IL1B gene variants, but not TNF, CXCL8, IL6 and IL10, modify the course of cystic fibrosis in Polish patients. [version 3; peer review: 1 approved, 2 approved with reservations]

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Abstract

**Background:** The main aim of this study was to evaluate whether selected polymorphic variants in genes from the inflammatory pathway can be predictors of pulmonary or digestive manifestation of cystic fibrosis, as well as of severity of lung disease.

**Materials and methods:** Using pyrosequencing and sequencing we have genotyped 12 variants in TNF (rs361525, rs1800629), CXCL8 (rs4073, rs2227306, rs2227307, rs188378669), IL1B (rs16944, rs1143634, rs1142639, rs1143627), IL6 (rs1800795) and IL10 (rs1800896) genes in a cohort of 55 Polish patients with diagnosed cystic fibrosis and controls. In our study group, a pulmonary manifestation of disease revealed 44 of subjects (80%), and digestive symptoms dominated in 11 (20%) of analyzed individuals. Severe lung dysfunction has occurred in 20 (36.4%) of patients.

**Results:** We proved, that two promoter variants of IL1B, rs1143627 (c.-118G > A) and rs16944 (c.-598T > C) are presented significantly more often in patients with severe character of lung disease compared to mild (82.5% vs. 62.8%, p-value 0.030, and 87.5% vs. 64.3%, p-value 0.008, respectively) in cystic fibrosis course. Haplotype AC formed by both changes had also a higher frequency (80%) in patients with severe course compared to the mild character (61.4%) of disease. However, the frequency of promoter variant TNF c.-308C > T (rs1800629) was presented at a significantly lower level in the patient's group compared to healthy controls (2.7% vs. 15%, p-value 0.001). Furthermore, the presence of methicillin-resistant *Staphylococcus*
*Staphylococcus aureus* significantly correlated with the lower FEV1% in patients (p-value 0.01).

**Conclusions:** Genetic variants, rs1143627 and rs16944, of *IL1B* are promising candidates as predictors of the severe character of lung disease in Polish patients with cystic fibrosis.

**Keywords**
cystic fibrosis, modifier genes, inflammatory mediators, IL1B

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Amendments from Version 2

Version 3 of this manuscript additionally include information about age of patients classified to severe and mild phenotype of the disease. Furthermore, the reference numbered 21 and the text was added to clarify the disease severity classification of patients. We also provided information about CF genotypes among patients group in the Table 4 containing analysed polymorphisms. Additionally, the information that patients were clinically stable at the time of study recruitment was added.

Any further responses from the reviewers can be found at the end of the article

Introduction

Recent scientific outcomes confirm that the clinical phenotype of cystic fibrosis (CF) (OMIM: 219700) is determined not only by classes of mutations in the CFTR gene (cystic fibrosis transmembrane regulator) but in association with environmental factors and genetic variations in modifier genes. The hypothesis about the role of modifier genes in CF was born based on the observations, that patients with the same CFTR genotype presented diverse manifestations and course of the disease. Today, over 2000 different CFTR mutations have been reported and F508del is by far the most common. Although mutations in the CFTR gene are well known and classified, the contribution of modulatory genes in CF is currently still investigated. Among analyzed candidate genes are those involved in the inflammatory process, as well as in immunity and antioxidant molecules. However, the results of the majority of global research on modifier genes’ role in CF are inconclusive.

CF is a multi-organ disease, whereas chronic pulmonary inflammation and respiratory failure consist of the main cause of death in those patients. There is evidence, that the inflammatory process in the lung is associated with an imbalance between pro- and anti-inflammatory mediators. Among important pro-inflammatory cytokines produced during the death in those patients. There is evidence, that the inflammatory process in the lung is associated with an imbalance between pro- and anti-inflammatory mediators. Among important pro-inflammatory cytokines produced during the response are tumor necrosis factor-alpha (TNF-α), interleukins (IL) 8, 6, 1, and 1B, while among cytokines inducing the opposite effect are transforming growth factor-beta 1 (TGFβ1) and IL10. Proteins, IL8 and TNF-α, play a crucial role in the pathophysiology of CF lung disease due to their participation in the recruitment and activation of neutrophils on the respiratory epithelial surface, which is a primary component of the innate immune response. Thus, genes CXCL8 and TNF coding for those cytokines, which expression is regulated by sequence variants, are highlighted as potential modifier genes in the severity of lung disease in CF. Although numerous polymorphic variants have been described in the CXCL8 gene, the association only between polymorphisms rs4073 (c.-251T>A), rs2227306 (c.781C>T), rs2227307 (c.396T>G), pulmonary function, and clinical severity markers in CF patients was confirmed in several studies. The most often analyzed changes in the TNF gene are located in the promoter region, such as c.-238G>A (rs361525) having a variable effect on gene expression and c.-308A>G (rs1800629) associated with increased gene transcription, worst pulmonary function, and early pulmonary symptoms in patients with CF, in contrast to studies performed by Schmitt-Grohé et al. and Khorrami et al. There is also proven, that some of TNF and CXCL8 polymorphisms are associated with Pseudomonas aeruginosa (PA) chronic colonization in CF patients. Furthermore, modulating effects on the CF also have shown differences in the course of the disease. Today, over 2000 different CFTR mutations have been reported and F508del is by far the most common. Although mutations in the CFTR gene are well known and classified, the contribution of modulatory genes in CF is currently still investigated. Among analyzed candidate genes are those involved in the inflammatory process, as well as in immunity and antioxidant molecules. However, the results of the majority of global research on modifier genes’ role in CF are inconclusive.

Results of up to now performed studies, searching for genes that modify the course and phenotype of CF, mostly concern the association with pulmonary exacerbation. However, based on our long-term observations of CF patients we state, that around 20% of CF patients manifest a pronounced exacerbation of symptoms from the digestive system. We hypothesized, that this possibly may be predicted by polymorphic changes at immunologically relevant genes. Within this context, we have selected 12 polymorphisms located in five genes CXCL8 (rs4073, rs2227306, rs2227307, rs188378669), TNF (rs361525, rs1800629), IL1B (rs16944, rs1143634, rs1142639, rs1143627), IL6 (rs1800795), and IL10 (rs1800896) for correlation analysis, as candidate genetic modulators of the pulmonary or digestive manifestation and severity of the disease among Polish CF patients.

Materials and methods

Patients and clinical data

The study was approved by the local Ethics Committee of the University of Medical Sciences in Poznan, Poland (resolution no. 675/15), and all experiments were performed following the relevant guidelines and regulations of this Committee. Written informed consent was obtained from each patient. 55 Polish patients (20 males and 35 females) between the ages of 20-52 with diagnosed CF were enrolled for this study. The patient group was collected in 12 months (from January to December 2016) in the Department of Pulmonology, Allergology and Lung Oncology of the Clinical Hospital of Poznan University of Medical Sciences in Poland. Diagnosis of CF in all patients was
performed by sweat chloride test results (> 60 mmol/L) or/and identification of CFTR gene mutations. Detailed information about each patient including sex, age, BMI, age of diagnosis, presence of F508del mutation, pulmonary function parameters, function of internal organs, complications, and hospitalizations were recorded. Additionally, a control group of 50 healthy individuals was collected. A detailed characteristics of the study cohort with clinical and demographic data are presented in Table 1.

Pulmonary function tests, using Jaeger MasterScreen system (Erich Jaeger GmbH; Würzburg, Germany) were performed to assess lung function. All spirometric examinations were carried out with the subject seated, using a nose clip and a

| Table 1. Baseline characteristics of study participants. |
|----------------------------------------------------------|
| **Subjects characteristic**                              | **Patients** | **Controls** |
|----------------------------------------------------------|--------------|--------------|
| Sex (M/F)                                                | 20 (36%)/35 (64%) | 19 (38%)/31 (62%) |
| Age (years)                                              | 28 (20-52) | 31 (22-45) |
| Ethnicity (Caucasian)                                   | 55 (100%) | 50 (100%) |
| BMI                                                      |             |              |
| Mean (range)                                             | 20.2 (15-27.8) | 23.3 (17.5-26) |
| Malnutrition                                             | 21 (38.2%) | 2 (4%) |
| Diagnosis (years)                                        | 7.8 (1-36) |              |
| Predominant manifestation of CF                         | -            |              |
| Pulmonary                                                | 44 (80%) |
| Digestive                                                | 11 (20%) |
| CF genotype (F508del)                                   | -            |              |
| Homozygote                                               | 13 (23.6%) |
| Heterozygote                                             | 31 (56.4%) |
| Nil                                                      | 11 (20%) |
| Notably severe character of CF                           | 20 (36.4%) |              |
| FEV1%                                                    | -            |              |
| Mean (range)                                             | 53.5 (10.6-106.8) |              |
| FVC%                                                     | -            |              |
| Mean (range)                                             | 67 (26.1-112.4) |              |
| TLC%                                                     | -            |              |
| Mean (range)                                             | 109.4 (78.7-151.6) |              |
| RV%                                                      | -            |              |
| Mean (range)                                             | 219.2 (88.8-391.6) |              |
| DLCO                                                     | -            |              |
| Mean (range)                                             | 59.7 (16.8-88) |              |
| Pulmonary complications (emphysema, hemoptysis)          | 25 (45.5%) |              |
| Lung transplantation/death                               | 14 (25.5%) |              |
| Diabetes/Impaired glucose tolerance                      | 29 (52.7%) |              |
| Exocrine insufficiency                                   | 38 (69%) |              |
| Liver dysfunction                                        | 19 (34.5%) |              |
| Hospitalizations per year                                | -            |              |
| Mean (range)                                             | 1.9 (0-8) |              |
disposable mouthpiece. Using spirometric measurements, values of expiratory forced vital capacity (FVC) and forced expiratory volume in one second (FEV1%) were obtained and were expressed as the percentage of predicted values according to European Community for Steel and Coal.20

At the same time the body plethysmography for assessing residual volume (RV), total lung capacity (TLC), and diffusing capacity of the lungs for carbon monoxide (DLCO) were performed.

Patients were divided in the context of lung function impairment, based on the FEV1% values, while they were clinically stable, 1 - within the norm (FEV1% ≥ 70) and mild pulmonary obstruction (FEV1% 40-70) (35 subjects in total), 2 - severe pulmonary obstruction (FEV1% ≤ 40) (20 subjects in total).21 The “severe” group of patients did not differ significantly in age from patients in the “mild” group (mean age was 27.11 and 30.75, respectively; p-value was 0.055).

In an attempt to analyze the correlation between the genotype and manifestation of CF, patients were divided into two subgroups depending on the dominant symptoms - the group with the manifestation primarily from the respiratory system (44 individuals) and the group of patients with prevalent gastrointestinal symptoms (11 individuals). The division was made by the specialists from the pulmonology field conducting the patients, based on the clinical data and interview. To the digestive predominant phenotype were enrolled patients with the coexistence of at least two of listed conditions: 1) diabetes or glucose metabolism impairment, 2) pancreatic insufficiency, 3) liver disease or cirrhosis, 4) nagging pain or dysfunction of the digestive system. All patients with GI predominant phenotype represented “mild” lung impairment. Specialists determining the patients phenotype were not involved in the analysis of genotype assessment. Results of genotyping did not influence the assessment of phenotypic description.

Genotyping
Genomic DNA of each patient was extracted from the peripheral blood samples (5 mL) using the standard method with guanidine isothiocyanate (GTC). Detection of the single nucleotide polymorphisms (SNPs) in five genes: CXCL8 (rs4073, rs2227306, rs2227307, rs188378669), TNF (rs361525, rs1800629), IL1B (rs16944, rs1143634, rs1142639, rs1143627), IL6 (rs1800795) and IL10 (rs1800896) was performed using pyrosequencing or Sanger sequencing. Primers for the pyrosequencing analysis were designed using PyroMark Assay Design Software (Biotage, Uppsala, Sweden) and for Sanger sequencing using Primer3Plus software. Primer details are shown in Table 2. Amplification of targeted DNA regions was carried out on Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) on the total volume of 30 uL containing 0.75 U of FIREPol® DNA Polymerase, 2.5 μL 10x buffer, 2.0 μL dNTP

| Table 2. Primer details. |
|--------------------------|
| **Gene** | **SNP** | **Primer Name** | **Primer sequence** | **Product length** |
| **TNF** | c.-238G > A (rs361525) | TNF_238_F | 5’-CTCCAGGGTCTCTACACAAAT-3’ | 188 bp |
| | | TNF_238_R | 5’-CATCTGGAGGAAGCGGTAGT-3’ | |
| | | TNF_238_Seq | 5’-CCCATCTCCCTGCT-3’ | |
| c.-308C > T (rs1800629) | TNF_308_F | 5’-GCCCTCCAGTTCTAGTCT-3’ | 184 bp |
| | | TNF_308_R | 5’-ATTCCCGAGGGGTTCTC-3’ | |
| | | TNF_308_Seq | 5’-GGCTGAACCCCTGC-3’ | |
| **CXCL8** | c.-251T > A (rs4073) | CXCL8_251_F | 5’-ATCTTTGTCTAACACCCTGC-3’ | 112 bp |
| | | CXCL8_251_R | 5’-AAGCTCCCAATTGCTGAATTA-3’ | |
| | | CXCL8_251_Seq | 5’-TAGAAATAAAAAGCATACA-3’ | - |
| c.781C > T (rs2227306) | CXCL8_781_F | 5’-GAAGGCAAATTTCTATGCTGAGG-3’ | 225 bp |
| | | CXCL8_781_R | 5’-CTCTGGAATTTCTCCTAGCCCTG-3’ | |
| | | CXCL8_781_Seq | 5’-CATAACTGACAAATC-3’ | - |
| c.396T > G (rs2227307) | CXCL8_396_F | 5’-GCGTTTTCTCTATGCTAAATGGAAG-3’ | 357 bp |
| | | CXCL8_396_R | 5’-CAAATCTGGCGCTGGTCAATAGA-3’ | |
| | | CXCL8_396_Seq | 5’-CTGCTTTTATAATTACACC-3’ | - |
| c.91G > T (rs188378669) | CXCL8_91_F | 5’-ATACAACTTTTTCCCCACAG-3’ | 246 bp |
| | | CXCL8_91_R | 5’-CCTAACACCTGGAACTTCTTTAA-3’ | |
mix (2.5 mM each dNTP), 1.5 mM MgCl₂ solution, 80 ng DNA and 0.2 μM of each primer. All reagents were obtained from Solis BioDyne (Tartu, Estonia). The amplification products were analyzed in 1.5% agarose gels electrophoresis. Pyrosequencing was performed by the PSQ™ 96MA system (Qiagen) using PyroMark™ Gold Q96 Reagents (Qiagen GmbH, Hilden, Germany), according to the manufacturer instructions. Direct sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on the Applied Biosystems 3500 and Series Genetic Analyzers.

Statistical analysis
Conformance of genotypes distribution of all analyzed polymorphisms with the Hardy-Weinberg equilibrium (HWE) was assessed using Fisher’s exact test. The pair-wise linkage disequilibrium (LD) of variants located in genes TNF, CXCL8, and IL1B was evaluated by Lewontin’s D’ using Haploview software version 4.2. The correlation analyses between genotypes and clinical data were performed using the chi-square test and Fisher’s exact test.

For all calculations, STATISTICA 12.0 software (Stat Soft, 2014) was used. The level of significance was set at p < 0.05.

Results
Allele frequencies and linkage disequilibrium analysis
A total of 55 CF Polish patients and 50 healthy controls were successfully genotyped for selected 12 polymorphisms located in genes TNF, CXCL8, and IL1B. Genotypes distribution for all SNPs met the requirements of HWE. No relevant differences in variant allele frequency between both groups were demonstrated. Only TNF c.-308C > T variant was observed significantly less often in the patient group (2.7%) compared to controls (15%), where the p-value was 0.001. All obtained frequencies of each genotype and allele are presented in Table 3.

Our haplotype analysis confirmed a strong LD (D’ = 1, r² = 0.928) between variants c.-251T > A (rs4073), c.781C > T (rs1142639) and c.-118G > A (rs1143627) in the CXCL8 gene, forming four haplotypes: TCT, ATG, TCG and ACG observed in our CF patient group with frequency 54.6%, 41.8%, 1.8% and 1.8%, respectively. Furthermore, two polymorphic changes located in the promoter region of IL1B gene, c.-118G > A (rs1143627) and c.-597 > T (rs114944) were observed in high LD (D’ = 0.904, r² = 0.75), constructing a haploblock, where haplotypes AC, GT, GC, AT frequency was 68.1%, 26.3%, 3.7% and 1.9%, respectively. Both haploblocks are presented on the Figure 1.

Table 2. Continued

| Gene | SNP | Primer Name | Primer sequence | Product length |
|------|-----|-------------|----------------|---------------|
| IL1B | c.-598T > C (rs16944) | IL1B_598_F* | 5’-TGAGGGTGTTGGTGCTCACCT-3’ | 112 bp |
|      |     | IL1B_598_R | 5’-AAGCTTCAATTTGGGAATTA-3’ |   |
|      |     | IL1B_598_Seq | 5’-TAGAAATAAAAAAGCATACA-3’ |  |
|      | c.315G > A (rs1143634) | IL1B_315_F | 5’-CGTGACATAACCCTGCTTTACC-3’ | 59 bp |
|      |     | IL1B_315_R* | 5’-GCTCCACATTTCAAGACTATCTT-3’ |  |
|      |     | IL1B_315_Seq | 5’-CATAACTGACAATTGAAAC-3’ |  |
|      | c.597+76G > A (rs1142639) | IL1B_597_F* | 5’-TTGAAGGTGCGACACATTA-3’ | 143 bp |
|      |     | IL1B_597_R | 5’-TCAGCCCTCTGGTCACACTTATT-3’ |  |
|      |     | IL1B_597_Seq | 5’-CAGACAACCACCTTCTC-3’ |  |
|      | c.-118G > A (rs1143627) | IL1B_118_F | 5’-GTGCGTTGCTGTACAGGAG-3’ | 86 bp |
|      |     | IL1B_118_R* | 5’-TCAGGCCCTACTTCTGCTCTTTGA-3’ |  |
|      |     | IL1B_118_Seq | 5’-CCCTCGCTGTTTTTAT-3’ |  |
| IL6  | c.-237G > C (rs1800795) | IL6_237_F* | 5’-TGACATTCTTTCCCTAGTGTG-3’ | 82 bp |
|      |     | IL6_237_R | 5’-TGGGGCTGATGGAGAACCT-3’ |  |
|      |     | IL6_237_Seq | 5’-TGTGAGTGGTGCCCTTTAGA-3’ |  |
| IL10 | c.-1117A > G (rs1800896) | IL10_1117_F | 5’-AACTGAGTCCCTCTACCTTCTA-3’ | 151 bp |
|      |     | IL10_1117_R | 5’-AGGCTGATAGGAGGCTCTCCTACT-3’ |  |
|      |     | IL10_1117_Seq | 5’-AAGGGCTTTCTTGGA-3’ |  |

*Primers labelled with biotin for pyrosequencing.
| SNP            | Genotype | Group of CF patients (n = 55) | Control group (n = 50) | 1000 Genomes database | Allele frequency CF patients vs CF database |
|---------------|----------|-------------------------------|------------------------|------------------------|------------------------------------------|
|               |          | Number (%)                   | HWE ** (p-value)       | Variant allele freq.   | HWE ** (p-value) | Variant allele freq. | Variant allele freq. (EU population) | p-value         |
| **TNF** c.-238G > A (rs361525) | GG       | 48 (87.3)                    | 0.614                  | 6.4%                   | 44 (88)       | 0.1                     | 7%                           | 6%              | p = 0.853               |
|               | GA       | 7 (12.7)                     |                        |                        | 5 (10)        |                        |                              |                 |
|               | AA       | 0 (0.0)                      |                        |                        | 1 (2)         |                        |                              |                 |
| **TNF** c.-308C > T (rs1800629) | CC       | 52 (94.5)                    | 0.835                  | 2.7%                   | 36 (72)       | 0.889                   | 15%                          | 13%             | p = 0.001               |
|               | CT       | 3 (5.5)                      |                        |                        | 13 (26)       |                        |                              |                 |
|               | TT       | 0 (0.0)                      |                        |                        | 1 (2)         |                        |                              |                 |
| **CXCL8** c.-251T > A (rs4073)  | TT       | 16 (29.1)                    | 0.419                  | 43.6%                  | 17 (34)       | 0.916                   | 42%                          | 42%             | p = 0.810               |
|               | TA       | 30 (54.5)                    |                        |                        | 24 (48)       |                        |                              |                 |
|               | AA       | 9 (16.4)                     |                        |                        | 9 (18)        |                        |                              |                 |
| **CXCL8** c.781C > T (rs2227306) | CC       | 16 (29.1)                    | 0.419                  | 43.6%                  | 17 (34)       | 0.916                   | 42%                          | 39%             | p = 0.810               |
|               | CT       | 30 (54.5)                    |                        |                        | 24 (48)       |                        |                              |                 |
|               | TT       | 9 (16.4)                     |                        |                        | 9 (18)        |                        |                              |                 |
| **CXCL8** c.396T > G (rs2227307) | TT       | 16 (29.1)                    | 0.419                  | 43.6%                  | 17 (34)       | 0.916                   | 42%                          | 42%             | p = 0.810               |
|               | TG       | 30 (54.5)                    |                        |                        | 24 (48)       |                        |                              |                 |
|               | GG       | 9 (16.4)                     |                        |                        | 9 (18)        |                        |                              |                 |
| **CXCL8** c.91G > T (rs188378669) | GG       | 54 (98.2)                    | 0.945                  | 0.9%                   | 50 (100)      | -                       | 0%                           | 0%              | p = 1.290               |
|               | GT       | 1 (1.8)                      |                        |                        | 0 (0)         |                        |                              |                 |
|               | TT       | 0 (0.0)                      |                        |                        | 0 (0)         |                        |                              |                 |
| **IL1B** c.315G > A (rs1143634)  | GG       | 34 (61.8)                    | 0.311                  | 20%                    | 35 (70)       | 0.578                   | 17%                          | 25%             | p = 0.576               |
|               | GA       | 20 (36.4)                    |                        |                        | 13 (26)       |                        |                              |                 |
|               | AA       | 1 (1.8)                      |                        |                        | 2 (4)         |                        |                              |                 |
| SNP               | Genotype | Group of CF patients (n = 55) | Control group (n = 50) | 1000 Genomes database | Allele frequency CF patients vs control group |
|-------------------|----------|------------------------------|------------------------|------------------------|-----------------------------------------------|
|                   |          | Number (%) | HWE ** (p-value) | Variant allele freq. | Number (%) | HWE ** (p-value) | Variant allele freq. | Variant allele freq. (EU population) |                                |
| **IL1B c.-598T > C** (rs16944) | TT       | 5 (9.1)    | 0.536          | 73%                    | 5 (10)       | 0.736          | 70%                    | 65%                                 | p = 0.662                      |
|                   | TC       | 20 (36.4)  |               |                        | 20 (40)       |               |                        |                                     |                                |
|                   | CC       | 30 (54.5)  |               |                        | 25 (50)       |               |                        |                                     |                                |
| **IL1B c.597+76G > A** (rs1143639) | GG       | 34 (61.8)  | 0.311          | 20%                   | 35 (70)       | 0.578          | 17%                    | 24%                                 | p = 0.576                      |
|                   | GA       | 20 (36.4)  |               |                        | 13 (26)       |               |                        |                                     |                                |
|                   | AA       | 1 (1.8)    |               |                        | 2 (4)         |               |                        |                                     |                                |
| **IL1B c.-118G > A** (rs1143627) | GG       | 5 (9.1)    | 0.974          | 70%                   | 4 (8)         | 0.594          | 69%                    | 65%                                 | p = 0.875                      |
|                   | GA       | 23 (41.8)  |               |                        | 23 (46)       |               |                        |                                     |                                |
|                   | AA       | 27 (49.1)  |               |                        | 23 (46)       |               |                        |                                     |                                |
| **IL6 c.-237G > C** (rs1800795) | GG       | 16 (29.1)  | 0.701          | 47%                   | 20 (40)       | 0.406          | 39%                    | 42%                                 | p = 0.226                      |
|                   | GC       | 26 (47.3)  |               |                        | 21 (42)       |               |                        |                                     |                                |
|                   | CC       | 13 (23.6)  |               |                        | 9 (18)        |               |                        |                                     |                                |
| **IL10 c.1117A > G** (rs1800896) | AA       | 16 (29)    | 0.185          | 43%                   | 20 (40)       | 0.238          | 40%                    | 45%                                 | p = 0.688                      |
|                   | AG       | 31 (56.5)  |               |                        | 20 (40)       |               |                        |                                     |                                |
|                   | GG       | 8 (14.5)   |               |                        | 10 (20)       |               |                        |                                     |                                |

**HWE – Hardy-Weinberg equilibrium (occurs when p > 0.05).
Association analysis of clinical data and genetic diversity

First, we have analyzed all 12 variants in designated, based on clinical data, groups of patients - with different manifestations of CF (with pulmonary or digestive dominant symptoms) and with variable courses of disease (mild or severe) to examine possible association.

Our study demonstrated that the presence of two polymorphisms, c.-598T > C (rs16944) and c.-118G > A (rs1143627), in $IL1B$ gene significantly correlate with character of disease (Table 4). Higher frequency of variant allele c.-598C was observed in patients with severe character of CF, compared to patients with mild course of disease (87.5% and 64.3%, respectively, $\chi^2 = 6.92; p = 0.008$). Similarly, variant allele c.-118A occurred with higher frequency in subjects presented severe character of CF versus those with mild course of disease (82.5% vs. 62.8%, respectively, $\chi^2 = 4.68; p < 0.05$).

Considering the fact, that analyzed changes formed in our study two haploblocks (in $CXCL8$ and $IL1B$ genes), an analysis of the haplotypes in the context of the course and manifestation of disease was performed. We proved, that only haplotype AC created by changes c.-118G > A and c.-598T > C in $IL1B$ gene is significantly more often observed in group with severe course of CF in comparison with mild course (80% and 61.4%, respectively; $\chi^2 = 4.055; p = 0.03$).

Discussion

Because CF is a multifactorial, life-shortening disorder, the determination of SNPs that would affect the general phenotype or course of the disease is essential, but also challenging due to previous inconclusive results. So far, most of the CF studies were focused on searching modifier genes responsible for the severe pulmonary phenotype of the disease. In our investigation we have analyzed the impact of selected 12 potential candidates of modulator changes, rs4073, rs2227306, rs2227307 and rs188378669 in $CXCL8$ gene, rs361525 and rs1800629 in $TNF$ gene, rs16944, rs1143634, rs1142639 and rs1143627 in $IL1B$ gene, rs1800795 in $IL6$ gene and rs1800896 in $IL10$ gene on CF phenotype in Polish patients, taking into account the severity of symptoms on the side of the digestive, but also, respiratory system. Our hypothesis was that candidate modulator changes may predict digestive character of CF.

We observed, that in most of our group of patients (80%) the dominating symptoms occurred from the respiratory system and only in 20% of CF patients from the digestive system. Severe character of lung disease, diagnosed based on the FEV1% values, was noted in 20 patients (36%) and mild in 35 individuals (64%). In those subgroups of patients, we have performed a correlation analysis with DNA changes. Obtained variant allele frequencies of analyzed genetic variants, did
Table 4. Distribution of analyzed polymorphisms in the studied group of CF patients with different manifestation and course of disease.

| SNP      | Genotype | CF manifestation | Course of disease |
|----------|----------|------------------|-------------------|
|          | Pulmonary (n = 44) | Digestive (n = 11) | Pulmonary vs. Digestive | Mild (n = 35) | Severe (n = 20) | Mild vs. Severe |
| TNF rs361525 | GG | 37 | 7.9% | 11 | 0% | p = 0.475 | 30 | 7.1% | 18 | 5% | p = 1.008 |
|          | GA | 7 | | | 0 | | 5 | | 2 | | |
|          | AA | 0 | | | 0 | | 0 | | 0 | | |
| TNF rs1800629 | CC | 42 | 2.3% | 10 | 4.5% | p = 1.128 | 33 | 2.9% | 19 | 2.5% | p = 1.146 |
|          | CT | 2 | | | 1 | | 2 | | 1 | | |
|          | TT | 0 | | | 0 | | 0 | | 0 | | |
| CXCL8 rs4073 | TT | 15 | 42% | 1 | 50% | p = 0.501 | 11 | 45.7% | 5 | 40% | p = 0.561 |
|          | TA | 21 | | 9 | | 16 | 14 | | | |
|          | AA | 8 | | 1 | | 8 | 1 | | | |
| CXCL8 rs2227306 | CC | 15 | 42% | 1 | 50% | p = 0.501 | 11 | 45.7% | 5 | 40% | p = 0.561 |
|          | CT | 21 | | 9 | | 16 | 14 | | | |
|          | TT | 8 | | 1 | | 8 | 1 | | | |
| CXCL8 rs2227307 | TT | 15 | 42% | 1 | 50% | p = 0.501 | 11 | 45.7% | 5 | 40% | p = 0.561 |
|          | TG | 21 | | 9 | | 16 | 14 | | | |
|          | GG | 8 | | 1 | | 8 | 1 | | | |
| CXCL8 rs18837869 | GG | 44 | 0% | 11 | 0% | p = 1.000 | 35 | 0% | 20 | 0% | p = 1.000 |
|          | GT | 0 | | 0 | | 0 | 0 | | 0 | | |
|          | TT | 0 | | 0 | | 0 | 0 | | 0 | | |
| IL1B rs1143634 | GG | 27 | 20.5% | 7 | 18.2% | p = 1.000 | 22 | 20% | 12 | 20% | p = 1.000 |
|          | GA | 16 | | 4 | | 12 | 8 | | 1 | | |
|          | AA | 1 | | 0 | | 1 | 0 | | | | |
| IL1B rs16944 | TT | 5 | 71.6% | 0 | 77.3% | p = 0.915 | 5 | 64.3% | 0 | 87.5% | p = 0.008 |
|          | TC | 15 | | 5 | | 15 | 5 | | 5 | | |
|          | CC | 24 | | 6 | | 15 | 5 | | 5 | | |
| SNP       | Genotype | CF manifestation | Course of disease |
|-----------|----------|-----------------|-------------------|
|           |          | Pulmonary (n = 44) | Digestive (n = 11) | Pulmonary vs. Digestive | Mild (n = 35) | Variant allele freq. | Severe (n = 20) | Variant allele freq. | Mild vs. Severe |
|           |          | Variant allele freq. | Variant allele freq. | p = 0.768 | 21 | 13 | 21.4% | 7 | p = 0.620 |
| IL1B rs1143639 | GG | 28 | 19.3% | 6 | 22.7% | 21 | 13 | 17.5% | 7 | p = 0.620 |
|           | GA | 15 | 5 | 0 | 1 | 1 | 17.5% | 7 | p = 0.620 |
|           | AA | 1 | 0 | 0 | 0 | 0 | 17.5% | 7 | p = 0.620 |
| IL1B rs1143627 | GG | 5 | 70.4% | 0 | 68.2% | 4 | 62.8% | 1 | 82.5% | p = 0.030 |
|           | GA | 16 | 7 | 4 | 13 | 5 | 82.5% | 14 | 0.030 |
|           | AA | 23 | 4 | 0 | 0 | 0 | 82.5% | 14 | 0.030 |
| IL6 rs1800795 | CC | 12 | 46.6% | 4 | 50% | 9 | 48.6% | 7 | 45% | p = 0.718 |
|           | CG | 23 | 3 | 4 | 18 | 8 | 45% | 14 | 0.718 |
|           | GG | 9 | 4 | 0 | 8 | 5 | 45% | 14 | 0.718 |
| IL10 rs1800896 | AA | 13 | 41% | 3 | 50% | 7 | 47.1% | 9 | 35% | p = 0.215 |
|           | AG | 26 | 5 | 3 | 23 | 8 | 35% | 14 | 0.215 |
|           | GG | 5 | 3 | 0 | 5 | 3 | 35% | 14 | 0.215 |
| CFTR F508del | del/del | 11 | 53.4% | 2 | 36.4% | 11 | 55.7% | 2 | 45% | p = 0.279 |
|           | del/- | 25 | 6 | 3 | 17 | 14 | 45% | 14 | 0.279 |
|           | +/- | 8 | 3 | 0 | 7 | 4 | 45% | 14 | 0.279 |
not much differ from reference values for European population in 1000 Genomes database, except change $\text{TNF}$ c.-308C > T (rs1800629) which occurred in our patients group less often (2.7%) than in the database (13%). Also interesting is, that variant $\text{CXCL8}$ c.91G > T, p.Glu31Ter (rs188378669) globally noted with variant allele frequency < 0.1%, was detected in our CF patients at level 0.9% (one heterozygote detected in a cohort of 55 individuals). In our previous study, we proved, that this variant is significantly more common in patients with inflammatory bowel disease (MAF = 2.12%, 15 heterozygotes detected in a cohort of 353 patients) compared to healthy Polish population (MAF = 0.25%, 1 heterozygote identified in a cohort of 200 individuals of Polish population), what may suggest its association with inflammatory diseases (unpublished data). Therefore, studies on a larger group of patients are undoubtedly necessary to verify the participation of this variant in CF, especially since there are no data on the relationship with this disease.

Our study revealed, that among all analyzed genetic changes two of them, c.-598T > C (rs16944) and c.-118G > A (rs1143627) located in the $\text{IL1B}$ gene, are significantly associated with the severe character of lung disease in polish CF subjects. Allele C in locus -598 was observed with frequency of 87.5% in patients with severe lung disease compared to patients with mild lung dysfunction (64.3%, $p = 0.008$, OR = 3.88, C.I. = [1.351-11.190]), while allele A in locus -118 was observed with frequency 82.5% and 62.8% in both groups, respectively ($p = 0.03$, OR = 2.78, C.I. = [1.079-7.194]). We confirmed high LD between both changes (rs1143627 and rs16944) creating haplotypes AC, GT, GC and AT, where AC was significantly more often observed in subjects with severe course of CF in comparison to mild.

Our findings concerning the impact of polymorphism rs16944 on CF phenotype are consistent with those obtained by de Vries et al. They also proved a significant correlation of the variant allele c.-598C of $\text{IL1B}$ gene with severe pulmonary dysfunction in total of 152 Australian CF patients. Similarly, Levy et al. have reported that $\text{IL1B}$ constitutes a clinically relevant modulator of CF lung disease in the study conducted among American patients. However, in their research other SNPs, rs1143634 and rs1143639 demonstrated a consistent association with severe pulmonary phenotype.

In contrast to those results, Corvol et al. did not find any correlation between variants c.-598T > C and c.-118G > A in $\text{IL1B}$ gene and lung function assessed by spirometry in 329 Caucasian CF children from France and Germany. Additionally, they did not confirm any linkage disequilibrium between those polymorphisms.

Studies mainly highlight the relationship between lung disease in CF and $\text{CXCL8}$ gene polymorphism. IL8 plays a crucial role in the pathophysiology of inflammation of the airways in CF patients caused by a deficiency or absence of the $\text{CFTR}$ protein. Our study did not confirm this association among Polish patients.

We are aware of several limitations of our research. Our study cohort included only 55 patients and 50 controls. In the next step, verification of our results should be performed on a larger group of patients. This may be crucial in the case of rare variants, as c.91G > T, p.Glu31Ter (rs188378669) in $\text{CXCL8}$ gene, candidate as a modifier of CF. Furthermore, other factors such as BMI, gender, or age of diagnosis was not taken into account in our statistical analyzes.

We should also highlight the strengths of our study. First, the study cohort was represented by detailed characterized patients and homogenous controls group. What is important, the effect of $\text{CFTR}$ mutation F508del on the manifestation and course of CF in the studied patients was excluded because the frequency of mutations in the subgroups was similar.

Although this study does not indicate any modulators of digestive manifestation of CF, it constitutes the first report of genes predicting the course of this disease in the Polish population.

Recent studies indicate the important role of the microbiome in the course and manifestation of cystic fibrosis. Scientists underline that both, genotype and microbiome profiles are crucial interconnected factors in disease progression.

Conclusions
Our data have shown, that from all analyzed pro-inflammatory cytokine genes, only $\text{IL1B}$, but not $\text{TNF}$, $\text{CXCL8}$, $\text{IL6}$, or $\text{IL10}$ clearly play a crucial role in CF manifestation, determining the severe character of lung disease. This is a confirmation of major global results, as well as the first report concerning modulator genes of CF manifestation among Polish patients. Unfortunately, none of the analyzed genetic variants was found as predictors of digestive manifestation of CF disease, which may suggest the participation of also other modulator genes in the final phenotype of the disease.

Data availability statement
All data underlying the results are available as part of the article and no additional source data are required.
Open Peer Review

Current Peer Review Status:  

Version 3

Reviewer Report 23 August 2024

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Agnieszka Swiatecka-Urban
University of Virginia, Charlottesville, Virginia, USA

The authors examined the association of polymorphic variants in genes from the inflammatory pathway with pulmonary or digestive manifestation of cystic fibrosis (CF), and the severity of CF lung disease.

It is an interesting and important study. However there are major issues that should be addressed before the work is accepted.

Major:
The size of the cohort. The authors should provide the rationale for the small number of patients enrolled in the study.

Variable genetic background: Only 23.6% of patients were homozygous for F508del. The remaining patients were compound heterozygotes or had undisclosed CFTR genotypes. The non-homogenous genetic background in the CFTR gene invalidates the conclusion that the gene modifiers were responsible for the severity of pulmonary and digestive CF disease. If the sample size was larger, a multivariate analysis could be used to tease out potential associations.

Healthy controls: The purpose of including healthy controls needs to be clarified and discussed in the manuscript.

The authors emphasize the strengths of the study but neglect to mention the study limitations.

Minor:
There are many typographical and syntax errors. I suggest that the manuscript is reviewed by a professional English language interpreter.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Version 2**

Reviewer Report 24 October 2022

https://doi.org/10.5256/f1000research.134254.r151823

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Daniel J. Smith  
Faculty of Medicine, The University of Queensland, Brisbane, Qld, Australia

The article aims to determine whether there are association with polymorphisms in the genes for common pro-inflammatory cytokines and severity of CF lung and GI manifestations.

The major findings, in general, are supported by findings from studies in other populations.

Major comments:
- The population size is relatively small for a study of this type and runs the risk of type 2 error (falsely exclude the null hypothesis).
The definition of severe lung disease is based purely on lung function. However, this does not take into account age, i.e. a patient who has an FEV1 of 50% at the age of 20 years of age has a more severe phenotype compared to a patient who has an FEV1 of 50% of predicted at the age of 50 years (it is likely the 50 year old had a substantially higher FEV1 %predicted when they were 20 years old). There are other tools available in the literature for classifying the severity of lung disease taking age into consideration.

Lung function and nutrition in people with CF are not independent. Often patients with the most severe lung disease, also have the worse nutritional status. Greater detail needs to be provided on how patients were classified to a pulmonary or GI predominant phenotype. This classification is subject to bias. It should be stated whether the specialists determining the patients phenotype were also involved in analysing the results of genotype assessment and whether all phenotyping was completed before the genotype results were available.

Information should be provided on the rates of the different polymorphism among people with different CF genotypes. If certain polymorphisms are over represented in people homozygous for the F508 mutation. It may be the CF genotype and not the polymorphism that explains the difference in phenotype.

Minor comments:
- Lung function will vary with clinical status. Were all patients clinically stable at time of recruitment? If not, best lung function in the last 12 months may be a better marker of severity of lung function impairment.

References
1. Schluchter M, Konstan M, Drumm M, Yankaskas J, et al.: Classifying Severity of Cystic Fibrosis Lung Disease Using Longitudinal Pulmonary Function Data. American Journal of Respiratory and Critical Care Medicine. 2006; 174 (7): 780-786 Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes
Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cystic Fibrosis Physician. Published in the field of CF modifier genes during PhD studies.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Nov 2022

Oliwia Zakerska-Banaszak

Dear Dr. Daniel J. Smith,

Thank You very much for reviewing our manuscript and for all valuable comments and suggestions, which we have analyzed step by step. We respond accordingly to Your comments:

- The population size is relatively small for a study of this type and runs the risk of type 2 error (falsely exclude the null hypothesis).

We agree, the study group is not big, it consists of only 55 patients and of a proportionally big control group of 50 healthy Polish subjects, and we are aware of this limitation of our research. We believe, we have collected as many patients, as we can in this study period and during this collaboration with the Department of Pulmonology, Allergology, and Lung Oncology, Poznan University of Medical Sciences. On the other hand, this group is well-characterized and enrolled in accordance with the assumptions of the study.

- The definition of severe lung disease is based purely on lung function. However, this does not take into account age, i.e. a patient who has an FEV1 of 50% at the age of 20 years of age has a more severe phenotype compared to a patient who has an FEV1 of 50% of predicted at the age of 50 years (it is likely the 50 year old had a substantially higher FEV1 %predicted when they were 20 years old). There are other tools available in the literature for classifying the severity of lung disease taking age into consideration.

We thank You very much for this comment. We agree, in the literature, there are available different tools for classifying the severity of lung disease. In our study, we were based also on the professional literature (Yankaskas JR et al. Cystic fibrosis adult care: consensus conference report. Chest 2004;125:1-39), where the classification was determined from FEV1 only, as follows: 40-69% mild, and <40% severe. Nevertheless, due to the importance of age in this classification and accordingly to your suggestion, we took age into consideration in both groups and compared them in this regard. For this purpose statistical analysis (unpaired t-test) was performed. Results revealed that the “severe” group of patients did not differ significantly in age from patients in the “mild” group (mean age was 27.11 and 30.75, respectively; p-value = 0.055).

Therefore, the reference (numbered 21) and the information in the text was added (page 6):
“The “severe” group of patients did not differ significantly in age from patients in the “mild” group (mean age was 27.11 and 30.75, respectively; p-value = 0.055”).

In the future study, we will be careful and meticulous in classifying patients with CF to take age into account.

- Lung function and nutrition in people with CF are not independent. Often patients with the most severe lung disease, also have the worse nutritional status. Greater detail needs to be provided on how patients were classified to a pulmonary or GI predominant phenotype. This classification is subject to bias. It should be stated whether the specialists determining the patients phenotype were also involved in analysing the results of genotype assessment and whether all phenotyping was completed before the genotype results were available.

Thank You for this important remark. Of course, nutritional status is correlated with the severity of the disease. We can observe (based on BMI) this association in our study – in the “mild” group 26% of patients were malnourished, while in the “severe” group, 60% of patients.

Patients were classified by the specialists from the pulmonology field conducting the patients, based on the long-term observation of the patient and personal clinical interview with patient, to a pulmonary or GI predominant phenotype (we did not designate groups of pulmonary or digestive type of the disease, we only assessed that the dominant symptoms in a given patient were from the respiratory or digestive system, which does not mean that it was not from the other system). To the GI predominant phenotype were enrolled patients with the coexistence of at least two of listed conditions: 1) diabetes or glucose metabolism impairment, 2) pancreatic insufficiency, 3) liver disease or cirrhosis, 4) nagging pain or dysfunction of the digestive system. All patients with GI predominant phenotype represented “mild” lung impairment. Specialists determining the patients phenotype were not involved in the analysis of genotype assessment. Results of genotyping did not influence the assessment of phenotypic description.

Therefore, in the text on page 6, the additional information was added to clarify:

“To the digestive predominant phenotype were enrolled patients with the coexistence of at least two of listed conditions: 1) diabetes or glucose metabolism impairment, 2) pancreatic insufficiency, 3) liver disease or cirrhosis, 4) nagging pain or dysfunction of the digestive system. All patients with GI predominant phenotype represented “mild” lung impairment. Specialists determining the patients phenotype were not involved in the analysis of genotype assessment. Results of genotyping did not influence the assessment of phenotypic description”.

- Information should be provided on the rates of the different polymorphism among people with different CF genotypes. If certain polymorphisms are over represented in people homozygous for the F508 mutation. It may be the CF genotype and not the polymorphism that explains the difference in phenotype.

Thank You for this valuable comment. Accordingly to Your suggestion, we provide information about CF genotypes among patients group in the Table 4 containing analysed polymorphisms.

- Lung function will vary with clinical status. Were all patients clinically stable at time of recruitment? If not, best lung function in the last 12 months may be a better marker of severity of lung function impairment.

Thank you very much for this valuable remark. Of course, all the patients were clinically
stable at the time of study recruitment.

Hence, additional information was added in the text on page 6: “Patients were divided in the context of lung function impairment, based on the FEV1% values, while they were clinically stable, 1 - within the norm (FEV1% ≥ 70) and mild pulmonary obstruction (FEV1% 40-70) (35 subjects in total), 2 - severe pulmonary obstruction (FEV1% ≤ 40) (20 subjects in total).”

We hope, our responses improve the understanding of the manuscript.
Yours sincerely,
Oliwia Zakerska-Banaszak

Competing Interests: We disclose any competing interest.
The way of classifying patients into different stages of disease severity on the basis of FEV\textsubscript{1} is not very clear to me. Why were the cut-off points of 70% and 40% of predicted used? In this part of method description there should be information if it was arbitrary classification or if it is consistent with the generally accepted system of grading, and in this case reference to an appropriate publication should be given.

The paragraph concerning the occurrence of PA and MRSA in the studied group does not fit into the subject of the paper, does not add any new information, and may be omitted in the final version.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

	extbf{Competing Interests:} No competing interests were disclosed.

	extbf{Reviewer Expertise:} Lung diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 19 May 2022

Oliwia Zakerska-Banaszak

Dear Reviewer, Prof. Przybyłowski,

We thank you very much for your comments and all the valuable suggestions concerning our manuscript.

According to Your questions:
1. The way of classifying patients into different stages of disease severity on the basis of FEV\textsubscript{1} is not very clear to me. Why were the cut-off points of 70% and 40% of predicted
were used?. In this part of the method description there should be information if it was arbitrary classification or if it is consistent with the generally accepted system of grading, and in this case reference to an appropriate publication should be given.

2. The paragraph concerning the occurrence of PA and MRSA in the studied group does not fit into the subject of the paper, does not add any new information, and may be omitted in the final version.

Our response:
1. In the literature on cystic fibrosis, disease severity is determined from FEV1 as follows: FEV1 > 70% severe, 40-69% moderate, and <40% severe. Such a division was presented, inter alia, in the work: Yankaskas JR. Cystic fibrosis adult care: consensus conference reprint. chest 2004; 125: 1-39.

2. The paragraph concerning the occurrence of PA and MRSA was omitted.

Thank You very much. We hope our revised version of the manuscript will be correct now.

Your sincerely,
Oliwia Zakerska-Banaszak

**Competing Interests:** We disclose any competing interests.

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