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# Deficiency of the mannan-binding lectin pathway of complement and poor outcome in cystic fibrosis: bacterial colonization may be decisive for a relationship

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## Introduction

Cystic fibrosis (CF) is the most common lethal inherited disease among Caucasians. The disease is considered monogenic, and is inherited as an autosomal recessive trait. The defect is located on chromosome 7 and causes a dysfunctional or non-existing chloride channel, called CF transmembrane regulator (CFTR), on epithelial cells [1]. In the airways, the mucus becomes highly viscous due to its high chloride content. More than 1200 different mutations are described. In Sweden, one out of approximately 5600 newborns has CF [2]. The cause of death in 90% of the CF cases is respiratory insufficiency due to chronic inflammation caused by bacterial colonization of the respiratory tract [3]. The most important pathogen is *Pseudomonas aeruginosa*. Chronic colonization by this pathogen is associated with poor prognosis [3]. However, prognosis is difficult to predict

## Summary

In cystic fibrosis (CF) prognosis concerning lung damage development is highly variable and difficult to predict. Mannan-binding lectin (MBL) deficiency has been reported to be associated with poor outcome in CF lung disease. MBL is a recognition molecule of the MBL pathway of the complement system and is encoded by a gene characterized by a high degree of polymorphism. Some genotypes result in low serum concentrations of MBL. MBL-associated serine protease 2 (MASP-2) is another protein belonging to the MBL pathway. A mutation resulting in low levels of MASP-2 in serum has been described recently. In the present study, 112 CF patients aged 4–54 years were investigated for MBL and MASP-2 genotypes, serum levels of MBL and MASP-2 and the MBL pathway function in serum. No correlation to reduced lung function or need for lung transplantation was seen, either for MBL deficiency, MASP-2 gene mutation or reduced MBL pathway function. However, in the 27 patients colonized with *Staphylococcus aureus*, MBL-deficient genotypes were associated with decreased lung function. As expected, MBL pathway function in serum was reduced both in MBL-deficient patients and in patients carrying a mutant MASP-2 allele. An unexpected finding was that CF patients had higher serum levels of MBL than healthy controls when corrected for MBL genotype. In conclusion, MBL pathway function was affected both by MBL and by MASP-2 genotypes. However, MBL or MASP-2 levels in serum did not affect the clinical outcome in the cohort of CF patients studied.

**Keywords:** complement, cystic fibrosis, lung function, MASP-2, MBL

as the clinical course of the lung disease differs widely within each CFTR genotype [4]. Polymorphisms of different genes coding for proteins involved in immunological and inflammatory responses have been suggested to modify the outcome of CF. Possible CF modifiers include glutathione-S-transferase [5], transforming growth factor- $\beta_1$  [6], tumour necrosis factor- $\alpha$  [7],  $\beta_2$ -adrenergic receptor [8] and mannan-binding lectin (MBL). Decreased MBL function has gained extensive interest as a potential modifier in CF, as two studies have shown an association between MBL deficiency and poor outcome for CF lung disease [9,10].

MBL is a recognition molecule of the complement system [11,12]. The complement system is important in host defence and can be activated through three major pathways: the classical pathway, the alternative pathway and the MBL pathway [13,14]. Complement activation leads to opsonization through deposition of C3b on microbial surfaces, and to

lysis of susceptible targets by the membrane attack complex, C5b-9<sub>n</sub>. Activation of the MBL pathway involves MBL binding to carbohydrate patterns on microorganisms. MBL circulates in complexes with MBL-associated serine proteases, MASP-1, MASP-2, MASP-3 and a 19 kDa protein known as MBL-associated protein (MAp19). When bound to a target, MBL/MASP-2 complexes are able to cleave C2 and C4 by action of the activated MASP-2, generating the C3 convertase C4bC2a, leading to cleavage of C3 into C3a and C3b.

MBL is encoded by a gene located on chromosome 10. A number of polymorphisms are present in the gene. In the promoter region, two loci are of particular interest: codon -221 (X/Y-type) and codon -550 (H/L-type). In the collagen-encoding region of exon 1, three single base substitutions are independently associated with low levels of MBL: the B-allele where a mutation at codon 54 exchanges glycine to aspartic acid, the C-allele where a mutation at codon 57 exchanges glycine to glutamic acid, and the D-allele where a mutation in codon 52 exchanges arginine to cysteine. Low levels of MBL in serum due to genetic variants are found in 10–15% of Caucasian populations. The condition has been reported to be connected with increased susceptibility to infection under certain circumstances. In children, MBL deficiency associated genotypes were over-represented in cases with severe infection admitted to hospital [15] and recurrent respiratory infections [16]. In patients immunocompromised due to chemotherapy, MBL-deficient patients were more prone to develop septicaemia and pneumonia [17]. Enhanced progression of systemic lupus erythematosus [18] and poor prognosis in patients with early rheumatoid arthritis [19] have also been described to be associated with MBL-deficient genotypes. Conversely, the MBL-sufficient genotype was reported to be associated with enhanced inflammation in patients with late onset of rheumatoid arthritis and advanced disease, suggesting that intact complement supports the inflammatory response [20].

MASP-2 deficiency due to a mutation in exon 3 of the MASP-2 gene encoding the first domain of the protein was recently described [21]. At amino acid position 120 (position 105 in the mature protein) glycine was exchanged for aspartic acid due to an A→G mutation in the corresponding codon. A patient with manifestations of severe autoimmune and infectious disease was homozygous for this mutation. In a healthy Danish population, the allele frequency was 5.5%. Heterozygosity was reported to be associated with moderately low serum levels of MASP-2.

The aim of the present study was to evaluate the clinical significance of MBL pathway deficiencies in CF, assuming that the previously described association between MBL deficiency and poor prognosis in CF lung disease would be confirmed. We also wished to assess the potential role of MASP-2 deficiency in CF. Contrary to expectations, we found no correlation between MBL pathway function and clinical outcome in CF, except in a small subgroup of patients colonized with *Staphylococcus aureus*.

## Patients and methods

### Patients

All patients at the CF centre of Lund University Hospital with genetically verified CF were asked to participate in the study. The median age of the patients was 20.5 years (range 4–54). Among the patients, 44 were under the age of 18 and were considered to be children. Lung transplantation had been performed in eight of the 112 patients. Lung transplantation was considered an end-point in CF lung disease, and those patients were therefore excluded when clinical parameters were analysed.

### Lung function

Pulmonary function was examined with spirometry according to guidelines of the American Thoracic Society [22]. The studies were performed at the Department of Clinical Physiology, Lund University Hospital during phases of clinical disease stability. Forced expiratory volume in 1 second (FEV<sub>1</sub>) was chosen as a measure of lung function, as this is the lung function parameter that most efficiently correlates with prognosis in CF [23]. The results were expressed as a proportion of the predicted values (FEV<sub>1</sub>%pred) based on height, age and gender. In children, FEV<sub>1</sub>%pred was calculated according to Solymar *et al.* [24] and in adults according to Quanjer *et al.* [25].

### Microbiological diagnosis

Samples for respiratory secretion cultures were taken when the patient attended a routine out-patient visit. Sampling, transport and culturing were performed according to routine procedures. Chronic colonization was defined according to European consensus [26], i.e. documentation of three consecutive positive cultures. All patients provided expectorate. In the 104 patients who were not lung-transplanted, 47 were chronically colonized with *P. aeruginosa*. In the remaining patients, 27 were colonized with *S. aureus*, 16 were not colonized, while the remaining 14 patients were intermittently colonized or chronically colonized with other bacteria.

### Genotyping

DNA was extracted by a salting out method [27]. In brief, nucleated cells were lysed and treated with protease K solution. The proteins were precipitated with saturated NaCl, and the DNA was then precipitated with absolute ethanol.

Analysis of MBL gene polymorphism due to mutations at codon 52 (D), 54 (B) and 57 (C) in exon 1 of the MBL gene and promoter variants at positions -550 (H/L) and -221 (X/Y) were determined by allele specific polymerase chain reaction (PCR) amplification, essentially as described previously [28]. The wild-type structural allele is designated A, while 0

**Table 1.** MBL genotype frequencies (%) in CF patients and healthy controls.

		CF <i>n</i> = 112	Controls <i>n</i> = 200		
MBL-sufficient genotypes	A/A	<i>HYA/HYA</i>	10.7	6.5	
		<i>HYA/LXA</i>	10.7	13.5	
		<i>HYA/LYA</i>	10.7	15.5	
		<i>LYA/LYA</i>	4.5	3.5	
		<i>LYA/LXA</i>	12.5	13.5	
		<i>LXA/LXA</i>	8.9	5.5	
	YA/0	<i>HYA/LYB</i>	9.8	10.0	
		<i>HYA/LYC</i>	0.0	0.5	
		<i>HYA/HYD</i>	6.3	7.5	
		<i>LYA/LYB</i>	4.5	6.0	
MBL-deficient genotypes	XA/0	<i>LXA/LYB</i>	4.5	5.0	
		<i>LXA/HYD</i>	8.0	1.5	
		<i>LXA/LYC</i>	0.0	0.5	
	0/0	<i>LYB/LYB</i>	1.8	3.0	
		<i>LYB/LYC</i>	0.9	0.0	
		<i>LYB/HYD</i>	0.9	1.0	
		<i>LYC/HYD</i>	0.9	1.0	
			<i>HYD/HYD</i>	0.9	2.0

is a description of the mutant alleles B, C and D, based on previously described associations between MBL genotype and serum concentrations, and confirmed in 200 healthy blood donors that were used as controls. The patients were stratified into two groups, MBL-deficient and MBL-sufficient according to MBL genotype. The MBL-deficient group consisted of people with two structural mutant alleles (0/0) or a structural mutant allele on one haplotype and on the other allele the haplotype containing the LX allele linked to the wild-type structural allele (*LXA/0*). People with combinations of other MBL haplotypes were considered MBL-sufficient (Table 1).

The primers used to amplify the region encoding the first domain CUB1 of MASP-2, were primers 5' forward – C GCG AGT ACG ACT TCG TCA AGG and biotinylated 5' reverse – CTC GGC TGC ATA GAA GGC CTC [21]. The PCRs were performed in 50 µl reactions containing approximately 100 ng DNA, 1.5 mM MgCl<sub>2</sub>, AmpliTaq Gold buffer (Applied Biosystems, NJ, USA), 125 µM of each dNTP (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.2 µM of each primer of which one was biotinylated (MWG Biotech AG, Ebersberg, Germany) and 1 U of AmpliTaq Gold polymerase.

We used a GeneAmp PCR System 2400 (Perkin Elmer) to perform a programme containing a denaturation step at 95°C for 5 min and then denaturation for 15 s at 95°C; in the annealing temperature a touchdown programme was used and started at 72°C for 30 s in 2 cycles, then decreased 1°C every second cycle until it reached 65°C and then extension at 72°C for 15 s. When the annealing temperature had

reached 65°C it was run for 45 cycles and the PCR was ended by a prolonged extension time at 72°C for 5 min. The MASP-2 primers generated a 259 base pairs (bp) fragment which was analysed by pyrosequencing (Biotage, Uppsala, Sweden) according to the manufacturer's description. In the pyrosequencing reaction 5'-GGA CAT TAC CTT CCG C was used as sequencing primer [29].

### Serum analyses

Concentrations of MBL were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) on microtitre plates coated with monoclonal anti-MBL antibodies (Mab 131-1, Immunolex, Copenhagen, Denmark) as described previously [30].

Concentrations of MASP-2 were determined by a sandwich-type time-resolved immunofluorometric assay on plates coated with antibody against MASP-2 and subsequent incubation with a biotinylated antibody against MAP19-MASP-2 and europium-labelled streptavidin [31].

MBL pathway function was assessed as deposition of complement complex C5b-9<sub>n</sub> by ELISA at Wieslab AB, Lund, Sweden, according to a method previously described [32]. In short, wells were coated with mannan. Diluted serum was added to the wells. Anti-C1q antibody was added to prevent classical pathway activation. The plate was incubated at 37°C resulting in MBL pathway activation, which was measured as bound C5b-9<sub>n</sub> using a specific labelled antibody. A calibrator serum said to contain 100 arbitrary units (AU)/l was included in each plate and the absorbance of the samples were related to the absorbance of the calibrator serum.

CRP was measured by a highly sensitive automated immunoassay system, Immulite®, using reagents from DPC (Los Angeles, CA, USA). As controls we used 200 healthy blood donors.

### Statistics and ethics

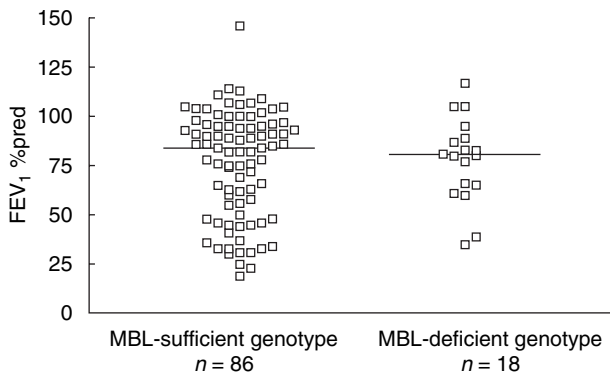
The Mann-Whitney *U*-test, Spearman's rank correlation test and Fisher's exact test were used when applicable. To calculate a 95% confidence interval, Hodges Lehman's method was used. Results were considered significant (\*) at *P* < 0.05 and highly significant (\*\*) at *P* < 0.01. The study was approved by the local Research Ethics Committee. Informed consent was obtained from all patients or, in the case of children, their parents. As controls were used 200 healthy blood donors, who gave their approval before samples were drawn.

## Results

### CF demography

Median lung function in the not lung-transplanted patients was 83% of normal values. The relative lung function decreased with age in the study group (Table 2). CFTR





**Fig. 2.** Lung function expressed as proportion of predicted forced expiratory volume in 1 second ( $FEV_1\%$ pred) in relation to MBL genotype. No difference in lung function according to MBL genotype was found in CF patients. Patients that had undergone lung transplantation were not included in the comparison. Horizontal lines indicate median values.

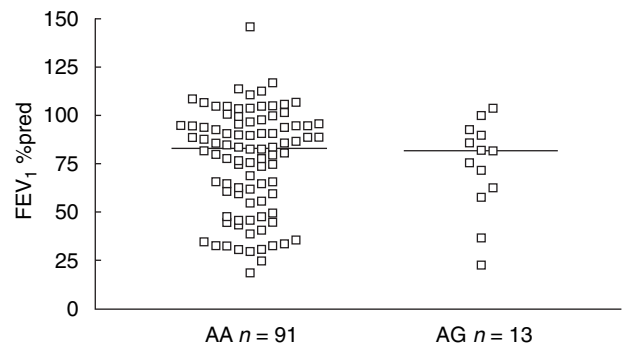
only 22% of the MBL-deficient patients were colonized with *P. aeruginosa* ( $P = 0.04$ , Fisher's exact test). In the 27 patients chronically or intermittently colonized by *S. aureus*, lung function was better in MBL-sufficient patients ( $n = 20$ ) than in MBL-deficient patients ( $n = 7$ ), i.e. 92% of the predicted  $FEV_1$  compared to 66% in the MBL-deficient patients colonized with *S. aureus* ( $P = 0.04$ , Mann-Whitney *U*-test).

### MBL and lung transplantation

No genotype was over-represented in patients that had been lung-transplanted. In the eight patients, two had MBL-deficient genotypes (LYB/CYB and LXA/LYB) and six had MBL-sufficient genotypes (HYA/HYA, HYA/LXA, LYA/LYA, LYA/LXA, LYA/LXA and LXA/LXA). The MBL-deficient patients were 20 and 46 years of age at lung transplantation, and the ages at transplantation of the MBL-sufficient patients were 20, 27, 27, 31, 32 and 32. The median serum level of MBL in lung-transplanted CF patients was 1252  $\mu\text{g/l}$ . In two patients, who were lung-transplanted during the study, MBL levels decreased after the transplantation from 1418  $\mu\text{g/l}$  to 838  $\mu\text{g/l}$  and from 3331  $\mu\text{g/l}$  to 2124  $\mu\text{g/l}$ , respectively.

### MASP-2 in CF

All patients were investigated for the exon 3 A→G MASP-2 gene mutation. Heterozygosity for the mutation was found in 14 patients, corresponding to an allele frequency of 6.3%. No homozygous MASP-2 deficiency was found. In the CF patients, the median MASP-2 concentration of 157  $\mu\text{g/l}$  (IQR 110–202  $\mu\text{g/l}$ ) in heterozygotes was significantly lower than the median MASP-2 concentration in patients with wild-types, which was 380  $\mu\text{g/l}$  (IQR 257–612  $\mu\text{g/l}$ ,  $P < 0.0001$ , Mann-Whitney *U*-test). In 200 healthy blood donors, the mutant allele frequency was 1.3%, as five hete-



**Fig. 3.** Lung function expressed as proportion of predicted forced expiratory volume in 1 second ( $FEV_1\%$ pred) in CF patients heterozygous for MASP-2 gene mutation (AG) compared to wild-types (AA). No difference in lung function according to MASP-2 genotype was found in CF patients. Patients that had undergone lung transplantation were not included in the comparison. AG refers to patients heterozygous for A→G mutation in exon 3 of the MASP-2 gene. AA refers to wild types. Horizontal lines indicate median values.

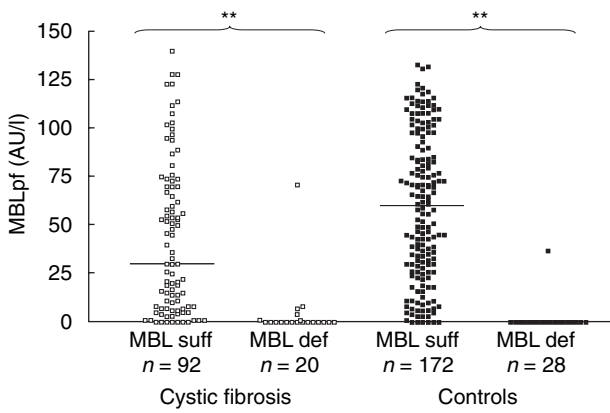
rozygous individuals were found among the 200 controls. The median MASP-2 concentration of the heterozygotes was 166  $\mu\text{g/l}$  (IQR 150–189  $\mu\text{g/l}$ ), whereas the median MASP-2 concentration in the wild-type controls was 353  $\mu\text{g/l}$  (IQR 273–443  $\mu\text{g/l}$ ). Surprisingly, the healthy controls heterozygous for the MASP-2 mutation had a higher median serum MBL level, 1321  $\text{mg/l}$ , than MASP-2 wild-type controls with the same MBL genotypes whose median level was 488  $\text{mg/l}$  ( $P = 0.03$ , Mann-Whitney *U*-test).

### MASP-2 and clinical data

No difference in lung function was found when patients heterozygotes for the MASP-2 mutation were compared to patients homozygous for the wild-type MASP-2. Thus, the median predicted  $FEV_1$  in patients with MASP-2 wild-types was 83% while heterozygotes had 82% of the predicted value (Fig. 3). The MASP-2 gene mutation was distributed equally among the lung-transplanted patients (7%) and the other patients (7%). Heterozygosity was found in one transplanted patient and in seven not-transplanted patients. In not-transplanted patients, the frequency of chronic *P. aeruginosa* colonization was not correlated to MASP-2 genotype. In heterozygotes, 38% were colonized, and in patients with wild-type genotypes 46% were colonized. In patients colonized by *S. aureus*, lung function was not related to MASP-2 genotype. Heterozygotes had a median  $FEV_1$  of 82% of the predicted value, and wild-type homozygotes had 86%.

### MBL pathway function

MBL pathway function was investigated using mannan-coated ELISA plates and was measured as deposition of C5b-9<sub>n</sub> in sera from all the CF patients and 200 healthy controls.



**Fig. 4.** MBL pathway function (MBLpf) in serum in relation to MBL genotype in cystic fibrosis patients and healthy controls. MBL suff refers to individuals with MBL-sufficient genotypes, and MBL def refers to individuals with MBL-deficient genotypes (Table 1). \*\* $P < 0.01$  (Mann-Whitney  $U$ -test). Horizontal lines indicate median values.

In both CF patients and healthy controls, the MBL genotype was correlated to MBL pathway function (Fig. 4). When MBL-deficient patients were excluded in the CF patients, MBL pathway function was decreased significantly in people that were heterozygous for the exon 3 A→G MASP-2 gene mutation compared to wild-type homozygotes (Fig. 5). None of the MASP-2 heterozygous patients had an MBL-deficient genotype. In the healthy controls, however, no correlation between MASP-2-genotype and MBL pathway function was seen. Healthy controls had higher levels of MBL pathway function than CF patients, despite their lower serum MBL levels. Median MBL pathway function was 45 AU/l in the 200 healthy controls, and 19 AU/l in the 112 CF patients ( $P = 0.001$ , Mann-Whitney  $U$ -test).

There was no correlation between MBL pathway function in serum and lung function ( $\rho = -0.06$ , Spearman's rank correlation test). Patients colonized by *P. aeruginosa* did not differ significantly from the other patients with regard to MBL pathway function. Median MBL pathway function was

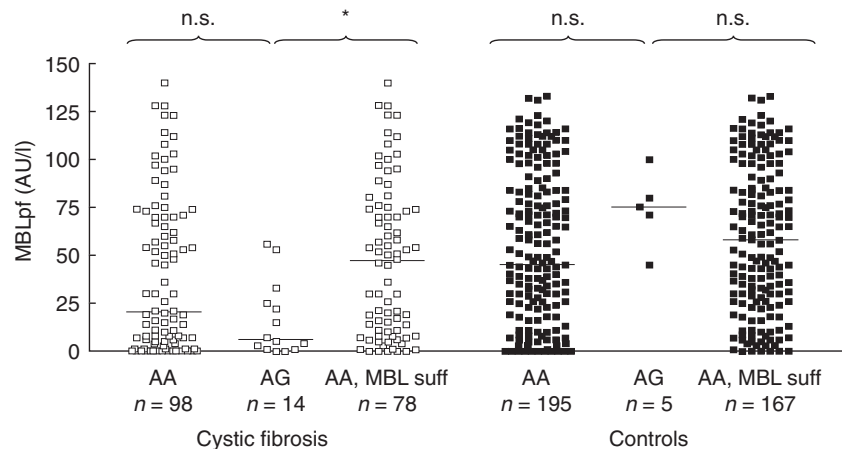
slightly lower in lung-transplanted patients than it was in patients that were not transplanted, 9 AU/l compared to 20 AU/l, but the difference was not significant ( $P = 0.4$ , Mann-Whitney  $U$ -test).

**Discussion**

In contrast to previous reports [9,10], we found no correlation between MBL pathway deficiency and reduced lung function in CF patients in this cross-sectional study. Interestingly, the MBL-deficient patients were colonized by *P. aeruginosa* to a lesser extent than the MBL-sufficient patients. Patients not colonized by *P. aeruginosa* in this group are colonized mainly by *S. aureus*, a pathogen that binds MBL, whereas *P. aeruginosa* does not [34]. A possible explanation for our findings could be that MBL deficiency might favour colonization by *S. aureus*. Furthermore, we found that in *S. aureus* colonized patients, MBL-deficient genotypes were associated with impaired lung function. This leads us to believe that microbiological aspects are important when validating MBL as a prognostic predictor in CF.

We consider the risk of a statistical type II error regarding the lack of a relationship between MBL pathway deficiency and reduced lung function to be very small, even though our cohort is not as large as those presented previously [9,10]: 104 patients compared to 149 and 164, respectively. The 95% confidence interval around the difference in median lung functions in MBL-deficient and MBL-sufficient patients is narrow, considering the broad range of the FEV<sub>1</sub> values. The  $P$ -value is also close to 1 when the groups are compared.

Management of CF lung disease has improved substantially in recent years, and as our cohort was investigated at a later time-point than those described in previous reports, it may be speculated that advances in treatment have reduced the impact of MBL pathway deficiency. Our cohort also has a broader age span than that of Garred *et al.* [10], which could influence the outcome of the study. However, we found no correlation between MBL-deficient genotypes and reduced lung function when excluding the youngest and the



**Fig. 5.** MBL pathway function (MBLpf) in serum in relation to MASP-2 genotypes in cystic fibrosis patients and healthy controls. AA refers to MASP-2 gene wild-type homozygotes. AG refers to individuals heterozygous for A→G mutation in exon 3 of the MASP-2 gene. AA, MBL suff refers to MASP-2 gene wild-type homozygotes that have an MBL-sufficient genotype. In the AG groups, all individuals had MBL-sufficient genotypes. \* $P < 0.05$  (Mann-Whitney  $U$ -test) and n.s. indicates a non significant difference. Vertical lines show median values.

oldest patients, to match the ages of previously reported cohorts.

Lung function in an individual normally increases until approximately 22 years of age, after which it declines. In this study, the median age was 20.5. The control groups used [24,25] have a high degree of variation in ages around 20, as different individuals reach their peak lung function at different ages, which could mean that lung function estimation in relation to the predicted value is difficult to define in many of the patients included. This would, however, not affect the outcome of the study, as this uncertainty concerns all patients, regardless of their MBL pathway parameters.

Heterozygosity for MASP-2 gene mutation was found in 12% of the patients, the allele frequency being 6.3%. The allele frequency found in the control population was 1.3%. We assume that the difference in gene frequency was a random finding, because a Danish control population of 100 individuals was reported previously to have an allele frequency of 5.5% [21]. More patients and controls are needed in order to determine the prevalence of the MASP-2 deficiency allele. Heterozygous individuals had lower serum levels of MASP-2 both in CF patients and controls. However, no correlation to clinical findings was found in the CF patients. In MBL-deficient patients colonized with *S. aureus*, lung function was reduced compared with the MBL-sufficient patients. A correlation of this kind was not seen for partial MASP-2 deficiency in *S. aureus* colonized patients, as heterozygotes and wild-type homozygotes had equal lung function.

Until now, MBL has been the only MBL pathway component that was possible to investigate for diagnostic purposes. In this study we show that sera from CF patients heterozygous for a specific mutation of the MASP-2 gene have significantly reduced MBL pathway function, when correction was made for MBL genotype. Surprisingly, this correlation was not seen in the controls. Until further evaluation of the MASP-2 impact on MBL pathway function has been performed, we wish to emphasize that MASP-2 should not be neglected in clinical studies of the MBL pathway, considering that the frequency of the MASP-2 deficiency allele may be as high as around 6%.

We found that CF patients have significantly higher MBL serum concentrations than healthy controls. MBL is a protein produced by the liver [35], but MBL production in intestinal mucosa has been reported in coeliac disease [36]. We speculate that the increased production of MBL in CF could emerge from the lungs as a part of an up-regulated innate immune response. MBL is known as a mild acute phase protein [35], and the elevated levels that we see in CF patients could, of course, be the result of this. To estimate ongoing acute phase reaction in the patients, high sensitivity CRP was measured. The median CRP level of the CF patients was 3.1 mg/l. In clinical practice, values below 5 mg/l are usually regarded as normal. In that sense, the CF patient group did not appear to have an ongoing acute phase reac-

tion. However, the median CRP in the controls was significantly lower, 0.9 mg/l. Furthermore, among the patients, CRP was higher in adults than in children. As the relative lung function is lower in adult CF patients than in CF children, the modest increase in CRP might possibly reflect a higher degree of inflammation. It could be that the increased concentration of MBL in serum is not an effect of increased synthesis in the liver, but reflects ectopic production.

In conclusion, MBL pathway dysfunction due to MBL deficiency or partial MASP-2 deficiency did not influence the outcome in CF lung disease in the cohort investigated. However, in the subgroup of *S. aureus* colonized patients, MBL deficiency genotypes were associated with reduced lung function. These results were not in accordance with those reported by others. A multi-centre study might contribute to further clarification of the role of the MBL pathway in CF.

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