [30, 31] also contribute to plasticity of SP-D structure and function. Reactive oxygen-nitrogen intermediates of the innate inflammatory response were not reported to have any appreciable impact on SP-D lectin activity when tested using similar pulldown methodology as that employed here; rather, these intermediates diminished the agglutinating activity of SP-D [30, 31]. Importantly, agglutination is not a critical determinant of zymosan mobility under the assay conditions used here. Taken together, this suggests that the functional heterogeneity reported here is not primarily due to post-translational modifications by reactive oxygen-nitrogen intermediates. However, a thorough assessment of the presence of such post-translational modifications in clinical BAL samples will be required to fully understand the impact of these processes on SP-D activity in vivo.

Lower zymosan binding activity was observed among SP-D from CF patients with clinically important infections. Although the number of patients with infections was limited owing to the lower rates of chronic infection in the pediatric CF population, the data nevertheless

demonstrate significant differences in the expression and lectin activity of SP-D in the face of infection. One interpretation of this finding, and that of reduced SP-D expression in infected patients, is that sequestration by adherence to bacteria and subsequent clearance during lavage processing explains the observed deficit. Although paired cellular and acellular samples were not available to address this possibility, a previous study of CF and non-CF patients with and without infection, indicates that poorly regulated inflammation rather than infection per se, is responsible for SP-D depletion during infectious disease [5]. That study also reported no difference in SP-D content between sonicated, cellular BALF and cleared acellular BALF, nor did EDTA treatment of BALF prior to clearance (to dissociate any potential SP-D complexes) have any effect on SP-D content. Our own unpublished data confirm the enhanced anti-viral properties of size excluded high oligomeric form alveolar proteinosis SP-D towards Influenza-A virus. Intriguingly, these highly oligomerised forms of SP-D also agglutinate *Escherichia coli* into very large aggregates (up to 50μm and occasionally 80 μm diameter; data not shown) and may play an important role in facilitating effective mucociliary clearance of bacteria from the lower airway; a process likely to be of critical importance in the CF airway. It is possible that enhanced mucociliary clearance of large aggregates may partially explain the depressed SP-D expression in the airway of CF patients with infection observed here and elsewhere. This would be predicted to lead to the preferential clearance of higher order oligomers of SP-D from the lower airway, which may also explain the relatively restricted lectin activity of SP-D in CF patients with infection, where lower order oligomers would dominate. Nevertheless, even among non-infected and control patients, considerable heterogeneity of SP-D lectin activity was observed. We have recently described functionally relevant heterogeneity of SP-D oligomeric form in preterm neonatal BALF using methodology similar to that described here [15]. Oligomeric diversity has also been observed in SP-D from asthmatic and chronic obstructive pulmonary disease patients [32, 33] but was not examined functionally. Few other systematic reports of SP-D activity in clinical populations have been published. One study reported maltose dependent agglutinating activity of BALF from tracheostomy patients towards

Pseudomonas aeruginosa as a surrogate measure of SP-D agglutinating activity [34]. Interestingly, although the agglutinating activity of lavage correlated with SP-D content, this was found to be a relatively weak association indicating either that other maltose inhibitable factors were contributing to the activity, or that not all SP-D detected by ELISA participated in the agglutinating activity. In light of data reported here and previously [15] we argue that it is important to rely not only on quantitative assays of SP-D content when considering the status of SP-D in a clinical population but also to consider what proportion of the recovered material is functionally active in any given assay. It is also important to consider what aspect of SP-D activity one wishes to measure. Both lectin and agglutinating activity are profoundly influenced by the oligomeric state of SP-D however how this influences the reported anti-inflammatory activity of SP-D is not well understood. Understanding this relationship will be an important step in fully understanding the influence of SP-D oligomeric diversity upon the range of reported SP-D activities

The importance of SP-D oligomeric state in controlling infection in vivo is also not well understood. Heritable variation in SP-D oligomerisation has been implicated in clinically relevant responses to respiratory syncitial virus [35, 36] and *Mycobacterium tuberculosis* [37]. In the case of MBL (another collectin subject to functionally relevant oligomeric variation), amino acid changes influencing multimerisation and carbohydrate binding activity act in combination with promoter polymorphisms to confer a state of functional and quantitative MBL insufficiency [38] associated with earlier and more severe lung pathology in CF [39]. Increased oligomeric complexity in SP-A has also been recently associated with better lung function in CF [40]. The only study published to date in CF identified no association between SP-D Met/Thr genotype and disease progression [28], however larger studies looking at the full range of factors influencing SP-D structure/function will probably be required to reach a firm conclusion.

In summary, our data show that SP-D is found in substantial quantities in the paediatric CF lung but exists as a functionally and structurally heterogeneous population in both this and the

control population. As our surgical control population did not have symptoms of airway disease at the time of sampling it will be interesting to examine SP-D expression and lectin activity in non-CF patients with suppurative lung disease to examine whether the link between infection, inflammation and SP-D activity observed here is a specific feature of CF. We propose that structural heterogeneity may be as important (if not more so) than proteolytic digestion in determining the functionality of SP-D in CF. Although factors influencing SP-D oligomerisation have been identified, how this may differ in health and disease and the contribution this makes to clinical outcome requires further investigation.

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6. FIGURE LEGENDS

Figure 1) SP-D expression in CF and control lavage fluid: A) SP-D was measured in CF cohort A (n=25) and controls (n=9) by sandwich ELISA. **B)** The CF cohort was divided into those samples in which clinically relevant microorganisms were recovered (infection status positive; n=10) and those which either yielded no microbiological culture or yielded microorganisms regarded either as part of the normal microflora or not clinically significant (infection status negative; n=15) as detailed in the materials and methods. Abbreviated microorganisms are as follows: *P. aer. = Pseudomonas aeruginosa, H. Infl. = Haemophilus influenzae, B. cep. = Burkholderia cepacia, S. malto. = Stenotrophomonas maltophilia, A. fum. = Aspergillus fumigatus, M. cat. = Moraxella catarrhalis*. Bars refer to medians.

Figure 2) SP-D expression negatively correlates with age in CF: Lavage SP-D concentration was plotted against patient age in CF cohort A (n=25). The r- and p-values refer to Spearman's rank correlation. As stated in the text, a similarly significant correlation between age and SP-D concentration was also observed in CF cohort B (data not shown; Spearman r=-0.47; p=0.04).

Figure 3) Assessing SP-D functional activity in CF and control lavage fluid: A) Native purified SP-D was incubated with a washed zymosan pellet in the presence of 10mM $CaCl_2$, 10mM EDTA or 10mM $CaCl_2$ with 100mM D-maltose. Following a 30 minute incubation with occasional agitation, zymosan was pelleted by centrifugation at 10,000g/1 minute. Supernatants were carefully aspirated to fresh tubes. The pellet was washed in the appropriate buffer and treated with 20mM EDTA. Both pellet (P) and supernatant (S) fractions were boiled for 2 minutes in SDS sample buffer with β-mercaptoethanol prior to separation by SDS-PAGE on 10% gels followed by Western blot for SP-D. SP-D mobilises with the zymosan pellet in the presence of calcium but not in the presence of maltose or EDTA. Data are representative of

three or more independent experiments. **B)** Control and CF lavage as well as positive control native purified SP-D were similarly assayed for their capacity to bind to zymosan in the presence of 10mM $CaCl_2$. The majority of native SP-D mobilised with the zymosan pellet. In the case of both control and CF, the major SP-D band migrated with an identical molecular weight as native purified SP-D (*). A significant proportion of SP-D in both control and CF lavage failed to bind to zymosan and was found in the supernatant fraction. In addition to the main SP-D monomer additional bands migrating 3-4 and 6-8kDa higher than the main SP-D monomer were observed in some samples under reducing conditions (closed arrows) but only in the non-bound supernatant fraction. A minor lower molecular weight band, probably corresponding to a proteolytic fragment of SP-D, was observed in a small number of samples (open arrow). Data are representative of at least 12 independent experiments. **C)** CF lavage was incubated with a washed zymosan pellet in the presence of 10mM $CaCl_2$, 10mM EDTA or 10mM $CaCl_2$ with 100mM D-maltose as described in panel A. Following separation of pellet and supernatant fractions by SDS-PAGE under reducing conditions, the majority of SP-D was seen to migrate with zymosan in a maltose and EDTA inhibitable fashion. Symbols refer to bands described in panel B. Data are representative of at least 3 (maltose) and 2 (EDTA + PMSF) independent experiments. **D)** Zymosan binding assays were undertaken for three CF lavage fluid samples at pH 5.6, 6.6 and 7.6. No substantial differences in the proportion of bound and non-bound SP-D were observed between assays carried out at different pH.

Figure 4) Assessing lavage SP-D binding activity towards zymosan and maltose agarose: CF lavage was incubated either with maltose agarose **(A)** or zymosan **(B)** for 30 minutes in the presence of 10mM $CaCl_2$. Native purified SP-D was included as a positive control. Following centrifugation, the supernatant (S) fraction was carefully aspirated. The pellet (P) fraction was washed and resuspended in 20mM EDTA. Both fractions were separated by SDS-PAGE under reducing conditions and blotted for SP-D. In addition to the main SP-D monomer, a minor

population of SP-D migrating 3-8kDa above this was evident in the non-bound supernatant. Additional bands migrating at approximately 95kDa are thought to represent partially non-reduced SP-D dimers. Data are representative of 3 independent experiments addressing 10 distinct lavage fluid samples.

Figure 5) SP-D zymosan binding activity in CF and control lavage: A) All available lavage samples were subjected to a zymosan binding assay and following SDS-PAGE of pellet and supernatant fractions, blots were probed for SP-D. The percentage of SP-D associated with the zymosan pellet was quantified by analysis of pellet and supernatant bands using ImageJ software. Data are presented for control (n=9) and CF cohort A (n=22; 3 CF samples had too little SP-D to be quantified by the zymosan binding assay). **B)** CF samples were separated into those with clinically important infections (infection status positive: n=7; 3 CF samples had too little SP-D to be quantified by the zymosan binding assay) or not (infection status negative: n=15) as described in the materials and methods. The identities of the infectious microorganisms are detailed in abbreviated form as detailed in the legend to figure 1b. Bars refer to medians.

Figure 6) SP-D binding activity in CF and control lavage: Data from the zymosan binding assay was combined with that from the ELISA to calculate the amount of lavage SP-D with zymosan binding activity. **A)** Data are presented for control (n=9) and CF cohort A (n=22; 3 CF samples had too little SP-D to be quantified by the zymosan binding assay). **B)** CF samples were separated into those with clinically important infections (infection status positive: n=7; 3 CF samples had too little SP-D to be quantified by the zymosan binding assay) or not (infection status negative: n=15) as described in the materials and methods. The identities of the infectious microorganisms are detailed in abbreviated form as detailed in the legend to figure 1b. Bars refer to medians.

Figure 7) Culture positive CF lavage with minimal endogenous functional SP-D does not substantially inhibit exogenous native SP-D binding activity towards zymosan: CF lavage which yielded positive microbiological culture and was found to contain SP-D with weak capacity to interact with zymosan was tested for its ability to inhibit exogenously added native SP-D binding to zymosan. Experiments were carried out in the presence or absence of 1mM PMSF to inhibit serine protease activity present in these samples. Lavage fluid was reconstituted with (+NhSP-D) or without (-NhSP-D) purified native SP-D. Positive controls in the absence of lavage were also included (lanes 1-4). Samples were incubated with zymosan in the presence of 10mM $CaCl_2$. Following centrifugation, pellet and supernatant fractions were separated by SDS-PAGE under reducing conditions and blotted for SP-D. The majority of exogenously added SP-D bound to zymosan in these experiments and PMSF had little impact on binding activity in the presence of 10mM $CaCl_2$. Data are representative of 3 independent experiments.

Figure 8) The percentage and amount of functional SP-D in CF lavage is inversely related to neutrophil elastase activity and CXCL8 expression: CF lavage samples with sufficient SP-D to enable analysis in the zymosan binding assay were separated into those samples with detectable elastase activity (elastase status positive; n=11) or not (elastase status negative; n=11) in order to assess differences in the percentage **(A)** and amount **(B)** of SP-D capable of binding to zymosan between these two groups. Bars refer to medians, p-values refer to the Mann Whitney test. The percentage **(C)** and amount **(D)** of SP-D capable of binding to zymosan was also plotted against CXCL8 concentration for each sample (n=22 in both cases). R- and p-values refer to Spearman's correlation.

Figure 9) Lavage SP-D with zymosan binding activity exhibits features of higher olgomeric form: A) CF and control lavage fluid was separated on 3-8% Tris-acetate

polyacrylamide gels under native conditions. Following transfer to nitrocellulose, blots were probed for SP-D. **B)** CF and control lavage as well as positive control native purified SP-D were subjected to a zymosan binding assay in the presence of 10mM $CaCl_2$. Zymosan bound (pellet;P) and non-bound (supernatant;S) fractions were isolated and prepared for either native PAGE (top panel) or SDS-PAGE under reducing conditions (bottom panel). Under native conditions the majority of zymosan bound material in the pellet fraction exhibited features of high oligomeric form and was unable to significantly migrate into the gel. By contrast, non-bound material from the supernatant migrated with characteristics of lower oligomeric form. The arrow in panel A highlights an intermediate oligomeric form of SP-D observed in unfractionated lavage which was less prevalent following the zymosan binding assay.

Figure 10) Two populations of SP-D of different oligomeric form and exhibiting distinct lectin activity are present in CF airway fluid: A) Six CF lavage fluid samples were subjected to Superose 6 gel filtration and fractions were assessed for SP-D content by ELISA. Two distinct peaks of different molecular weight were observed in all samples tested. The elution profile of three standards - blue dextran (BD), thyroglobulin (TG) and alcohol dehydrogenase (ADH) - and their molecular weights are indicated at the top of the panel. **B)** For five of the samples separated by gel filtration, individual fractions were also assessed for calcium dependent SP-D binding to mannan in a solid phase assay. Larger molecular weight SP-D oligomers exhibited preferential binding to mannan but little or no binding was observed from the lower molecular weight fractions. **C)** The inclusion of EDTA in mannan binding assays was sufficient to inhibit all SP-D/mannan interactions.

Figure 1

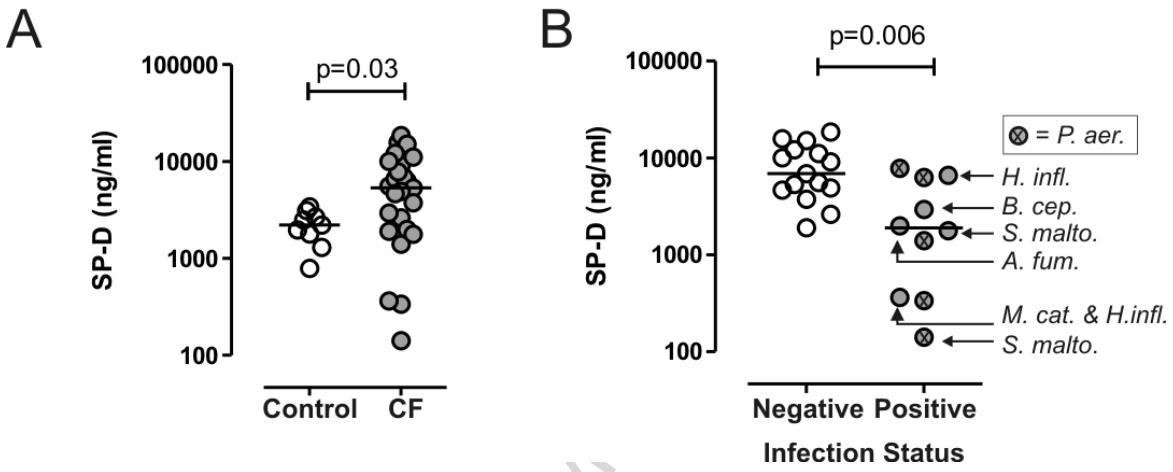


Figure 2

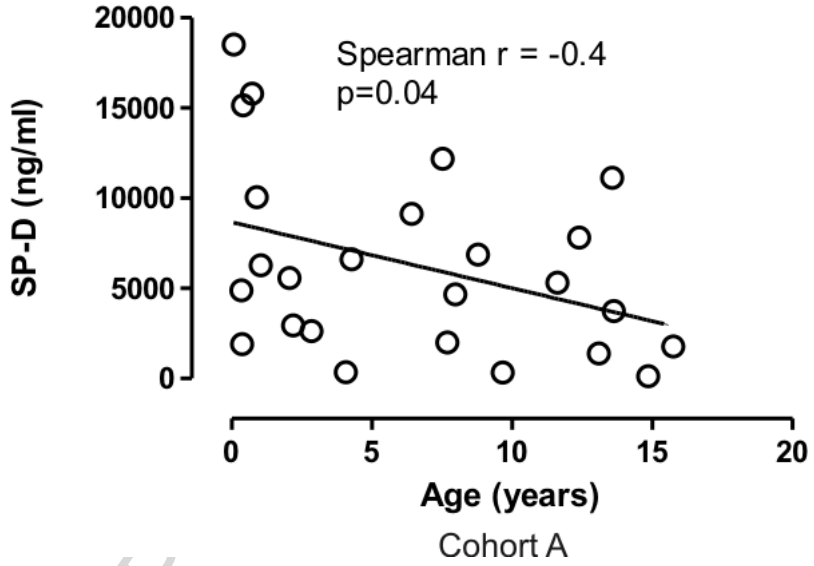


Figure 3

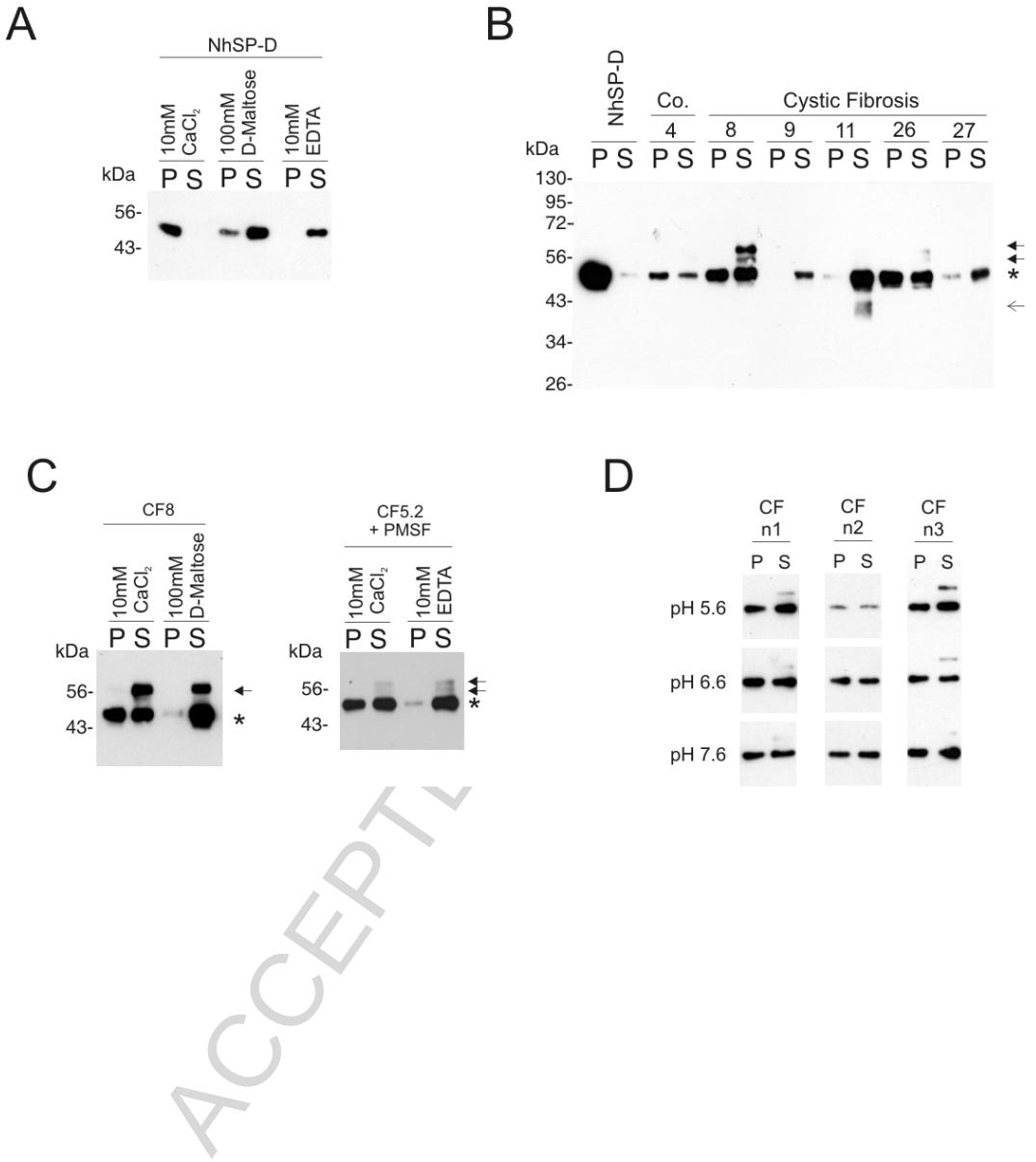


Figure 4

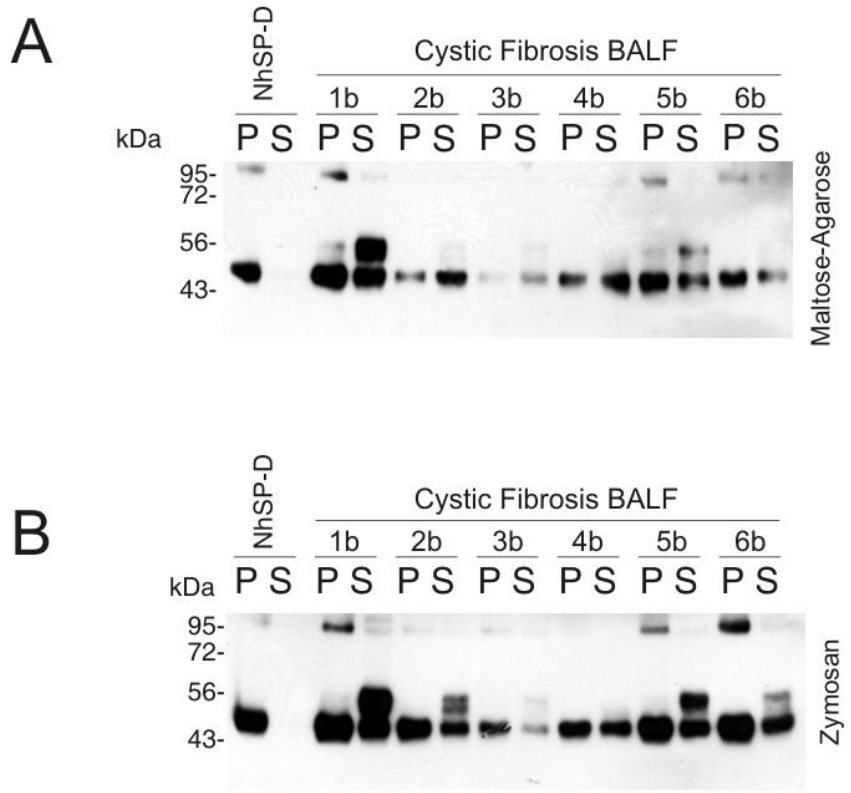


Figure 5

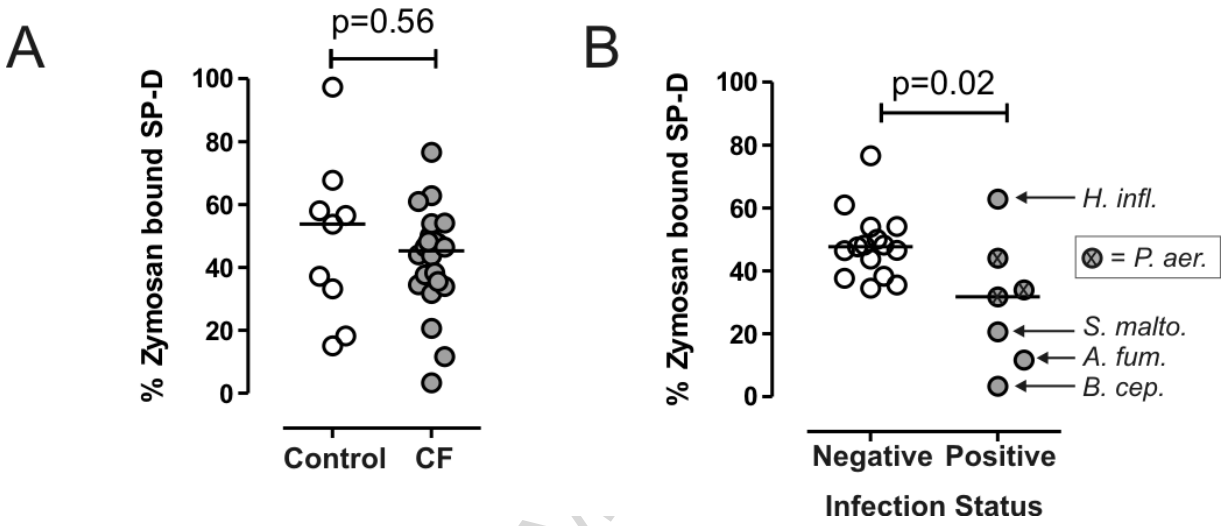


Figure 6

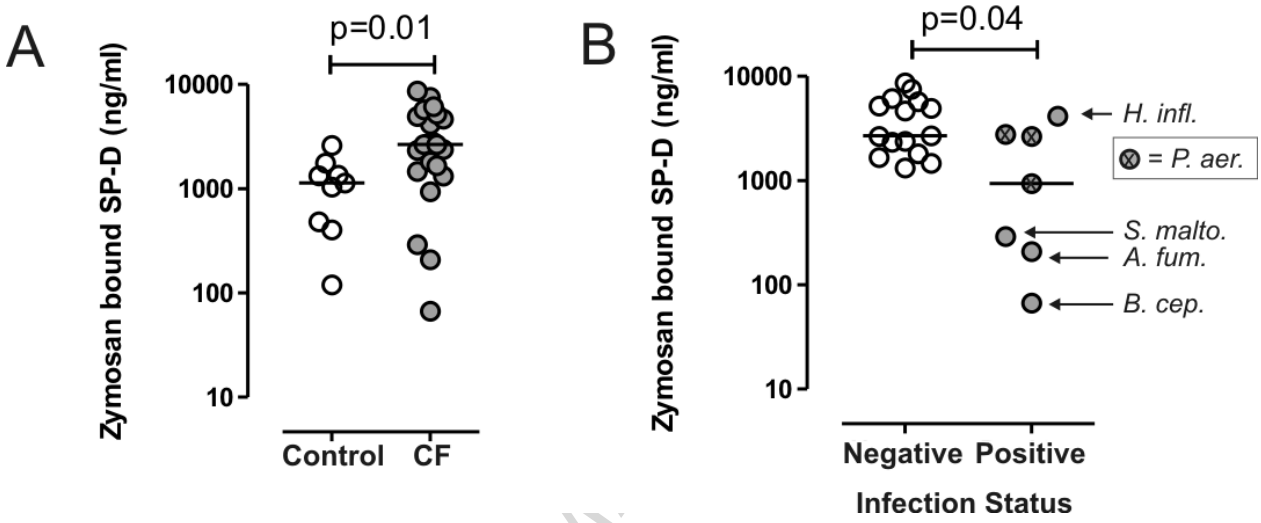


Figure 7

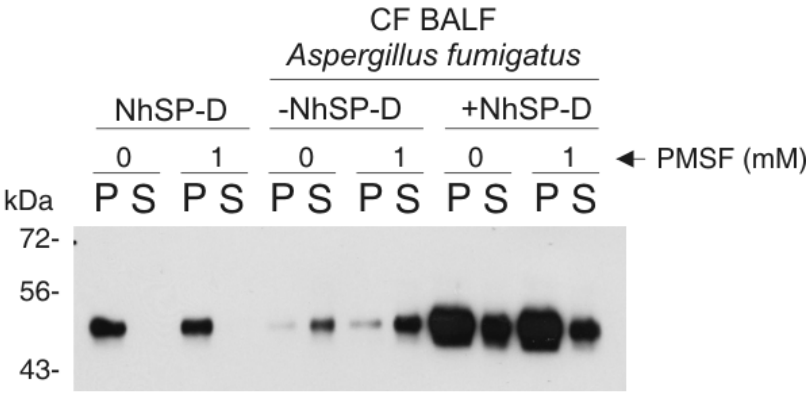


Figure 8

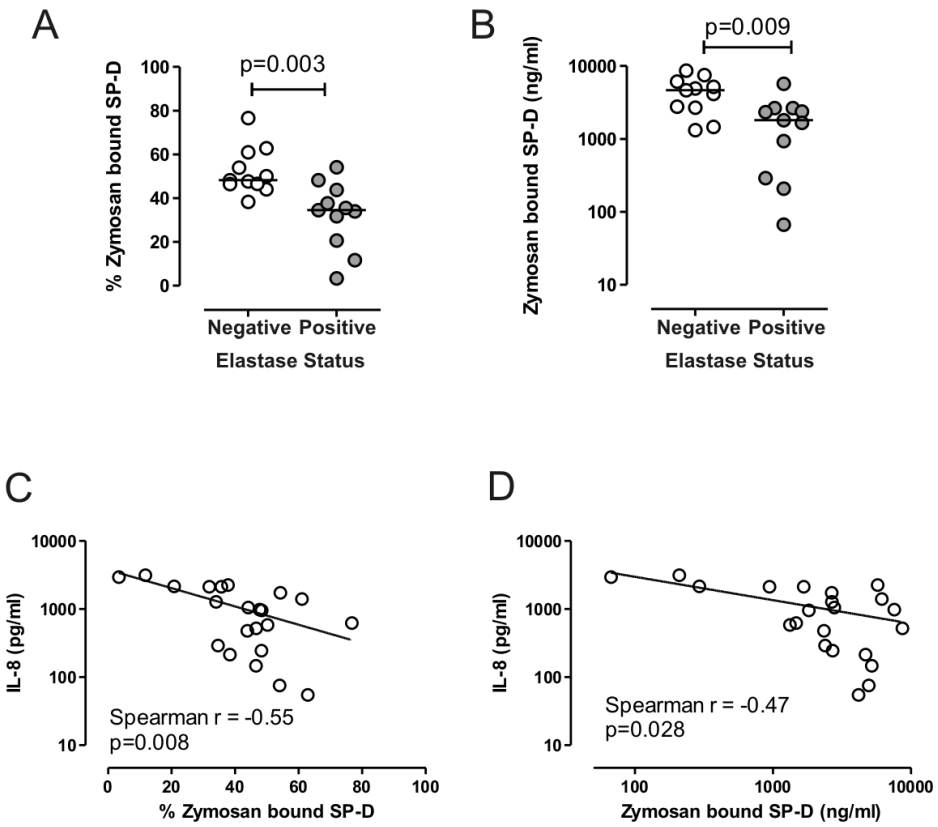


Figure 9

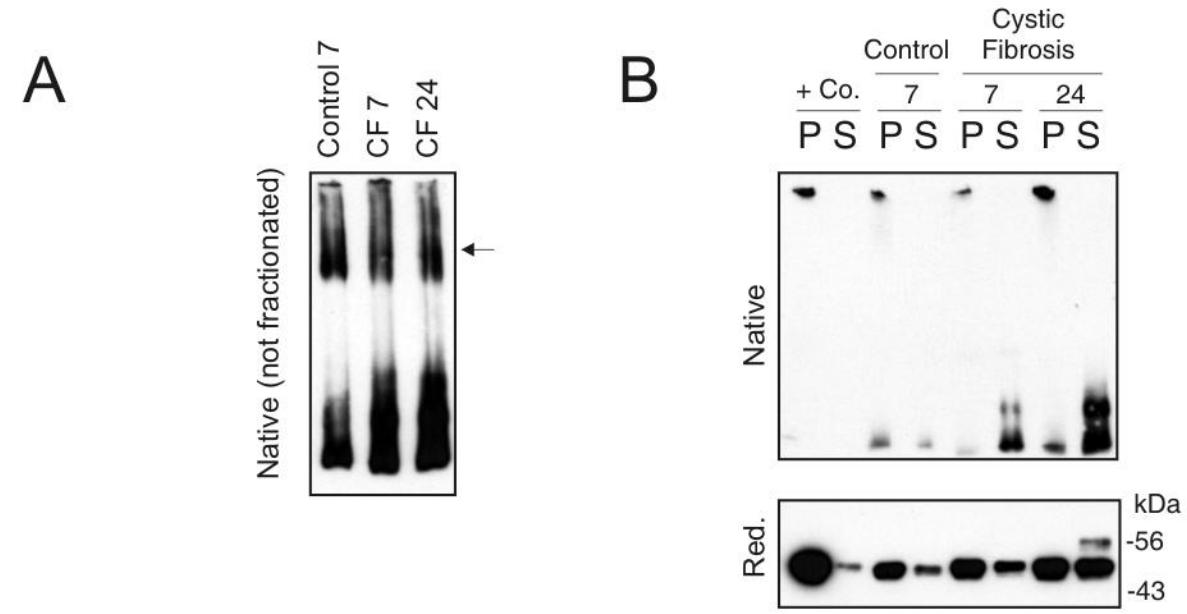
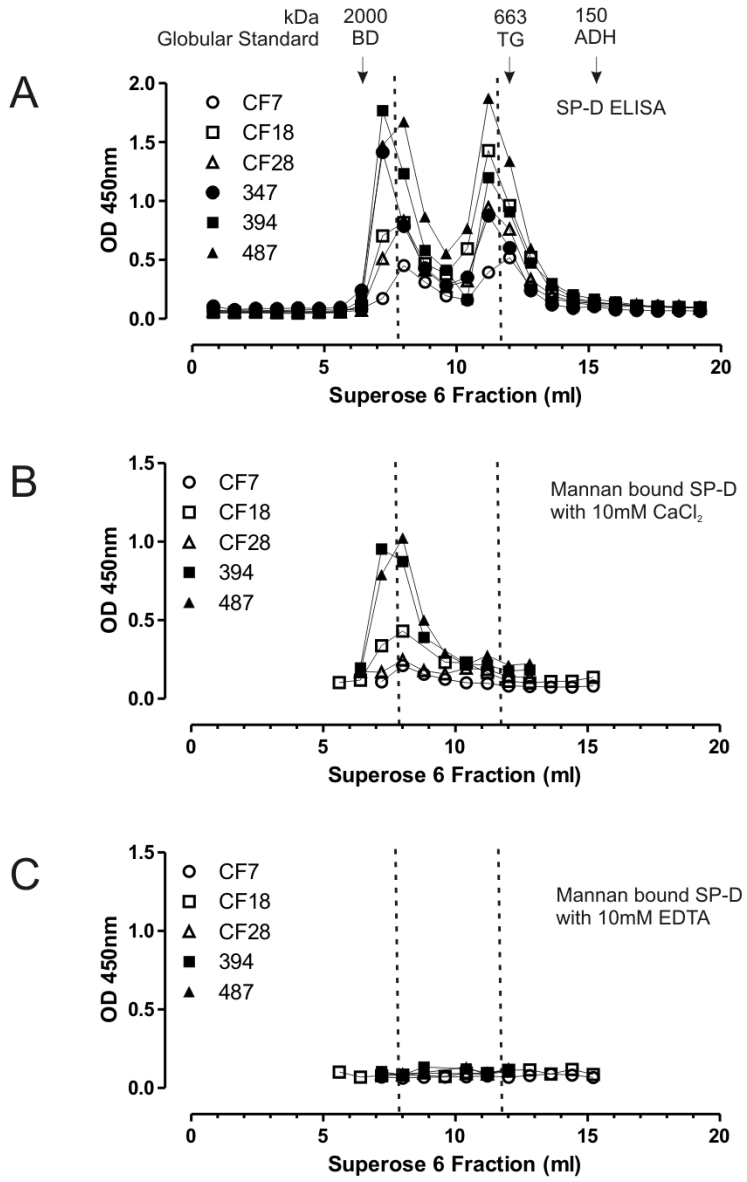


Figure 10



FUNCTIONAL HETEROGENEITY OF PULMONARY SURFACTANT PROTEIN-D IN CYSTIC FIBROSIS .

Highlights

1. SP-D is abundantly expressed in CF lung fluid
2. SP-D in CF lung fluid is structurally and functionally heterogenous
3. SP-D expression and function correlates negatively with infection and inflammation
4. Oligomeric form is a major determinant of SP-D function in CF and control patients