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Cytokine Expression and Promoter Methylation Signatures in COPD

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung condition and a leading cause of morbidity and mortality worldwide. Despite well-established links to smoking, emerging evidence highlights the involvement of complex immune and epigenetic mechanisms in its pathogenesis. This study aimed to investigate the expression, secretion, and promoter methylation status of key pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) of patients with mild and severe COPD, compared with healthy individuals.

PBMCs were isolated from 90 participants divided into three groups: severe COPD, mild COPD, and healthy controls. Quantitative polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and methylation-specific PCR were employed to evaluate gene expression, protein secretion, and promoter methylation of inflammatory cytokines.

Patients with COPD exhibited significant upregulation of pro-inflammatory cytokines (*Interleukin1β* (*IL-1β*), *IL-6, IL-18, IFN-γ*, and *TNF-α*) at both transcript and protein levels, with more pronounced alterations in the severe group. Conversely, anti-inflammatory mediators (*IL-10* and *TGF-β*) were significantly downregulated. Promoter methylation analysis revealed hypomethylation in pro-inflammatory cytokine genes and hypermethylation in anti-inflammatory ones, correlating with disease severity.

The findings demonstrate that COPD progression is associated with a shift toward a hyper-inflammatory, hypo-regulatory immune phenotype sustained by epigenetic modifications. These results support the potential for integrating cytokine-methylation signatures into clinical staging and for targeting epigenetic and immune pathways in future therapeutic strategies.

Keywords: Chronic obstructive pulmonary disease; Cytokines; Epigenetics; Inflammation; Methylation

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INTRODUCTION

Around 6% of mortality from all causes is attributable to chronic obstructive pulmonary disease (COPD), making it the third leading cause of death globally.[1] Because of its chronic respiratory symptoms and permanent airflow restriction, COPD is a serious worldwide health issue that significantly affects society.[2,3] The etiology of COPD is now known to be complicated despite the fact that it was originally thought to be a disorder linked to cigarette smoking.[4] Cigarette smoke and other harmful chemicals and particles have long been associated with COPD.[5] Studies have shown that air pollution, infections, genetic predispositions, occupational hazards, and early life disadvantages can all affect its development.[6,7]

Chronic inflammation lingers over time and can harm healthy tissues, in contrast to acute inflammation, which is a temporary, beneficial response to an infection, oxidative stress or injury to cells.[8,9] The development and progression of many diseases, including cancer, infertility, autoimmunity, and infections, are significantly influenced by chronic inflammation, a protracted, dysregulated immune response.[10–13] People with COPD may experience an inflammatory cascade in their airways and lung tissues as a result of gas exposure, cigarette smoking, or other hazardous particles. Because of this, powerful inflammatory cytokines are produced, leading to tissue death and persistent inflammation.[14] It has been demonstrated that neutrophils and macrophages both have a role in the development of COPD by entering the respiratory tract, releasing a large number of inflammatory chemicals, and upregulating chemokines such as monocyte chemotactic protein-1 (MCP-1) and CCL-2.[15] These elements can initiate the production of oxygen radicals and elastase. This will make lung tissue deterioration worse by increasing pulmonary vascular system permeability.[16,17] Moreover, interleukin-1β (IL-1β) and TNF-α will start an inflammatory chain reaction that makes COPD symptoms worse.[18] Although inflammation is a well-established feature of COPD, most previous studies have examined cytokine expression, protein levels, or epigenetic alterations in isolation. Moreover, limited data are available on the integrated assessment of cytokine transcription, promoter methylation, and secretion across different stages of COPD severity, particularly in peripheral blood immune cells. Therefore, the present study addresses this gap by providing a combined immune–epigenetic analysis of mild and severe COPD using peripheral blood mononuclear cells (PBMCs). As a result, we hope to learn more about the gene expression, methylation, and cytokine secretion profiles in PBMCs from COPD patients with mild and severe symptoms, and then compare these profiles.

MATERIALS AND METHODS

Study Design

This non-interventional, applied basic study was conducted in line with the priorities of prevention, epidemiology, treatment, and rehabilitation of inflammatory airway diseases, including asthma and COPD. The study population consisted of individuals with mild and severe COPD. Additionally, a healthy control group was included for comparison purposes. A total of 90 participants were enrolled in the study, including 30 patients with severe COPD, 30 with mild symptoms, and 30 healthy individuals as controls. Blood samples (8 mL) containing an anticoagulant were collected after obtaining written informed consent. Participants were randomly selected using the RandList software. All blood samples were transferred to the laboratory within 2 h for isolation of PBMCs. Smoking history was recorded for all participants at enrollment. Patients in the mild and severe COPD groups had comparable smoking exposure histories (Smoking by pack- year), while healthy controls had no history of chronic lung disease. Individuals with acute infections, autoimmune disorders, malignancies, or recent systemic corticosteroid use were excluded to minimize potential confounding effects on immune and cytokine profiles. Demographic variables such as age and sex were comparable across study groups. These criteria were applied to reduce the influence of major risk factors on cytokine expression and methylation outcomes. Nevertheless, residual confounding effects related to smoking intensity and medication use cannot be entirely excluded.

Cytokine Quantification in PBMCs

Cytokine levels were measured in PBMCs using an ELISA kit (Abcam, UK) according to the manufacturer's protocol. Optical density was measured at 450 nm using a microplate reader.

RNA Extraction

RNA was extracted using the miRCURY RNA Isolation Kit (EXIQON, DENMARK) according to the manufacturer's instructions. RNA purity and concentration were assessed using a NanoDrop spectrophotometer. The A260/A280 ratio was used to evaluate protein contamination.

cDNA Synthesis

Using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA), 1 µg of RNA was reverse transcribed using random hexamer primers. Thermal conditions included incubation at 65°C and subsequent steps per the kit protocol.

qPCR Procedure

Gene expression levels were quantified via real-time PCR using gene-specific primers. *GAPDH* was used as an internal housekeeping control. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Table 1 lists the primer sequences in detail (supplementary).

Promoter Methylation Analysis

DNA methylation of inflammatory cytokine gene promoters was assessed using bisulfite conversion followed by MSP-PCR. Bisulfite reagent was added according to the kit protocol. Unmethylated cytosines were converted to uracil; methylated cytosines remained unchanged. MSP-PCR was used to amplify the target region. Methylation-specific primers were designed using Methyl Primer Express v1.0 (ABI), and SssI enzyme was used to generate methylated DNA as a positive control.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism v8.00. Pre-specified pairwise comparisons between study groups (Control vs Mild COPD, Control vs Severe COPD, and Mild vs Severe COPD) were conducted using Student's *t* test. A p value<0.05 was considered statistically significant.

RESULTS

Gene Expression of Inflammatory and Anti-inflammatory Cytokines

The expression levels of key inflammatory and anti-inflammatory cytokine genes involved in the pathogenesis of COPD were analyzed in PBMCs from patients with mild and severe COPD and compared with those of healthy controls (Figure 1). The mRNA expression of pro-inflammatory cytokines, including *IL1B*, *IL18*, *IFNγ*, and *TNFα*, was significantly increased in patients with severe COPD compared with healthy controls (p<0.05). In patients with mild COPD, a similar upward trend was observed for these cytokines; however, the differences did not reach statistical significance for *IFNγ*, *TNFα*, and *IL18*. In contrast, the expression of anti-inflammatory cytokines *IL10* and *TGFβ* was significantly reduced in severe COPD patients, with a less pronounced and mostly non-significant decrease in the mild COPD group.

Promoter Methylation Status of Cytokine Genes

Promoter methylation analysis of cytokine genes revealed significant epigenetic alterations in COPD patients (Figure 2). The promoter regions of the pro-inflammatory cytokine genes (*IL1β*, *IL18*, *IFNγ*, *TNFα*) exhibited significantly reduced methylation in COPD patients compared to healthy controls (p<0.05), particularly in those with severe disease. In contrast, the promoter methylation of anti-inflammatory genes (*IL10* and *TGFβ*) was significantly elevated in COPD patients, again with more pronounced changes observed in the severe COPD group (p<0.05). Statistical analysis further indicated that the methylation differences between mild and severe COPD patients were significant.

Cytokine Secretion Levels–pro-inflammatory Cytokines

As shown in Figure 3, secretion levels of key pro-inflammatory cytokines were assessed in serum samples. IL-1β secretion was significantly increased in both mild (p=0.03) and severe COPD (p=0.0001 vs controls; p=0.005 vs mild COPD). The mean ± SD for severe COPD was 214.86 ± 110.3, for mild COPD 140.81 ± 87.44, and for the control group 94.27 ± 74.29 (Figure 3a). IFN-γ levels were elevated in mild COPD patients compared to healthy controls, though this increase did not reach statistical significance. However, IFN-γ levels were significantly higher in severe COPD patients compared to both controls (p=0.0003) and mild COPD patients (p=0.037) (Figure 3b). IL-6 levels were significantly elevated in mild (p=0.039) and severe COPD patients (p=0.0002) (Figure 3c). IL-18 levels were increased in both mild and severe COPD groups; however, only the increase in severe COPD patients reached statistical significance (p=0.0006), while the

elevation in mild COPD was not statistically significant (Figure 3d). Finally, TNF-α secretion was also upregulated in both COPD groups, with statistically significant increases observed in the severe group compared to controls (p=0.0003) (Figure 3e).

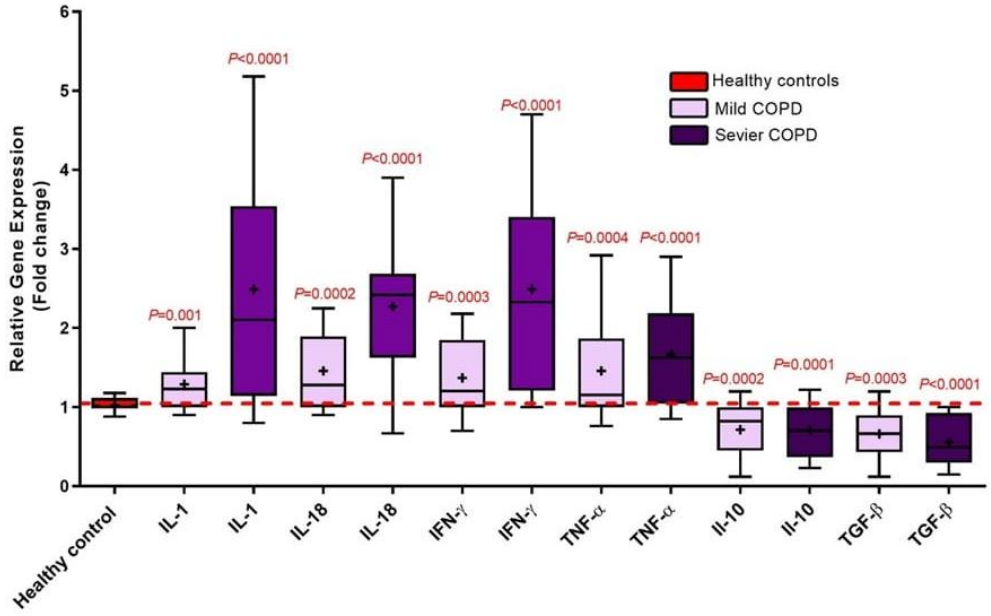

Figure 1. Relative mRNA expression levels of pro- and anti-inflammatory cytokines in PBMCs from mild and severe COPD patients compared with healthy controls. Data are presented as mean ± SD. Gene expression was normalized to *GAPDH* and calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical comparisons were performed using Student's *t* test. $p<0.05$ was considered statistically significant. Pairwise comparisons between groups were performed using Student's *t* test. ns indicates non-significant differences.

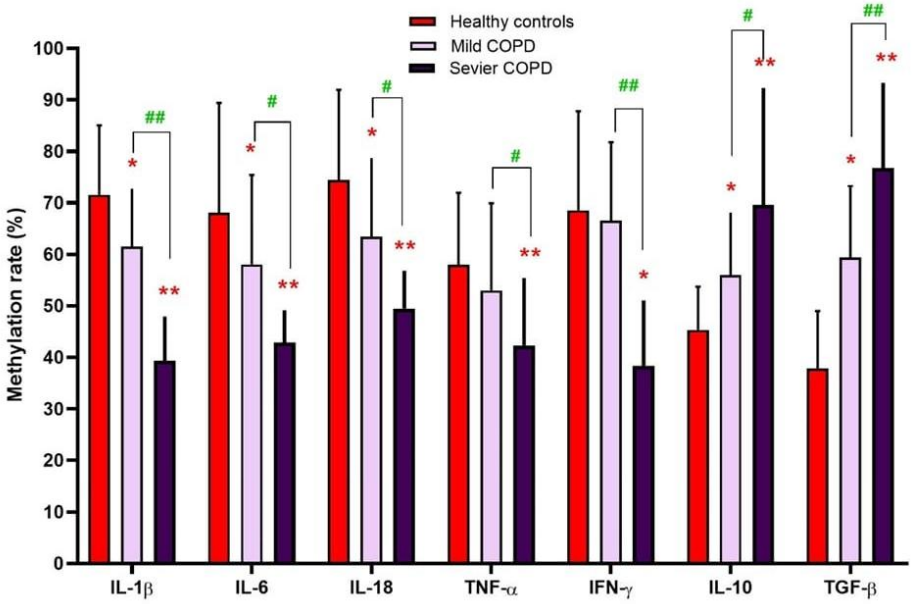

Figure 2. Promoter methylation status of inflammatory and anti-inflammatory cytokine genes in PBMCs from mild and severe COPD patients and healthy controls. Data are shown as mean ± SD. Differences between groups were analyzed using Student's *t* test, with $p<0.05$ indicating statistical significance.

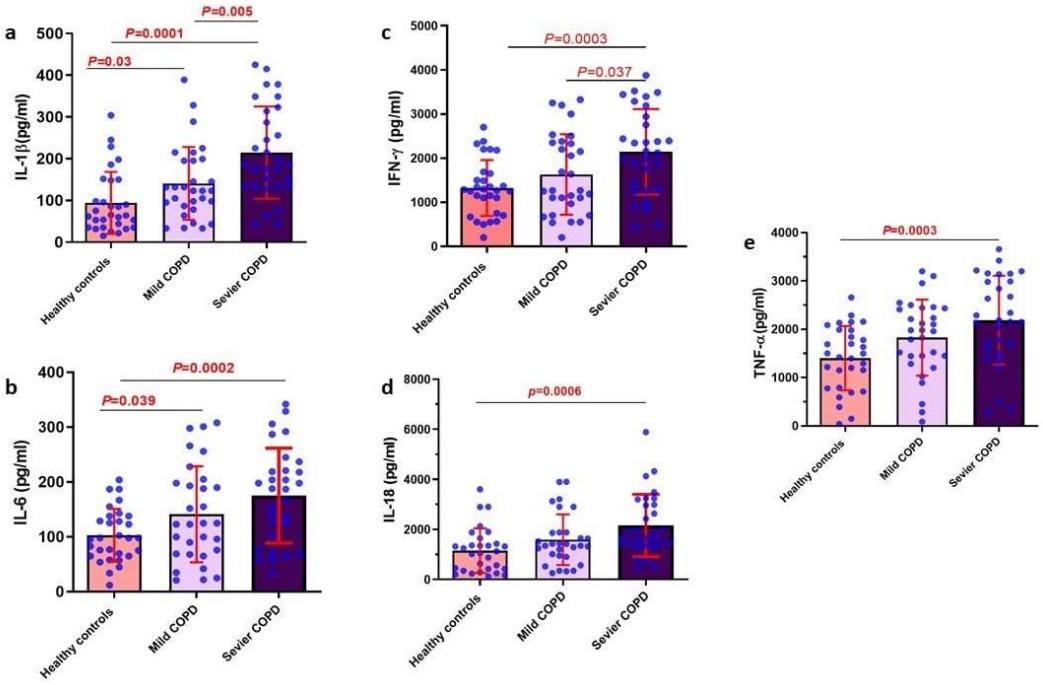

Figure 3. Serum levels of pro-inflammatory cytokines in mild and severe COPD patients compared with healthy controls: (A) IL-1β, (B) IFN-γ, (C) IL-6, (D) IL-18, and (E) TNF-α. Data are expressed as mean ± SD. Statistical analysis was performed using Student's *t* test. $p<0.05$ was considered statistically significant.

Cytokine Secretion Levels–anti-inflammatory Cytokines

As illustrated in Figure 4, anti-inflammatory cytokine secretion levels were reduced in COPD patients. IL-10 levels showed a non-significant decrease in mild COPD patients, whereas a significant reduction was observed in severe COPD patients compared to both controls (p=0.0021) and mild COPD patients. Similarly, TGF-β levels were decreased in mild COPD patients (non-significant), with a statistically significant reduction in severe COPD patients relative to controls (p=0.0002). The mean and SD are shown in Table 2.

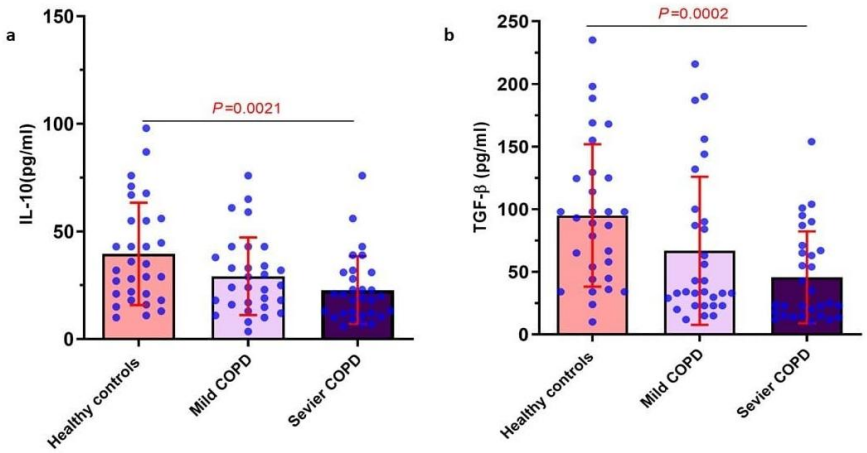

Figure 4. Serum secretion levels of anti-inflammatory cytokines IL-10 and TGF-β in mild and severe COPD patients compared with healthy controls. Data are presented as mean ± SD. Statistical comparisons were conducted using Student's *t* test, with $p<0.05$ indicating statistical significance.

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Table 2. Cytokine gene expression levels (Mean ± SD) in study groups

Cytokine	Control (n=30)	Mild COPD (n=30)	Severe COPD (n=30)	*p* (Control vs Mild)	*p* (Control vs Severe)	*p* (Mild vs Severe)
IFN-γ	1325±633	1630±911.5	2147±968.4	ns	0.0003	0.037
IL-1β	94.27±74.29	140.81±87.44	214.86±110.3	0.03	0.0001	0.005
IL-6	102.99±48.06	141.43±87.54	175.49±86.5	0.039	0.0002	ns
TNF-α	1402.27±665.1	1828.9±785.6	2189.93±917	ns	0.0003	ns
IL-18	1151.93±889	1590±1014	2160±1242	ns	0.0006	ns
IL-10	39.58±23.8	29.18±18.05	22.8±15.69	ns	0.0021	0.04
TGF-β	95.02±56.89	66.79±59.14	45.65±36.73	ns	0.0002	ns

Abbreviations: IFN-γ: interferon-γ; IL: interleukin; ns: non-significant; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor-β.

DISCUSSION

In the present study, we investigated the expression and secretion of key inflammatory and anti-inflammatory cytokines in patients with mild and severe COPD. Our findings demonstrated that IL-1β, IL-18, IFN-γ, and TNF-α were significantly upregulated in patients with severe COPD compared with healthy controls. In contrast, patients with mild COPD exhibited a similar upward trend in pro-inflammatory cytokine expression, although these changes were not consistently statistically significant. In parallel, anti-inflammatory cytokines, including IL-10 and TGF-β, were reduced in COPD patients, with the most pronounced and significant decreases observed in the severe disease group.

Dhillon et al observed hypertensive rats that were left unattended for as long as 12 weeks in environments with either filtered air (FA) or tobacco smoke (TS) at a concentration of 90 mg/m^3. In comparison to FA, these rats' bronchoalveolar lavage had significantly more macrophages, neutrophils, and lymphocytes during every week that was studied. Tissue attenuation and central airway resistance both increased significantly after TS exposure, suggesting physiological airway blockage. Although tissue attenuation remained markedly higher for up to 12 weeks following TS exposure, it peaked at 4 weeks, and then began to drop. There were significant positive associations found between the level of tissue attenuation and the proinflammatory cytokines IL-6, IL-1β, TNF-α, and IFN-γ. Increased distal airway mucinous metaplasia and central airway squamous epithelial metaplasia were linked to these alterations. In this COPD mouse model, these results show that TS exposure causes an aberrant inflammatory response and progressive airway blockage.[19]

High-mobility group 1 (HMGB1) has been identified as a key inflammatory factor that may be responsible for the progression of COPD. The pathophysiology of COPD includes an intricate inflammatory process during which macrophages perform important regulatory activities. According to Mu et al's research, the cigarette smoke-induced heightened proinflammatory M1 macrophage polarization is linked to elevated HMGB1 in the setting of COPD. This polarization is linked to apoptotic induction and inhibition of cell growth, indicating a function for HMGB1 in the disease's inflammatory process. The research also linked HMGB1 to the chemokine signaling in macrophages and stimulation of the NF-κB signaling pathway, which most likely heightens the inflammatory reaction that is typical of COPD. These results highlight HMGB1's pivotal role in the pathophysiology of COPD and position it as a key target for therapeutic intervention meant to alter inflammation and macrophage polarization.[20]

The function of mitochondria and pathological conditions such as inflammation, immunological reactions, and necrosis are significantly influenced by mitochondrial transcription factor A (mtTFA).[27] Patients with COPD had considerably lower levels of mtTFA

expression in their skeletal muscle.[28] Using an in vitro experimental paradigm that mimics COPD exacerbations, Kaur and Batra performed extensive research to understand the role of CpG promoter methylation of NF-κB and STAT3-driven pathway genes. The transcription factors NF-κB and STAT3 play a crucial role in controlling inflammatory responses when exposed to cigarette smoke. The researchers discovered that cytokines and chemokines (IL-6, IL-8, CCL5, and MCP-1) were secreted at significantly higher levels in the COPD exacerbation group compared to the control group. In their model of COPD exacerbation, they discovered a connection between the hypomethylation of genes in the NF-κB-mediated pathway and their stimulation.[29] Furthermore, B cells' capacity to produce IL-10 was reduced after being exposed to cigarette smoke extract (CSE) in vitro. Finally, memory B cells treated with CSE exhibited lower *IRF4* and *HIF1A* mRNA levels. Smokers and COPD patients may have a different onset and progression of the disease due to a decrease in the number and effectiveness of regulatory B cells (B-regs).[30]

Certain genetic variations can modify cytokine expression in COPD, and changes in the quantity or effectiveness of cytokines can affect the susceptibility to the disease.[31] Macrophage activity can regulate the initiation and resolution of various inflammatory processes.[32] M1 macrophages are activated and differentiated by a series of signaling cascades that are triggered when $CD8^+$, T_H1, and B cells release IFN-γ to the IFN-γ receptor.[33] These cells then create a range of cytokines, such as TNF-α, IL-1β, and IL-6, according to the tissue site. On the other hand, M2 macrophages help remodel airways by mending and altering damaged tissues. They are activated by many cell variables such as IL-4, IL-10, and IL-13.[34,35] Patients with COPD had higher serum and nasal levels of IL-1β, according to Obling et al.[36] The Zhang study suggests that an imbalance in IL-1, IL-2, IL-8, and TNF-α may increase the incidence of psychological depression in older COPD patients.[37] Furthermore, they discovered that TNF-α is a biomarker for COPD and that patients with the condition have greater levels of TNF-α and IL-6.[38] In a similar vein, this study found that healthy people had higher levels of anti-inflammatory cytokines than people with moderate COPD, and that severe COPD showed a larger decline than mild COPD. Compared to controls, COPD patients with depression had higher levels of TNF-α and IL-1β, according to a study by Małujło-Balcerska et al. Recurrent depressive disorder (RDD) patients with COPD had substantially lower levels of diiodothyronine diiodinase (DIOs) compared to controls.[39] The same approach was applied with 213 COPD patients and those at risk for the disease, along with 100 healthy controls who were matched for age and sex, to examine the relationship between polymorphisms of *TGFB1* codon 10, *TGFB1* codon 25, *IL10*, *TNFA,* and *IFNG*. The researchers demonstrated that COPD risk was enhanced by *TGFB1* and *IL10* polymorphisms, while no correlation was observed with *TNFA* (G-308A) and *IFNG* (+847 T/A) polymorphisms.[40] Thus, cytokines, the principal regulators of immune responses, are pivotal in COPD pathogenesis.

Our findings suggest that combined cytokine–methylation signatures in circulating immune cells could complement current clinical staging. These signatures may help classify patients according to inflammatory endotypes, track disease progression, and identify individuals who are more likely to benefit from anti-inflammatory or epigenetic therapies. The observed reduction in IL-10 and TGF-β, together with enhanced NF-κB/STAT3–linked inflammatory activity, provides a rationale for targeted therapeutic strategies. These approaches may aim to restore regulatory pathways, such as enhancing IL-10–producing B cells, or to selectively inhibit upstream inflammatory drivers including HMGB1, DNMT activity, and key NF-κB/STAT3 signaling nodes. Because epigenetic modifications are potentially reversible, our methylation findings provide a rationale for evaluating DNA methylation–modifying therapies in COPD. Such interventions could be tested alone or in combination with conventional treatments, particularly in well-phenotyped patient subgroups.

From a clinical perspective, the combined cytokine–methylation signatures identified in this study may have important implications for patient stratification and disease monitoring in COPD. The progressive shift toward a hyper-inflammatory and epigenetically deregulated immune profile with increasing disease severity suggests that these molecular patterns could serve as accessible biomarkers for distinguishing mild from severe COPD. Moreover, profiling cytokine expression and promoter methylation in peripheral blood immune cells may help identify patients who are more likely to benefit from targeted anti-inflammatory or epigenetic-based therapies. Such approaches could

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complement existing clinical and functional assessments, contributing to more personalized disease management strategies in COPD. Taken together, our results extend previous findings by demonstrating that immune dysregulation in COPD is not driven solely by activation of inflammatory pathways, but also by epigenetic silencing of counter-regulatory mechanisms. This integrated immune–epigenetic imbalance may help explain the persistence and progression of inflammation in advanced COPD.

This study has several limitations. First, the relatively modest sample size may limit the generalizability of the findings and warrants validation in larger, multi-center cohorts. Second, the cross-sectional design precludes causal inference regarding the relationship between cytokine expression, promoter methylation, and disease progression. Third, analyses were restricted to PBMCs, which may not fully reflect local inflammatory and epigenetic changes within lung tissue. Additionally, potential confounding factors such as medication use, smoking history intensity, and comorbidities were not stratified in detail and may have influenced cytokine profiles. Future longitudinal studies integrating airway-derived samples and functional epigenetic assays are needed to further clarify the mechanistic role of cytokine-methylation interactions in COPD progression. In addition, multiple pairwise comparisons were performed without formal adjustment for multiple testing, which may increase the risk of a type I error and should be considered when interpreting the results.

In this non-interventional investigation, we demonstrate that a systemic immunological disequilibrium associated with COPD worsens as the disease progresses. Patients' PBMCs, particularly those with severe COPD, demonstrated (i) a consistent upregulation of pro-inflammatory cytokine transcripts and proteins (IL-1β, IL-18, IFN-γ, TNF-α, and IL-6), (ii) a concurrent decrease in anti-inflammatory mediators (IL-10, TGF-β), and (iii) a reciprocal epigenetic pattern where pro-inflammatory cytokine promoters were hypomethylated and anti-inflammatory ones were hypermethylated. Collectively, our results suggest that as COPD worsens, epigenetic reprogramming perpetuates and potentially maintains in a pro-inflammatory phenotype.

In conclusion, the severity of COPD is linked to a transition toward a hyper-inflammatory, hypo-regulatory cytokine milieu, which is reflected in and probably sustained by directional changes in promoter methylation. More accurate endotyping, prognostication, and customization of anti-inflammatory and epigenetic treatments for COPD may be made possible by integrating transcriptomic, epigenetic, and secretory cytokine profiles from accessible immune cells.

STATEMENT OF ETHICS

Ethical issues (including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been fully addressed by the authors. The research project was approved with the ethical code IR.TBZMED.REC.1400.264 on 06/21/2021.

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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