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Human SP-A- genes, structure, function- and lung diseases

Von

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aus

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For my Familiy and Friends

Erklärung

Diese Dissertation wurde im Sinne von §13 Abs. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. Griese betreut und von Herrn Prof. Dr. Alexander Pfeifer vor der Fakultät für Chemie und Pharmazie vertreten.

Ehrenwörtliche Versicherung

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Abbrevations

A	**Alanine**
A	**Adenosine**
aa	**Amino acid**
AFM	**Atomic force microscopy**
$AgNO_3$	**Silver nitrate**
AM	**Alveolar macrophages**
AP	**Alveolar Proteinosis**
APS	**Ammonium persulfate**
BAL	**Bronchoalveolar lavage**
BMI	**Body mass index**
Bro	**Chronic bronchitis and asthma**
BSA	**Bovine serum albumin**
C	**Control**
C	**Cytosine**
Ca	**Calcium**
CF	**Cystic fibrosis**
CRD	**Carbohydrate recognition domain**
D	**Aspartatic acid**
ddNTP	**Dideoxynucleotide**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide**
E	**Glutamic acid**
EDTA	**Ethylenediaminetetraacetic acid**
ELISA	**Enzyme linked immunosorbent assay**
Fev1	**Forced expiratory volume in one second**
FL	**Fluorescine**
G	**Guanine**
GOLD	**Global initiative for chronic obstructive lung disease**
HCl	**Hydrochloric acid**
H_2SO_4	**Sulfuric acid**
HS	**Healthy subjects**
IgG	**Immunoglobulin G**
K	**Lysine**
L	**Leucine**
LC	**Light cycler**
M	**Methionine**
MALDI-TOF	**Matrix assisted laser desorption/ionisation - time of**
$MgCl_2$	**flight**

min	**Magnesium chloride**
N	**Minute**
Na_2CO_3	**Asparagine**
$Na_2S_2O_3$	**Sodium Carbonate**
NaOH	**Sodium sulphite**
NCF	**Sodium hydroxide**
P	**Non-cystic fibrosis**
P.aer.	**Proline**
PAGE	***Pseudomonas aeruginosa***
PCR	**Polyacryl-amide-gelelectrophorese**
PPR	**Polymerase chain reaction**
Q	**Pattern recognition receptor**
R	**Glutamine**
s	**Arginine**
S	**Second**
SAP	**Serine**
SDS	**Shrimp alkaline phosphatase**
SFTPA	**Sodium dodecyl sulfate**
SFTPD	**SP-A gene**
SNP	**SP-D gene**
SP-A	**Single nucleotide polymorphism**
SP-D	**Surfactant protein A**
T	**Surfactant protein D**
T	**Threonine**
Taq	**Thymine**
TBS	***Thermophilus aquaticus***
TBS-T	**Tris-buffered saline**
TEMED	**TBS with TWEEN**
TLR	***N,N,N',N'-tetramethylethylenediamine***
TMB	**Toll like receptor**
UTR	***3, 3', 5, 5'-tetramethylbenzidine***
V	**Untranslated region**
W	**Valine**
WHO	**Tryptophan**
	World health organisation

1) Introduction

1.1) The surfactant

Pulmonary surfactant covers the alveoli and terminal airspaces as a complex film made of lipids (90% by mass) and proteins. The vital biophysical functions of surfactant are known for a long time and this knowledge has been very successfully translated into novel therapeutic options, e.g. exogenous surfactant therapy of neonatal and adult respiratory distress syndrome (1). The anti-microbial and immunomodulatory properties of surfactant have also been noticed for a long time (2-5), however the precise molecules and their functions involved have only been recently unravelled.

On average we inhale with every second or third breath one micro-organism into the lungs (6) Thus there must be a reliable defence system in the airways and alveoli that can eliminate incoming micro-organisms, but also prevent a significant inflammatory reaction which would destroy the lungs pivotal function of gas exchange.

Pulmonary surfactant is synthesized by alveolar type II cells and stored as intracellular inclusion organelles called 'lamellar bodies'. Then it is secreted into the airspaces where complex structures like tubular myelin and mono- and multilayer films are built (7,8) (Fig. 1). The major surfactant associated proteins surfactant protein A (SP-A) and SP–D are hydrophilic. These are known to play a crucial role in pulmonary immunity (9-12). The surfactant proteins SP-B and SP–C are very hydrophobic proteins. They are closely linked to alveolar surface-tension, phospholipid packaging and organization of surfactant structure.

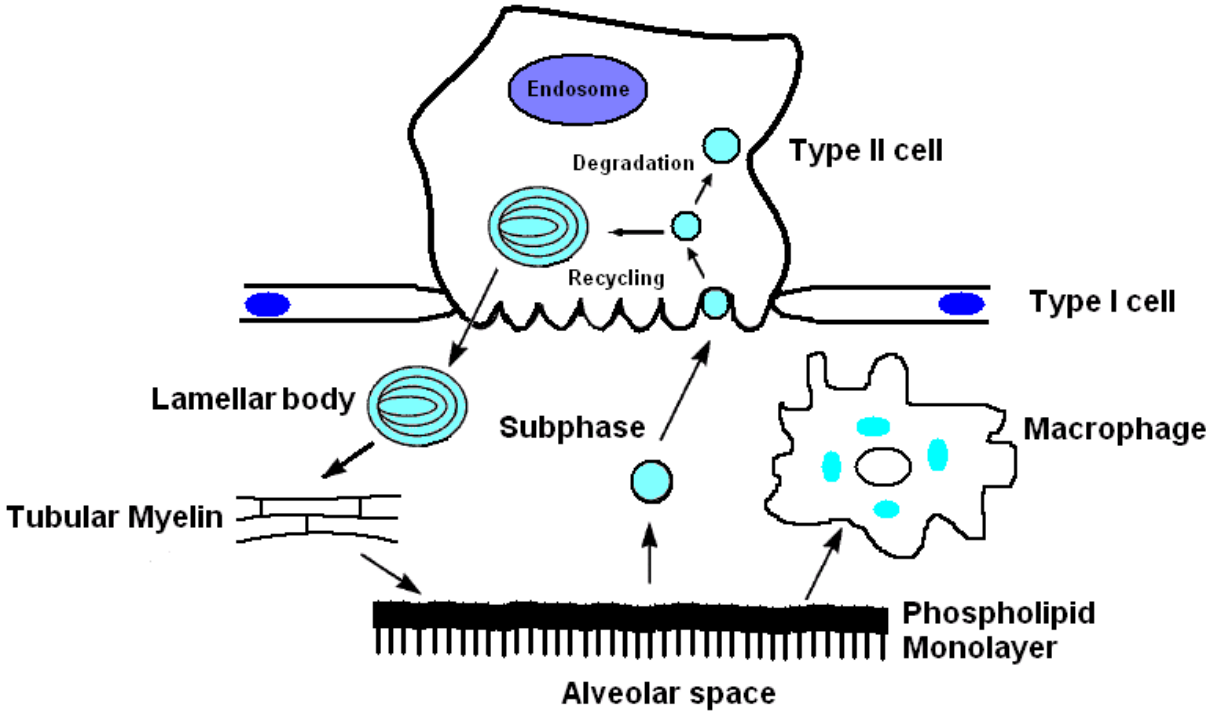


Figure 1.1.1**: Surfactant synthesis.** The pulmonary surfactant (phospholipids and proteins) is synthesized by the type II alveolar cell and stored as lamellar bodies. After secretion into the alveolar space, lamellar bodies develop tubular myelin. Used surfactant is recycled or degraded by the type II alveolar cells (90% under normal conditions) and alveolar macrophages.

1.2) Surfactant protein A

1.2.1) SP-A gene structure (*SFTPA1/2*)

The human SP-A-gene locus consists of two functional genes, *SFTPA1* and *SFTPA2*, and a pseudogene (Ψ) and is located on chromosome 10q22-q23. 1. The *SFTPA1* and *SFTPA2* genes are inversely orientated (Fig. **1**) and expressed independently (13). Each functional gene stretches across 4. 6 kb and consists of four coding exons (1-4) and three non-translated exons (A-C) in the 5' untranslated region (UTR) (Fig. **2**) (14). The pseudogene contains besides exon 4 of the *SFTPA* genes a large number of highly repetitive sequences and is not translated. From the primary transcript arise through different splicing of the exons A-D different mRNAs (15), but these mRNAs lead to similar protein sequences. The leading sequence of the mRNA consisting of parts of the untranslated exons A-D influences the expression rate or the mRNA stability. The major transcripts are the AD' type for SP-A1 and the ABD and ABD' types for SP-A2. The D and D' splice sites differ by three nucleotides with D' being shorter (16). These SP-A transcripts are translated *in vitro (17)* and *in vivo (18)*.

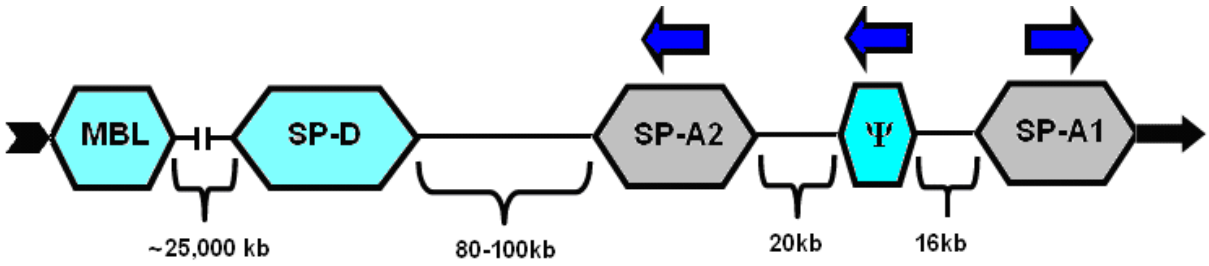


Figure 1.2.1.1: **Gene Locus SFTPA and SFTPD.** The orientation of the SP-A-genes is shown. The arrow (→) marks the direction of transcription (according to (19)). MBL stands for mannose-binding-protein, Ψ for the pseudogene.

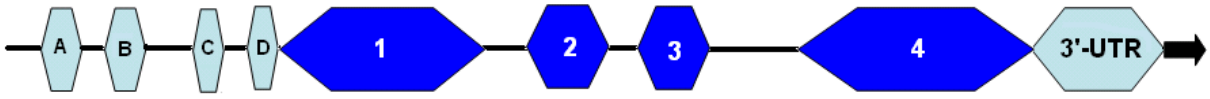


Figure 1.2.1.2: **Gene structure SP-A.** Intron-exon-structure of a SP-A-gene is demonstrated. Coding exons 1-4 are highlighted [according to (20)]. 3'-UTR stands for the 3' untranslated region.

1.2.2) SP-A mRNA

A number of studies using human fetal lung explant culture have investigated the response of SP-A protein and its mRNA to a variety of stimulatory and inhibitory regimens. Of particular interest was the observation of Ballard and co-workers (21) that there was a considerable variability in the amount of

both SP-A and its message among different specimens. The observed variability in the level of SP-A mRNA among specimens may have been a reflection of differences in SP-A genotypes of the fetal lungs used in their experiments. The results of those studies show that, in general, the protein level tends to follow the mRNA level (22,23). For example, the response to glucocorticoids of both SP-A and its message is biphasic, showing increased expression at low concentrations of glucocorticoids and inhibition of expression at high concentrations (24,25). The lung levels of total SP-A mRNA varies remarkably among adult individuals (26). The ratio of SP-A1:SP-A2 mRNA among individuals differs from the initially assumed (27) ratio of 2:1 (28). The majority of individuals studied by Karinch *et al*. had a SP-A1/SP-A2 ratio greater than one examining their lung tissues (29), indicating an excess of SP-A1 mRNA.

1.2.3) SP-A Protein Structure

The gene products of both functional genes are propeptides containing 248 amino acids. After separation of the signal sequence, monomers of 228 amino acids evolve (30). The amino acidic sequences of SP-A1 and SP-A2 are homologous by 98% (31). The proteins deviate from each other by 6-10 residues (32). At the amino acidic positions 9, 19, 66, 73, 81 and 83 they differ constantly and at the positions 50, 91, 219 and 223 there are polymorphisms which cause similar or different amino acids at these positions. Four domains can be differentiated in the primary structure (33). The N-terminus is followed by the collagen-like region which is linked to the globular head by the neck region (Fig. **3**). The intermolecular disulfide bonds at the N-terminal end enable the aggregation of trimers to higher oligomers (Fig. **4**). In this formation SP-A participates in the regulation of the surfactant metabolism and phospholipid aggregation (34,35). The collagen-like domain is recognized by the collectin receptor C1q (36). The neck region arranges the trimer association of the globular domain and influences the binding to phospholipids and the bacterial lipid A (37). Aggregates are formed with lipids in the presence of calcium ions (38). Tubular myelin is a characteristically organized lipid aggregate formed in the presence of SP-A, calcium ions, SP-B and phospholipids that accelerates the development of the surface active film at the air-water interface in the lungs (39). The globular domain contains the recognition site for carbohydrates (CRD). Besides its interaction with microorganisms, lectins or other molecules with attached sugar sequences, the CRD is also a ligand for phospholipids (40), macrophage membrane proteins (41) and the calcium-dependent binding and endocytosis of SP-A by type-II pneumocytes (42).

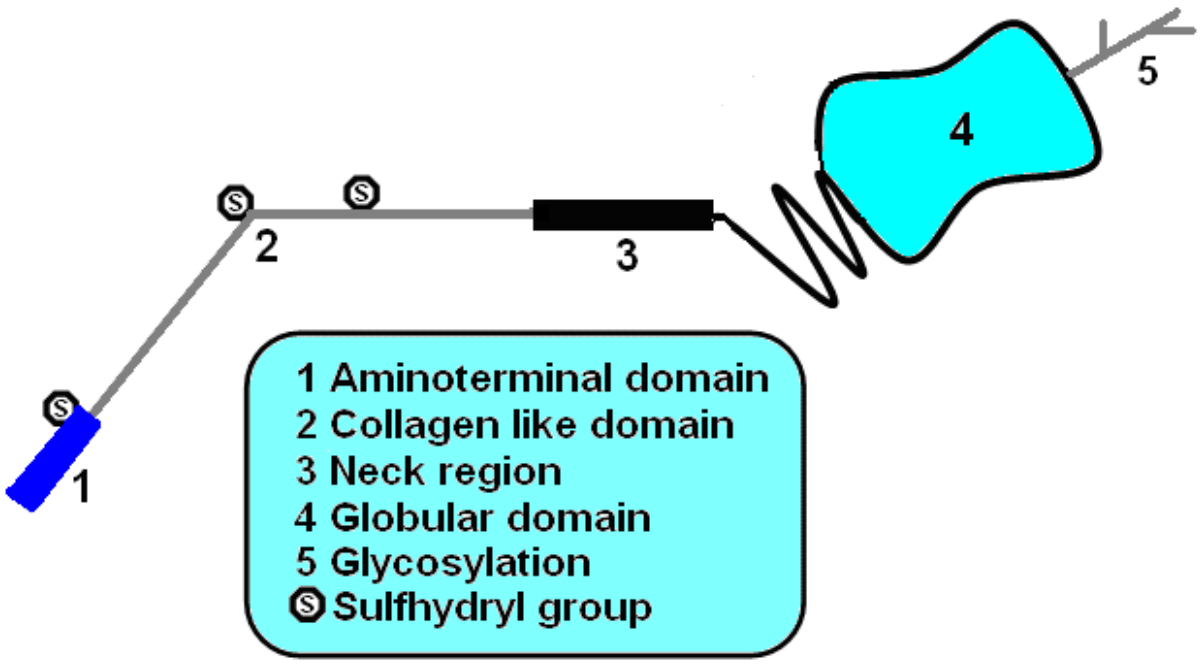


Figure 1.2.3.1: **Schematic representation of the primary protein structure of SP-A monomers**. Free sulfhydryl and hydroxyl groups are involved in forming intermolecular bonds during oligomerization (see Fig. 1.2.3.1).

1.2.4) Molecular Structure and Sites of Expression

SP-A belongs to a family of innate host defence proteins termed collectins because of the presence of a collagenous and a lectin-like domain (11). In the lungs, SP-A is the most abundant surfactant protein by weight. The SP-A monomer consists of 228 amino acids and has a molecular weight of 26-32 kDa. The three dimensional structure is shown in Fig. **6**. Different post-translational modifications are responsible for this molecular range. SP-A is glycosilated at position 187 to enable additional interactions with viruses and mycobacteria as well as with other SP-A molecules (43-45). But the lack of glycosylation does not affect either the collagen domain stability or conformational changes induced by calcium in the globular domain (46). SP-A accumulates *in vivo* predominantly as octadecamers composed of six trimeric subunits forming a flower bouquet-like structure (47,48). SPD is also a hydrophilic surfactant protein belonging to the family of collectins. Recent studies showed that in contrast to SP-A, SP-D monomers preferentially assembly as dodecamers composed of four trimeric subunits forming a cruciform structure (49,50).

Assuming that mRNA levels in the lungs reflect protein levels, the variable SP-A1:SP-A2 mRNA ratio indicates that single gene products may exist. These can form functional homotrimers and/or homooligomers. The extra cysteine of SP-A1 at position 85 can be involved in the formation of a SP-A intertrimeric or intratrimeric disulfide bond. It may account, in part, for differences in the oligomerization pattern between SP-A1 and SP-A2 variants (51). The majority of SP-A2 oligomers

exist in the dimer and trimer forms, whereas the majority of SP-A1 were found in larger molecular forms (e. g. trimers, hexamers) (52).

Human SP-A mRNA is expressed in alveolar epithelial type II cells (53), Clara cells (54), in tracheal and bronchial submucosal gland cells, in breast (55) and Eustachian tube epithelium (56), peritoneum, synovium (57), small and large intestine (58), colon, salivary gland, prostate, pancreas and thymus (59). In human tissues other than lung, except for vaginal mucosa, peritoneum, synovium and Eustachian tube epithelium, only SP-A mRNA was found and no protein expression could be detected (60,61). Recently, Lee *et al*. found SP-A mRNA and protein expressed in paranasal sinus mucosa (62). Functional SP-A is involved in various aspects of surfactant biology, for example constitution of tubular myelin and innate immunity (63-67).

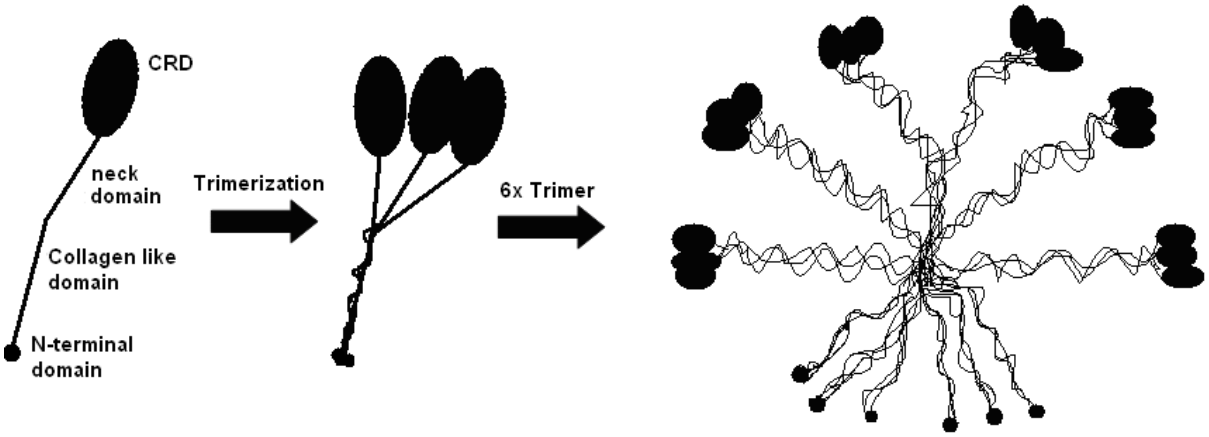


Figure 1.2.3.1**: Oligomerization of SP-A.** Three polypeptide chains assemble to trimers. For SP-A those can be hetero- and homotrimers concerning SP-A1 and -A2. Up to six SP-A trimers form the characteristically bouquet-of-flowers.

1.2.5) SP-A variants

1.2.5.1) SP-A Polymorphisms and Genotypes

Since the decryption of the *SFTPA* DNA-sequences many single base substitutions have been detected (68,69). For *SFTPA1* are 54 and for *SFTPA2* are about 80 substitutions listed in the SNP database of the University of California. Sequence variability within the coding region of both genes results in a number of allelic variants for each gene (70,71). These SP-A alleles have been denoted as $6A^n$ for the exons of the SP-A1 gene and as $1A^n$ for the SP-A2 gene (72).

Tables 1.2.5.1.1 and 1.2.5.1.2 show the relevant polymorphisms of SFTPA 1/2 to determine the individual alleles separated for each gene (73,74). Populations differ in frequencies of these alleles (75). This is demonstrated by the comparison of the American and Finnish population's haplotype frequencies, as illustrated in Tables 1.2.5.1.1-1.2.5.1.3. Five SP-A1 alleles (6A, $6A^{2\ 5}$) and six SP-A2 alleles (1A, $1A^{0\text{-}3}$, $1A^6$) occur at a generally moderate frequency in the populations studied, whereas

the remaining more than 30 alleles described yet, are found less frequently (74,76). Previously, only a small number of single nucleotide polymorphisms (SNP) were used to determine the alleles of the genes (74,77).

Table 1.2.5.1.1: **Alleles of SP-A1**

Allele	**Amino acid exchange**										
	N9T	**V19A**	**H39H**	**V50L**	**P62P**	**P91A**	**T133T**	**S140S**	**Y184Y**	**Q223K**	**Allele frequency**
6A	a	c	c	c	g	c	g	c	t	c	0.04 (Fi) 0.09 (US)
6A^2	a	t	c	g	a	c	a	c	t	c	0.60 (Fi) 0.54 (US)
6A^3	a	t	c	c	a	c	a	c	t	c	0.28 (Fi) 0.28 (US)
6A^4	a	t	c	c	g	c	a	c	t	c	0.09 (Fi) 0.09 (US)
6A^5	a	t	t	c	a	c	a	c		c	<0.01 (Fi)

The table shows the alleles of the SP-A1 gene named 6A-6a^5 and their frequency in different populations (Fi: Finnish population; US: American population). The alleles are defined by genotypes (bases:a, c, g, t) concerning specific polymorphisms, e.g. N9T which means an amino acidic exchange from asparagine to threonine at position 9 of the protein sequence caused by a single base polymorphism in the DNA sequence.

Table 1.2.5.1.2: **Alleles of SP-A2**

Allele	**Amino acid exchange**								
	N9T	**V19A**	**V50L**	**P62P**	**P91A**	**T133T**	**S140S**	**Q223K**	**Allele frequency**
1A	c	c	g	g	c	a	c	c	0.04 (Fi); 0.12 (US)
1A^0	a	c	g	g	g	a	c	c	0.57 (Fi); 0.57 (US)
1A^1	c	c	g	g	g	a	t	a	0.16 (Fi); 0.22 (US)
1A^2	c	c	g	g	g	a	c	c	0.13 (Fi); 0.09 (US)
1A^3	a	c	g	g	g	a	t	a	0.03 (Fi); 0.08 (US)
1A^4	c				g		t	a	<0.01 (Fi)
1A^5	c	c	g	g	c	a	t	c	0.07 (Fi)
1A^6	c	c	g	g	g	a	t	c	<0.01 (Fi)

The table presents the same pattern as table 1a for the SP-A2 gene. The alleles are named 1A-1A^6 and the allele frequencies are shown of the same populations as for the SP-A1 gene (Fi: Finnish population; US: American population)

Table 1.2.5.1.3: **Haplotypes of the SP-A genes**

Frequency	6A/1A	$6A^2/1A^0$	$6A^2/1A^1$	$6A^2/1A^2$	$6A^2/1A^3$	$6A^3/1A^0$	$6A^3/1A^1$	$6A^3/1A^2$	$6A^4/1A^5$
Fi (74)	0.04	0.56	0	0	0.03	0.02	0.16	0.10	0.07
US (78)	0	0.33	0.1	0.07	0.02	0.16	0.05	0.04	0.01

Haplotypes are created by combining different alleles. The table displays some frequencies of such haplotypes in different populations. Fi stands for Finnish population (74), US for American (79).

A number, but not all of polymorphisms for each SP-A gene are listed in the database of the University of California, Santa Cruz (UCSC). Some of those SNPs are not validated by population studies, e.g. S140S and P62P (for SP-A2) as you can see in Table A.1 in the appendix. Therefore it remains unclear whether one can extrapolate these data based only on a few subjects, to populations.

1.2.5.2) Allelic Variants and mRNA

The different alleles and their combinations result in different mRNA splice variants which differ in their stability and expression levels. The haplotype $6A^2/1A^0$ was shown to be associated with low to moderate levels of mRNA (80). The $6A^3$ allele-derived mRNA was more prone to the destabilization effect of glucocorticoid treatment than $6A^2$ or $1A^0$ derived mRNA in a cell culture model (81). Both alleles, $1A^0$ and $1A^1$, are also associated with the splice variant ABD, whereas 1A and $1A^2$ are associated with ABD' (82). Therefore the leader sequences resulting by those splice variants could be responsible for the different outcome of the mRNA. The SP-A2 splice variant ABD has a higher activity, higher mRNA stability and higher translation efficiency compared to SP-A1 variants (83). Five structural elements near the 5'-end of the mRNA determine the translational efficiency (84,85). These are *1*) the m7G cap, *2*) the primary sequence or context surrounding the AUG codon, *3*) the presence of the AUG codons in the 5'-UTR, *4*) the secondary structure of the mRNA, and *5*) the 5'-UT leader length. It is likely that the composition and/or the length of the leader sequence play an important role in the translation efficiency and may account for efficiency differences among hSP-A 5'-UTR splice variants. The most frequently observed SP-A2 variants (ABD and ABD') have a longer leader sequence (ABD and ABD' are 100 and 97 nt, respectively) than SP-A1 (A'D') variants (62 nt). Other less frequently found variants with even longer leader sequences, for example AB'D' (137 nt), exhibit translational efficiency equivalent to A'D' or lower (86). These observations support the notion that the leader sequence composition is more important than simply the length of this sequence. It is speculated that the sequence composition of exon B contributes to an enhanced activity of the ABD variant, *via* binding of positive regulatory factors to exon B recognition sites (87). A previous study has implicated possible regulatory AP-1-like binding sequences within intron 1 and between exons A and B of human SP-A1 and SP-A2 (88). Whether the AP-1 site in exon B' plays a role in the regulation of SP-A concentrations remains to be determined.

1.2.5.3) Alleles and Protein Function

The amino acids 215glu, 222glu, 234asn and 235asp are involved in mannose binding of SP-A with the participation of calcium ions (89). In line with this, functions of the CRD, i. e. the binding of SP-A to type-II-pneumocytes, the regulation of surfactant metabolism and the ability to lipid aggregation, may be prevented by exchanging one of these amino acids to alanine (90). In addition, a number of the variant amino acids are located near to the mannose-binding sites. For example, in the SP-A1 allele 6A4 (arg219trp) is the alkaline arginine replaced by a hydrophobic tryptophan. This may also affect the functions of the CRD. The affinity of binding of SP-A to the collectin receptor of phagocytes can be influenced by the substitution of the val50leu located in the collagen like domain of the SP-A molecule. When in the twenty-first collagen triplet of the allele 1A variant the amino acid proline is encoded instead of alanine (ala91pro), an amino acidic sequence is generated in 1A as regularly observed for SP-A2 alleles. During processing hydroxylated prolines stabilize the triple-helix by hydrogen bonds (91). Proline hydroxylation affects also both the arrangement of disulfide bonding and the extent of oligomerization, but not conformational changes in the globular domain (92). Therefore the SP-A1 protein resulting from the 1A allele is more stable during processing than the other SPA1 variants (93). *In vitro* expressed SP-A1 and SP-A2 allelic variants were recently tested for their ability to enhance phagocytosis in rat alveolar macrophages. Generally, the SP-A2 variants were more effective than SP-A1 variants (94). Although the $1A^0$ variant exhibited higher activity compared with 1A, the coexpressed variant $1A^0/6A^2$ was less active than the $1A/6A^4$. This can be explained by the activity level for these coexpressed variants being modulated by the SP-A1 variant present in the SP-A1/SP-A2 oligomer (95). In conclusion, genetic SP-A variants result in different SP-A protein concentrations and different functional properties of the protein. A major goal is to link those variants to specific advantages or disadvantages with respect to disease related issues in humans. For this purpose many *in vitro* studies and mouse models have been generated which will provide new insights into the functional differences between SP-A1 and SP-A2.

1.2.6) SP-A *in vitro* studies

Most of the *in vitro* studies have been performed with human SP-A isolated from bronchoalveolar lavage fluid (BALF) of patients with *pulmonary alveolar proteinosis* (PAP). *Alveolar proteinosis* is a condition of greatly enhanced accumulation of surfactant components in the alveolar space. A few studies examined murine SP-A. Of interest, so far no studies have taken the different gene products, SPA1 and SP-A2, or other protein variations, into consideration.

1.2.6.1) SP-A and Pathogens

SP-A is a member of the mannose-type subfamily of C type, i.e. calcium-dependent, lectins. As such it can bind with its CRD in its globular domain (Fig. 1.2.3.1) to mannose and glucose, and shows little interaction with galactose (96). Most microbial ligands naturally contain these or adequate saccharides

on their surface. SP-A can bind to di-mannose-repeating units present in certain Gram-negative capsular polysaccharides of Klebsiella bacteria (97). Moreover, SP-A shows CRD dependent binding to the N-linked sugars of specific cell wall glycoproteins, including major glycoprotein allergens of *Aspergillus fumigatus* (98). It also binds in a CRD-dependent manner to gpA, a heavily mannosylated glycoprotein associated with the trophozoites and cysts of *Pneumocystis carinii* (99,100). As an exception, some interactions of SP-A with viruses require the binding of viral lectins to the complex oligosaccharides on SP-A molecules (101). On the other hand SP-A can interact with lipids through its collagen like domain. SP-A binds to dipalmitoylphosphatidylcholine (DPPC), the major surfactant phospholipid (102,103), the lipid A domain of gram-negative LPS (104,105) and to several glycolipids and neutral glycosphingolipids (106-108). Furthermore SP-A was found to have direct effects on the survival of Gram-negative bacteria through mechanisms leading to increased permeability of the bacterial cell membrane (109). Exposure of the facultative intracellular fungal pathogen *Histoplasma capsulatum* to SP-A resulted in increased cell permeability and enhanced killing of the pathogen (110). These results suggest that SP-A plays a crucial role in the host defence.

1.2.6.2) SP-A and Immune Cells

In vitro data show that SP-A affects a variety of immune cell functions (reviewed in (111-113)). Within the alveolar space of the lungs, the abundantly present surfactant lipids have an inhibitory effect on immune cell function, whereas SP-A may have both stimulatory (114) and/or inhibitory effects (115). SP-A binds pathogen associated molecular patterns as described above by its globular head domain (CRD) and at the same time initiates phagocytosis through presentation of its collagen like domain to phagocytes. This effect is supposed to be mediated *via* receptors, e. g. cC1q, and activation of NFκB. Alternatively, if there are only a few pathogens present, the globular head domain interacts with the phagocytes, leading to an inhibition of NFκB. The latter effect is mediated *via* SIRPα thereby activating the tyrosine phosphatase SHP-1, with downstream blockade of signalling through src-family kinases and P38 map kinase, leading to a suppression of proinflammatory mediator production. It is assumed that SP-A acts in a dual manner, to enhance or suppress inflammatory mediator production depending on binding orientation illustrated in Fig. **5** (116). It appears that in the normal lung a fine-tuned balance exists between inhibition and stimulation of immune cells through the maintenance of an appropriate ratio of the various surfactant components (117,118). For instance, SP-A has been shown to enhance the uptake of a variety of bacteria by both alveolar macrophages and monocytes (reviewed in (119,120)). Additionally SP-A can act indirectly as an activation ligand to stimulate the phagocytosis of microorganisms (121). In this case, binding of SP-A to the cell but not the pathogen is required. The pathogen may be opsonized by another ligand, for instance IgG, or may bind directly to the phagocytic cell *via* a cell surface receptor such as the mannose receptor. SP-A has also been reported to inhibit the phagocytosis of some pathogens including *Pneumocystis carinii* (122) and *M. tuberculosis* (123). However, other studies found that SP-A enhanced the uptake of both organisms (124-126). Among other factors, such differences may be related to the different SP-A preparations used in the studies.
Many other functions and properties of various immune cells have been demonstrated to be differentially influenced by SP-A (reviewed in (127)). For example, SP-A stimulates the directional

actin polymerization and the chemotaxis of alveolar macrophages (128), but not the migration of monocytes. The production of cytokines in response to the inflammatory bacterial cell wall component LPS (129), intact *Candida albicans* (130), and to both intact bacteria and LPS, is inhibited by SP-A in the system of mixed buffy-coat cells (131). In alveolar macrophages, SP-A has been shown to directly enhance the production of tumour necrosis factor α and granulocyte-macrophage-colonystimulating factor (132,133). Similarly in human peripheral blood mononuclear cells, SP-A increased the production of tumour necrosis factor α, interleukin (IL) -1β, IL-6, IL-8 and interferon-γ (134). Moreover the production of reactive oxygen and nitrogen species by alveolar macrophages and monocytes can be modulated by SP-A (135-137). Neutrophils are responsive to SP-A *in vitro* as well. Thereby, SP-A enhances the phagocytosis of *E. coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* by neutrophils (138), the killing of *Aspergillus fumigatus* conidia and also neutrophil migration (139). In eosinophils, Cheng and co-workers observed that SP-A suppresses ionomycin-induced production of IL-8 (140), suggesting that SP-A modulates the inflammatory activities of these cells in response to allergen challenge. Further studies defined a role for SP-A in attenuating lymphocyte responses (141,142) and inhibiting dendritic cell maturation (143). Migration and maturation of dendritic cells result in their movement to the lymphoid tissue compartment where they can activate T cells and link the innate and the adaptive immune response (143).

In highly enriched populations of peripheral blood lymphocytes, SP-A inhibits plant lectin and anti-CD3 stimulated T-lymphocyte proliferation (144). Partially, this inhibition is secondary to the inhibition of the production of IL-2, a potent mitogen for lymphocytes. To conclude, SP-A acts as a potent modulator of immune cell functions and enhances and/or suppresses inflammatory mediator production (145). The direction of these modulating influences is determined by the presence or absence of microorganisms resulting in opposite physical presentation of the CRD or the collagen like domain of SP-A to immune cells.

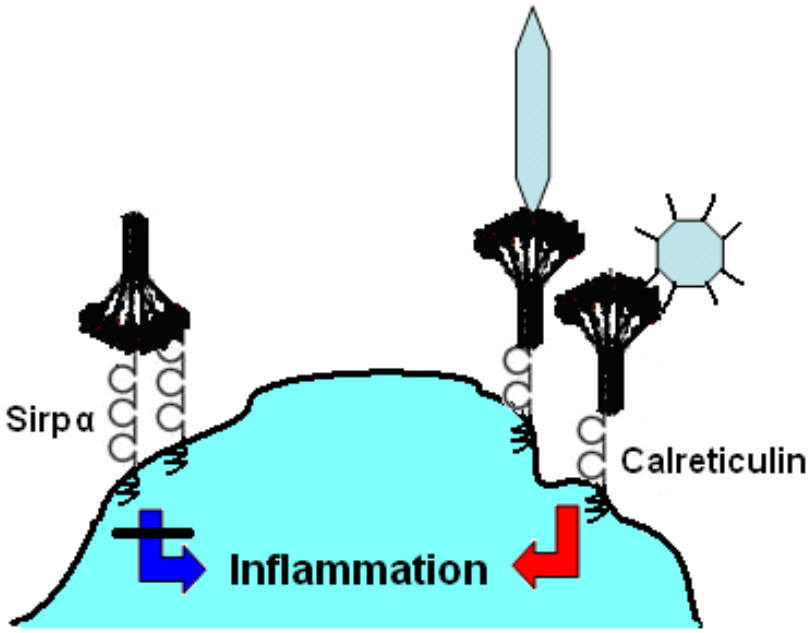


Figure 1.2.6.2.1: **Stimulation and inhibition of inflammation by SP-A.** SP-A acts in a dual manner, to enhance or suppress inflammatory mediator production depending on binding orientation (146). SP-A binds to SIRPα via their globular head domains to initiate a signalling pathway that blocks pro-inflammatory mediator production. In contrast, their collagenous tail stimulate pro-inflammatory mediator production via binding to calreticulin/CD91. SIRPα stands for signal inhibitory regulatory protein α, SHP for a protein tyrosine phosphatase, P38 for a map kinase, NFkB for a nuclear factor and CD91 for a heat shock protein receptor.

1.2.7) Mouse Models

Under resting conditions, the SP-A knock-out mouse reveals normal surfactant homeostasis and respiratory function, although the surfactant aggregates lack tubular myelin figures (147). A modest increase in the surfactant pool size was shown in 8-week-old SP-A (-/-) mice compared with their wild-type counterparts (148). On the other hand, SP-A deficient mice have a significant defect in host defence (149). The SP-A (-/-) mice show delayed microbial clearance of intratracheally administered group B *Streptococcus* (GBS) (150), *Haemophilus influenza (151)*, respiratory syncytial virus (RSV) (152), and *Pseudomonas aeruginosa (153)*. An increased susceptibility to *Pneumocystis carinii* is also reported (154). The uptake of these microbes by alveolar macrophages in SP-A (-/-) mice is significantly reduced. In addition, decreased production of superoxide and hydrogen peroxide by alveolar macrophages in GBS and RSV infections are observed in SP-A (-/-) mice (155). Furthermore, SPA (-/-) mice had increased alveolar protein levels after four days of hyperoxia, but pressure-volume curves and the amount of alveolar protein were similar compared to SP-A (+/+) mice (156). After exercise, both groups showed no alterations of the amount of alveolar saturated phosphatidylcholine (157). This indicates that no functional deficits develop through SP-A dependent alterations in surfactant. The surface tension-lowering properties of surfactant from the SP-A (-/-) postnatal pup are compromised when the concentration of surfactant components (e. g. lipids) or other molecules (e. g. Ca^{2+}) are suboptimal (158). In the full-term or postnatal SP-A (-/-) pup the composition of surfactant components is similar to that of the wild-type. Mice with sequential targeted deficiency of SP-A and SP-D develop progressive alveolar lipoproteinosis and emphysema (159). These doubly deficient mice indicate a functional overlap between SP-A and SP-D in regulating the surfactant homeostasis. SP-A overexpressing mice showed no alteration of thedistribution of the surfactant phospholipid between the air space and lung tissue, although alveolar levels of SP-A were as much as 7-to 8-fold higher in transgenic mice than in controls (160). Furthermore, overexpression of SP-A does not appear to disrupt lung function, since the transgenic mice show normal exercise tolerance, lung compliance, and *ex vivo* surfactant function. Additionally surfactant isolated from the transgenic mice is more resistant to protein inhibition than that from control littermates in the presence of protein inhibitors (161).
The results show a predominant role of SP-A for host defence and immunologic processes of the lungs. Moreover, SP-A is of importance for the biophysical surfactant functions especially during increased pulmonary stress.

1.2.8) SP-A receptors

An important task has been the identification of specific receptors for SP-A and SP-D. Both proteins bind to a multitude of different ligands. A number of potential receptors have been identified including surfactant protein receptor 210 (SP-R210) (162,163), glycoprotein-340 (GP-340) (164,165), signal inhibitory regulatory peptide (SIRP-α) (166), Toll-like receptors, receptors for C1q, CD14/sCD14 (167), the complex of CD91 and calreticulin (168).
Future studies are required to define the mechanisms by which receptor expression is regulated and to determine whether specific cell types express different receptors under basal conditions.

1.2.8.1) CD14

Both SP-A and SP-D can directly bind CD14 (a major LPS receptor) as well as recombinant soluble CD14 (rsCD14). The binding of rsCD14, pre-incubated with SP-A, to smooth LPS is significantly reduced but the association of rsCD14 with rough LPS is augmented, suggesting different actions of SP-A upon distinct serotypes of LPS (169). Thus, a direct interaction of SP-A with CD14 constitutes a likely mechanism by which SP-A modulates LPS-elicited cellular responses. SPA binds the peptide portion containing the leucine-rich repeats of CD14 whereas SP-D binds the carbohydrate moiety of CD14 (170).

1.2.8.2) Calreticulin-CD91 complex

SP-A (via its collagen region), has been shown to interact with an approximately 60 kDa, Ca^{2+}-binding, multifunctional protein, called calreticulin (CRT), originally described by Malhotra et al. (171) as cC1qR or collectin receptor. Isolated as a 60 kDa acidic glycoprotein from U937 cells and human tonsil lymphocytes (and also shown to be present on leukocytes, platelets and endothelium) that formed dimers of 115 kDa when solubilised under non-denaturing conditions, this receptor molecule was shown to bind C1q via its collagen region (hence the name cC1qR) and related members of the collectin family-MBL, SPA and bovine conglutinin (thus the name collectin receptor) (172). The surface expression of cC1qR was also shown to be upregulated by interaction with SP-A (173). CRT, when bound to collagen region of C1q, MBL, SP-A and SP-D, uses the endocytic receptor CD91 as an adaptor molecule for phagocytosis and signal transduction (174,175). The role of CRT-CD91 in the clearance of apoptotic cells has been extended to SP-A when the collectin is engaged as opsonin via its lectin domain (176). Recently, the CRT-CD91 pair has also been shown to orchestrate SP-A mediated induction of pro-inflammatory cytokines (177). Based on a series of elegant experiments, Gardai et al. (178) have demonstrated that SP-A can initiate either proinflammatory or anti-inflammatory mechanisms, depending on the orientation of the molecule.

1.2.8.3) SIRPα

SP-A is a potent modulator of macrophage function and may suppress clearance of apoptotic cells through activation of the transmembrane receptor signal inhibitory regulatory protein alpha (SIRPα) (179). SP-A has been shown to opsonise apoptotic cells in vitro and to facilitate their phagocytosis by resting alveolar macrophages (AM) (180-182). This effect has been confirmed in vivo and is mediated through CD91 and calreticulin on the phagocyte surface (183). An opposing effect may be seen when SIRPa is activated by SPA. Ligation of this receptor blocks Fcg receptor and complement-mediated cellular phagocytosis (184,185). Thus, in the naive lung, binding of SP-A to SIRPα on the AM may tonically inhibit apoptotic cell engulfment, resulting in the inefficient uptake observed for these cells. During inflammation, this inhibitory effect may be lifted to facilitate apoptotic cell removal.

1.2.8.4) SP-R210

One receptor that has been characterized on the surface of macrophages and type II cells and found to bind SP-A even in the presence of a 100-fold excess of mannan (186). Polyclonal antiserum raised against this 210-kDa receptor (SP-R210) was also able to prevent SP-A from inhibiting phosphatidylcholine secretion by phorbol ester-treated type II cells and to inhibit the SP-A dependent uptake of bacillus Calmette-Guerin by macrophages (187). Mediated by this specific receptor SP-A inhibited T cell proliferation in vitro (188). The domain responsible is a segment of the collagen-like domain, so the CRD is not involved (189).

1.2.8.5) Toll like receptors

Toll like receptors (TLR) are important cell-associated and intracellular pattern recognition receptors (PRR). There are currently 12 identified mammalian TLRs, of which TLR2 and TLR4 are among the most widely studied and are considered the major transmembrane TLRs (190). TLR2 and TLR4 play roles in initiating immune responses against pathogens. TLR2 forms a heterodimer with TLR6 or TLR1 to recognize diacyl and triacyl lipopeptides, respectively. TLR2 binds to zymosan, a particle composed of yeast cell wall components and peptidoglycan, a component of the Gram-positive bacteria cell wall (191,192). SP-A has been shown to up-regulate certain PRRs, such as scavenger receptor (193) and the mannose receptor (194). SP-A also differentially regulates the expression of TLR2 and TLR4 during primary human monocyte differentiation into macrophages (195). Despite up-regulation of TLR2 expression, SP-A markedly diminishes the macrophage proinflammatory response generated by both TLR2 and TLR4 agonists (196). The underlying mechanism is related to altered phosphorylation of a central regulator of cellular function, Akt, as well as downstream intermediates in the MAPK pathway and the activation of NFκB (197).

1.2.9) SP-A and human lung diseases

Primary SP-A deficiency in humans has not been demonstrated yet. Similarly, mutations in SP-A1/2 which cause monogenic disease states have not been found. However, in various human lung diseases altered levels of SP-A have been observed in the lungs, i. e. BAL fluid, and also in serum (198). Extensive genetic analyses show correlations between certain SP-A alleles and altered risks for lung disease (199-202). Taken together these data suggest that SP-A acts as a modifier gene influencing the manifestation, progression or pattern of human lung diseases. Few of these studies presented below allow genotype/phenotype correlations. More knowledge is required on the exact relations between genetic variants of SP-A, protein levels, the macromolecular organization of SP-A and its function. In a wide range of paediatric and adult lung diseases, deviations of the concentrations and integrity of SP-A have been found. These include the *Acute Respiratory Distress Syndrome* (ARDS), patients with chronic lung diseases like *Cystic fibrosis* (CF), various interstitial lung diseases and others.

In the following, the human studies on SP-A are summarised and the most important issues are discussed.

1.2.9.1) Neonatal and Adult Respiratory Distress Syndrome

The composition of surfactant components in prematurely born infants that died from *Respiratory Distress Syndrome* (RDS) differs from that in term infants or in prematurely born healthy infants (203). Reduced levels of SP-A which slowly recover by and by are known for infants with RDS (204). There is an association of low levels of SP-A and a lack of tubular myelin in infants with RDS (205). Recently it was shown that the SP-A2 allele $1A^0$ occurs at a significantly higher frequency in white infants older than 28 weeks gestation and RDS (206). Reduced levels of surfactant components including SP-A and altered surfactant composition in infants with or at risk for RDS have been shown (207-209). In agreement with animal experiments, these data support a physiological role of SP-A for the surface tension lowering function of surfactant during RDS. Abnormalities in surface tension lowering properties or host defence functions due to secondary alterations of SP-A levels have also been implicated in the pathophysiology of the *Adult Respiratory Distress Syndrome (ARDS) (210-212)*. Specific genetic defects have not been identified. However, certain SP-A polymorphisms, e. g. SNPs determining the $6A^2$ allele, were identified as risk factors (213,214). Of note, SP-A levels are reduced in (bronchoalveolar lavage) BAL fluid (BALF) from ARDS patients (215). In these patients SP-A concentrations in sera are significantly elevated compared to healthy controls after the onset of ARDS (216). Leakage out of the alveolar space has been suggested as the responsible mechanism for this. Thus, serum levels of SP-A were proposed as disease activity or prognostic marker for ARDS and other severe lung injuries (217). In patients with ARDS different genotypic subgroups with respect to SP-A may be responsible for the susceptibility to lung injury, and this difference may have an impact on the success of various therapeutic modalities.

1.2.9.2) Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA), an allergic and inflammatory disorder of the airways, is caused by the pathogenic fungi *Aspergillus fumigatus*. A series of investigations by Madan *et al*. established that SP-A has an important role in strengthening the host defence against *A. fumigatus* and its allergic disorders (218,219). A significantly higher frequency of the AGA allele (A1660G) of SP-A2 was observed in patients with ABPA in comparison with controls.

1.2.9.3) Idiopathic Pulmonary Fibrosis

Derangement in pulmonary surfactant or its components are common observations in IPF. In patients with IPF, serum SP-A concentrations reflect the disease activity of IPF (220). The associations between IPF and genetic variants of SP-A1 and SP-A2 have been examined in a Mexican population (221). One SP-A1 allele ($6A^4$) was found in higher frequency in non-smoker and smoker IPF subgroups compared to healthy controls.

1.2.9.4) Pulmonary Alveolar Proteinosis

PAP is characterized histologically by intra-alveolar accumulation of large quantities of fine granular eosinophilic and periodic acid-Schiff positive material (222). SP-A levels in BALF in patients with PAP is increased in comparison with controls, but the ratio of SP-A to total protein in BALF is almost similar, while the ratio to phospholipid is higher (223). Therefore, measurement of SP-A levels could be used as a biochemical diagnostic tool in the clinical laboratory to detect PAP. But SP-A in PAP showed not-reducible intramolecular bonds which are not existent in SP-A from healthy subjects or other patient groups (224).

1.2.9.5) Cystic Fibrosis

SP-A is increased in BALF of infants and young children with *Cystic fibrosis* (CF) in the absence of pulmonary inflammation (225), whereas in older children and adults with CF SP-A is consistently decreased in BALF (226-228). Furthermore, proteolytic degradation of SP-A was shown in CF, supporting the hypothesis that inflammation may play a role in decreasing SP-A function in CF airways and that lower levels are not a primary abnormality associated with this disorder (229,230). Chronic airway inflammation caused by *Pseudomonas (P.) aeruginosa* is an important feature of CF. SP-A enhances the phagocytosis of *P. aeruginosa* in some (231), but not in all *in vitro* studies (232). A direct modification of the chance to acquire *P. aeruginosa* or to eliminate it from the CF lungs has not been demonstrated in humans so far. There is also a suggestive linkage of the fibrotic lung phenotype and regions on chromosome 10, where the SP-A genes are located *(233)*. A genome-wide linkage scan in CF knock-out mice shows an association between intestinal distress and a locus on chromosome 10

in male CF mice (234). A recent study showed an association between the haplotype $6A^3/1A^1$ and lower pulmonary function as well as poor pulmonary scores (235).

1.2.9.6) Asthma and chronic bronchitis

The possible involvement of pulmonary surfactant in the pathophysiology of respiratory diseases with a predominant disturbance in the conducting airways, such as asthma, has only recently been addressed (236). In patients with asthma the SP-A levels were found to be unchanged (237). Van de Graaf *et al*. (238) reported that BALF levels of SP-A were decreased in patients with asthma. Accordingly, it has been reported that mite-allergen-induced airway inflammation leads to decreased levels of SP-A and SP-D in BALF from sensitized mice (239). In contrast, Cheng and co-workers (240) found increased levels of SP-A in bronchial and alveolar lavages in mild, stable asthmatics compared with controls. The discrepancy of these findings might be due to different time points and methods of sampling of the lavage fluids, and requires further clarification. A very early step in the induction of allergic inflammation is allergen uptake by dendritic cells, antigen processing and subsequent antigen presentation to T lymphocytes. SP-A has been shown to bind to pollen grains (241). In addition, it has been demonstrated that SP-A interacts with mite allergens in a carbohydrate-specific and calcium-dependent manner (242). Moreover, SP-A was found to inhibit allergen-specific IgE binding to the mite allergens.

1.3) Surfactant protein D

SP-D belongs as SP-A to the family of C-type lectins called 'collectins' (243-245). The primary structures of both collectins are very similar and contain four regions, (i) a cysteine-containing N-terminus, (ii) a triple-helical collagen region composed of repeating Gly-X-Y triplets, (iii) an α-helical coiled coil neck region, and (iv) a globular structure at the C-terminus comprising the C-type lectin or CRD (246) (Fig. 2). Surfactant protein D is encoded by a single gene on chromosome 10 located near SP-A and other members of the collectin family (247). It forms multimers like SP-A, which increase its affinity to immune cells and pathogens (248). The SP-D gene (*SFTPD*) is translated into a protein consisting of 345 amino acids (249). These monomers form trimers and four trimers assemble to a cruciform structure (Fig. 1.3.1). As SP-D has the largest and most flexible collagen domain among the collectins, it forms networks with various bound organisms (250). Surfactant protein D is primarily expressed and secreted by type II alveolar cells but is also detected in Clara cells and in the tracheal and bronchial glands of the lower airways (251,252). Although SP-D was first identified in the respiratory tract, current studies demonstrate the expression of SP-D in almost all mucosal surfaces, including epithelial cells in exocrine ducts, the mucosa of the gastrointestinal and genitourinary tract and recently in human tear fluid, protecting the eyes against *Pseudomonas aeruginosa* invasion(253-255). SP-D knock-out mice exhibit an enhanced pulmonary Th2 response, leading to a predisposition

of allergic dysregulation and inflammation (256). These data clearly show that despite many structural similarities between the two proteins, their roles in physiology are distinct. Therefore we will discuss all structural and functional data separately for each of the proteins to facilitate the understanding of their differential roles.

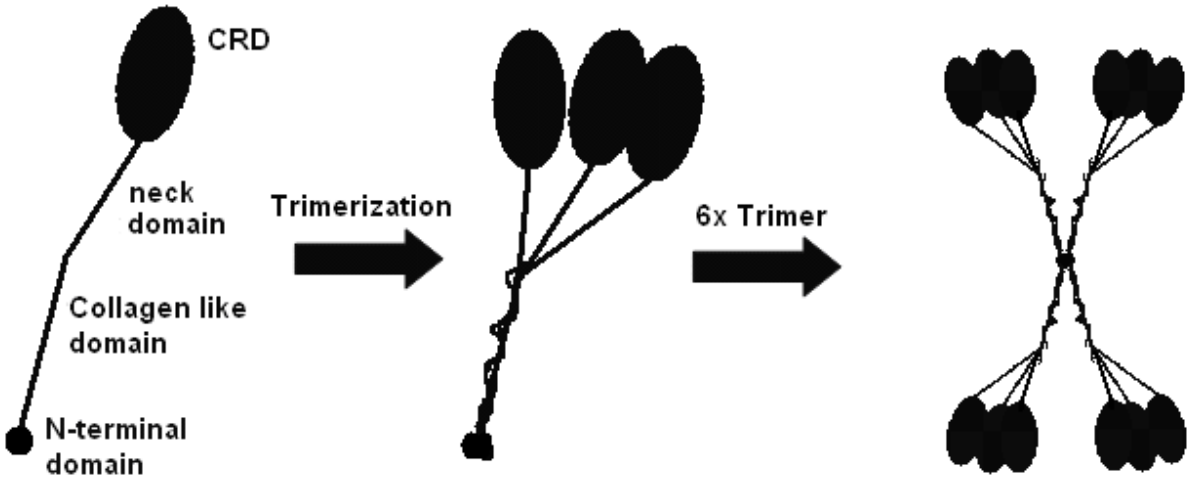


Figure 1.3.1: **Oligomerization of SP-D.** Three polypeptide chains assemble to trimers. Up to four SP-D trimers form the characteristically cruciform structure.

1.3.1) SP-D in human lung diseases

Primary deficiency in humans has been demonstrated for SP-D (257). The composition of surfactant components in prematurely born infants that died from *Respiratory Distress Syndrome* (RDS) differs from that in term infants or in prematurely born healthy infants (258). Reduced levels of SP-D which slowly recover by and by are known for infants with RDS (259). Two studies demonstrated that ARDS patients have reduced SP-D levels in BALF compared with control subjects (260,261). Increased SP-D serum levels are associated with worse clinical outcome and greater risk of death in ARDS.

The levels are also severely decreased in CF patients, except in infants and young children (262-264). The CF-associated protease human leucocyte elastase (265), proteinase 3, cathepsin G (266) and *Pseudomonas* elastase (265) degrade SP-D proteolytically. The proteolytic damage may impair the host defence functions in lungs of CF patients (267).

Chronic *interstitial lung diseases* (ILD) involve the most peripheral compartment of the lungs and are associated with chronic injury to the cells of the alveolar region, i.e. hypertrophy of type-II pneumocytes, and damage to the surfactant system (268). Under these conditions alterations of SP-D levels have been demonstrated, as well as abnormalities in surface tension-lowering properties or host defense functions. SP-D levels are elevated in patients with systemic sclerosis and polymyositis/dermatomyositis.

1.4) Aim of the study

Surfactant associated protein-A (SP-A) is the most abundant pulmonary surfactant protein and belongs to the family of innate host defence proteins termed collectins. Besides pulmonary host defence, SP-A is also involved in the formation of pulmonary surfactant, as it is essential for the structure of tubular myelin. The human SP-A gene locus includes two functional genes, SFTPA1 and SFTPA2 which are expressed independently, and a pseudo gene. SP-A polymorphisms play a role in respiratory distress syndrome, allergic bronchopulmonary aspergillosis and idiopathic pulmonary fibrosis. The levels of SP-A are decreased in the lungs of patients with CF, respiratory distress syndrome and further chronic lung diseases. Future areas for clinical research include disease specific SP-A expression pattern and their functional consequences, the differential roles of SP-A1 and SP-A2 in human lung diseases, and therapeutic approaches to correct altered SP-A levels.

The aim of the study was to elucidate the role of SP-A in human lung disease. There were numerous studies about the structure and function of SP-A *in vitro* or *ex vivo* using PAP-SP-A. Although studies had proved that SP-A structure arising from in vitro samples does not equal *ex vivo* samples (269) the relation between genetic polymorphisms in SFTPA and SP-A structure was analyzed with *in vitro* samples. PAP-SP-A has been shown to contain additional intramolecular bonds and is existent in different isoforms (270). Therefore it is not representative for SP-A of healthy individuals. This study was designed to analyze the relation between genetics, structure and function of SP-A in a CF, chronic bronchitis and asthma and healthy control population *ex vivo*.

2) Material and Methods

All reagents were purchased from Merck (Darmstadt, Germany) if not otherwise entitled. *Aqua injectabili* was purchased from Delta Select (Munich, Germany). The tips were purchased by Greiner BioOne (Frickenhausen, Germany), the tubes from Eppendorf (Hamburg, Germany) and the pipettes from Qiagen (Hilden, Germany).

2.1) Study populations

From all patients clinical data was collected and they gave written consent that their material can be analyzed for scientific studies.

2.1.1) Cystic Fibrosis study population

The CF patients whose samples were analyzed in this study were 371 patients from the Dr.-von-Haunersches children's hospital, Munich, not included in the BEAT study group while 82 were from a previous study (BEAT) including CF centres in Berlin (17 patients), Essen (18 patients), Hannover (12 patients), Cologne (26 patients) and Munich, the Dr.-von-Haunersches children's hospital (9 patients). For the BEAT study BAL and serum of each patient was analyzed. Because BALs are rarely used for diagnostic and therapeutic reasons in routine clinical management of CF patients only four additional BAL samples were available from the Hauner CF population. The diagnosis of CF had been confirmed by repeated sweat test with chloride concentrations exceeding 60 mmol/l. Genetic analysis for the 30 most prevalent CFTR mutations in Germany was available for 404 of the 453 patients.

2.1.2) Bronchitis study population

All 72 patients included in the bronchitis study population were chronic bronchitis and asthma patients from the Dr.-von-Haunersches children's hospital, Munich. All patients were diagnosed after the definition criteria. The chronic bronchitis patients had a daily cough for at least 3 months. In all patients primary ciliary dyskinesia, CF, gastro-oesophageal reflux disease, anomalies of the lungs and airways and immunodeficiency were excluded. The asthma patients had an increased responsiveness of the bronchi to various stimuli, had manifested as narrowing of the airways in exercise or cold air provocation tests and were responsive to β-agonists.

2.1.3) Control study population

The control study population includes only persons who had no airway diseases or abnormalities. 25 persons were recruited from the Dr.-von-Haunersches children's hospital, Munich, and 10 from the children's hospital in Hannover.

No.	Patients	Clinical data	SNPs	ELISA		Gel chromatography		Agglutination-assay			
				BAL	Serum	BAL	Serum	F BAL	F Serum	W BAL	W Serum
1	10 CF-BEAT										
2	15 CF-BEAT 1 CF-Hauner										
3	2 CF-BEAT 1 CF-Hauner										
4	12 CF-BEAT										
5	4 CF-Hauner										
6	10 Bro-Hauner 7 C-Hauner										
7	1 CF-Hauner 4 Bro-Hauner 4 C-Hauner										
8	11 Bro-Hauner 1 C-Hauner										
9	10 C Hannover										
10	41 CF-BEAT										
11	202 CF-Hauner 1 CF-BEAT										
12	154 CF-Hauner 1 CF-BEAT 47 Bro-Hauner 14 C-Hauner										
13	102 CF-London										

Figure 2.1.1: **Scheme of the study design.** The figure shows the data analyzed and methods used in this study and the number of patients included. CF stands for cystic fibrosis patients, Bro for bronchitis and asthma patients and C for the control group. BEAT means a special study population including patients from CF centres in Berlin (17 patients), Essen (18 patients), Hannover (12 patients), Cologne (26 patients) and Munich, the Dr.-von-Haunersches children's hospital (9 patients). Hauner means that those are patients from the Dr.-von-Haunersches children's hospital not included in the BEAT study group. F stands for fractions of gel chromatography, W for whole samples. In the left column the identification number of each group is listed. The thick lines enframe the two major study populations, the structure and function population (orange: Gel chromatography and Agglutination-assay; exact groups see table 2.1.1) and the genetic population (blue: SNPs and ELISA; exact groups see table 2.1.1**)**. The clinical data were used for both groups. Grey areas indicate variables not assessed in these groups of subjects.

Table 2.1.1: **Methods and group distribution.**

Clinical data	**MALDI-TOF and Probes**	**ELISA**	**Gel chromatography**	**Agglutination assay F**	**Agglutination assay W**
No. 1-9	No. 1-8	No. 1-11	No. 1-9	No. 1-6	No. 1-5
No. 11-13	No. 10-12	No. 13			

The table shows the patient groups analyzed by the different methods. The group numbers are the identification numbers of figure 2.1.1. In addition within these groups the grey shaded areas of Fig. 2.1.1. indicate variables not assessed in these subjects.

2.2) DNA extraction from whole blood with a QIAmp-Kit

To extract the DNA from whole blood samples of the patients and controls a kit from QIAmp (Qiagen, Hilden, Germany) was used after the provided protocol. In a 50 ml Falcon tube 3 ml whole blood were mixed with 500 µl Protease and 5 ml lyzation buffer. The solution was incubated in a water bath for 10 min (minutes) at 70 °C. Then 5 ml Ethanol were added and everything was mixed by a vortex. The solution was transferred to a column (QIAmp) placed in a clean collection 50 ml Falcon tube. After closing the cap the column was centrifuged at 3000 rpm for 3 min. The filtrate was discarded and the column placed back into the tube. Afterwards 5 ml buffer AW1 were added to the column and the column was centrifuged at 5000 rpm for 1 min. 5 ml buffer AW2 were added followed by another centrifugation at 5000 rpm for 15 min. The column was placed in a new tube while the collection tube and the filtrate were discarded. 1 ml elution buffer was pipetted on the membrane of the column and incubated for 5 min. Afterwards the column was centrifuged at 5000 rpm for 2 min. The last step was repeated. The DNA solution was transferred in an Eppendorf tube (Eppendorf, Hamburg, Germany). The concentration of the solution was measured with SybrGreen and then adjusted to a concentration of 20 ng/ml.

2.3) Bronchoalveolar lavage (BAL)

BAL was performed as described (271) with 4 × 1 ml/kg body weight normal saline warmed to body temperature. The first aliquot of the recovered BAL fluid was treated separately; all other samples were pooled for analysis. The total cell count was measured in a hemocytometer; the differential cell count was assessed from cytoprep slides. Aliquots of the cell free BAL supernatant of the pooled BAL sample were used for the analysis of total protein, and the surfactant proteins by enzyme linked immunosorbent assay (ELISA).

2.4) SP-A structure

2.4.1) Gel Chromatography with Superose 6 column

Table 2.4.1.1: **Population**.

Patient group	Serum	BAL	Pairs
Cystic Fibrosis	34	42	30
Bronchitis	14	21	10
Control	11	18	7

The table shows the number of the samples analyzed by gel chromatography (total n=94) from the three patient groups.

Buffers

ÄKTA buffer

20 mM Tris
150 mM NaCl
1 mM EDTA
pH 7.4

Procedure

To determine structural differences in BAL and serum between the study populations a gel chromatography method with a superpose 6 column (Amersham Pharmacia Biotech, Uppsala, Sweden) in an ÄKTA Purifier 900 (Amersham Pharmacia Biotech, Uppsala, Sweden) was chosen. In this method SP-A molecules were separated with regard to their oligomerization form. The larger the oligomers the earlier they were eluted. The column was calibrated by using bluedextran (2000 kDa),

holotransferrin (669 kDa) and bovine serum albumin (66 kDa). The column was equilibrated with ÄKTA buffer and then loaded with either 0.5 ml serum or 1 ml BAL. 39 fractions were collected in Eppendorf safe lock tubes á 0.8 ml volume (Eppendorf, Hamburg, Germany). Flow rate was 0.4 ml per min; the void volume was 6.8 ml. The fractions 6-25 were loaded on a Slot-Blot for further analysis. The other fractions were excluded by a prior experiment to analyze whether SP-A or SP-D is detectable in the fractions. Because of a constantly negative result the fractions from 1-5 (void volume) and from 26 to 39 were not further analyzed.

2.4.2) Dot-Blot

Buffers

Blocking buffer
2 g TWEEN
6 g Fish gelatine
200 ml TBS

TBS (Tris-buffered saline)
20 mM Tris-HCl
150 mM NaCl
pH 7.5

TBS-T (Tris-buffered saline with Tween)
20 mM Tris-HCl
150 mM NaCl
pH 7.5
0.05 % Tween20

Procedure

After gel chromatography the SP-A amount of the eluted fractions had to be determined. For this a Slot-Blot method was used. The membrane (BioRad, Munich, Germany) was incubated for 1 min in 100% methanol and then with 3 BioRad filters (BioRad, Munich, Germany) washed with H_2O for 5 min. Further everything was equilibrated in TBS/methanol (4:1). Cells were washed with 200 µl TBS. 100 µl Standard recombinant human SP-A (gift of Dr. Wolfram Steinhilber, Nycomed, Konstanz, Germany) and 200 µl of the fractions were loaded in the cells and washed by 200 µl TBS. Membrane was blocked with blocking buffer for 3 h. After washing 3 times with TBS for 10 min the membrane was incubated with a polyclonal recombinant rabbit-anti-human SP-A antibody (Nycomed, Constance, Germany) and 1 % bovine serum albumin (BSA, Paesel+Lorei, Duisburg, Germany) overnight. Again the membrane was washed 3 times for 10 min with TBS-T and then incubated with a second goat-anti-rabbit antibody (1:10,000, Biozol, Eching, Germany) and 1 % BSA for 2 h. The membrane was washed three times with TBS-T for 10 min. For signal detection the membrane was incubated for 1min in a mixture of 500 µl of each reagent from Super Signal West Dura (Pierce, Bonn, Germany) and 1 ml of each reagent from ECL Western Blot Detection Reagents (GE Healthcare, Munich, Germany). Signals were measured with a DIANA III camera (raytest, Straubenhardt, Germany) by 15 min duration of exposure and evaluated by AIDA Software (raytest, Straubenhardt, Germany).

2.4.3) Reproducibility of gel chromatography and Dot-Blot

The reproducibility of the gel chromatography and the Slot-Blot assay were tested (see fig. 2.4.3.2 and 2.4.3.3). Four serum and 2 BAL samples of 5 different individuals were analyzed twice by gel chromatography and Slot Blot at intervals of four weeks. Two serum samples of one person were each twice analyzed at one day and one sample analysis was repeated after 4 days. Also from the same person another serum sample was taken after these four days and twice analyzed by gel chromatography and Slot-Blot. For testing the reproducibility of the Slot-Blot assay separately the SP-A standard curve was compared (see fig. 2.4.3.1). The reproducibility of the slot-blot assay was good, as determined by the coefficient of variation at the highest (41.6 ng/ml; rel CV 6.1 %) concentration of the standard curve.

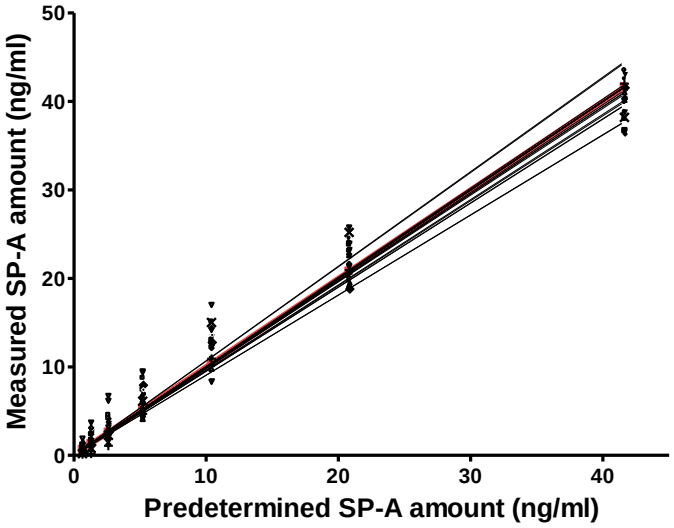


Figure 2.4.3.1: **SP-A standard variance.** The graph shows on the x-axis the predetermined SP-A amount in ng/ml and on the y-axis the measured SP-A amount in ng/ml (n = 10). The variance was insignificant.

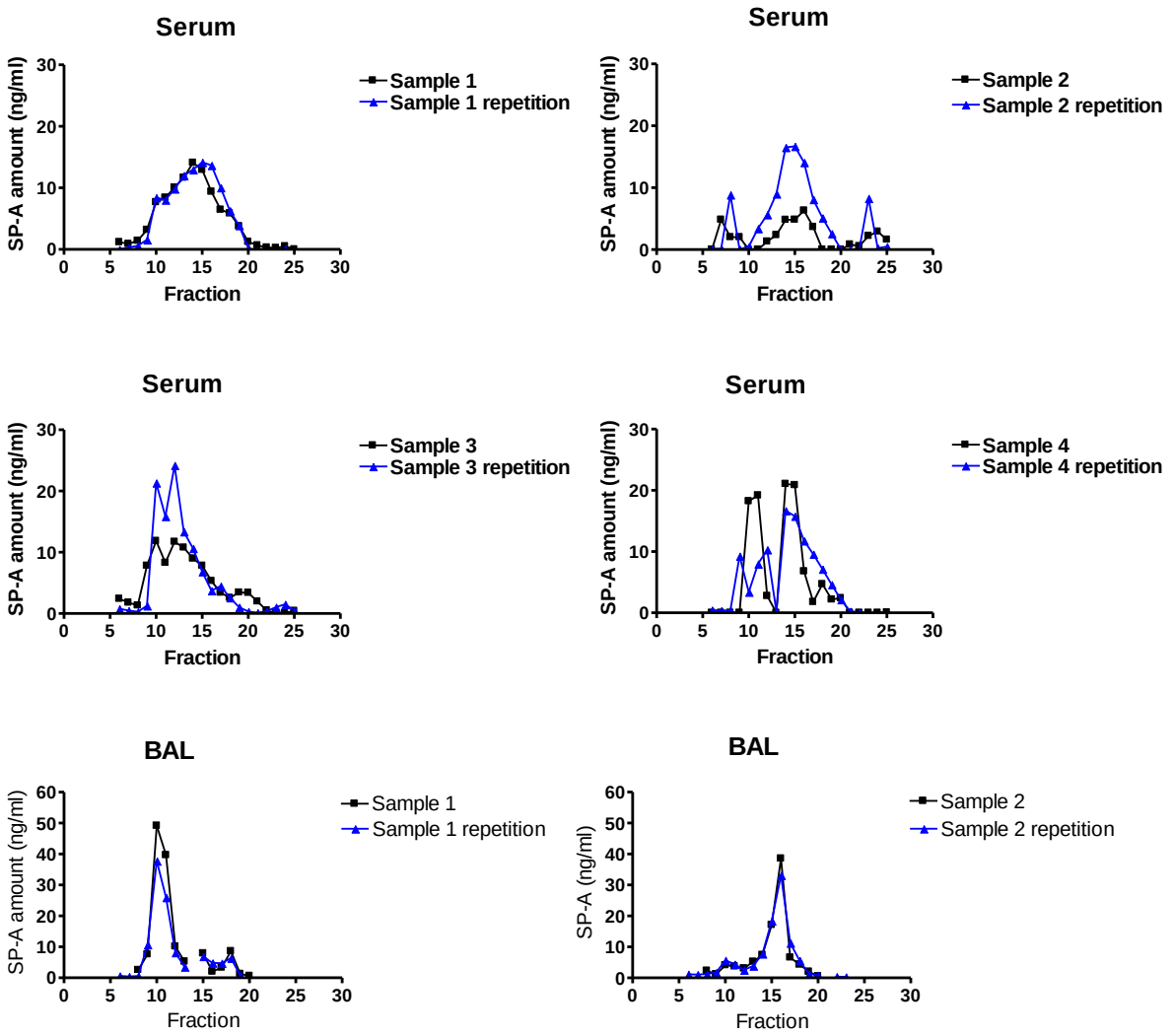


Figure 2.4.3.2: **Reproducibility of gel chromatography.** The graphs show the fraction number on the x-axis and the SP-A amount in ng/ml on the y-axis. All samples were measured twice in an interval of 4 weeks. The curve form varied only little in comparison to the SP-A amount, but the variances are insignificant.

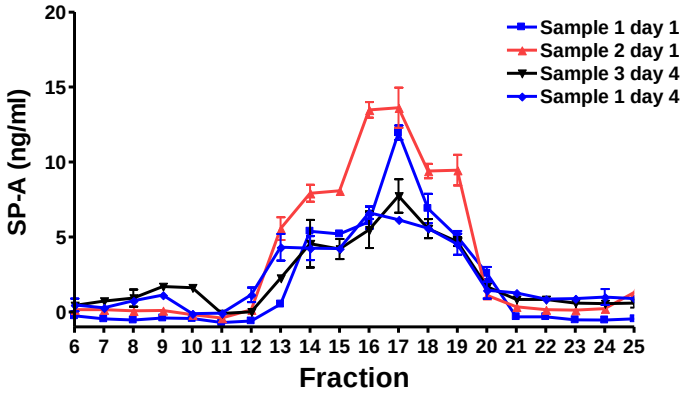


Figure 2.4.3.3: **Reproducibility of serum gel chromatography.** The graph shows the reproducibility of serum samples from one person. On the x-axis is the fraction number and on the y-axis is the SP-A amount in ng/ml plotted. The curve form varied only little in comparison to the SP-A amount, but the variances are insignificant.

2.4.4) Calibration of the Superose column

After validation of the reproducibility the column was calibrated with different molecules, Bluedextran, Holotransferrin and bovine serum albumin (BSA), of a known molecular weight which had been used in published studies (272,273).

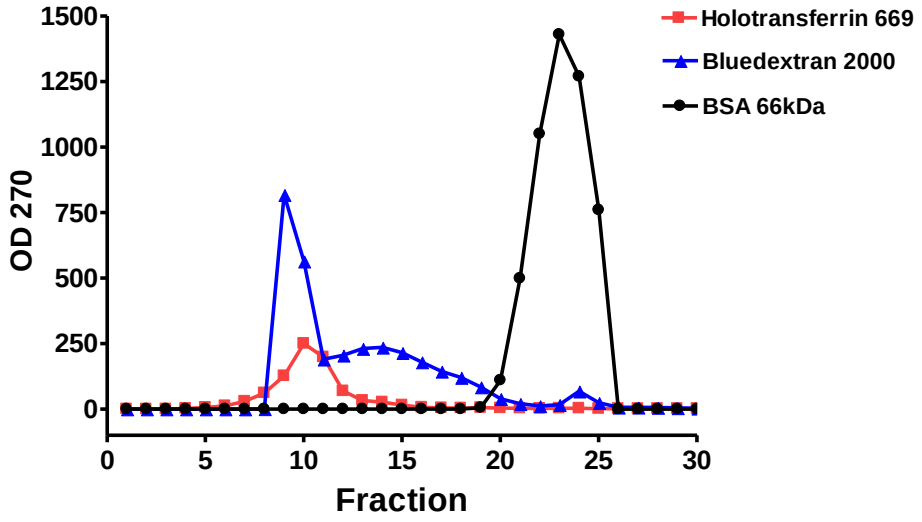


Figure 2.4.4.1: **Calibration of Superose 6 column.** The graph plots on the x-axis the number of the fractions and on the y-axis the OD 270. The calibration curves of Holotransferrin with a molecular weight of 669 kDa, Bluedextran with a molecular weight of 2000 kDa and BSA with a molecular weight of 66 kDa are shown.

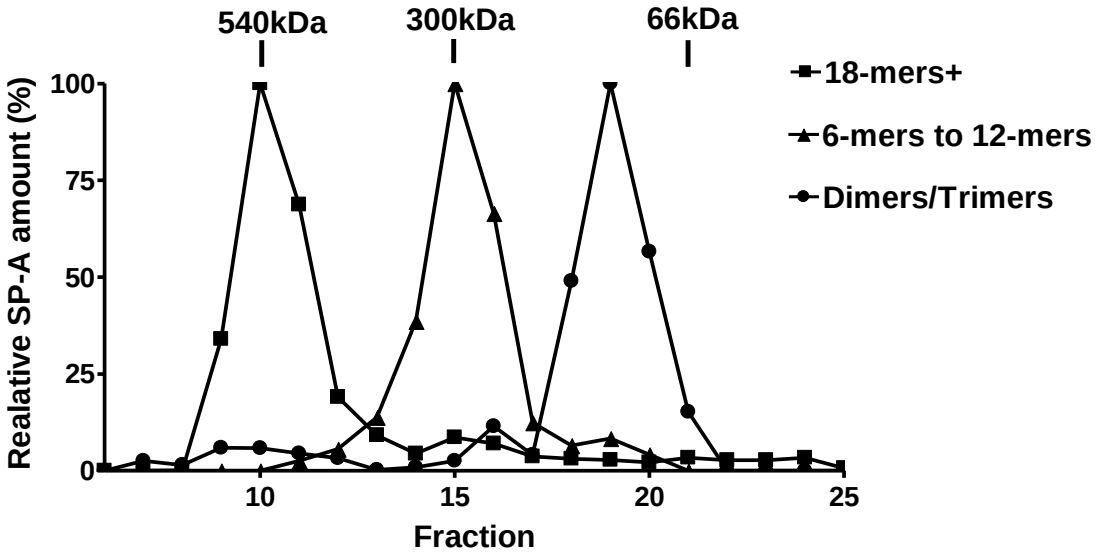


Figure 2.4.4.2: **Column calibration and SP-A peaks.** The graph illustrates the defined peaks of SP-A after gel chromatography and the calibration. On the x-axis the number of the fractions and on the y-axis the relative SP-A amount in % is plotted. With regard to the molecular weight of the calibration proteins the first peak should contain octadecamers or more complex oligomers, the second sixmers to twelvemers and the last peak dimers and trimers.

2.4.5) SP-A isolation

For testing the SP-A isolation assay, first BAL samples from controls, in particular fraction 10 derived from gel chromatography of 12 controls (group no. 9 and controls C Hauner 1 and 4), were pooled and used in 2.4.5, 2.4.6 and 2.4.7. Because the complete pooled sample was spent in the first test, for 2.4.8 12 BAL fractions (F 10) of CF patients (group no.2) were pooled which showed the same high relative SP-A amount in the first peak and had also comparable SP-A levels in BAL. Because the self-agglutination ability of SP-A derived from CF patients was less effective than of SP-A derived from controls the test was only done with fraction 10 (first peak) of CF patients which showed nevertheless a high self-agglutination ability.

Buffers

TBS
20 mM Tris-HCl
150 mM NaCl
pH 7.5

Procedure

For investigating the SP-A structure with another method, SP-A was isolated of the fractions from the first peak (F10) after gel chromatography by beads. The beads were coupled to an anti-SP-A antibody by biotin-streptavidin binding and could therefore pull the SP-A down in the fractions. The fractions were taken from 12 BAL control samples. All 10 samples from group no. 9 (C Hannover) and 2 samples from group no. 6 (C Hauner) were used, see also Fig. 2.1.1. The streptavidin-coated Dynabeads M-280 (Invitrogen, Karlsruhe, Germany) were separated from the solution by holding the tube near a magnet (magnetic bubbler), and the solution was discarded by pipetting. The beads were washed twice with 1ml of water followed by 1ml of TBS. Then the beads were resuspended in 200 µl of TBS and add 100 µl biotinylated mouse anti-human SP-A antibody (HYB 238-04, Antibodyshop, Gentofte, Denmark). This suspension was mixed for 1h under constant movement. Afterwards the beads were washed sequentially with 1 ml TBS, 1 ml TBS with 1% Triton X-100 (SIGMA, Munich, Germany) to inhibit unspecific binding, and 3 times with 1ml of TBS. The beads were resuspended in 450µl of TBS and stored at 4°C till use. Then the beads were incubated with 600 µl of a fraction or 500 µl of BAL or serum samples under constant movement for 1 h at RT. The beads were washed three times with TBS to remove calcium to inhibit self-agglutination and binding to other proteins or pathogens. Thereafter the beads were incubated for 30 min with 400 µl 100 mM glycine-HCL buffer pH 2.5 at RT to dissolve SP-A. The SP-A solution was separated from the beads and the pH was changed to about 5 (isoelectric point of SP-A) with 1 M NaOH. At last the beads were washed for reactivation 3 times with glycine-HCL buffer and resuspended in 450 µl TBS and store at 4°C.
In the supernatant after the pull-down, almost none SP-A was left in solution. Also after dissolving and separating the beads from the SP-A solution only a small amount of SP-A was left with the beads and detected in the wash solution.

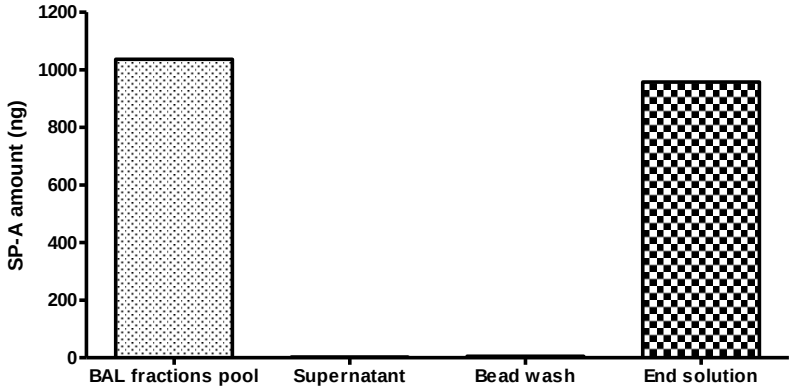


Figure 2.4.5.1: **Efficiency of the SP-A isolation assay.** The graph shows the amount of SP-A in ng (y-axis) in the different solutions before (BAL fractions pool), during (supernatant, bead wash) and after (end solution) the SP-A isolation measured by Slot-Blot. SP-A was isolated of the fractions from the first peak of 12 controls after gel chromatography by beads. The beads were coupled to an anti-SP-A antibody by biotin-streptavidin binding and could therefore pull the SP-A down in the fractions. Almost the complete SP-A amount from the starting pool solution could be isolated.

2.4.6) Protein gel

Buffers

Separation gel
10 ml acryl amide
5 ml separation gel buffer
5 ml *Aqua dest.*
200 µl SDS (20%)
100 µl APS (20%)
8 µl TEMED

Collecting gel
0.75 ml acryl amide
1 ml collecting gel buffer
4.1 ml *Aqua dest.*
60 µl SDS (20%)
30 µl APS (20%)
6 µl TEMED

Sample buffer
7 ml Tris/SDS pH 7.8
3 ml glycerol
1.2 ml Bromphenolblue
1 g SDS
10 ml *Aqua dest.*

Electrophoresis buffer
30.2 g Tris-base
144 g glycine
10 g SDS
1 l *Aqua dest.*

Separation gel buffer
1.5 M Tris
0.4 % SDS
pH 8.8

Collecting gel buffer
0.5 M Tris-base
0.4 % SDS
pH 6.8

Procedure

For determining the purity of the isolated SP-A sample (derived from step 2.4.5) it was first separated by gel chromatography. The denaturizing SDS-polyacryl-amide-gelelectrophoresis (SDS-PAGE) was executed after the protocol of Lämmli (275). The polymerization of the gel was started by adding APS as a radical starter and catalyzed by TEMED. The polyacryl-amide-gel consisted of a collecting gel where the proteins were focused and a separation gel in which the proteins were separated. The SDS which contained the sample buffer destroyed non-covalent interactions in native proteins. By SDS binding to the protein it influenced the migration behaviour of the complex, so the proteins were separated by molecular size only. The protein lysates were dissolved in 6x sample buffer and incubated at 70 °C for 10 min. Then the samples were loaded on the collecting gel which was cast on a 15 % separation gel. Also a molecular weight standard (Novex Sharp Protein Standard LC5800, Invitrogen, Karlsruhe, Germany) was loaded.

2.4.7) Silver staining

Solutions

Fixing solution
500 ml ethanol
100 ml acetic acid
400 ml *Aqua dest.*
0.5 ml formaldehyde (37%) (Roth, Karlsruhe, Germany)

Washing solution
500 ml ethanol
500 ml *Aqua dest.*

Sensitizing solution
0.1 g sodium sulphite ($Na_2S_2O_3$)
500 ml *Aqua dest.*

Dyeing solution
0.4 g silver nitrate ($AgNO_3$)
200 ml *Aqua dest.*
150 µl formaldehyde (37%)

Developer solution
15 g sodium carbonate (Na_2CO_3)
1 mg sodium sulphite($Na_2S_2O_3$)
250 ml *Aqua dest.*
125 µl formaldehyde (37%)

Stop solution
60 ml acetic acid
220 ml ethanol
220 ml *Aqua dest.*

Procedure

To determine the purity of the SP-A sample the gel was stained with silver. To fix the proteins on the gel the gel was incubated over night in the fixing solution. Then it was washed 2 times for 25 min with the washing solution. For a better silver binding the gel was sensitized for 1 min with the sensitizing solution and afterwards washed 3 times for 20 s with *Aqua dest.*. For staining the gel was incubated

20 min with the dyeing solution and washed again 3 times for 20 s. To make the protein bands visible the gel was incubated in the developer solution for 3-5 min. Next it was washed one time for 20 s before it was laid for 10 min in the stop solution. At last it was washed 3 times for 10 min to remove surplus staining and kept in 1 % acetic acid at 4 °C.

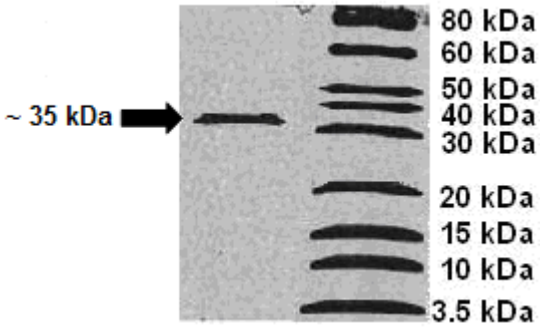


Figure 2.4.7.1: **Silver stained protein gel of isolated reduced SP-A.** The figure shows the relevant part of the silver stained protein gel. In the left lane ran the sample taken from the SP-A isolation and in the right lane the protein marker. Next to the marker the molecular weight of the marker bands are shown. The SP-A isolation band is between the 30 and 40 kDa.

There was only one band for the SP-A isolation sample at about 35 kDa. SP-A monomers have a molecular weight of 28-35 kDa. Therefore the purity of the SP-A isolation sample is more than 90 %, but there seems to be only glycosylated SP-A monomers. The gel chromatography results of the samples can be seen in the appendix D.

2.4.8) Single molecule force spectroscopy

Procedure

To finally examine the structure of SP-A in a fraction after gel chromatography the method of single molecule force spectroscopy based on atomic force microscopy was used. SP-A was isolated of pooled fractions from the first peak (F10) after gel chromatography of BAL from 12 CF patients out of group no. 2 (see figure 2.1.1) by beads. Only one molecule was attached to a gold layer and then pulled of by a cantilever, while the applied force is measured. The protein solution was incubated for 20 min on a glass substrate with freshly evaporated gold surface at a concentration of 1 mg/ml. After washing with PBS, the cantilevers (Olympus, Mannheim, Germany) were calibrated using the equipartition theorem, and the automated recording of force extension traces was started with a pulling speed of 1 mm/s. In classical statistical mechanics, the equipartition theorem is a general formula that relates the temperature of a system with its average energies and makes quantitative predictions. The original idea of equipartition was that, in thermal equilibrium, energy is shared equally among its various forms; for example, the average kinetic energy in the translational motion of a molecule should equal the average kinetic energy in its rotational motion. For measuring an atomic force microscope (custom-built, Biophysics, Ludwig-Maximilians-University, Munich) was used.

2.5) SP-A function

2.5.1) SP-A self-agglutination-assay for demonstration of supra-quaternary organisational structure of SP-A

Table 2.5.1.1: **Sample size.**

Patient group	BAL and serum pairs, fractions	BAL, fractions	Serum fractions	BAL and serum pairs	BAL	Serum
Cystic Fibrosis	10	8	20	27	15	4
Bronchitis	10					
Control	7					

The table shows the number of the samples analyzed by the SP-A self-agglutination-assay from the three patient groups after gel chromatography (fractions) and from whole BAL or serum samples.

Procedure

Because it is known that there are structural differences of SP-A between individuals, it was necessary to elucidate if there are functional differences between different oligomeric states or between different oligomeric compositions. For this a new agglutination assay with carboxylate-modified, streptavidin-labeled latex beads (SIGMA Aldrich, Munich, Germany) was established. 50 µl Streptavidin-beads ($1.82x10^{10}$ beads/ml) were incubated with 200 µl biotinylated rabbit anti-goat antibody (1.3 mg/ml, Dianova, Hamburg, Germany) for 18 h at 23°C in 0.2 ml Eppendorf tubes (Eppendorf, Hamburg, Germany). The mixture was washed 2 times with Hank solution (8 g/l NaCl, 1 g/l glucose, 0.4 g/l KCl, 0.35 g/l $NaHCO_3$, 0.14 g/l $CaCl_2$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 0.06 g/l $Na_2HPO_4 \cdot 2H_2O$, 0.06 g/l KH_2PO_4 and 0.06 g/l $MgSO_4 \cdot 7H_2O$, pH 7.4; Apotheke Innenstadt, University Munich, Germany) and resuspended in 600 µl Hank solution. Then 5 µl of the samples (end concentration: 100 ng/ml SP-A from peak fractions) were incubated with 2 µl bead suspension (6.5 mM Ca^{2+}), 3 µl *Aqua injectabili* and 0.01 µg goat anti-human-SP-A antibody (N19, Santa Cruz, Heidelberg, Germany) for 3 h at 4°C on a microscope slide (Menzel, Braunschweig, Germany) under a cover slip (Menzel, Braunschweig, Germany). To avoid non-specific agglutination of the SP-A, all assays were done in the presence of 0.1 % (v/v) Triton X-100. Under the light-optical microscope (Axioskop, Zeiss, Aalen, Germany) the biggest, pure bead-agglutinate was searched and photographed. The picture was viewed by the software Adobe Photoshop (Adobe, Munich, Germany) at a size of 764x573 pixel and the size of the determined agglutinate was measured in pixel by drawing a square around it.

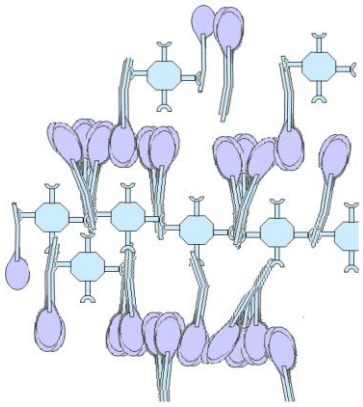


Figure 2.5.1.1: **Scheme of the SP-A self-agglutination-Assay**. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD.

2.5.2) SP-A self-agglutination-assay establishment

Because of the structural differences of SP-A between individuals, it was necessary to analyze whether there are functional differences between different oligomeric states. For this analysis the new agglutination assay with streptavidin beads was used. First the assay-construction was tested. The conditions of the assay were varied with respect to the reagent addition as listed in table 2.5.2.1 to prove that the assay functions because of the binding chain bead-anti-goat antibody-anti-human SP-A antibody-SP-A and not because of other influences. The results state that the beads only agglutinate when also the anti-goat antibody, the goat-anti-human SP-A antibody and SP-A is present. Secondly to prove that the assay was calcium and carbohydrate dependent the assay conditions were changed as shown in table 2.5.2.2. Without calcium and/or with mannose no agglutination could be observed. The tests were repeated three times.

Table 2.5.2.1: **SP-A self-agglutination-assay conditions.**

Condition	**Reagent**				**Agglutination**
	SP-A	**SP-D**	**Goat-anti-human-SP-A antibody**	**Anti-goat antibody**	
1	**+**	**-**	**+**	**+**	**+**
2	**-**	**+**	**+**	**+**	**-**
3	**+**	**-**	**-**	**+**	**-**
4	**+**	**-**	**+**	**-**	**-**
5	**-**	**-**	**+**	**+**	**-**

The table shows five different conditions of the bead-assay. The beads were incubated alternatively with anti-goat antibody, goat-anti-human antibody, SP-A or SP-D in a buffer containing calcium ions. The “+” means that the reagent was used. After incubation the mixture was analyzed by light microscopy and agglutination was only detected under condition 1 when SP-A was added. The experiments were repeated three times.

Table 2.5.2.2: **Tests for SP-A self-agglutination-assay dependency.**

Beads	**Goat-anti-human-SP-A antibody**	**Anti-goat antibody**	**Serum**	**Ca^{2+}**	**Agglutination**
+	**+**	**+**	**+**	**-**	**-**
+	**+**	**+**	**+**	**+**	**+**
+	**+**	**+**	**-**	**+**	**-**
+	**+**	**+**	**-**	**-**	**-**
+	**-**	**+**	**+**	**+**	**-**
+	**-**	**-**	**+**	**+**	**-**
-	**+**	**+**	**+**	**+**	**-**
+	**+**	**+**	SP-D	**-**	**-**
+	**+**	**+**	SP-D	**+**	**-**
+	**+**	**+**	**-**	**+** with Mannose	**-**
+	**+**	**+**	**+**	**+** with Mannose	**-**
+	**+**	**+**	**+/**without SP-D	**+**	**+**
+	**+**	**+**	**+/+**Triton X	**+**	**-**

The table shows twelve conditions of the bead assay with varying reagents: beads, anti-goat antibody, goat-anti-human antibody, human serum and calcium ions (Ca^{2+}). The "+" means that the reagent was used for incubation. Without SP-A in serum, serum and calcium ions and also with Mannose no agglutination was detected. The experiments were repeated three times. For the last condition a concentration of 0.1 % (v/v) Triton X-100 was used.

Figure 2.5.2.1: **SP-A self-agglutination-assay with serum lacking SP-D.** The microscope picture with a magnification of 10 times was taken under a light microscope. It illustrates that SP-A and not SP-D was causing the agglutination.

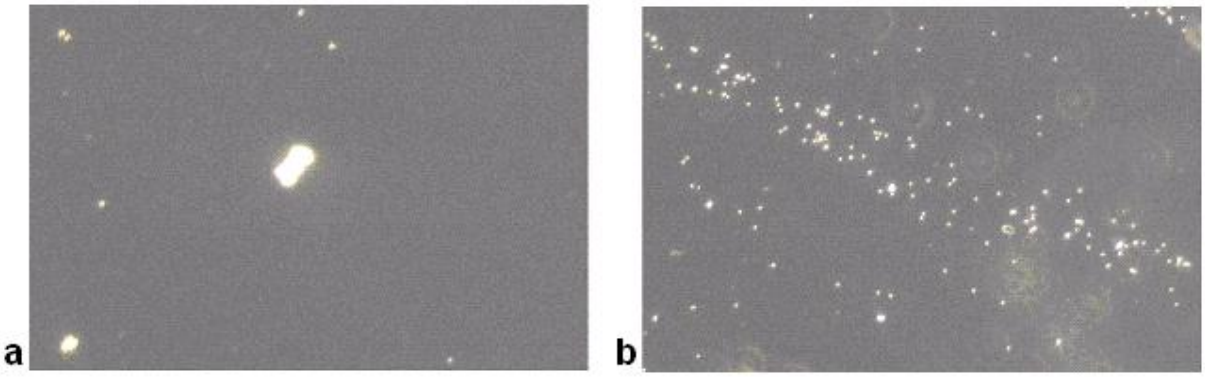


Figure 2.5.2.2: **Agglutinated and spread beads.** The microscope pictures with a magnification of 10 times were taken under a light microscope. Picture a shows an agglutinate while picture b illustrates beads without agglutination. These pictures were representative for condition testing and dependency testing.

After proving that the assay was SP-A dependent the SP-A self-agglutination-assay was further analyzed by incubation with an increasing serum amount. As illustrated in figure 3.2.2.2 the more SP-A containing serum is added the bigger the bead agglutinates are. The test was repeated three times with two different sera. This supports the SP-A dependency of the SP-A self-agglutination-assay.

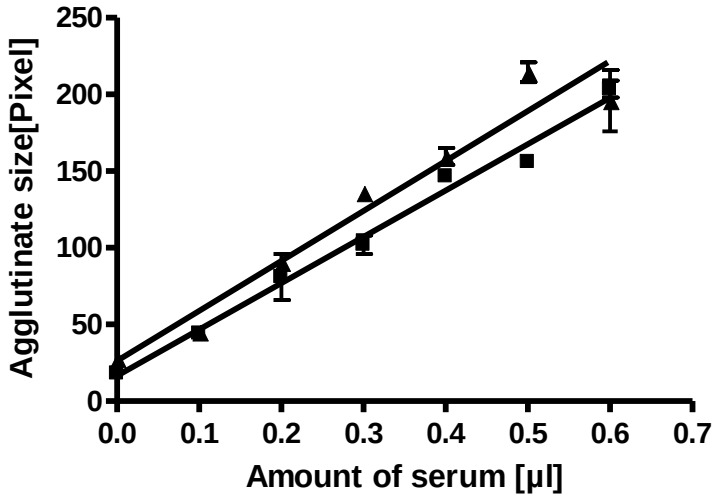


Figure 2.5.2.3: **SP-A dependent agglutination.** The graph illustrates the SP-A dependency of the bead agglutination. The agglutinate size is plotted against the amount of serum containing SP-A which was incubated with the beads, anti-goat antibody and goat-anti-human antibody in a buffer with calcium ions. Two different sera were used (▲and ■) in the experiments which were repeated three times.

Afterwards it was tested what part of the SP-A protein is responsible for agglutination. The goat-anti-human SP-A antibody binds at the N-terminal end of SP-A, so binding at this end is unlikely. Therefore the hypothesis was that the agglutination is caused by self-agglutination of the CRD. Therefore the calcium and carbohydrate dependency was tested specifically. Figures 2.5.2.3 and 2.5.2.4 show that

with increasing Mannose concentration or addition of EDTA (removing calcium) significantly smaller agglutinates are formed. Both tests were repeated five times for three or four serum samples respectively.

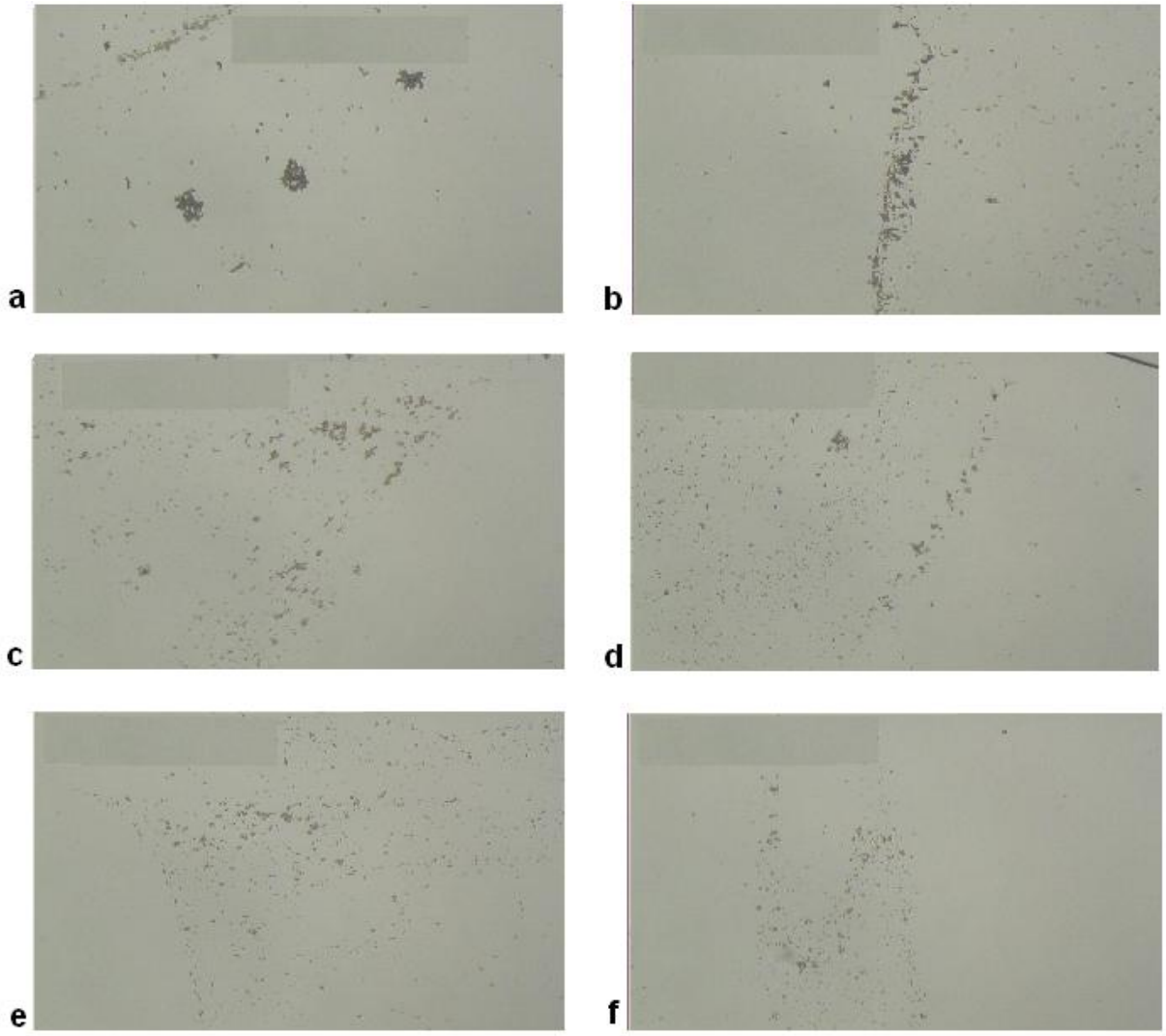


Figure 2.5.2.4: **Carbohydrate-dependency of the bead-assay.** The pictures were taken under a light microscope by a 10 time magnification. The beads were incubated with anti-goat antibody, goat anti-human SP-A antibody, 5 µl serum and an increasing amount of Mannose (a: 0 mM Mannose; b: 10 mM Mannose; c: 20 mM Mannose; d: 30 mM Mannose; e: 40 mM Mannose; f: 50 mM Mannose). The agglutinate size decreased corresponding to an increasing Mannose concentration.

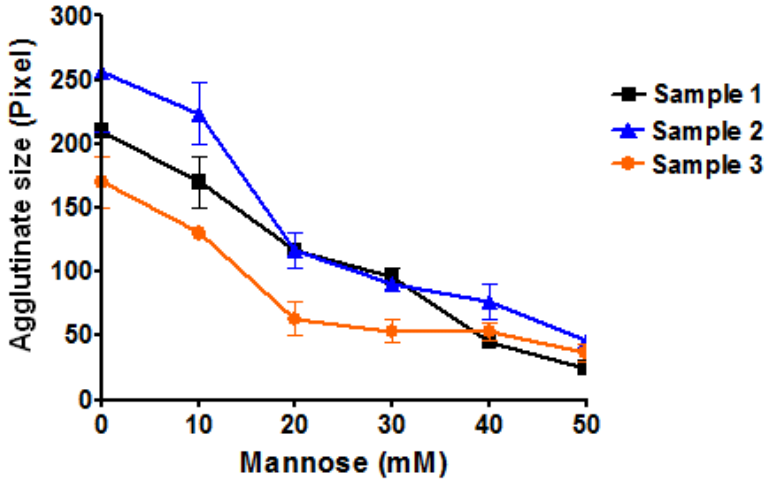


Figure 2.5.2.5: **Mannose inhibition of SP-A agglutination.** The figure shows a graph of the agglutinate size arisen from beads incubated with anti-goat antibody, goat-anti-human antibody and human serum from three different test persons under varying mannose concentration in a buffer containing calcium ions. The higher the mannose concentration the smaller the bead agglutinates. The experiments were repeated five times.

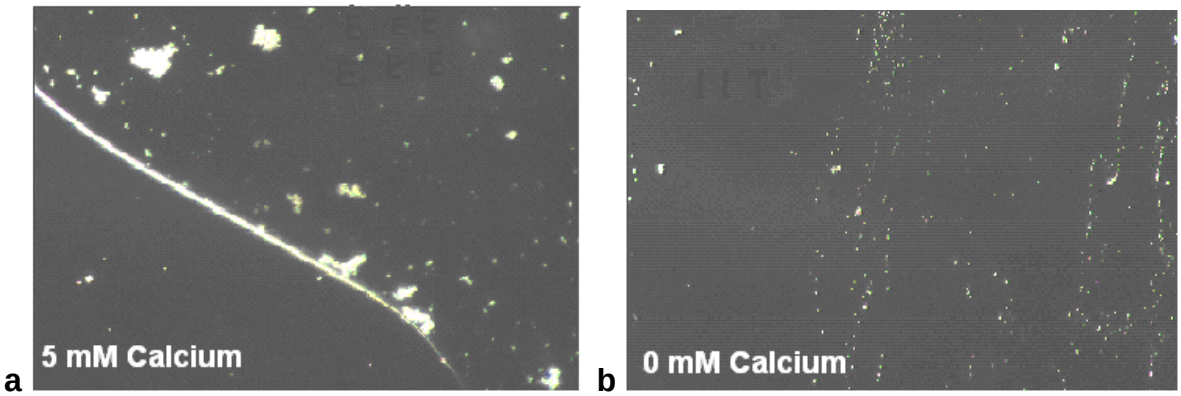


Figure 2.5.2.6: **Calcium-dependency of the bead-assay.** The pictures show the Calcium-dependency of the agglutination-assay. The beads were incubated with anti-goat antibody, goat-anti-human antibody and human serum from three different test persons and either 5 mM Calcium (a) or 0 mM Calcium (b).

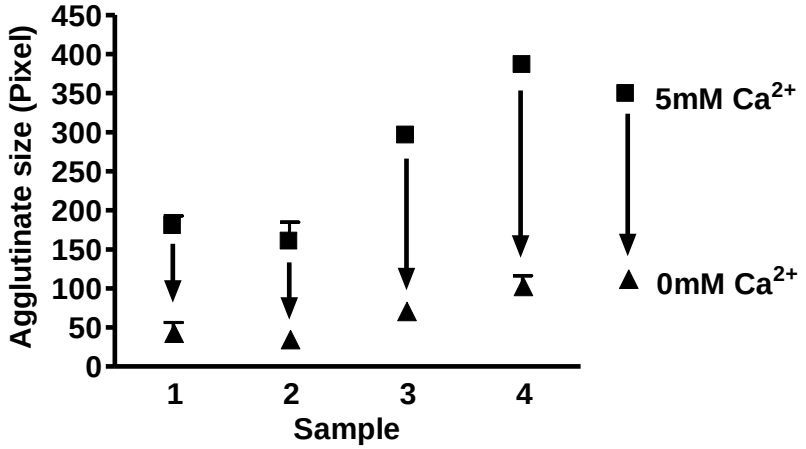


Figure 2.5.2.7: **Calcium-dependency of the bead-assay.** The graph displays the dependency on calcium ions of the bead agglutination caused by SP-A. Four different serum samples were incubated with beads, anti-goat antibody, goat-anti-human antibody in a buffer containing calcium ions first without EDTA (■) and then with 50 mM EDTA (▲). After repeating the experiments five times it became obvious that addition of EDTA leads to smaller bead agglutinates.

2.6) SP-A genetics, clinical and biochemical data

2.6.1) Body Mass Index (BMI)

The BMI of the patients and controls was determined according to the formula:

BMI = weight (kg)/(length (m))^2

and classified at enrolment according to the World Health Organisation (WHO). The enrolment criteria are listed in the tables below separated by age.

Table 2.6.1.1: **BMI classification for women older than 19.**

Age [years]	Underweight	Normal weight	Overweight
19-24	<19	19-24	>24
25-34	<20	20-25	>25
35-44	<21	21-26	>26
45-54	<22	22-27	>27
55-64	<23	23-28	>28
>64	<24	24-29	>29

The table shows the cut-off values used to define weight status groups (underweight, normal, overweight) from the global database on body mass index (http://www.who.int/bmi/index.html).

Table 2.6.1.1: **BMI classification for men older than 19.**

Age [years]	Underweight	Normal weight	Overweight
19-24	<20	20-25	>25
25-34	<21	21-26	>26
35-44	<22	22-27	>27
45-54	<23	23-28	>28
55-64	<24	24-28	>28
>64	<24	24-29	>29

The table shows the cut-off values used to define weight status groups (underweight, normal, overweight) from the global database on body mass index (http://www.who.int/bmi/index.html).

Table 2.6.1.2: **BMI classification for Girls between 7-18 years of age.**

Age [years]	Severe underweight	Underweight	Normal weight	Overweight	Severe Overweight
7	< 12	12-13	13.1-18	18.1-23	>23
8	< 12	12-13	13.1-19	19.1-22	>22
9	< 13	13-15	15,1-19.9	20-23	>23
10	< 13	13-14	14.1-20	20.1-23	>23
11	< 14	14-15	15.1-20	20.1-23	>23
12	< 15	15	16-21	21.1-23	>23
13	< 15	15.1-16	16.1-22	22.1-24	>24
14	< 16	16-17	17.1-23	23.1-26	>26
15	< 17	17-18	18.1-23	23.1-27	>27
16	< 17	17-18	18.1-23	23.1-24	>24
17	< 17	17-18	18.1-23	23.1-25	>25
18	< 17	17-18	18.1-23	23.1-25	>25

The table shows the cut-off values used to define weight status groups (underweight, normal, overweight) from the global database on body mass index (http://www.who.int/bmi/index.html).

Table 2.6.1.3: **BMI classification for Boys between 7 to 18 years of age.**

Age [years]	Severe underweight	Underweight	Normal weight	Overweight	Severe Overweight
7	< 13	13-14	14.1-19	19.1-21	>21
8	< 13	13-14	14.1-19	19.1-22	>22
9	< 13	13-14	14,1-19	19.1-22	>22
10	< 14	14-15	15.1-21	21.1-25	>25
11	< 14	14	15-21	21.1-23	>23
12	< 15	15	16-22	22.1-25	>25
13	< 16	16	17-22	22.1-24	>24
14	< 16	16-17	17.1-23	23.1-26	>26
15	< 17	17-18	18.1-23	23.1-26	>26
16	< 18	18-19	19.1-24	24.1-26	>26
17	< 18	18-19	19.1-24	24.1-26	>26
18	< 18	18-19	19.1-24	24.1-26	>26

The table shows the cut-off values used to define weight status groups (underweight, normal, overweight) from the global database on body mass index (http://www.who.int/bmi/index.html).

2.6.2) Pseudomonas aeruginosa infection

The status of *Pseudomonas aeruginosa* infection for CF patients was determined after the criteria shown in the following table.

Table 2.6.2.1: **Classification criteria for Pseudomonas aeruginosa infection.**

	Criteria
Chronic	7 out of 8 samples of the last 2 years must be positive
Negative	No positive result in the last 2 years
Real-negative	Never a positive result
Intermittent	Minimum one positive result, maximum 6 in the last 2 years
First initial infection	First positive result

2.6.3) Lung Function Analysis

2.6.3.1) Lung disease status of bronchitis patients

The lung disease status of the bronchitis study population was determined according to the classification of the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The enrolment criteria are listed in the table below and are used for classification in adults.

Table 2.6.3.1.1: **Classification of lung disease status in Bronchitis patients.**

Stadium	Fev_1 [%pred.]
mild	≥ 80
moderate	50 ≤ Fev_1 [%pred.] > 80
severe	< 50

The table shows the cut-off values used to define severe, moderate and mild groups.

2.6.3.2) Lung disease status of cystic fibrosis patients

As there is a strong dependency on age and sex it is difficult to normalize lung function values. Several approaches are available and were used. These are listed in table 2.6.3.2.1.

Table 2.6.3.2.1: **The different methods for lung function classification in CF.**

Method
Pattern analysis – see paragraph 2.6.3.3
Fev1 (% pred.)$_{age\ 20}$ – see paragraph 2.6.3.4
Mean ΔFev1 (% pred.) – see paragraph 2.6.3.5
Last Fev1 (% pred.) value available – here not used

2.6.3.3) Pattern analysis (“extremes”) of CF lung disease according to Schluchter

One specific type of case-control genetic association study design compares patients at the extremes of phenotype (i.e., those with severe versus mild disease) because it provides additional power to detect gene modifiers. In such studies, it is particularly important to be able to accurately identify patients at the extremes of phenotype.

First the data from the last 3 years were examined, in particular the mean lung function value per year for three years. These data were taken to determine the lung disease status with respect to the enrolment from Schluchter et al. {Schluchter, 2006 3711 /id} (see table 2.6.3.4.1). Categorization by severity group was based on age-specific cut-off values for Fev1 (% pred.). These values derived approximately from quartiles of lung function for patients <34 years of age in the U.S. Cystic Fibrosis Foundation Patient Registry.
The mean value of each year was calculated and all three values had to fit in the enrolment criteria for one group (mild young: age 15-24 years; mild old: age ≥ 25 years; severe: age 8-25 years) to determine a status. If a patient did not match these criteria he had to be excluded. All patients ≥ 34 years of age were considered "mild" regardless of their Fev1, on the basis of survival.
Longitudinal Fev1 data were analyzed using a mixed model, assuming that the mean Fev1 (% pred.) follows a linear regression versus time for each patient with random patient-specific slope and intercept, with a separate population regression line for each of the three severity groups. This means that first for each patient a linear regression was calculated and then a mixed model of the patients' data grouped by their lung disease status to check the classification ability of the method (see figure 3.3.2.1.1). 305 CF patients from 453 could be classified. {Schluchter, 2006 3711 /id}

2.6.3.4) Mixed-Regression-model Fev1 (% pred.) estimated for age 20

The results from Schluchter et al. developed in their first study (method 2.6.3.3) indicated a good separation of lung disease severity groups mild and severe from the extrapolated Fev1 (% pred.) at age 20 (Fev1 (% pred.)$_{age20}$). The graph of our results showed also a very good data ability to distinguish the disease status groups at age 20 (see figure 3.3.2.1.1). Therefore as a second approach and to strengthen data resulting from further analysis respecting the lung disease status we also estimated with respect to the linear regressions a value for Fev1 (% pred.)$_{age20}$ for each patient (see above). For the regression calculation this time the complete lung function data of each patient as mean values per year was used. This estimated value is used again to classify the patients (Fev_1 (% pred.)$_{age20}$ ≥ 84 is mild and ≤54 is severe). All classification results derived by this method (paragraph 2.6.3.4) matched the ones analyzed by the method in paragraph 2.6.3.3. This method helped to classify 82 of those patients not definable by the initial approach (paragraph 2.6.3.3.). Now 387 out of 453 CF patients were classified. Children younger than 8 years (n = 35), patients with less than one Fev1 (% pred.) value per year over the last 3 years (n = 10) and patients who didn't match the criteria (n = 21) could not be classified.

In addition Fev1 (% pred.) estimated for age 20 was also used directly as stand alone variable to describe overall pulmonary disease course of a particular CF patient (e.g. Fig. 3.1.3.3).

Table 2.6.3.4.1:**Classification of the lung disease status in CF patients.**

Age	Mean Fev1 (% pred.) per year	
	mild	severe
8		≤ 80
9		≤ 79
10		≤ 78
11		≤ 77
12		≤ 75
13		≤ 72
14		≤ 69
15	≥ 97	≤ 67
16	≥ 92	≤ 63
17	≥ 90	≤ 60
18	≥ 87	≤ 59
19	≥ 86	≤ 57
20	≥ 84	≤ 54
21	≥ 82	≤ 50
22	≥ 77	≤ 45
23	≥ 70	≤ 39
24	≥ 68	≤ 36
25	≥ 67	≤ 34
26	≥ 62	
27	≥ 58	
28	≥ 54	
29	≥ 52	
30	≥ 50	
31	≥ 45	
32	≥ 32	
33	≥ 32	
≥ 34	x	

The table shows the cut-off values used to define severe and mild groups, based on the mean Fev1 % predicted per year. The x means that patients of 34 years of age or older are grouped into the mild lung disease group no matter of their Fev1 % pred. value.

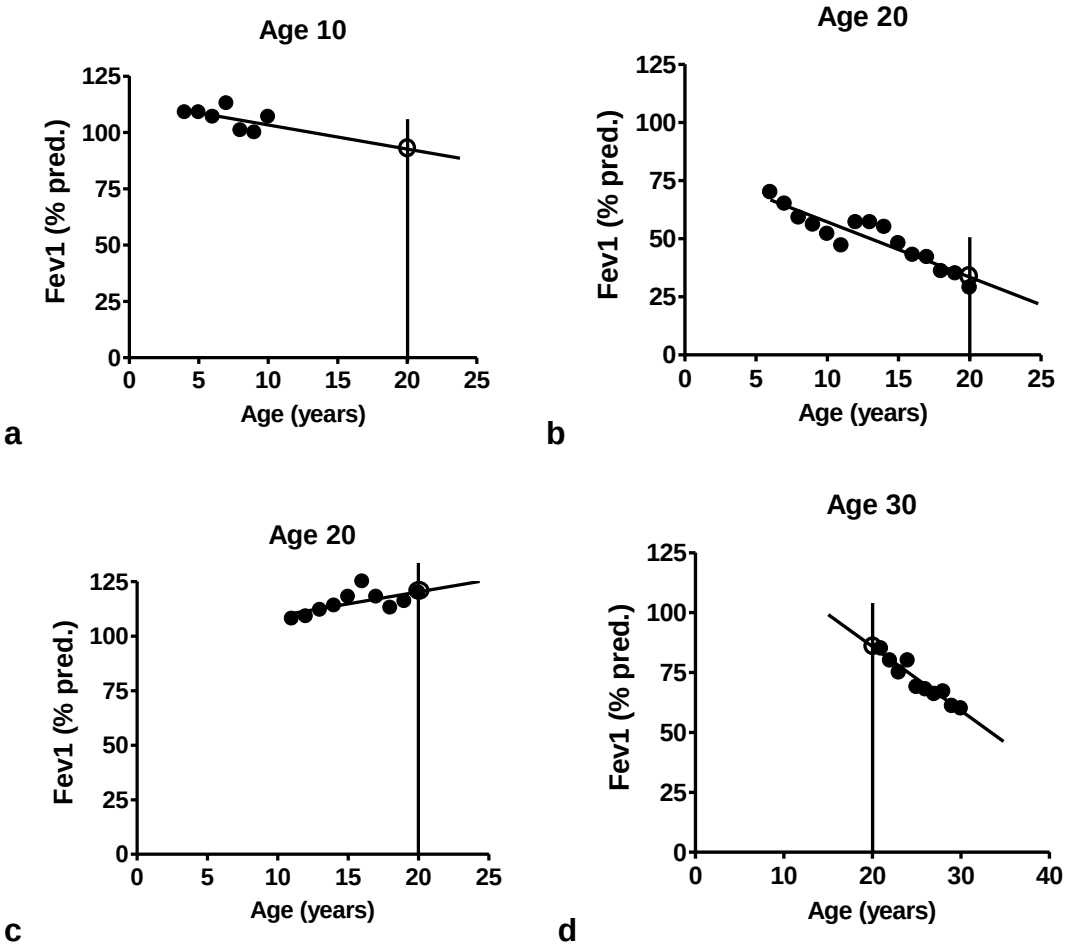


Figure 2.6.3.3.1: **Examples for lung disease classification.** All graphs show the mean lung function data per year of CF patients at different ages (10 years: a, 20 years: b and c, 30 years: d). The patient described in graph a could not be classified according to the first method of pattern analysis because he was too young. Using linear regression a Fev1 (% pred.)$_{age20}$ of > 84 % could be extrapolated. Therefore this patient could be classified as having a mild lung disease. The patient described in graph b could be classified with both methods as having a severe lung disease because the last three Fev1 (% pred.) values were like the Fev1 (% pred.)$_{age20}$ below 54 %. Graphs c and d show CF patients with mild lung diseases because the Fev1 (% pred.)$_{age20}$ was > 84 % and the last three Fev1 (% pred.) values were higher than the limiting values shown in table 2.6.3.3.1.

2.6.3.5) Comparing Mean Δ Fev1 (% pred.)

A third approach to analyze the lung function data was to calculate the mean Δ Fev1 (% pred.) per year of each patient over all data available. For example there were Fev1 (% pred.) data available for one patient over 4 years. Then the Δ Fev1 (% pred.) for each year was calculated. Then the annual Δ Fev1 (% pred.) were averaged and a mean Δ Fev1 (% pred.) value per year was calculated using the four Δ Fev1 (% pred.) calculated before. As many as possible Δ Fev1 (% pred.) values of a patient were used. The average available lung function data spanned over 7 years.

2.6.4) Classification of IgG and IgE levels

Because the IgG and IgE levels are age-dependent the measured level values had to be converted into a comparable characteristic. Therefore the ratio of the level compared to the upper standard value for the correspondent age was calculated. The upper standard values are shown in table 2.6.4.1.

Table 2.6.4.1: **Upper standard values of IgG and IgE.**

Age (years)	**IgG (mg/dl)**	**IgE (IU/ml)**
1-5	1200	60
5-9	1507	90
9-14	1507	200
> 14	1800	200

The table shows the upper standard values of the IgG and IgE levels depending on age.

2.6.5) Selection of single nucleotide polymorphisms (SNP) for analysis

First the gene structure and sequence of *SFTPA*1, *SFTPA*2 and *SFTPD* were taken from the databank of the UCSC Genome Browsers, University of California, U.S.A.. In the database the known SNPs of the genes are also marked. For each SNP the kind of SNP (deletion, insertion and exchange), the validation status and the mean rate of heterozygosis is stated. SNPs were chosen for each gene if the SNP was likely to be found in Caucasians (validation status) and appeared frequently (mean heterozygosis > 0.1), so that over the length of the gene the analyzed SNPs were evenly distributed. The oligonucleotides necessary for polymerase chain reaction (PCR) were designed with "Primer Express" (Applied Biosystems, Darmstadt, Germany).

2.6.6) Polymerase chain reaction (PCR)

The PCR is a method to amplify *in vitro* specific DNA fragments. The fragment that should be amplified is defined by two synthetic oligonucleotides called primer. These primers are complementary to sequences which flank the relevant fragment and serve as the starting point for the DNA-polymerase.

The PCR comes off in three themselves repeating steps. In the first step, the denaturation, the reaction mixture is heated up to 95 °C to separate the DNA strands. The temperature of the second step, the annealing or hybridization, is variable and depends on the length and the base composition of the primers. It should usually be about 5 °C lower than the melting temperature of the primers to prevent unspecific hybridization. After primer annealing the primers are extended by the DNA-polymerase (elongation) at 72 °C. Because of the high temperature at the denaturation step heat-

stable enzymes are needed. Such polymerases are found in thermophilic bacteria which live in hot springs. The *Taq*-polymerase out of the bacterium *Thermophilus Aquaticus* is the most common used.

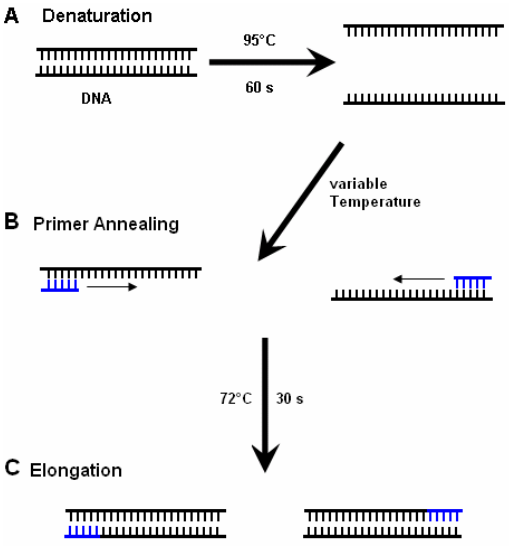


Figure 2.6.6.1: **Scheme of a PCR.** The figure shows the three steps of a PCR from denaturation (A) over primer annealing (B) to elongation (C) to amplify a certain DNA-fragment.

2.6.7) Probes

Table 2.6.7.1: **Probe sequences.**

Gene	SNP	Probe sequences
SFTPA1	V19A	LC RED640-TGAAGGACGTTTGTGTTGGAAGCCCT-P 5-CCTCTGGTGCTGCGTGCGA-FL
	L50V	LC RED705-GCCCTCCAGGTACTGTGCTGCAGA-P 5-GATGGTGTCAAAGGTGACCT-FL
	R219W	LC RED640-CTCCCCTCGGTACCAGTTGGTGTAG-P 5-CTCTTTTCTCCAACCTGCGG-FL
SFTPA2	N9T	LC RED640-CTTGATGGCAGCCTCTGGTGC-P 5-CTCTGGCCCTCACCCTCA-FL
	A91P	LC RED705-CTTCTCTCCACGCTCTCCAGGGACAC-P 5-CCTCTCTGGCCAGGCTCCC-FL
	Q223K	LC RED705-CACAGATGGGCAGTGGAATGACAG P 5-GGAAAAGAG CAGTGTGTGGAG AT-FL
SFTPD	M31T	LC RED640-TGCTTGCACCCTGGTCATGTGTAGC P 5-CTCCCACAGAACAACGCCCA-FL
	A180T	LC RED705-GTGAGCAGTGGAAAGAGCTGGGCT P 5-CTGG+AAAC+ACAGGGGCAGCA
	S290T	LC RED640-CTCTGCCGCTGAGAATGCCGC P 5-GACAGT+TGGCC+ACTCCAC-FL

The table shows the sequences of the probes used to genotype SNPs in *SFTPA*1, *SFTP*A2 and *SFTP*D by melting analysis.

Table 2.6.7.2: **Primer sequences for probe assay.**

Gene	SNP	Primer sequences and Annealing temperature	
***SFTPA*1**	V19A	GCTCACGGCCATCCCTCCT CTAGGGGCCTGGACAGATGG	58°C
	L50V	GCTCACGGCCATCCCTCCT CTAGGGGCCTGGACAGATGG	58°C
	R219W	GAGACTTCCGCTACTCAGAC GCCGAAGGCCAGAGAGCG	60°C
SFTPA2	N9T	CCCGCCCTGCCTCTCGCT TGAAAAGGGGTCTGTGTCCC	59°C
	A91P	TGGGTCCGCCTGGAGAAACA ATTGCCCTTGGGGTACCTGC	59°C
	Q223K	ACTTCCGCTACTCAGATGGGA ACAGACCAAGTGGATCCTGG	58°C
SFTPD	M31T	TCTTCCTCCTCTCTGCACTG GACCAGGCAGGCCACTC	61°C
	A180T	GCCTCGCAGGCCCTAA TGTAGGCATTGACAGCTCCAA	58°C
	S290T	GTGCACACAGGCTGATGG CCCTCTGTCTTGGAATCAGTCAT	59°C

The table lists the sequences and annealing temperatures of the primers used to amplify DNA parts surrounding the SNPs which were chosen to be genotyped by melting analysis with probes.

Procedure Probes

For determining the genotypes of the study populations some SNPs (see table 2.6.6.2) which cause an amino acidic exchange were analyzed by melting curves with hybridized probes in a Light Cycler (Roche, Mannheim, Germany). First the relevant DNA fragment was amplified. Then the fluorescent and fluorescein marked probes given by Heiko Witt (Charité Berlin, Germany) and purchased from Metabion (Planegg-Martinsried, Germany) were hybridized with the DNA fragment. A signal could only be detected if both markers were located near each other so that the laser energy could be transferred from the Fluorescein to the fluorescent marker. During the melting progress the probes detached from the DNA fragment and the energy transfer was interrupted. By determining the temperature when the detachment took place the base of the relevant SNP locus could be detected. The probe could be designed either be complementary to the wildtype allele or the mutated allele. The melting temperature was higher if the complementary allele was existent in the DNA fragment. After extraction of the DNA from whole blood with Qiagen Maxi Kit (Qiagen, Hilden, Germany) the solutions of 202 CF patients were adjusted to a concentration of 20 ng/µl. The DNA samples were amplified in a 25 µl reaction mixture containing 50 ng of template DNA, 2.5 µl of 10 x PCR buffer without $MgCl_2$, 2 µl $MgCl_2$ (25 mM), 1.25 µl dNTPs (10 mM), 1 µl of each primer (10 µM) and 0.1 µl HotstarTaq® (Qiagen, Hilden Germany). The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 48 cycles at 95 °C for 20 s (seconds), 40 s at annealing temperature (see table) and 72 °C for 90 s and a final extension at 72 °C for 120 s in an Eppendorf cycler (Eppendorf, Hamburg, Germany). 1 nmol lyophilized probe was resupended in 200 µl H_2O. Then 13 µl FL-probe 1 (Fluoresceine-probe) and 16 µl LC-probe (Light Cycler probe, LC 750) were mixed with 13 µl FL-probe

2 and 16 µl LC-probe (LC 640). 1.5 µl of the probe mixture and 4 µl PCR-product were pipetted in LC-glass capillaries (Roche, Mannheim, Germany). Afterwards melting curves were run in a LightCycler with 95 °C for 60 s, 40 °C for 40 s and an increase to 80 °C with a ramp of 0.1 °C/s.

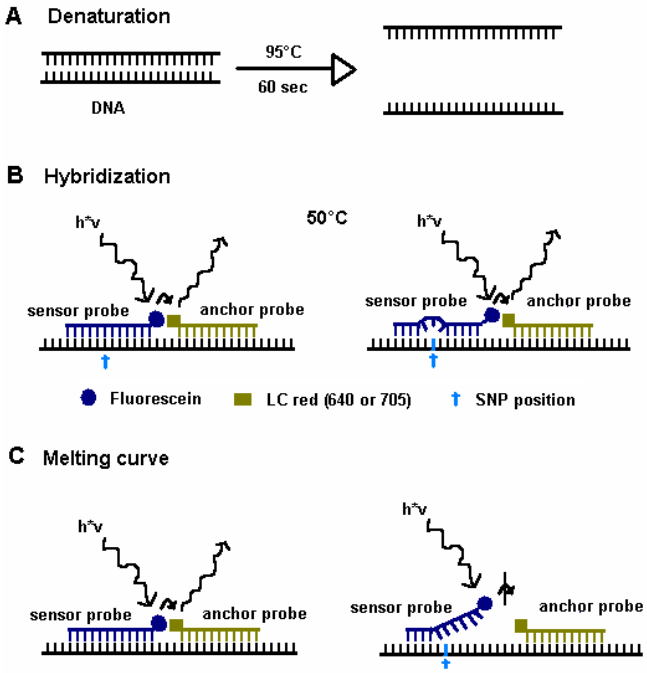


Figure 2.6.7.1: **Scheme of the probe procedure.** First the relevant DNA fragment was amplified. Then the fluorescent and Fluorescein marked probes were hybridized (B) with the DNA fragment after the DNA fragment was denaturized (A). A signal could only be detected if both markers were located near each other that the laser energy could be transferred from the Fluorescein to the fluorescent marker. During the melting progress the probes detached from the DNA fragment and the energy transfer was interrupted (C). By determining the temperature when the detachment took place the base of the relevant SNP locus could be detected. The probe could be designed either be complementary to the wildtype allele or the mutated allele. The melting temperature was higher if the complementary allele was existent in the DNA fragment.

2.6.8) Matrix Assisted Laser Desorption/Ionisation-Time-Of-Flight (MALDI-TOF)

Table 2.6.8.1: **Population**.

Patient group	Number analyzed
Cystic Fibrosis	482
Bronchitis	46
Controls	25

The table shows the number of the DNA samples analyzed by MALDI-TOF.

Procedure

For a bigger throughput of genotyping the method of Matrix Assisted Laser Desorption/Ionisation-Time-Of-Flight (MALDI-TOF) was used. The DNA samples were adjusted to a concentration of 1 ng/ml. The isolated DNA was removed from the 96-well 1.2 ml Storage Plates (ABgene®, Darmstadt, Germany), and the fragment of interest was PCR amplified in a heat sealed Thermo-Fast® 384 PCR Plate (ABgene®, Darmstadt, Germany). The reaction mixture contained 3.82 µl H_2O, 0.625 µl PCR buffer (Qiagen, Hilden, Germany), 0.325 µl $MgCl_2$ (Qiagen, Hilden, Germany), 0.1 µl dNTP mix (Qiagen, Hilden, Germany) and 0.1 µl Hotstar Taq® (Qiagen, Hilden, Germany) in a total volume of 5 µl. The cycling conditions were as follows: denaturation for 15 min at 94 °C, 45 cycles of 20 s denaturation at 94 °C, 30 s primer annealing at 56 °C and 60 s elongation at 72 °C, an additional elongation phase of 3 min at 72 °C and a holding temperature of 20 °C. The amplicons were processed with a SAP enzyme mix (Promega, Mannheim, Germany) containing 1.53 µl H_2O, 0.17 µl SAP buffer (10x, Promega, Mannheim, Germany) and 0.3 µl SAP enzyme solution. The enzyme was activated for 40 min at 37 °C and then inactivated at 85 °C for 5 min. Furthermore a primer extension reaction was run. The reaction mixture contained 0.755 µl H_2O, 0.2 µl iPlex Buffer plus (10x, Sequenom, Hamburg, Germany), 0.1 µl i-Plex termination mix (10x, Sequenom, Hamburg, Germany), 0.1 µl Primer (Metabion, Planegg-Martinsried, Germany) and 0.0205 µl iPlex enzyme (10x, Sequenom, Hamburg, Germany) in a total volume of 2 µl. The cycling conditions were as follows: denaturation for 30 s at 94 °C, 40 cycles of 5 s denaturation at 94 °C, 5 s primer annealing at 52 °C and 5 s elongation at 80 °C, 5 cycles of 5 s at 52°C and 5 s at 80°C, an additional elongation phase of 3 min at 72 °C and a holding temperature of 20 °C. In this reaction an oligonucleotide was designed to bind directly to the 5'end of the identified SNP. Dideoxy nucleotides (ddNTPs) were substituted for one of the 4 deoxy nucleotides (dNTPs) in the reaction mix. Therefore, if a SNP was present the complementary ddNTP will be incorporated and different allele specific fragments are created. The extension products could be spotted on microchips and analysed in the MALDI-TOF Mass Spectrometer (Sequenom, Hamburg, Germany). A laser fired on the spotted products on the microchip and the DNA was accelerated in a vacuum to a detector. Smaller molecules (e.g. a 24-mer) were faster than larger molecules (e.g. a 26-mer) and were detected earlier. The mass of every extended product was determined and could be "translated" into one allele of the SNP.

Table 2.6.8.1: **SNPs analyzed by MALDI-TOF.**

***SFTPA*1**	***SFTPA*2**	***SFTPD***	**Other genes**
rs1059047	rs1059046	rs911887	CD14_CMIN159T
rs4253527	rs17886395	rs721917	TLR1 TMIN2192C
rs10351	rs17880428	rs2243639	TLR1 A742G
rs4253518	rs17881665R	rs3088308	TLR2 AMIN15607G
Cys85Arg	rs1975006	rs12784549	TLR2 T596C
rs1059057	rs17886376R	rs17885228	TLR2 TMIN16934A
rs4253526	rs1965708	rs7078012	TLR3 C6300T
rs4253511	rs17882297	rs726014	TLR4 GMIN2570A_2
rs4253524	rs17886221	rs726289	TLR4 AMIN2570G
rs1059056	rs17880349	rs911887	TLR4 C8851T
rs1136451	rs17885057	rs17880779	TLR4 TMIN1607C
		rs17881639	TLR6 TMIN2079A
		rs17885295	TLR9 TMIN2871C
		rs17886333	TLR10 A2322G
		rs3088308	TLR10 G1031T

The table shows all SNPs that were genotyped in the *SFTPA*1, *SFTPA*2 and *SFTPD* genes as well as in other genes which proteins are important factors in immunity.

2.6.9) SP-A and SP-D ELISA

Buffer

TBS	Carbonate buffer
20 mM Tris-HCl	15 mM Na_2CO_3
150 mM NaCl	35 mM $NaHCO_3$
pH 7.5	pH 9.6

Blocking buffer: 1% BSA in TBS

Procedure

To measure the SP-A and SP-D level in human serum and BAL the microplates (Nunc Maxisorp C 96, Nunc, Langenselbold, Germany) were coated overnight at 4 °C with either anti-human SP-A (HYB 238-04, AntibodyShop, Gentofte, Denmark) or anti-human SP-D (HYB 246-08, AntibodyShop, Gentofte, Denmark) dissolved in carbonate buffer to a concentration of 1 µg/ml. Then the wells were blocked with 200 µl blocking buffer for 2 h at 37 °C. The plates were washed three times with TBS. 100 µl of the samples were dissolved in 350 µl of 1 mM EDTA, 1 % BSA and 10 mM Tris (pH 7.4). Afterwards the samples were put in the ultrasonic bath (Sonorex RK100, Bandelin, Mörfelden-Walldorf, Germany) for 1 min and 50 µl Triton X-100 (SIGMA, Munich, Germany) was added. They were mixed and 10 min at 10,000 g centrifuged (Haeraeus, Hanau, Germany). 50 µl of the standard were dissolved in 200 µl of 1 mM EDTA and 10 mM Tris (pH 7.4) and put in the ultrasonic bath for 1 min. Then 25 µl Triton X-100 was added and the standard was centrifuged for 10 min at 10,000 g. At last the standard was filled up to 1,000 µl with blocking buffer. 100 µl of the samples and the standard were loaded on the plate. Thereafter 100 µl of the anti-human SP-A (Rabbit anti-human SPA, Dianova, Hamburg, Germany) or SP-D (HYB 246-08, AntibodyShop, Gentofte, Denmark) dissolved 1:100 in blocking buffer were pipetted into each well and incubated for 1 h at 37 °C. The plates were washed three times with TBS and then each well was incubated with 100 µl goat anti-rabbit antibody (Biozol, Eching, Germany) dissolved 1:5000 in blocking buffer for 1 h at 37 °C. Again the plates were washed three times with TBS. 100 µl TMB solution were added to each well and after 10-15 min 100 µl of 1 N H_2SO4. After another 10-15 min the plates were read in the photometer (anthos ht III, anthos, Krefeld, Germany) at 405 nm.

2.7) Absence of SP-D in BAL

2.7.1) Sequencing

If there are more than one SNP in a short DNA-segment or if unknown SNPs are searched for DNA fragments are sequenced. The fragment which should be analyzed is first amplified in a 6 times PCR to synthesize a sufficient product amount. Then the reactions mixture is purified to remove surplus primers and nucleotides. Through sequencing after Sanger the concerning nucleotide-order can be determined. In this method fluorescent dideoxynucleotides are used which lead to a chain extension stop. The reaction mixture is purified by alcohol precipitation. The fragment length and the ending nucleotide are detected by a laser-photo-unit during a sequencing-gel electrophoresis through the specific fluorescent dyes of the dideoxynucleotides. With a computer program like CEQ™ 8000 Genetic Analysis system (Beckman Coulter, Krefeld, Germany) the sequences can be illustrated.

2.7.2) Procedure SP-D- sequencing

After we identified subjects with no SP-D in their BAL common polymorphisms known to be associated with low serum SP-D levels were assessed in those subjects from whom informed consent could be obtained and DNA was available.

The M31T polymorphism in exon 1, A180T in exon 4 and S290T in exon 7 were analyzed. DNA was available from 10 of the 12 children who had no SP-D detectable in their BAL by ELISA (Hain Lifescience, Nehren, Germany) (n = 6) and Western blotting (n = 4). For comparison 8 children were selected with the same clinical characteristics, but detectable SP-D levels in serum.

To identify these polymorphisms exon 1, exon 4, and exon 7 were amplified in a 25 µl reaction mixture containing 50 ng of template DNA, 2.5 µl of 10x PCR buffer without $MgCl_2$, 2 µl $MgCl_2$ (25 mM), 1.25 µl dNTPs (10 mM), 1 µl of each primer (10 µM) and 0.1 µl HotstarTaq® (Qiagen, Hilden, Germany). The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 48 cycles at 95 °C for 20 s, 58 °C for 40 s and 72 °C for 90 s and a final extension at 72 °C for 120 s. The reaction mixtures were purified with a DNA purification kit (Qiagen, Hilden, Germany). Then sequencing was performed after Sanger using a kit from Applied Biosystems (Darmstadt, Germany).

Additionally, part of the promotor was sequenced after Sanger (-600 till +79) in 10 patients with the forward primer: ttacaagaacagtgcacaagac and reward primer: gctgtaggtcattgtgcaac. The amplification with these primers was performed as described above.

2.8) Statistics

For parametric testing one-way ANOVA and Tukey-test and for non-parametric testing the Kruska-Wallis test and Dunns-test were chosen to compare different groups with each other. For comparing only two groups t-test was used. All these tests were analyzed by Graph Pad Prism software (San Diego, CA, U.S.A.).

The SNP data resulting from the genotyping by MALDI-TOF and probes were correlated with the clinical data and ELISA level measurements by χ^2-test on the webpage http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Catego.htm and the results were corrected according to Bonferroni. Additionally some SNP genotypes were analyzed in Graph Pad Prism by one-way ANOVA and Tukey-test or t-test respectively.

3) Results

For these studies samples from 453 patients with CF, 72 chronic bronchitis and asthma patients and 35 controls subjects were used, as detailed in fig 2.1.1 in the methods section. As not from all subjects all variables were assessed in each instance, in addition the exact number of subjects included or materials available is given in the figure and table legends.

3.1) SP-A structure and function

3.1.1) Clinical data of the structure and function population

The BAL and serum samples of 46 CF patients, 19 chronic bronchitis and asthma patients and 13 controls that were genotyped by MALDI-TOF as well as BAL and serum samples from additional 9 chronic bronchitis patients and 9 controls were analyzed by gel chromatography with a Superose 6 column. The clinical data for these study populations were also completed.

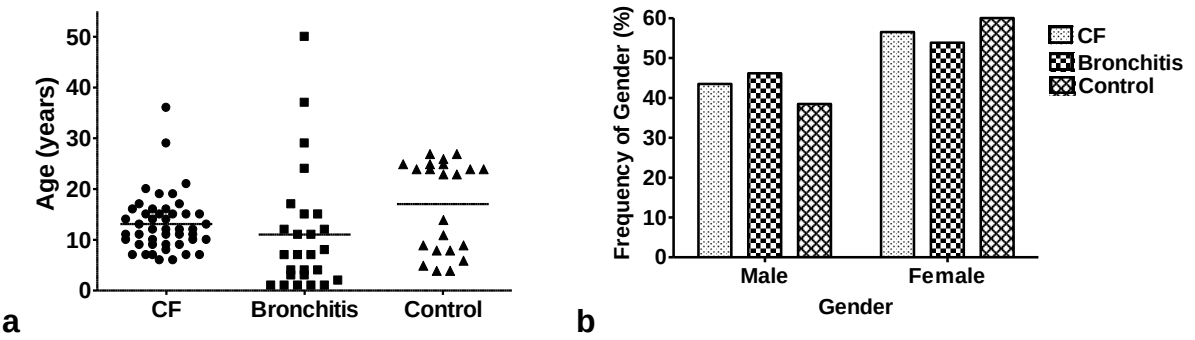


Figure 3.1.1.1: **Age and Gender distribution.** The graphs illustrate the age (a) and gender (b) distribution of all 46 CF, 28 bronchitis patients and 22 controls analyzed by gel chromatography. Analyzed by One way ANOVA there were no significant differences between the patient groups.

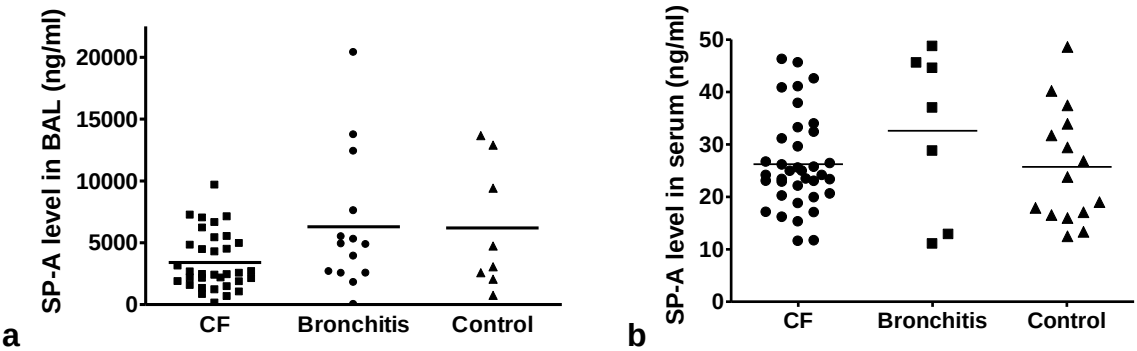


Figure 3.1.1.2: **SP-A level in BAL and serum.** The graphs show the distributions of the BAL (a) and serum (b) levels of the patients analyzed by ELISA. Analyzed by One way ANOVA there were no significant differences between the patient groups. The serum SP-A levels of 34 CF, 14 bronchitis patients and 8 controls and the BAL SP-A levels of 37 CF, 7 bronchitis and 15 controls were measured.

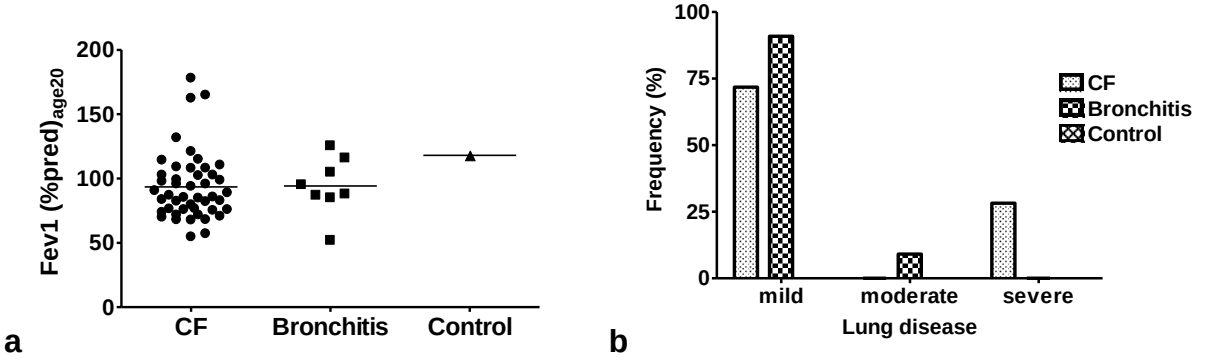


Figure 3.1.1.3: **Lung function data and lung disease.** The graphs plot the distribution of the Fev1 (% pred.)$_{age20}$ (a) and the lung disease status (b) of the patients analyzed by gel chromatography. Analyzed by One way ANOVA there were no significant differences between the patient groups regarding the Fev1 (% pred.)$_{age20}$ data at the sampling point. The controls and most of the bronchitis patients had a mild lung disease while 28% of the CF patients had a severe lung disease. The Fev1 (% pred.) data at sampling point was available for 46 CF, 8 bronchitis patients and one control.

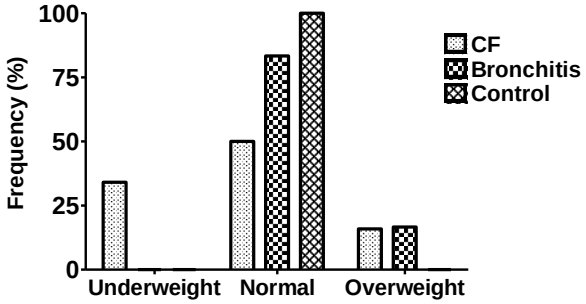


Figure 3.1.1.4: **The weight distribution.** The graph illustrates the weight distribution classified by the BMI of all patients analyzed by gel chromatography. 34 % of the CF patients had underweight and 16 % overweight. 17 % of the bronchitis patients had also overweight while the remaining patients and all controls had normal weight. The BMI data was available for all 42 CF, 10 bronchitis patients and 19 controls.

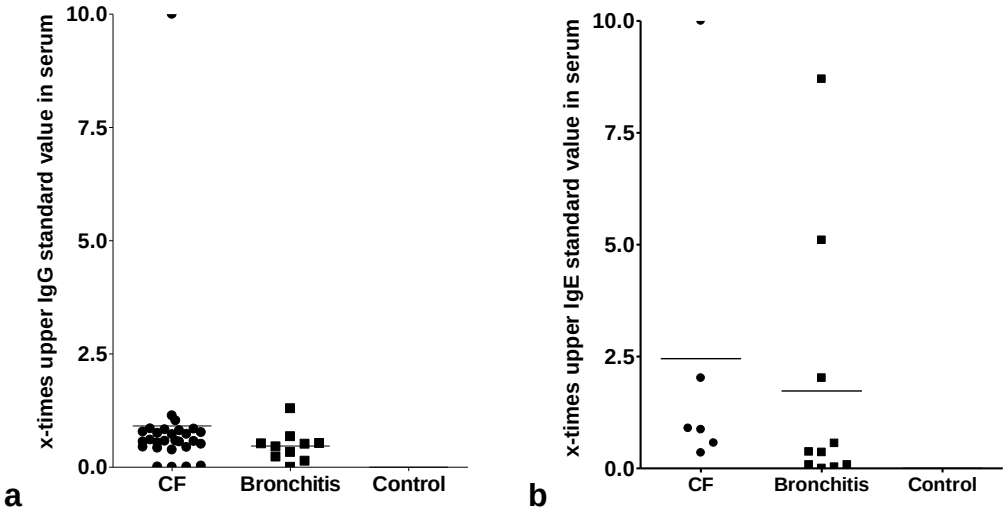


Figure 3.1.1.5: **IgG and IgE levels in serum.** The graphs show the distribution of the ratio of the IgG (a) and IgE (b) levels in serum of the patients in comparison to the upper standard values. There was no significant difference analyzed by t-test. IgG levels were available for 20 CF and 10 bronchitis patients and IgE levels for 11 CF and 7 Bronchitis patients.

Table 3.1.1.1:**Clinical characteristics of the CF patients included in the structure and function population.**

Characteristic		n
Lung disease (% mild/% severe)	72/28	33/13
Lung disease not determined (%)	12	7
Fev1 (% pred.)$_{age20}$	94 ± 26	46
∆Fev1 (% pred.)	- 3.34 ± 9.8	42
***P. aer.* infection at sample point (%)**	20	46
ABPA (%)	7	46
Neutrophils in BAL (%)	28 ± 19	39

In the table the clinical characteristics of the CF patients are listed as mean values with standard deviation or the percentage.

The CF patients included in this population had mild lung disease as indicated by a normal mean FEV1 and the majority had a normal weight expressed as BMI, a low rate of P. aeruginosa positive culture status and normal level of serum IgG, a marker of the systemic inflammatory response. In contrast to the controls and the subjects with chronic bronchitis, the CF patients had a moderately increased neutrophilic airway inflammation. The total levels of SP-A in serum or BAL were not different between the patients with CF and those with bronchitis and the controls (Fig. 3.1.1.1-3.1.1.5 and table 3.1.1.1).

3.1.2) SP-A structure analysis by gel chromatography

To determine structural differences in BAL and serum between the study populations a gel chromatography method with a superpose 6 column was chosen. In this method SP-A molecules were separated with regard to their oligomerization form. 1 ml BAL and 500 µl serum were loaded. The larger the oligomers the earlier they were eluted. After gel chromatography the SP-A amount of the eluted fractions (800 µl) was determined by Slot-Blot. The void volume of the column was about 6.8 ml (between fraction 8 and 9). The peaks were defined as a relative SP-A amount of more than 20 % of the total SP-A eluate from the column and a distance of 5 fractions (each 0.8 ml) between each peak (mainly in fraction 10, 15 and 20). The peak areas ranged from fraction 9-12, 13-17 and 18-22. For calculation also the first peak from fraction 9-12 and the last peak from fraction 18-22 were defined.

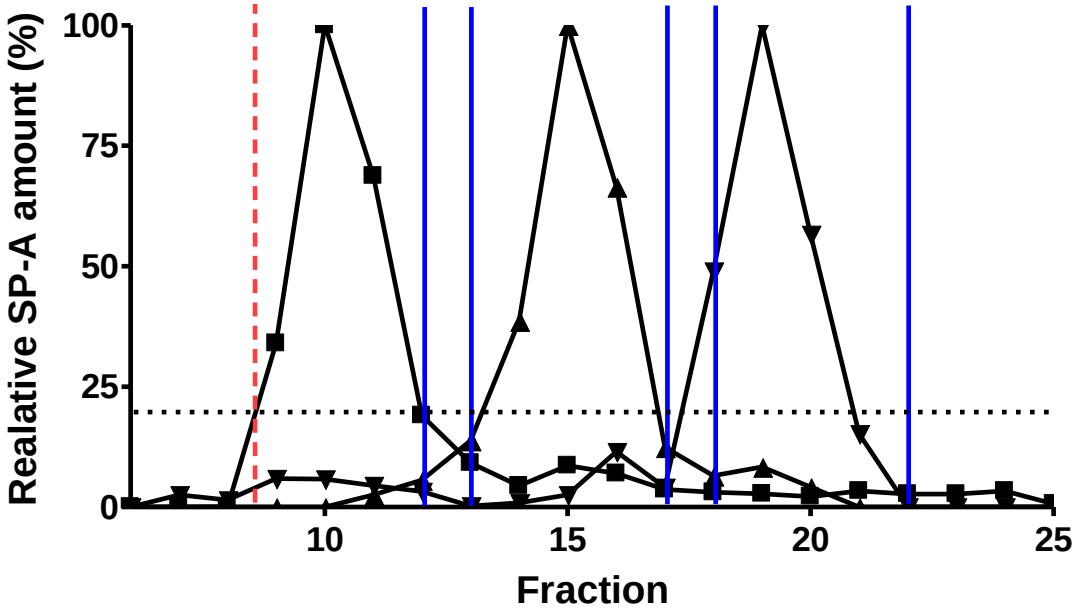
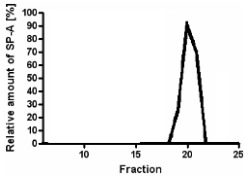
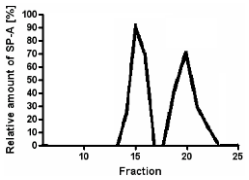
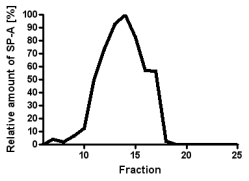
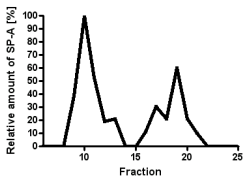
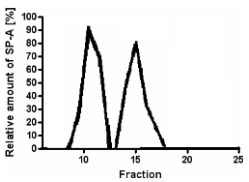
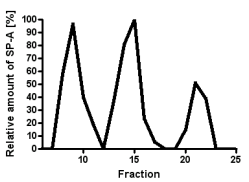
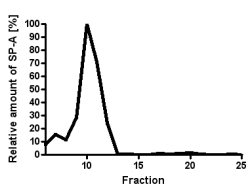


Figure 3.1.2.1: **Peak definition.** The graph illustrates a scheme of the defined, possible peaks of SP-A after gel chromatography and the calibration with proteins of a known molecular weight. On the x-axis the number of the fractions and on the y-axis the relative SP-A amount in % is plotted. With regard to the molecular weight of the calibration proteins the first peak should contain octadecamers or more complex oligomers, the second sixmers to twelvemers and the last peak dimers and trimers.

3.1.3) Different peak distribution in study populations

After analysis of the 59 serum (34 CF, 14 Bro and 11 C) and 81 BAL samples (42 CF, 21 Bro and 18 C) by gel chromatography a wide variability of oligomeric SP-A forms could be observed. For an easier handling the peak distribution was coded as 1 if there was a peak at the first, second or third peak position and 0 if there was none (all individual results are shown in appendix D). This coding system is illustrated in Table 3.1.3.1.

Table 3.1.3.1: **Peak distribution patterns and translation.**

Graphs of SP-A peak distribution	Coded as
	**001**
	**011**
	**010**
	**101**
	**110**
	**111**
	**100**

The table illustrates the different peak distribution patterns of SP-A resulting by analysis of BAL and serum samples with gel chromatography and the translation.

In BAL most commonly forms that contained the first peak were observed and there was no obvious difference of distributions between the study populations, but the bronchitis and control populations had more SP-A in the last peak (see figures 3.1.3.1).

In serum all control samples showed the second peak and partly the last peak, but none contained the first peak. In sera of patients with CF or chronic bronchitis and asthma there was more variability, but only the CF samples contained mainly the first peak (see figure 3.1.3.2).

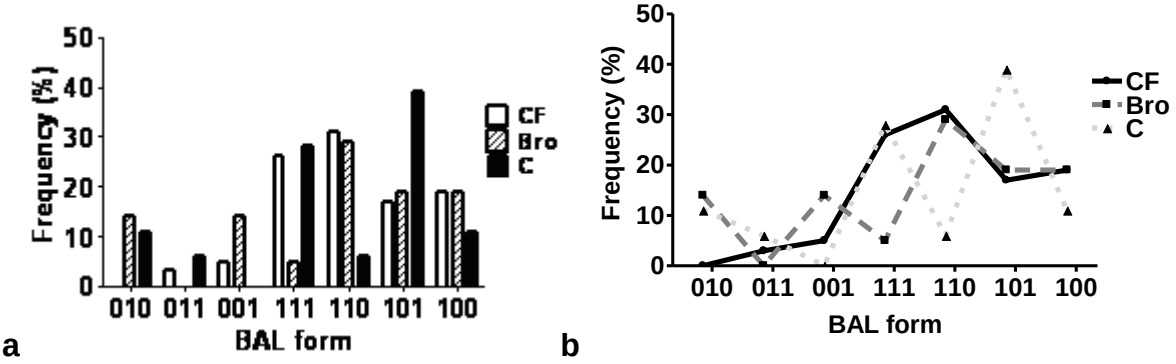


Figure 3.1.3.1: **Oligomeric distribution of SP-A in BAL.** The graphs illustrate the distribution frequency (y-axis) in correlation with the oligomeric form distribution (x-axis). The BAL samples of 42 CF patients, 21 chronic bronchitis and asthma patients (bronchitis) and 18 controls were separated by gel chromatography and then the fractions were analyzed with regard to their SP-A content by Slot-Blot. The most common forms contained the first peak and there was no obvious difference of distributions between the study populations. Analyzed by Fisher's exact test there were no significant differences between the patient groups with respect to the frequency comparing each form separately, but comparing the complete distribution frequency there is a highly significant difference ($p < 0.0001$).

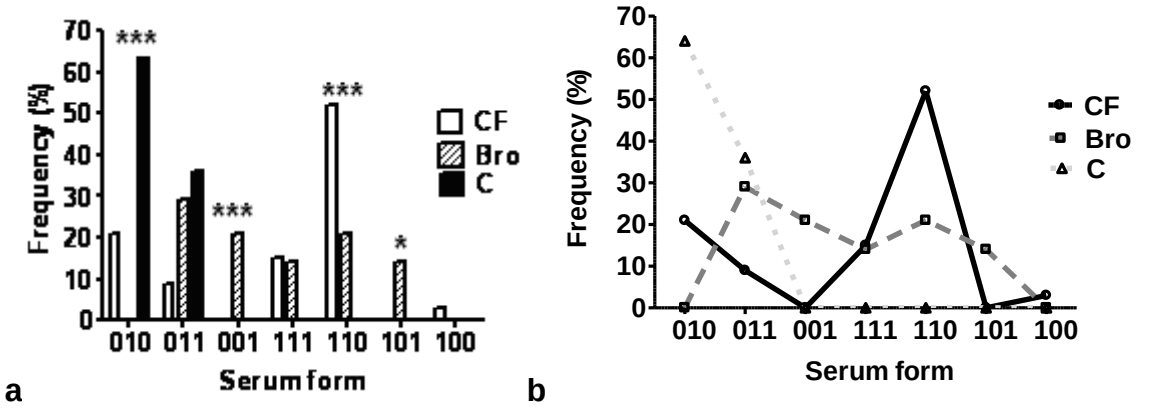


Figure 3.1.3.2: **Oligomeric distribution of SP-A in serum.** The graphs illustrate the distribution frequency (y-axis) in correlation with the oligomeric form distribution (x-axis). The serum samples of 34 cystic fibrosis (CF) patients, 14 chronic bronchitis and asthma patients (Bro) and 11 controls (C) were separated by gel chromatography and then the fractions were analyzed with regard to their SP-A content by Slot-Blot. In controls all samples showed the second peak and partly the last peak, but none contained the first peak. In sera of CF, chronic bronchitis and asthma patients there were more variability, but only the CF samples contained mainly the first peak. Analyzed by Fisher's exact test there was a significant difference between the patient and control groups regarding the frequency of the SP-A form 010 ($p = 0.0008$), 001 ($p = 0.006$), 110 ($p = 0.0027$) and 101 ($p = 0.036$). Comparing the complete distribution of all forms between the patient and control groups there is also a significant difference ($p < 0.0001$).

Next the corresponding pairs of 47 BAL and serum samples from 30 CF, 10 bronchitis patients and 7 controls were examined (see table 3.1.3.2). The most frequent pairs contained the first peak in BAL and serum. There were some sample pairs with the same form in BAL and serum, but also pairs with the opposite forms. No correlation between the SP-A distributions in BAL and serum could be found.

Table 3.1.3.2: **BAL and serum pair distribution.**

CF		Bronchitis		Control	
Serum	**BAL**	**Serum**	**BAL**	**Serum**	**BAL**
111	111	111	101	111	011
111	110	111	001	011	111
111	110	110	100	011	111
111	010	110	100	011	100
111	010	110	010	011	001
110	111	101	101	010	101
110	111	101	010	010	001
110	111	011	101		
110	111	011	100		
110	110	001	001		
110	110				
110	110				
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110	101				
110	100				
110	100				
110	100				
110	100				
100	110				
011	111				
011	101				
011	100				
010	111				
010	111				
010	110				
010	110				

The table shows the distribution of BAL and serum forms for each disease group and controls. The yellow cells mark same SP-A structure distributions in BAL and serum levels and the red cells opposite distributions. Generally the concordance between BAL and serum was very low. In the CF group there were 5 matches and 1 opposite distributions in 47 patients, in the bronchitis group were 2 matches and 2 opposite distributions in 10 patients and in the control group there were no matches and 2 opposite distributions in 7 controls.

Because different oligomeric distributions between individuals and study populations were observed in the following the correlations between the lung function and the oligomeric SP-A distribution form in BAL and serum were analyzed.

A slight increase regarding Fev1 (% pred.)$_{age20}$ can be observed the more SP-A is existent in the first peak in BAL (111 to 100, see figure 3.1.3.3). In BAL samples where all three peaks could be observed (111) SP-A is present in dimers/trimers, smaller oligomeric forms like hexamers and only partly in complex oligomeric forms like octadecamers. In samples where only the first peak is present (100) SP-A is mainly existent in complex forms. The slightly better lung function of the 101 form in comparison to the 110 form could be explained in BAL by comparing the peaks in each form. In the 101 form the first peaks were bigger in contrast to the first peaks in the 110 forms (mean percentage of first peak in 101 = 45 % and 110 = 39%).

In serum the distributions show the same correlation except for the 101 form (see figure 3.1.3.4). There were no correlations between the Δ Fev1 (% pred.)/year or the lung disease (mild/severe) and forms in serum or BAL.

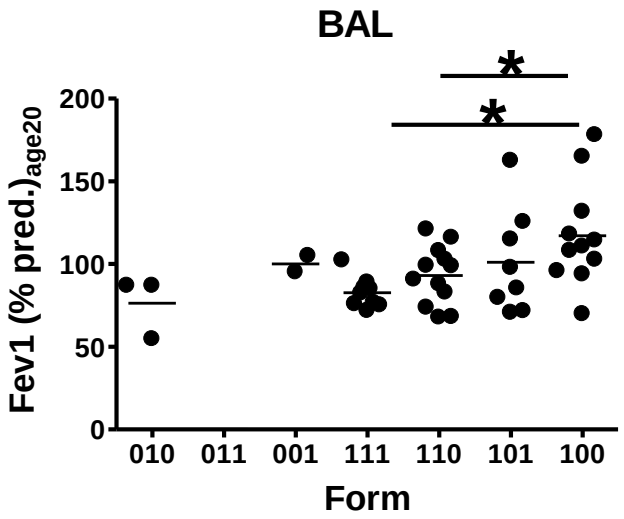


Figure 3.1.3.3: **Oligomeric SP-A distribution in BAL and lung function in CF.** The graph plots the correlation between the lung function (y-axis) and the oligomeric SP-A distribution in BAL. An increase in Fev1 (% pred.)$_{age20}$ can be observed the more SP-A is existent in the first peak (111 to 100). In BAL samples where all three peaks could be observed (111) SP-A is present in dimers/trimers, smaller oligomeric forms like hexamers and only partly in complex oligomeric forms like octadecamers. In samples where only the first peak is present (100) SP-A is mainly existent in complex forms. 43 BAL samples of CF patients were analyzed. For analysis One way ANOVA with Tukey as post-test was used. * means a p-value < 0.05.

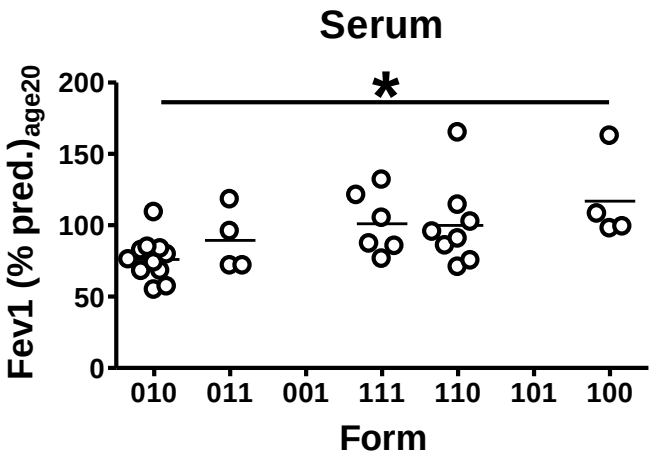


Figure 3.1.3.4: **Oligomeric SP-A distribution in serum and lung function in CF.** The graph plots the correlation between the lung function (y-axis) and the oligomeric SP-A distribution in serum. As in BAL samples a slight, but not significant increase in Fev1 (% pred.)$_{age20}$ can be observed the more SP-A is existent in the first peak (111 to 100). In the samples where all three peaks could be observed (111) SP-A is present in dimmers/trimers, smaller oligomeric forms like hexamers and only partly in complex oligomeric forms like octadecamers. In samples where only the first peak is present (100) SP-A is mainly existent in complex forms. 34 serum samples from CF patients were analyzed by One way ANOVA with Tukey as post-test. * means a p-value < 0.05.

In conclusion there were no correlations between serum and BAL SP-A distribution forms. The forms showed only a significantly different distribution comparing serum samples from bronchitis patients and controls. In serum and BAL of CF patients the forms containing the first peak were correlated with a better lung function (Fev1 (% pred.)$_{age20}$).

3.1.4) Influence of SP-A structure on self-agglutination

Finally the self-agglutination abilities of BAL and serum were analyzed and compared to the oligomeric distribution forms of SP-A derived from gel chromatography using 100 ng SP-A from the 43 BAL and 34 serum samples of CF patients. While in serum the different forms indicated a marginal association with the self-agglutination ability, in BAL the differences were more distinctive.

In serum only the 010 and 110 forms showed a significant difference regarding the self-agglutination ability (see figure 3.1.4.1).

In BAL the 100 form agglutinated significantly better than the 111 and 110 form and the 101 form significantly better than the 111 form (see figure 3.1.4.1). Altogether the results imply that the 100 form agglutinates best with the rest showing a declining ability in the following rank order: 100>101>110>111>010.

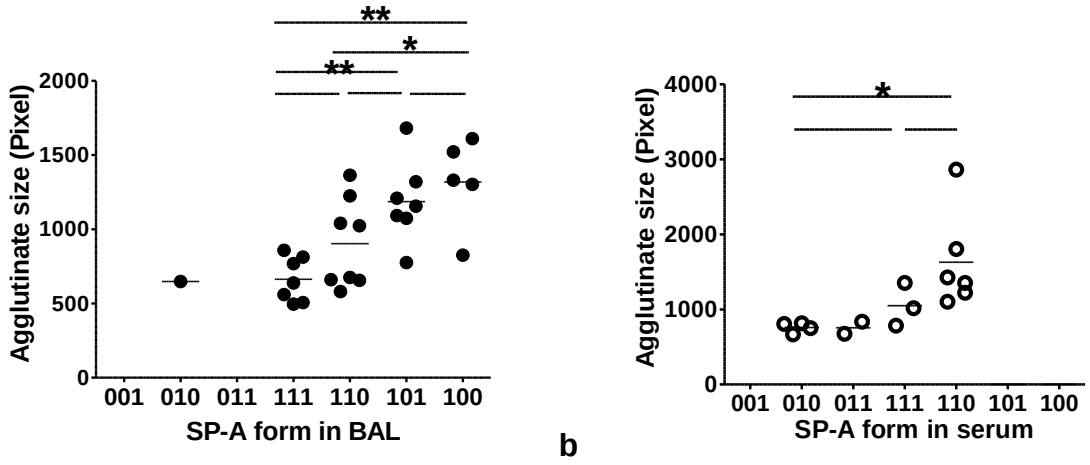


Figure: 3.1.4.1: **Self-agglutination ability of different structure compositions.** The graphs illustrate the self-agglutination ability differences between different oligomeric structure compositions (forms) in BAL (a) and serum (b) analyzed by gel chromatography. 42 BAL samples and 31 serum samples of CF patients were analyzed. The SP-A amount in the BAL or serum sample was adjusted to 100 ng for the self-agglutination analysis. While in serum the different forms indicated an approximate association with the self-agglutination ability, in BAL the differences were more distinctive. In serum only the 010 and 100 forms showed a significant difference regarding the self-agglutination ability. In BAL the 100 form agglutinated significantly better than the 111 and 110 form and the 101 form significantly better than the 111 form. Altogether imply the results that the 100 form agglutinates best with the rest showing a declining ability in the following order: 100>101>110>111>010. * stands for a p-value < 0.05 and ** for a p-value < 0.01 analyzed by One way ANOVA.

Next it was important to analyze if these correlations were caused by the structure composition of the whole samples, or if the individual fraction was responsible for the activity and if the fractions differed in activity. Therefore individual fractions (e.g. F10, F15 or F20) were isolated from various patients' samples with different forms, brought to the same final concentration and compared for their ability to self-agglutinate.

Whereas the individual fractions F10, F15, and F20 were very different in their activity, it did not matter what the source of these fractions was, as the activity within a certain fraction was the same. From these data we can conclude that the relative composition of the whole sample with respect to representation of the different oligomeric forms is responsible for its activity and not potential differences among the oligomeric forms within a particular fraction.

In BAL the self-agglutination ability in all fractions showed a wide variability without any correlation to the corresponding distribution form, while in serum the self-agglutination ability was on a same level in all forms for each fraction (see figure 3.1.4.2).

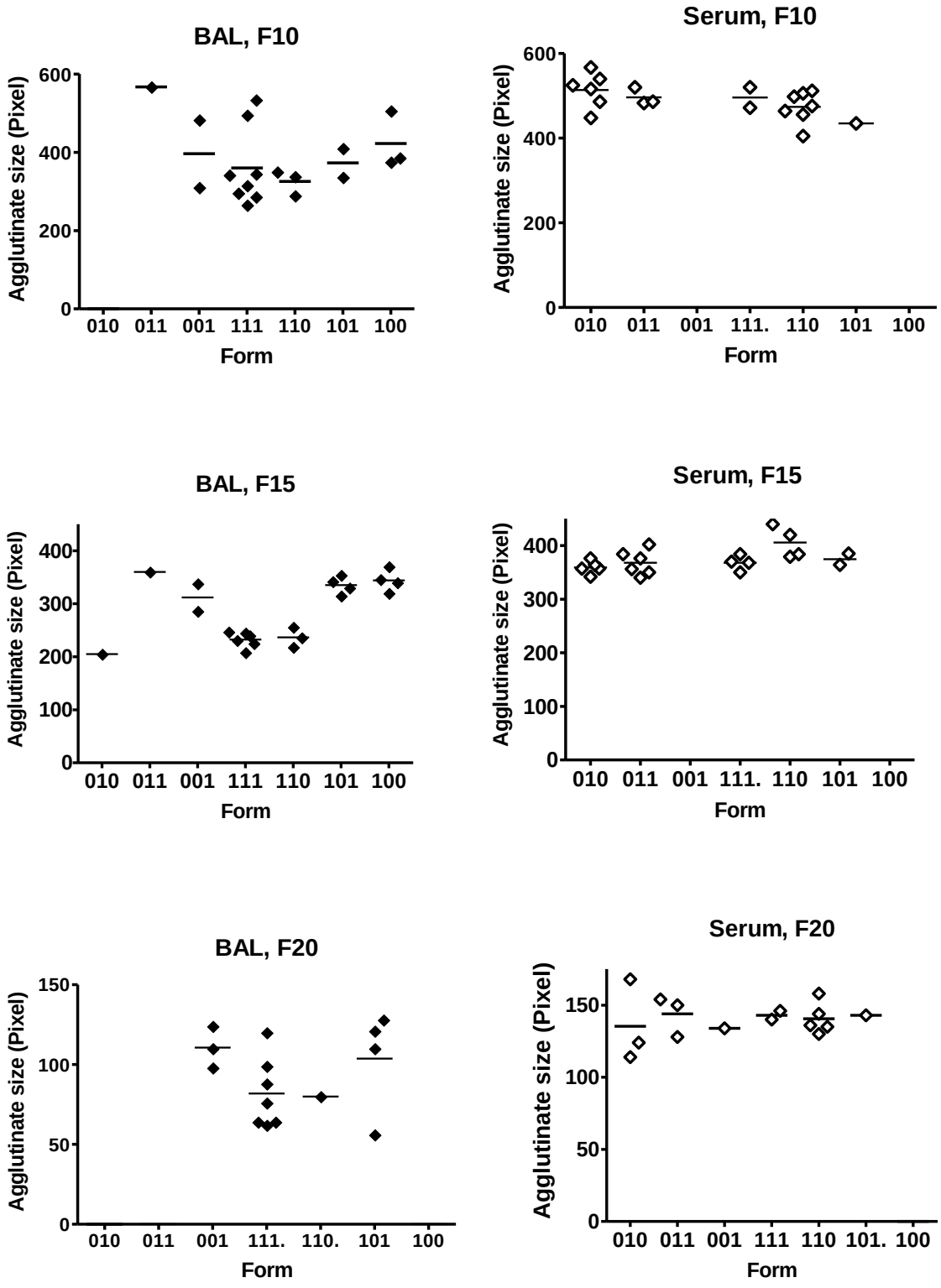


Figure 3.1.4.2: **Self-agglutination ability of fractions regarding the SP-A structure distribution in CF.** The graphs plot the SP-A structure distribution on the x-axis (form) against the self-agglutination ability on the y-axis (agglutinate size) for fraction 10, 15 and 20 derived from gel chromatography of BAL and serum samples of all study populations. In BAL and serum the increasing order correlated to the increasing lung function (Fev1 (% pred.)$_{age20}$) could not be supported. In BAL and serum there was no correlation analyzed by One way ANOVA ($p > 0.05$). The SP-A amount in the BAL or serum sample was adjusted to 100 ng for each test.

These data suggest that the correlation of Fev1 (% pred.)$_{age20}$ with the different oligomeric forms is related to the degree of the presence of active oligomeric forms within a sample. Therefore the relative contribution of higher oligomers within a sample was calculated, i.e. the percentage of SP-A present in the first peak.

3.1.5) Oligomerization in CF patients is influenced by lung disease, genetics and SP-A level

3.1.5.1) SP-A oligomerization and lung disease

Another approach to evaluate the distribution of SP-A oligomers, was to calculate the relative amount of SP-A in the first and the last peak. This was done by integrating the area under the peak, when the fraction number was plotted against the amount of SP-A in ng/ml; then the proportional amount of SP-A per peak was calculated. Only the first and the last peak were chosen for correlation analysis because the extremes of phenotype provide additional power to detect genetic modifiers or clinical influences.

There was a significant association of the lung disease status in CF patients and the percentage of SP-A that is existent as octadecamers or more complex structures (first peak) in BAL ($p = 0.02$, see figure 3.1.5.1.1), while in serum an association was slightly detectable but not significant.

Then the correlation between the course of lung disease (ΔFev1 (% pred.)) and the percentage of SP-A that was present in the first peak was analyzed and there was also a significant association in BAL and serum ($p = 0.0008$ and 0.0038, see figures 3.1.5.1.2 and 3.1.5.1.3). The more SP-A is existent in octadecamers or more complex structures the better is the lung function and the course of lung disease in CF patients.

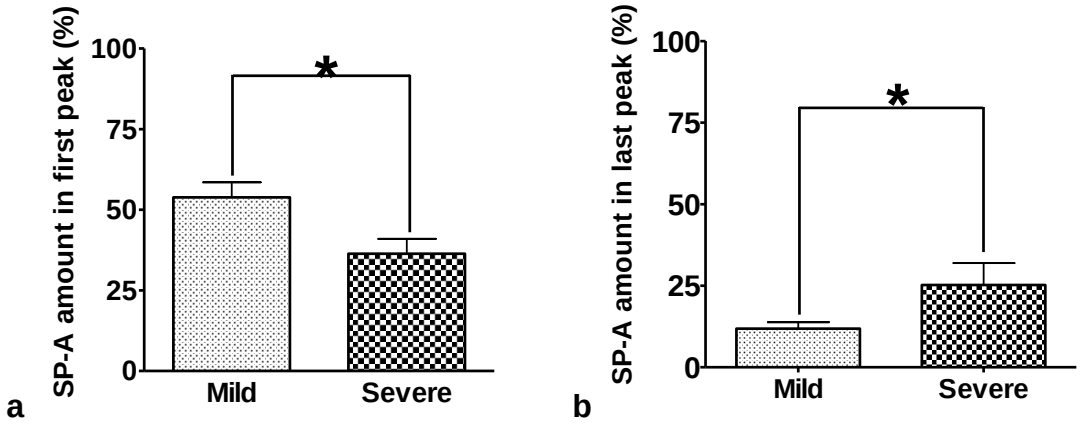


Figure 3.1.5.1.1: **BAL SP-A structure and lung disease in CF.** 42 BAL samples of CF patients were separated by gel chromatography. For the analysis of the 38 CF patients which could be classified regarding to their lung function the t-test was used. The graphs plot the lung disease status on the x-axis against the amount of SP-A existent in the first (a) or the last peak (b) respectively. There was a significant association of the lung disease status in CF patients and the percentage of SP-A that is existent as octadecamers or more complex structures (first peak) in BAL analyzed by t-test ($p = 0.02$, *).

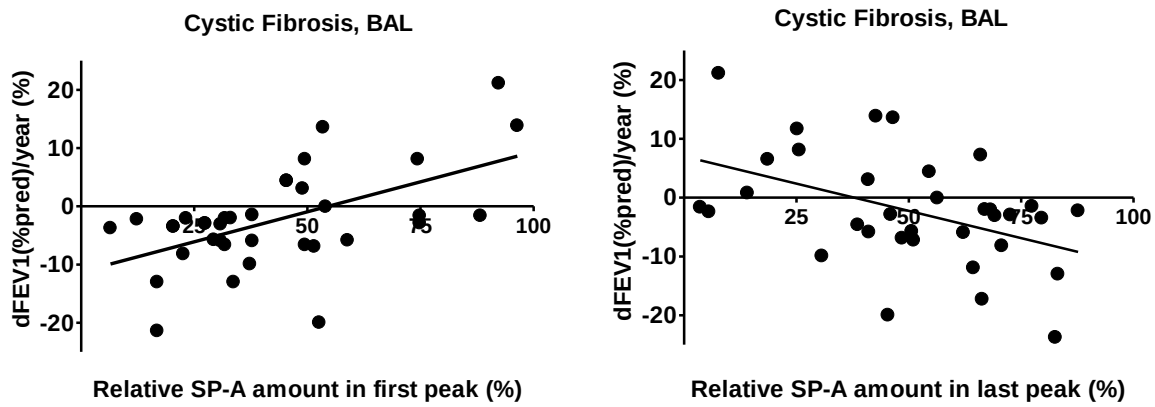


Figure 3.1.5.1.2: **BAL SP-A structure and course of lung disease in CF.** 42 BAL samples of CF patients were separated by gel chromatography. 38 CF patients with lung function data could be included for analysis. The graphs plot the course of lung disease on the y-axis against the amount of SP-A existent in the first (left) or the last peak (right) respectively. The correlation between the course of lung disease (∆Fev1 (% pred.)/year) and the percentage of SP-A that was present in the first peak was analyzed by linear regression and there was a significant association ($p = 0.009$, $r^2 = 0.8$) also by excluding too extreme values above or under a ∆Fev1 (% pred.)/year of 10 or -10 respectively.

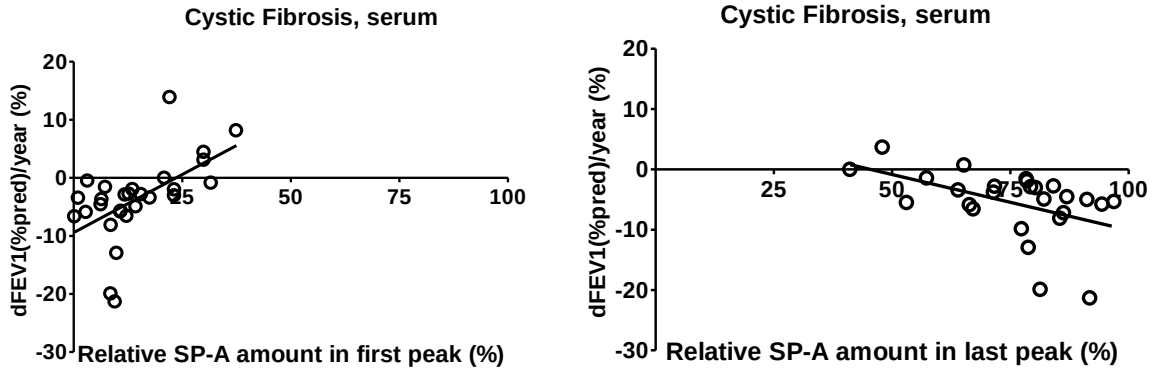


Figure 3.1.5.1.3: **Serum SP-A structure and course of lung disease in CF.** 34 serum samples of CF patients were separated by gel chromatography. For 30 patients lung function data was available. The graphs plot the course of lung disease on the y-axis against the amount of SP-A existent in the first (left) or the last peak (right) respectively. The correlation between the course of lung disease (∆Fev1 (% pred.)) and the percentage of SP-A that was present in the first peak was analyzed by linear regression and there was a significant association ($p = 0.004$, $r^2 = 0.5$) also by excluding too extreme values above or under a ∆Fev1 (% pred.)/year of 10 or -10 respectively ($p = 0.008$, $r^2 = 0.6$).

There was no correlation between the Fev1 (% pred.)$_{age20}$ and the SP-A structure distribution in BAL or serum (see figure 3.1.5.1.4).

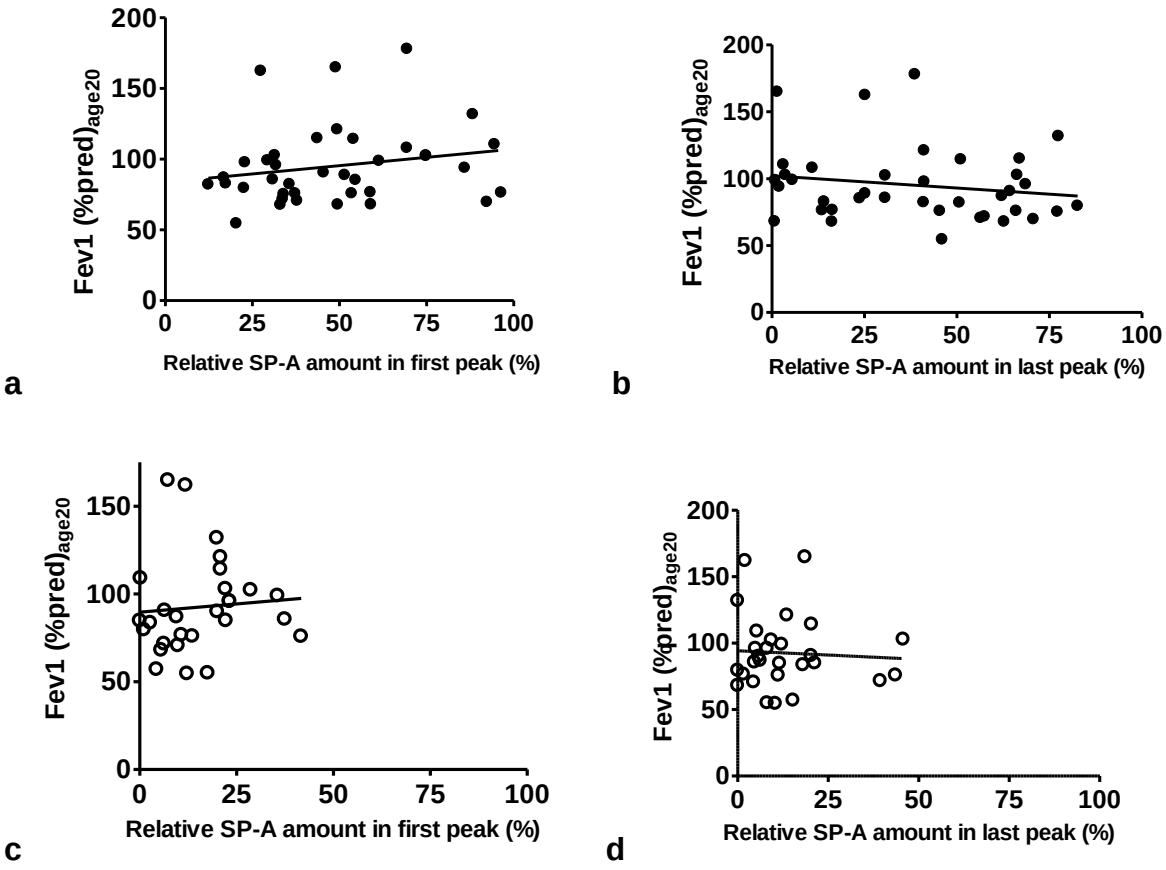


Figure 3.1.5.1.4: **BAL and serum SP-A structure and Fev1 (% pred.)$_{age20}$.** The graphs show the relative SP-A amount in the first peaks (a, c) and the last peaks (b, d) of BAL (a, b) and serum (c, d) in relation to the Fev1 (% pred.)$_{age20}$. There was no significant correlation analyzing the 38 (BAL) and 30 (serum) CF patients by linear regression.

In conclusion a higher relative SP-A amount in the first peak is associated with a milder lung disease and a better course of lung disease.

3.1.5.2) SP-A oligomerization and self-agglutination

Also the self-agglutination abilities of BAL and serum were analyzed and compared to the relative amount of oligomerization of SP-A derived from gel chromatography using 100 ng SP-A from the 43 BAL and 34 serum samples of CF patients. Like the oligomeric distribution forms also the relative SP-A amounts in the first and last peak in BAL showed a correlation with the self-agglutination ability (see fig.3.1.5.2.1). In serum there was no such correlation (see fig.3.1.5.2.1).
There was no correlation between the self-agglutination of the SP-A fractions 10, 15 and 20 and the relative SP-A amount in the first and last peak.

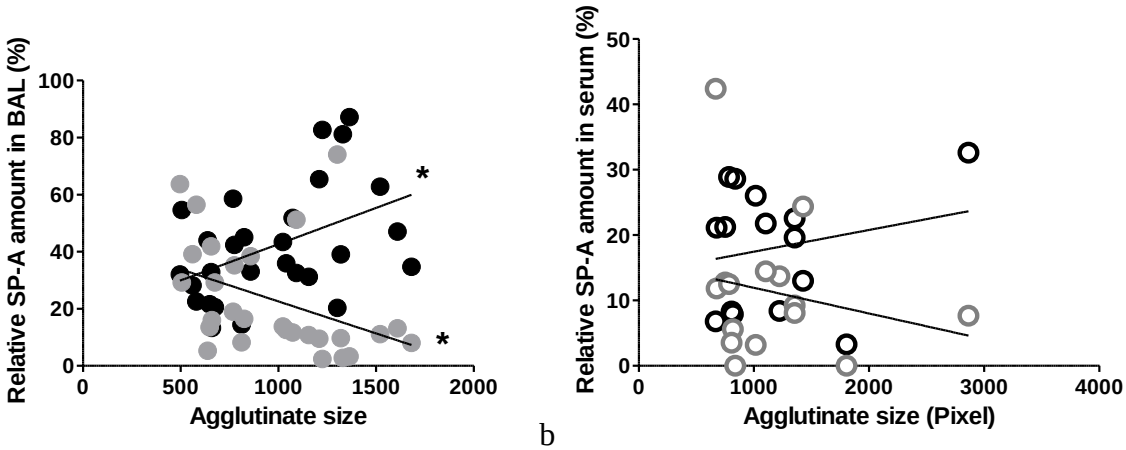


Figure 3.1.5.2.1: **BAL and serum SP-A structure and self-agglutination.** The graphs illustrate the relative amount of SP-A in the first (black) and last (grey) peak after column separation in BAL (a) and in serum (b) with regard to the self-agglutination ability. The SP-A amount in the BAL or serum sample was adjusted to 100 ng for the self-agglutination analysis. On the x-axis the size of the SP-A agglutinate is shown, while the y-axis plots the relative SP-A amount. There was a significant correlation between the relative SP-A amount in the first and last peak in BAL and the self-agglutination ability (first peak: r^2=01971; p=0.0174; last peak: r^2=01591; p=0.0355). For serum, only trends, but no significant correlations were observed. This was analyzed by linear regression of 28 (BAL) and accordingly 15 (serum) data of CF patients.

The more SP-A was present as higher oligomers (in the first peak) and the less in smaller oligomers (last peak) the bigger was the agglutinate.

SP-A self-agglutination is dependent on the amount of particular SP-A oligomers present in a sample, i.e. the relative strength of SP-A molecular forms in a sample. This supports the finding that also the correlation of the oligomeric distribution patterns with the Fev1 (% pred.)$_{age20}$ is caused by the composition of the different oligomeric forms and not by differences of the oligomeric forms in the fractions because the self-agglutination ability is supposed to be adequate to the functional ability of SP-A.

3.1.5.3) Influence of SP-A self-agglutination ability on Fev$_1$ (% pred.)$_{age20}$ and ΔFev$_1$ (% pred.)/year

Because of the correlation between SP-A structure and lung function and between the SP-A structure and the self-agglutination ability it was also searched for a correlation between the self-agglutination ability and lung disease severity. For Fev1 (% pred.)$_{age20}$ there was in BAL (p = 0.0137) and serum (p = 0.0338) a significant correlation (shown in fig. 3.1.5.3.1 a and b). Additionally, for ΔFev$_1$ (% pred.)/year there was also a significant correlation in BAL (p = 0.0213) and weakly in serum (p = 0.0.497) which is shown in fig. 3.1.5.3.2 a and b. These results indicate the better the self-agglutination ability of SP-A the better is the lung function. There was no correlation between the lung disease status (mild/severe) and the agglutination size.

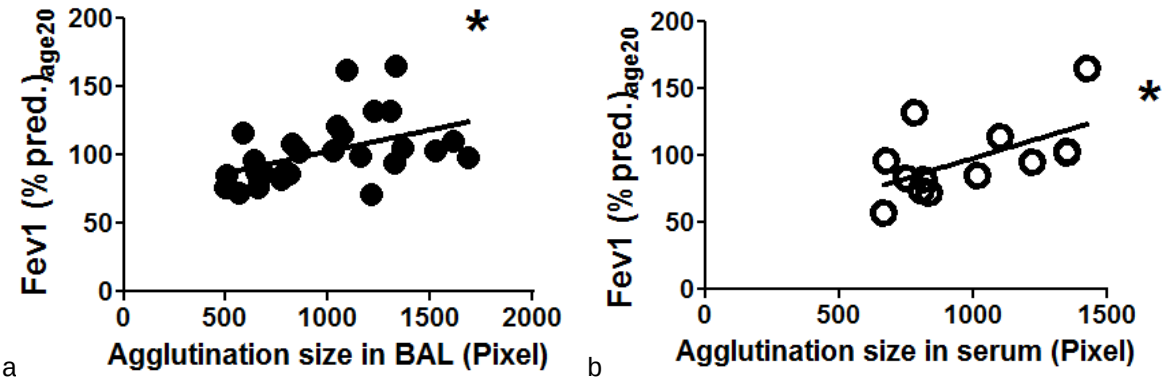


Figure 3.1.5.3.1: Correlation of Agglutinate size and Fev1 (% pred.)$_{age20}$. The graphs show on the x-axis the SP-A agglutination size in BAL (a) and serum (b) in Pixel and in the y-axis the Fev1 (% pred.)$_{age20}$. 28 BAL samples and 12 serum samples of CF patients were used. The p-values analyzed by linear regression were 0.0137 (r^2 = 0.2119) for BAL and 0.0338 (r^2 = 0.3480) for serum.

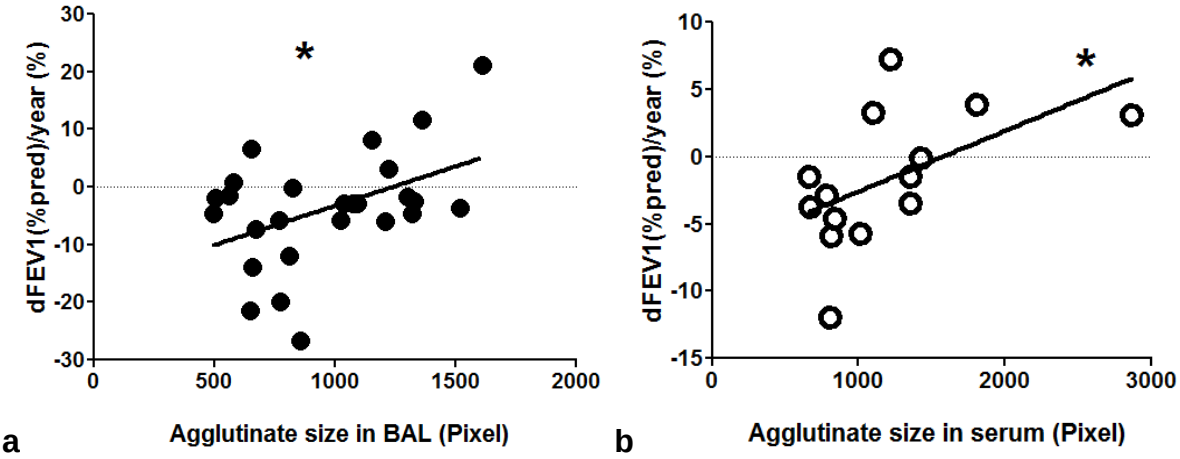


Figure 3.1.5.3.2: The Agglutinate size and ΔFev$_1$ (% pred.)/year. The graphs plot on the x-axis the agglutinate size in BAL (a) or serum (b) and on the y-axis the ΔFev$_1$ (% pred.)/year. 26 BAL samples and 14 serum samples of CF patients could be included. The p-values analyzed by linear regression were 0.0213 (r^2 = 0.2017) for BAL and 0.0497 (r^2 = 0.2841) for serum.

These results indicate the better the self-agglutination ability of SP-A the better is the lung function.

3.1.5.4) SP-A oligomerization and genetics

Because of the different oligomeric SP-A distribution patterns it was searched for a genetic background in the CF structure group (no.:1-5, 7; n=46). Therefore the *SFTPA*1 and *SFTPA*2 genotypes from MALDI-TOF were correlated with the percentage of SP-A (>20% yes/no) which was present in the first peak in relation to the percentage of SP-A which was present in the last peak. Only the extremes (first and last peak) were examined to have a better capability of to find significant correlations.

In BAL, but not in serum, two SNPs, rs1136451 (*SFTPA*1, see figure 3.1.5.4.1 a) and rs17881665 (*SFTPA*2, see figure 3.1.5.4.1 b), were significantly associated with the formation of octadecamers or more complex structures presented by the first peak (p = 0.0108 and 0.0122). The wildtype alleles of both SNPs were associated with octadecamers or more complex structures. An analysis of a correlation between both SNPs indicated a linkage disequilibrium (p = 0.00001). The *SFTPA*1 SNP is a polymorphism in the coding region of the gene (P62P), but it doesn't lead to an amino acidic exchange. The *SFTPA*2 SNP is located in the 5'-UTR of the gene where also splicing and other regulative sequences influencing the SP-A expression exist.

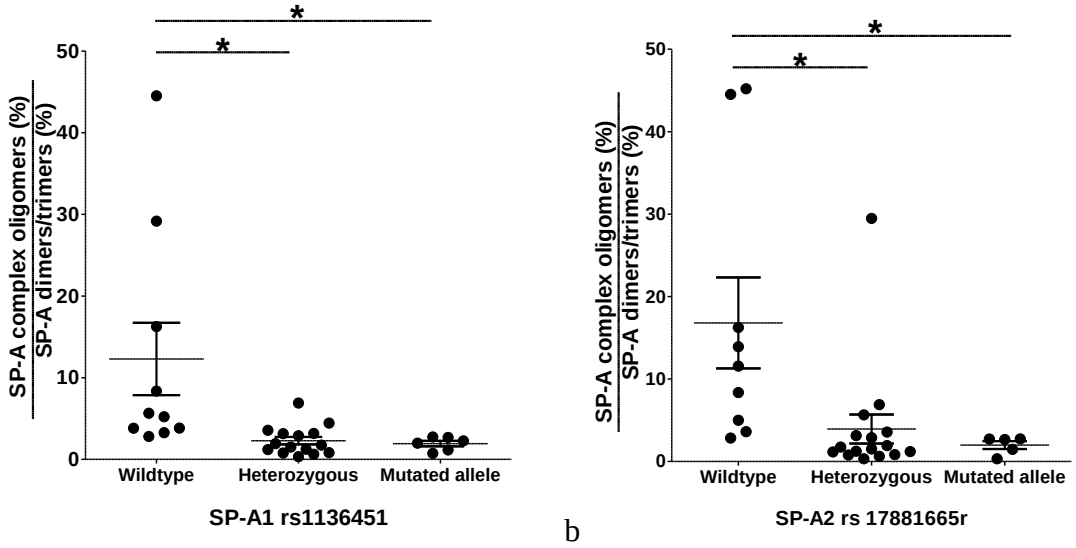


Figure 3.1.5.4.1: a) SNP rs1136451 and BAL SP-A structure. and b) SNP rs17881665 and BAL SP-A structure. 11 *SFTPA*1 and 11 *SFTPA*2 SNPs were genotyped by MALDI-TOF and correlated with the SP-A structure in BAL (ratio of the relative amount of SP-A existent in octadecamers to the relative amount of SP-A existent in dimers/trimers). The genotype (wildtype, heterozygous, mutated allele; x-axis) was plotted against the ratio of the relative amount of SP-A in the first peak compared to the relative SP-A amount in the last peak on the y-axis. The wildtype allele of the SNP rs1136451 in the coding region of *SFTPA*1 was significantly associated with the formation of octadecamers or more complex structures (p = 0.0108) analyzed by One-way ANOVA with Tukey as post-hoc test. The wildtype allele of the SNP rs17881665 in the 5'-UTR of *SFTPA*2 was also significantly associated with the formation of octadecamers or more complex structures (p = 0.0122) analyzed by One-way ANOVA with Tukey as post-hoc test.

No other correlation between these SNPs and other parameters (e.g. lung function) could be found. This supports again the results that the composition of the oligomers and not the nominal amount of a particular oligomeric type (e.g. monomers, trimers, etc.) is important for the functional ability of SP-A. There were also no other significant correlations between any other SNPs and parameters in this group.

Because both SNPs don't lead to an amino acid exchange, the only possible influence on the protein could be on the expression level. Therefore the next step was the analysis of the correlation between

the SP-A level and the SP-A structure. There was a significant association (p = 0.0006, see figure 3.1.5.4.3) indicating that the higher the SP-A level was in BAL the bigger was the percentage of SP-A which was existent in octadecamers or more complex structures.

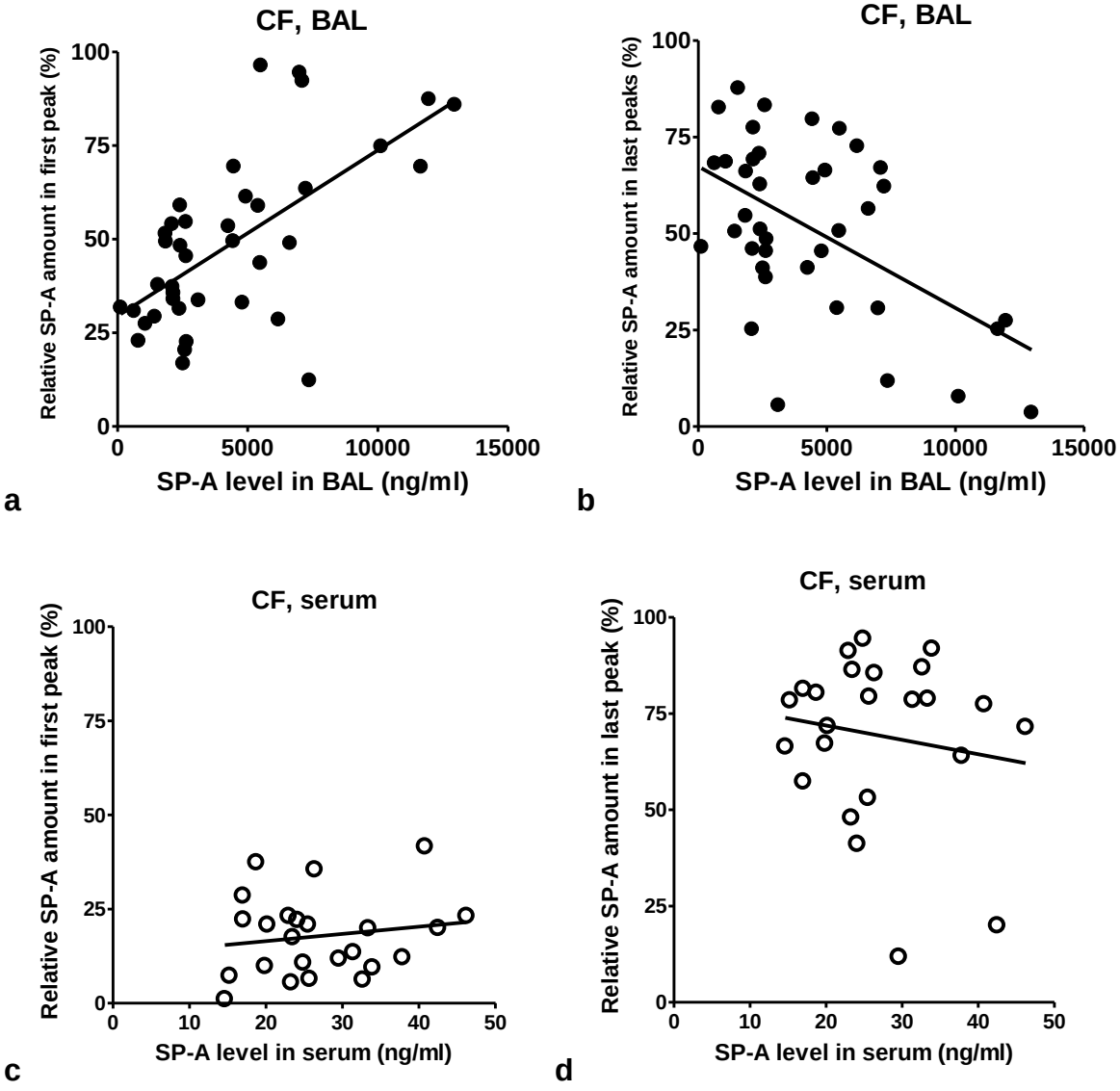


Figure 3.1.5.4.3: **Correlation of BAL SP-A level and structure in CF.** The graphs illustrate the correlation of the SP-A level in BAL (a, b) or serum (c, d) (x-axis) and the SP-A structure (y-axis) as the relative amount of SP-A in the first (a, c) or the last peak (b, d) after gel chromatography.
There was a significant association (p = 0.0006, r^2 = 0.5) analyzed by linear regression indicating that the higher the SP-A level was in BAL the bigger was the percentage of SP-A which was existent in octadecamers or more complex structures (in the first peak) in BAL.
In serum there was no correlation (p = 0.46, r^2 = 0.02). 38 BAL samples and 24 serum samples were analyzed.

3.1.6) Single molecule forced microscopy

To finally examine the structure of SP-A in a fraction after gel chromatography the method of single molecule force spectroscopy based on atomic force microscopy was used. Only one molecule was attached to a gold layer and then pulled of by a cantilever, while the applied force is measured.

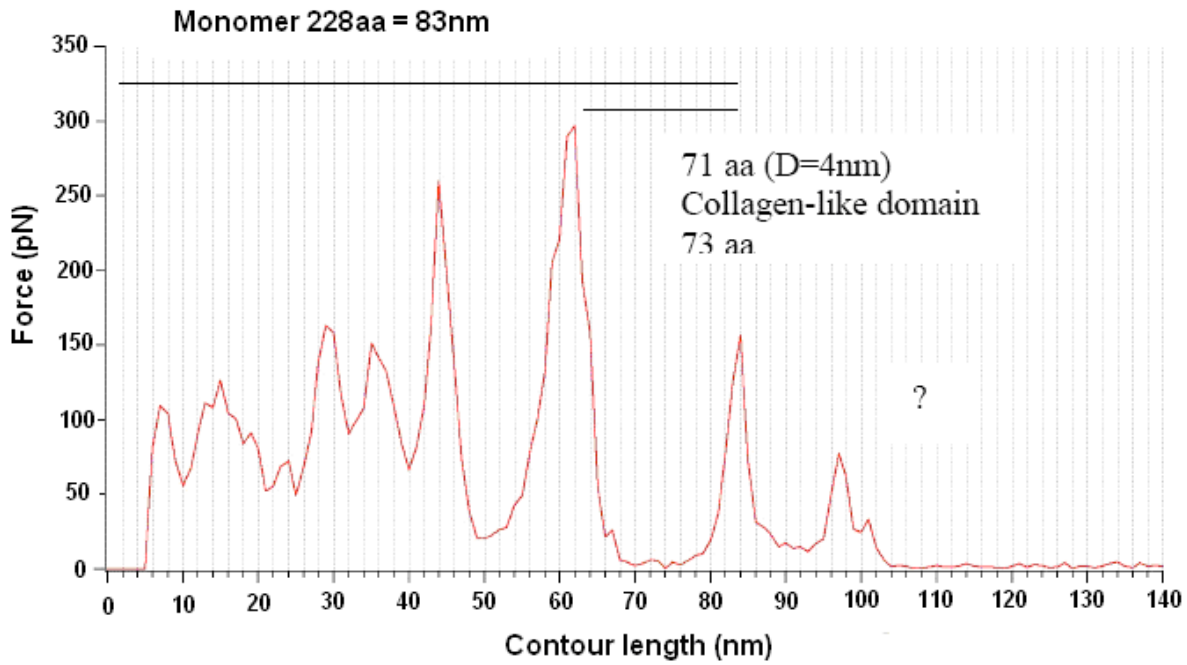


Figure 3.1.6.1: **Force spectrum of SP-A.** The graph illustrates the superimposed curves of unfolding force spectrums of SP-A fragments. SP-A was isolated of pooled fractions from the first peak (F10) after gel chromatography of BAL from 12 CF patients by beads at pH 2.5. Only one molecule was attached to a gold layer and then pulled of by a cantilever, while the applied force is measured.

About 2,000 measurements were completed. There were curves about 100 nm long which would correspond to about 270 aa. These curves could depict SP-A monomers. There were also curves corresponding to about 66 aa which could display the collagen-like domain. Because there were no longer curves it must be concluded that there were no multimers in the solution. There were also a lot of smaller curves which had to be fitted and put in order. After all a force spectrum of a SP-A monomer could be developed by superimposition of the curves.

3.2) SP-A self-agglutination

For a better understanding of the SP-A self agglutination assay used to correlate the SP-A function with different parameters and to analyze possible differences of SP-A function between patient groups (CF, chronic bronchitis and controls) further experiments were executed.

3.2.1) Samples analyzed by SP-A self-agglutination-assay

Because it is known that there are structural differences of SP-A between individuals, it was necessary to elucidate if there are functional differences between different oligomeric states. Therefore the BAL and serum sample fractions arisen from gel chromatography of 10 CF patients, 10 chronic bronchitis and 7 control patients were further analyzed with a SP-A self-agglutination-assay (100 ng SP-A per sample was analyzed). These patients correspond to group no. 1-6 in scheme 2.1.1.in the methods. The DNA from all patients was genotyped by MALDI-TOF, the samples were analyzed by gel chromatography and the clinical data were collected.

Table 3.2.1.1: **Sample distribution of the BAL and serum analysis.**

Sample	N
BAL and serum pairs	8
BAL	20
Serum	7

The table shows the number of samples available for the SP-A self-agglutination analysis of the BAL and serum samples.

3.2.2) Relation between SP-A self-agglutination and structure

The self-agglutination assay was used to determine the functional capability of SP-A regarding its CRD. Because different oligomeric structures of SP-A were found in BAL and serum of all study groups the fractions number 10, 15 and 20 of the gel chromatography representing the three peaks were analyzed by the self-agglutination assay. In all three study populations, CF, asthma and chronic bronchitis patients and controls, there was a significant difference regarding the self-agglutination ability between the fractions in BAL and serum (see figure 3.2.2.1). There was a significant decrease regarding the self-agglutination ability from SP-A octadecamers or more complex structures present in fraction 10 over the hexamers present in fraction 15 to the dimers and trimers present in fraction 20. Comparing the fractions between BAL and serum in the patient groups there was also a significant difference (see figure 3.2.2.2). The serum SP-A structures had significantly better self-agglutination ability than the corresponding structures in BAL. While in CF patients this difference was highly significant for all structures in the bronchitis study population the significance decreased from fraction 10 over fraction 15 to fraction 20. In controls all fractions and therefore all SP-A structures in serum and the corresponding structures in BAL showed the same self-agglutination ability. At last the

fractions in BAL and serum were compared between the study populations (see figure 3.2.2.3 and 3.2.2.4). For all BAL fractions the SP-A oligomers derived from controls agglutinated significantly better than the SP-A oligomers derived from patients. Also all BAL structures from the bronchitis study population agglutinated better than the structures derived from CF patients, but the difference was only significant in fraction 15. In contrast to these findings in serum there were no differences regarding the self-agglutination ability between the study populations.

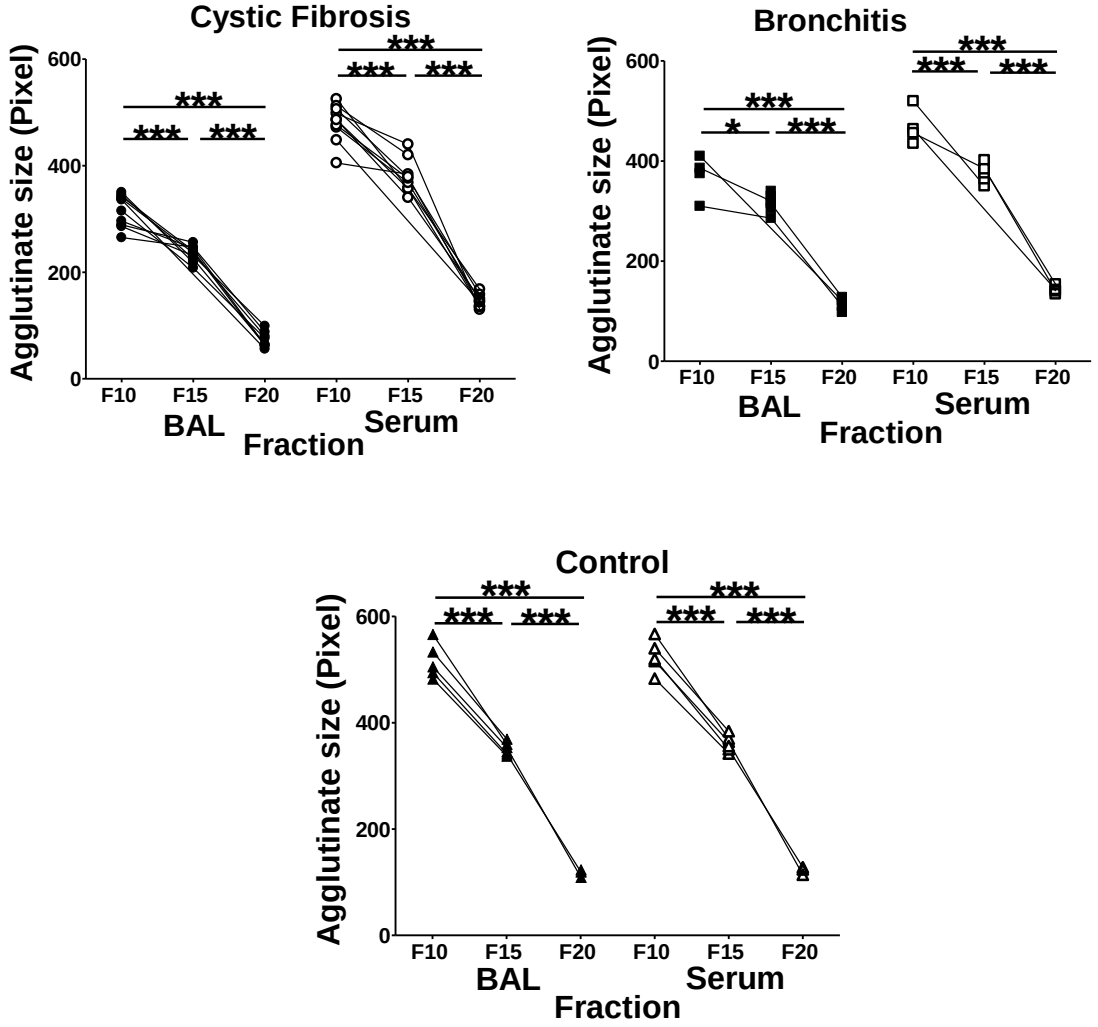


Figure 3.2.2.1.: **SP-A self-agglutination and fractions.** The graphs show the self-agglutination ability (y-axis) of different SP-A structures derived from BAL and serum (x-axis) of the study populations. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD. The SP-A amount was adjusted to 100 ng. In all study populations (10 CF, 10 Bro, 7 C) there was a significant difference (*** means $p < 0.0001$ and * means $p < 0.05$) between the self-agglutination ability of the different structures (F10, F15 and F20) analyzed by One way ANOVA. The octadecamers or more complex structures showed the best self-agglutination ability followed by the hexameric structures and then the dimers/trimers.

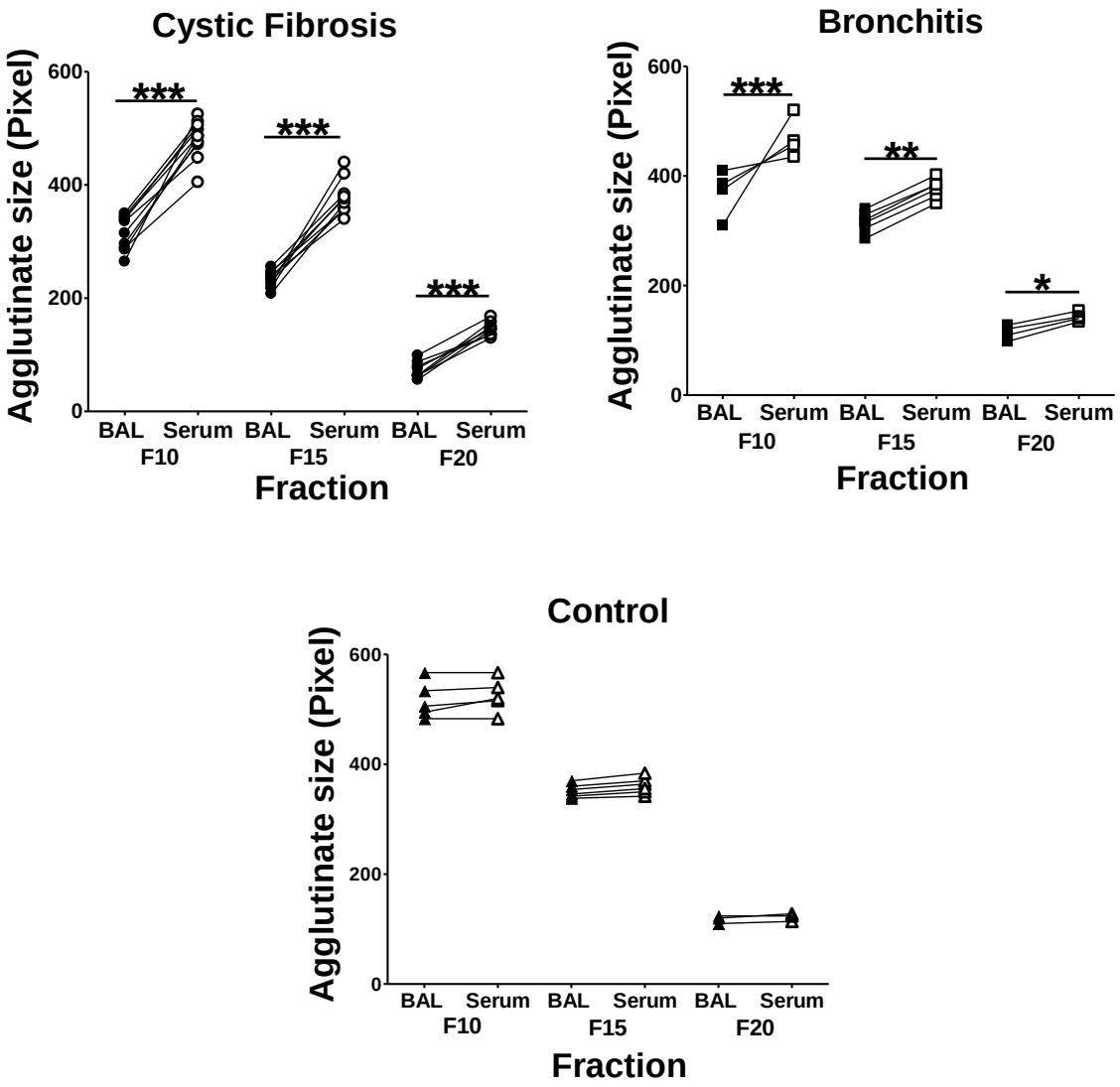


Figure 3.2.2.2: **Self-agglutination ability of SP-A out of BAL vs. serum.** The graphs illustrate the self-agglutination ability (y-axis) of different SP-A structures derived from BAL and serum (x-axis) of the study populations. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD. The SP-A amount was adjusted to 100 ng. Only in the patient populations (10 CF, 10 Bro, 7 C) all SP-A structures derived from serum fractions agglutinated significantly better than the corresponding structures derived from BAL (* stands for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$) analyzed by One way ANOVA. While in CF patients this difference was highly significant for all structures in the bronchitis study population the significance decreased from fraction 10 over fraction 15 to fraction 20. In controls all fractions and therefore all SP-A structures in serum and the corresponding structures in BAL showed the same self-agglutination ability.

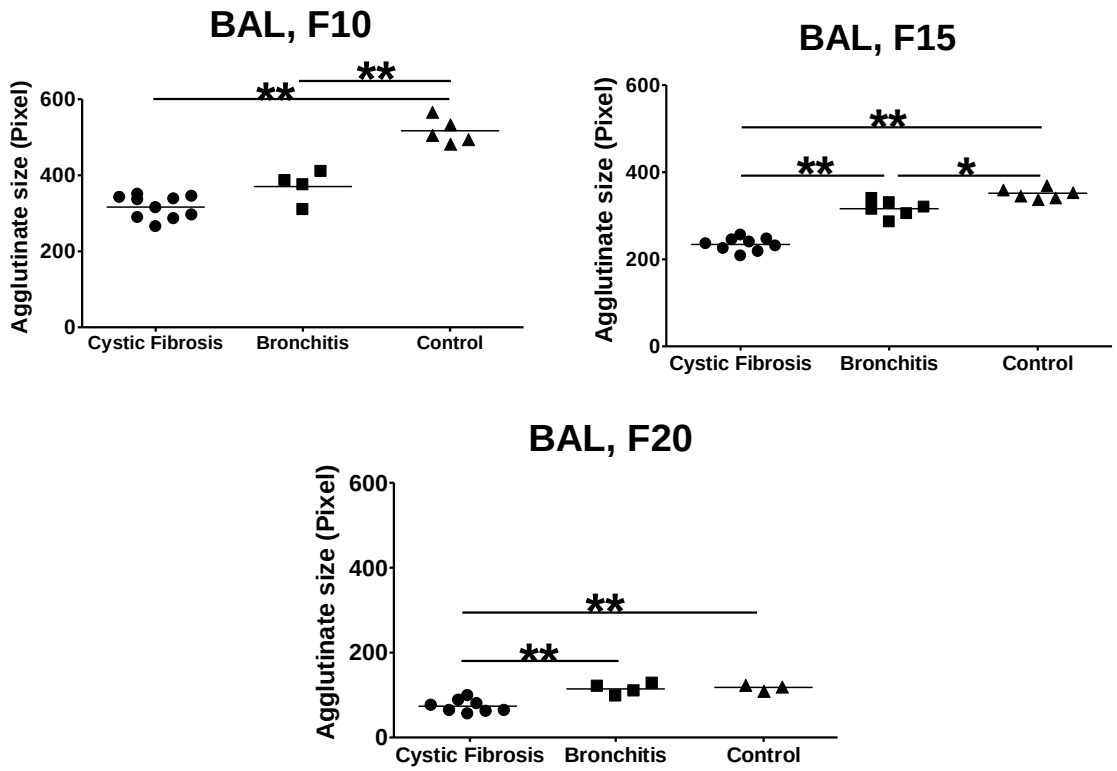
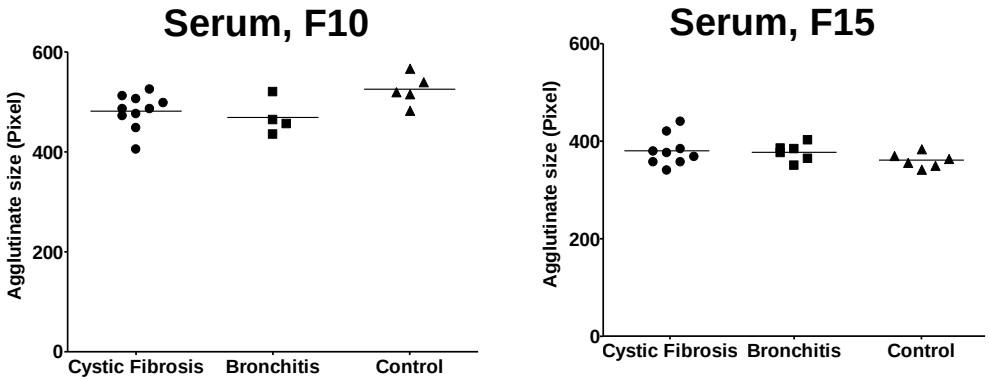


Figure 3.2.2.3: **Self-agglutination ability of BAL SP-A structures.** The graphs show the self-agglutination ability (y-axis) of different SP-A structures derived from BAL comparing the study populations. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD. The SP-A amount was adjusted to 100 ng. For all BAL fractions the SP-A oligomers derived from controls agglutinated significantly better than the SP-A oligomers derived from patients except fraction 20 bronchitis samples showed the same self-agglutination ability as controls. Also all BAL structures from the bronchitis study population agglutinated better than the structures derived from CF patients, but the difference was only significant in fraction 15 and 20 (* stands for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$). All experiments were analyzed by One way ANOVA.



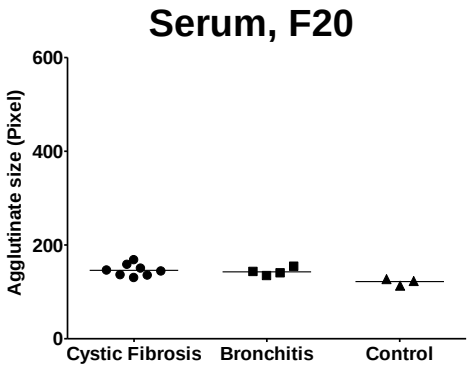


Figure 3.2.2.4: **Self-agglutination ability of serum SP-A structures.** The graphs show the self-agglutination ability (y-axis) of different SP-A structures derived from serum comparing the study populations. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD. The SP-A amount was adjusted to 100 ng. In contrast to BAL in serum there was no difference between the study populations regarding the self-agglutination ability of the different fractions analyzed by One way ANOVA ($p > 0.05$).

In summary the more complex the SP-A structure the better was the self-agglutination ability. All SP-A structures self-agglutinate in serum better than in BAL in samples of the patients groups while in control samples BAL and serum SP-A showed the same abilities. There was also a difference between the self-agglutination ability of BAL samples from CF, Bro and control derived SP-A, but none in serum. SP-A from control BAL agglutinated better than from bronchitis BAL and this better than from CF BAL. This indicates that the analysis of the SP-A structure composition was important because the structure had strong effects on the SP-A self-agglutination ability.

3.3) SP-A-genetics, clinical and biochemical data

For these studies samples from 453 patients with CF, 72 chronic bronchitis and asthma patients and 25 controls subjects were used, as detailed in fig 2.1.1 in the methods section. As not from all subjects all variables were assessed in each instance, in addition the exact number of subjects included or materials available is given in the figure and table legends.

3.3.1) Clinical data of the genetic population

In this study the CF population consisted of two cohorts. The first cohort included 371 patients from the Dr.-von-Haunersche children's hospital, Munich. The second cohort included 82 patients from a previous study (BEAT) including CF patients from Berlin (17 patients), Essen (18 patients), Hannover (12 patients), Cologne (26 patients) and Munich, the Dr.-von-Haunersche children's hospital (9 patients) not included in the first cohort. From the BEAT study group BAL, serum and DNA of each patient, for the other CF patients only serum and DNA as available were analyzed. There were also 72 bronchitis and asthma patients and 25 controls without any lung disease genotyped and BAL and serum as available analyzed.

Table 3.3.1.1: **Clinical data of study populations.**

Clinical characteristic	CF	N	Bronchitis	N	Control	N
Number	453		72		25	
Female (%)	49	230	51	35	56	14
Age at sampling point (years)	15 ± 9	453	6 ± 6	72	26 ± 17	25
BAL SP-A level (ng/ml)	3747 ± 2959	86	4639 ± 4867	20	4774 ± 4232	8
Serum SP-A level (ng/ml)	34 ± 40	292	30 ± 20	5	17 ± 4	5
Fev1 (% pred.)$_{age20}$	85 ± 42	387	92 ± 23	32	118	1
Lung disease status (mild/moderate/severe)	278/0/109	387	23/9/0	42		
Mean ∆Fev1 (% pred.)/year	-3 ± 15	376				
BMI (underweight/normal/overweight)	154/225/47	426	6/35/11	52	0/22/0	22
IgG level in serum (mg/ml)	292 ± 773	384	116 ± 247	51	29	2
x-times upper IgG standard value	0.6 ± 0.3	384	0.5 ± 0.3	51	0.7 ± 0.1	2
IgE level in serum (IU/ml)	1027 ± 502	372	653 ± 415	51	423,9	2
x-times upper IgE standard value	1.6 ± 4	372	0.9 ± 1.7	51	0.42 ± 0.1	2
ABPA (% yes)	35 (n = 153)	432	0	72	0	25

The table shows the clinical data as means ± standard deviation for all three study groups. Included were all patients genotyped by MALDI-TOF and probes. N means the actual number of the available data.

3.3.2) Lung disease

3.3.2.1) Classification of the lung disease status in the CF population

The lung disease status of the CF patients was calculated according to Schluchter (1,2). First the Fev1 (% pred.) values over the last 3 years were examined and taken to determine the lung disease status. Enrolment by severity group was based on age-specific cut-off values for Fev1 (% pred.), derived from quartiles of lung function for patients <34 years of age in the U.S. Cystic Fibrosis Foundation Patient Registry (2). The mean values of each year was calculated and all three values had to fit in the enrolment criteria for one group (mild young: age 15-24 years; mild old: age ≥ 25 years; severe: age 8-25 years) to determine a status. If a patient didn't match these criteria he had to be excluded. There were 35 children who were too young for classification and 31 patients with less than 3 Fev1 % pred. values or who didn't match the criteria. All patients ≥ 34 years of age were considered "mild"

regardless of their Fev1 % pred., on the basis of survival. The classification distribution of the CF study population is shown in table 3.3.2.1.1.

Table 3.3.2.1.1: **Classification distribution of the CF study population.**

Disease Group	Number of patients
Mild young	213
Severe	109
Mild old	65
Total classified (from n = 453)	387

The table shows how many CF patients could be classified as having a mild or severe lung disease status. Children younger than 8 years (n = 35), patients with less than one Fev1 (% pred.) value per year over the last 3 years (n = 10) and patients who didn't match the criteria (n = 21) could not be classified.

The data from the patients of the three lung disease groups (mild young, mild old and severe young) show a definite distribution (see Fig. 3.3.2.1.1). Therefore the classification into these groups after Schluchter is also capable to accurately identify patients at the extremes of phenotypes in our study group. (2)

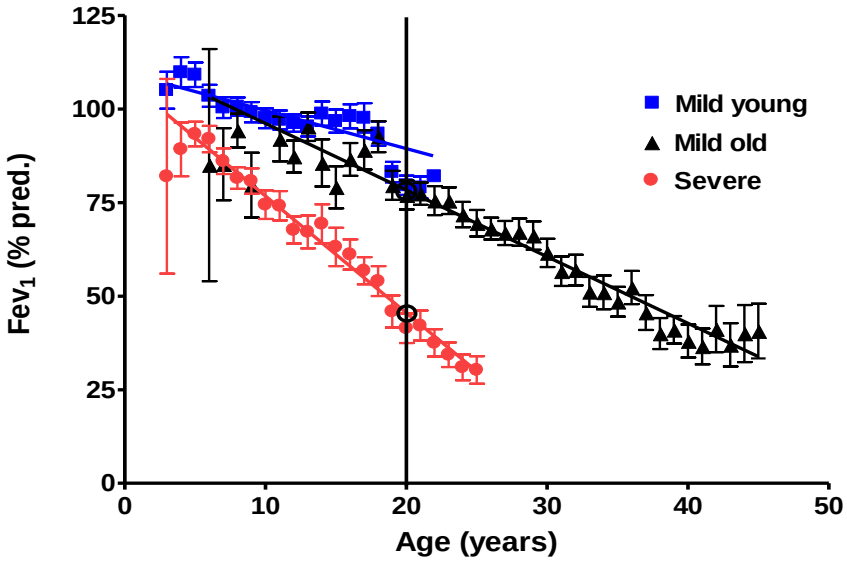


Figure 3.3.2.1.1: **Lung function distribution.** The graph shows on the x-axis the age in years of the CF patients and in the y-axis the lung function as Fev1 (% pred.). The CF patients were grouped after classification of their lung disease with regard to the calculations of Schluchter et al. (Zitat) and the mean Fev1 % pred. for each age was plotted. The bar at age 20 indicates the significant difference of lung function curves between the mild old and severe group. Therefore the classification into these groups after Schluchter is also capable to accurately identify patients at the extremes of phenotypes in our study group and a good separation of lung disease severity groups (mild/severe) from the extrapolated Fev1 (% pred.) at age 20 is possible.

3.3.2.2) Classification of the lung disease status in the bronchitis population

The lung disease status of the bronchitis study population was determined after the enrolments of the Global Initiative for Chronic Obstructive Lung Disease (GOLD). Only 32 patients could be classified because most patients were too young for a lung function test. Therefore no lung function data was available to classify the lung disease status.

Table 3.3.2.2.1: **Classification distribution of the bronchitis study population.**

Disease Group	Number of patients
Mild	23
Moderate	9
Severe	0
Total classified	32

The table shows how many chronic bronchitis and asthma patients could be classified as having a mild, moderate or severe lung disease status.

3.3.3) Genetic influence on BMI and genotype differences between patient groups

For determining the genotypes of the study populations some SNPs which cause an amino acidic exchange were analyzed by melting curves with hybridized probes in a Light Cycler. Also for a bigger throughput of genotyping the method of Matrix Assisted Laser Desorption/Ionisation-Time-Of-Flight (MALDI-TOF) was used.

The SNP data resulting from the genotyping by MALDI-TOF and probes were correlated with the clinical data and ELISA level measurements by χ^2-test on the webpage http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Catego.htm and the results were corrected according to Bonferroni.

No correlation between any genotyped SNPs and BMI with respect to the patient groups (CF, chronic bronchitis and controls) could be found.

The frequency of the alleles was significantly different between the study groups CF, bronchitis and controls, for SP-D M31T ($p = 0.03$), SP-A2 rs1965708 ($p = 0.0001$) and SP-A2 rs1975006 ($p = 0.0001$). The SP-A SNPs were linked. For the SP-D SNP the CF population showed a significantly higher amount of the mutated allele, while for the SP-A SNPs the CF population had a significantly smaller amount of the mutated allele.

3.3.4) SP-A and SP-D level measurements

For a correlation analysis between the clinical and genetic data and the SP-A and SP-D levels the levels were measured by ELISA (see figure 3.3.4.1). The mean SP-A levels in BAL were 3747 ng/ml for the CF patients, 4639 ng/ml for the bronchitis patients and 4774 ng/ml for the controls. The mean SP-A levels in serum were 34 ng/ml for the CF patients, 30 ng/ml for the bronchitis patients and 17 ng/ml for the controls. The mean SP-D levels in BAL were 699 ng/ml for the CF patients, 34 ng/ml for the bronchitis patients and 505 ng/ml for the controls. The mean SP-D levels in serum were 261 ng/ml for the CF patients, 382 ng/ml for the bronchitis patients and 388 ng/ml for the controls.

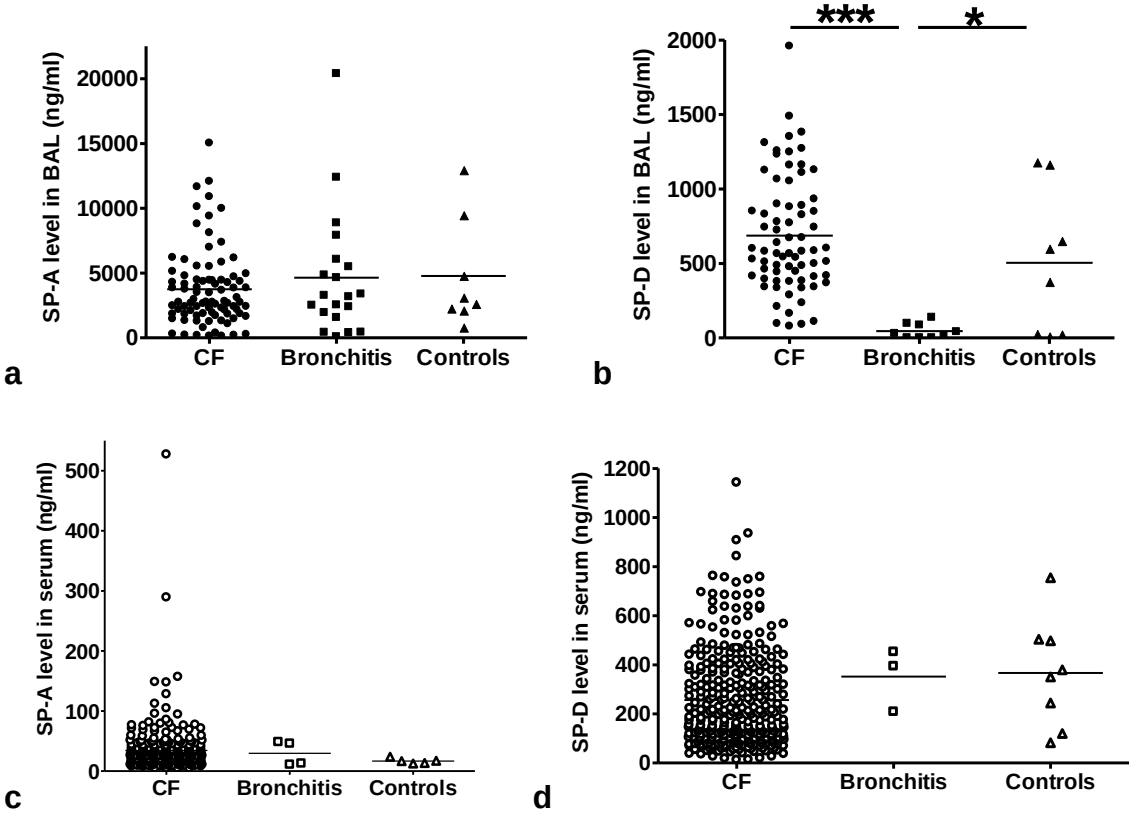


Figure 3.3.4.1: **The SP-A and SP-D BAL and serum levels.** The graphs illustrate the BAL (a, b) and the serum (c, d) levels of SP-A (a, c) and SP-D (b, d) of the study populations. The SP-D levels in BAL differed significantly ($p_{CF\text{-}Bro}$ = 0.0003, $p_{Bro\text{-}C}$ = 0.02) between the patient groups analyzed by One way ANOVA. No other correlation was found. BAL samples of 86 CF, 20 Bro patients and 8 controls were analyzed as well as serum samples from 292 CF, 4 Bro patients and 8 controls.

In a former study by Bielecki ("Surfactantproteine A und D im Serum und in Bronchoalveolärer Lavage bei Patienten mit Cystischer Fibrose", MD, 2007) the consistency of the SP-A serum levels in CF patients over time was shown. This consistency allows us to compare results arising from sera taken at different points of time from CF patients with clinical stability.

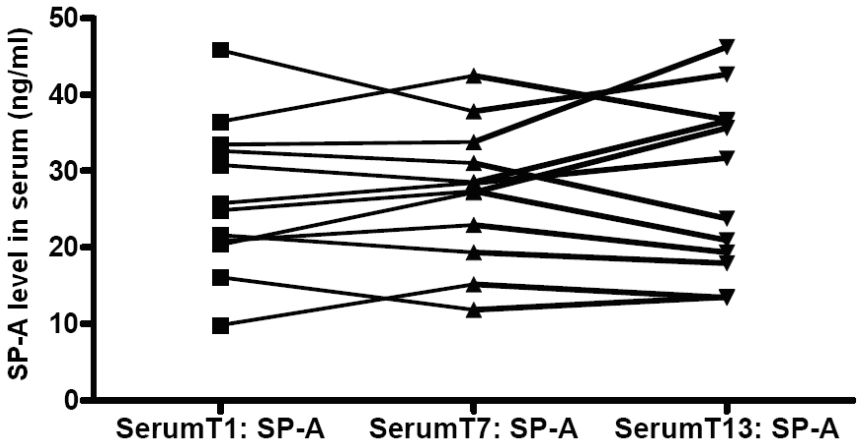


Figure 3.3.4.2: **Time developing of SP-A level in serum of CF patients.** The graph shows on the y-axis the SP-A level in serum (ng/ml) and on the x-axis three different points in time which stand for the beginning of the measurements (T1), about a year later (T7) and about two years from the beginning (T13). Each line shows the development of the SP-A level in serum of one CF patient with respect to time. These results of 11 BEAT-CF patients also included in this study show that the level are consistent over time (Doktorarbeit Eva Bielecki, 2007).

Analyzing the SP-D BAL and serum levels of all available patients and controls regarding age there was a significant correlation between the serum levels and the age ($p = 0.001$, see figure 3.3.4.3. For further analysis of the SP-D level with respect to the age in CF patients see 3.3.5.6.2.

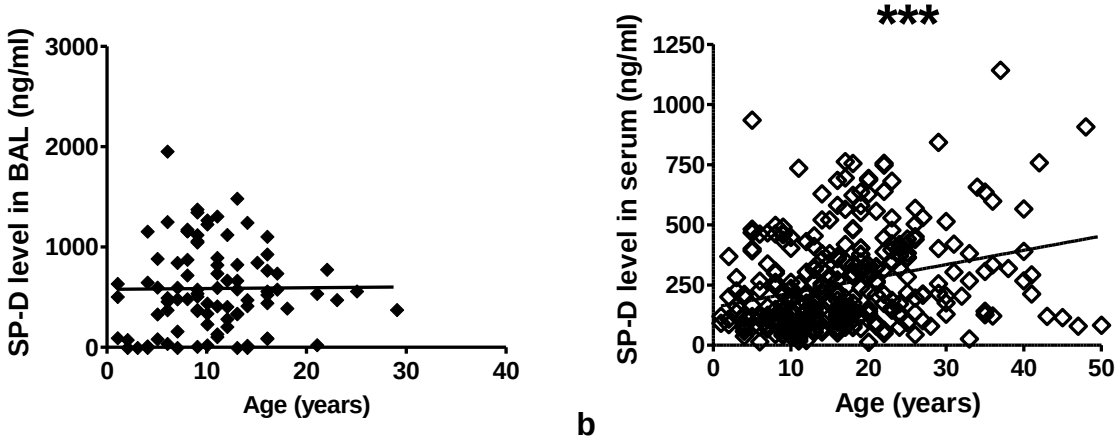


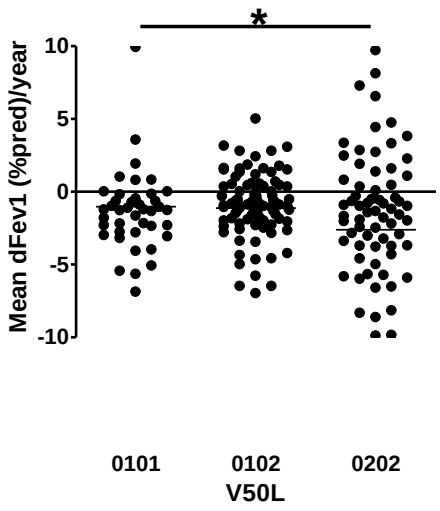
Figure 3.3.4.3: **SP-D level and age.** The graphs show the correlations between SP-D levels in BAL (a) and serum (b) on the y-axis and the age on the x-axis. There was a significant association between the serum levels and the age ($p < 0.0001$, $r^2 = 0.08$), but no association in BAL ($p = 0.9$) analyzed by linear regression. BAL samples of 86 CF, 20 Bro patients and 8 controls were analyzed as well as serum samples from 292 CF, 4 Bro patients and 8 controls.

The results in section 3.3.5 are only related to the group of the CF patients, as not sufficient clinical and biochemical data of the chronic bronchitis patients and controls were available for meaningful statistical analysis.

3.3.5) Genetics in CF

3.3.5.1) SP-A SNPs and clinical data

There was a significant association between the mean ∆ Fev1 (%pred.) / year and V50L a SNP in the *SFTPA1* gene (p = 0.0038, see figure 3.3.5.1.1) while the mutated allele was associated with a worse course of lung disease. There was also a significant associations of SP-A2 N9T (p = 0.004) with ABPA illness regarding whether the CF patients had an ABPA illness or not analyzed by χ^2-test. For SP-A2 N9T the wildtype allele was significantly associated with the group that had never ABPA.



Figures 3.3.5.1.1: **Significant SNP association with the mean ∆ Fev1 (%pred.) / year in CF.** The graph plots the genotypes (0101 for the wildtype, 0102 for heterozygous and 0202 for the homozygous mutation) on the x-axis and the mean ∆ Fev1 (%pred.) / year on the y-axis. The graph shows a significant correlation of the SNP SP-A1 V50L (p = 0.038). * stands for a p-value < 0.05 analyzed by One way ANOVA. 376 CF patients were analyzed.

The other characteristics, age at the first *P. aer.* infection, age at the first *P. aer.* mucoid infection, chronic or non-chronic *P. aer.* infection, age at the first chronic *P. aer.* infection, BMI and Fev1 (% pred.) estimated for age 20, had no significant correlations with any SNPs after Bonferroni correction.

3.3.5.2) SP-A level and genetics in CF

For SP-A there were significant associations with the BAL and serum level. The SP-A BAL level was significantly associated with the SP-A1 SNP rs1136451 (p = 0.04) and the SP-A2 SNP rs17881665r (p = 0.03) (see fig. 3.3.5.2.1). Here the mutated allele was associated with lower SP-A levels like it was shown before with the CF part analyzed by gel chromatography. This significant correlation existed only by analyzing the SNP data with the χ^2-test and grouping after BAL levels (group 1: 0-1,000 ng/ml; group 2: 1,000-10,000; group 3: >10,000) to compare the extremes. The correlation of the BAL level data by grouping regarding the genotype was not significant. The SP-A serum level was significantly associated with SP-A1 N9T (p = 0.028) (see fig. 3.3.5.2.2).

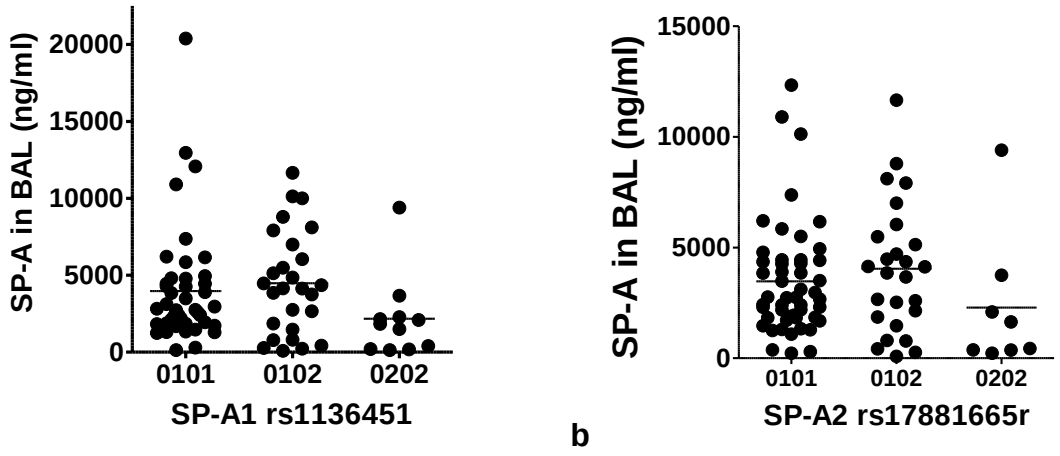


Figure 3.3.5.2.1: **SNP associations with the SP-A level in BAL.** Both graphs plot the association of the SNP genotypes (0101 for the wildtype, 0102 for the heterozygous and 0202 for the homozygous mutation, x-axis) and the SP-A level in BAL (y-axis). Graph a illustrates the association for SP-A1 rs1136451 and graph b for SP-A2 rs17881665r. 86 BAL samples of CF patients were analyzed. Although the analysis by χ^2-test showed a significant association, these grouping after genotype showed none analyzed by One way ANOVA.

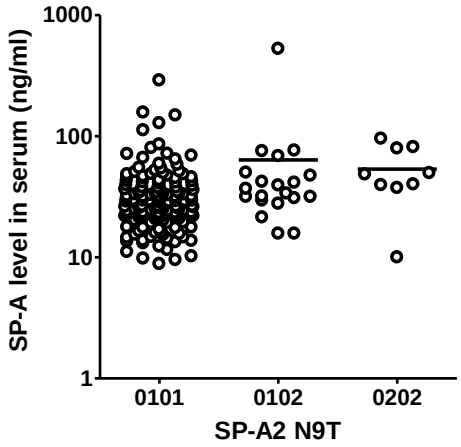


Figure 3.3.5.2.2: **SNP association with the SP-A level in serum.** The graph shows the association of the SP-A2 N9T genotype (0101 for the wildtype, 0102 for the heterozygous and 0202 for the homozygous mutation, x-axis) with the SP-A level in serum (y-axis) with a p-value of 0.028 analyzed by One way ANOVA. 292 serum samples of CF patients were analyzed.

3.3.5.3) Fev1 (% pred.) estimated for age 20 correlates with SP-A level

The SP-A levels in BAL and serum were measured by ELISA and correlated to the clinical data of the patients. In the CF study population there was a significant association between the SP-A level in BAL and the Fev1 (% pred.)$_{age\ 20}$ ($p = 0.009$, see figure 3.3.5.6.1). The higher the SP-A levels were in BAL the bigger were the values of the Fev1 (% pred.)$_{age\ 20}$. In serum there was no correlation with this characteristic ($p = 0.33$). The SP-A serum level was associated with the lung disease ($p = 0.05$, see figure 3.3.5.6.2) while patients with severe lung disease had a higher serum level.

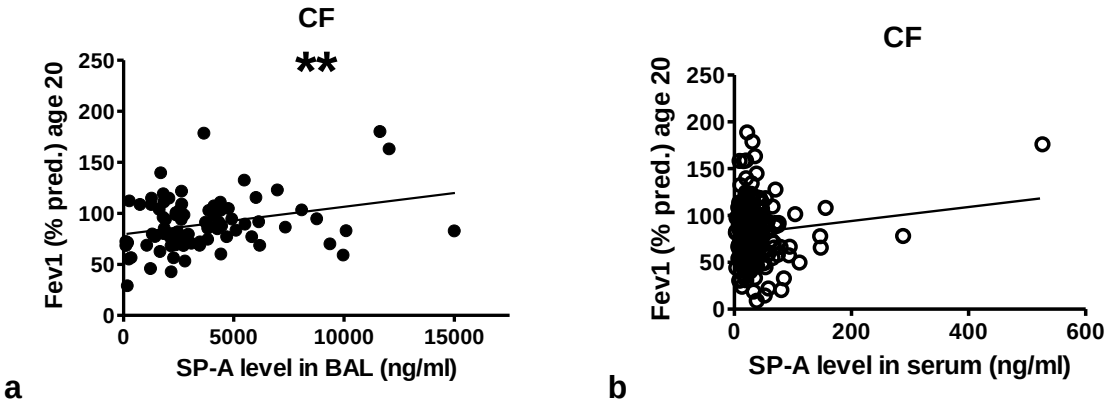


Figure 3.3.5.3.1: **SP-A level and Fev1 (% pred.)$_{age\ 20}$.** Both graphs plot the SP-A level in BAL (a) and serum (b) of the CF study population against the Fev1 (% pred.)$_{age\ 20}$. There was a significant association between the SP-A level in BAL and the Fev1 (% pred.)$_{age\ 20}$ ($p = 0.007$, $r^2 = 0.1$) analyzed by linear regression. In serum there was no correlation with this characteristic ($p = 0.07$). 292 serum samples and 85 BAL samples of CF patients were analyzed.

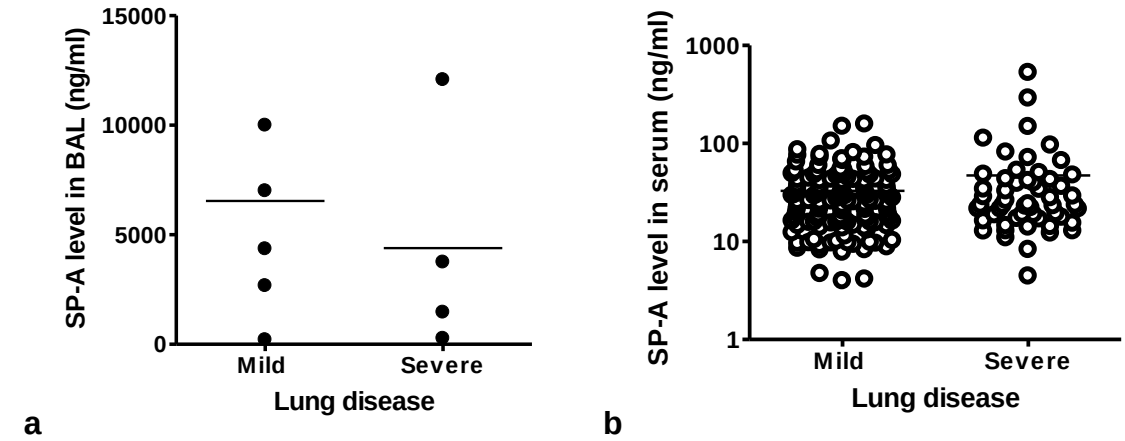


Figure 3.3.5.3.2: **Lung disease and SP-A level.** The graphs illustrate the SP-A level in BAL (a) and serum (b) of CF patients grouped according to the lung disease (mild/severe). 292 serum samples and 12 BAL samples of CF patients were analyzed. In serum there was a significant correlation (t-test, $p = 0.05$). In BAL was no correlation.

There were no correlations between the ΔFev1 (% pred.)/year, ABPA (yes/no), BMI, age, gender, IgG or IgE in serum and the SP-A BAL or serum level.

3.3.5.4) SP-D genetics in CF

Because the Fev1 (% pred.)$_{age\ 20}$ had been shown to be an equivalent classification criteria for lung disease of CF patients (2) and the lung disease groups were significantly associated with SP-D M31T ($p = 0.0005$, see figure 3.3.5.4.1) the correlations were again analyzed after grouping the patients regarding to their genotype. Both showed the same correlation that the mutated allele leads to a worse lung disease outcome ($p = 0.0091$). Analyzing the mean ΔFev1 (% pred.)/year regarding to the genotype of *SFTPD* M31T there was also a significant correlation ($p = 0.019$, see figure 3.3.5.4.1).

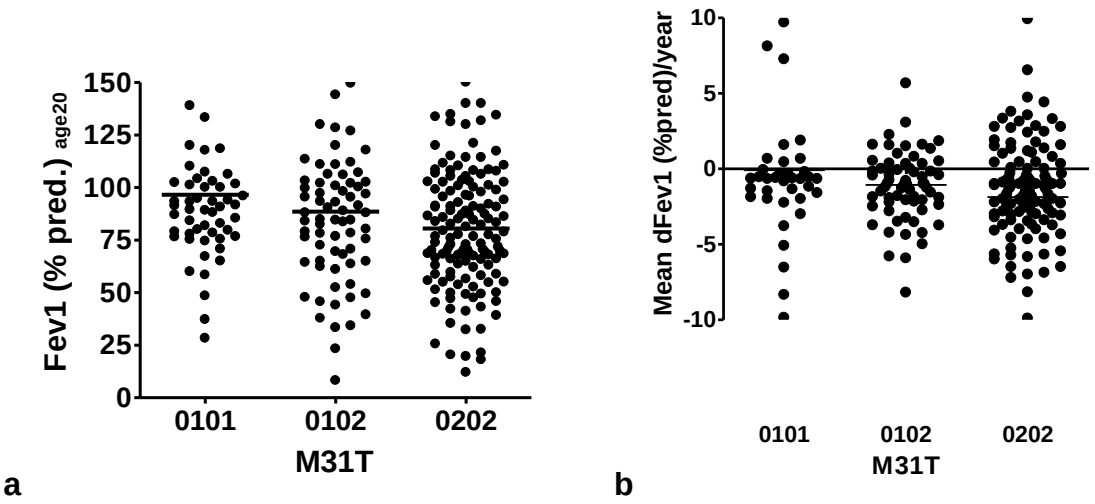


Figure: 3.3.5.4.1: **Significant SNP associations with lung disease.** The graphs plot the genotype (0101 for the wildtype, 0102 for the heterozygous and 0202 for the homozygous mutation) of the M31T SNP on the x-axis and the Fev1 (%pred.)$_{age\ 20}$ (a) or the mean ΔFev1 (% pred.)/year on the y-axis. The graphs show a significant association analyzed by One way ANOVA (p = 0.0091 and p = 0.019). 376 CF patients were analyzed.

3.3.5.5) SP-D SNPs influencing the protein level in CF

There was no significant association between the SP-D BAL level and any of the SP-D SNPs genotyped by MALDI-TOF and probes.

The SP-D serum level was significantly associated with the SNPs SP-D M31T (p = 0.04) and SP-D A180T (p = 0.013). In BAL the same correlation could be observed, but the difference was not significant (see fig. 3.3.5.5.1). For both SNPs the mutated allele is associated with lower SP-D levels.

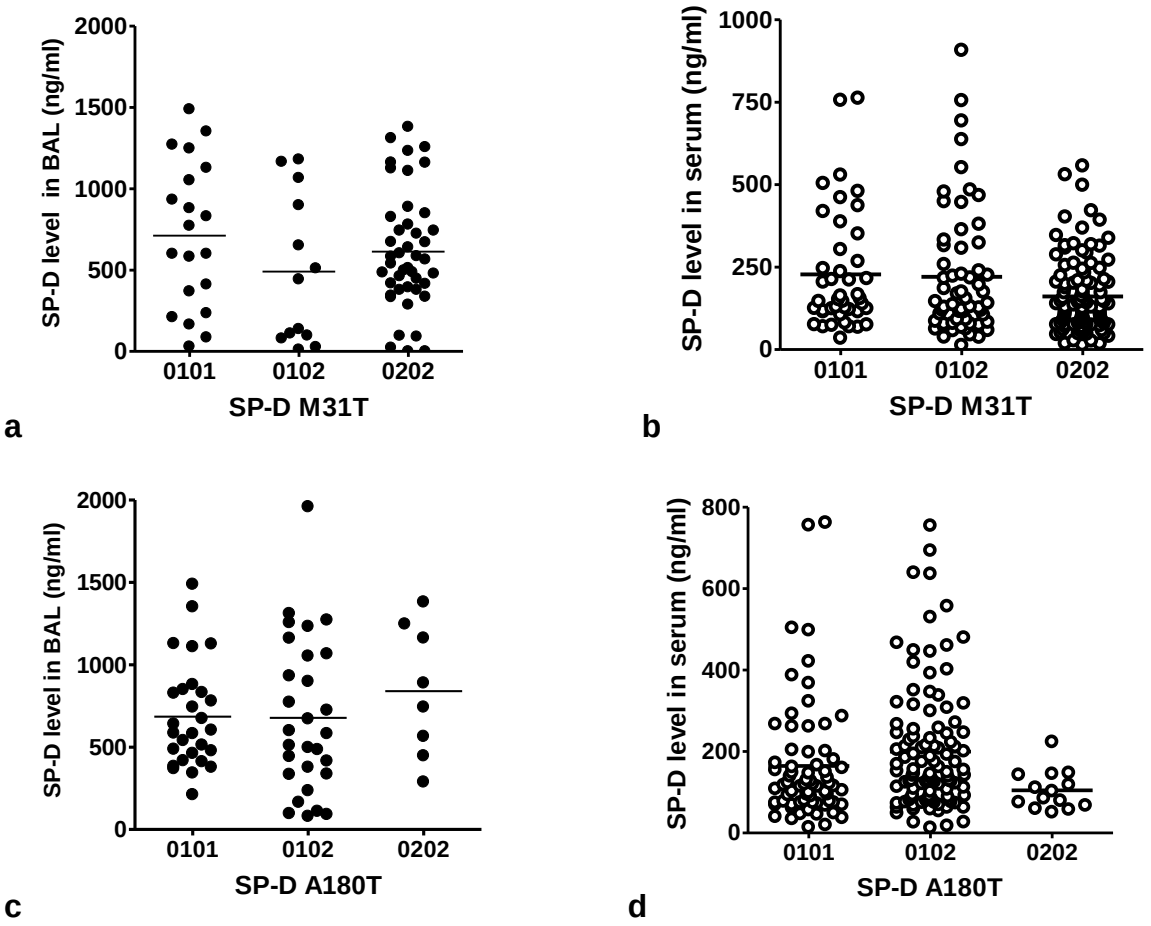


Figure 3.3.5.5.1: **SNP associations with the SP-D level in serum.** All four graphs plot the association of the SNP genotypes (0101 for the wildtype, 0102 for the heterozygous and 0202 for the homozygous mutation, x-axis) and the SP-D level in serum (b, d) and in BAL (a, c) (y-axis). Analyzed were 86 BAL and 292 serum samples of CF patients. Graphs a and b illustrate the association for SP-D M31T with a p-value of 0.04 in serum and graph c and d for SP-D A180T with a p-value of 0.013 in serum analyzed by One way ANOVA. There was no significant correlation with the SP-D level in BAL.

3.3.5.6) Influence of lung disease, age and BMI on SP-D levels

After analysis of associations between the SNP data and the SP-D levels in BAL and serum measured by ELISA, also the correlation between the levels and clinical data was investigated.

The SP-D level in serum of the CF study population was significantly associated with the lung disease (mild/severe, p = 0.005, see figure 3.3.5.6.2) and the Fev1 (% pred.)$_{age20}$ (p = 0.0027, see figure 3.3.5.6.1). The higher the SP-D level was in serum the smaller was the value of the Fev1 (% pred.)$_{age20}$. In BAL there was no correlation (p = 0.9). In line with this also the ΔFev1 (% pred.)/year was significantly associated with the serum level (p = 0.01, see figure 3.3.5.6.3) indicating that the lower the SP-D level in serum was the better was the course of lung disease.

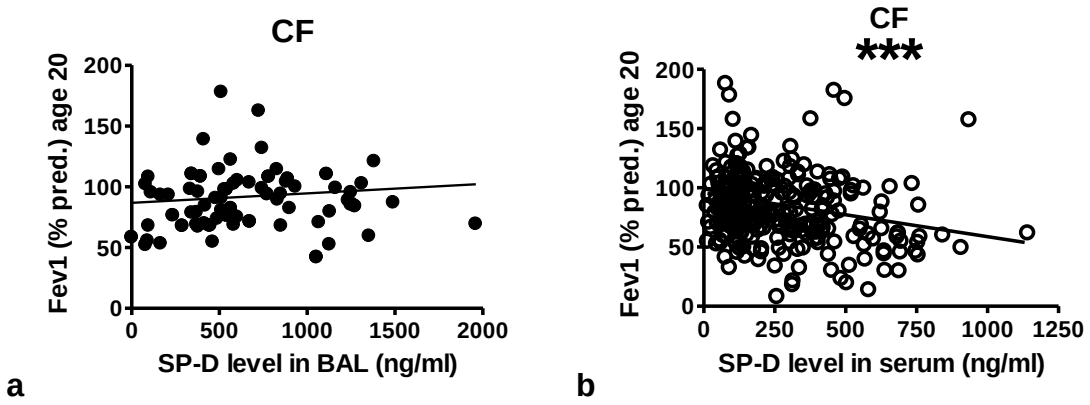


Figure 3.3.5.6.1: **SP-D level and Fev1 (% pred.) estimated for age 20.** Both graphs plot the SP-D level in BAL (a) and serum (b) of the CF study population against the Fev1 (% pred.)$_{age\ 20}$. There was a significant association between the SP-A level in serum and the Fev1 (% pred.)$_{age\ 20}$ also after excluding too extreme values above 200 % ($p = 0.003$, $r^2 = 0.3$) analyzed by linear regression. In BAL there was no correlation with this characteristic ($p = 0.9$) analyzed by linear regression. 86 BAL and 292 serum samples of CF patients were analyzed.

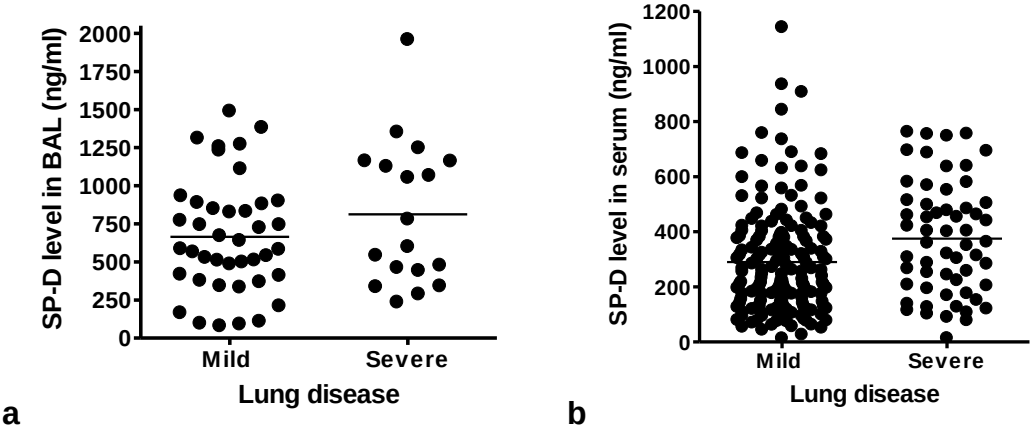


Figure 3.3.5.6.2: **SP-D level and lung disease.** The graphs show the SP-D level in BAL (a) and serum (b) and the lung disease (mild/severe). Analyzed by t-test there was a significant correlation between the serum level and the lung disease ($p = 0.005$). 86 BAL and 292 serum samples of CF patients were analyzed.

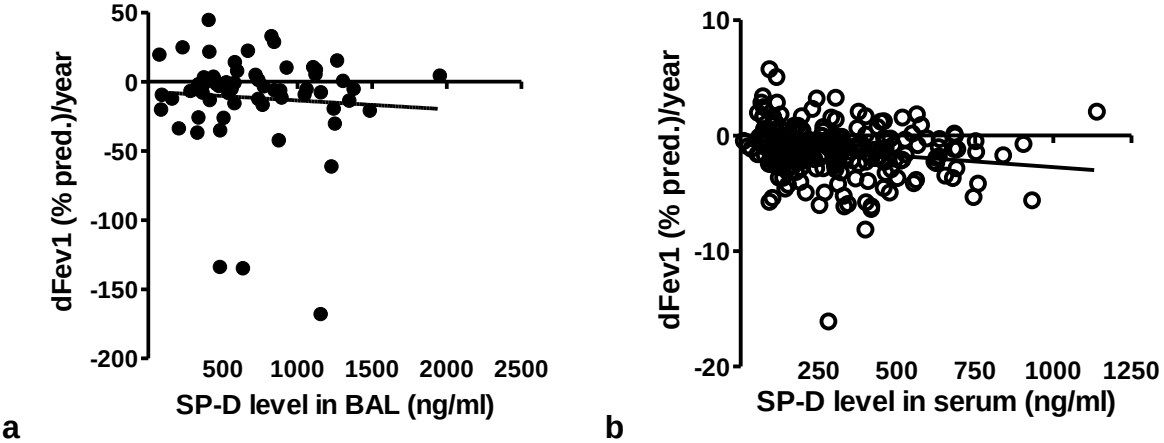


Figure 3.3.5.6.3: **SP-D level and ΔFev1(% pred.)/year.** Both graphs show the SP-D level in BAL (a) and serum (b) in relation to the ΔFev1(% pred.)/year. Analyzed by linear regression only in serum was a significant correlation ($p = 0.01$). 86 BAL and 292 serum samples of CF patients were analyzed.

Analyzing the SP-D levels in BAL and serum with respect to the age of the individuals there was a significant association between increasing SP-D serum level and increasing age ($p = 0.001$), but no correlation between the SP-D BAL level and age. The same result was found analyzing only the CF patients regarding to SP-D level in serum ($p = 0.00003$) and BAL ($p = 0.19$) and the age (see figure 3.3.5.6.4). Only in CF patients the correlation between the BAL SP-D level and the age seems to be oppositional to the correlation between the serum SP-D level and age, leading to the assumption that with increasing age the SP-D level in BAL decreases in CF patients. Additionally by analyzing the BMI groups underweight, normal weight and overweight and the SP-D levels there was a significant difference between the serum levels of the underweight and the overweight group as well as between the normal weight group and the overweight group (both $p < 0.01$), but no significance regarding the BAL levels (see figure 3.3.5.6.5). While the underweight and normal weight groups had a similar SP-D level distribution in serum, the overweight group had significantly lower serum SP-D levels.

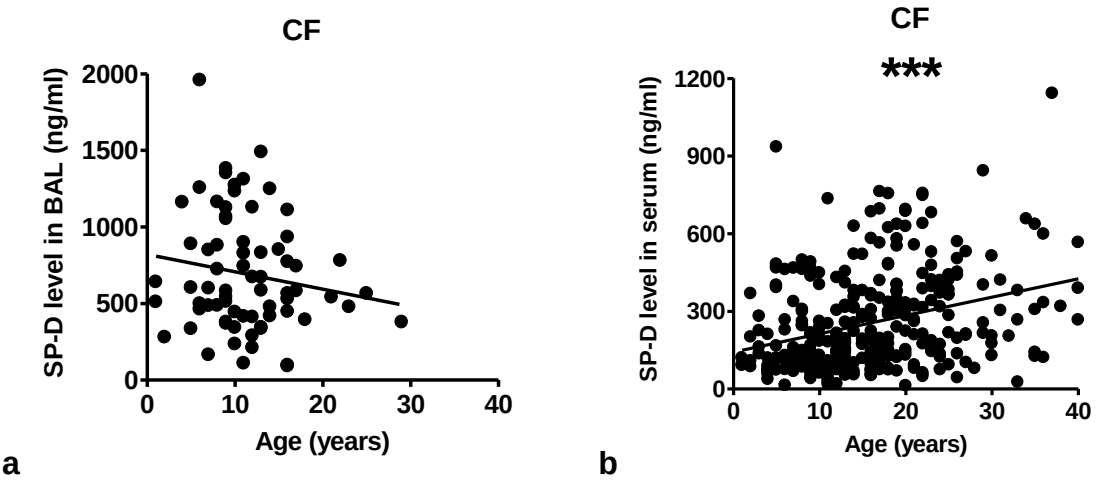


Figure 3.3.5.6.4: **SP-D level and age in CF patients.** The graph b illustrates the significant correlation ($p = 0.00003$, $r^2 = 0.1$) between the SP-D level in serum and the age of CF patients, whereas graph a indicates an oppositional, not significant ($p = 0.19$) correlation between the SP-D level in BAL and the age of CF patients analyzed by linear regression.

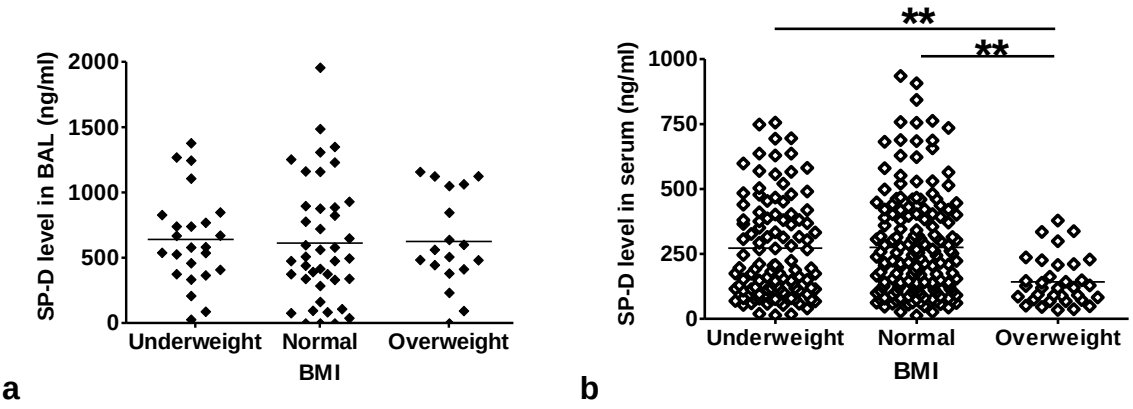


Figure 3.3.5.6.5: **SP-D level and BMI.** The graphs plot the SP-D levels in BAL (a) and serum (b) on the y-axis against the BMI classified in underweight, normal weight and overweight on the x-axis. There was a significant difference between the serum levels of the underweight and the overweight group as well as between the normal weight group and the overweight group (both $p < 0.01$), but no significance regarding the BAL levels analyzed by One way ANOVA. BAL samples of 86 CF, 20 Bro patients and 8 controls were analyzed as well as serum samples from 292 CF, 4 Bro patients and 8 controls.

3.4) Absence of SP-D in BAL

For this study samples from 10 chronic bronchitis and asthma patients (out of group no.12 in scheme 2.1.1, methods) were used. After we identified subjects with no SP-D in their BAL common polymorphisms known to be associated with low serum SP-D levels were assessed in those subjects from whom informed consent could be obtained and DNA was available.

The M31T polymorphism in exon 1, A180T in exon 4 and S290T in exon 7 were analyzed. DNA was available from 10 of the 12 children who had no SP-D detectable in their BAL by ELISA (n = 6) and Western blotting (n = 4). For comparison 8 children were selected with the same clinical characteristics, but detectable SP-D levels in serum.

No significant genetic association could be found between the common polymorphisms known to be associated with low serum SP-D levels and the lack of SP-D in BAL or serum respectively.

3.5) TLR SNPs

The SNP TLR4 A-2570G was significantly associated with the lung disease (mild/severe) in CF patients analyzed by χ^2-test ($p = 0.0003$). No other correlation between any clinical characteristics could be found.

4) Discussion

Surfactant associated protein A (SP-A) is the most abundant pulmonary surfactant protein and belongs to the family of innate host defence proteins termed collectins. Besides pulmonary host defence, SP-A is also involved in the formation of pulmonary surfactant, as it is essential for the structure of tubular myelin. The human SP-A gene locus includes two functional genes, *SFTPA1* and *SFTPA2* which are expressed independently, and a pseudo gene. The largest amount of SP-A1 proteins assemble to larger molecular complexes, whereas SP-A2 forms mainly dimers and trimers. SPA polymorphisms play a role in respiratory distress syndrome, allergic bronchopulmonary aspergillosis and idiopathic pulmonary fibrosis. The levels of SP-A are decreased in the lungs of patients with cystic fibrosis, respiratory distress syndrome and further chronic lung diseases. Future areas for clinical research include disease specific SP-A expression pattern and their functional consequences, the differential roles of SP A1 and SP-A2 in human lung diseases, and therapeutic approaches to correct altered SP-A levels.

The aim of the study was to elucidate the role of SP-A in human lung disease. The study was designed to analyze the relation between genetics, structure and function of SP-A in a cystic fibrosis (CF), chronic bronchitis and asthma and healthy control population *ex vivo*. Therefore the DNA was genotyped by MALDI-TOF and probes, the SP-A serum and BAL levels were measured by ELISA, BAL and serum SP-A was separated by gel chromatography and analyzed by Slot-Blot, the functional capability of SP-A was measured by an agglutination-assay with respect to the CRD and clinical data was collected.

4.1) Study populations

The BAL and serum samples of 42 CF patients, 19 chronic bronchitis and asthma patients and 8 controls that were genotyped by MALDI-TOF as well as BAL and serum samples from additional 9 chronic bronchitis patients and 10 controls were analyzed by gel chromatography with a Superose 6 column. Because it is known that there are structural differences of SP-A between individuals, it was necessary to elucidate if there are functional differences between different oligomeric states. Therefore the BAL and serum samples fractions of 10 CF patients, 10 chronic bronchitis and 7 control patients and the whole BAL and serum samples of all patients analyzed by gel chromatography were further analyzed with a SP-A self-agglutination-assay. The DNA from 454 CF patients, 72 chronic bronchitis and asthma patients and 25 controls was isolated and genotyped by MALDI-TOF.

4.2) SP-A structure and function

4.2.1) Clinical data of the structure and function population

The BAL and serum samples of 42 CF patients, 19 chronic bronchitis and asthma patients and 8 controls that were genotyped by MALDI-TOF as well as BAL and serum samples from additional 9 chronic bronchitis patients and 10 controls were analyzed by gel chromatography. Because it is known that the SP-A ratio of SP-A1:SP-A2 decreases with respect to the age (3) and that there are differences between the oligomerization characteristics of SP-A1 and SP-A2 (4) the patients and controls were selected with respect to the mean age of the study populations. The female percentage was almost even. The mean SP-A level in BAL of CF patients was lower as in the other populations as expected, but not in serum which could be caused be the high variability of the values. The lung function was equal between the bronchitis and CF populations. A problem here was that either because of the young age of controls or no available data there was only one measurement of lung function for controls. The BMI was available for all study populations and underweight could only be detected in the CF population as expected (5). The lung disease status shows that there are only in the CF population severe disease status. The bronchitis patients had in spite of a few exceptions a mild lung disease status. Therefore the bronchitis patients are more comparable to controls with respect to lung disease. Leucocytes, neutrophils and eosinophils could be more frequently detected in CF patients while the bronchitis patients and controls were on the same low level. This supports the idea that the bronchitis group has less inflammation activity in lung than CF patients.

4.2.2) SP-A structure analysis by gel chromatography

To determine structural differences in BAL and serum between the study populations a gel chromatography method with a superpose 6 column was chosen. In this method SP-A molecules were separated with regard to their oligomerization form. The larger the oligomers the earlier they were eluted. After gel chromatography the SP-A amount of the eluted fractions was determined by Slot-Blot. The peaks were defined as a relative SP-A amount of more than 20 % and a distance of 5 fractions between each peak (mainly in fraction 10, 15 and 20). The peak areas ranged from fraction 9-12, 13-17 and 18-22. For calculation also the first peak from fraction 9-12 and the last peak from fraction 18-22 were defined. The peak area and position definition was evaluated after the main appearance of the SP-A peaks. Because the minimum value of 20 % was arbitrary it was considered by determining the distribution form resulting in more than one possible form for some patients.

4.2.2.1) Reproducibility of structure analysis

The reproducibility of the gel chromatography and the Slot-Blot assay was tested to exclude distribution variations caused by inhomogeneous testing. Regarding the distribution forms the reproducibility was very good, only the SP-A amount in the fractions seemed to vary. Therefore the relative SP-A amount per fraction was taken for correlation analysis to diminish the varying level.

Because the form could be also reproduced after some weeks with freezing and thawing and was consistent for different samples of the same individual the samples were comparable for all study populations.

4.2.2.2) Calibration of the Superose 6 column

The calibration of the column after Griese et al. and Hickling et al. (6,7) identified the SP-A forms in the first peak as octadecamers and more complex structures, in the second peak as hexamers to dodecamers and in the last peak as dimers and trimers regarding the molecular weight. This calibration is reliable because Hickling et al. determined the Stokes radii and sucrose density gradient of the molecular weight markers and based on this calculated the molecular weight of the structures in the peaks.

4.2.3) Different peak distribution in study populations

In BAL the most common forms contained the first peak and there was no obvious difference of distributions between the study populations, but the bronchitis and control populations had more SP-A in the last peak. In the BAL of CF patients elastase and oxidants lead to proteolysis of SP-A (8). Smaller oligomers had been shown to be more susceptible to oxidation (9) therefore the dimers and trimers in the CF BAL samples could have been destroyed by elastase and oxidants. Hickling et al. found a higher percentage of complex oligomers like octadecamers in BAL from healthy persons compared to BAL from birch pollen allergic persons (7). The higher variability found in BAL structures in this study could be a consequence of analyzing more samples, Hickling et al. analyzed only 11 allergic persons and 4 healthy persons compared to 21 bronchitis patients and 18 controls in this study. The results of the control subjects arising from the study of Griese et al. (6) match with the results of this study. They showed in the publication only the mean distribution of 6 from 17 measured control BALs. Altogether the distributions showed the same variability. These two studies were the only ones analyzing the degree of oligomerization of SP-A *ex vivo* by gel chromatography.
In serum all control samples showed the second peak and partly the last peak, but none contained the first peak. In sera of CF, chronic bronchitis and asthma patients there was more variability, but the CF samples contained mainly the first peak. The distal airspaces of the lung are lined with a continuous epithelium comprising two major types of alveolar epithelial cells (type I and type II pneumocytes) joined by tight junctions (*zonulae occludentes*). The normal air-blood barrier of human lungs appears to differentially restrict the passage of large proteins as evident by the finding that concentrations of macromolecules like IgM in BAL were extremely low compared with the smaller proteins like BSA (10). During inflammation the barrier can be disrupted for example by cationic aa and peptides which arise from protein degradation which was shown in *acute respiratory syndrome* (11). This mechanism could cause the appearance of octadecamers and more complex structures in serum of CF patients.
The differences in the degree of SP-A oligomerization between individuals is also caused by the composition of SP-A1 and SP-A2. Recombinant, human SP-A1 forms lower supratrimeric structures

as SP-A2, but the proteolysis-resistance is higher for rhSP-A1. Co-expressed SP-A1/A2 forms more complex oligomers and leads to a higher degree of self-agglutination compared to SP-A1 and -A2 alone (4). A gradual decrease ($P < 0.05$) in SP-A1/SP-A ratio was observed in healthy subjects (HS) with increased age, although no significant change was observed in total SP-A content among age groups in a study from Tagaram et al. The CF ratio was significantly higher compared with AP, HS and non-cystic fibrosis (NCF), even though SP-A1 and total SP-A were decreased in CF compared with most of the other groups, but there were only 16 CF patients analyzed compared to 42 in this study. A higher ratio would indicate that SP-A in CF BAL is mainly existent in smaller oligomers which would be contradictory to the results of the analysis by gel chromatography, but this was not further analyzed in the study by Tagaram et al. (3). The statements regarding the structure of SP-A and the ratio of SP-A1/SP-A2 were exclusively made based on recombinant SP-A assays and native SP-A had shown different oligomerization patterns than a mixture of recombinant SP-A1/SP-A2 (4). Therefore the conclusions regarding the ratio and the structure are not reliable and this is an important field which has to be investigated in the future.

Next the 47 BAL and serum pairs were examined. The most frequent pairs contained the first peak in BAL and serum. There were some sample pairs with the same form in BAL and serum, but also pairs with the opposite forms. In controls the complex oligomers were only in BAL not in the corresponding serum as was stated before. In CF and bronchitis patients appeared a more variable distribution of forms in BAL and serum indicating a higher exchange rate.

Because different oligomeric distributions between individuals and study populations were observed also the correlation between the lung function and the oligomeric SP-A distribution form in BAL and serum were analyzed. In BAL a slight increase in Fev1 (% pred.) can be observed the more SP-A is existent in the first peak (111 to 100). In BAL samples where all three peaks could be observed (111) SP-A is present in dimers/trimers, smaller oligomeric forms like hexamers and only partly in complex oligomeric forms like octadecamers. In samples where only the first peak is present (100) SP-A is mainly existent in complex forms. The slightly better lung function of the 101 form in comparison to the 110 form could be explained in BAL by comparing the peaks in each form. In the 101 form the first peaks were bigger in contrast to the first peaks in the 110 forms (mean percentage of first peak in 101 = 45 % and 110 = 39%).

In conclusion there were no correlations between serum and BAL SP-A distribution forms. The forms showed only a significantly different distribution comparing serum samples from bronchitis patients and controls. In serum and BAL of CF patients the forms containing the first peak were correlated with a better lung function $(\text{Fev1 (\% pred.)})_{age20}$. These findings suggest that the more SP-A is existent in octadecamers or more complex structures in BAL the better is the lung function. In serum the distributions show the same correlation. The better lung function of the 101 form in comparison to the 110 form is most likely a result of the small sample size for the 101 form.

4.2.4) Influence of SP-A structure on self-agglutination

The capacity of SP-A from serum and BAL to induce agglutination was linked to the organizational structure of naturally occurring macromolecular forms. While in serum the different forms indicated an

approximate association with the self-agglutination ability, in BAL the differences were more distinctive. In serum only the 010 and 110 forms showed a significant difference regarding the self-agglutination ability. In BAL the 100 form agglutinated significantly better than the 111 and 110 form and the 101 form significantly better than the 111 form. Altogether, the results imply that the 100 form agglutinates best with the rest showing a declining ability in the following order: 100>101>110>111>010. The weaker self-agglutination ability of distributions containing smaller oligomeric structures (111 in comparison to 100) and the complex oligomeric structures in the first peak could be the result of the actual amount of SP-A which is existent in these forms. While in the 100 distribution almost the entire SP-A in the sample is existent in complex forms like octadecamers in 111 a smaller part of the SP-A is existent in complex forms. This decreases the self-agglutination ability.

Focussed on the fractions in BAL and serum the increasing order correlated to the increasing lung function could not be supported. In BAL the self-agglutination ability in all fractions showed a wide variability without any correlation while in serum the self-agglutination ability was on a same level in all forms for each fraction. Only in BAL there seemed to be a correlation between the appearance of hexamers in SP-A distribution forms containing octadecamers and more complex forms and weaker agglutination ability.

These data suggest that the correlation of Fev1 (% pred.)$_{age20}$ with the different oligomeric forms is related to the degree of the presence of active oligomeric forms within a sample.

4.2.5) Oligomerization in CF patients is influenced by lung disease, genetics and SP-A level

Another approach for the SP-A oligomer distribution evaluation was to calculate the relative SP-A amount in the first and the last peak by integrating the area beneath the peak, when the fraction number is plotted against the amount of SP-A in ng/ml, and then calculating the proportional amount of SP-A per peak. Only the first and the last peak were chosen for correlation analysis because the extremes (first and last peak) provide additional power to detect genetic modifiers or clinical influences.

4.2.5.1) SP-A oligomerization and lung disease

There was a significant association of the lung disease status in CF patients and the percentage of SP-A that is existent as octadecamers or more complex structures (first peak after separation on column) in BAL ($p = 0.02$), while in serum an association was slightly detectable but not significant. Then the correlation between the course of lung disease (ΔFev1 (% pred.)) and the percentage of SP-A that was present in the first peak was analyzed and there was also a significant association in BAL and serum ($p = 0.0008$ and 0.0038).

In conclusion a higher relative SP-A amount in the first peak is associated with a milder lung disease and a better course of lung disease. This supports the findings correlating the distribution forms in BAL

that the more SP-A is existent in octadecamers or more complex structures in BAL the better is the lung function.

4.2.5.2) SP-A oligomerization and self-agglutination

Also the self-agglutination abilities of BAL and serum were analyzed and compared to the relative amount of oligomerization of SP-A derived from gel chromatography using 100 ng SP-A from the 43 BAL and 34 serum samples of CF patients. Like the oligomeric distribution forms also the relative SP-A amounts in the first and last peak in BAL showed a correlation with the self-agglutination ability (see fig.3.1.5.2.1). In serum there was no such correlation (see fig.3.1.5.2.1).
There was no correlation between the self-agglutination of the SP-A fractions 10, 15 and 20 and the relative SP-A amount in the first and last peak.
These findings support the theses that SP-A self-agglutination is dependent on the amount of particular SP-A oligomers present in a sample, i.e. the relative strength of SP-A molecular forms in a sample.

4.2.5.3) Influence of SP-A self-agglutination ability on Fev_1 (% pred.)$_{age20}$ and ΔFev_1 (% pred.)/year

Because of the correlation between SP-A structure and lung Fev1 (% pred.)$_{age20}$ and between the SP-A structure and the self-agglutination ability it was also searched for a correlation between the self-agglutination ability and lung disease severity. For Fev1 (% pred.)$_{age20}$ there was in BAL ($p = 0.0137$) and serum ($p = 0.0338$) a significant correlation (shown in fig. 3.1.5.3.1 a and b). Additionally, for ΔFev_1 (% pred.)/year there was also a significant correlation in BAL ($p = 0.0213$) and weakly in serum ($p = 0.0.497$) which is shown in fig. 3.1.5.3.2 a and b. These results indicate the better the self-agglutination ability of SP-A the better is the lung function. There was no correlation between the lung disease status (mild/severe) and the agglutination size.
These results indicate the better the self-agglutination ability of SP-A the better is the lung function.

4.2.5.4) SP-A oligomerization and genetics

Afterwards it was searched for a genetic background of the oligomeric formation of SP-A. Therefore the *SFTPA*1 and *SFTPA*2 genotypes from MALDI-TOF were correlated with the percentage of SP-A which was present in the first peak in relation to the percentage of SP-A which was present in the last peak. Two SNPs, rs1136451 (*SFTPA*1) and rs17881665 (*SFTPA*2), were significantly associated with the formation of octadecamers or more complex structures ($p = 0.0015$ and 0.004). The wildtype alleles of both SNPs were associated with octadecamers or more complex structures. An analysis of a correlation between both SNPs indicated a linkage disequilibrium ($p = 0.00001$). The *SFTPA*1 SNP is a polymorphism in the coding region of the gene (P62P), but it doesn't lead to an aa exchange. In a previous study by Floros et al. (12) it was shown that P62P is associated with the expression level of

SP-A. The *SFTPA*2 SNP is located in the 5'-UTR of the gene where also splicing and other regulative sequences influencing the SP-A expression exist. Therefore the *SFTPA2* SNP either influence the SP-A structure or was just associated because of the linkage disequilibrium.
The next step was the analysis of the correlation between the SP-A level and the SP-A structure. There was a significant association ($p = 0.0006$) indicating that the higher the SP-A level was in BAL the bigger was the percentage of SP-A which was existent in octadecamers or more complex structures. This indicates that the SNPs are associated with the SP-A structure because they are associated with the SP-A level in BAL which influences the structure. Because the *SFTPA1* SNP is localized in the coding region a direct influence on the expression level is not likely. There could exist another coupling with an *SFTPA1* SNP in a regulative sequence of the gene responsible for the association.
The results show that the SP-A oligomerization is associated with one *SFTPA1* and one *SFTPA2* SNP which are coupled.

4.2.6) SP-A isolation

For investigating the SP-A structure with another method, SP-A was isolated of the fractions from the first peak after gel chromatography by beads. The beads were coupled to an anti-SP-A antibody by biotin-streptavidin binding and could therefore pull the SP-A down in the fractions. A pool of 12 fractions (F10) from control BAL samples was used. In the supernatant after the pull-down, almost none SP-A was left in solution. Also after dissolving and separating the beads from the SP-A solution only a small amount of SP-A was left with the beads and detected in the wash solution. It was important for the following AFM-based force spectroscopy to have a preferably pure sample of SP-A. Therefore the isolation had to be done without calcium to prevent the binding of other proteins to SP-A. Several washing steps were chosen to remove the unbound proteins like one with the addition of Triton X-100 to inhibit unspecific binding. The constant movement during incubation assured an optimal binding of the antibody or SP-A. The pH had to be adjusted to the iso-electric point of SP-A to keep it stable.
Most of the SP-A amount could be isolated out of the fractions and thus the SP-A isolation assay was proved to be effective.

4.2.6.1) Protein gel and silver staining

For determining the purity of the isolated SP-A sample it was first separated by gel chromatography on an SDS PAGE and then the gel was stained with silver.
There was only one band for the SP-A isolation sample at about 35 kDa. SP-A monomers have a molecular weight of 28-35 kDa. Therefore the purity of the SP-A isolation sample is more than 90 %. This indicates that the SP-A isolation assay shows not only an efficient binding capability, but also enables an isolation with high purity. Normally there is more than one band on a reducing gel because

of different posttranslational modifications of SP-A which led to different molecular weights from 28 to 35 kDa, but there seems to be only one form that was isolated.

4.2.6.2) Single molecule forced microscopy

To finally examine the structure of SP-A in a fraction after gel chromatography the method of single molecule force spectroscopy based on atomic force microscopy was used. Only one molecule was attached to a gold layer and then pulled of by a cantilever, while the applied force is measured.
About 2,000 measurements were completed. There were curves about 100 nm long which would correspond to about 270 aa. These curves could depict SP-A monomers. There were also curves corresponding to about 66 aa which could display the collagen-like domain. Because there were no longer curves it must be concluded that there were no multimers in the solution. There were also a lot of smaller curves which had to be fitted and put in order. After all a force spectrum of a SP-A monomer could be developed by superimposition of the curves. This implicates that the oligomers were hydrolyzed before because at least dimers or trimers should have been found. This happened most probably during the isolation because the samples showed a good reproducibility by gel chromatography. The last step of the isolation with an acid glycine buffer could cause the hydrolyzation of the bisulfide bonds which link the SP-A monomers to form oligomers.
After all the single force microscopy would be only useful for analysis of the monomeric SP-A domains, but not of the oligomeric structure.

4.3) SP-A self-agglutination

4.3.1) SP-A self-agglutination-assay

Because of the structural differences of SP-A between individuals, it was necessary to analyze whether there are functional differences between different oligomeric states. For this a new agglutination assay with streptavidin beads was established. The streptavidin beads were coupled to biotinylated rabbit anti-goat antibodies which bound goat anti-human SP-A antibodies. These anti-SP-A antibodies bound SP-A at its N-terminal end, so SP-A could self-agglutinate by its CRD.

4.3.2) SP-A self-agglutination-assay establishment

The agglutination-assay construction was tested. The conditions of the assay were varied with respect to the reagent addition to prove that the assay functions because of the binding chain bead-anti-goat antibody-anti-human SP-A antibody-SP-A and not because of other influences. The results state that the beads only agglutinate when also the anti-goat antibody, the goat-anti-human SP-A antibody and SP-A is present. Also an increasing agglutinate size could be measured with respect to an increasing SP-A level. In this way the assay construction was proved to function. Secondly the calcium and carbohydrate dependency of the assay was analyzed by changing the assay conditions with regard to

the calcium and carbohydrate amount. Without calcium and/or with mannose no agglutination could be observed. The pH and the ion concentration were at physical conditions. This was important because intact SP-A self-agglutinates at low pH (≤ 4) and/or high ion concentrations (0.1-0.4 M Cl^-) (13,14).
Thus the agglutination-assay was specific for measuring the self-agglutination of SP-A caused by its CRD.

4.3.3) Relation between SP-A self-agglutination and structure

In line with the results of the assay establishment the agglutination assay was used to determine the functional capability of SP-A regarding its CRD. Because different oligomeric structures of SP-A were found in BAL and serum of all study populations the fractions number 10, 15 and 20 of the gel chromatography representing the three peaks were analyzed by the agglutination assay. In all three study populations, CF, asthma and chronic bronchitis patients and controls, there was a significant difference regarding the agglutination ability between the fractions in BAL and serum. There was a significant decrease regarding the agglutination ability from SP-A octadecamers or more complex structures present in fraction 10 over the hexamers present in fraction 15 to the dimers and trimers present in fraction 20. Sanchez-Barbero et al. (9) found out that the complex oligomerization is essential for collagen triple helix stability at physiological temperatures, protection against proteases, and SP-A-induced ligand aggregation. On the other hand it is not essential for the binding of SP-A to ligands and anti-inflammatory effects of SP-A. This corresponds to the results of this study because the SP-A out of fraction 10 showed the better agglutination abilities, but also the other fractions could cause agglutination.
Comparing the fractions between BAL and serum in the patient groups there was also a significant difference. The serum SP-A structures had significantly better agglutination ability than the corresponding structures in BAL from patients. While in CF patients this difference was highly significant for all structures in the bronchitis study population the significance decreased from fraction 10 over fraction 15 to fraction 20. In controls all fractions and therefore all SP-A structures in serum and the corresponding structures in BAL showed the same agglutination ability. The reason for this could be another way for SP-A to move from lung into serum except for the already discussed way of passive diffusion. Restricted passive diffusion of large proteins via the paracellular route plays an insignificant role in the net absorption or secretion of these proteins across normal alveolar epithelium (15). Receptor-mediated transport through transcytosis could also play a role as for BSA (16). Therefore the more effective the SP-A is able to bind to a receptor also represented by its agglutination ability the more is transported. In endothelial cells numerous vesicles and membrane invaginations (including caveolae and clathrin-coated pits) that are expected to play an important role in internalization of proteins and transcellular movement of cargo proteins (17). SP-A binds to high-affinity binding sites (receptors) at the type II pneumocyte cell membrane (18,19) and has been demonstrated in association with coated pits and coated vesicles, suggesting receptor-mediated endocytosis via the coated-pit pathway (20,21). Thus receptor-mediated transport could explain the difference between the agglutination ability of SP-A derived from BAL compared to SP-A derived from serum in one patient.

At last the fractions in BAL and serum were compared between the study populations. For all BAL fractions the SP-A oligomers derived from controls agglutinated significantly better than the SP-A oligomers derived from patients. Also all BAL structures from the bronchitis study population agglutinated better than the structures derived from CF patients, but the difference was only significant in fraction 15. In contrast to these findings in serum there were no differences regarding the agglutination ability between the study populations. A gradual decrease ($P < 0.05$) in SP-A1/SP-A ratio in BAL was observed in healthy subjects (HS) with increased age, although no significant change was observed in total SP-A content among age groups in the study from Tagaram et al. (3). The CF ratio was significantly higher compared with AP, HS and non-cystic fibrosis (NCF), even though SP-A1 and total SP-A were decreased in CF compared with most of the other groups. The ratio was higher in culture-positive vs. culture-negative samples from CF and NCF ($P = 0.031$). This indicates that here the more complex forms are associated with culture-negative cells. As SP-A is involved in the immunity of the lung and the more complex structures have better agglutination abilities the ratio which influences the degree of oligomerization could influence the immunological function of SP-A. These findings are consistent with the agglutination results of this study. The lack of difference in serum supports the theory of the receptor-mediated transport because the transporting criteria would be the same for all study populations and therefore the SP-A which could be transported, shows the same agglutination abilities.

In summary the more complex the SP-A structure the better was the self-agglutination ability. All SP-A structures self-agglutinate in serum better than in BAL in samples of the patients groups while in control samples BAL and serum SP-A showed the same abilities. There was also a difference between the self-agglutination ability of BAL samples from CF, Bro and control derived SP-A, but none in serum. SP-A from control BAL agglutinated better than from bronchitis BAL and this better than from CF BAL. This indicates that the analysis of the SP-A structure composition is important because the structure has strong effects on the SP-A self-agglutination ability.

4.4) SP-A-genetics, clinical and biochemical data

4.4.1) Clinical data of the genetic populations

The CF population was with 454 patients the largest, while in the bronchitis population 72 and in the control population only 25 individuals were included. The problem here was to collect all the samples. The controls or bronchitis patients were mostly only once in the hospital and then not enough blood samples were taken to be also available for scientific research. Because of this also the mean ages at sampling point of the study populations differ. The percentage of female patients and controls was almost even. The mean BAL level of the CF population was as expected lower than the BAL levels of the other populations, but the serum level was higher. The difference was only marginal between the CF and bronchitis population, but significant between the CF and control population. This is most probably caused by the small number of controls. Normally as previous studies had shown the serum level of CF patients is lowered (22-24).

4.4.2) Lung disease

Determining the lung disease status for the chronic bronchitis and asthma patients was could be done without any exclusion as long as there was lung function data available. In CF patients there had to be at least 3 values to determine the disease status. One problem for all populations was the age of the patients. Children younger than 5 weren't tested for lung function and if they were, the value is only partly useful. Sometimes the children don't do their best while measurement because they have to learn the procedure. To get most reliable data the measurements of children under 5 were excluded. The next problem aroused by determining the status either by the last three lung function data or by estimating the value at age 20 which was chosen because the patients are fully grown and it allows a good prediction for the future. Also the lung disease groups differed significantly regarding their Fev1 at this age. Patients had to be excluded by determining the lung disease status because they didn't fit into the cut-off-values and would have dampened the statement of a correlation analysis. To include these CF patients an additional approach was chosen. The mean change of the lung function per year were calculated and also used for correlation analysis.

4.4.3) Genetic influence on BMI and genotype differences between patient groups

For determining the genotypes of the study populations some SNPs which cause an aa exchange were analyzed by melting curves with hybridized probes in a Light Cycler. Also for a bigger throughput of genotyping the method of Matrix Assisted Laser Desorption/Ionisation-Time-Of-Flight (MALDI-TOF) was used. The SNP data resulting from the genotyping by MALDI-TOF and probes were correlated with the clinical data and ELISA level measurements by χ^2-test and the results were corrected according to Bonferroni.

There were significant p values after comparison of the study populations, CF, bronchitis and controls, for SP-D M31T ($p = 0.03$), SP-A2 rs1965708 ($p = 0.0001$) and SP-A2 rs1975006 ($p = 0.0001$). For SP-D M31T the allelic variant in the N-terminal region at position 11 (methionine exchange for threonine; Met11Thr) was associated with susceptibility to Tbc in a Mexican population (25), whereas methionine at position 11 was associated with severe RSV bronchiolitis in infants (26). Recently, Leth- Larson *et al*. demonstrated that individuals with the Thr/Thr 11 genotype had significantly lower SP-D serum levels compared with Met/Met 11 genotype carriers (27). While the blood of individuals with the Met/Met 11 alleles contains the multimeric SP-D forms, the Thr/Thr 11 genotypes lack these forms. Multimerization of SP-D is suggested to play a crucial role for the interaction with immune cells and pathogens (28). In this study the CF population was associated with the threonine allele. Therefore the lack of multimeric forms and the lower SP-D levels could influence the disease.

SP-A knock-out mice demonstrated a crucial role of SP-A in the regulation of innate immunity and adaptive immunity (29). In mice with CF, linkage scans showed an association of the degree of fibrosis and a locus on chromosome 10 in mice. A recent study showed an association between the haplotype $6A^3/1A^1$ and lower pulmonary function as well as poor pulmonary scores (30,31) and SP-A2 rs1965708 is one SNP used to describe the $1A^x$ haplotypes. It causes an aa exchange at position 223

from glutamine to lysine and the lysine allele from the haplotype $1A^1$ was associated with CF. No other SNPs of the haplotype were associated with the disease, but the haplotypes are based on the allele distributions in an American population. Differences could occur because of the different genetic background and therefore association analyses should first be done according to each analyzed SNP and then haplotypes can be tried according the associated SNPs. The SP-A2 rs1975006 is located in an intron of the gene and has not been analyzed before. It could be linked to another relevant SNP, but in this study no linkage was found.
According to this, the SP-A2 SNPs rs1965708 and rs1975006 could influence CF by causing a worse lung disease outcome.

4.4.4) SP-A and SP-D level measurements

In a former study by Bielecki ("Surfactantproteine A und D im Serum und in Bronchoalveolärer Lavage bei Patienten mit Cystischer Fibrose", MD, 2007) the consistency of the SP-A serum levels in CF patients over time was shown. This consistency allows us to compare results arising from sera taken at different points of time from CF patients with clinical stability. The SP-D levels in BAL differed significantly ($p_{CF\text{-}Bro}$ = 0.0003, $p_{Bro\text{-}C}$ = 0.02) between the patient groups. The bronchitis patients had significantly lower SP-D levels in BAL than the CF patients or controls. SP-D is known to be degraded by elastase in BAL of CF patients (32), but the elastase levels at sampling point were low. This could explain the higher levels of CF in the range of controls. The bronchitis patients included some patients with a lack of SP-D in BAL which was further analyzed and discussed in 4.5.

4.4.5) Genetics in CF

4.4.5.1) SP-A SNPs and clinical data

There was a significant association between the mean Δ Fev1 (%pred.) / year and V50L a SNP in the *SFTPA1* gene (p = 0.0038) while the mutated allele was associated with a worse course of lung disease. The SP-A1 SNP V50L was used to describe the SP-A1 haplotypes $6A^x$. CF patients with the haplotype $6A^3$ had a significantly lower Fev1 (%pred.) than patients with other haplotypes. According to this the V50L SNP which causes an aa exchange from valine to leucine at position 50 in the SP-A1 protein could directly influence the lung disease pattern. An aa exchange could cause a conformational change and therefore influence the immunological capability of SP-A. This could affect the lung disease.
Also the appearance of an ABPA infection was significantly associated with a *SFTPA2* SNP N9T analyzed by χ^2-test (p = 0.004). For SP-A2 N9T the wildtype allele was significantly associated with the group that had never ABPA. N9T is relevant for the haplotype $1A^0$ and $1A^3$. The $1A^0$ allele is associated with RDS (33), but there is nothing known about associations of the SNP itself. It is located in the coding area and causes an aa exchange from asparagine to threonine at position 9 in the

amino-terminal domain. This domain is important for the stimulation of inflammation and could therefore influence ABPA infection.
The other characteristics, age at the first *P. aer.* infection, age at the first *P. aer.* mucoid infection, chronic or non-chronic *P. aer.* infection, age at the first chronic *P. aer.* infection, BMI and Fev1 (% pred.) estimated for age 20, had no significant correlations with any SNPs after Bonferroni correction.

4.4.5.2) SP-A level and genetics in CF

For SP-A there were significant genetical associations with the BAL and serum level. The SP-A BAL level was significantly associated with the SP-A1 SNP rs1136451 ($p = 0.002$) and the SP-A2 SNP rs17881665r ($p = 0.002$). Here the mutated allele was associated with lower SP-A levels like it was shown before with the CF part analyzed by gel chromatography. The SNPs regarding the SP-A level were discussed in 4.2.9. This significant correlation existed only by analyzing the SNP data with the χ^2-test and grouping after BAL levels (group 1: 0-1,000 ng/ml; group 2: 1,000-10,000; group 3: >10,000) to compare the extremes. The correlation of the BAL level data by grouping regarding the genotype was not significant. The SP-A serum level was significantly associated with SP-A1 N9T ($p = 0.028$). This SNP was also associated with ABPA infection (see above). Because of the location of the SNP in the coding region it is more likely that the SNP indirectly influences the SP-A level. Because the wildtype seems to protect against ABPA infection and is associated with lower serum levels the explanation could be that infection destroys the lung-blood barrier and therefore increases the SP-A level in serum.

4.4.5.3) Fev1 (% pred.) estimated for age 20 correlates with SP-A level

The SP-A levels in BAL and serum were measured by ELISA and correlated to the clinical data of the patients. In the CF study population there was a significant association between the SP-A level in BAL and the Fev1 (% pred.) estimated for age 20 ($p = 0.009$). The higher the SP-A levels were in BAL the bigger were the values of the Fev1 (% pred.) estimated for age 20. This is consistent with the finding that higher BAL levels lead to more complex oligomeric structures in BAL which are associated with a better lung disease outcome in CF patients. In serum there was no correlation with this characteristic ($p = 0.33$). There was also no correlation between the SP-A BAL or serum level and any other clinical characteristic such as BMI, age, gender, IgG or IgE in serum.

4.4.5.4) SP-D genetics in CF

After comparison of the lung disease groups, mild young, mild old and severe, for the CF population one SNP showed a significant allele distribution, SP-D M31T ($p = 0.0005$).
Because the Fev1 (% pred.) estimated for age 20 had been shown to be an equivalent classification criteria for lung disease of CF patients (2) and the lung disease groups were significantly associated

with SP-D M31T this correlation was again analyzed in prism after grouping the patients regarding to their genotype. The same correlation could be shown that the mutated allele leads to a worse lung disease outcome ($p = 0.0091$).
Analyzing the mean ΔFev1 (% pred.)/year regarding to the genotype of *SFTPD* M31T there was also a significant correlation ($p = 0.019$).
As mentioned above the SP-D M31T influences the multimerization of the SP-D monomers which affects the immunological effect. Patients with a severe lung disease status had significantly more frequent the threonine allele. This supports the idea that the lack of higher multimers caused by the threonine at position 31 influences the lung disease because of the weaker pathogen defence.

4.4.5.5) SP-D SNPs influencing the protein level in CF

The SP-D serum level was significantly associated with the SNPs SP-D M31T ($p = 0.04$) and SP-D A180T ($p = 0.013$). These SNPs were also shown to be associated with the protein level by Leth-Larsen et al., 2005 (34). They genotyped 143 individuals regarding these SNPs and measured the SP-D level in serum. The p-value for the association of M31T with the serum level was 0.015, and the alleles of A180T were linked to the M31T alleles.
In line with the study by Leth-Larsen also in this study the mutated alleles of both SNPs were associated with lower serum levels.

4.4.5.6) Influence of lung disease, age and BMI on SP-D levels

The SP-D level in serum of the CF study population was significantly associated with the lung disease (mild/severe, $p = 0.0005$) and the Fev1 (% pred.) estimated for age 20 ($p = 0.0027$). The higher the SP-D level was in serum the smaller was the value of the Fev1 (% pred.) estimated for age 20. In BAL there was no correlation ($p = 0.9$).
The SP-D SNP M31T was associated with the serum levels, the lung disease group and lung disease pattern. This is consistent with the results from the ELISA measurements. Therefore the threonine allele at aa position 31 in the SP-D gene causes a low serum level and a worse lung disease outcome.
Analyzing the SP-D levels in BAL and serum with respect to the age of the individuals there was a significant association between increasing SP-D serum level and increasing age ($p = 0.001$), but no correlation between the SP-D BAL level and age. Additionally by analyzing the BMI groups underweight, normal weight and overweight and the SP-D levels there was a significant difference between the serum levels of the underweight and the overweight group as well as between the normal weight group and the overweight group (both $p < 0.01$), but no significance regarding the BAL levels. While the underweight and normal weight groups had a similar SP-D level distribution in serum, the overweight group had significantly lower serum SP-D levels.
There were two studies indicating a genetically and phenotypically association of SP-D with obesity (35,36). In these studies weight and BMI related significantly inversely to serum SP-D. Additionally a significant age-dependent increase in serum SP-D was prominent in lean persons (36).

In conclusion the SP-D level in serum is associated with the *SFTPD* SNP M31T, is a marker for the severity of lung disease and is also influenced by age and weight.

4.5) Absence of SP-D in BAL

After we identified subjects with no SP-D in their BAL common polymorphisms known to be associated with low serum SP-D levels were assessed in those subjects from whom informed consent could be obtained and DNA was available. The M31T polymorphism in exon 1, A180T in exon 4 and S290T in exon 7 were analyzed. DNA was available from 10 of the 12 children who had no SP-D detectable in their BAL by ELISA (n = 6) and Western blotting (n = 4). For comparison 8 children were selected with the same clinical characteristics, but detectable SP-D levels in serum.
No significant genetic association could be found between the common polymorphisms known to be associated with low serum SP-D levels and the lack of SP-D in BAL or serum respectively. Because the SNPs are only associated with low serum levels and are not the only cause for it the small sample size could be the reason for the lack of an association in this study.

4.6) TLR SNPs

The SNP TLR4 A-2570G was significantly associated with the lung disease (mild/severe) in CF patients analyzed by χ^2-test ($p = 0.0003$). No other correlation between any clinical characteristics could be found. TLR4 is a lipopolysaccharide receptor and initiates the innate immune response against gram-negative bacteria (37). Other polymorphisms have been shown to attenuate receptor signalling (38), but TLR4 A-2570G has not been described before. Additionally no linkage between this SNP and any other TLR4 SNP analyzed could be found. The TLR4 SNP is located in the 5'-UTR of the gene. It could influence the expression rate of TLR4 and therefore the immune response. A lower protein level could affect the lung disease.

5) Summary

Surfactant associated protein-A (SP-A) is the most abundant pulmonary surfactant protein and belongs to the family of innate host defence proteins termed collectins. Besides pulmonary host defence, SP-A is also involved in the formation of pulmonary surfactant, as it is essential for the structure of tubular myelin. The human SP-A gene locus includes two functional genes, SFTPA1 and SFTPA2 which are expressed independently, and a pseudo gene. SP-A polymorphisms play a role in respiratory distress syndrome, allergic bronchopulmonary aspergillosis and idiopathic pulmonary fibrosis. The levels of SP-A are decreased in the lungs of patients with CF, respiratory distress syndrome and further chronic lung diseases.

The aim of the study was to elucidate the role of SP-A in human lung disease. This study was designed to analyze the relation between genetics, structure and function of SP-A in a CF, chronic bronchitis and asthma and healthy control population *ex vivo*.

Beginning with the analysis of SP-A oligomeric forms, there were no correlations between serum and BAL SP-A distribution forms. The forms showed only a significantly different distribution comparing serum samples from bronchitis patients and controls. In serum and BAL of CF patients the forms containing the first peak were correlated with a better lung function (Fev1 (% pred.)$_{age20}$). Also higher relative SP-A amount in the first peak is associated with a milder lung disease and a better course of lung disease. Additionally, SP-A self-agglutination is dependent on the amount of particular SP-A oligomers present in a sample, i.e. the relative strength of SP-A molecular forms in a sample. All SP-A structures self-agglutinate in serum better than in BAL in samples of the patients groups while in control samples BAL and serum SP-A showed the same abilities. There was also a difference between the self-agglutination ability of BAL samples from CF, Bro and control derived SP-A, but none in serum. SP-A from control BAL agglutinated better than from bronchitis BAL and this better than from CF BAL. These results are also supported by the fact that a better agglutination ability of SP-A was significantly associated with lung function. Therefore the degree of the presence of active oligomeric forms within a BAL or serum sample seems to be important for a better lung function outcome.

The SP-A oligomerization is associated with one *SFTPA1* rs1136451 and one *SFTPA2* SNP rs17881665 which are coupled, while the mutated allele seems to cause a lack of complex oligomers. The wildtype alleles of the SP-A2 SNPs rs1965708 and rs1975006 were associated with CF compared to the bronchitis and control group. There was a significant association between the mean Δ Fev1 (%pred.) / year and V50L a SNP in the *SFTPA1* gene ($p = 0.0038$) while the mutated allele was associated with a worse course of lung disease. The SP-A BAL level was significantly associated with the SP-A1 SNP rs1136451 ($p = 0.002$) and the SP-A2 SNP rs17881665r ($p = 0.002$). The SP-A serum level was significantly associated with SP-A1 N9T ($p = 0.028$).

In addition in the CF study population there was a significant association between the SP-A level in BAL and the Fev1 (% pred.) estimated for age 20 ($p = 0.009$). The higher the SP-A levels were in BAL the bigger were the values of the Fev1 (% pred.) estimated for age 20. There was no correlation between the SP-A BAL or serum level and any other clinical characteristic such as BMI, age, gender, IgG or IgE in serum.

In conclusion, these results indicate a very important role for SP-A in human lung immunity. Future areas for clinical research include disease specific SP-A expression pattern and their functional consequences, the differential roles of SP-A1 and SP-A2 in human lung diseases, and therapeutic approaches to correct altered SP-A levels.

For SP-D the SNP M31T influences the multimerization of the SP-D monomers which affects the immunological effect. M31T is like *SFTPD* A180T associated with the serum SP-D level. In line with the study by Leth-Larsen also in this study the mutated alleles of both SNPs were associated with lower serum levels. The SP-D level in serum is also influenced by age and weight. Nevertheless it is a good marker for the severity of lung disease.

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Appendix

A: SP-A1 and SP-A2 polymorphism data

Table A.1: **Polymorphism data from the Universitiy of California, Santa Cruz** [1]

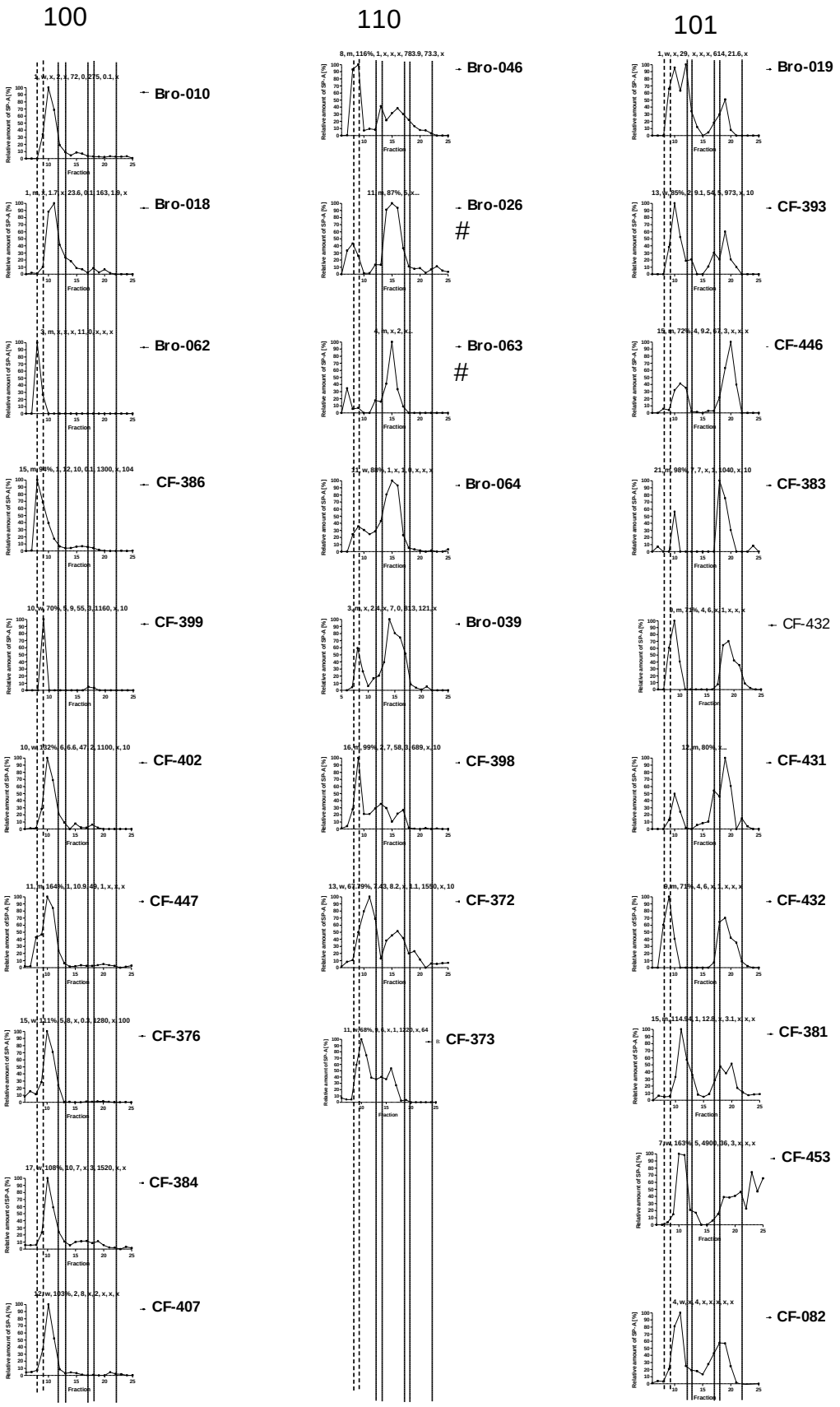
Polymorphism		Reference SNP ID	Alleles	Number of populations studied	Allele frequency
N9T	SP-A1	Not listed	C/A	-	-
	SP-A2	rs1059046		2	0.032/0.968
V19A	SP-A1	rs1059047	C/T	2	0.047/0.953
	SP-A2	Not listed		-	-
H39H	SP-A1	Not listed	C/T	-	-
	SP-A2	Not listed		-	-
V50L	SP-A1	Not listed	G/C	-	-
	SP-A2	rs17883551		1	Not available
P62P	SP-A1	rs1136451	G/A	2	0.136/0.864
	SP-A2	rs2434114		0	Not available
P91P	SP-A1	Not listed	G/C	-	-
	SP-A2	rs17886395		2	Not available
T133T	SP-A1	rs1059057	G/A	2	0.043/0.957
	SP-A2	Not listed		-	-
S140S	SP-A1	rs3997777	C/T	0	Not available
	SP-A2	rs1965707		0	Not available
Y184Y	SP-A1	rs4253526	C/T	2	0.032/0.968
	SP-A2	Not listed		-	-
Q223K	SP-A1	rs1965708	A/C	1	0.272/0.728
	SP-A2	rs17881479		2	Not available

The table shows the reference number for each SNP used in studies to characterize the alleles of the SP-A genes, the alleles, the number of populations where it was observed in, and the allele frequencies.

[1] http://genome.cse.ucsc.edu/

B: SP-A BAL and serum structure distributions

B.1: **BAL forms**



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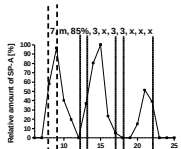

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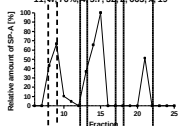

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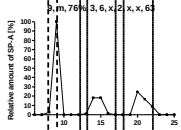

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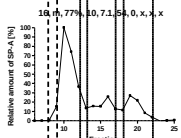

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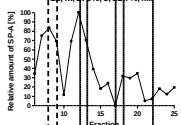

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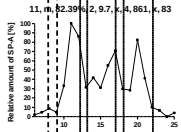

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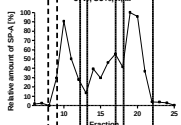

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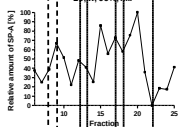

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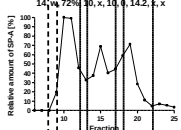

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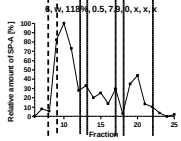

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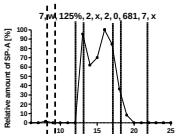

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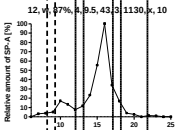

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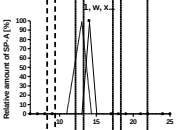

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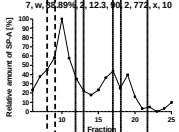

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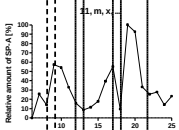

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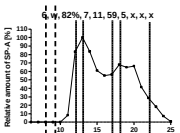

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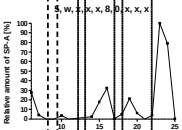

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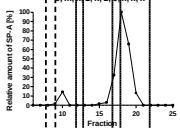

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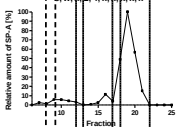

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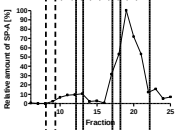

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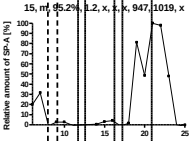

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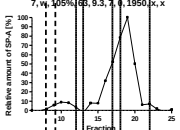

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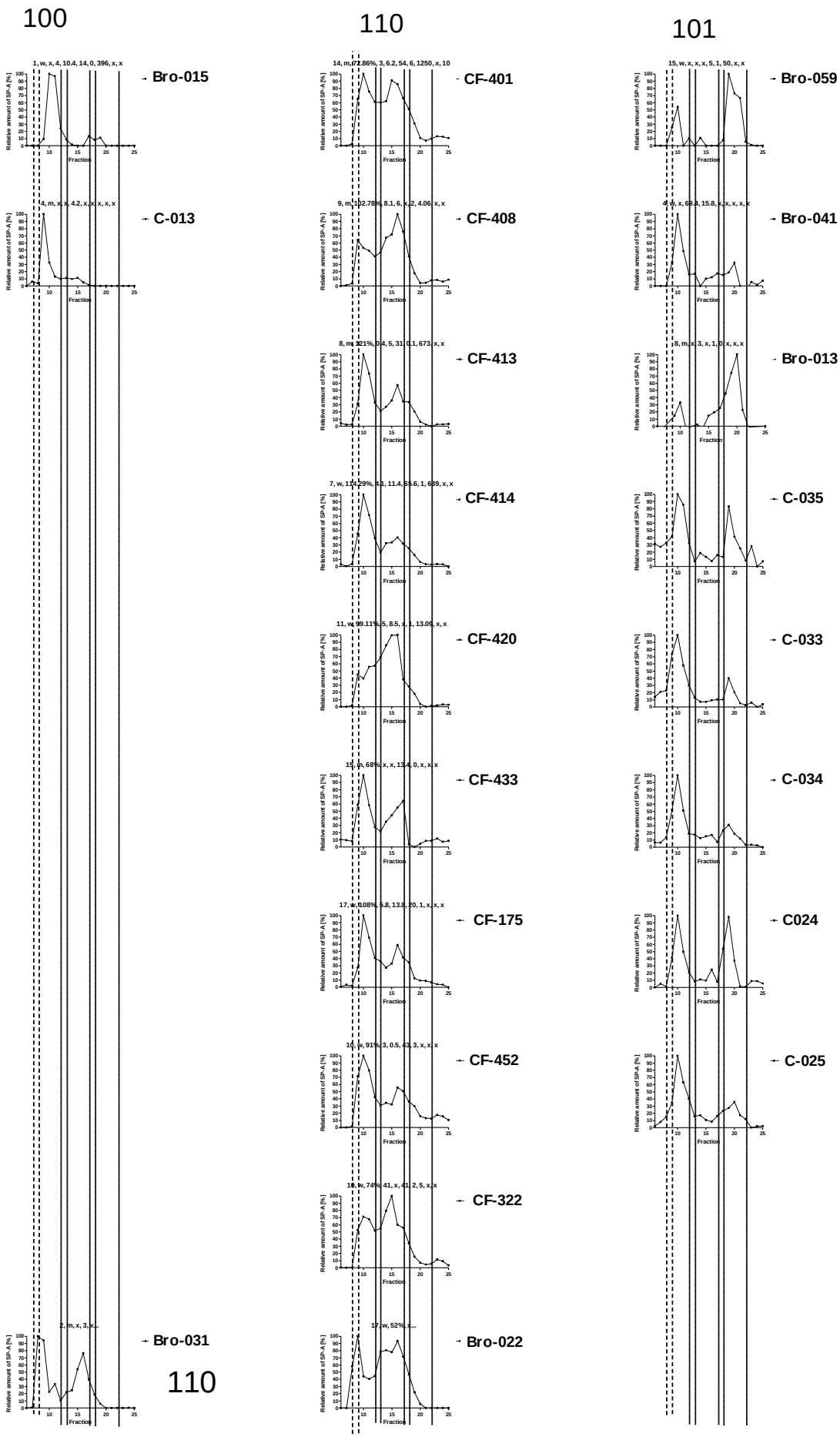
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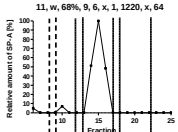
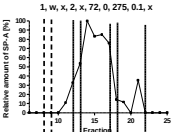
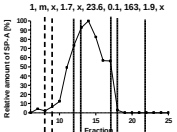
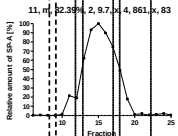
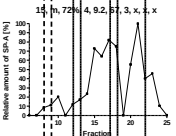
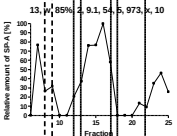
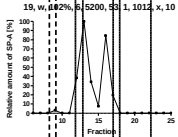
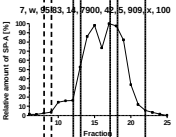
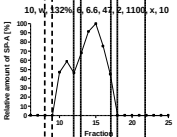
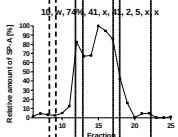
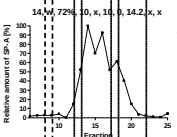
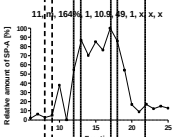
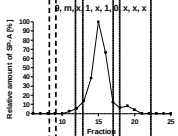
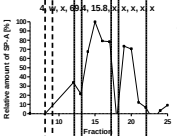
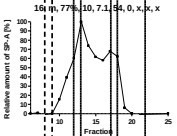
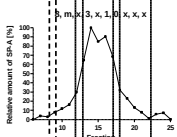
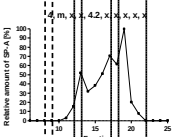
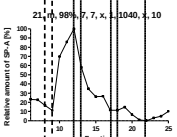
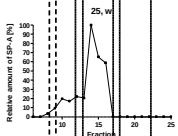
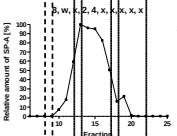
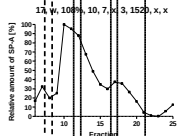
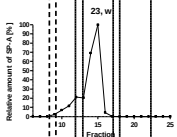
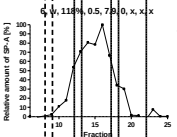
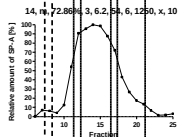
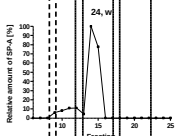
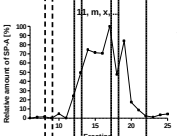
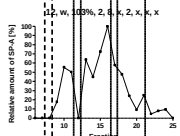
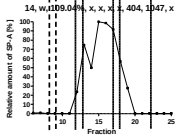
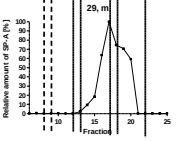
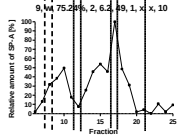
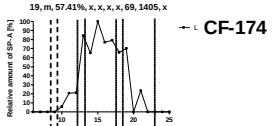
110
101
Bro-015
C-013
Bro-031
110
CF-401
CF-408
CF-413
CF-414
CF-420
CF-433
CF-175
CF-452
CF-322
Bro-022
Bro-059
Bro-041
Bro-013
C-035
C-033
C-034
C024
C-025
Relative amount of SP-A [%]
Fraction

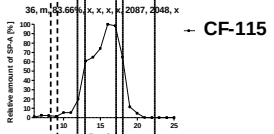
B.2: **Serum forms**

010	011	110
 CF-373	 Bro-014	 Bro-030
 CF-419	 CF-446	 CF-393
 CF-430	 CF-442	 CF-402
 CF-322	 CF-210	 CF-447
 C-009	 Bro-070	 CF-449
 C-011	 C-026	 CF-383
 C-023	 C-010	 CF-384
 C-017	 C-014	 CF-401
 C-008	 C-025	 CF-407
 CF-354	 Bro-079	 CF-423

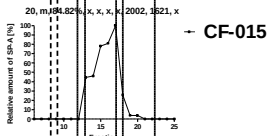
010



CF-174

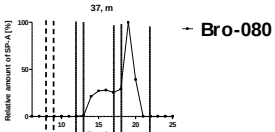


CF-115



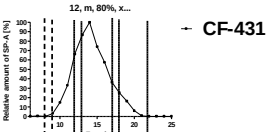
CF-015

011



Bro-080

110



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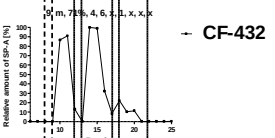

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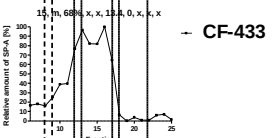

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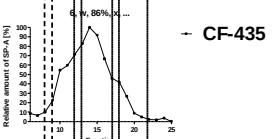

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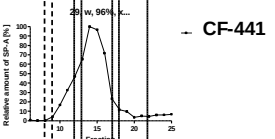
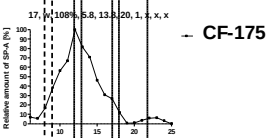
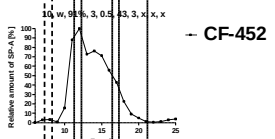


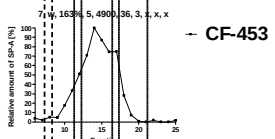
CF-441



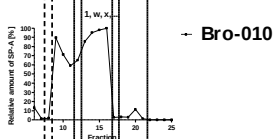
CF-175



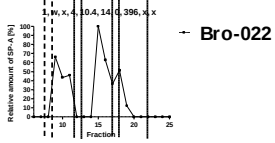
CF-452



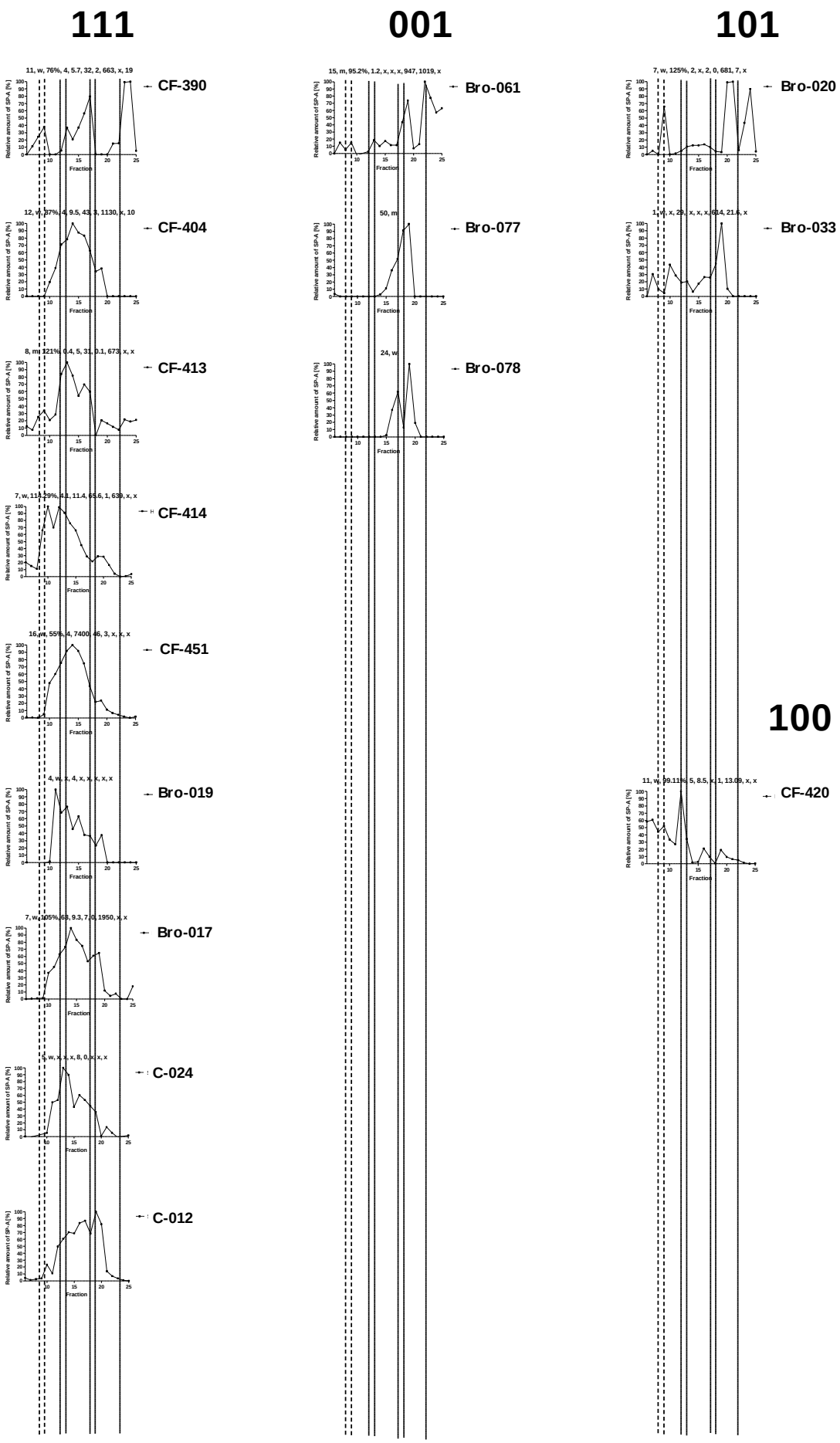
CF-453



Bro-010



Bro-022


111
001
101
100
11, w, 76%, 4, 5.7, 32, 2, 663, x, 19
CF-390
12, w, 87%, 4, 9.5, 43, 3, 1130, x, 10
CF-404
8, m, 121%, 0.4, 5, 31, 0.1, 673, x, x
CF-413
7, w, 111, 29%, 4.1, 11.4, 65.6, 1, 639, x, x
CF-414
16, w, 55%, 4, 7400, 46, 3, x, x, x
CF-451
4, w, x, 4, x, x, x, x, x
Bro-019
7, w, 105%, 63, 9.3, 7.0, 1950, x, x
Bro-017
5, w, x, x, x, 8, 0, x, x, x
C-024
C-012
15, m, 95.2%, 1.2, x, x, x, 947, 1019, x
Bro-061
50, m
Bro-077
24, w
Bro-078
7, w, 125%, 2, x, 2, 0, 681, 7, x
Bro-020
11, w, x, 29, , x, x, x, 614, 21.6, x
Bro-033
11, w, 99.11%, 5, 8.5, x, 1, 13.09, x, x
CF-420
Relative amount of SP-A [%]
Fraction

Curriculum vitae

Name	Stefanie May Heinrich
Date of birth	05-20-1981
Place of birth	Scottsdale, U.S.A.
Nationality	German
Marital status	Married

Work experience

March 2009-current
UKGM, Gießen
Institute of Human Genetics

08-01-2005 to Februar 2009
Dr.-von-Haunersche's Kinderspital
Department of pulmonology, Munich

Education

03-04-2005
Diploma in Bio-Chemistry
"About the influence of mutations, polymorphisms and variations of the *ITGA4*- and *NOS2A*-genes on the predisposition to multiple sclerose"; department of human genetics

WS 2000/2001 – WS 2004/2005
Study of Bio-Chemistry at the "Ruhr-Universität" Bochum, Germany with focus an human genetics

Internships

17.03.2003 – 04.04.2003
Department of nuclear medicine at the Marienhospital, Herne

05.05.2003 – 27.06.2003
Department of human genetics, faculty of medicine, "Ruhr-Universität", Bochum

Schools

1991-2000
Antonius-Kolleg, private registered *Gymnasium*, Neunkirchen-Seelscheid

1987-1991
Janusz-Korczak-primary school, Marienfeld

Publications

"Surfactant protein A in cystic fibrosis: supratrimeric structure and pulmonary outcome", Heinrich et al. (Paper in progress)

"Assessment of SP-A dependent agglutination", Heinrich, Griese, BMC Pulmed, 2010

"Children with absent surfactant protein D in bronchoalveolar lavage have more frequently pneumonia", Griese, Heinrich, et al., Pediatr Allergy Immunol. 2008

"Surfactant protein A - from genes to human lung diseases", Heinrich, Griese, Curr Med Chem, 2006

Poster and Presentations

"Genomewide analysis of paraffin embedded HER2-positive breast tumors", Gamerdinger, Heinrich et al., 2011, Poster for Congress of Human Genetics, Regensburg

"Whole-Genome Genotyping with Bead-Arrays", Heinrich, presentation at the university of Marburg, seminar for human biology students, 2010

"Small deletions in monogenic primary dystonias", Köhler, Heinrich et al., 2010, Poster for congress of human genetics, Hamburg

"Simple terminal deletions of the X chromosome are frequently complex rearrangements", Fritz, Jung, Heinrich et al., 2010; poster for congress of human genetics, Hamburg

"SP-A – structure and function in chronic bronchitis and cystic fibrosis", Heinrich, 2008; poster for congress of pediatrics, Munich

"The influence of the surfactant proteins A and D on the disease pattern in cystic fibrosis patients.", Heinrich, 2008; presentation at the Symposium of Mukoviszidose e.V., Mickeln

"The roles of the surfactant proteins A (SP-A) and D (SP-D) in cystic fibrosis", Heinrich, 2006; presentation at the Symposium of Mukoviszidose e.V., Mickeln