A dataset of 26 candidate gene and pro-inflammatory cytokine variants for association studies in idiopathic pulmonary fibrosis: frequency distribution in normal Czech population

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia with poor diagnosis and a median survival of 2–3 years from initial diagnosis (1, 2). The cellular inflammation drives the fibrotic response in lung and plays a major role in IPF pathogenesis (3). Inflammatory cells (majorly, type 2 alveolar epithelial cells) release TGF-β, the key mediator of pulmonary fibrosis, that regulates several profibrotic cytokines/chemokines, their receptors, receptor subunits, and growth factors inducing process of epithelial–mesenchymal transition (EMT) (3, 4). Among the pro-inflammatory and profibrotic cytokines involved in IPF pathogenesis, interleukin (IL)-1 (4), IL-1β (5), IL-4, IL-5 (6), IL-6Rα (4), angiogenic IL-8/CXCL-8 (7), IL-13, its receptor IL-13 Rα2 (8), and IL-33 (9) have been implicated in accelerated inflammation and irreversible damage to lung architecture with loss of alveolar-capillary barrier basal membrane leading to persistent fibrosis. Genes encoding these factors exhibit nucleotide variation that could affect the severity of immune/inflammatory reactions and extent of any subsequent dysregulated fibroproliferative activity in disease development. Furthermore, variants in mucin–encoding genes (10–13) and in genes for pathogen-associated molecular patterns (PAMPs) receptors of innate immunity known as toll-like receptors (TLRs) (14, 15) have been also implicated in IPF immunopathogenesis and related to rapid progression of the disease. Investigations of these “candidate” gene variant(s) e.g., in case-control association studies may, therefore, provide novel insight into underlying mechanism of IPF susceptibility/disease outcome and, further, may aid to develop novel diagnostic approaches and eventually therapeutic interventions based on genetic information (16).

A candidate gene study typically involves genotyping 5–50 single nucleotide polymorphisms (SNPs) within gene(s) for its coding and non-coding/regulatory regions (17). Irrespective of the number of tested gene variants; for a standard conductance, data collection, and transparent reporting of a genetic association study, the recommendations of STrengthening the REporting of Genetic Association studies (STREGA) and STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) should be considered (16). In case-control studies, knowledge of frequency distribution of candidate gene loci/variants among normal (healthy control) population(s)


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is necessary and could be useful also for genetically related population(s) to determine the gene variants associated with disease and/or its clinical course.

The role of inflammatory and profibrotic mechanisms involving gene variation has been investigated in IPF and spectrum of susceptible polymorphic gene variants, including those in genes of immune reactions and signaling processes, have been recently reported from both genome-wide association studies (GWAS) and population-based case-control studies (14, 18, 19) performed mostly in US Caucasians, and also in some other ethnicities. The nominated gene variants, summarized in Table 1, are of different functions and implicate some yet-unanticipated pathways in IPF pathogenesis, including endoplasmic reticulum stress and unfolded protein response, cellular senescence, DNA-damage response, and already known Wnt–β-catenin signaling (20). The role of inflammatory and profibrotic mechanisms involving gene variation has been investigated in IPF and spectrum of disease and/or its clinical course.

No complex data have been yet reported on IPF-related variants in Slavonic populations, including Czechs. Starting our investigations of plausible multiple IPF susceptibility polymorphisms primarily in Czech and also related populations, we adopted allele-specific MALDI-TOF mass spectrometry-based SNPs genotyping assay for determination of gene variation in the relevant targets. Several IPF susceptible SNPs in genes of various functional categories were multiplexed, and in the first phase genotyped in probands from normal (healthy) Czech population using Sequenom MassARRAY platform. In the current dataset manuscript, we, besides genotyping methodology, report the genotype, allele, and phenotype (carriage rate) frequencies for plausible IPF susceptibility variants among normal population of Czech Republic of Western Slavonic (Caucasian) ancestry.

### Table 1 | List of candidate SNPs investigated in the study.

| S.No. | Location | Gene | SNP ID | Position | Region | Functional category | Reference |
|-------|----------|------|--------|----------|--------|---------------------|-----------|
| 1     | 2q14.1   | IL-1α| rs1800587 | 112,785,383 | 5′-flanking region | Pro-inflammatory cytokine (25) |
| 2     | 2q14.1   | IL-1β| rs16944  | 112,837,290 | Promoter | Pro-inflammatory cytokine (25) |
| 3     | 2q14.1   | IL-1β| rs1143634 | 112,832,813 | Exon (dl) | Pro-inflammatory cytokine (25) |
| 4     | 2p21     | PRKCE| rs628877  | 45,698,441 | Intron | Cellular signaling pathways |
| 5     | 3q26     | LRRC34| rs6793595 | 169,800,667 | Exon (dl) | Protein-protein interaction (26) |
| 6     | 3q22.1   | TF   | rs1799999 | 133,756,968 | Exon (dl) | Iron ion transport |
| 7     | 4q13.3   | IL-8 | rs4073   | 73,740,307 | Promoter | Pro-inflammatory cytokine (18) |
| 8     | 4q22.1   | FAM13A| rs2609055 | 88,890,044 | Intron | GTPase activator activity (26) |
| 9     | 4q35     |TLF3 | rs3775291 | 186,082,920 | Exon (dl) | Innate immunity (14) |
| 10    | 5p15     | TERT | rs2736100 | 1,286,401 | Intron 2 | Telomerase maintenance (26) |
| 11    | 5q31.1   | IL-13| rs1800925 | 132,657,117 | Promoter | Pro-inflammatory cytokine (27) |
| 12    | 5q31.1   | IL-4 | rs2234234 | 132,672,952 | Promoter | Pro-inflammatory cytokine (19) |
| 13    | 5q31.1   | IL-4 | rs2234235 | 132,673,462 | Promoter | Pro-inflammatory cytokine (19) |
| 14    | 5q31.1   | IL-4 | rs2070674 | 132,674,018 | Promoter | Pro-inflammatory cytokine (19) |
| 15    | 6p21.2   | CDK9V1A| rs2395655 | 36,677,919 | Promoter | Cell cycle regulation (19) |
| 16    | 6p21.2   | CDK9V1A| rs735590 | 36,677,428 | Promoter | Cell cycle regulation (19) |
| 17    | 6p24     | DSP  | rs2076295 | 7,562,999 | Intron | Binds intermediate filaments |
| 18    | 10q24    | DBFC1| rs11191865 | 103,913,084 | Intron | Telomerase maintenance (26) |
| 19    | 11p15.5  | MUC2 | rs7934606 | 1,100,037 | Intron | Mucin production in lungs (26) |
| 20    | 11p15.5  | MUC5B| rs35706950 | 1,219,991 | Promoter | Mucin production in lungs (12, 21) |
| 21    | 11p15.5  | TOLLIP| rs5743890 | 1,304,599 | Intron | Innate immunity (21) |
| 22    | 13q34    | ATP11A| rs1278769 | 112,882,313 | 3′ UTR | Phospholipid translocation (26) |
| 23    | 16p12.1  | IL-4Rα| rs1801275 | 27,363,079 | Exon (dl) | Pro-inflammatory cytokines (19) |
| 24    | 17p13.1  | TP53 | rs12951053 | 7,674,089 | Intron | Cell cycle regulation (28) |
| 25    | 17p13.1  | TP53 | rs12602273 | 7,679,695 | Intron | Cell cycle regulation (28) |
| 26    | 17q21    | MAPT | rs19861997 | 45,979,401 | Promoter | Fibrotic pathway (29) |
| 27    | 17q22.3  | ACE  | rs477405 | 65,471,587 | Promoter | Fibrotic pathway (29) |
| 28    | 17q23.3  | ACE  | rs4459609 | 65,471,587 | Promoter | Fibrotic pathway (29) |
| 29    | 19p13    | DPP9 | rs12610495 | 4,717,660 | Intron | Serine protease encoding (26) |

**ACE**, angiotensin-converting enzyme; **ATP11A**, ATPase, class VI, type 11A; **AZGP1**, alpha-2-glycoprotein 1, zinc-binding; **CDK9V1A**, cyclin-dependent kinase inhibitor 1A, the gene encoding p21; **COMP**, cartilage oligomeric matrix protein; **DPP9**, dipeptidyl-peptidase 9; **DSP**, dipeptidyl-peptidase 9; **FAM13A**, family with sequence similarity 13, member A; **IL-13**, interleukin-13; **IL-4**, interleukin-4; **LRRC34**, leucine rich repeat containing 34; **MAPT**, microtubule-associated protein tau; **MUC2**, mucin 2; **MUC5B**, mucin 5B; **OBFC1**, oligonucleotide/oligosaccharide-binding fold containing 1; **PKCE**, protein kinase C epsilon; **SFTR**, surfactant protein; **SPPL2C**, signal peptide peptidase-like 2C; **TERT**, telomerase reverse transcriptase; **TF**, transcription factor; **TGF-β**, transforming growth factor-β; **TNF-α**, tumor necrosis factor-α; **TLR3**, Toll-like receptor; **TOLLIP**, toll interacting protein; **TP53**, tumor protein **S3**, **dl**, synonymous mutation; **dl**, non-synonymous mutation; **U TR**, untranslated region.
Materials and Methods

Characteristics of the Study Group

Ninety-six unrelated healthy subjects (45 males, 51 females), free of any disease as assessed by physician's enquiry about their personal and family history, were enrolled. The mean age ± standard deviation was 34.5 ± 8.9 years and ranged from 18–57 years. All were Caucasians, and as assessed by surname and tracking personal history of Czech (Western Slavonic) ancestry living in Moravian region of the Czech Republic. All probands were informed about the purpose of the study and provided informed consent; the study was realized with the approval of the institutional ethics committee. Genomic DNA was isolated from peripheral blood leukocytes by standard salting out method (30).

A list of reference single nucleotide polymorphism database ID (rsSNP) was prepared for IFP-associated gene variants identified from available literature (GWAS and case-control studies) (Table 1). SNP genotyping using Sequenom iPLEX and MALDI-TOF-based MassARRAY platform allows analysis of up to 40 SNPs in a single well reaction. Primers were designed using Assay Design Suite v2.0 (ADS v 2.0) available under online tools of Agena Bioscience (https://www.mysequenom.com/Tools).

Assay Design, PCR Amplification, and Genotyping

For online genotyping assay design of multiplexed SNPs using ADS v2.0, an input list of SNP IDs (rsSNP of each target) was provided and following steps were followed: (1) the sequence for each rsSNP was retrieved from database and formatted accordingly, (2) the proximal SNPs for each rsSNP were identified from database, and (3) optimal primer areas were identified that result in a unique amplicons containing a target for the extension primer. To avoid extension primer rejection due to insufficient known bases, the proximal base was replaced with inosine. (4) PCR and extension primers were designed and checked for false priming, hairpin/dimer formation. The primer multiplexes with mass separation of analytes (alleles) were created. The PCR primer plex was prepared for PCR amplification, and extension primer mix was prepared for single base extension (SBE)-based iPLEX reaction. The SBE pool plex consists of multiplexed primers that anneal adjacent to polymorphic site for each reaction present together in the multiplexed assay pool. Thus, several individual DNA polymorphisms with their corresponding SNP sites could be analyzed in a single reaction. Due to the inverse relationship between peak intensity and extend primer mass, the extension target site could be identified with peak representing increase in mass of extend primer. The assay peak spectrum and call cluster plot resulting from MALDI-TOF MS analysis were analyzed with MassARRAY Typer 4.0.20 software that traces primer masses to the assayed alleles.

Statistical Analysis

Each SNP was tested for Hardy–Weinberg Equilibrium (HWE) by Pearson's goodness-of-fit, Chi-square (χ2) test. SNPs within HWE (p > 0.05) and sufficiently common (Minor Allele Frequency, MAF > 5%) in the general population were included. Phenotype frequency (carriage rate) was calculated as proportion of individuals carrying one or two copies of a particular allele on one or both (maternal or paternal) chromosomes.

Results

Using online Assay Design Suite v2.0 for primer designing, twoplexes were generated. Plexes I and II consisted of 22 and 7 SNPs, respectively. For a successful assay, inosine was used instead of proximal SNP in the extend primer sequence for rs2736100, rs2243250, and rs4277405 of plex I.

In assay control of multiplexed SNP genotyping, SNP rs2395655 in CDKN1A showed low assay-success rate (< 95%) and two SNPs DSP rs2076295 and TOLLIP rs5743890 were found as positive in no template control. These SNPs failed the quality control assessments and were removed from further analysis (Table 2). The genotyping assays success rates for all other analyzed SNPs were 98–100%. In our Czech healthy control population, all analyzed SNPs were in HWE, except for IL-4Rα rs1801275 that exhibited minor deviation (p = 0.04) reflecting a small anomaly, so the locus was not excluded from analysis (Table 2).

Among analyzed SNPs in cytokines, their receptor and subunits, IL-4 rs2243248 exhibited highest genotype (TT = 0.85), allele frequency (T = 0.93) and carriage rate (T = 1.00). Besides cytokines, we also report allele frequency of rs3775291 in TLR3, an innate immune system receptor. In present report, four out of 26 SNPs, viz., TP53 rs12951053, TP53 rs12602273, TF rs1799899, and IL-4 rs2243248 showed complete absence of their respective homozgyous genotype CC, GG, AA, and GG, and exhibited high phenotype frequency (1.00) for allele A, C, G, and T, respectively (Table 2). For MUC5B rs35709590*T risk allele, allelic and phenotype frequencies were found as 9% and 17%, respectively.

The genotype frequency and allele frequency for the 26 analyzed SNPs are available online at ALlele FREquency Database with Sample UID: SA004336Q (http://alfred.med.yale.edu/alfred/pophetgraph.asp?sampleuid=SA004336Q&cutoff=0.25) and will be publicly available at dbSNP database with the release of dbSNP Build (B144) (http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=LIGP).
| S.No. | Genetic variant | Assay rate (%) | Genotype | n = 96 | HWE | Allele | Phenotype frequency |
|-------|-----------------|----------------|----------|--------|------|--------|---------------------|
| 1     | *IL-1* α rs1800587 | 100            | CC       | 39     | 0.86 | C      | 0.88               |
|       |                 |                | CT       | 45     |      | T      | 0.59               |
|       |                 |                | TT       | 12     |      |        |                    |
| 2     | *IL-1* β rs16944 | 100            | GG       | 46     | 0.35 | G      | 0.88               |
|       |                 |                | GA       | 38     |      | A      | 0.52               |
|       |                 |                | AA       | 12     |      |        |                    |
| 3     | *IL-1* β rs1143634 | 100            | CC       | 56     | 0.49 | C      | 0.93               |
|       |                 |                | CT       | 33     |      | T      | 0.42               |
|       |                 |                | TT       | 7      |      |        |                    |
| 4     | *PRKCE* rs628877 | 99*            | GG       | 61     | 0.11 | G      | 0.93               |
|       |                 |                | GT       | 27     |      |        |                    |
|       |                 |                | TT       | 7      |      |        |                    |
| 5     | *LRRC34* rs6793295 | 100            | TT       | 45     | 0.23 | T      | 0.94               |
|       |                 |                | TC       | 45     |      | C      | 0.53               |
|       |                 |                | CC       | 6      |      |        |                    |
| 6     | *TF* rs1799899 | 100            | GG       | 81     | 0.41 | G      | 1.00               |
|       |                 |                | GA       | 15     |      | A      | 0.16               |
|       |                 |                | AA       | 0      |      |        |                    |
| 7     | *IL-8* rs4073 | 100            | AA       | 19     | 0.50 | A      | 0.73               |
|       |                 |                | AT       | 51     |      | T      | 0.80               |
|       |                 |                | TT       | 26     |      |        |                    |
| 8     | *FAM134* rs2609255 | 100            | TT       | 53     | 0.89 | T      | 0.94               |
|       |                 |                | GT       | 37     |      | G      | 0.45               |
|       |                 |                | GG       | 6      |      |        |                    |
| 9     | *TLR3* rs3775291 | 100            | GG       | 41     | 0.88 | G      | 0.89               |
|       |                 |                | GA       | 44     |      | A      | 0.57               |
|       |                 |                | AA       | 11     |      |        |                    |
| 10    | *TERT* rs2736100 | 100            | TT       | 29     | 0.91 | T      | 0.80               |
|       |                 |                | GT       | 48     |      | G      | 0.70               |
|       |                 |                | GG       | 19     |      |        |                    |
| 11    | *IL-13* rs1800925 | 100            | CC       | 49     | 0.68 | C      | 0.91               |
|       |                 |                | CT       | 38     |      | T      | 0.49               |
|       |                 |                | TT       | 9      |      |        |                    |
| 12    | *IL-4* rs2243248 | 98*            | TT       | 80     | 0.44 | T      | 1.00               |
|       |                 |                | GT       | 14     |      | G      | 0.15               |
|       |                 |                | GG       | 0      |      |        |                    |
| 13    | *IL-4* rs2243250 | 98*            | CC       | 64     | 0.24 | C      | 0.95               |
|       |                 |                | CT       | 25     |      | T      | 0.32               |
|       |                 |                | TT       | 5      |      |        |                    |
| 14    | *IL-4* rs2070874 | 100            | CC       | 66     | 0.91 | C      | 0.97               |
|       |                 |                | CT       | 27     |      | T      | 0.31               |
|       |                 |                | TT       | 3      |      |        |                    |
| 15    | *CDKN1A* rs733590 | 100            | TT       | 36     | 0.71 | T      | 0.86               |
|       |                 |                | CT       | 47     |      | C      | 0.63               |
|       |                 |                | CC       | 13     |      |        |                    |
| 16    | *OSFC1* rs11191865 | 100            | GG       | 33     | 0.70 | G      | 0.81               |
|       |                 |                | AG       | 45     |      | A      | 0.66               |
|       |                 |                | AA       | 18     |      |        |                    |
| 17    | *MUC2* rs7934606 | 100            | GG       | 41     | 0.16 | G      | 0.93               |
|       |                 |                | AG       | 48     |      | A      | 0.57               |
|       |                 |                | AA       | 7      |      |        |                    |
| 18    | *MUC5B* rs35705950 | 100           | GG       | 80     | 0.16 | G      | 0.98               |
|       |                 |                | GT       | 14     |      | T      | 0.17               |

(Continued)
TABLE 2 | Continued

| S.No. | Genetic variant | Assay rate (%) | Genotype | n = 96 | HWE | Allele | Phenotype frequency |
|-------|----------------|----------------|----------|--------|------|--------|---------------------|
| 19    | ATP11A rs1278769 | 100            | GG       | 48     | 0.79 | G      | 0.91               |
|       |                 |                | GA       | 39     |      | A      | 0.50               |
|       |                 |                | AA       | 9      |      |        |                    |
| 20    | IL-4R α rs1801275 | 100            | AA       | 68     | 0.04 | A      | 0.94               |
|       |                 |                | AG       | 22     |      | G      | 0.29               |
|       |                 |                | GG       | 6      |      |        |                    |
| 21    | TP53 rs12951053  | 100            | AA       | 81     | 0.41 | A      | 1.00               |
|       |                 |                | CA       | 15     |      | C      | 0.16               |
|       |                 |                | CC       | 0      |      |        |                    |
| 22    | TP53 rs12602273  | 100            | CC       | 81     | 0.41 | C      | 1.00               |
|       |                 |                | GC       | 15     |      | G      | 0.16               |
|       |                 |                | GG       | 0      |      |        |                    |
| 23    | MAPT rs1981997   | 100            | GG       | 67     | 0.81 | G      | 0.97               |
|       |                 |                | GA       | 26     |      | A      | 0.30               |
|       |                 |                | AA       | 3      |      |        |                    |
| 24    | ACE rs4277405    | 100            | TT       | 36     | 0.51 | T      | 0.88               |
|       |                 |                | CT       | 48     |      | C      | 0.63               |
|       |                 |                | CC       | 12     |      |        |                    |
| 25    | ACE rs4459609    | 100            | AA       | 37     | 0.83 | A      | 0.86               |
|       |                 |                | CA       | 48     |      | C      | 0.61               |
|       |                 |                | CC       | 13     |      |        |                    |
| 26    | DPP9 rs12610495  | 100            | AA       | 54     | 0.69 | A      | 0.93               |
|       |                 |                | GA       | 35     |      | G      | 0.44               |
|       |                 |                | GG       | 7      |      |        |                    |

*One sample for PRKCE rs628877.
**Two samples each for IL-4 rs2243250 and IL-4 rs2243248 were not genotyped.

HWE, Hardy–Weinberg equilibrium.

Discussion

The present dataset reports the genotype distribution, genotype, allele, and phenotype frequency of 26 gene variants involved in immune-related pathomechanisms of IPF in normal Czech population using Sequenom MassARRAY based genotyping platform. Besides the relevance to the delineation of immunogenetic component of IPF, the knowledge of frequency distribution of gene variants in normal populations is of considerable importance for their evaluation as genetic markers in susceptibility, manifestation, prognosis, and potentially treatment of diseases in different populations (32).

A SNP rs35705950 in the putative promoter of MUC5B has been shown to exhibit strong association with both familial interstitial pneumonia and IPF (33). The observed rs35705950*T risk-allele frequency of 9% in normal Czech population was in concordance with other reports in normal Caucasians of European-American descents, as 9–11% in American (33), 10% in UK Caucasians (34), 11% in French (22), and 4.3% in Germans (24) populations among Europeans. Interestingly, the MUC5B promoter polymorphism is observed less frequently in normal Asian populations, such as 0.8% in Japanese (24), 0.7% in Chinese (23), and <1% in Koreans (11). Overall, mucin glycoprotein encoding MUC5B has role in normal lung function by regulating immune function, microbial population, airway infection, and mucociliary clearance in lungs (35, 36).

Among analyzed cytokines, IL-4 has significant role in IPF pathogenesis by regulating fibroblast functions, such as chemotaxis, proliferation, collagen synthesis, myofibroblast differentiation, and T_h1/T_h2 equilibrium (19). The angiogenic IL-8 was shown as predictive for early stage of IPF (37) and as poor IPF survival (38). Additionally, IL-13 and IL-13 pathway markers (39) and the innate immune signaling receptor TLR3 have been suggested as potential markers of rapidly progressive form of IPF. Several recent studies have suggested that defective TLRs are linked to dysregulated fibrogenesis and have key role in myofibroblast activation, increased profibrotic cytokines, collagen deposition, fibrosis, and tissue destruction and, thus, promoting the progression of disease during the later phase of IPF (14, 15, 40, 41).

Of the four variants that exhibited absence of homozygous genotypes in this data report: (1) the frequency of TP53 rs12951053 CC genotype has been reported as 6% in Caucasian HC (28), 1.2% in European and Africans and relatively higher in Asian (11.9% in Han Chinese and 11.6% in Japanese) populations (http://snp-nexus.org/temp/snpnexus_10220/results.html); (2) For TP53 rs12602273, CC genotype frequency has been reported as 3% in Caucasian healthy controls (28); (3) For TF rs1799899, AA genotype frequency has been reported as 0.6% in European, and 0.0% among African, Han Chinese, and Japanese populations (http://snp-nexus.org/temp/snpnexus_10168/results.html); and (4) For IL-4 rs2243248, low GG genotype frequency (IL-4, -1098 G/T) has been reported in another independent study for...
characterization of IL-4 gene polymorphism in a relatively small cohort of IPF patients of same ethnicity (19).

The present findings are widely applicable in IPF genetics research in other related populations as well. In a current research initiative in immunogenetics by HLA-NET network, a working group for population definitions and sampling strategies in population genetics analyses strongly recommend the usage of geographical and/or cultural criteria (with anthropological considerations) to describe human populations instead of a priori misclassifications of racial and ethnic groups (42). In this context, Central Europe also in Germans and Austrians, as we could recently exemplify relevant for IPF gene case-control studies not only in Czech but also in neighboring populations, namely Slovak and Polish, and also in Germans and Austrians, as we could recently exemplify in preliminary investigations of immune-related IPF susceptible variants in Czech and German population cohorts (10, 13).

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Conclusion

The present data on a spectrum of 26 gene variants including 10 variants of immune and inflammatory response (cytokines/chemokines and TLR) and their frequency distribution in normal Czech (Western Slavonic, Caucasian) population has wider application as standard control along with cases in association studies for IPF. It is also relevant in other fibrotic lung diseases among Czech and genetically related/neighboring population(s) and in the wider context for further delineation of the role of immune and inflammatory reactions in this debilitating disease.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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