

Gene and miRNA expression profiles in PBMCs from patients with severe and mild emphysema and PiZZ alpha1-antitrypsin deficiency

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Introduction: COPD has complex etiologies involving both genetic and environmental determinants. Among genetic determinants, the most recognized is a severe PiZZ (Glu342Lys) inherited alpha1-antitrypsin deficiency (AATD). Nonetheless, AATD patients present a heterogeneous clinical evolution, which has not been completely explained by sociodemographic or clinical factors. Here we performed the gene expression profiling of blood cells collected from mild and severe COPD patients with PiZZ AATD. Our aim was to identify differences in messenger RNA (mRNA) and microRNA (miRNA) expressions that may be associated with disease severity.

Materials and methods: Peripheral blood mononuclear cells from 12 COPD patients with PiZZ AATD (6 with severe disease and 6 with mild disease) were used in this pilot, high-throughput microarray study. We compared the cellular expression levels of RNA and miRNA of the 2 groups, and performed functional and enrichment analyses using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene-ontology (GO) terms. We also integrated the miRNA and the differentially expressed putative target mRNA. For data analyses, we used the R statistical language R Studio (version 3.2.5).

Results: The severe and mild COPD–AATD groups were similar in terms of age, gender, exacerbations, comorbidities, and use of augmentation therapy. In severe COPD–AATD patients, we found 205 differentially expressed genes (DEGs) (114 upregulated and 91 downregulated) and 28 miRNA (20 upregulated and 8 downregulated) compared to patients with mild COPD–AATD disease. Of these, hsa-miR-335-5p was downregulated and 12 target genes were involved in cytokine signaling, MAPK/mk2, JNK signaling cascades, and angiogenesis were much more highly expressed in severe compared with mild patients.

Conclusions: Despite the small sample size, we identified downregulated miRNA (hsa-miR-335) and the activation of pathways related to inflammation and angiogenesis on comparing patients with severe vs mild COPD–AATD. Nonetheless, our findings warrant further validation in large studies.

Keywords: alpha1-antitrypsin deficiency, COPD, gene expression, miRNAs, integrative analysis

Introduction

COPD is the third leading cause of death worldwide. It is a complex disease influenced by both genetic and environmental determinants, the most important of which is cigarette smoking. However, only 10%–20% of smokers develop clinically significant disease, and there is a significant variability across the smokers with similar cigarette exposure. Some of these heterogeneities may be the result of genetic variations.¹

A known genetic risk factor for COPD is severe alpha1-antitrypsin deficiency (AATD). SERPINA1 is the gene responsible for the synthesis of the AAT protein, which is a serine protease inhibitor (serpin) and an acute-phase glycoprotein.² Normal serum AAT levels are the result of 2 copies of the common M allele of the SERPINA1 gene. The M allele of AAT is present in >90% of the population, and is called Pi*M. Over 100 SERPINA1 mutations have been identified to date, but only some are implicated in disease pathogenesis. The most frequent deficient alleles are S (Glu264Val) and Z (Glu342Lys), which are called Pi*S and Pi*Z, respectively. In particular, carriers of 2 Z alleles (PiZZ) have strongly reduced circulating levels of AAT (up to 10%–20% of normal, which is 1.3–2 g/L). Because of Z AAT misfolding and intracellular accumulation, the Z allele is the major contributor to pulmonary emphysema and liver disease in people of European ancestry.^{3–4}

The evolution of emphysema in clinical terms and life expectancy are directly related to the accelerated loss of forced expiratory volume in 1 s (FEV₁). Previous studies identified tobacco consumption and exacerbations as the main factors related to FEV₁ decline.⁵ Lung function decline is faster in AATD compared with non-AATD COPD patients, although this is not homogeneous in all individuals.^{6–8}

Recent genome-wide association studies (GWAS) and meta-analyses of GWAS have begun to shed light on biological pathways that contribute to the development of COPD.^{9–11} The analysis of the miRNA expression is increasingly used in clinical research. miRNAs belong to the family of small non-coding RNAs (~21–25 nucleotides long), but they are involved in mRNA regulation.¹² miRNAs modulate gene expression by either destabilizing transcripts or inhibiting protein translation. In fact, miRNAs may target up to one-third of the transcriptome. Therefore, miRNA expression patterns in cells and tissues are considered to be biomarkers for both the diagnosis and prognosis of different diseases, including COPD.^{13–16} mRNA and miRNA signatures can assist clinicians in prognosis assessment and therapeutic approaches.^{12–18} However, the heterogeneity in study designs makes comparisons across studies difficult. Therefore, this field is naturally progressing toward the integration of miRNA with gene expression.¹⁹

In this context, some SERPINA1 gene expression studies have been performed.⁶ We propose that gene expression profiling may provide new insights into the molecular mechanisms of AATD-related COPD and eventually lead to the identification of novel therapeutic targets.^{20–21} Here, we explored the use of gene expression patterns in peripheral blood mononuclear cells (PBMCs) collected from PiZZ cases with severe and mild

COPD. We aimed to identify differentially expressed putative mRNA and miRNAs in PBMCs isolated from patients with different severities of lung disease.

Materials and methods

Design and study sample

This study was a pilot high-throughput microarray experiment. On the basis of the presence of poor lung function at diagnosis (FEV₁<45% predicted), we included 6 patients considered to have rapid progression of emphysema (severe disease group). This severe group was compared with a group of 6 subjects matched for age and gender but with significantly better lung function at diagnosis (FEV₁>65% predicted). We considered these patients to have slow disease progression (mild disease group). All the patients were recruited from the outpatient clinic of the respiratory department of the Vall d'Hebron University Hospital (Barcelona, Spain) and all had undergone genotyping by sequencing of all coding exons of SERPINA1. Signed informed consent was obtained from all subjects for participation in the study, and the study was approved (project ID: PR (AG)157/2016) by the Ethics Committee of the Research Institute of Hospital Vall d'Hebron (Barcelona, Spain).

All the patients presented clinical stability, defined as no respiratory ambulatory exacerbations or hospital admission in the previous 2 months. They were followed and treated according to guidelines.²² Some patients were receiving intravenous therapy with plasma purified AAT (Prolastin or Trypsone; Grifols, Barcelona, Spain) at a dose of 120 mg/kg for 15 days.

As previous studies have shown that AAT therapy may indirectly reduce the expression of Z-AAT,²³ blood samples were taken immediately before the next infusion in patients receiving augmentation therapy.

Variables and data collection

Sociodemographic, clinical (phenotype, age at diagnosis, current age, age at onset of symptoms, tobacco consumption, comorbidities, exacerbations, and treatments), and lung function data at the time of AATD diagnosis were collected from medical records. Lung function tests were performed according to standardized recommendations and international guidelines.^{24–25}

Blood processing and RNA isolation

Blood processing and RNA isolation was carried out at the Biobank at the Vall d'Hebron Research Institute (VHIR), Barcelona, Spain. PBMCs were isolated from AATD patients using Ficoll gradient centrifugation. Total RNA and miRNA were isolated from PBMCs using the QuickGene RNA tissue

SII (RT-S2) kit and QuickGene 810 FUJIFILM device. miRNA was also isolated from PBMCs using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and the miRNeasy Mini Handbook Kit (Qiagen). RNA and miRNA concentrations were determined with a NanoDrop ND2000 spectrophotometer and quality was checked using a Bioanalyzer (Agilent Technologies, Hilden, Germany) with Eukaryotic NanoChip before microarray analysis.

RNA and miRNA microarray hybridization

The RNA and miRNA microarrays were performed at the High Technology Unit (UAT) of the VHIR. In this experiment, the Affymetrix GeneTitan microarray platform and the Genechip Human Gene Array Plate 2.1 were used for RNA hybridization. This array analyzes gene expression patterns on a whole-genome scale on a single array with probes covering many exons on the target genomes, thereby providing expression summarization at the exon or gene level. The starting material was 200 ng of total RNA of each sample. Briefly, sense single-stranded DNA (ssDNA) suitable for labeling was generated from total RNA with the GeneChip WT Plus Reagent Kit from Affymetrix (High Wycomb, UK) according to the manufacturer's instructions. Sense ssDNA was fragmented, labeled, and hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit from the same manufacturer.

The Affymetrix GeneTitan microarray platform and the Genechip miRNA array plate 4.1 were used for miRNA hybridization. The starting material was 400 ng of total RNA of each sample. miRNA in the sample was labeled using the Flash Tag Biotin HSR RNA Labeling Kit following the manufacturer's instructions.

We used a unique miRNA and 2 mRNA microarray batches in our experiments. Distribution of severe and mild patient samples within the batches was similar.

Statistical analysis for comparison of patient characteristics

The results of the statistical analyses are expressed as mean (SD) for quantitative variables and as valid percentage for groups using the nonparametric Mann–Whitney *U* or chi-square tests (Fisher's exact test when the expected frequencies were <5).

Data preprocessing and analysis of differentially expressed genes

First, a raw data quality control was performed before normalization in order to analyze sample quality, hybridization

quality, signal comparability and biases, array correlation, and probe sets' homogeneity.

Background correction and quantile normalization were performed for the raw array data using the oligo package (<http://www.bioconductor.org/>)²⁶ and robust multi-array average (RMA package for R).²⁷ For each sample, the expression values of all probes for a given gene were reduced to a single value by taking the average expression value. Principal component analysis (PCA) was performed and a hierarchical clustering plot was made after normalization. Batch effects were analyzed using surrogate variable analysis (SVA package)²⁸ and Combining Batches of Gene Expression Microarray Data (ComBat) functions.²⁹

Before differentially expressed genes (DEGs) analysis, present probes without the corresponding gene symbols and expression intensities below the 3rd quartile value were removed for further statistical analysis. The Limma (Linear Models for Microarray Data) package³⁰ was used to perform statistical testing of differential mRNA and miRNA expression between the 2 study groups. Differentially expressed mRNAs and miRNAs were defined as having a *P*-value of <0.05 with at least ± 1.5 -fold change (FC) ($\log FC \pm 0.58$) between the groups. *P*-values adjusted for the false discovery rate (FDR) were also calculated.

DEGs annotation and pathway-enrichment analyses

DEGs were annotated identifying the beginning and the end of the chromosome as well as the gene symbol with the BioMart R package (<http://www.biomart.org>) and the Affymetrix annotation files.

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene-ontology (GO) terms³¹ were enriched for DEGs using the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) database. The major GO terms associated with biological processes was manually summarized based on gene-term enrichment provided for each functional group at a Fisher's exact *P*-value of 0.05. The same statistical approach was used for KEGG analysis.

miRNA and mRNA data integration

Integration of miRNA DEGs and their target mRNA DEGs was performed using the multiMiR R package with the web server at <http://multimir.ucdenver.edu>. This is a comprehensive collection of predicted and validated miRNA–target interactions and their associations with diseases and drugs.³² All the analyses were performed using the R Studio (version 3.2.5).

Quantitative reverse transcription polymerase chain reaction validation

For all the samples, an independent analysis of EGR3 and EREG expression was performed by the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method. The levels of expression of selected genes were analyzed using the TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies, Madrid, Spain). The expression of the reference gene, hypoxanthine phosphoribosyl transferase (Life Technologies, #Hs02800695_m1), was used for normalization. Relative gene expression was calculated according to the Δ cycle threshold method using StepOne software. Statistical significance was defined as $P < 0.05$ as measured using the Student's *t*-test.

Results

Blood PBMCs were isolated from 12 PiZZ patients: 6 with severe emphysema with a mean FEV₁(%) of 44% (SD=6.7%) and 6 with mild disease, with FEV₁(%) of 93% (SD=29%). Both study groups presented similar characteristics in terms of age, gender, exacerbations, comorbidities, and time from the last infusion. Severe patients were diagnosed at a younger age than mild patients (42.7 years [SD=6.4] vs 51.2 [SD=4.0], $P=0.041$) and all received inhaled corticosteroids (Table 1).

Table 1 Patients' characteristics

Variables	Mild emphysema N=6	Severe emphysema N=6	P-value
Age (years) at the time of the study	61.7 (7.7)	61.0 (10.5)	0.937
Age (years) at diagnosis	51.2 (4)	42.7 (6.4)	0.041
Gender			0.999
Male (%)	4 (66.7)	4 (66.7)	
Female (%)	2 (33.3)	2 (33.3)	
Smoking history			0.135
Never (%)	2 (33.3)	0	
Current (%)	0	2 (33.3)	
Former (%)	4 (66.7)	4 (66.7)	
FEV ₁ %	93 (29)	44 (6.7)	0.002
AAT blood levels (mg/dL)	22 (5)	27 (5.1)	0.265
Other comorbidities			
Bronchiectasis	1 (16.7)	1 (16.7)	0.098
Cardiovascular diseases	1 (16.7)	2 (33.3)	0.125
Inhaled respiratory treatments (%)	4 (66.7)	6 (100)	0.225
Beta2 agonists (%)	4 (66.7)	6 (100)	0.065
Inhaled corticosteroids (%)	0	6 (100)	0.025
Replacement therapy (RT) (%)	2 (33.3)	4 (66.7)	0.248
Time from last RT infusion (days)	15	15	0.999
Time from blood collection to sample process (minutes)	74.2 (49.6)	68.3 (23.2)	0.589

Notes: Data are expressed as mean (SD) or n (valid percentage). Bold values indicate $p < 0.05$.

Abbreviations: AAT, alpha1-antitrypsin; FEV₁, forced expiratory volume in 1 s.

Two patients from the severe group were current smokers and, therefore, did not receive replacement therapy. None of the patients in either group received systemic corticosteroids. The time from blood collection and processing in the laboratory was compared between groups without statistical differences.

mRNA analysis

Initially, the mRNA expression of 53,617 probe sets corresponding to 12 samples were included. Data were normalized and after filtering, a total of 10,143 probes were used for the following analysis. We found no batch effects according to the time of blood processing, study group, or gender (data not shown).

Normalized PCA results showed that the first axis explained 16% of the variance. Two samples corresponding to current smokers from the severe group presented a different gene expression profile according to the second axis that explained 10.5% of the variability. To avoid a potential gene expression bias, these 2 samples were removed from the following analyses. Finally, 10 AATD samples (4 from the severe and 6 from the mild disease groups) were included in the DEGs analysis.

A total of 205 DEGs differed between severe and mild patients, of which 114 genes were upregulated and 91 down-regulated in severe patients using non-adjusted *P*-value of < 0.05 and FCs $> \pm 1.5$ (logFC $> \pm 0.58$) (Figure 1).

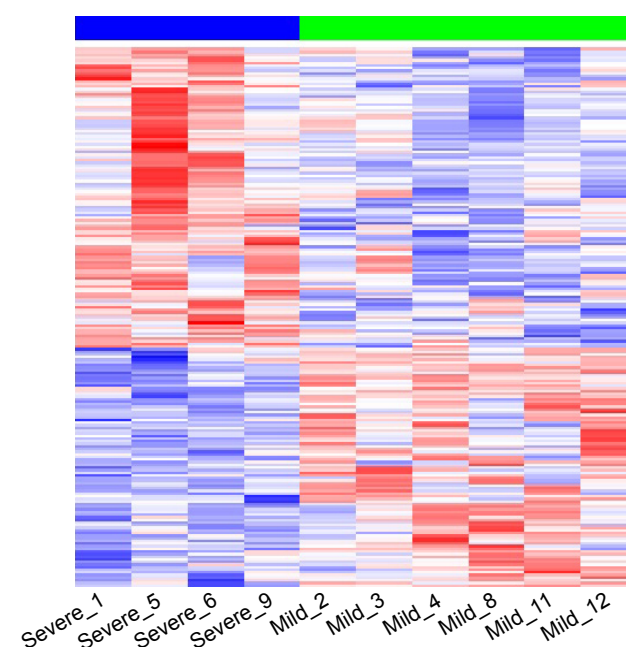


Figure 1 Differentially expressed genes in severe (blue line) vs mild (green line) patients.

Notes: Two-dimensional hierarchical clustering of the 10 samples according to the expression of 205 genes differentially expressed (by row) comparing severe (n=4) vs mild (n=6) patients (by column), with a *P*-value of < 0.05 and fold changes (FC) $> \pm 1.5$ (logFC $> \pm 0.58$) using the Limma (Linear Models for Microarray Data) method. The functional classification of genes (color-coded) is shown. Red represents a high level of gene expression while blue represents a low level of expression.

FDR *P*-values were calculated, but no differences were found in gene expression between the 2 groups. Therefore, for all the following analyses, we used non-adjusted *P*-values.

The top dysregulated mRNAs are shown in Table 2. Upregulated genes with highest difference between the 2 groups ($\log_{2}FC > 1$) included HLA-DRB5, OLFM4, HBEGF, LTF, MMP8, CXCL8, SOCS3, CAMP, G0S2, CCL3L3, EREG, EGR3, NR4A2, JUP, and IL1B genes.

These genes are involved in systemic and local inflammation, immunity, proliferation, differentiation, apoptosis, and oncogenesis. We found that these groups of genes are enriched in different pathways such as the mitogen-activated protein kinases (MAPK) cascade pathway, the Jun amino-terminal kinases (JNK) cascade, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, the NOD-like receptor pathway, and the Toll-like receptor signaling pathway using GO and KEGG terms (Tables 3 and 4).

Table 2 Top dysregulated mRNA comparisons in peripheral blood mononuclear cells from severe vs mild AATD emphysema patients

Symbols	logFC	P-value
Upregulated		
HLA-DRB5	2.46	0.025
OLFM4	2.42	0.003
HBEGF	2.01	0.002
LTF	1.79	0.047
MMP8	1.69	0.028
CXCL8/IL-8	1.57	0.019
SOCS3	1.56	0.000
CAMP	1.53	0.014
G0S2	1.50	0.015
CCL3L3/CCL3L1	1.38	0.001
EREG	1.37	0.018
EGR3	1.31	0.004
NR4A2	1.31	0.004
OSM	1.28	0.007
CRISP3	1.24	0.043
IL1B	1.18	0.014
JUP	1.16	0.008
PTGS2	1.11	0.038
IDI	1.11	0.003
CLEC5A	1.10	0.003
NR4A3	1.08	0.027
SNAI1	1.00	0.003
CD24	1.00	0.034
Downregulated		
XCL2	-1.01	0.000
PKD4	-1.01	0.047
RNU1-59P	-1.05	0.013
ZNF208	-1.12	0.000

Note: Table 2 shows different miRNAs using a *P*-value < 0.05 and $\log_{2}FC > \pm 1$.

Abbreviations: AATD, alpha1-antitrypsin deficiency; IL-8, interleukin-8; $\log_{2}FC$, fold change logarithm; MMP, matrix metalloproteases protease; mRNA, messenger RNA.

miRNA DEGs analysis

The miRNA data were analyzed using the same pipeline as in mRNA data analysis. Initially, a quality control of 6,658 miRNA probes was performed and after filtering, 1,165 miRNA were included in the Limma analysis. A total of 28 miRNAs were found to be differently expressed in PBMCs from severe compared to mild AATD emphysema patients, with 20 miRNAs being upregulated and 8 being downregulated in severe patients (Table 5).

All the dysregulated miRNAs are shown in Table 5. Upregulated miRNA included hsa-miR-146b-3p, hsa-miR-146b-5p, hsa-miR-193b-3p, and hsa-miR-365a-5p, whereas downregulated miRNAs included hsa-miR-486-3p and hsa-miR-335-5p. The latter 2 have previously been reported to be related to respiratory diseases.

miRNA and mRNA interactions

As shown in Table 6, we identified 3 putative miRNA interacting targets between differentially expressed miRNAs and DEGs. Our analysis showed significant downregulation of hsa-miR-335-5p in severe patients, which was significantly linked to 12 upregulated transcripts, enriched in the cytokine-mediated signaling pathway, the MAPK/mk2 signaling cascade, the JNK cascade, and angiogenesis (Table 6).

qRT-PCR validation of DEGs

We randomly selected EREG and EGR3 genes to be verified by qRT-PCR. We have chosen these 2 genes with lower FCs, hoping to reproduce our findings by using an independent method. In accordance with microarray data, the expression of both genes was significantly higher in PBMCs from severe than from mild AATD emphysema patients (Figure 2).

Discussion

One of the important issues in developing biomarkers for clinical purposes is the selection of appropriate cells or tissues from which candidates can be identified. Because of the systemic nature of COPD/emphysema³³ and because of unique properties of AAT protein,³⁴ we hypothesized that genome-wide expression profiling of peripheral blood cells may be used to identify signatures of AATD-related emphysema phenotypes. PBMCs are attractive candidates because they are easy to obtain and simple to prepare. Therefore, here we performed the gene expression profiling of PBMCs collected from severe and mild emphysema patients with PiZZ AATD. Our objective was to identify putative differences in mRNA and miRNAs expression that might be associated with disease severity. The microarray analysis of PBMCs isolated from 10 emphysema patients with

Table 3 Gene ontology functional enrichment analysis of the DEGs between peripheral blood mononuclear cells isolated from severe vs mild AATD emphysema patients

GO ID	Term	Odds ratio	P-value	DEGs
Upregulated				
0043408	Regulation of MAPK cascade	4.8	9.03e-07	ATF3, CCR1, HBEGF, DUSP1, EREG, ID1, IL1B, OSM, PARK2, TPD52L1, VEGFA, WNT7A, ARHGEF5, TRIB1, DACT1, AVPI1, CARD9, DUSP16, CCL3L3, CD24
0001525	Angiogenesis	4.68	4.41e-05	ADM, ANPEP, EGR3, EREG, NR4A1, ID1, IL1B, CXCL8, MFGE8, PTGS2, VEGFA, WNT7A, PLXDC1
0001817	Regulation of cytokine production	4.17	7.3e-05	CAMP, CD14, CLC, EREG, HLA-DRB5, IL1B, LTF, PTGS2, NR4A3, SOCS1, CLEC5A, CARD9, ATG9A, CD24
0007254	JNK cascade	6.17	9.2e-05	IL1B, PARK2, TPD52L1, WNT7A, ARHGEF5, TRIB1, DACT1, CARD9
001937	Cyclooxygenase pathway	39.6	0.00012	PTGDS, PTGS2, HPGDS
0019221	Cytokine-mediated signaling pathway	3.55	0.00019	CCR1, EGR1, EREG, CXCL2, HLA-DRB5, IL1B, CXCL8, OSM, PARK2, SOCS1, SOCS3, CCL3L3, CD24
0007249	I-kappaB kinase/NFkB signaling	4.51	0.00068	CD14, IL1B, LTF, PARK2, PTGS2, OLFM4, PLK2, CARD9
Downregulated				
0043949	Regulation of cAMP-mediated signaling	25.7	0.00345	CXCL10, PTGIR
0035490	Regulation of leukotriene production involved in inflammatory response	Inf	0.00382	SERPINE1
0055118	Negative regulation of cardiac muscle contraction	265	0.00762	PDE5A
0007599	Hemostasis	3.75	0.0034	A2M, SERPINE1, PTGIR, TFPI, PDE5A, P2RY12, HIST2H3D
0006950	Response to stress	2.44	0.000726	A2M, HLA-DQB1, HLA-DRB1, IGFBP4, CXCL10, OAS3, SERPINE1, PDK4, PTGIR, XCL2, TFPI, HIST1H2BG, HIST1H2BF, HIST2H2BE, PDE5A, MTSS1, DCLRE1A, PDCD6, KLRG1, CRTAM, P2RY12, TNIP3, ZKSCAN3, DUSP19, HIST2H3D, GTF2H2C_2

Abbreviations: AATD, alpha1-antitrypsin deficiency; cAMP, cyclic adenosine monophosphate; DEGs, differentially expressed genes; GO, gene ontology; JNK, jun amino-terminal kinases; MAPK, mitogen-activated protein kinases; NFkB, nuclear factor kappa B.

PiZZ AATD revealed that cells from patients with severe disease presented 205 differentially expressed mRNAs (114 upregulated and 91 downregulated) and 28 miRNAs (20 upregulated and 8 downregulated) compared to those from patients with mild disease. Moreover, we found an interaction between downregulated hsa-miR-335-5p and 12 upregulated target gene mRNAs. These latter genes are related to the cytokine-mediated signaling pathway, the MAPK/mk2 signaling cascade, the JNK cascade, and angiogenesis. It is important to remark that all our patients were

clinically stable and took their usual respiratory treatment at inclusion in the study.

In the cells of severe patients, we found a higher expression of the *EREG*, *EGR3*, and *TRIB1* genes, which are involved in inflammatory response-related signal transduction pathways including MAPK.³⁵ EGR3 is a zinc finger transcription factor, an immediate-early response protein activated by cellular stressors.³⁶ As a major transcription factor, EGR3 alters the expression of several target genes, including repair enzyme systems, angiogenic factors, cytokines (interleukin-8 [IL-8])

Table 4 KEGG functional enrichment analysis of the DEGs between peripheral blood mononuclear cells isolated from severe vs mild AATD emphysema patients

KEGG ID	Term	Odds ratio	P-value	DEGs
04640	Hematopoietic cell lineage	5.7	0.0014	ANPEP, CD14, HLA-DRB1, HLA-DRB5, IL1B, CD24
00590	Arachidonic acid metabolism	5.9	0.00654	PTGDS, PTGS2, HPGDS, PLA2G2C
04621	NOD-like receptor pathway	5.9	0.00625	CXCL2, IL1B, CXCL8, CARD9
04060	Cytokine-cytokine receptor interaction	2.89	0.00786	CCR1, CXCL2, IL1B, CXCL8, OSM, XCL2, VEGFA, CCL3L3
05310	Asthma	8.29	0.00769	HLA-DQB1, HLA-DRB1, HLA-DRB5
04062	Chemokine signaling pathway	2.69	0.036	CCR1, CXCL2, CXCL8, CXCL10, XCL2, CCL3L3
04620	Toll-like receptor signaling pathway	3.14	0.046	CD14, IL1B, CXCL8, CXCL10

Abbreviations: AATD, alpha1-antitrypsin deficiency; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; NOD, nucleotide-binding oligomerization domain.

Table 5 Dysregulated miRNA in peripheral blood mononuclear cells from severe compared with mild AATD emphysema patients

miRNA ID	logFC	P-value
Upregulated		
hsa-miR-708-5p	2.46	0.009
hsa-miR-4440	2.42	0.007
hsa-miR-193b-3p	2.01	0.036
hsa-miR-3934-5p	1.79	0.030
hsa-miR-7846-3p	1.69	0.003
hsa-miR-873-5p	1.57	0.008
hsa-miR-27a-5p	1.56	0.003
hsa-miR-3064-3p	1.53	0.018
hsa-miR-146b-3p	1.50	0.003
hsa-miR-193b-5p	1.38	0.009
hsa-miR-873-3p	1.37	0.035
hsa-miR-146b-5p	1.31	0.006
hsa-miR-2278	1.31	0.021
hsa-miR-1255a	1.28	0.005
hsa-miR-1225-5p	1.24	0.035
hsa-miR-135a-3p	1.18	0.004
hsa-miR-4723-5p	1.16	0.037
hsa-miR-874-3p	1.11	0.008
hsa-miR-365a-5p	1.11	0.003
hsa-miR-29b-1-5p	1.10	0.003
Downregulated		
hsa-miR-543	-1.00	0.018
hsa-miR-139-3p	-1.00	0.013
hsa-miR-485-3p	-1.02	0.049
hsa-miR-335-5p	-1.01	0.047
hsa-miR-139-5p	-1.01	0.040
hsa-miR-486-3p	-1.05	0.049
hsa-miR-185-3p	-1.12	0.020
hsa-miR-328-3p	-1.20	0.025

Note: Table 5 shows different miRNAs using P-value <0.05 and logFC >±1.

Abbreviations: AATD, alpha1-antitrypsin deficiency; logFC, fold change logarithm; miRNA, microRNA.

and tumor necrosis factor-alpha [TNF α]), apoptotic factors (Fas), cell cycle factors (p21 and p53), metabolic factors, and matrix metalloproteases (MMPs). In parallel to EGR3, we also found a higher expression of cytokines (IL1B, CCL3L3, and CXCL8). Hence, there may be a direct link between EGR3 and cytokine expression, which has already been validated as a target gene in respiratory inflammation and different cancers.³⁷

The activation of members of the MAPK family triggers the activation of transcription factors such as the nuclear factor kappa B (NF κ B) subunit 1.³⁸ Activation of NF κ B eventually results in the expression of a battery of genes that regulate inflammatory, apoptotic, and proliferative responses associated with the pathogenesis of emphysema.³⁹ Previous studies have shown the activation of NF κ B in the bronchial biopsies and inflammatory cells of non-AATD

Table 6 miRNA–target interactions between differentially expressed miRNAs and mRNA in severe vs mild AATD emphysema patients

Downregulated miRNA ID	Upregulated target gene	P-value	logFC
hsa-miR-335-5p	REG	0.018	2.2
	OLFM4	0.003	1.4
	NR4A2	0.004	1.2
	JUP	0.008	1.2
	SLC2A3	0.012	0.97
	NR4A1	0.018	0.97
	NFIL3	0.001	0.84
	ST14	0.002	0.73
	TRIB1	0.015	0.70
	RNF157	0.002	0.63
	EGR3	0.004	0.60
	MAFB	0.018	0.60
Upregulated miRNA ID	Downregulated target gene	P-value	logFC
hsa-miR-874-3p	HIST1H4H	0.001	-1.2
hsa-miR-193b-3p	HIST2H3D	0.002	-0.6

Notes: Integration of validated and predicted interactions between miRNAs and their target genes using multiMiR R package. Comparisons were performed using a P-value <0.05 and FC >±1.5 (logFC >±0.58).

Abbreviations: AATD, alpha1-antitrypsin deficiency; logFC, fold change logarithm; miRNA, microRNA.

COPD patients.⁴⁰ Gene expression profiling studies have revealed that inflammatory cytokine- and chemokine-related genes (ie, fractalkine/CX3CL1) are significantly increased in stable non-AATD-COPD patients, becoming more pronounced during COPD exacerbations.^{21,41}

Transcripts involved in the JNK cascade pathway were also upregulated in severe compared to mild COPD patients. Recent data by Pastore et al⁴² revealed that JNK and c-JUN play a role in SERPINA1 gene transcriptional upregulation in AATD-related liver disease. To date, however, there are no studies investigating the impact of JNK on the pathogenesis of AATD.

Changes in miRNA expression are associated with the development and progression of cancer, and cardiovascular and inflammatory pulmonary diseases.^{13,43} We found 8 miRNAs to be downregulated in PBMCs from severe patients, including hsa-miR-486-3p and hsa-miR-335-5p, which have previously been reported to be related to respiratory diseases. Downregulation of hsa-miR-335-5p involves the activation of pathways related to inflammation and angiogenesis. Therefore, our analysis suggests a potential novel link between decreased miR-335-5p expression and the severity of AATD-related emphysema. The integrative analysis revealed that miR-335-5p interacts with other miRNAs previously described in emphysema. For example, high expression of hsa-miR 146-5p in cells from severe

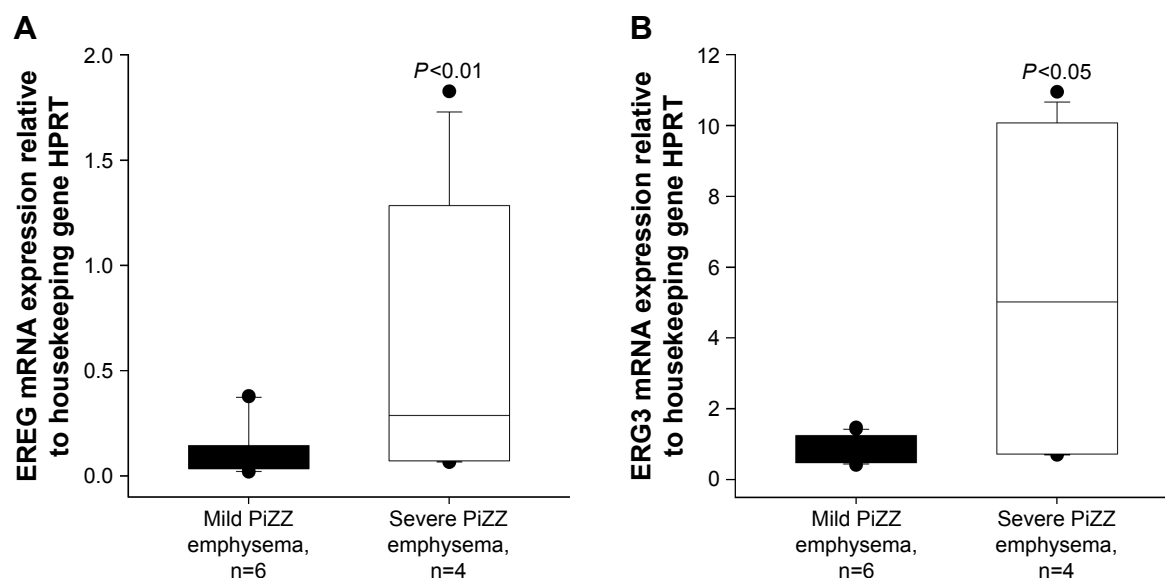


Figure 2 Quantitative reverse transcription polymerase chain reaction.

Note: Validation of EREG (**A**) and EGR3 (**B**) expression compared with housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT).

Abbreviation: mRNA, messenger RNA.

patients supports the findings by Ezzie et al,¹⁸ who stated that miR-146a expression is higher in lung tissue from patients with COPD than in non-COPD smokers. In contrast, Sato et al⁴⁴ reported reduced expression of miR-146a in cultured fibroblasts from COPD subjects. miR-146a was down-regulated in sputum of patients with COPD and smokers compared with never-smokers, and in smokers with COPD in comparison to smokers without COPD.⁴⁵ Overall, recent studies support the hypothesis that miR-146a and miR-146b play a role in the regulation of inflammatory pulmonary disorders.⁴⁶

The results of the present study provide additional support for the role of miRNAs in COPD and improve the understanding of the molecular mechanisms underlying the expression of peripheral inflammatory cells in patients with severe and mild AATD-related emphysema.

Limitations

We found significant differences in the expression patterns of genes between severe and mild emphysema patients with PiZZ AATD. However, the results from this small pilot study must be complemented by the inclusion of a control group of patients with non-AATD-related COPD. A control group would help to clarify whether the activation of pathways related to inflammation, immune response, and angiogenesis are the cause or the effect of more progressed emphysema. Golpon et al³⁵ found some degree of shared gene profiles between “usual” non-AATD-related emphysema

and AATD-related emphysema. These authors observed statistically significant differences in the modulation of genes associated with protein and energy metabolism and immune function, which allow differentiation between these 2 emphysema types at the lung tissue level. We are aware that differences in the genetic signatures of PBMCs isolated from severe vs mild PiZZ AATD patients need to be validated in a larger study population.

In our analysis, 12 samples were initially included, but 2 current smokers were excluded from the patient group with severe AATD emphysema. According to the quality control analysis, the gene expression profile of these individuals was markedly different compared with the other samples. Cigarette smoke is a complex mixture of >4,000 chemical compounds that can induce a variety of biological responses, including oxidative stress, apoptosis, and inflammation, among others.⁴⁷ In general, most studies report a global downregulation and upregulation of miRNA and mRNA abundance in response to cigarette smoking.^{18,48} We plan to perform a separate study to analyze the gene expression profiles in PBMCs isolated from smokers with severe and mild COPD.

The cellular gene expression pattern may be influenced by multiple factors, such as patient age, gender, genetic background, and treatment. Replacement therapy could have influenced gene expression. Some studies have suggested that replacement therapy may reduce airway inflammation.^{49–51} However, Olfert et al⁵² found that replacement therapy

reduces the concentration of TNF- α , but has no effect on IL-6, IL1B, or C-reactive protein. In our study, 4 severe and 2 mild emphysema AATD patients received augmentation therapy every 15 days. To minimize the potential effect of replacement therapy, blood samples were taken just before the patients received the next infusion. However, it would be important to include PBMCs from patients who have never received augmentation therapy.

Conclusion

The findings of this analysis provide novel information about the different gene profile of PBMCs isolated from patients with severe and mild emphysema with PiZZ AATD. We specifically identified downregulated miRNA (hsa-miR-335) in severe AATD patients. The hsa-miR-335-5p regulates important transcription factors involved in the activation of pathways directly related to inflammation and angiogenesis and could play an important role in the pathogenesis of this rare condition. Nevertheless, further analyses are needed to validate these results.

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Disclosure

The authors report no conflicts of interest in this work.

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