UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF MEDICINE

SECTION OF GENERAL PATHOLOGY

PhD SCHOOL OF LIFE AND HEALTH SCIENCES

DOCTORAL PROGRAM IN

INFLAMMATION, IMMUNITY AND CANCER

CYCLE XXXIII/2017

HIGH-THROUGHPUT CHARACTERIZATION OF CIRCULATING LEUKOCYTE ALTERATIONS IN MALE AND FEMALE COPD PATIENTS

S.S.D. MED/04

Coordinator: Prof.ssa GABRIELA CONSTANTIN

Signature _____

Tutor: Prof.ssa FLAVIA BAZZONI

Signature _____

Doctoral Student: NATALIA TROIANOVA

Signature _____

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease that causes obstructed airflow from the lungs. Nowadays, COPD is the fourth leading cause of death in the world and its morbidity and mortality are constantly increasing. COPD is characterized by emphysema and chronic bronchitis, usually present simultaneously, with a wide range of severity. COPD was considered as a lung-restricted disease for many years, but from 2000 increasing new evidence, including a number of comorbid conditions accompanying this pathology and the presence of systemic inflammation, indicate that COPD should be considered a systemic disease. Systemic inflammation is the most common systemic manifestation of COPD, and it is associated with a progressive worsening of symptoms and comorbidities. In addition to soluble inflammatory markers, such as TNFα, several research groups focused their attention on peripheral blood leukocytes to assess the changes occurring in these cells in COPD and their relation to the inflammatory pattern in the lungs. However, the data about peripheral leukocyte composition and functional changes in COPD are quite controversial.

COPD was traditionally considered as a disease affecting principally males, but rising evidence show that it probably equally concerns male and female populations. However, there are differences in symptoms severity, disease progression and treatment benefits between male and female COPD. Yet, despite the clear evidence of sexual dimorphism in COPD manifestation and progression, there are, to our knowledge, no publications about the difference in the immune state between male and female COPD.

Given the lack of data assessing gender-related differences in COPD systemic immune system manifestations and the discordance in the data about circulating leukocyte alterations in COPD, we aimed at thoroughly characterize circulating leukocyte alterations in COPD as compared to age-matched controls.

Additionally, results have been stratified according to gender in order to identify male- and/or female-specific COPD immunophenotype.

Blood samples from 50 COPD patients and 63 age and sex-matched controls have been collected and analyzed. Flow cytometry analysis revealed the existence of well-defined gender-related pattern of circulating leukocytes in COPD. Specifically, while neutrophilia and increased neutrophil to lymphocyte ratio are hallmarks of COPD in male, on the contrary, female COPD are characterized by a generalized leukopenia. Remarkably, we demonstrated that T cell exhaustion is more prominent in female COPD, nevertheless it increases with disease severity in both male and female groups. Moreover, several observations suggest that that T cells decrease in both male and female COPD may be related to PD1-dependent apoptosis. Additionally, we showed that absolute numbers and/or frequencies of several leukocyte populations correlate with different clinical parameters of COPD disease severity.

Given the relevance of the increase in neutrophil absolute count and frequency in male COPD, we subsequently characterized circulating neutrophil phenotype, function and transcriptome of male COPD and related controls. Despite the fact that phenotype and function of neutrophils purified from COPD subjects were comparable to those from controls, differential gene expression analysis highlighted a number of differentially expressed genes. On the basis of the correlation of the differentially expressed genes with the disease severity, we identified a neutrophil-specific COPD signature. Finally, this proposed COPD signature was successfully validated on a larger cohort of male COPD subjects.

Contents

ABBREVIATIONS

ANOVA – analysis of variance

APC – allophycocyanin

BAFF – B cell-activating factor belonging to the TNF family

BAL – bronchoalveolar lavage

BMI – body mass index

BrDU – bromodeoxyuridine

CCR – CC-chemokine receptor

COPD – chronic obstructive pulmonary disease

CRP – C-reactive protein

CT – computerized tomography

CTL – cytotoxic T-lymphocytes

CVD – cardiovascular diseases

CXCR – CXC-chemokine receptor

DC – dendritic cells

DLCO – diffusing capacity of the lungs for carbon monoxide

ECLIPSE – evaluation of COPD longitudinally to identify predictive surrogate endpoints

EDTA – ethylenediamine tetra-acetic acid

ERV – expiratory reserve volume

FACS – fluorescense-activated cell sorting method

FBS – fetal bovine serum

FEV1 – forced expiratory volume in 1 second

FITC – fluorescein isothiocyanate

fMLP – formyl-methionyl-leucyl phenylalanine

FPKM – fragments per kilobase of transcript per million mapped reads

FSC – forward scatter

FVC – forced vital capacity

GO – gene ontology

GOLD – Global Initiative for Obstructive Lung Disease COPD grade units

GTPase – guanosine triphosphatase

HBSS – Hank's balanced salt solution

HLA-DR – major histocompatibility complex class II

IC – inspiratory capacity

IL – interleukin

KCO – carbon monoxide transfer coefficient

LPS – lipopolysaccharide

LRT – likelihood ratio test

MDSC – myeloid-derived suppressor cells

MMEF – mean mid-expiratory flow

MMP – matrix metalloproteinase

MPO – myeloperoxidase

MRC – Medical Research Council scale

mRNA – messenger RNA

NE – neutrophil elastase

NK – natural killer cells

NKT – natural killer T cells

NLR – neutrophil-to-lymphocyte ratio

$O_2^{\cdot -}$ – superoxide anion

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffer salt

PC – principal component

PCA – principal component analysis

PD1– programmed cell death 1

PDL1 – programmed cell death ligand 1

PE – phycoerythrin

PerCP – peridinin chlorophyll protein

PMA – phorbol myristate acetate

PMN – polymorphonuclear neutrophils

RNA – ribonucleic acid

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute medium

RV – residual volume

SD – standard deviation

TCM – central memory T cells

TEM – effector memory T cells

TEMRA – effector memory CD45RA+ T cells

Th – T helper cells

TLC – total lung capacity

TNFα – tumor necrosis factor alpha

Treg – T regulatory cells

UMI – Unique Molecular Indices

WBC – white blood cells

BACKGROUND

COPD description

Chronic obstructive pulmonary disease, or COPD, refers to a group of diseases characterized by progressive airflow obstruction and shortness of breath. At the lung level, COPD is manifested in chronic bronchitis, bronchiolitis (small airway disease), and emphysema. According to the recent report of the World Health Organization, COPD is the fourth leading cause of death in the world. The most common reason for COPD development is smoking. However, the results of a 25-years follow-up study revealed that only about 25% of continuous smokers develop COPD in 25 years (Løkke et al. 2006) and the reason why some smokers develop COPD and the others do not is still unknown. Air pollution and exposure to biomass smoke, such as using biomass fuel for heating and cooking, are the other important risk factors, significantly associated with COPD development. There is also a genetic component in COPD. The most known genetic factor of COPD development is an α1-antitrypsin deficiency – a rare condition that affects about 1:2000 to 1:5000 individuals and frequently leads to progressive lung emphysema (Stoller and Aboussouan 2012). There are also several other genetic mutations that were associated with an increased risk of COPD development (Hobbs and Hersh 2014).

The disease pathophysiology includes inflammation in small and large airways, usually provoked by cigarette smoke particles or other airborne irritators, that leads to airway walls thickening, mucus hypersecretion and mucous metaplasia – the hallmarks of chronic bronchitis. Massive immune response and other factors, including uncontrolled trypsin or other enzymes activity due to genetic defects in protease balance (Brode, Ling, and Chapman 2012), cause the destruction of alveolar walls that leads to the development of emphysema. Most people with COPD have both emphysema and chronic bronchitis, but the prevalence and severity of each condition vary from person to person (Bhat et al. 2015).

The degree of COPD severity can be estimated by different means:

I. COPD severity is evaluated on the basis of self-assessed symptoms and some generic physical activity measurements, such as a 6-minute walking test. Self-assessed COPD symptoms include cough intensity (usually on a scale from 0 to 10), sputum production (usually reported in tablespoons/day), and dyspnea grade (usually reported as MRC score from 0 to 4). Questionnaires and self-assessed symptom severity scales are very variable, as the same person can give different answers to the question in slightly different conditions.

II. The most precise COPD severity assessment method is a computerized tomography (CT) scan, that can provide objective information about emphysema grade, chronic bronchitis severity (airway walls thickness) and, with some limitations, bronchiolitis (Segal and Martinez 2018). However, a CT scan is not widely used for COPD patients as it is an expensive procedure.

III. The easiest and most used method to assess COPD severity is spirometry test, which gives information about the patient's breathing capacity. The spirometry test includes static and dynamic lung volumes measurements. Static lung volumes refer to the lung size and elasticity and include inspiratory capacity (IC) – the amount of air that can be inhaled after the end of a normal expiration, total lung capacity (TLC) – the volume in the lungs at maximal inflation, and residual volume (RV) – the volume of air remaining in the lungs after a maximal exhalation (Figure 1).

The main reason for the alteration of the static lung volumes in COPD is lung hyperinflation that occurs in most COPD patients. Both static and dynamic lung hyperinflation may take place. Static hyperinflation is a feature of severe emphysematous patients and is caused by lung elasticity loss. Dynamic lung hyperinflation is very common and is attributed to air trapping, which is caused by too slow exhalation due to airway obstruction. Unlike static hyperinflation,

which can be easily estimated by IC/TLC or RV/TLC alterations, dynamic hyperinflation requires specific additional tests (Shin et al. 2015). Although dynamic hyperinflation has been considered a major contributor to reduced quality of life and poor exercise capacity in COPD (Krieger 2009), static hyperinflation, as expressed by IC/TLC or RV/TLC, was reported to be an independent predictor of mortality in COPD (Casanova et al. 2005; Budweiser et al. 2014; Shin et al. 2015).

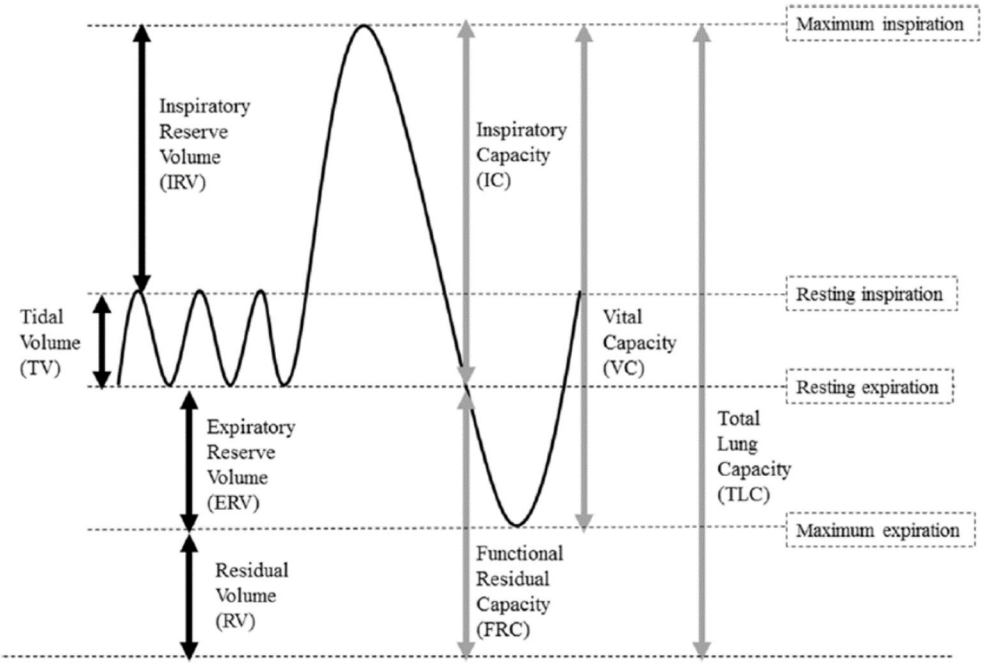

Figure 1. Static lung volumes (Lutfi 2017)

Alterations of dynamic lung volumes (Figure 2) is a direct consequence of airway obstruction, as narrowed bronchial lumen limits the speed of exhalation. The most important spirometric parameter for COPD severity assessment is the forced expiratory volume in 1 second (FEV1) – the maximal volume of air that can be forcefully exhaled in 1 second. This parameter varies from person to person and depends on sex, age height, weight, and race. Therefore, it is usually substituted by normalized value, expressed as a percent of predicted values for the subject of the same age, sex, and body composition (FEV1%). FEV1% continuously declines with COPD progression as airway

obstruction raises. Another important severity indicator is FEV1/FVC – a ratio between FEV1 and forced vital capacity (total volume of air that can be possibly forcefully exhaled). FVC also declines with COPD progression, but not as much as FEV1, which leads to a decrease of FEV1/FVC. This phenomenon helps to distinguish COPD from restrictive lung diseases, such as lung fibrosis, where FEV1 and FVC decline at the same levels, leading to low FEV1%, but stable FEV1/FVC ratio. FEV1/FVC ratio is widely used as a diagnostic tool with an established cutoff of 0.7. People with FEV1/ FVC < 0.7 are considered to have COPD. Additional information that can be obtained by spirometry is an exhalation flowrate. The mean flow rate of 25% and 75% of total air exhaled is called mean mid-expiratory flow (MMEF) and can indicate the presence of small airway disease (Estenne et al. 2002). To distinguish COPD from asthma, another obstructive lung disease with prevalently allergic etiology, all spirometry measurements are conducted after bronchodilator use. Unlike asthma, that can be generally kept under control with the use of bronchodilators, in COPD expiratory parameters, such as FEV1, FEV1/FVC ratio, and MMEF, can be poorly restored after bronchodilators use. This irreversible airflow limitation is a hallmark of COPD pathology.

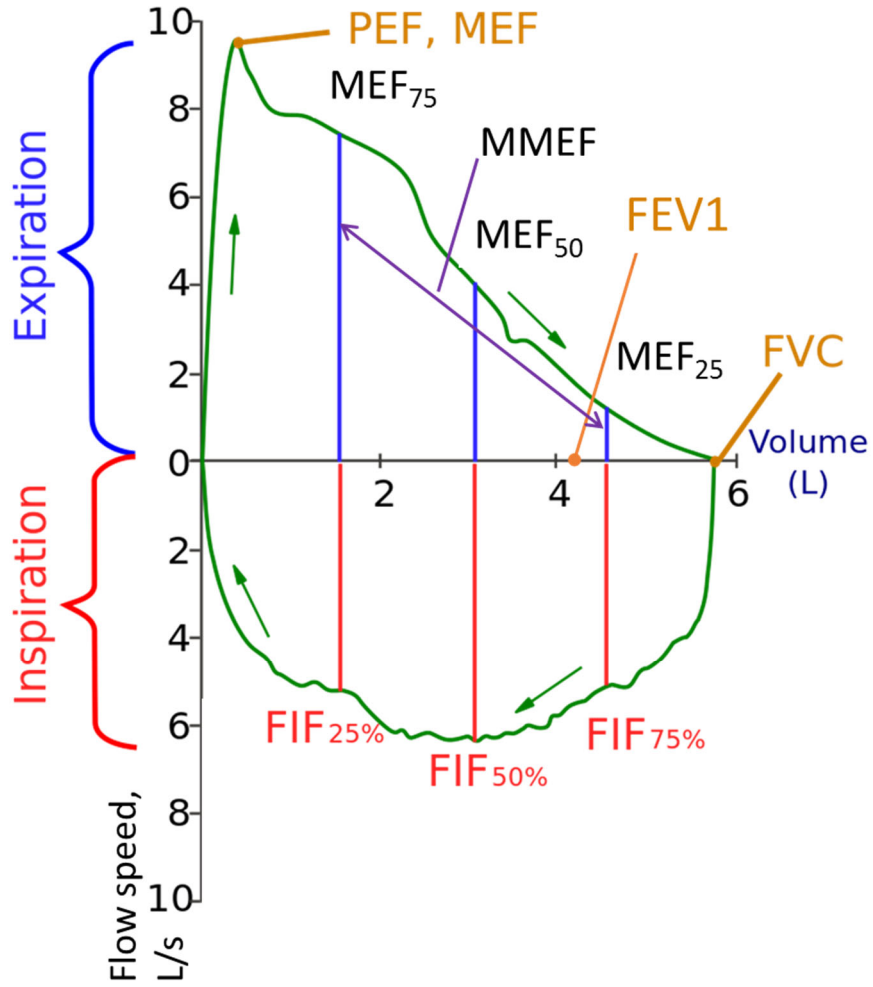

Figure 2. Dynamic lung volumes. Adapted from Mhetre et al. (Mhetre, Patil, and Abhyankar 2018)

FEV1% is used as the main parameter to define the severity of COPD. According to Global Initiative for Obstructive Lung Disease (GOLD) COPD severity can be defined as MILD (FEV1% ≥ 80%), MODERATE (50% ≤ FEV1% <80%), SEVERE (30% ≤ FEV1% < 50%), and VERY SEVERE (FEV1% < 30%) (Hadfield 2017). However, severity classification based only on FEV1% value is incomplete and does not consider pathophysiologic derangements and possible histopathologic conditions that lead to airway obstruction, as well as the other important features, such as symptoms (as wheeze and dyspnea), physical capacity (6-min walking distance and quadriceps maximum voluntary contraction), and exacerbation risk, that all remain very important for

prognosis and the choice of treatment strategy (Segal and Martinez 2018). This is the reason why researchers and physicians are still in search for a better COPD assessment. From the 2011 GOLD organization updated COPD assessment guide introducing A-D groups, defined according to dyspnea score (MRC) and the number and the severity of exacerbations in the previous year. Nevertheless, effective guidelines for COPD assessment are still lacking (Segal and Martinez 2018).

COPD heterogeneity and patients' phenotyping

As COPD is defined just as spirometry-based identification of airway obstruction, it is not surprising that this pathology is highly heterogeneous. Indeed, emphysema prevalent and chronic bronchitis prevalent are not the only phenotypes that can be distinguished within COPD. There is also Asthma-COPD overlap phenotype that combines features of both obstructive diseases and is characterized by higher symptom severity and poorer prognosis, genetically determined α1-antitrypsin deficiency-related phenotype, very distinct from the other COPD by early onset and trypsin-dependent lung tissue destruction, eosinophilic COPD, characterized by high blood eosinophil count and massive eosinophils infiltration into lungs during exacerbations (Segal and Martinez 2018) and possibly some others.

The benefit of different treatment approaches is not equal in these phenotypes.

COPD systemic features

COPD was considered as a lung-restricted disease for many years, but from 2000 to nowadays some new evidence of its systemic manifestations has been provided. This includes a number of comorbid conditions accompanying this pathology. These comorbidities can be divided into three main groups. First – the ones directly related to lung function impairment, such as cardiovascular diseases and erythrocyte abnormalities: ischemic heart disease, ventricular

failure, and normocytic anemia. The second group – diseases related to lung immunity disruption: lung infections and lung cancer. The third group includes the diseases, which are not directly related to COPD pathology development, but frequently appear in COPD patients: diabetes, metabolic syndrome, muscle weakness, depression, osteoporosis (A. G. N. Agustí et al. 2003; Huertas and Palange 2011; P. J. Barnes and Celli 2009). Recent publications refer to systemic inflammation as one of the most common COPD systemic manifestations, that is also linked to worsening of the symptoms and comorbidities progression (Austin et al. 2016; A. Agustí et al. 2012). The main aspects of local and systemic inflammation in COPD are described below.

Local and systemic inflammation in COPD

Cellular component of local inflammation

Inflammatory response in the lungs, usually related to lung tissue irritation by cigarette smoke particles or other inhaled compounds, is known to play the major pathological role in COPD progression. This includes, on the first place, neutrophil infiltration and massive matrix metalloproteinases (MMPs), myeloperoxidase (MPO) and reactive oxygen species (ROS) production by these cells, that cause direct destruction of lung tissue, and T cells (predominantly $CD8^+$ CTL) migration to the lungs, where they perform cytotoxic activity (Peter J Barnes and Cosio 2004). New studies also include B cells as key players in COPD pathology, demonstrating that they not only infiltrate the lungs of COPD patients, but also form lymphoid follicles along the airways, which number correlates with poor prognosis (Hogg et al. 2004; Brusselle et al. 2009; Polverino et al. 2016).

Protein biomarkers in local inflammation

In a detailed review of the last year Dong et al (Dong et al. 2020) summarized what is known about inflammatory markers in sputum of COPD patients. Brief results of their investigation are presented below and are summarized in the Table 1.

Many authors report an increase of CRP level in the sputum, which also correlates with the lung function impairment and with the amount of produced sputum.

Among interleukins both IL-6 and IL-8 levels were shown to be elevated in COPD patients in comparison to controls, independently of the smoking habit, and their concentrations correlated inversely with FEV1%. Other studies also demonstrated a direct correlation of both IL-6 and IL-8 with FEV1% decline speed. IL-6 level was also able to distinguish between COPD and asthma patients.

Several authors have reported an increased MMP-8 and MMP-9 activity in COPD-derived sputum. For MMP-9 the correlation with COPD severity was also shown.

TNFα was also shown to be increased in the sputum of COPD patients and to correlate with the disease progression. However, in two different studies was shown no significant difference between healthy smokers and non-smokers and no significant difference between healthy smokers and COPD, respectively.

As well as other agents secreted by activated neutrophils, neutrophil elastase (NE) level is increased in COPD induced sputum. It also was shown to correlate with COPD severity and annual FEV1 decline.

Table 1. Protein biomarkers in the sputum of COPD patients

Molecule	Change in sputum	Additional observations
CRP	Increased concentration	• Correlates with the lung function impairment and with the amount of produced sputum
IL-6	Increased concentration	• Is elevated independently of the smoking habit • Correlates inversely with FEV1% • Correlates with FEV1% decline speed • Can distinguish between COPD and asthma patients
IL-8	Increased concentration	• Is elevated independently of the smoking habit • Correlates inversely with FEV1% • Correlates with FEV1% decline speed
MMP-8	Increased activity	
MMP-9	Increased activity	• Correlates with COPD severity
TNFα	Increased concentration	• Correlates with COPD severity • Some authors report no difference between COPD and healthy smokers
Neutrophil elastase (NE)	Increased concentration	• Correlates with COPD severity • Correlates with FEV1% decline speed

Soluble markers of systemic inflammation

The role of systemic inflammation in COPD pathology and the reflection of the immune process occurring in the lungs on peripheral blood level are now actively being studied. In particular, it has been suggested that cellular and transcriptomic changes in the lung parenchyma may be reflected in peripheral blood (Teresa and Bhat 2013; Faner et al. 2019). However, it is unclear if systemic inflammation is secondary to local lung inflammation and occurs as a consequence of inflammatory cytokines hypersecretion in the lungs, or systemic inflammation develops independently due to other diseases or conditions and can potentiate COPD development in susceptible subjects (Sevenoaks and Stockley 2006; Peter J. Barnes 2016).

Several inflammatory markers were found to be elevated in the blood of COPD patients. Serum levels of C-reactive protein was found elevated in COPD, both in the acute and stable phase (İn et al. 2016; W. Q. Gan 2004; Moayyedkazemi and Rahimirad 2018), Moreover, high CRP level was reported to be an independent risk factor for overall mortality (Xiong et al. 2017).

Metanalysis performed by Gan et al in 2004 identified increase serum levels of fibrinogen and TNFα in stable COPD patients, thus suggesting the presence of chronic systemic inflammation (W. Q. Gan 2004).

Agusti et al have also shown a significant increase of IL-6, IL-8, fibrinogen, and TNFα plasma levels in COPD in comparison to both smoking and non-smoking controls on the ECLIPSE cohort of about 1800 COPD patients and about 500 controls (A. Agustí et al. 2012).

Cellular component of systemic inflammation

Several research groups focused their attention on peripheral blood leukocytes to assess the changes occurring in these cells in COPD and their relation to the inflammatory pattern in the lungs. However, the data about peripheral leukocyte composition and functional changes in COPD are quite controversial.

The first reported change in circulating leukocytes was an increase of neutrophil-to-lymphocyte ratio (NLR) in COPD, which was shown to correlate with mortality (Duffy et al. 2006; Pascual-González et al. 2018). Subsequently, the increase in NLR in stable COPD was confirmed by others and in some studies an inverse correlation between NLR and FEV1% in COPD patients was also demonstrated (Pascual-González et al. 2018; Furutate et al. 2016; İn et al. 2016). Several authors also reported NLR as a marker of COPD exacerbation, on-going or past (Lee et al. 2016; Taylan et al. 2017; Pascual-González et al. 2018). However, NLR elevation is not a feature specific to COPD, since an increased NLR value was also demonstrated in several other pathologies, such as

bacterial infection, breast cancer, type-2 diabetes, systemic lupus erythematosus, myocardial infarction and rheumatoid arthritis (Li et al. 2015). For instance, the increase of NLR during bacterial infection may explain the predictive capacity of NLR for COPD exacerbations.

COPD related changes in total leukocyte count, as well as count and rate of main leukocyte populations available by the routine blood test were described in several studies. However, there is no consensus in the literature concerning these populations. Most authors report an increase of neutrophil count in stable COPD (Bilir et al. 2016; İn et al. 2016; Koch et al. 2007), while some report no significant difference (Taylan et al. 2017). Similarly, lymphocyte count decrease is reported in several papers (Bilir et al. 2016; İn et al. 2016; Taylan et al. 2017), while some authors report no difference (Koch et al. 2007). The same discrepancy persists for total leukocyte count, which is reported to be elevated in about 50% of papers analyzed (Fratta Pasini et al. 2016; Bilir et al. 2016; Koo et al. 2017; Taylan et al. 2017; A. Agustí et al. 2012) and not altered in another 50% (İn et al. 2016; Stoll et al. 2015; Koch et al. 2007; Rumora et al. 2008; Scrimini et al. 2013). The possible reasons for such a discrepancy in the literature data are proposed in the discussion of this thesis.

COPD was initially considered to be a neutrophilic disease. Indeed, neutrophil infiltrate is the main cellular component of BAL in COPD and it was shown to correlate with the severity of airflow obstruction (O'Donnell 2006). Also, matrix metalloproteinases and the main neutrophil cytokine IL-8 were detected at high levels in BAL of COPD patients (El-Shimy et al. 2014). An increase of neutrophil count in peripheral blood of COPD patients was demonstrated in many studies. However, very few of them report significant correlations between circulating neutrophil count and disease parameters (Ellingsen et al. 2016) in contrast to NLR that was widely reported as an important severity and exacerbation risk marker (Pascual-González et al. 2018).

In addition to neutrophil count assessment, there are several publications describing alterations in neutrophil physiology in COPD, although these works are often not consistent. With respect to neutrophil chemotaxis, Yoshikawa et al reported impaired neutrophil chemotaxis in COPD (Yoshikawa et al. 2007), while Milara et al showed enhanced chemotactic activity of COPD neutrophils (Milara et al. 2012). Jaroenpool et al have shown a significant impairment of phagocytic ability of COPD neutrophils (mild-moderate cohort) in comparison to neutrophils of non-smoking and smoking controls. They have also demonstrated an increased ROS production by activated neutrophils of COPD patients and non-COPD heavy smokers in comparison to non-smoking controls (Jaroenpool et al. 2016). Oudijk et have shown an increased mRNA expression of IL-1β, IL-RA, MIP-1β, and CD83 in response to stimulation in neutrophils derived from severe and very severe COPD in comparison to the ones from moderate COPD or healthy controls. These expression levels correlated negatively with FEV1% in the COPD cohort (Oudijk et al. 2005).

All main lymphocyte subpopulations, T, B, and NK cells, were demonstrated to be altered in circulation in COPD pathology.

Despite known T cells involvement in COPD pathology and their increased number in the lungs of COPD patients, the data about circulating T cells are less certain. Chen et al demonstrated a disbalance in the $CD8^+$ T population in COPD with a higher prevalence of IFNγ-producing Tc1 and lower prevalence of IL-4 producing Tc2 (Chen et al. 2016), which goes in line with the general concept considering COPD as a Type-1 disease. Roberts et al have also shown differences in gene expression patterns between COPD- and control-derived circulating $CD4^+$ T cells (Roberts et al. 2015). Additionally, they have reported a decrease of $CD4^+$ T cells count in a moderate-severe cohort of COPD patients in comparison to non-COPD smoking-, sex-, and age-matched controls.

As well as for T cells, very few is known about circulating B cells changes in COPD, despite strong evidence of their involvement in COPD pathophysiology and demonstrated lung infiltration by B cells in COPD. Brandsma et al have shown a decrease of B cell percentage among lymphocytes of COPD patients in comparison to the control group, as well as a decrease of memory cell rate among the B cells in the COPD group, although this subtype was increased in smokers (Brandsma et al. 2009). Also, several papers reported an increased level of autoantibodies and B cell-activating factor (BAFF) in the blood of COPD patients (Núñez et al. 2011; Gao et al. 2020). The frequency of high-titer anti-tissue autoantibodies was higher in the more severe COPD group (GOLD3-4) and the group with low gas transfusion capacity (DLCO%<50%). The level of BAFF, an essential factor for B cell survival, maturation, and differentiation, was increased in plasma of COPD patients in comparison to smoking or non-smoking controls and it correlated with FEV1% in the COPD cohort. BAFF was also shown to inhibit apoptosis of $CD8^+$ T cells and to induce IFNγ expression by $CD4^+$ T cells and perforin and granzyme B expression by $CD8^+$ T cells derived from peripheral blood of COPD patients (Gao et al. 2020).

The rate of NK and NKT cells among $CD3^+$ cells was shown to be increased in BAL fluid of COPD patients in comparison to either smoking or non-smoking controls. The increase in the NKT rate was mostly due to $CD8^+$ NKT (Eriksson Ström et al. 2018).

Data about circulating $CD56^+CD3^+$ NKT cells in COPD are controversial. Tang et al reported an increase of NKT count in COPD patients in comparison to healthy non-smokers (Tang et al. 2013), while Urbanowicz et al reported a decrease of NKT percentage in PBMC (Urbanowicz et al. 2009). Invariant NKT (iNKT) cell percentage of total leukocytes was also reported to be decreased in COPD together with mucosal-associated invariant T (MAIT) cells (Szabó et al. 2017).

Dendritic cells are found in COPD-associated lung lymphoid follicles together with T and B cells (Brusselle et al. 2009). However, circulating DC alterations in COPD are poorly described. Stoll et al have reported no difference in DC composition and pDC and cDC absolute count between COPD severe patients and current smokers. However, they have shown differences in surface molecules expression by DCs, particularly an increased rate of $OX40L^+$ cells among cDC and decreased rate of $PD\text{-}L1^+$ cells among pDC (Stoll et al. 2015). Kalathil et al also observed no difference in pDC absolute count and rate among DC between COPD and non-smoking healthy controls (Kalathil et al. 2014).

Monocytes have a dual role in immune response, being key players in the development as well as in the resolution of inflammation. Monocytes migrating to the lungs of COPD patients increase lung tissue destruction via production of proinflammatory cytokines, MMPs, and ROS. At the systemic level circulating monocytes of COPD patients showed an activated phenotype, including higher levels of CCR5 expression in resting conditions (Ravi et al. 2017) and increased secretion of MMP-9 upon LPS stimulation (Aldonyte et al. 2003), as compared to monocytes of healthy donors. Impaired chemotaxis (Ravi et al. 2017) together with an activated profile in resting conditions may suggest monocytes from COPD patients to be rather "senescent" than "activated". The phenomenon of immunosenescense was defined as an age-associated decline of the immune system efficiency due to global changes in the immune system towards an increased rate of more mature, sometimes primed or activated, cells with low proliferation capacity, low plasticity and often lower functional efficiency.

Myeloid-derived suppressor cells, or MDSC, were detected in COPD by several authors. However, most of them did not check the suppressive capacity of these cells. MDSC are a heterogeneous group of monocyte- and granulocyte-derived mature and immature cells sharing one common feature: the ability to suppress T cell activation/proliferation. To the moment, there is no clear

marker to distinguish MDSC from their non-suppressive counterpart. For monocytes most papers report that HLA-DR$^{low/-}$ CD14^{+} monocytes have a suppressive capacity (Bronte et al. 2016), while for neutrophils none of the indications proposed, such as low density or FATP2 expression, can be unequivocally considered as a marker of suppressive cells (Veglia et al. 2019; Scapini et al. 2016; Marini et al. 2017). Kalathil et al have shown an increased percentage of low-density neutrophils (CD14^{-}HLA-DR^{-}CD11b^{+}CD33^{+} cells) in PBMC of COPD patients compared to controls (Kalathil et al. 2014) but did not assess their suppressive capacity.

Gender-related differences in COPD

Changing paradigm from "male disease"

COPD was traditionally considered as a male disease (Watson et al. 2004), but rising evidence shows that it probably equally concerns male and female populations. Also, according to the data from American Lung Association, the number of women dying of COPD in the United States surpasses the number of men starting from the year 2000, even though the age-adjusted death rate per 100'000 is still higher in men (American Lung Association 2013). They also reported a high prevalence of chronic bronchitis in females in comparison to males (56.7 cases of 1000 for female vs 29.6 cases for male) with almost no difference in emphysema prevalence.

COPD diagnostics is based on anamnesis, including smoking history, symptom assessment, and spirometry. Since COPD symptoms are common for several lung diseases, including asthma, the main diagnostic tool is a spirometry test. However, the belief that COPD is a male disease can lead to underdiagnosis of women to have COPD. Thus, Whatson et al reported that generally women are prescribed to undergo the spirometry test 20% less often than men (Watson et al. 2004).

Symptoms and quality of life

With the group of 3265 male and female COPD patients Watson et al. reported that women with COPD suffer from more severe dyspnea despite younger mean age and less pack-year smoking burden (Watson et al. 2004). Physicians have usually related it to the fact that the same pack-year smoking index would mean a higher tobacco products burden per kilogram of body mass, as women generally weigh less than men. However, Tam et al have recently shown that an increase of airway resistance, induced by a long-term cigarette smoke exposure, is attenuated in ovariectomized mice, thus pointing to the role of sex hormones in COPD development (Tam et al. 2016).

Phenotype differences

In addition to the abovementioned higher prevalence of chronic bronchitis in COPD female patients than in male, women were described to have less severe emphysema, particularly in the periphery of the lung, and stronger airway wall thickness increase (Martinez et al. 2007). Concerning the dynamics of COPD development, smoking women have a faster FEV1% decline than smoking men (Wen Qi Gan et al. 2006), which probably contributes to a much higher prevalence of women (71%) among the early-onset COPD group (Silverman et al. 2000). Interestingly, several studies have demonstrated that female non-smokers are more likely to develop COPD than male, which is reflected in both higher prevalence of females in the COPD non-smoker group (>80%, (Birring et al. 2002) and a higher rate of diagnosed COPD in the non-smoking female population than in male (>5% and <3% for white females and males, respectively (Fuller-Thomson, Chisholm, and Brennenstuhl 2016).

The treatment effectiveness is also not equal in male and female COPD population. Thus, females with COPD demonstrated less benefit from inhaled corticosteroid treatment than males, as well as females with asthma in comparison to the male group. Similarly, it was reported that emphysematous

female COPD patients demonstrate poorer airway obstruction reversibility than male patients (Han et al. 2007).

AIM

A great deal of controversy on the composition of circulating leukocytes in COPD patients exists, and no consensus has been reached for any of the parameters analyzed. So far, studies aimed at characterizing circulating leukocyte composition and status in a gender-specific manner have not been undertaken, regardless the significant gender-related difference of several features of this pathology. The hypothesis is that such controversial results may be derived from the composition of the cohorts investigated. The aim of this study was to identify the role of gender in circulating leukocyte composition and state in COPD patients.

The main goals of the present project are the following:

1. Thorough characterization of the total number, frequency, phenotypic and functional characteristics of circulating leukocyte in stable male and female COPD patients;
2. Identification of potential correlations between defined leukocyte traits and clinical/diagnostic features;
3. Transcriptomic analysis of circulating polymorphonuclear neutrophils and identification of a potential COPD molecular signature.

MATERIALS AND METHODS

Demographics and ethics

We conducted a case-control study in a cohort of COPD patients recruited at Verona University Hospital between 2017 and 2019, belonging to the Respiratory Medicine operating unit, and control cohort free of COPD recruited from the general population. Research protocol was approved by the Ethic Committee of the Azienda Ospedaliera Universitaria Integrata Verona (protocol n. 42052/2015), in agreement with the principles of the Declaration of Helsinki, and written informed consent was acquired from all the subjects before their enrollment. Personal data were anonymized and numeric ID was assigned to each participant and used instead of the name.

The diagnosis of COPD was based on the presence of symptoms, such as chronic cough, dyspnea, sputum production, history of exposure to risk factors for the disease, and spirometry exam. FEV1/FVC < 0.70 was used to confirm the presence of persistent airflow limitation with accordance to Global Initiative for Chronic Obstructive Lung Disease (GOLD) recommendations.

We collected demographic data including age, sex, body mass index (BMI), working history, physiological anamnesis (in particular smoking habit), pathological anamnesis and medications taken by each person; we investigated clinical features of COPD of each patient (duration, dyspnea MRC score, presence of nocturnal dyspnea, purulence presence and quantification of the sputum, number of exacerbations in the last 12 months); Lung function parameters were evaluated for COPD patients, such as FEV1 – forced expiratory volume in 1 second, FVC – forced vital capacity, FEV1/FVC ratio, TLC – total lung capacity, RV – residual volume, RV/TLC ratio, ERV – expiratory reserve volume, IC – inspiratory capacity, IC/TLC ratio, DLCO – diffusing lung capacity for carbon monoxide , KCO – diffusing coefficients corrected for alveolar

volume. All above mentioned parameters were obtained as absolute values and as percent of predicted values for the subject of the same age, sex and height.

Exclusion criteria for COPD patients and control subjects:

- younger than 55 years old
- the presence of active tumor pathology or a history of cancer within the previous three years
- the presence of any autoimmune disease
- the presence of infectious and/or inflammatory diseases in the acute phase
- the presence of any lung disease other than COPD (asthma, lung fibrosis or other)
- the intake of immunosuppressive or corticosteroid drugs orally or systemically.

Leukocyte count and distribution assessment

A venous sampling was performed for each donor using K_2EDTA vials. Leukocyte count was obtained from routine test of automated differential blood cell count analysis performed in operative unit of Laboratory of Clinical Chemistry, Hematology and Clinical Molecular Biology of Hospital Borgo Roma. Estimation of different leukocyte populations count from Fluorescense-Activated Cell Sorting (FACS) analysis was performed by multiplying the population percentage of total leukocytes obtained by flow cytometry using specific cell surface markers by total leukocyte count obtained by routine blood test. Neutrophil-to-Lymphocyte ratio (NLR) was calculated by dividing neutrophil count by lymphocyte count.

Immunophenotyping and flow cytometry

Human peripheral blood samples were stained with various monoclonal antibodies (mAbs) to determine the frequency of leukocyte subsets in each individual subject using flow cytometry. Briefly, 50 µl of fresh whole blood was used for each staining experiment, and the samples were incubated with fluorochrome-conjugated antibodies for 15 min at room temperature in the dark. Erythrocytes were lysed by incubation with BD Pharm Lyse™ lysing Solution (BD Bioscience, San Diego, CA, USA) for 5 minutes followed by washing and resuspension in FACS buffer (PBS + 2% FBS + 2mM EDTA) and immediately analyzed on 8-color MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Auburn, CA, USA). Flow cytometric analysis was performed following published guidelines (Cossarizza et al. 2019). Data were analyzed using FlowJo software (FlowJo 10.6.2, Ashland). A list of all fluorochrome-conjugated antibodies used is reported in Table 2.

Maturation and activation status of PMN was performed on PMN collected from Ficoll gradient separation as described below.

Table 2. List of all fluorochrome-conjugated antibodies used for cytometric analysis

Marker	Clone	Flurochrome	Company
CCR6	REA190	APC	Miltenyi
CCR7 (CD197)	REA546	VioBlue	Miltenyi
CD10	HI10a	PE	BioLegend
CD11b	ICRF44	PE PEcy7	BioLegend
CD11c	REA618	APC	Miltenyi
CD127	HIL-7R-M21	PEcy7	BD
CD14	REA599	VioBlue	Miltenyi
CD141	AD5-14H12	APC	Miltenyi
CD16	3G8	PerCP-cy5.5 APC/Cy7	BioLegend
CD161	REA631	PE-vio770	Miltenyi
CD19	LT19	PE-vio770	Miltenyi
CD1c	AD5-8E7	PE	Miltenyi
CD25	BC96	APC-cy7	BioLegend
CD3	BW264/56	PerCP-Vio700 PE-vio770	Miltenyi
CD35	E11	Vio-blue	Miltenyi
CD303	AC144	FITC	Miltenyi
CD4	M-T466	VioBlue PE	Miltenyi
CD45	HI30	BV510	BD
CD45RA	T6D11	FITC	Miltenyi
CD45RO	UCHL1	PerCP-Vio700	Miltenyi
CD56	MEM-188	FITC	BioLegend
CD62L	145/15	APC	Miltenyi
CD66b	G10F5	FITC	BioLegend
CD8	REA734	FITC APC-vio770	Miltenyi
CRTH2	BM-16	PE	Miltenyi
CXCR3	REA232	VioBright FITC	Miltenyi
HLA-DR	REA805	VioBlue	Miltenyi
HLA-DR	L243	APC/Cy7	BioLegend
MDC8	DD-1	FITC	Miltenyi
PD1	REA1165	APC	Miltenyi
PDL1	MIH1	BV421	BD

PMN and PBMC purification from peripheral blood

Separation of peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) out of whole blood was performed by density gradient centrifugation of blood diluted 1:1 in pyrogen-free PBS over Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Purification of PMN, collected from the bottom of the tubes, from RBC contamination was performed by RBC sedimentation in 4% dextran solution in PBS followed by 50 seconds mixing in hypotonic solution (0.2% NaCl) RBC lysis. After RBC lysis PMN were washed with PBS.

PMN purification on magnetic beads

PMN purification for suppressive capacity test and RNA sequencing was performed by magnetic separation based CD66b-dependent positive selection using Miltenyi magnetic separation Kit (Miltenyi Biotec, Auburn, CA, USA). PMN, collected from Ficoll gradient separation as described in corresponding paragraph, were incubated with FITC-conjugated anti-CD66b antibody for 15 min at room temperature. Then cells were washed with FACS buffer and incubated with anti-FITC magnetic beads for another 15 min at room temperature. After this, cells were washed again with FACS buffer and applied on magnetic columns, washed and eluted by FACS buffer according to manufacturer instructions. The final purity of granulocytes was >99.9%, as assessed by flow cytometry.

T cell proliferation analysis

For proliferation analysis T cells were isolated from the whole blood of COPD patients and control donors using Pan-T Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Then BrDU incorporation-based test was performed with BrDU Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Briefly, 40 000 cells/well T cells were plated in the wells of 96-well plate precoated with anti-CD3 antibody (Clone UCHT1, Biolegend, San Diego, CA,

USA) for three hours at 37 C. 200 000 cells/well PMN or the same volume of full RPMI were added to the T cells. Then anti-CD28 antibody (Clone CD28.8, Biolegend, San Diego, CA, USA) and full RPMI to the total volume of 200 µl were added. Cells were cultured for 72 hours. After 72 hours of co-incubation, BrDU for total concentration of 10µM was added in all wells and the cells were incubated at 37C for another 18 hours to allow dividing cells to incorporate BrDU. After 18 hours cells were dried and stained with anti-BrDU antibodies and optical density was measured using VICTOR Multilabel Plate Reader (PerkinElmer Life Sciences, Boston, MA, USA). All samples were plated in triplicates.

Induced superoxide anion ($O_2^{\cdot-}$) production

Superoxide anion production by neutrophils was assessed by Cytochrome C reduction test. This method of reactive oxygen species calculation is based on ferricytochrome C reduction to ferrocytochrome C by receiving an electron from $O_2^{\cdot-}$, which leads to its spectrophotometric absorbance alteration. To perform the test, PMN were resuspended in HBSS (pH 7.4) containing 80 mM ferricytochrome C type III (MilliporeSigma, St. Louis, Missouri, United States) to 2×10^6 cells/mL and 20 ng/mL of PMA (MilliporeSigma, St. Louis, Missouri, United States) or 1 nM of fMLP (MilliporeSigma, St. Louis, Missouri, United States) was added to induce ROS production. Cytochrome C reduction was evaluated at 550 nm from the moment of stimulation and every 10 minutes to the total evaluation time of 2 hours.

Reagents and cell culture

RPMI 1640 medium (Corning, NY, USA) containing 10% FBS (≤10 EU/mL endotoxin, MilliporeSigma, St. Louis, Missouri, United States) was used for cell culture. Cultures were maintained in a 37°C incubator containing 5% CO2.

PBS solution was prepared by dissolving 10x DPBS (Corning, NY, USA) in sterile pyrogen-free water (B. Braun, Melsungen, Germany)

EDTA (MilliporeSigma, St. Louis, Missouri, United States)

RNA sequencing

Total RNA was extracted from resting granulocyte samples purified from whole blood of male COPD and age and matched healthy controls included in the "discovery cohort" (3 COPD patients and 4 controls) and "validation cohort" (26 controls and 24 COPD patients) using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Demographic and clinical data for discovery and validation cohorts are represented in supplementary tables Table 11 and Table 12, respectively. For the "discovery cohort" PMN were purified by positive magnetic selection as described in the dedicated paragraph above (PMN purity >99.9%), while for "validation cohort" PMN purified from peripheral blood by Ficoll separation were used (PMN purity 99.6% [99.3 – 99.8]). An on-column DNase digestion with the RNase-free DNase I set (Qiagen), was performed to remove any contaminating DNA.

RNA quantification was performed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA).

Transcriptomic analysis of the "discovery cohort"

RNA purified from granulocytes of donors enrolled in the "discovery cohort" was used to set cDNA libraries according to SmartSeq2 protocol (Picelli et al. 2014). The choice of SmartSeq2 method, which allows sequencing from as little as 500pg of RNA, was dictated by the fact of low RNA yield from granulocytes. RNA-seq libraries were than sequenced on Illumina Next Seq500 (Illumina, San Diego, California, USA).

Trimming for raw reads was performed with TrimGalore. a minimum of 15 million fragments per sample were analyzed. After quality filtering according to the Illumina pipeline, reads were aligned to the GrCh38.p12 reference human genome (Genome Reference consortium) and the *H. sapiens* transcriptome (Ensembl, version 94) using HISAT2 aligner (Kim et al. 2019). Summed exon reads count per gene were estimated using HTSeq-count

(Anders, Pyl, and Huber 2014) with an intersection-nonempty mode for overlapping exons alignments and gene ID as feature ID. Differential gene expression analysis was performed with DESeq2 using the Wald test for two groups comparison and Likelihood ratio test (LRT) for multiple groups comparison. Genes considered as differentially expressed based on adjusted p value $p_{adj}<0.05$. Normalized gene expression levels were expressed as fragments per kilobase of exon for million reads mapped (FPKM).

Transcriptomic analysis of the "validation cohort"

To validate the data of COPD signature obtained on the "discovery cohort", 3'Seq sequencing was performed on PMN samples from 24 male COPD patients and 26 male controls.

RNA-seq library preparation was accomplished using the 3' mRNA-Seq Library Prep Kit (Lexogen GmbH, Germany); unique molecular indices (UMI) were used during the library preparation. Sequencing was performed on Illumina Next Seq500 (Illumina, San Diego, California, USA).

Trimming for raw reads was performed with bbduk. After quality filtering according to the Illumina pipeline, reads were aligned to the GrCh38.p12 reference human genome (Genome Reference consortium) and the *H. sapiens* transcriptome (Ensembl, version 94) using STAR aligner according to manufacturer recommendations. UMI-based deduplication was performed using UMI tools. Summed exon reads count per gene were estimated using HTSeq-count (Anders, Pyl, and Huber 2014) with an intersection-nonempty mode for overlapping exons alignments.

Differential gene expression analysis was performed using the negative binomial distribution-based method implemented in DESeq2 on the summed exon reads count per gene (Love, Huber, and Anders 2014). Gene expression normalization for sequencing depth was performed by internal DESeq2 method.

Gene ontology

Gene Ontology enrichment analysis was performed using Panther tools with GO database of the Gene Ontology Consortium as reference database. GO terms were considered enriched with $p_{adj} < 0.05$. Semantic similarity of GO terms with Revigo tools was used to remove the redundant GO terms and visualize the summarized GO enrichment (Supek et al. 2011).

Statistics

All continuous variables are expressed as median [Q1-Q3] for all types of data distribution unless otherwise specified. The Shapiro-Wilk test was performed to control the distribution type. The Fisher's exact test was used to compare proportions in different groups. Student T-test or Mann-Whitney U-test for normally and not-normally distributed data, respectively, were performed to compare the two independent groups. Analysis of variance (ANOVA) was used to compare more than two groups for normally distributed variables; Tukey post-test was used to identify significantly different groups. Where needed (as for ANOVA test), non-normally distributed variables were logarithmically transformed to normalize the distribution. The correlation coefficients and significance of correlations were analyzed using the Spearman Rank test. On correlation plots, linear regression lines with 95% CI are plotted to indicate the trend of the correlation. Linear regression analysis was also performed to assess more complex associations. All *p*-values were two sided. $P < 0.05$ was considered as significant. Wolfram Mathematica 12.0.0.0 software was used for statistical analysis.

RESULTS. PART 1. CIRCULATING LEUKOCYTE POPULATIONS IN COPD

Demographic data

Demographic data for recruited COPD patients and age-matched controls are presented in Table 3.

Table 3. Demographic data for recruited COPD and control subjects

	Control	COPD	P Value
N	63	50	
Age	76.5 [66.3-80]	76 [70-80]	0.8 $
Sex (Male/Female)	35/28	31/19	0.5 χ
Smoking (never/ex/current/NA)	42/18/3/0	5/31/12/2	1×10^{-4} χ
Smoking pack-year index	0 [0-12]	30 [20-49.6]	6.3×10^{-11} $
FEV1/FVC	0.77 [0.74-0.79]	0.50 [0.39-0.58]	3.3×10^{-21} t
FEV1%	115 [103-127.5]	66 [47.5-79.5]	2.4×10^{-20} t
BMI	26.7[24.2-29.4]	26.5 [24-31.5]	0.8 t
Charlson index (except COPD)	2 [0-2.25]	2 [1-3]	0.4 $
Hypertension (present/not)	34/25	38/10	0.023 χ
Diabetes (present/not)	10/53	7/43	1.0 χ
CVD (present/not)	11/45	14/36	0.4 χ
Dyslepidemia (present/not)	16/40	17/29	0.4 χ
Arthrosis (present/not)	17/44	13/33	1.0 χ
Hypothyroidism (present/not)	7/53	4/42	0.8 χ

$ – Mann-Whitney test, χ – Fisher's exact test, t – Student T-test

Gender-related differences in WBC and NLR

Leukocyte counts and neutrophil-to-lymphocyte ratio (NLR) are well recognized inflammatory markers associated with COPD. A recent comprehensive systematic review shows that the NLR value is higher in stable COPD patients than in healthy controls (Pascual-González et al. 2018).

In order to provide a direct evaluation of leukocytes homeostasis, we studied the frequency and immunological characteristics of peripheral blood leukocytes derived from COPD patients. Total white blood cell (WBC) absolute numbers were comparable between the cohorts of COPD patients (6.5 [5.5–7.4] × 10^6 cells/mL) and control subjects (6.1 [5.3–6.9] × 10^6 cells/mL) (Figure 3 A). However, when the same analysis was performed stratifying subjects by gender, male COPD patients were characterized by a significant increase (6.9 [5.8–7.6] × 10^6 cells/mL *vs* 5.9 [5.3–6.6] × 10^6 cells/mL, p = 0.002), whereas female COPD showed a significant decrease (5.6 [4.2–6.8] × 10^6 cells/mL *vs* 6.3 [5.3–7.9] × 10^6 cells/mL, p = 0.038) in total leukocyte absolute numbers as compared to age and sex-matched controls (Figure 3 C). Additionally, the neutrophil-to-lymphocyte ratio (NLR) was assessed (Figure 3 B). NLR was higher in the COPD group compared to control, although the difference did not reach significance (2.4 [1.7–3.6] *vs* 2.1 [1.7–2.7], p = 0.070). As it is shown in Figure 3 D, no difference in NLR value between COPD and controls could be detected in the female cohort. Only the male COPD cohort showed a significant increase in NLR value as compared to controls (2.7 [2.0–4.1] *vs* 2.2 [1.7–2.7], p = 0.024) (Figure 3), indicating that the increased NLR value detected in the total COPD group versus the control cohort reflects significant difference confined exclusively in the male population.

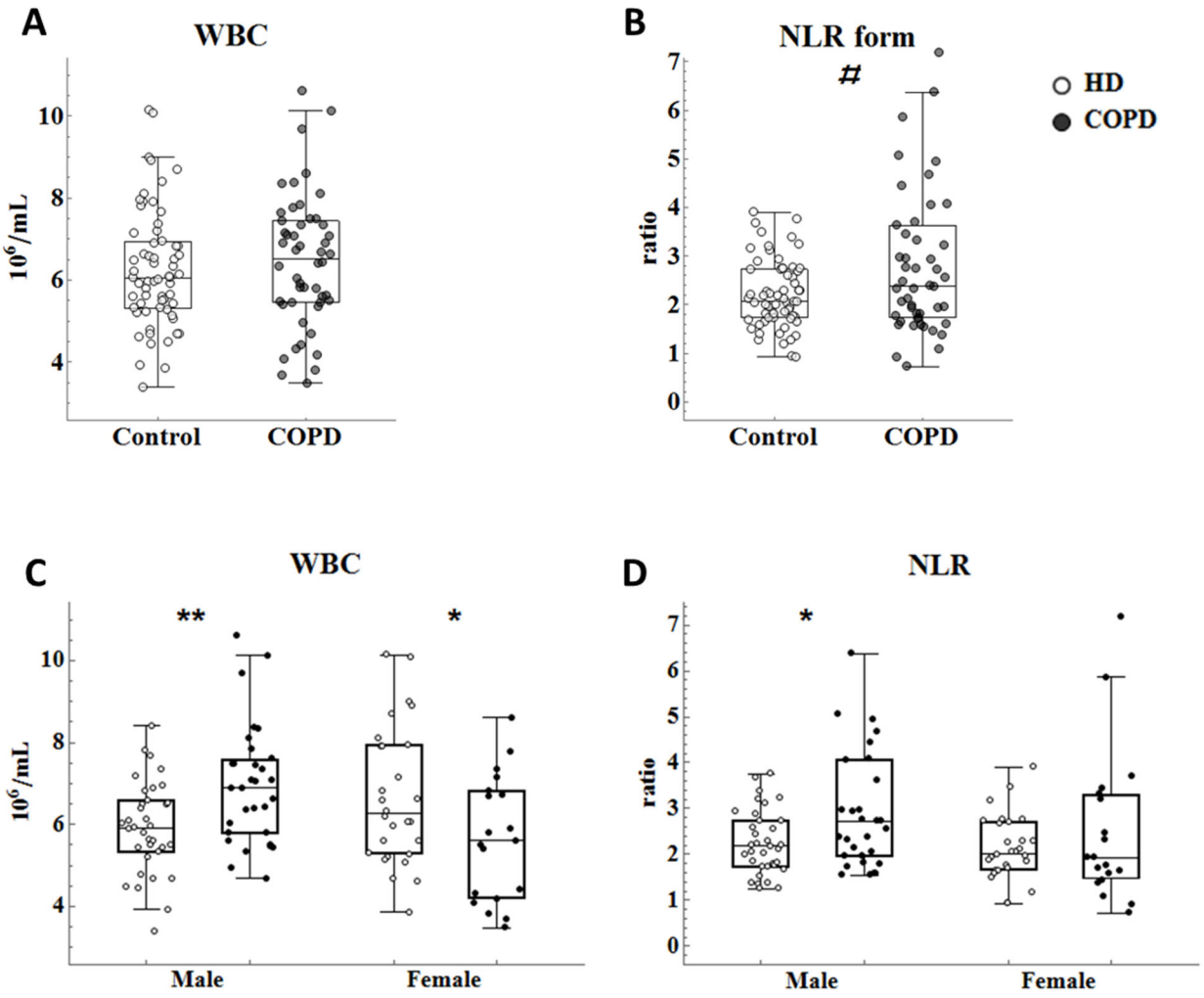

Figure 3. White blood cells count (A, C) and neutrophil-to-lymphocyte ratio (B, D) value in gender-mixed (A-B) and gender-stratified (C-D) COPD and control groups; light dots correspond to control group values and dark dots – to COPD. # – p = 0.07, * – p < 0.05, ** – p < 0.01.

Leukocyte composition of males and females with and without COPD

An analysis of the absolute count (Figure 4 A) and of the frequency of leukocyte populations (Figure 4 B) showed that the increased WBC and NLR in the male COPD cohort as compared to sex-matched controls (Figure 3 C-D) can be attributed to the increase in the number of neutrophils (4.48 [3.30-5.04] × 10^6 cells/mL vs 3.47 [2.88-3.87] × 10^6 cells/mL, p = 0.002). Neutrophil frequency was significantly increased (Mean±SD value 64.0±8.3% *vs* 59.7±6.5%, p = 0.03) and lymphocyte frequency – significantly decreased

(Mean±SD value 24.3±7.3% *vs* 28.0±5.7%, p = 0.03) in male COPD group, while lymphocyte count was similar in COPD and controls. Absolute counts and frequencies of other general leukocyte populations were equally represented in male COPD and control groups (Figure 4 A and B respectively). On the opposite, the reduction of WBC in female COPD versus the respective control (Figure 3 C) results from a consistent reduction of each leukocyte population analyzed, even though significance was reached only for lymphocyte and monocyte populations (Figure 4 A). The leukocyte frequencies in female COPD were, therefore, indistinguishable from that of controls (Figure 4 B). The exact values of main leukocyte populations count and rate for male and female cohorts are reported in supplementary tables Table 9 and Table 10, respectively.

Collectively, the data show that gender is a relevant factor conditioning circulating leukocyte homeostasis in COPD and that a general leukopenia characterizes COPD in the female population.

In order to better characterize all the leukocyte subsets, we performed a flow cytometry analysis of circulating leukocytes. Lymphoid and myeloid subpopulations were identified according to the gating strategy shown in Figure 5 and Figure 6, respectively.

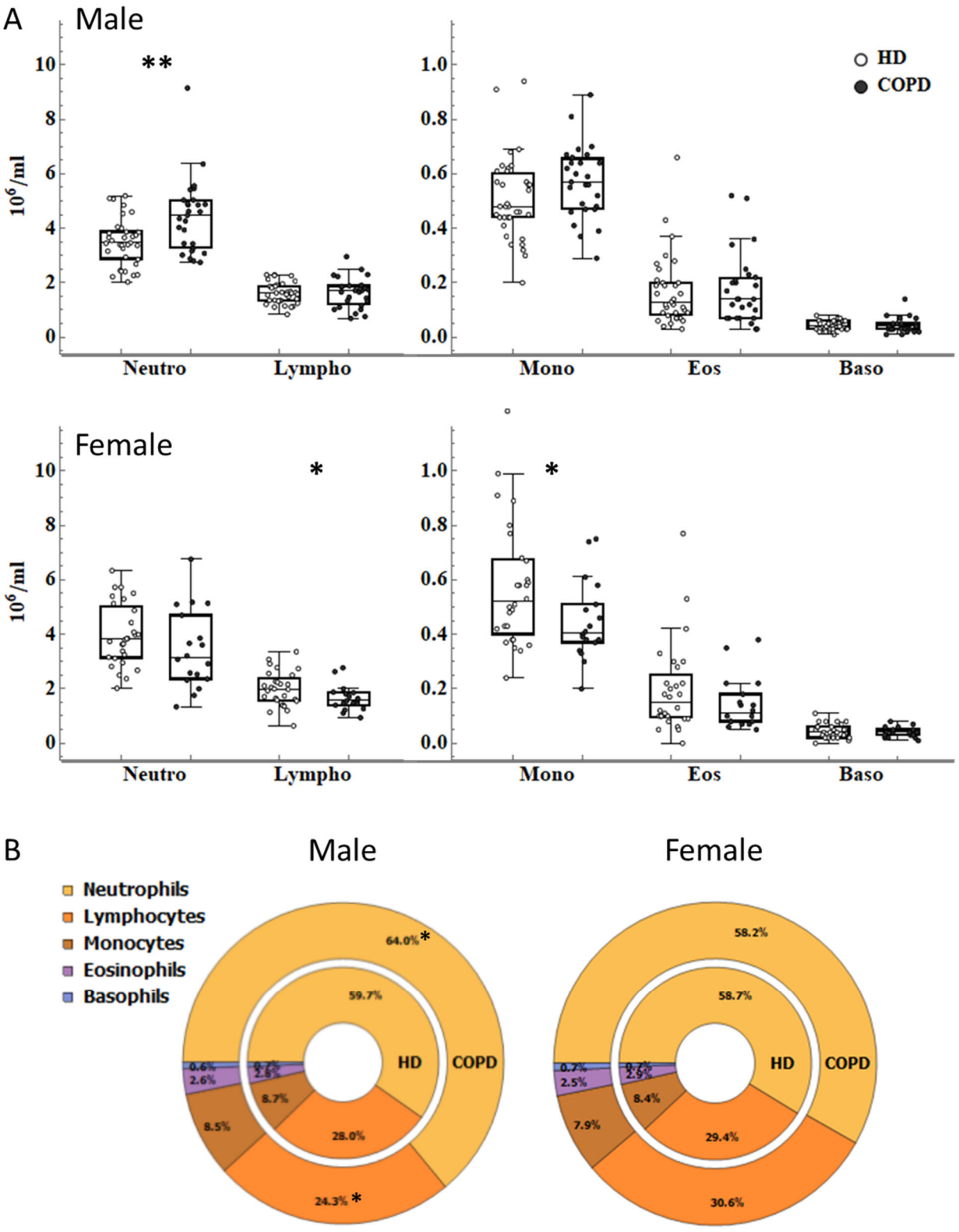

Figure 4. Leukocyte composition in COPD and control males and females
A – absolute concentrations of main leukocyte populations, B – main leukocyte populations frequencies of total WBC (sectors represent the mean percentage of each population of total WBC). * – $p<0.05$, ** – $p<0.01$.

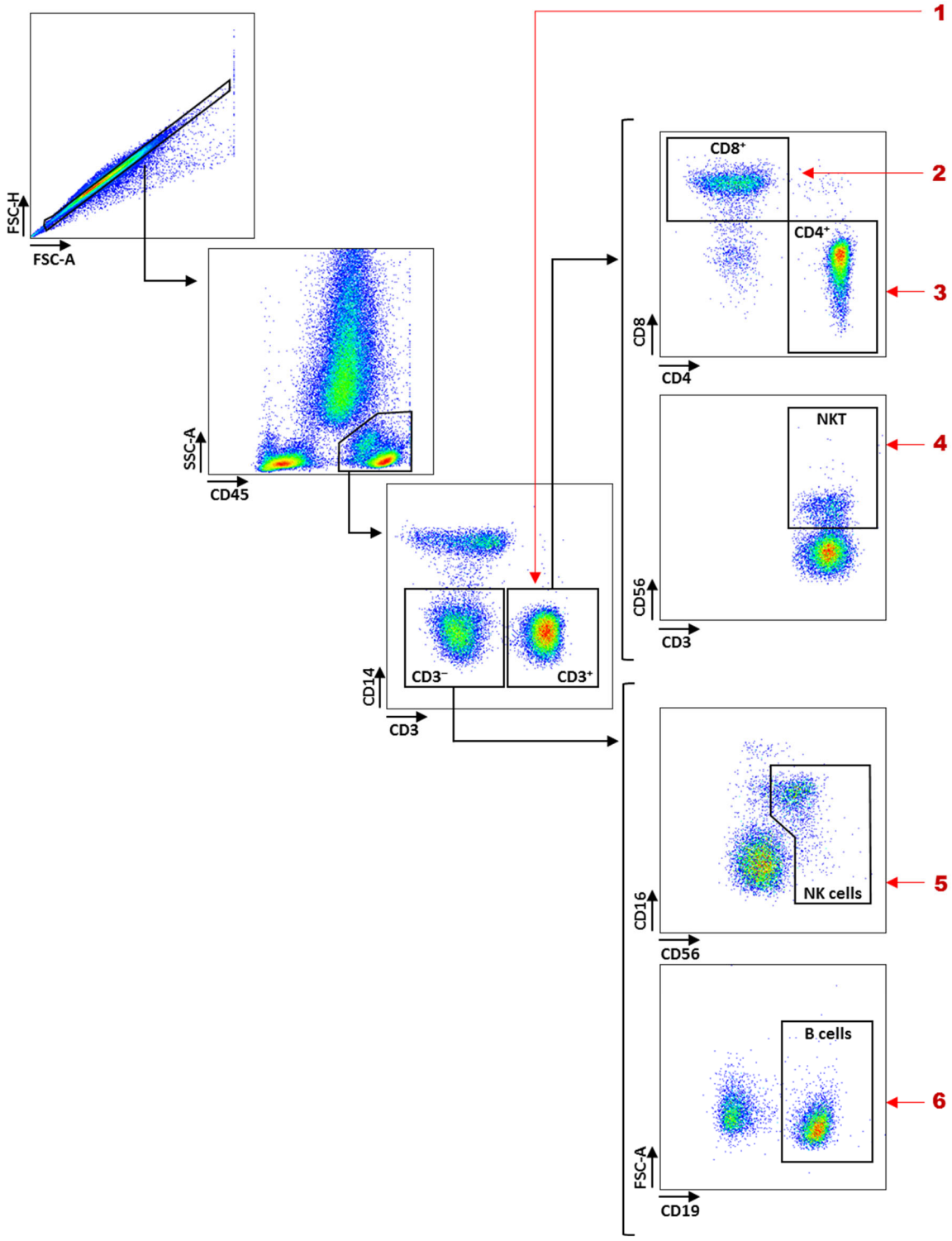

Figure 5. Gating strategy for lymphocyte subtypes

Singlets were gated based on FSC-A and FSC-H parameters. Circulating leukocytes were defined as $CD45^+$ cells. From them, after an exclusion of neutrophils by high granularity and lower CD45 expression and an exclusion of $CD14^+$ monocytes (to reduce the noise in target populations identification), T cells (1) were defined as $CD3^+$ cells. Among the T cells $CD8^+$ CTL (2), $CD4^+$ T helpers (3), and $CD56^+$ NKT cells (4) were identified. NK cells (5) were identified as $CD3^-CD56^+$ and B cells (6) – as $CD3^-CD19^+$.

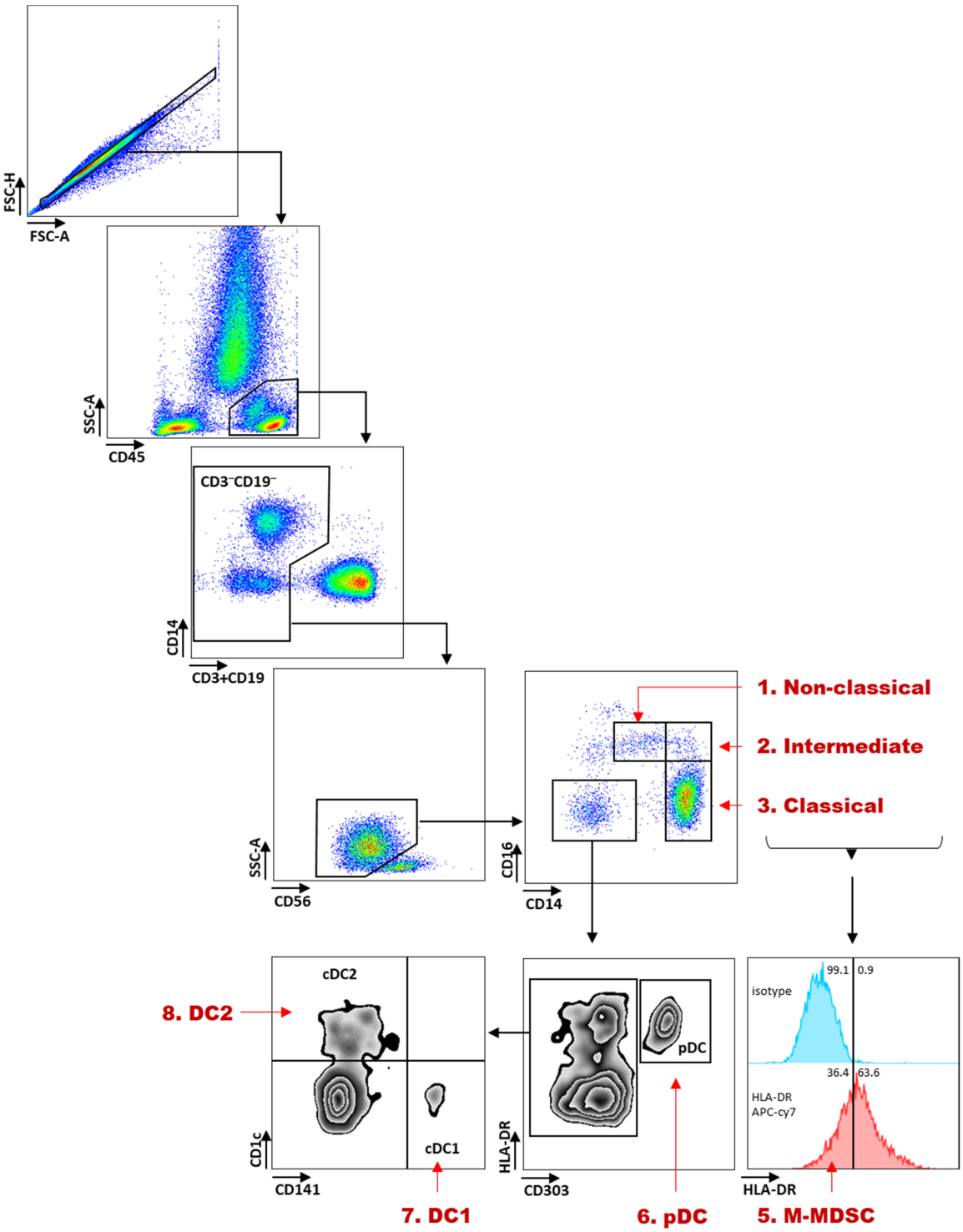

Figure 6. Gating strategy for myeloid subtypes. Singlets were gated based on FSC-A and FSC-H parameters

Circulating leukocytes were defined as CD45$^+$ cells. From them, after an exclusion of neutrophils by high granularity and lower CD45 expression and an exclusion of T, B, and NK cells by their lineage markers (CD3, CD19, and CD56, respectively), Non-classical CD14$^{-/low}$CD16$^+$ monocytes (1), intermediate CD14$^+$CD16$^+$ monocytes (2) and classical CD14$^+$CD16$^-$ monocytes (3) were defined. Total monocytes were stained for HLA-DR expression and monocytic MDSC population (5) was defined as HLA-DR$^{-/low}$. From CD14$^-$CD16$^-$ cells pDC (6) were defined as CD303$^+$ population, cDC1 (7) – as CD141$^+$ cells and cDC2 (8) – as CD1c$^+$ cells.

Characterization of the circulating myeloid cell subtypes in COPD

Analysis of the three monocyte subtypes (classical, intermediate and non-classical) showed no significant difference in monocyte composition in either male or female cohorts (Figure 7 A).

$CD14^{+}HLA\text{-}DR^{-}$ monocytic MDSC were also similar in number and percentage of total monocytes in COPD and control groups in both males and females (Figure 7 B).

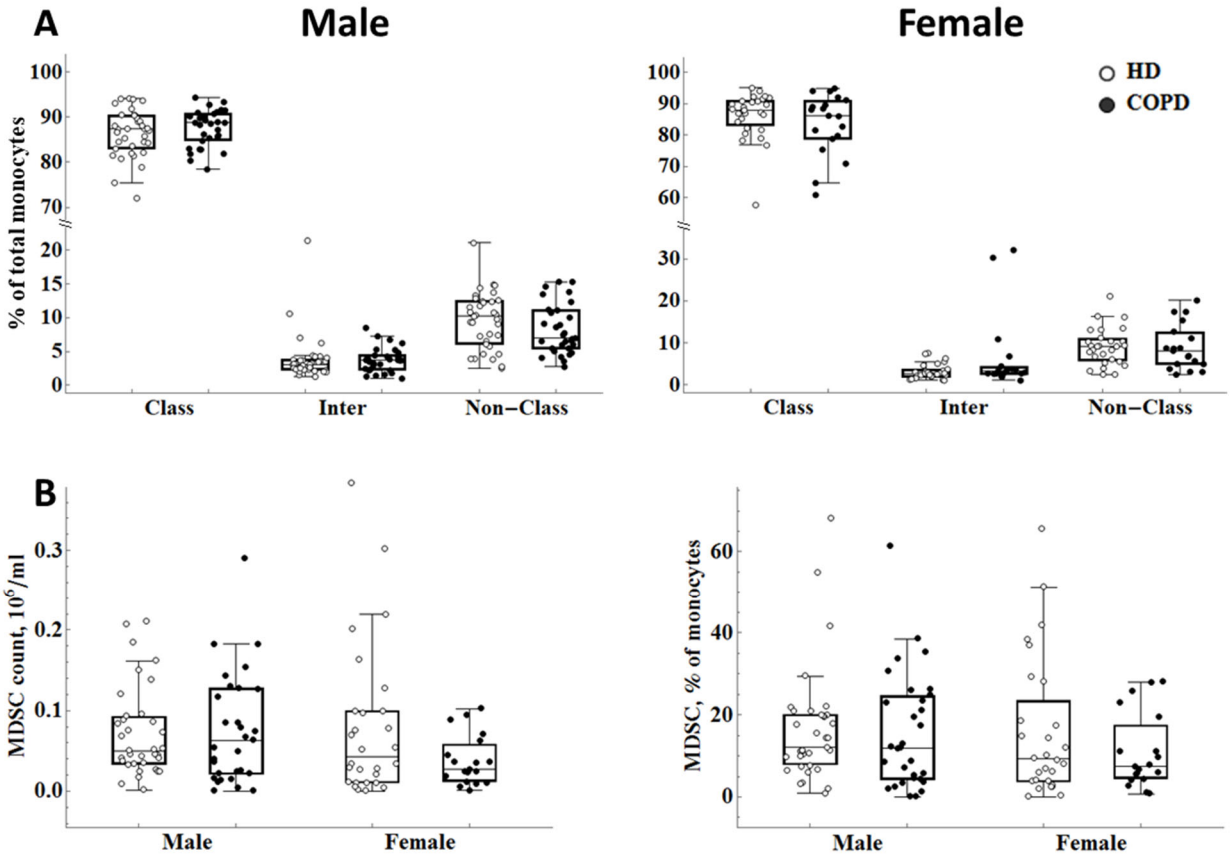

Figure 7. Monocyte subpopulations in COPD and control groups in male and female cohorts

A – relative concentration of main monocyte subpopulations in total monocytes; B – $CD14^{+}HLA\text{-}DR^{low/-}$ monocytic MDSC count and rate of total monocytes; Class – classical $CD14^{++}CD16^{-}$ monocytes, Inter – intermediate $CD14^{++}CD16^{+}$ monocytes, Non-class – non-classical $CD14^{low/-}CD16^{+}$ monocytes.

Finally, with respect to dendritic cells, no differences in DC count between male/female COPD and control groups were observed (Figure 8 A). However,

while DC subtypes were equally represented in male COPD, a disbalance in conventional DC composition was observed in female COPD in comparison to control group (Figure 8 B). DC1 rate was increased while DC2 rate was decreased in female COPD vs controls, although none of these changes was statistically significant.

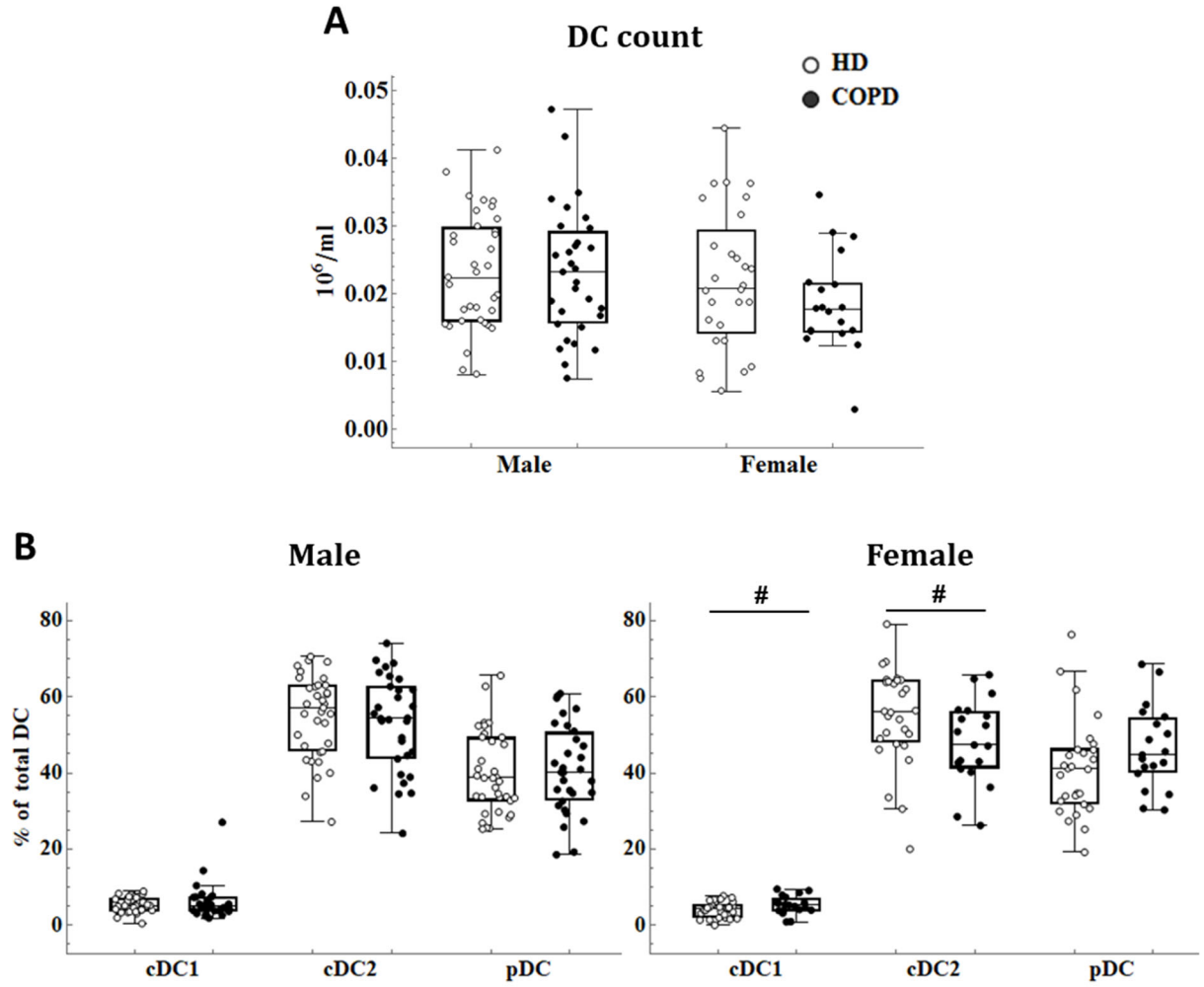

Figure 8. Total count and composition of dendritic cells in COPD and control groups in male and female cohorts
A – DC absolute concentration in whole blood; B – DC composition, represented as the rate of each subset in total DC; # – $p<0.07$.

Characterization of the circulating lymphoid cell subtypes in COPD

For the male cohort, where no difference in lymphocyte count was observed between COPD and controls (Figure 4 A), no changes were detected for lymphocyte subpopulations as well (Figure 9 A). In females the decrease of total

lymphocyte count was reflected in all lymphocyte populations, reaching significance, though, only for the B cells (Figure 9 A). This observation further strengthens the evidence that nearly all myeloid and lymphoid populations are involved in general leukocyte count reduction in female COPD. T cell subpopulations counts, namely CTL, T helpers, and NKT cells, were not significantly different between COPD and control groups for both male and female cohorts, although T helpers and NKT in females demonstrated the same trend of decreasing in COPD as other lymphocyte populations (Figure 9 B).

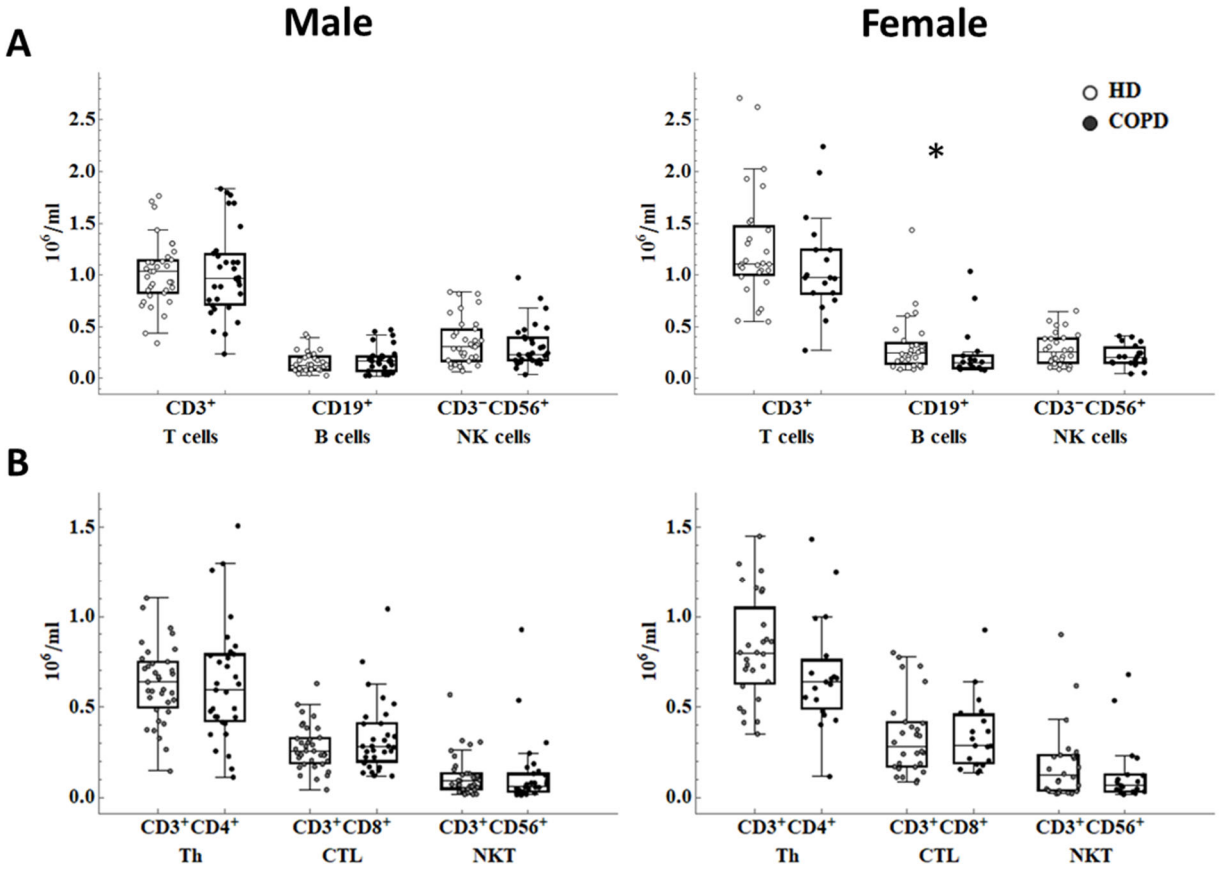

Figure 9. Lymphocyte subpopulations (A) and T cell subpopulations (B) concentrations; * – $p<0.05$.

T cell subsets analysis performed according to the gating strategy, depicted in Figure 10, revealed a significant increase of Treg cells frequency in T helpers in the COPD group in both male and female cohorts in comparison to respective

controls with no difference in other T helper subsets frequencies (Figure 11 A). Interestingly, Treg absolute count did not differ between COPD patients and controls. However, an increase of 40-70% in the percentage indicates an imbalanced T cell composition in the COPD group. Additionally, an imbalance in memory cell distribution among CTL was observed in female COPD, with a decreased rate of conventional $CD45RO^+$ central (TCM) and effector (TEM) memory cells and a higher prevalence of terminally differentiated $CD45RA^+$ TEMRA cells (Figure 11 C). None of such changes were observed in the male group. No changes were observed for $CD4^+$ memory cell frequency in ether group (Figure 11 B).

A summary of the significant differences identified between COPD and control groups in male and female cohorts is reported in Figure 12.

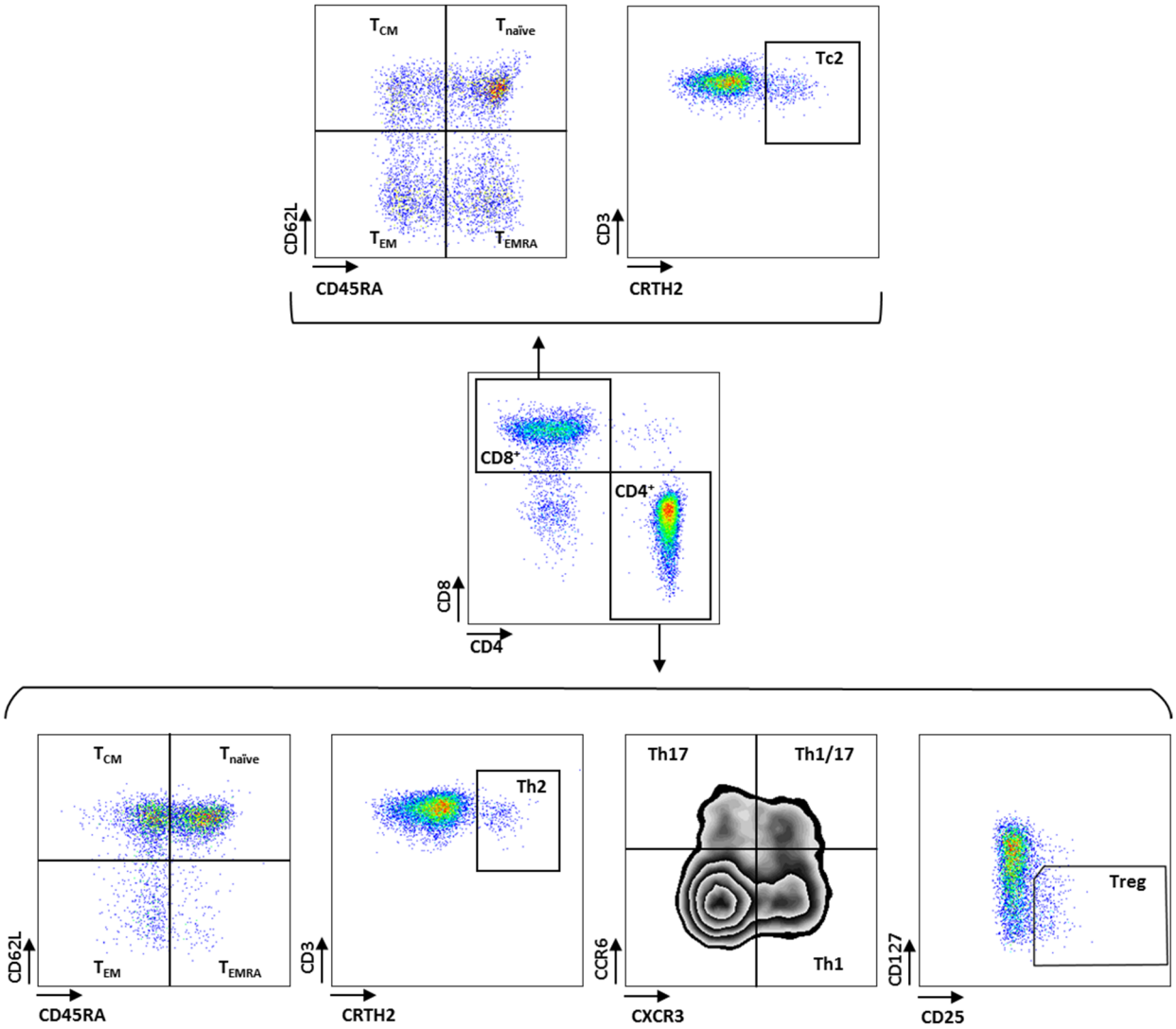

Figure 10. Gating strategy for T cell subtypes
Memory T cells were defined as: CD62L^{+}CD45RA^{-} for central memory (T_{CM}), CD62L^{-}CD45RA^{-} for effector memory (T_{EM}), CD62L^{-}CD45RA^{+} for senescent effector memory RA^{+} (T_{EMRA}) and CD62L^{+}CD45RA^{+} for naïve T cells. Type-2 committed CD4 and CD8 cells (Th2 and Tc2) were defined by an expression of prostaglandin D2 receptor CRTH2. Other T helper subsets were defined by CCR6 and CXCR3 expression with Th1 defined as CXCR3^{+}CCR6^{-} cells, Th17 defined as CXCR3^{-}CCR6^{+} and Th1/Th17 hybrid population defined as CXCR3^{+}CCR6^{+}. CXCR3^{-}CCR6^{-} population consisted prevalently of T naïve cells but included also Th2 subset. Treg cells were defined as CD127^{-}CD25^{+} cells.

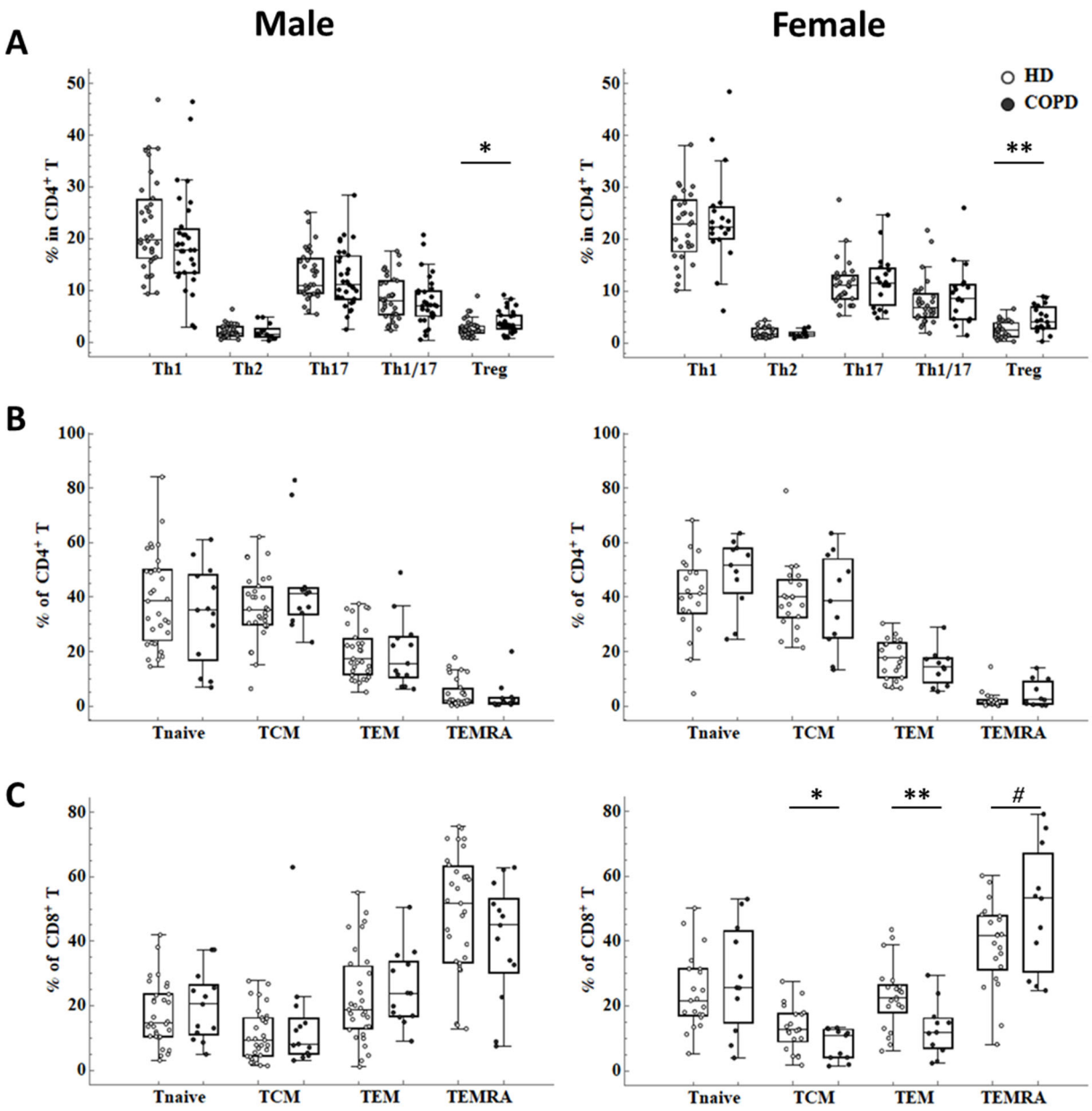

Figure 11. T helper subsets frequencies in CD4+ T cells (A) and memory cell subtypes frequencies in CD4+ (B) and CD8+ (C) T cells; # – $p<0.1$, * – $p<0.05$, ** – $p<0.01$.

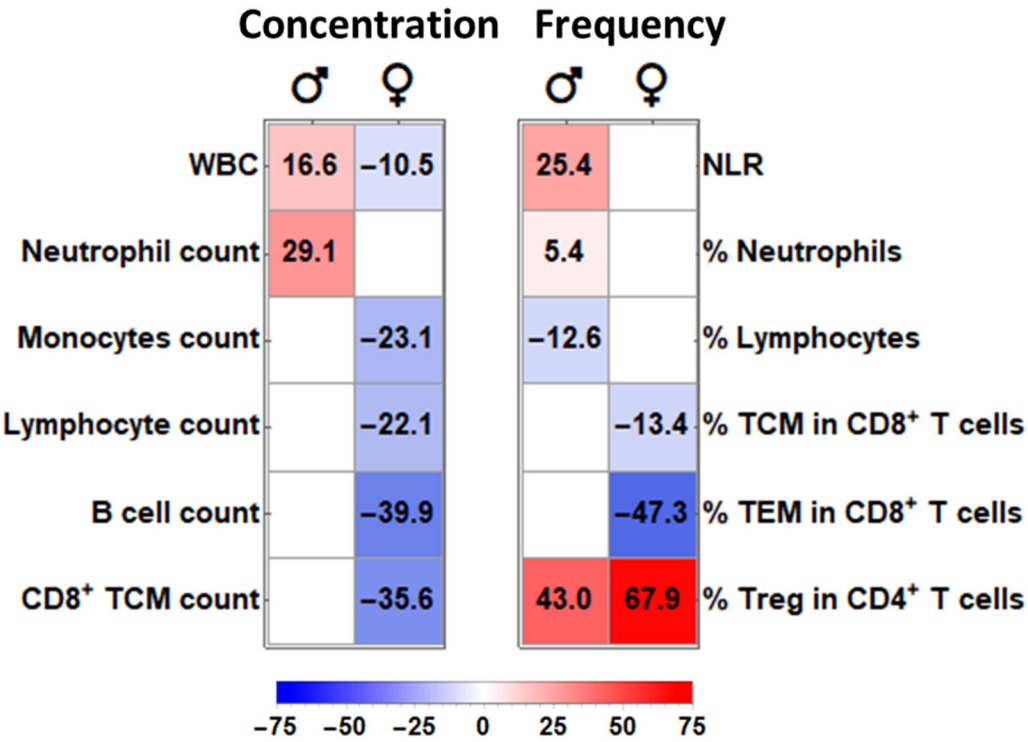

Figure 12. All differences between COPD and control groups with $p<0.05$ in male and female cohorts

The difference between the medians is reported in each box as percentage of control values ($\Delta = \frac{\text{Median}_{\text{COPD}} - \text{Median}_{\text{Control}}}{\text{Median}_{\text{Control}}} \times 100\%$). Boxes are left empty when difference between COPD and control group are not significant ($p \geq 0.05$). Color represents the direction and the magnitude of the difference with more intensive red for an increase in COPD and more intensive blue for a decrease with maximum and minimum difference of 75% and -75%, respectively.

General differences between male and female cohorts

Male and female COPD patients recruited in this study did not differ significantly by symptom severity or any of assessed spirometry parameters represented as a percentage of predicted values, with an exception of the higher FVC% in the female group in comparison to males (Table 4).

Table 4. COPD severity indicators and smoking status of male and female COPD

	COPD Male		COPD Female		
	n	**Median (Q1-Q3)**	**n**	**Median (Q1-Q3)**	**P value**
FEV1 %	30	59 (43±82)	19	70 (50.25±76)	0.5 [t]
GOLD	30	2 (1±3)	19	2 (2±2.75)	0.7 [$]
FVC %	30	94.5 (77±111)	19	114 (100.75±122.75)	0.03 [t]
FEV1/FVC	30	0.5 (0.38±0.6)	19	0.5 (0.4±0.55)	0.8 [t]
IC/TLC	25	0.37 (0.32±0.45)	17	0.35 (0.31±0.43)	0.92 [t]
MMEF%	19	23 (13.5±29.25)	12	18 (13±24.5)	0.4 [t]
Dyspnea grade	29	1 (1±3)	19	1 (1±2)	0.3 [$]
DLCO%	24	79 (57.5±95.5)	15	74 (59.25±88.25)	0.6 [t]
KCO%	24	95 (67.5±110)	15	82 (69.5±87.25)	0.2 [t]
Smoking (current/ex/no)	29	6/22/1	19	5/10/4	0.7 [χ] for current vs ex+no

[$] – Mann-Whitney test, [χ] – Fisher's exact test, [t] – Student T-test

Other than spirometry parameters, smoking is an important confounder for COPD studies. With respect to this point, male and female cohorts did not differ significantly in the frequency of ex- and current-smokers (Table 4). However, female COPD patients had significantly lower cigarette smoke burden, represented as a pack-year index, than the male ones (Figure 13).

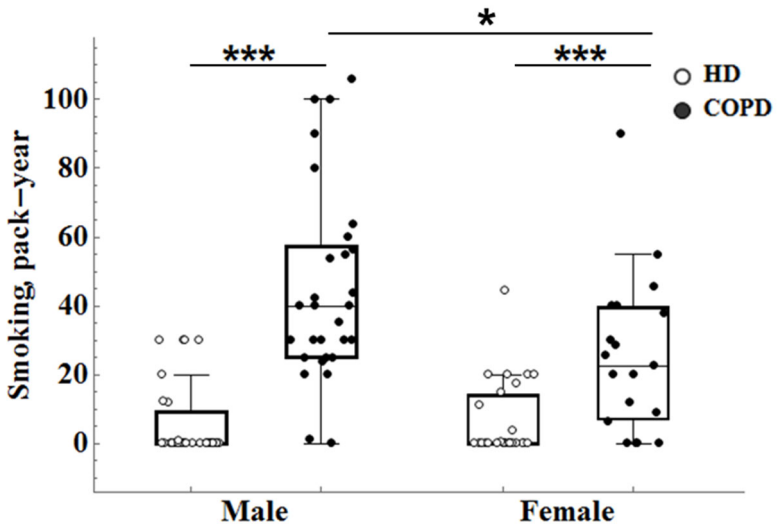

Figure 13. Smoking pack-year index in male and female COPD patients and controls; * – p<0.05, *** – p<0.001.

On the basis of this observation, we performed a multivariate analysis of variance (ANOVA) to assess if the smoking index (pack-year) has an impact on the differences described above. The total cohort was grouped by pack-year index into 3 groups: very light smokers with pack/years ≤ 15, light smokers with pack/year >15 and < 30, and heavy smokers with pack/year ≥ 30. Frequencies of very light, light, and heavy smokers are reported in Table 5.

Table 5. Frequencies of very light, light, and heavy smokers in COPD and control male and female subjects

	Very light (≤ 15 pack-years)	Light (15-30 pack-years)	Heavy (≥ 30 pack-years)
COPD male	2	6	21
COPD female	7	5	7
Control male	28	6	1
Control female	21	5	1

In addition to the influence of smoking, it is known that some of the frequent COPD comorbidities may cause systemic changes, including peripheral blood leukocytes alterations (Pettersson et al. 2011). On these bases, comorbidities were included in the ANOVA test to assess the possible influence of these

pathological conditions on the parameters that were shown to be significantly different between COPD and control groups. Comorbidities included in this analysis were diabetes, dyslipidemia, arthrosis, hypertension, cardiovascular diseases (CVD) and hypothyroidism (Table 6 and Table 7 for males and females, respectively). Smoking burden did not alter any of the systemic markers found to be significantly different in the COPD versus control group in either cohort. Pathological conditions, such as diabetes, dyslipidemia, and CVD, did modify per se the concentration of some of the leukocyte subsets previously identified. Nevertheless, and most importantly, the presence of COPD was an independent factor responsible for the statistically significant variation of the blood cell parameters identified in male and female cohorts, also after an adjustment to smoking habit and comorbidity conditions in most cases.

Particularly, in male cohort COPD impact on WBC, neutrophil, and monocyte count, as well as on NLR and on Treg rate in T helpers, was significant after the adjustment to any of the confounders analyzed. Significance of COPD impact on neutrophil and lymphocyte percentage of total leukocytes was lost after the adjustment for Charlson index, dyslipidemia or arthrosis. However, dyslipidemia and arthrosis did not impact neutrophil and lymphocyte percentage. The impact of COPD on cytotoxic lymphocyte (CTL) rate in T cells lost the significance after the adjustment for most of confounders, except diabetes and hypothyroidism, even though the p value in most cases was still less than 0.1. Interestingly, the Charlson index was an independent predictor of the difference in CTL rate in male group.

In female cohort alterations in T cells subsets and DC1/DC2 ratio increase in COPD were still significant after the adjustment for any of the comorbidities analyzed. The impact of COPD on monocyte and total leukocyte count lost the significance after the adjustment for hypertension and CVD (with the p value still close to 0.05 from monocytes and $p = 0.08$ for WBC). Diabetes and Charlson

index impact were not assessed in female cohort due to no COPD subjects with diabetes and very few subjects with Charlson index higher than 2. The decrease of B cell count in female COPD group lost the significance after the adjustment for any of the confounders analyzed, suggesting that B cell decrease is rather a part of overall leucopenia in female COPD than an independent alteration.

Table 6. The impact of COPD comorbidities on significant leukocyte alterations in males.

		None	Smoking*	Charlson**	Diabetes	Dyslipid-emia	Arthrosis	Hyper-tension	CVD	Hypo-thyroidism
WBC	COPD	**0.002**[t]	**0.003**	**0.022**	**0.0008**	**0.002**	**0.002**	**0.002**	**0.001**	**0.002**
	Con-founder	-	0.95	0.5	**0.013**	0.21	0.60	0.7	0.9	0.5
	Inter-action	-	0.3	0.5	**0.03**	0.36	0.07	0.9	0.5	0.8
% Neutro in WBC	COPD	**0.02**[t]	**0.02**	0.2	**0.02**	0.2	0.14	**0.04**	**0.02**	**0.02**
	Con-founder	-	0.3	0.2	**0.008**	0.7	0.5	**0.02**	**0.010**	0.06
	Inter-action	-	**0.008**	0.4	0.2	0.4	0.3	0.4	0.3	0.6
% Lympho in WBC	COPD	**0.007**[t]	**0.02**	0.2	**0.02**	0.13	0.14	0.07	**0.02**	**0.02**
	Con-founder	-	0.6	**0.03**	**0.002**	0.6	0.3	**0.03**	**0.007**	**0.02**
	Inter-action	-	**0.002**	0.3	0.2	0.11	0.2	0.6	0.4	0.8
NLR	COPD	**0.02**[$]	**0.018**	0.07	**0.004**	**0.028**	**0.026**	**0.014**	**0.007**	**0.006**
	Con-founder	-	0.6	0.8	**0.013**	0.20	0.96	0.15	**0.029**	0.10
	Inter-action	-	**0.044**	0.9	0.3	0.061	0.08	0.3	0.5	0.8
Neutro	COPD	**0.002**[$]	**0.002**	**0.016**	**0.0003**	**0.003**	**0.002**	**0.001**	**0.0006**	**0.0009**
	Con-founder	-	0.6	0.9	**0.003**	0.8	0.8	0.3	0.10	0.5
	Inter-action	-	**0.03**	0.9	0.13	0.14	**0.03**	0.4	0.6	0.6
% Treg of CD4 T cells	COPD	**0.012**[$]	**0.033**	**0.010**	**0.029**	**0.015**	**0.008**	**0.017**	**0.018**	**0.026**
	Con-founder	-	0.98	0.5	0.3	0.4	0.8	0.5	0.9	0.6
	Inter-action	-	0.2	0.4	0.9	0.2	**0.005**	0.13	0.2	**0.045**

The table represents the P values of the impact of COPD presence, the confounder and their interaction on the selected parameters, assessed by ANOVA. P values indicated in the column "None" are the unadjusted p values of pairwise comparison of COPD and control groups (by Student or Mann-Whitney test).

[t] TTest

[$] Mann-Whitney test

[*] Smoking burden parameter was introduced as belonging to very light smoking, light smoking or heavy smoking group;

[**] Charlson index parameter was introduced as belonging to a low comormidity group (Charlson 0-2) and high comorbidity group (Charlson ≥ 3).

Table 7. The impact of COPD comorbidities on significant leukocyte alterations in females.

		None	Smoking*	dyslipidemia	arthrosis	Hypertension	CVD	Hypothyroidism
WBC	COPD	**0.038**[t]	**0.053**	0.06	**0.031**	0.08	0.08	**0.030**
	confounder	-	0.7	0.4	0.12	0.4	0.2	0.054
	Interaction	-	0.07	0.6	0.7	0.98	0.7	0.5
Mono	COPD	**0.03**[$]	**0.021**	**0.042**	**0.026**	0.05	0.056	**0.026**
	confounder	-	0.07	**0.013**	0.3	0.5	0.9	0.57
	Interaction	-	0.5	0.6	0.9	0.7	0.9	0.8
Lympho	COPD	**0.03**[t]	**0.033**	**0.039**	**0.028**	**0.041**	**0.043**	**0.03**
	confounder	-	0.2	0.4	0.5	0.3	0.9	0.4
	Interaction	-	0.7	0.6	0.4	0.8	0.9	0.6
B cells	COPD	**0.03**[$]	0.10	0.16	0.07	0.08	0.08	0.08
	confounder	-	0.6	0.4	0.06	0.4	0.9	0.12
	Interaction	-	0.6	0.6	0.7	0.9	0.8	0.9
CD8 TCM count	COPD	**0.016**[$]	**0.049**	**0.023**	**0.030**	**0.043**	**0.032**	**0.042**
	confounder	-	0.4	0.2	0.13	0.9	0.6	0.9
	Interaction	-	0.6	**0.043**	0.6	0.15	**0.021**	0.6
% Treg of CD4 T cells	COPD	**0.004**[t]	**0.002**	**0.0011**	**0.0005**	**0.0016**	**0.0018**	**0.0017**
	confounder	-	0.2	0.2	0.4	0.8	0.8	0.8
	Interaction	-	0.6	0.6	0.10	0.4	0.8	0.2
TCM % of CD8 T	COPD	**0.047**[$]	0.056	**0.038**	**0.042**	**0.042**	**0.012**	**0.030**
	confounder	-	0.7	0.3	0.2	0.3	**0.036**	0.13
	Interaction	-	0.7	0.15	0.9	0.2	**0.0004**	0.12
TEM % of CD8 T	COPD	**0.009**[t]	**0.008**	**0.004**	**0.007**	**0.0098**	**0.0086**	**0.0087**
	confounder	-	0.6	0.09	0.7	0.9	0.4	0.2
	Interaction	-	0.10	**0.035**	0.06	0.2	0.13	0.9

The table represents the P values of the impact of COPD presence, the confounder and their interaction on the selected parameters, assessed by ANOVA. P values indicated in the column "None" are the unadjusted p values of pairwise comparison of COPD and control groups (by Student or Mann-Whitney test).

[t] TTest

[$] Mann-Whitney test

[*] Smoking burden parameter was introduced as belonging to very light smoking, light smoking or heavy smoking group;

Correlations to COPD clinical parameters

To understand whether the changes in circulating leukocyte composition are associated with COPD disease severity, we searched for correlations between the absolute number and percentage of each cell type identified by flow-cytometry and different clinical indicators of COPD severity. Specifically, FEV1 %, FEV1/FVC, MMEF %, IC/TLC, dyspnea grade, and GOLD stage were chosen as representative of COPD severity. FEV1 %, FEV1/FVC, MMEF % and IC/TLC decrease with COPD progression, whereas dyspnea grade and GOLD stage increase (Figure 14). Thus, a direct correlation of the count or frequency of a cell type with FEV1%, FEV1/FVC, IC/TLC, and/or MMEF %, as well as an inverse correlation with GOLD and/or dyspnea indicates a progressive decrease of the given cell type along with increased disease severity. On the contrary, an inverse correlation of number and/or frequency of a given cell type with FEV1 %, FEV1/FVC, IC/TLC, and/or MMEF %, as well as a direct correlation with GOLD and/or dyspnea marks a progressive increase of the indicated cell type with COPD severity.

Spearman rank test revealed that a number of circulating leukocyte populations correlate with one or more parameters of COPD severity. Figure 15 illustrates a comprehensive heat map of the complete results of the correlation analysis.

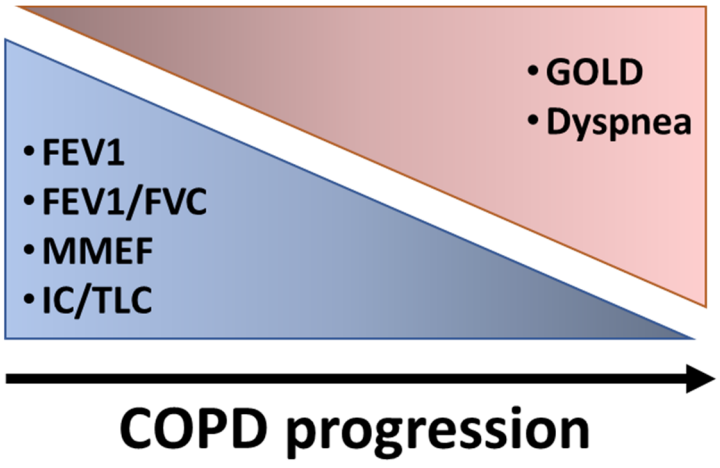

Figure 14. Alteration of main COPD severity indicators during the disease progression

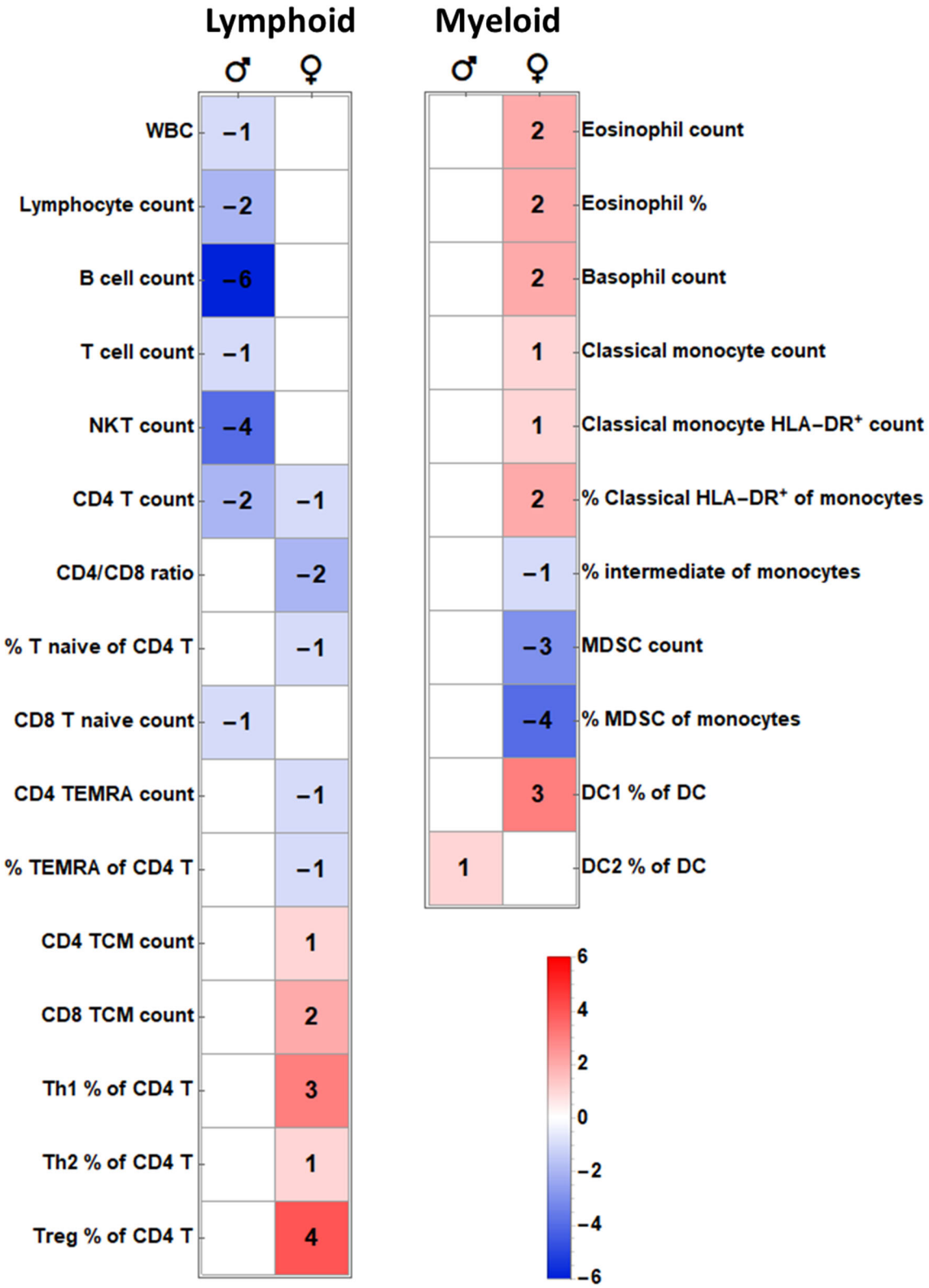

Figure 15. The number of significant correlations of various leukocyte populations count and rate with COPD progression, calculated separately for men (♂) and women (♀). The numbers in the squares indicate the number of COPD progression indicators that correlate significantly with a given parameter (of 6 indicators analyzed: FEV1%, FEV1/FVC, MMEF%, IC/TLC, GOLD stage, dyspnea MRC score). The plus or minus sign represents an increase or a decrease of the parameter with COPD progression (which means either

a decrease of FEV1%, FEV1/FVC, MMEF%, and/or IC/TLC or an increase of GOLD stage and/or dyspnea MRC score). Empty squares correspond to no significant correlations.

Total white blood cell count, which is significantly higher in male COPD group as compared to respective controls (Figure 3 C), progressively decreases alongside with the increase in COPD severity (Figure 15), since it directly correlates with FEV1/FVC in this group (Figure 16 A). This is likely a consequence of the decrease in total lymphocyte count observed at later stages of the disease. Indeed, neutrophil absolute count, which is significantly increased in male COPD as compared to relative controls (Figure 12), does not correlate with any of the selected indicators of COPD severity. At the same time, lymphocyte count directly correlates with FEV1% and inversely correlates with dyspnea MRC score (Figure 16 B). Among lymphocytes, the highest number of correlations was observed for B cell count (Figure 16 C) and NKT cell count (Figure 16 D). Specifically, B cell count directly correlates with FEV1%, FEV1/FVC, IC/TLC and MMEF%, while it inversely correlates with GOLD stage and dyspnea MRC (Figure 16 C). Similarly, NKT cell count directly correlates with FEV1%, FEV1/FVC, IC/TLC and inversely correlates with GOLD stage (Figure 16 D). Among the T cells, total T cell count (Figure 16 E) and T helper ($CD4^+T$) count (Figure 16 F) decreased along with COPD severity.

With respect to the myeloid compartment, an imbalance in dendritic cell composition associated with the disease severity was observed in male COPD (Figure 17), with an increase of DC2 rate and respective decrease of pDC/DC2 ratio during progression towards more severe stages of COPD. Contrary to DC2, pDC and DC1 rates themselves were not associated with COPD severity.

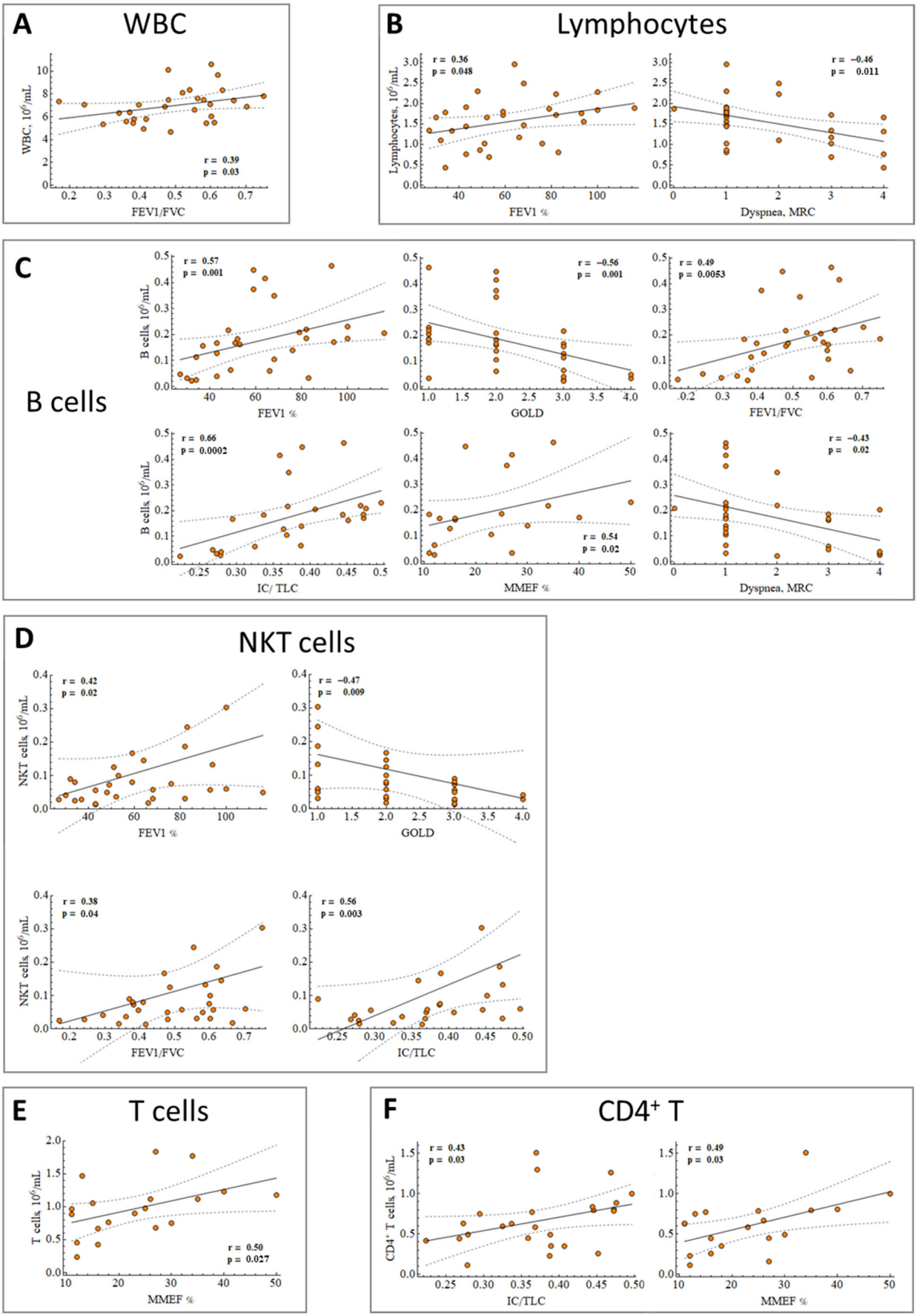

Figure 16. Significant correlations of total WBC (A) and lymphocyte subpopulations (B-G) with COPD severity indicators in male COPD group

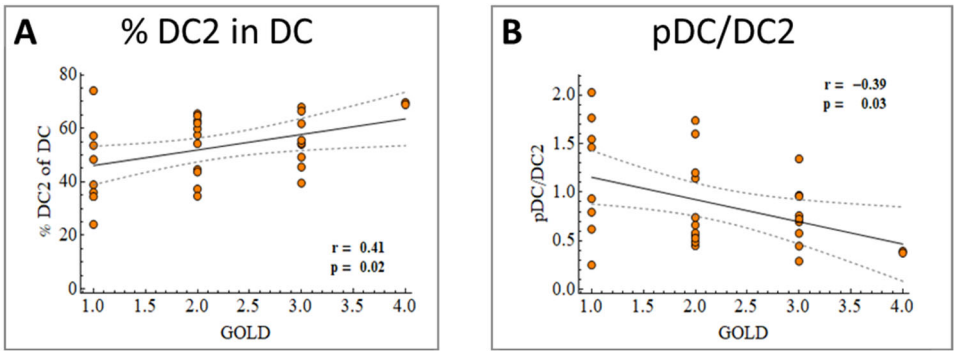

Figure 17. Significant correlations of DC2 rate in DC (A) and pDC/DC2 ratio (B) with the GOLD stage in male COPD group

In the female cohort, none of the leukocyte populations identified as significantly decreased in COPD as compared to control subjects (Figure 12) correlated with any of the clinical parameters analyzed. Nevertheless, correlation analysis showed a direct correlation of CD4/CD8 ratio with FEV1% and MMEF% (Figure 18 A). The progressive decrease in CD4/CD8 ratio along with the disease severity more likely results from a CD4 T cell count decrease (Figure 18 B) as CD8 T cell count does not correlate with COPD progression.

In addition, we observed an increase in frequency of some CD4 subtypes along with disease severity rise. Specifically: frequency of Th1 in CD4 T cells inversely correlated with FEV1% and MMEF%, and concurrently directly correlated with GOLD stage (Figure 18 C); frequency of Th2 in CD4 T cells inversely correlated with IC/TLC (Figure 18 D); frequency of Treg in CD4 T cells inversely correlated with FEV1%, FEV1/FVC and MMEF%, while directly correlated with GOLD stage (Figure 18 E).

The rate of naïve cells among CD4 T directly correlated with FEV1% (Figure 19 A). Terminally differentiated CD4 TEMRA cells number and rate among CD4 T cells correlated directly with FEV1/FVC ratio (Figure 19 B-C), also decreasing with the disease severity rise, whereas central memory $CD8^+$ T increased with COPD progression (Figure 19 D). In case of CD4 TEMRA count and rate, a pronounced bimodal distribution is observed, even despite a quite few

samples, with a group of patients with relatively high FEV1/FVC and CD4 TEMRA rate higher than 6 % and a group of patients with low FEV1/FVC and CD4 TEMRA rate lower than 3 %.

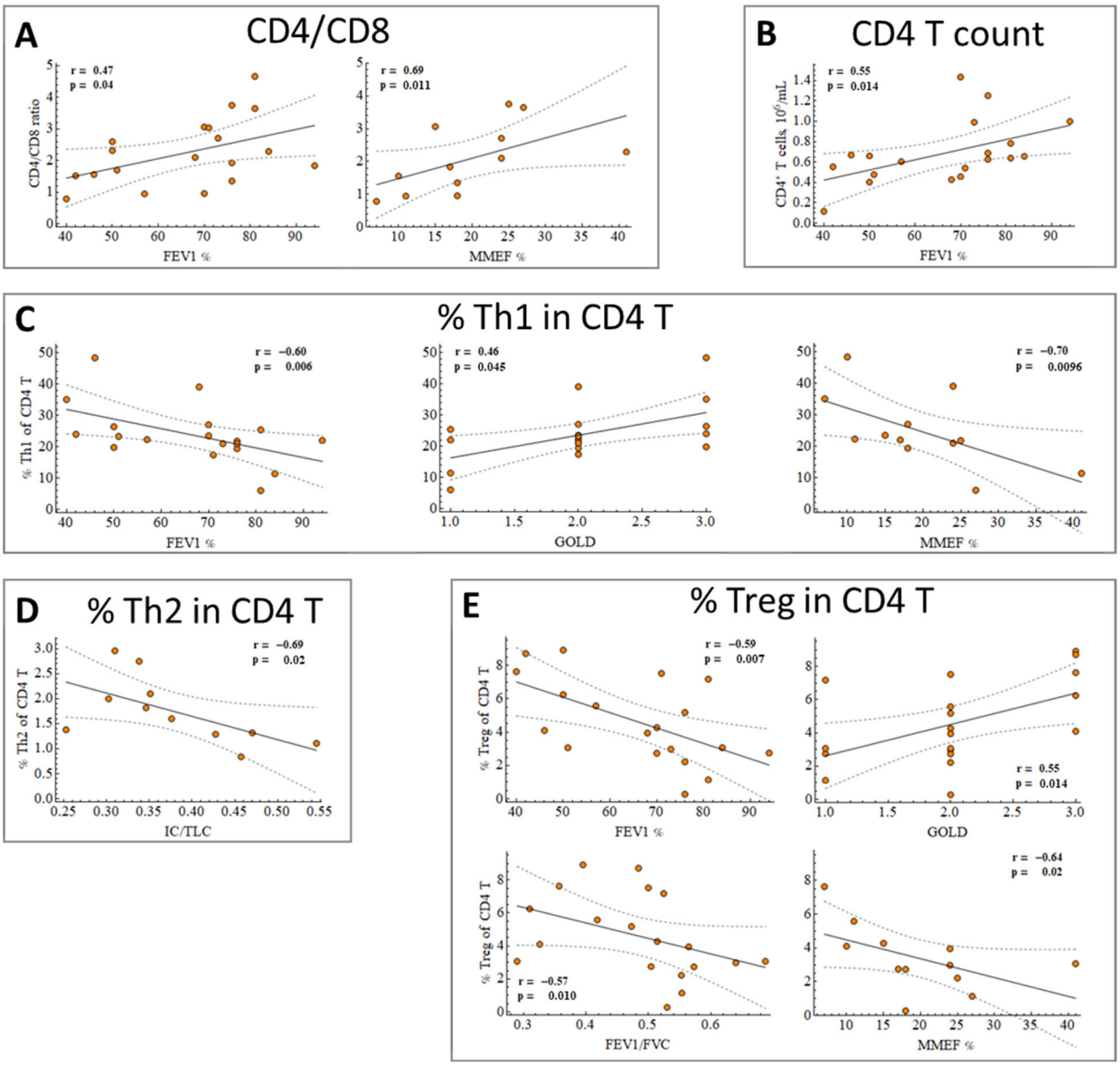

Figure 18. Significant correlations of T cell subpopulations with COPD severity indicators in female COPD group

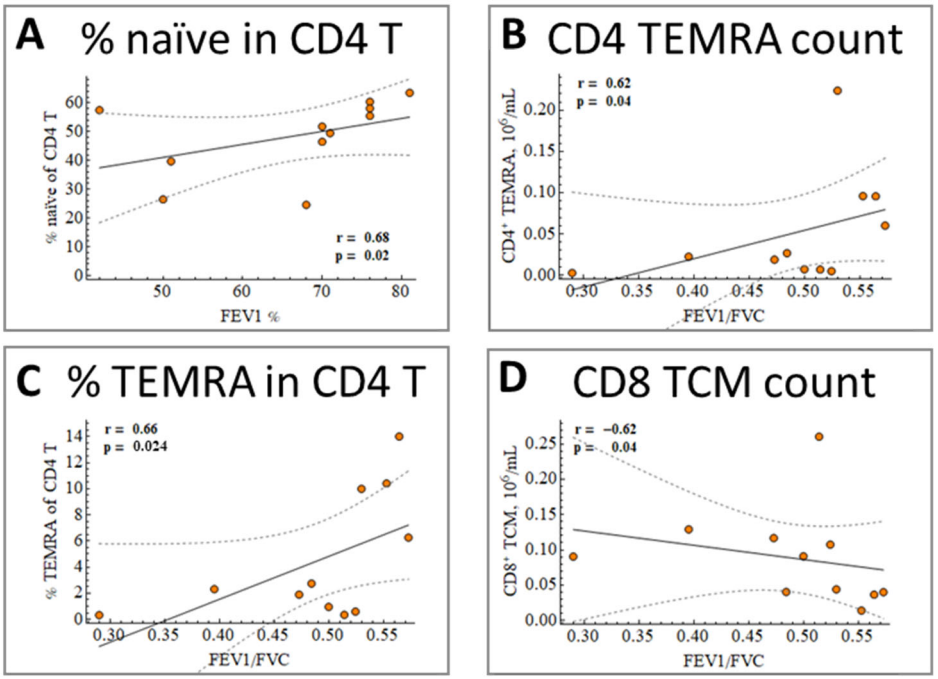

Figure 19. Significant correlations of memory T cell subpopulations with COPD severity indicators in female COPD group

In female COPD, several populations of the myeloid compartment increase with the disease severity (Figure 15). Indeed, absolute counts of eosinophils (Figure 20 A), classical monocytes (Figure 20 B), and basophils (Figure 20 C) increased with the disease severity. Within monocytes, frequencies of different subpopulations changed along with the disease severity progression: on one hand a reduction of the frequency of intermediate monocyte (Figure 20 D) and frequency and count of the immunosuppressive $CD14^{+}CD16^{-}HLA\text{-}DR^{-/low}$ M-MDSC were observed (Figure 20 E-F), while, on the other hand, a parallel increase in the frequency and number of conventional classical monocytes ($CD14^{+}CD16^{-}HLA\text{-}DR^{+}$) was detected (Figure 20 G-H). Interestingly, both absolute count and frequency of M-MDSC correlated exclusively with the parameters related to dynamic lung volumes, in case of MDSC rate – with all the four dynamic lung volume parameters analyzed (FEV1%, FEV1/FVC, MMEF%, and GOLD; Figure 20 E-F). Additionally, the DC1 percentage among total dendritic cells correlated directly with COPD progression in female COPD (Figure 20 I). DC1 percentage was also increased, even though not significantly (Mean±SD of 5.3±2.4 vs 4.0±2.0, p=0.06), in the COPD female group in comparison to control.

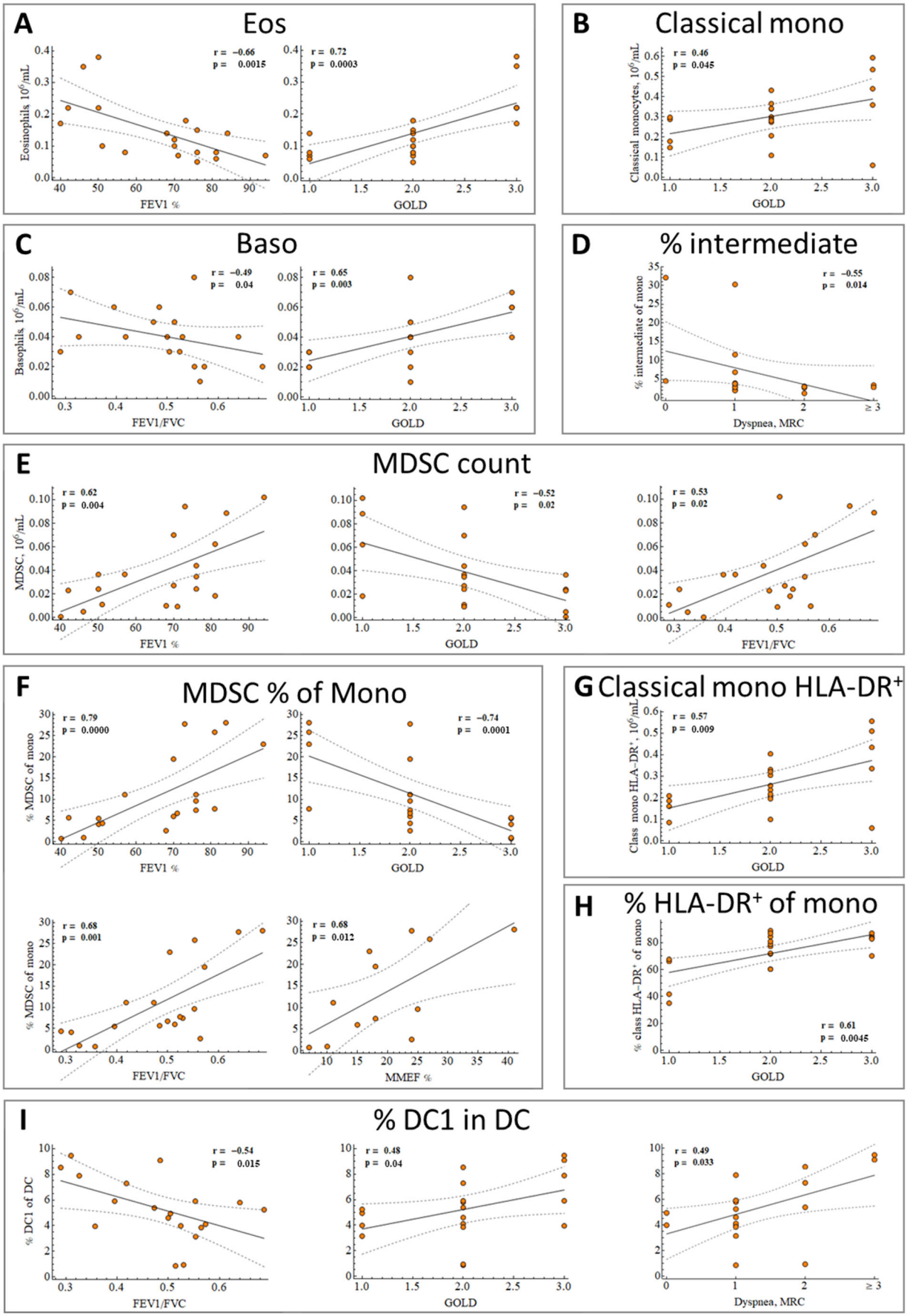

Figure 20. Significant correlations of myeloid subpopulations with COPD severity indicators in female COPD group

Interestingly, some of the parameters that correlated to GOLD stage in female COPD cohort, such as classical monocyte, eosinophil and basophil count and DC1 rate among total DC, also strongly correlated with each other in this group. None of these correlations were observed in male COPD (Figure 21).

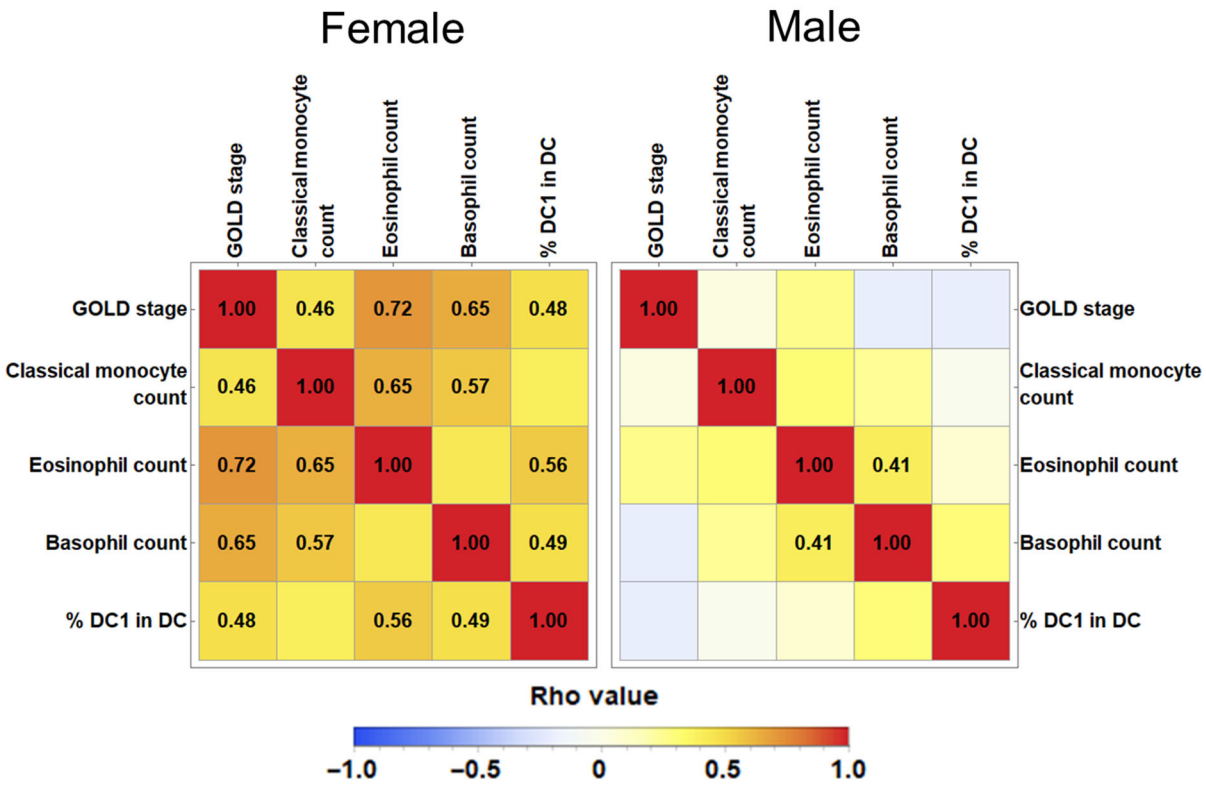

Figure 21. Correlation between COPD GOLD stage, classical monocyte count, eosinophil count, basophil count and DC1 rate in DC in female and male COPD cohorts. Numbers in the wells correspond to Spearman rho value of significant correlations, while empty wells correspond to no significant correlation.

T cell activation and exhaustion

Reduction of T cell count, which we observe with COPD severity rise in some T cell subtypes in both males and females, was described in other inflammatory pathologies, and it has been proposed to be determined by apoptosis of exhausted cells (Tavakolpour et al. 2020). To assess the level of exhaustion of COPD T cells we examined PD1 expression on the total, $CD4^+$, and $CD8^+$ T cells. Cytofluorimetric analysis showed that total T lymphocytes, as well as CD4 T and

CD8 T populations exhibit higher frequency of PD1⁺ cells only in the female COPD cohort as compared to the age-matched female controls (Figure 22).

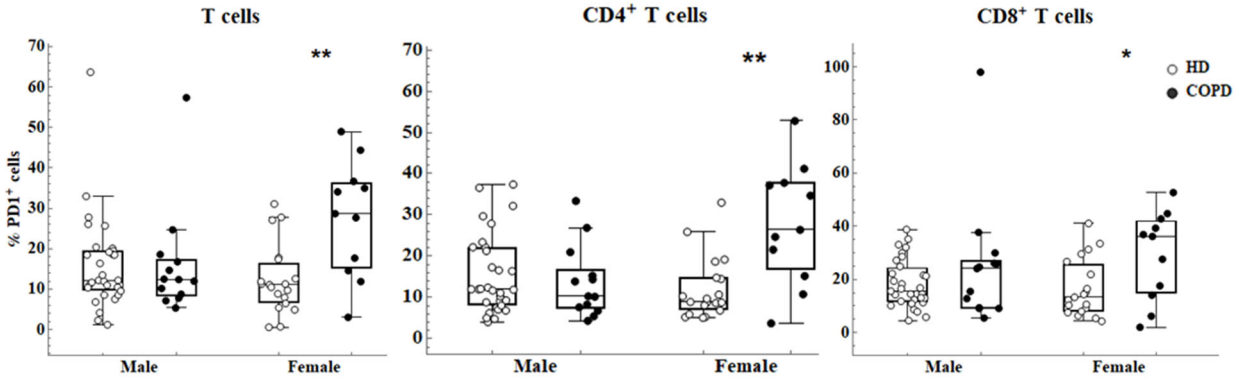

Figure 22. Percentage of PD1 expressing cells among total, CD4⁺, and CD8⁺ T cells in male and female COPD and control subjects

Despite no significant difference in the number of PD1-expressing lymphocytes in the male COPD group as compared to male controls, the level of membrane PD1 expression on T cells increased with COPD severity in this group (Figure 23), directly correlating with the GOLD stage and inversely correlating with IC/TLC, FEV1/FVC (Figure 24 A). PD1 expression level on CD4⁺ and CD8⁺ T cells also increased in males with COPD severity rise, in case of CD4⁺ T cells correlating directly with the GOLD stage and inversely with FEV1/FVC ratio, and in case of CD8⁺ T cells – inversely correlating with IC/TLC and FEV1/FVC (Figure 24 B and C, respectively).

In female COPD PD1 expression in T cells also correlated with COPD severity (Figure 23), as indicated by the significant negative correlations of PD1⁺ cell rate in total T cells and PD1 expression level on CD4⁺ T cells with FEV1% (Figure 25 A and B, respectively).

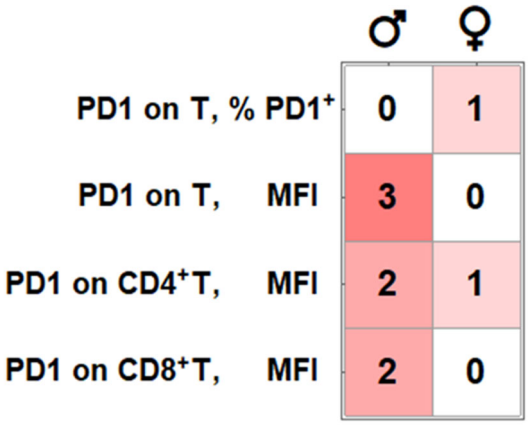

Figure 23. The number of significant correlations between PD1 expression on T cells and COPD severity indicators (decrease of FEV1%, FEV1/FVC, IC/TLC, increase of GOLD stage and dyspnea score).

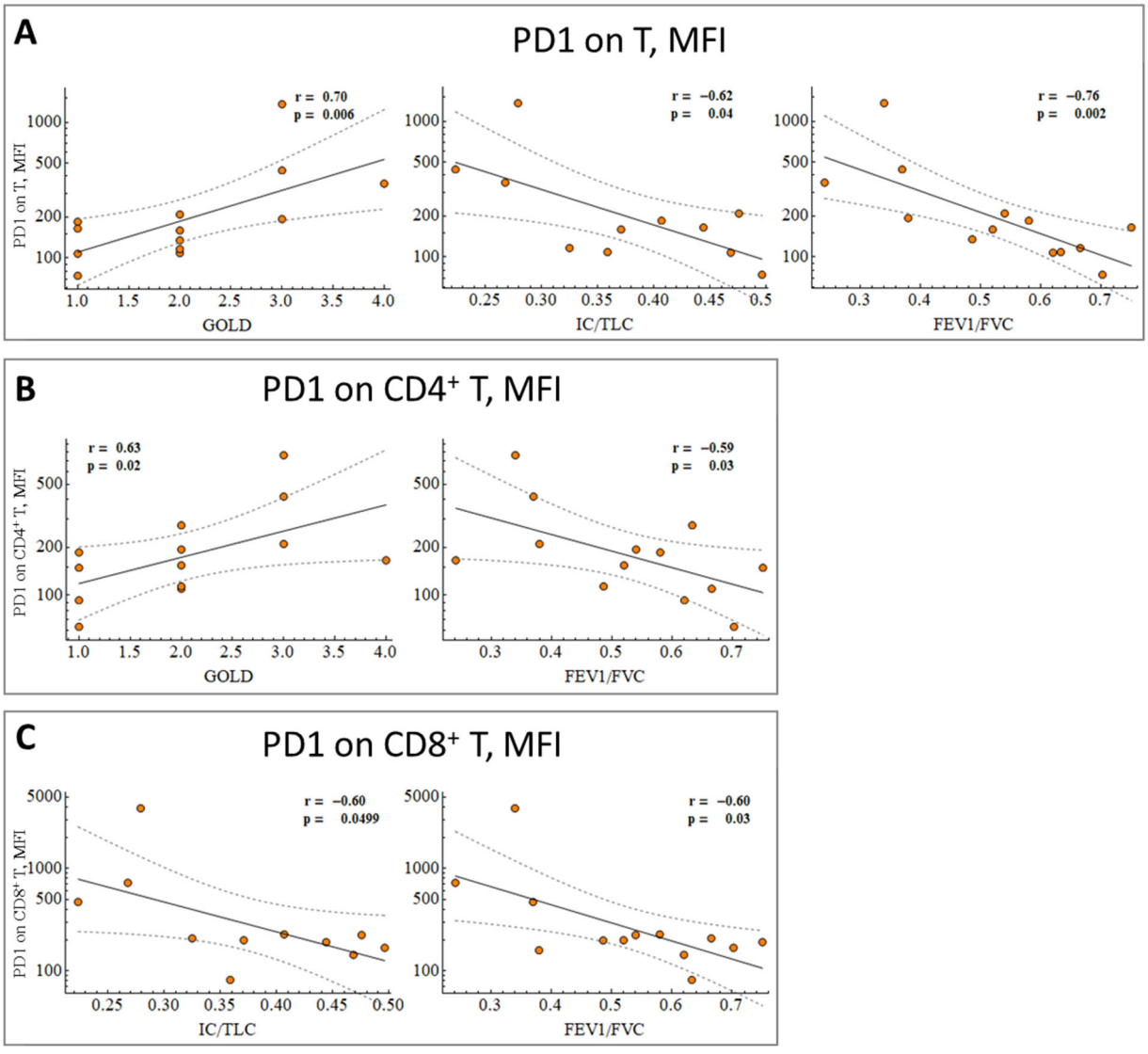

Figure 24. Correlation plots for the significant correlations between PD1 expression on T cells and COPD severity indicators in male COPD group. For clarity, PD1 expression level is represented in Log scale.

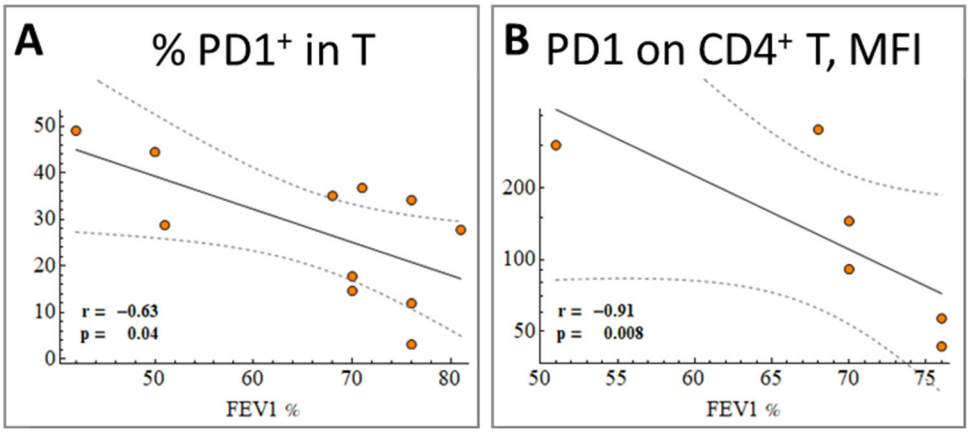

Figure 25. Correlation plots for the significant correlations between PD1 expression on T cells and COPD severity indicators in female COPD group. For clarity, PD1 expression level is represented in Log scale.

PD1 expression on the T cells significantly inversely correlated with the T cell count, which points to the relationship between T cell exhaustion and their decline in number (Figure 26). The significant correlation was observed only on the mixed cohort, while in male and female COPD groups separately it did not reach significance, more likely due to an insufficient number of subjects. The rate of PD1⁺ cells among the T cells also directly correlated with Treg rate and count in the pooled COPD cohort (Figure 27 A and B, respectively), indicating that inhibitory cell rate increases together with T cell susceptibility to inhibitory signals.

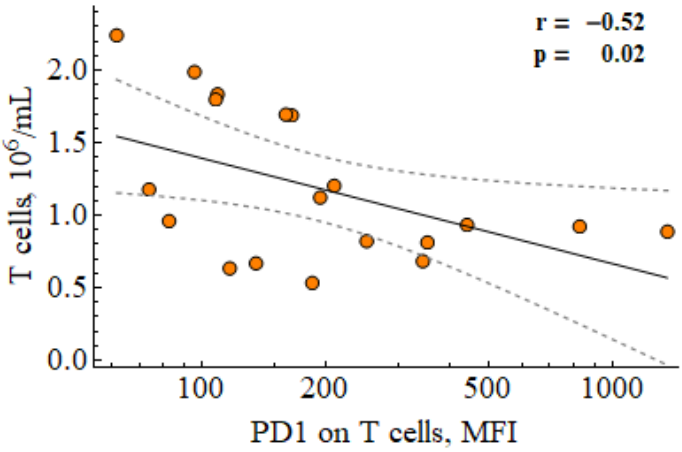

Figure 26. Correlation between PD1 expression on T cells and T cell concentration in the blood in total COPD group. For clarity, PD1 expression level is represented in Log scale.

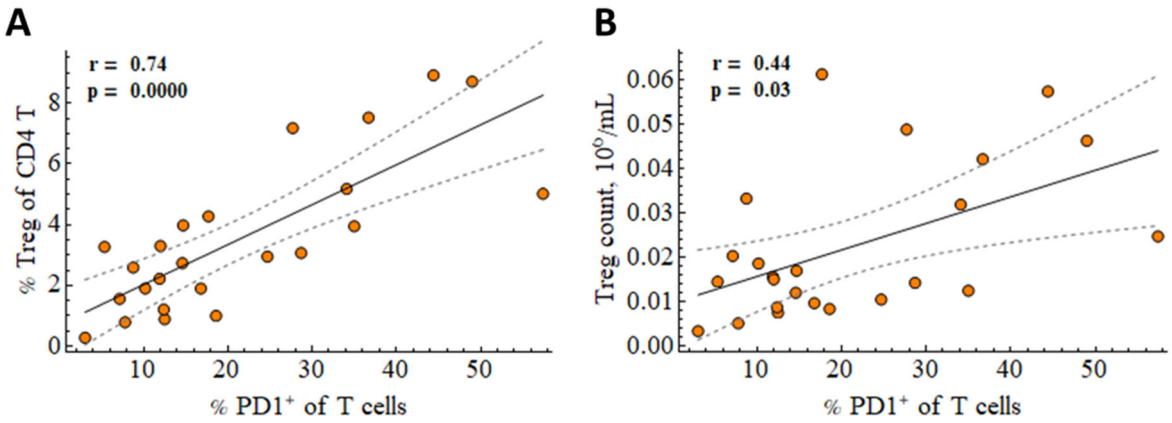

Figure 27. Correlations of frequency of PD1+ cells among T cells with Treg rate among CD4+ T cells (A) and Treg count (B).

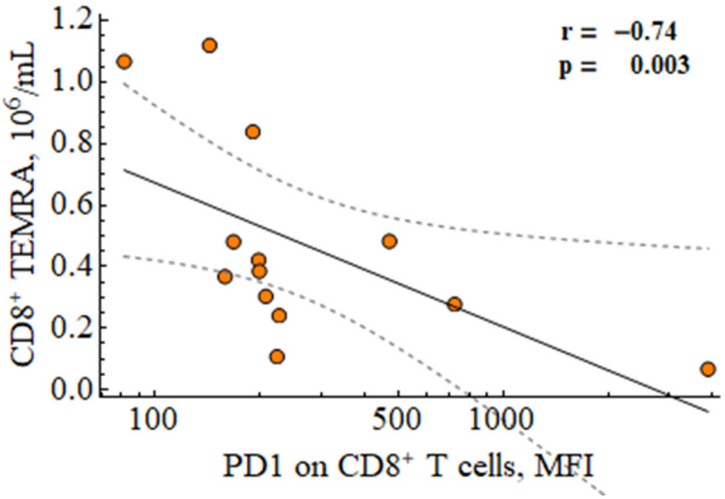

Figure 28. Correlation of PD1 expression on CD8+ T cells with CD8+ TEMRA cell count in male COPD. For clarity, PD1 expression level is represented in Log scale.

In addition to PD1 expression, the expression of CXCR3 and CCR6 homing molecules was measured on the cell surface of $CD4^+$ and $CD8^+$ T cells. Both CXCR3 and CCR6 expression levels were decreased on T helper subpopulations of COPD patients in comparison to the ones of control; the significance, however, was reached only for the male population (Figure 29 A, C). CXCR3 on CD8 T cells was also decreased in male COPD compared to control (Figure 29 B). HLA-DR surface expression was higher on $CD4^+$ T cells in female COPD, although the difference did not reach the statistical significance level (172.5 [154.6 – 282.0] *vs* 26.8 [0 – 115.3], p = 0.056).

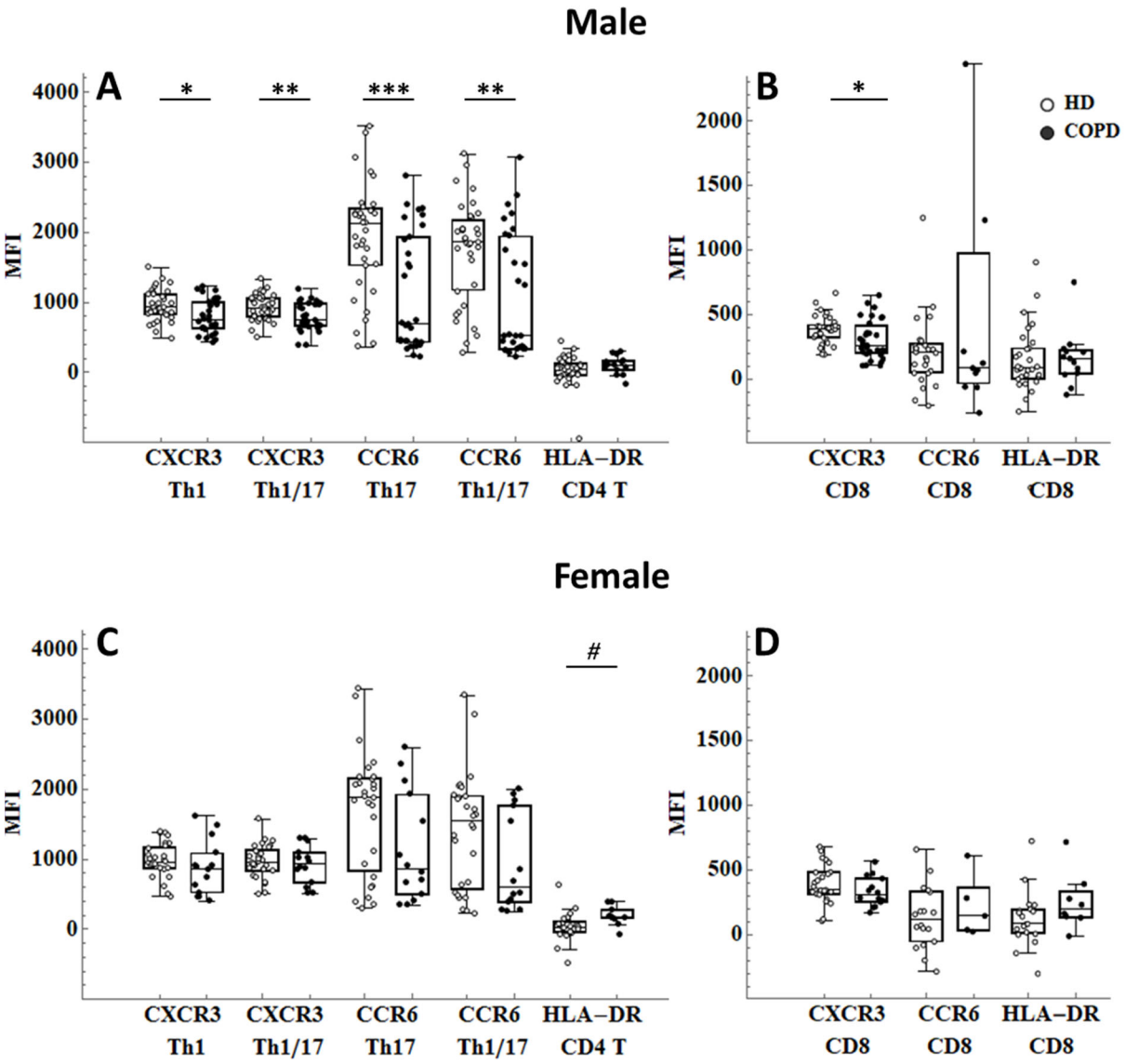

Figure 29. Homing (CXCR3 and CCR6) and activation (HLA-DR) molecules expression on surface of $CD4^+$ (A, C) and $CD8^+$ (B, D) T cells in male (A-B) and female (C-D) cohorts.

RESULTS. PART 2. NEUTROPHIL CHARACTERIZATION

Activation markers expression on surface of neutrophils

The neutrophil population was significantly increased in male COPD patients in comparison to the control group and did not differ in number in females. To assess the differences of male and female COPD neutrophils from respective controls we focused on phenotypical and functional characterization of peripheral blood neutrophils.

To estimate the activation status of neutrophils we measured the surface expression of well-established neutrophil activation markers: L-selectin (CD62L), complement receptor 1 (CD35), Fc-gamma immunoglobulin receptor III (FcyRIII, or CD16), and CD11b and CD11c (αM and αX, respectively). The expression of activation markers was measured by flow cytometry on mature ($CD10^+$) peripheral blood neutrophils, gated according to the strategy depicted in Figure 30.

Activated neutrophils are known to shade CD62L (Ivetic, Hoskins Green, and Hart 2019) and to decrease also surface CD16 expression upon activation (Kuijpers et al. 1991), together with an increase of CD11b (Ivetic, Hoskins Green, and Hart 2019). CD35 is expressed on granule membranes and appears on the cell surface as a result of the membranes fusion upon degranulation (van Staveren et al. 2018). The percentage of $CD10^+$ mature cells among the total neutrophils was even higher in COPD patients compared to controls, thus the neutrophil increase in male COPD in comparison to control is not due to immature neutrophils mobilization from the bone marrow (data not shown). As shown in Figure 31, the level of activation of circulating neutrophils of COPD patients was comparable to neutrophils derived from control donors with a slight increase of surface expression of CD11b in male COPD neutrophils ($p=0.052$) and significant increase of CD16 expression on female neutrophils ($p=0.03$).

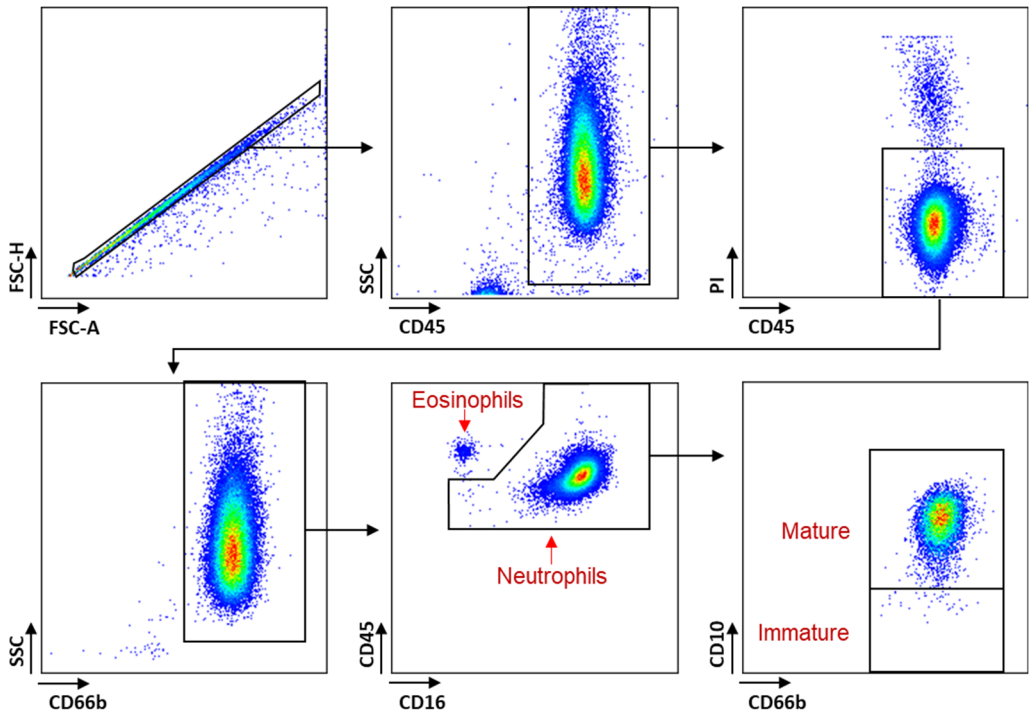

Figure 30. Gating strategy for mature neutrophils. CD66b^{+} neutrophils were gated from alive CD45^{+} high-density fraction leukocyte population by exclusion of CD45^{bright}CD16^{-} eosinophils. After this, mature neutrophils were defined as CD10^{+} cells.

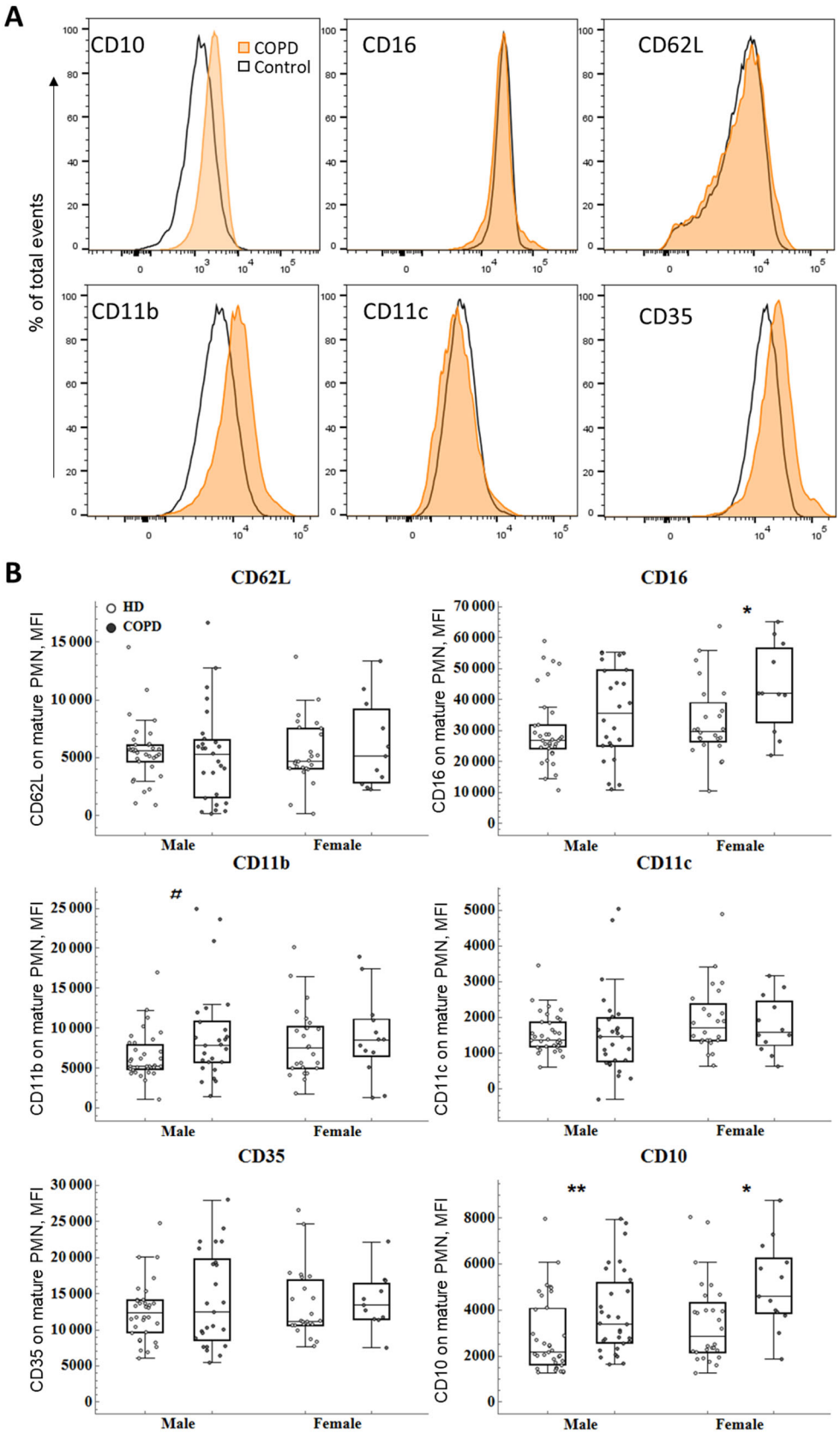

Figure 31. Surface expression of maturation and activation markers on neutrophils, derived from peripheral blood of COPD and control subjects

A – Representative histograms for surface expression of the markers on neutrophils of control subjects (empty histograms) and COPD patients (filled histograms),

B – Box Whisker charts for main activation and maturation markers surface expression represented as median fluorescence intensity and interquartile distribution in control and COPD neutrophils; # – p=0.052, * – p<0.05, ** – p<0.01.

Functional characterization of PMN

Functional characterization of the PMN included reactive oxygen species (ROS) production.

ROS production in response to conventional neutrophil stimuli phorbol myristate acetate (PMA) and formyl-methionyl-leucyl phenylalanine (fMLP), as assessed by cytochrome C reduction test in five COPD patients and six controls, was similar in COPD- and control-derived PMN (Figure 32).

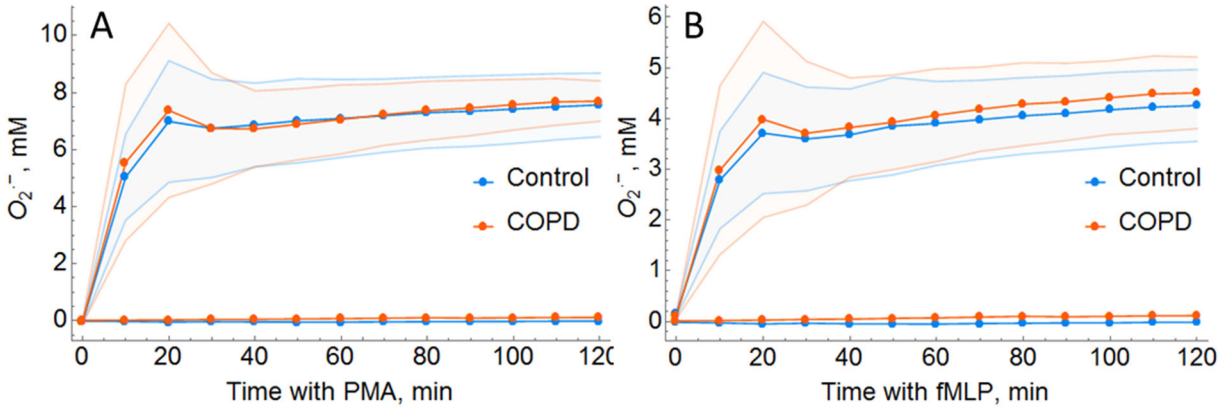

Figure 32. ROS production by stimulated COPD and control neutrophils
Plots represent Mean O_2 production (mM) by 2×10^5 cells/mL neutrophils stimulated by 20 ng/mL PMA (A) or 1nM fMLP (B) at different time points after stimulation. SD are plotted as filled areas of respective colors.

In addition to neutrophil activation identified as ROS production, neutrophil suppressive capacity was assessed. Neutrophils have been demonstrated to gain immunosuppressive functions under specific circumstances, such as cancer and severe acute inflammation, as they have been shown to be able to suppress activated T cell proliferation (Delano et al. 2007; Cuenca et al. 2011).

The suppressive capacity of neutrophils from COPD was assessed by BrDU incorporation-based test.

Purified T cells were incubated alone or with purified granulocytes (in 1:5 ratio) and the intensity of cell division was estimated by an amount of BrDU incorporated into DNA during cell divisions.

BrDU incorporation-based test showed no difference in proliferation capacity of T cells incubated with PMN purified from COPD or control subjects (Figure 33).

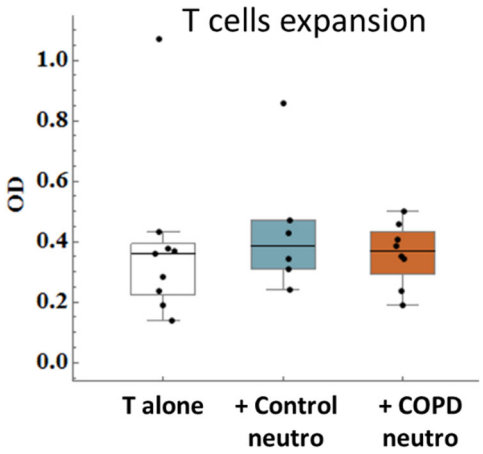

Figure 33. TCR stimulation induced T cell proliferation after 90h of incubation alone or with either control- or COPD-derived PMN. OD – optical density, which corresponds to an amount of BrDU incorporated by cells and bound to antibody.

Collectively, we can conclude that neutrophils from COPD have a higher surface expression of CD10 and, in case of COPD females, CD16, than those from control, while the ability to produce ROS following activation was comparable. Additionally, no T cell suppression was observed in any of the groups.

Transcriptomics for purified male granulocytes

To thoroughly characterize PMN purified from COPD, we performed RNA sequencing of purified PMN of four male control subjects and three COPD patients.

Differential gene expression analysis revealed 231 genes to be significantly differentially expressed in COPD vs control (Figure 34). Among these, 93 genes were upregulated and 138 downregulated in PMN from COPD as compared to PMN from control subjects. Gene ontology enrichment analysis of the differentially expressed genes revealed a high enrichment in processes related to activation/degranulation and GTPase activity (Figure 35). Different types of GTPases are known to play an important role in neutrophil function, participating in the pathways essential for migration and adhesion (McCormick, Chu, and Vermeren 2019). Thus, despite a similar level of activation markers expression and induced ROS production in vitro, PMN from COPD differ from PMN of control subjects in their gene expression pattern, which includes genes related to activation, degranulation, and GTPase activity.

.

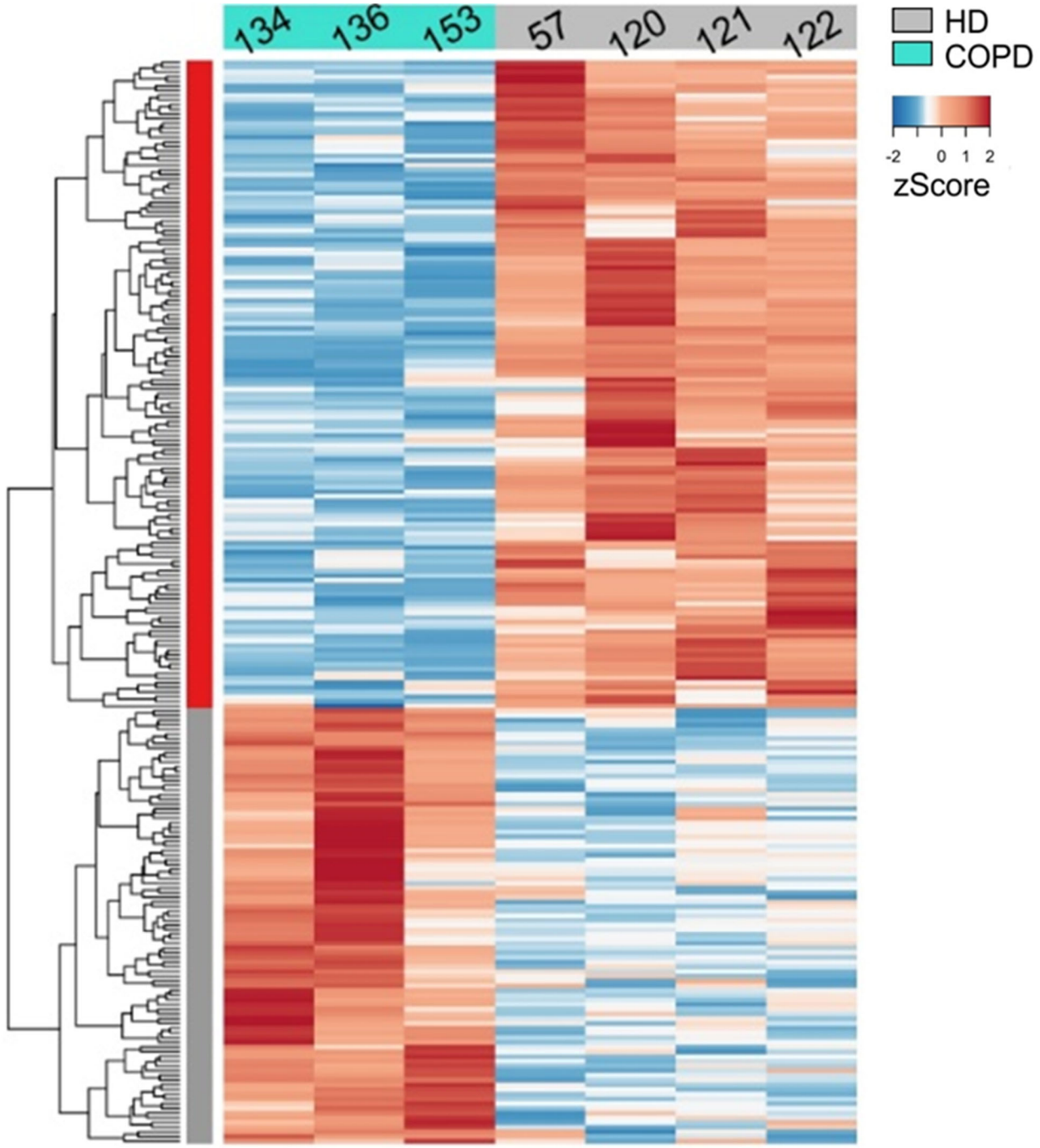

Figure 34. Heat map of gene expression in not-activated peripheral blood neutrophils derived from male COPD patients and male non-COPD controls

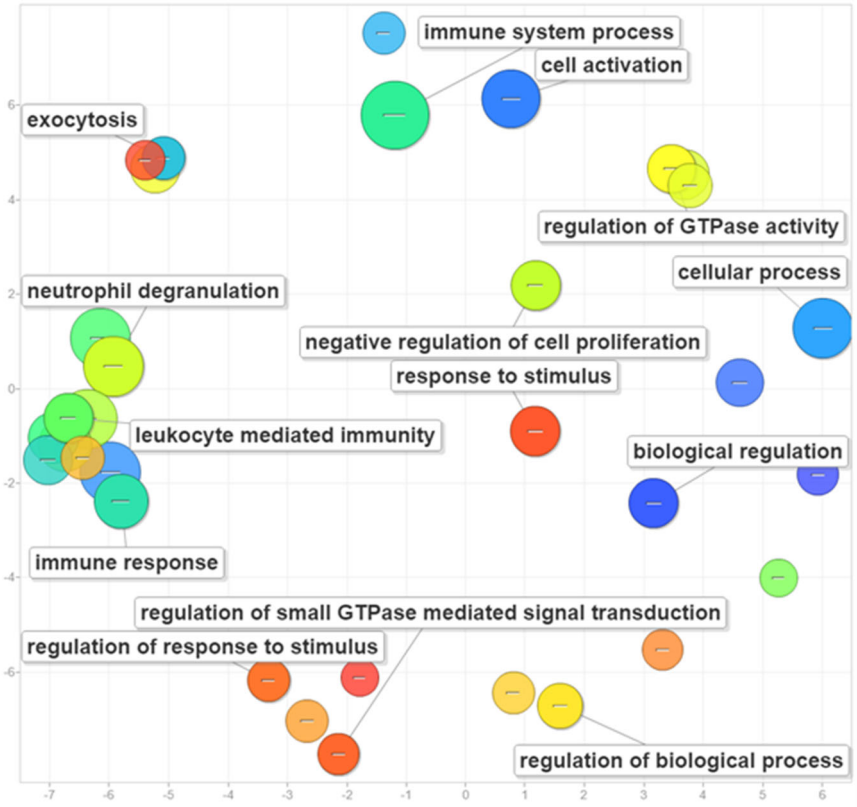

Figure 35. REVIGO semantic plot for GO enrichment analysis of genes, differentially expressed in peripheral blood neutrophils of COPD patients in comparison to age-matched controls. Each ball corresponds to significantly enriched GO term, while the size of the balls reflects log10 P value of GO term enrichment.

To estimate the extent of upregulation and downregulation signals in COPD, we calculated the expression score for the genes significantly upregulated and downregulated in COPD granulocytes as compared to controls. The score was calculated as follows:

1. Standardized gene expression (SGE) was calculated for each of the differentially expressed genes (Brkic 2013, Brkic 2006, Klrou 2004), according to the formula $\frac{X-\bar{X}_{Control}}{SD_{X_{Control}}}$, where X is the expression level of the gene of interest, $\bar{X}_{Control}$ is the mean value of the same gene in the control group and $SD_{X_{Control}}$ is the standard deviation of the same gene in the control group.

2. The “positive” and “negative” scores were obtained by summing up SGE of all the genes identified as significantly upregulated or downregulated in COPD as compared to control, respectively.

The scores obtained for each of the seven subjects sequenced are shown in Figure 36. As expected, in both cases there is a significant difference in the score between COPD and control group. However, the negative counterpart of the differentially expressed genes is more pronounced.

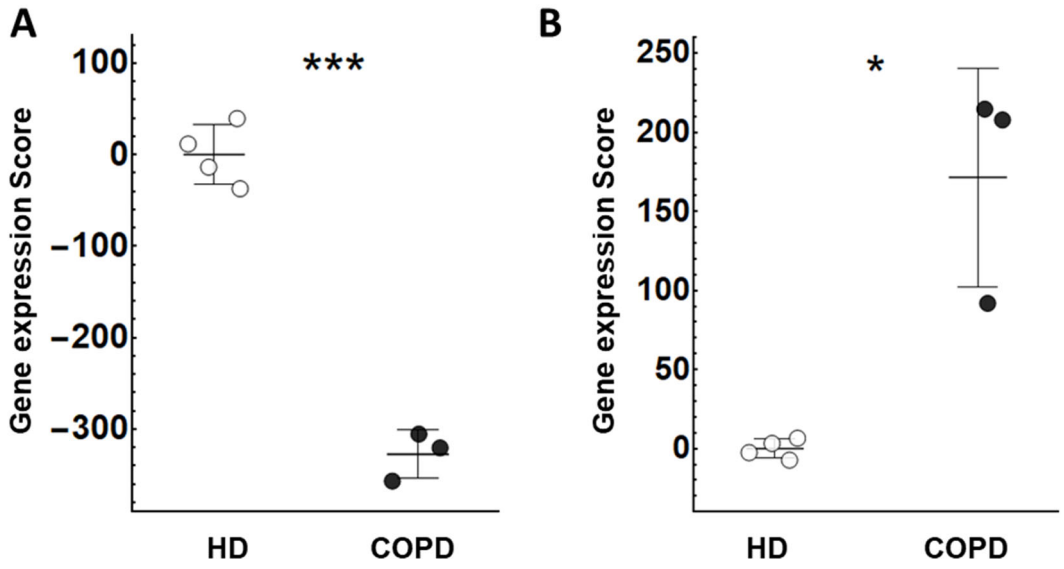

Figure 36. Overall gene expression scores obtained for genes significantly downregulated (A) and upregulated (B) in COPD. Mean±SD and individual samples values are presented; * – $p \leq 0.05$, *** – $p \leq 0.001$.

Differentially expressed genes together with the scores identified in the discovery cohort, were subsequently validated in a second cohort (validation cohort) consisting in 24 COPD and 26 control subjects. PMN RNA from the validation cohort were sequenced and 87 of 93 genes upregulated in the discovery cohort and 100 of 138 genes downregulated in the discovery cohort were validated.

Consistently with the discovery cohort, the “negative” COPD score was significantly lower in COPD patients than in controls (Figure 37 A) and “positive” COPD score – significantly higher (Figure 38 A). “Negative” score

alone distinguished COPD patients from age-matched control donors with the sensitivity of 70.8 % and specificity of 65.2 % for the cutoff of -27.5. Area under the Receiver Operating Characteristic (ROC) curve for the model, based only on downregulated genes score, reached 0.75 (Figure 37 B).

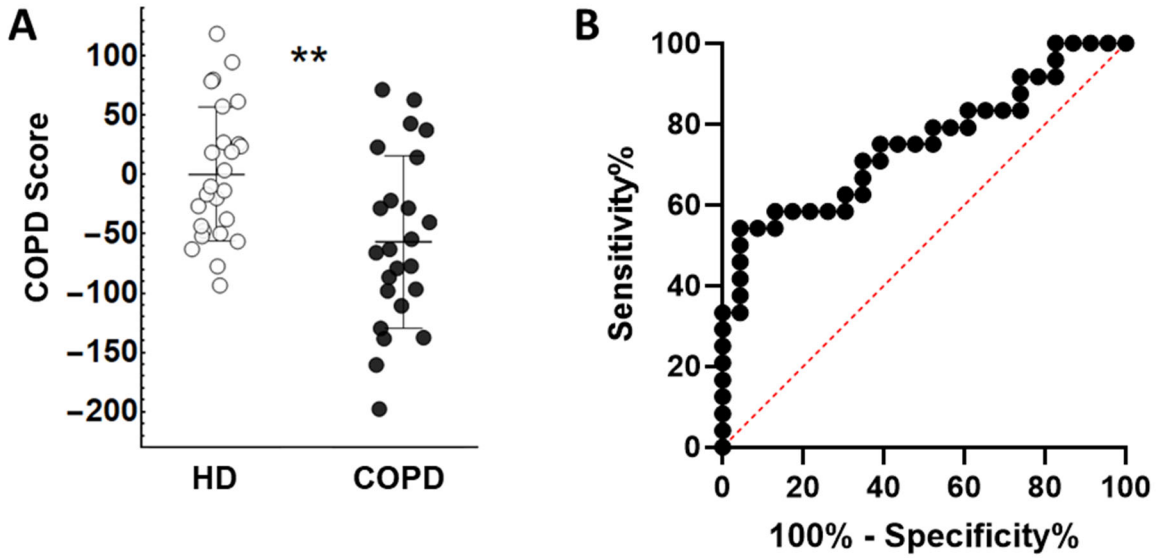

Figure 37. “COPD score” value for downregulated genes in validation cohort of 27 male controls and 26 male COPD patients
A – COPD score values (Mean±SD and individual samples values are presented), B – ROC curve for COPD discrimination by the score values; ** – $p<0.01$.

Same but with lesser extent was true for the “positive” COPD score – the area under the ROC curve for the classifier based on upregulated genes was 0.68, while sensitivity reached 62.5 % and specificity – 65.2 % for the cutoff of 13.9.

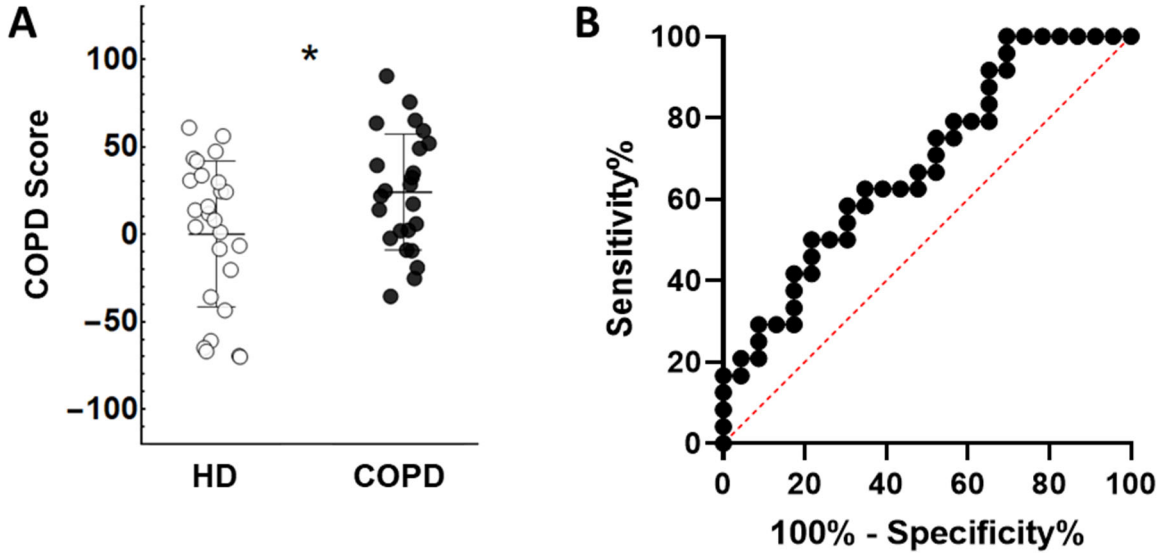

Figure 38. "COPD score" value for upregulated genes in validation cohort of 27 male controls and 26 male COPD patients
A – COPD score values (Mean±SD and individual samples values are presented), B – ROC curve for COPD discrimination by the score values; * – p<0.05.

"Negative" COPD score significantly correlated with the age in COPD, but not in control group (Figure 39). After the adjustment for the age, "negative" score was found to be significantly associated with emphysema level in COPD group, as assessed either by gas transfusion capacity or by direct CT scans analysis (Table 8). However, more patients with available CT scans are needed to validate this indication.

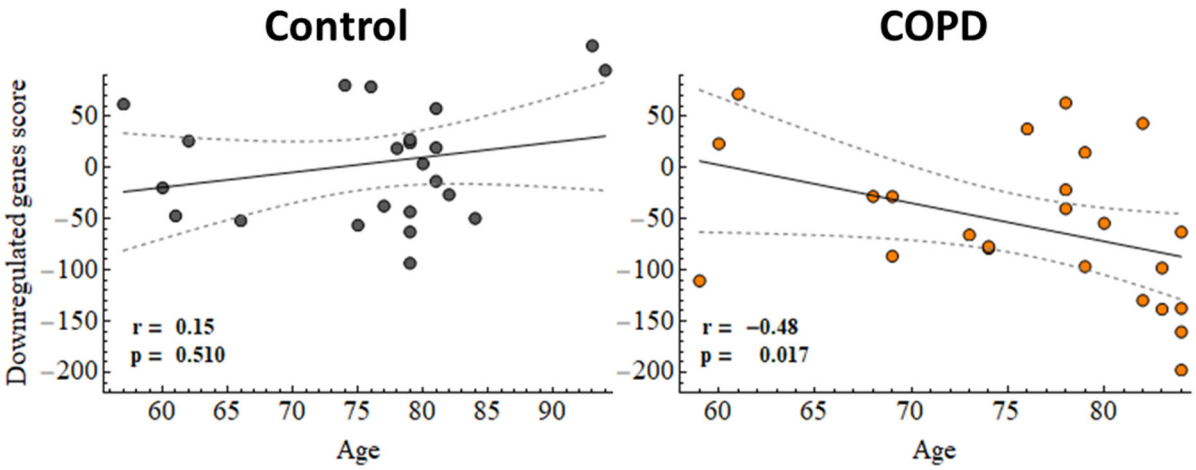

Figure 39. Downregulated genes score correlation with the age in male control and COPD subjects

Table 8. The association of COPD score for downregulated genes with emphysema severity indicators adjusted for age

	P value	**$R^2_{adjusted}$ ***	**n**
DLCO % adjusted for Age:	0.054	0.54	19
KCO % adjusted for Age:	0.021	0.58	19
Emphysema level adjusted for Age:	0.0056	0.96	7

* $R^2_{adjusted}$ for the model including Age, emphysema indicator and their interaction

Collectively, transcriptomic profile of the granulocytes from COPD patients revealed a COPD-specific signature, that distinguished these cells from the control counterpart, despite a comparable phenotypic and functional features. The pronouncement of the negative counterpart of this signature is likely to be associated with emphysema severity.

CONCLUSION

The main results of this work can be summarized as follows:

1. Gender plays a central role in driving changes of the composition of circulating leukocyte in stable COPD subjects: while neutrophilia and increased neutrophil to lymphocyte ratio are the hallmarks of COPD in male, female COPD, on the contrary, are characterized by a generalized leukopenia.

2. A decrease of the absolute number of lymphoid population (B cells, T cells, NKT, CD4T cells and CD8 naïve T cells) in male, and an increase of the absolute number of myeloid population (eosinophils, basophils, classical monocytes) in female correlate with the degree of COPD severity.

3. Reduction of different populations of T cells along with COPD severity was observed in both males and females. Such a decrease is likely due to T cell exhaustion, as suggested by increase in $PD1^+$ cells.

4. Neutrophils purified form male COPD patients do not display significant changes at the functional level (as assessed by induced ROS generation and activation state). Nevertheless, they are characterized by a distinct transcriptional profile. Analysis of COPD granulocyte transcriptome allowed us to identify a specific molecular “signature”.

DISCUSSION

Leukocyte composition alterations

The number of accompanying comorbidities and the presence of systemic inflammation associated with symptoms worsening and comorbidities progression make COPD to be considered as a systemic disease. Several research groups focused their attention on peripheral blood leukocytes composition and immune-phenotype to assess the changes occurring in these cells in COPD and their relation to the inflammatory pattern in the lungs. The overall picture derived from these studies is quite conflicting, and the results of these studies range from reporting a significant increase in total leukocyte count (Taylan et al. 2017; Koo et al. 2017; Bilir et al. 2016; A. Agustí et al. 2012; Fratta Pasini et al. 2016) and in neutrophil count (Bilir et al. 2016; İn et al. 2016), to describing no difference of assessed leukocyte concentrations between the COPD and control groups (Sun et al. 2018; Koch et al. 2007; Scrimini et al. 2013).

Our data obtained on the male COPD cohort are consistent with most other reports: we observed an increase of WBC and neutrophil count, which also was the main driver for significant NLR increase in the male group. Surprisingly, these findings have not been confirmed in the female cohort. Conversely, we demonstrate for the first time that circulating leukocytes in female COPD are characterized by a significant leukopenia, resulting from a decrease of almost every leukocyte population. Other than highlighting that alteration of the immune system homeostasis in subjects with COPD occurs in a gender-specific manner, this finding helps to reconcile the conflictual data of the literature. In fact, one of the reasons for such discrepancy may be the heterogeneity of the composition of the COPD cohorts under investigation. Indeed, retrospectively we analyzed the gender composition of the cohorts recruited in these contradictory studies and found out that the studies reporting a significant

increase in WBC and neutrophil count have been performed on cohorts composed predominantly of male subjects, while no difference in circulating leukocyte parameters have been reported for studies performed on gender-balanced cohorts.

Remarkably, WBC count in female COPD was not increased as compared to their respective controls, but it even occurred to be significantly decreased. The reduction of leukocyte number was observed in all the main leukocyte types and reached significant values for monocytes, lymphocytes and B cells. Decrease in WBC count can be consequent to different conditions. For instance, one of the most frequent conditions that can possibly lead to WBC count decrease is hypothyroidism. Hypothyroidism was previously reported to cause leukopenia, more likely through a general bone marrow depression (Kawa et al. 2010). ANOVA analysis of the female cohort recruited in this study, confirmed a significant reduction in WBC count in females affected by hypothyroidism, regardless the presence of COPD pathology. Nevertheless, we can exclude this pathological condition as the main driver of leukopenia in female COPD, since WBC counts remained significantly lower in female COPD vs control even after removing subjects affected by hypothyroidism (not shown).

Lymphopenia occurs also in a number of conditions characterized by acute inflammation, such as respiratory viral infection and sepsis (Mazzoni et al. 2020; Venet et al. 2010), and autoimmune diseases (Schulze-Koops 2004). Inflammation-related lymphopenia has been shown to result from three main mechanisms: (I) lymphocyte migration from circulation to inflamed tissue (Cavaillon and Adib-Conquy 2010); (II) bone marrow deprivation (Binder et al. 1997); (III) lymphocyte exhaustion followed by their apoptosis through PD1-PDL1, Fas-FasL, or other pathways (Tavakolpour et al. 2020; Aggarwal, Gollapudi, and Gupta 1999; Cavaillon and Adib-Conquy 2010). Lymphocyte apoptosis was associated with the elevated levels of IL-6 and TNFα.

TNFα-induced apoptosis was demonstrated for monocytes and T cells (Aggarwal, Gollapudi, and Gupta 1999; Shen et al. 2005). Both TNFα and IL-6 plasma levels are known to be elevated in COPD patients (Yasuda et al. 1998; W. Q. Gan 2004), suggesting that an increased lymphocyte apoptosis may be responsible for a decline in lymphocyte number in COPD. PD1-PDL1 interaction is one of the main triggers of apoptosis of activated and exhausted T cells (Mandal, Mehta, and Prajapati 2018). Kalathil et al have previously shown an increase of PD1 expression on the surface of circulating CD4 T cells, as well as an increase in Treg percentage and count in COPD patients compared to healthy controls (Kalathil et al. 2014). Here we confirm an increased PD1 expression on circulating CD4 T cells and report the same for CD8 T cells (at least in female and severe male COPD). We also observed a significant correlation between PD1 expression on T cells and immunosuppressive Treg subset frequency and count. Remarkably, we showed that PD1 expression level on T cells significantly inversely correlated with T cell count, indicating that PD1-triggered T cell apoptosis is involved in COPD lymphopenia.

CD4 T cell count decreased with COPD progression in both male and female cohorts, while CD8 T cell count was similar in COPD and control and did not correlate with COPD severity. These results are consistent with the findings of Tang et al. and Roberts et al, who demonstrated a decrease of CD4 T cell count in moderate-to-severe COPD patients in comparison to gender-, age-, and smoking matched controls and no difference in CD8 T cells (Tang et al. 2013; Roberts et al. 2015). Possible pathological role of CD4 T cells in emphysema development was shown by Taraseviciene-Stewart et al, who demonstrated that adoptive transfer of syngeneic CD4+ lymphocytes that had been sensitized to endothelial cell antigens resulted in the development of emphysema in naïve rats (Taraseviciene-Stewart et al. 2005). Roberts et al. in their paper relate circulating CD4 T cell decrease to their possible migration to the inflamed lungs.

However, it does not explain an absence of changes in CD8 T cell count, as both CD4 and CD8 T cells infiltrate the lungs of COPD patients.

We observed a significant increase of Treg percentage in CD4 T cells in both male and female COPD patients in comparison to respective controls. In the female COPD group, Treg increase correlated with most of the clinical parameters related to the disease progression, such as FEV1%, GOLD, FEV1/FVC, and MMEF%, while in males such correlations were not observed. There are controversial data about circulating Treg in COPD. Kalathil et al reported an increase of the percentage and absolute concentration of $CD127^{-}CD25^{+}Foxp3^{+}$ Treg cells in COPD in comparison to control (Kalathil et al. 2014), while Hou et al reported a decrease in total count and percentage among CD4 T cells of two Treg subpopulations ($CD25^{+++}CD45RA^{+}$ and $CD25^{+++}CD45RA^{-}$) in COPD in comparison to healthy donors and an increase of a third and prevalent Treg subpopulation ($CD25^{++}CD45RA^{-}$) percentage and count (Hou et al. 2013). Such inconsistent data may reflect differences in Treg definition: we identified Treg as $CD3^{+}CD4^{+}CD127^{-}CD25^{+}$ cells, without assessing Foxp3 intracellular expression, according to Seddiki et al, who have shown a strong correspondence of this phenotype to Foxp3 expression (Seddiki et al. 2006). Nonetheless, correlation of this population with those described in other works needs to be investigated, and might explain the discrepancy of the results.

We also observed a decrease of homing molecules CXCR3 and CCR6 expression on the surface of T cells in the total and male, but not in the female cohort. Chemokine receptor CXCR3 is known as a type-1 immune response molecule. It is expressed on Th1-committed $CD4^{+}$ T cells and activated Th17 cells acquiring Th1 functions – Th1/17 population (Cosmi et al. 2011), but also on other leukocytes. In $CD8^{+}$ CTL, CXCR3 expression characterizes TEM and TEMRA memory cell subpopulations (Willinger et al. 2005). In 2007 Koch et al.

reported an increase of $CXCR3^+$ cells proportion in CD8 T cells in currently smoking COPD patients compared to smoking or non-smoking healthy donors (Koch et al. 2007). In our cohort, we observed a decrease of CXCR3 expression by CD8 T cells in COPD patients, which was significant for the total and male cohorts, but not significant in females. The discrepancy with the data reported by 2007 Koch et al. can be given by the difference in age, as their small cohort consisted of much younger subjects. We also demonstrated a significant decrease of CXCR3 expression on Th1 and Th1/17 cells of male but not female COPD patients, as well as CCR6 repression on Th17 and Th1/17 cells. The decrease of homing molecules expression may be either a response to anti-inflammatory signals that decrease activation and/or migratory capacity of T cells or a result of the recent migration of competent cells to inflammatory sites so that the cells left in the circulation express lower levels of homing molecules. Interestingly, Th1/17 cells – activated Th17 cells that are able to produce both IFNγ and IL-17 and appear to be involved in different chronic inflammatory diseases (Cosmi et al. 2011), demonstrated a decrease of both CXCR3 and CCR6 surface expression. Grumelli et al have previously shown an increased surface density of CXCR3 chemokine on lung T cells of emphysematous patients in comparison to controls (Grumelli et al. 2004). This favors the hypothesis of the excessive migration of $CXCR3^{bright}$ cells to the lungs rather than CXCR3 downregulation.

In addition to CD4 T cells, we demonstrated a decrease of NKT count with COPD progression in the male cohort. To our knowledge, there are no publications assessing NKT count alterations in COPD. However, Urbanowitz et al have previously shown NKT percentage in PBMC to be decreased in moderate-severe COPD patients in comparison to smoking or non-smoking controls (Urbanowicz et al. 2009).

The involvement of B cells in COPD pathology is actively studied. Several articles report an increase in B cell count in different lung areas in COPD. Moreover, B cell-enriched lymphoid follicles were found in the lungs of COPD patients. The number of these lymphoid follicles was associated with an emphysema-prevalent phenotype and with a poor prognosis of the disease (Polverino et al. 2016). However, very little is known about circulating B cells in COPD. Brandsma et al. have reported a decreased B cell percentage in COPD patients in comparison to either smoking or non-smoking healthy donors (Brandsma et al. 2009). We demonstrated that B cell count was decreased in females with COPD independently of the GOLD stage, while in males there was no significant difference in B cell count between COPD and control group. However, B cell count in males correlated significantly with all the six COPD severity indicators analyzed, while none of such correlations were observed in females. This is explained by non-linear dynamics in B cell count change during COPD progression in the male group with a significant increase of B cells number at GOLD1-2 and a significant decrease at GOLD3-4 stages (data not shown). Why the dynamics of B cell count changes in COPD differs in males in females remains unclear. The explanation can be related to sexual dimorphism in gene expression signature of B cells, demonstrated by Fan et al on healthy males and females (Fan et al. 2014), which means that male and female B cells may respond differently to similar stimuli.

Intriguingly, systemic changes occurring in females, but not males, with COPD progression resemble the immune signature of asthma. The increase of eosinophil, basophil, and classical monocyte count, which we observed in the COPD female cohort with the disease progression, was previously shown for asthma (Tomita et al. 1995; Hill et al. 2014), where peripheral blood classical monocytes expansion potentiated airway hyper-responsiveness (Kowal et al. 2012). Hayashi et al also demonstrated a relation between Type-2 shift of immune response and an increase of DC1 ($CD141^+$ DC) and a decrease of DC2

(CD1c$^+$ DC) in atopic diseases (Hayashi et al. 2013). In the COPD female cohort, we also observe an increase of DC1 rate in the COPD group (p=0.055, data not shown) that correlates with the disease progression. Moreover, the abovementioned parameters correlate not only with COPD severity but also with each other, while none of these changes occur in male COPD, except the classical monocyte count increase, which, however, correlates neither with COPD progression (Figure 15) nor with eosinophil or basophil count (Figure 21).

The relations between asthma and COPD and their association with gender are under active investigation. Several authors have reported that women not only develop asthma more frequently than men, but also more frequently demonstrate asthma-COPD overlap syndrome (Wheaton et al. 2016; de Marco et al. 2013). Roberts et al. also reported that women with confirmed diagnosis of COPD had significantly higher frequency of allergy in their respiratory medical history, than men. In our cohort we also observed higher prevalence of allergy in COPD women than in men with no such difference in the control group (1 over 30 in men and 5 over 19 in women with COPD, p=0.027).

One of the most unexpected results was a decrease of M-MDSC cell count and rate in severe female COPD in comparison to mild control groups. The role of MDSC decrease in COPD pathology is yet totally unknown. There are several papers describing MDSC in COPD. However, all of them describe different populations: low-density neutrophils (CD14$^-$HLA-DR$^-$CD11b$^+$CD33$^+$ cells in PBMC, (Kalathil et al. 2014)), immature monocyte precursors (Lin$^-$CD14$^-$HLA-DR$^-$CD11b$^+$CD33$^+$, (Tan et al. 2014)) and mature HLA-DR$^{-/low}$ classical monocytes (CD14$^+$HLA-DR$^-$CD11b$^+$CD33$^+$, (Tan et al. 2014)). Tan et al reported no significant difference in in MDSC proportion among PBMC on a small cohort of COPD patients and smoking and of non-smoking controls (donor's sex is not reported) (Tan et al. 2014). In the present work, we also did not observe significant difference in MDSC count or rate between COPD

patients and control group, even in female cohort, where MDSC count and rate correlated with COPD severity indicators. A possible explanation may be that, similarly to B cells in male cohort, MDSC change along with COPD severity in a non-linear manner. In fact, we observed that the rate of MDSC in the monocytes remained similar to control values up to GOLD2 and significantly decreased at GOLD3-4 COPD stages (data not shown). This hypothesis should be confirmed in a larger cohort.

Neutrophils phenotypical and transcriptomic alterations

An increase of neutrophil count in stable COPD, widely described in the literature, occurred to be a characteristic of only male population. This goes in line with the finding of Deitch et al. who have shown in rat model that testosterone may be responsible for stronger neutrophil activation during non-infectious inflammatory states in males than in females (Deitch et al. 2006).

In the present work we have demonstrated a significant CD10 (neutral endopeptidase) surface expression increase in COPD patients in comparison to controls in both male and female cohorts, as well as CD11b increase on male COPD neutrophils and CD16 increase on female COPD neutrophils. Long-lasting phenotypical changes in resting circulating neutrophils were recently demonstrated in the paper of Moorlag et al. on healthy donors receiving BCG vaccination (Moorlag et al. 2020). They demonstrated a shift of circulating neutrophils towards expressing higher CD10, CD11b, and CD16 and lower CD62L 3 months after the vaccination. They describe these changes to be most likely mediated by epigenetic modifications at the promoter sites of genes essential for antimicrobial function, as they also demonstrated increased levels of H3K4me3 upon BCG vaccination. The increased surface expression of CD11b that we observed on a trend level for male COPD group was also shown by Blidberg et al. on prevalently male COPD patients compared to sex-matched

controls, although CD11b increase in their study did not reach significance as well (Blidberg et al. 2012). Chronic inflammation-induced trained immunity in neutrophils may be a part of a pathological loop of well-established neutrophil-related lung injury in COPD and may partially explain why smoking cessation in COPD patients often does not lead to the remission.

Despite quite few phenotypical changes and no functional changes observed in neutrophils from COPD, the transcriptomic analysis of the discovery cohort revealed a set of 231 genes that differ significantly between COPD and control. This COPD-specific gene signature (reduced to 187 genes) was than validated on validation cohort of 50 donors, showing a significant difference in the cumulative gene scores between COPD and control granulocytes. In addition, after the adjustment for age, the cumulative score of the genes, identified in the discovery cohort as downregulated in COPD, was significantly associated with emphysema grade in COPD patients of validation cohort. However, more samples with available CT scans are needed to validate this indication and to assess the predictive capacity of this signature for emphysema grade.

According to the results of gene enrichment analysis, the most affected biological processes in COPD PMN were neutrophil degranulation and GTPase activity. GTPases play an important role in neutrophil biology and are involved in a vast majority of neutrophil immune functions – migration, degranulation, phagocytosis, ROS production, and NET formation (McCormick, Chu, and Vermeren 2019; Tackenberg et al. 2020). GTPases are also involved in neutrophil apoptosis, which was shown to be significantly decreased in COPD-derived neutrophils in comparison to healthy controls (Zhang et al. 2012).

Based on our findings, it can be concluded that, although the main COPD manifestations are related to the lungs, there are prominent changes in circulating leukocytes related to COPD development and its severity. These changes include alterations in cell count, cell composition disbalance, changes

in the level of surface expression of activation markers and homing molecules, and transcriptomic modifications. Interestingly, the changes occurring in male and female cohorts were not similar. For the future, it would be very interesting to investigate whether also granulocytes from female COPD are characterized by a transcriptomic signature and, in case, identify shared or distinct traits of male and female COPD score.

SUPPLEMENTARY MATERIALS

Table 9. Main leukocyte populations count and rate of total WBC in male COPD and control groups

Male cohort	Median [Q1-Q3] population count, 10^6 cells/mL			Mean±SD population percentage in total WBC, %		
	Control	COPD	P value	Control	COPD	P value
Neutrophil	3.47 [2.88-3.87]	4.48 [3.3-5.04]	0.0023 $	59.71±6.49	64.01±8.25	0.025 t
Lymphocyte	1.62 [1.35-1.87]	1.66 [1.12-1.86]	0.6 t	28.04±5.66	24.28±7.33	0.026 t
Monocyte	0.48 [0.44-0.60]	0.56 [0.47-0.64]	0.2 t	8.71±1.92	8.52±2.18	0.5 $
Eosinophil	0.13 [0.08-0.20]	0.14 [0.07-0.21]	0.9 $	2.81±2.05	2.56±1.67	0.6 $
Basophil	0.04 [0.03-0.06]	0.04 [0.03-0.05]	0.5 $	0.73±0.25	0.63±0.35	0.07 $

$ – Mann-Whitney test, χ – Fisher's exact test, t – Student T-test

Table 10. Main leukocyte populations count and rate of total WBC in female COPD and control groups

Female cohort	Median [Q1-Q3] population count, 10^6 cells/mL			Mean±SD population percentage in total WBC, %		
	Control	COPD	P value	Control	COPD	P value
Neutrophil	3.82 [3.14-5.01]	3.08 [2.40-4.49]	0.15 t	58.69±8.07	58.20±10.75	0.7 $
Lymphocyte	1.99 [1.57-2.39]	1.55 [1.30-1.85]	0.029 t	29.35±6.10	30.63±10.48	0.6 t
Monocyte	0.52 [0.40-0.68]	0.4 [0.35-0.51]	0.03 $	8.44±2.36	7.93±2.05	0.4 $
Eosinophil	0.15 [0.10-0.25]	0.12 [0.08-0.18]	0.3 $	2.86±2.25	2.52±1.05	0.8 $
Basophil	0.04 [0.02-0.06]	0.04 [0.03-0.05]	0.6 t	0.66±0.35	0.72±0.33	0.6 t

$ – Mann-Whitney test, χ – Fisher's exact test, t – Student T-test

Table 11. Demographic and clinical data of the “discovery cohort” participants

	COPD 1	**COPD 2**	**COPD 3**	**Control 1**	**Control 2**	**Control 3**	**Control 4**
Age	73	74	83	74	93	94	81
Smoking	EX	EX	EX	EX	NEVER	NEVER	NEVER
Smoking pack-year index	2.9	30	43.8	150	0	0	0
FEV1%	43	82	32	115	102	97.1	108
Charlson index	3	2	0	3	0	2	1

Table 12. Demographic and clinical data of the “validation cohort” participants

	Control	**COPD**	**P Value**
N	26	24	
Age	79 [74.3-80]	78 [71-82.8]	0.95 $
Smoking (never/ex/current/NA)	19/4/0/3	1/15/6/2	1 × 10-4 χ
Smoking pack-year index	0 [0]	40 [30-63.6]	1.2 × 10-6 $
FEV1%	115 [100-131.3]	59 [43-81.3]	2.0 × 10-8 t
Charlson index	1 [0-3]	2 [1-3]	0.08 $

REFERENCES

Aggarwal, Sudeepta, Sastry Gollapudi, and Sudhir Gupta. 1999. "Increased TNF-Alpha-Induced Apoptosis in Lymphocytes from Aged Humans: Changes in TNF-Alpha Receptor Expression and Activation of Caspases." *Journal of Immunology (Baltimore, Md. : 1950)* 162 (4): 2154–61.

Agustí, A. G.N., A. Noguera, J. Sauleda, E. Sala, J. Pons, and X. Busquets. 2003. "Systemic Effects of Chronic Obstructive Pulmonary Disease." *European Respiratory Journal* 21 (2): 347–60. https://doi.org/10.1183/09031936.03.00405703.

Agustí, Alvar, Lisa D. Edwards, Stephen I. Rennard, William MacNee, Ruth Tal-Singer, Bruce E. Miller, Jørgen Vestbo, et al. 2012. "Persistent Systemic Inflammation Is Associated with Poor Clinical Outcomes in Copd: A Novel Phenotype." Edited by Juan P. de Torres. *PLoS ONE* 7 (5): e37483. https://doi.org/10.1371/journal.pone.0037483.

Aldonyte, Ruta, Lennart Jansson, Eeva Piitulainen, and Sabina Janciauskiene. 2003. "Circulating Monocytes from Healthy Individuals and COPD Patients." *Respiratory Research* 4 (1): 11. https://doi.org/10.1186/1465-9921-4-11.

American Lung Association. 2013. "Trends in COPD (Chronic Bronchitis and Emphysema): Morbidity and Mortality." *American Lung Association*.

Anders, Simon, Paul Theodor Pyl, and Wolfgang Huber. 2014. "HTSeq - A Python Framework to Work with High-Throughput Sequencing Data." *BioRxiv*, August, 002824. https://doi.org/10.1101/002824.

Austin, Victoria, Peter J. Crack, Steven Bozinovski, Alyson A. Miller, and Ross Vlahos. 2016. "COPD and Stroke: Are Systemic Inflammation and Oxidative Stress the Missing Links?" *Clinical Science* 130 (13): 1039–50. https://doi.org/10.1042/CS20160043.

Barnes, P. J., and B. R. Celli. 2009. "Systemic Manifestations and Comorbidities of COPD." *European Respiratory Journal* 33 (5): 1165–85. https://doi.org/10.1183/09031936.00128008.

Barnes, Peter J. 2016. "Inflammatory Mechanisms in Patients with Chronic Obstructive Pulmonary Disease." *Journal of Allergy and Clinical Immunology* 138 (1): 16–27. https://doi.org/10.1016/j.jaci.2016.05.011.

Barnes, Peter J, and Manuel G Cosio. 2004. "Characterization of T Lymphocytes in Chronic Obstructive Pulmonary Disease." *PLoS Medicine* 1 (1): e20. https://doi.org/10.1371/journal.pmed.0010020.

Bhat, Tariq A, Louis Panzica, Suresh Gopi Kalathil, and Yasmin Thanavala. 2015. "Immune Dysfunction in Patients with Chronic Obstructive

Pulmonary Disease." In *Annals of the American Thoracic Society*, 12:S169–75. American Thoracic Society. https://doi.org/10.1513/AnnalsATS.201503-126AW.

Bilir, Bulent, Nejat Altıntaş, Murat Aydın, Mustafa Oran, Savas Özsu, and Ümit Tutar. 2016. "The Predictive Role of Neutrophil to Lymphocyte Ratio in Chronic Obstructive Pulmonary Disease." *Electronic Journal of General Medicine* 13 (2): 105–10. https://doi.org/10.15197/ejgm.1554.

Binder, Daniel, Jörg Fehr, Hans Hengartner, and Rolf M. Zinkernagel. 1997. "Virus-Induced Transient Bone Marrow Aplasia: Major Role of Interferon-α/β during Acute Infection with the Noncytopathic Lymphocytic Choriomeningitis Virus." *Journal of Experimental Medicine* 185 (3): 517–30. https://doi.org/10.1084/jem.185.3.517.

Birring, Surinder, Christopher Brightling, Peter Bradding, James J. Entwisle, Dhiraj D. Vara, Jonathan Grigg, Andrew J. Wardlaw, and Ian D. Pavord. 2002. "Clinical, Radiologic, and Induced Sputum Features of Chronic Obstructive Pulmonary Disease in Nonsmokers: A Descriptive Study." *American Journal of Respiratory and Critical Care Medicine* 166 (8): 1078–83. https://doi.org/10.1164/rccm.200203-245OC.

Blidberg, Kristin, Lena Palmberg, Barbro Dahlén, Ann Sofie Lantz, and Kjell Larsson. 2012. "Increased Neutrophil Migration in Smokers with or without Chronic Obstructive Pulmonary Disease." *Respirology* 17 (5): 854–60. https://doi.org/10.1111/j.1440-1843.2012.02181.x.

Brandsma, Corry-Anke Anke, Machteld N. Hylkema, Marie Geerlings, Wouter H. van Geffen, Dirkje S. Postma, Wim Timens, and Huib A.M. M Kerstjens. 2009. "Increased Levels of (Class Switched) Memory B Cells in Peripheral Blood of Current Smokers." *Respiratory Research* 10 (1): 108. https://doi.org/10.1186/1465-9921-10-108.

Brode, Sarah K., Simon C. Ling, and Kenneth R. Chapman. 2012. "Alpha-1 Antitrypsin Deficiency: A Commonly Overlooked Cause of Lung Disease." *Canadian Medical Association Journal* 184 (12): 1365–71. https://doi.org/10.1503/cmaj.111749.

Bronte, Vincenzo, Sven Brandau, Shu-Hsia Chen, Mario P. Colombo, Alan B. Frey, Tim F. Greten, Susanna Mandruzzato, et al. 2016. "Recommendations for Myeloid-Derived Suppressor Cell Nomenclature and Characterization Standards." *Nature Communications* 7 (1): 12150. https://doi.org/10.1038/ncomms12150.

Brusselle, G. G., T. Demoor, K. R. Bracke, C-A. Brandsma, and W. Timens. 2009. "Lymphoid Follicles in (Very) Severe COPD: Beneficial or Harmful?" *European Respiratory Journal* 34 (1): 219–30. https://doi.org/10.1183/09031936.00150208.

Budweiser, Stephan, Martina Harlacher, Michael Pfeifer, and Rudolf A. Jörres. 2014. “Co-Morbidities and Hyperinflation Are Independent Risk Factors of All-Cause Mortality in Very Severe COPD.” *COPD: Journal of Chronic Obstructive Pulmonary Disease* 11 (4): 388–400. https://doi.org/10.3109/15412555.2013.836174.

Casanova, Ciro, Claudia Cote, Juan P. De Torres, Armando Aguirre-Jaime, Jose M. Marin, Victor Pinto-Plata, and Bartolome R. Celli. 2005. “Inspiratory-to-Total Lung Capacity Ratio Predicts Mortality in Patients with Chronic Obstructive Pulmonary Disease.” *American Journal of Respiratory and Critical Care Medicine* 171 (6): 591–97. https://doi.org/10.1164/rccm.200407-867OC.

Cavaillon, Jean-Marc, and Minou Adib-Conquy. 2010. “Immune Status in Sepsis: The Bug, the Site of Infection and the Severity Can Make the Difference.” *Critical Care* 14 (3): 167. https://doi.org/10.1186/cc9046.

Chen, Long, Gang Chen, Ming-Qiang Zhang, Xian-Zhi Xiong, Hong-Ju Liu, Jian-Bao Xin, Jian-Chu Zhang, Jiang-Hua Wu, Zhao-Ji Meng, and Sheng-Wen Sun. 2016. “Imbalance between Subsets of CD8 + Peripheral Blood T Cells in Patients with Chronic Obstructive Pulmonary Disease.” *PeerJ* 4 (August): e2301. https://doi.org/10.7717/peerj.2301.

Cosmi, L, F Liotta, E Maggi, S Romagnani, and F Annunziato. 2011. “Th17 Cells: New Players in Asthma Pathogenesis.” *Allergy* 66 (8): 989–98. https://doi.org/10.1111/j.1398-9995.2011.02576.x.

Cossarizza, Andrea, Hyun Dong Chang, Andreas Radbruch, Andreas Acs, Dieter Adam, Sabine Adam-Klages, William W. Agace, et al. 2019. “Guidelines for the Use of Flow Cytometry and Cell Sorting in Immunological Studies (Second Edition).” *European Journal of Immunology* 49 (10): 1457–1973. https://doi.org/10.1002/eji.201970107.

Cuenca, Alex G., Matthew J. Delano, Kindra M. Kelly-Scumpia, Claudia Moreno, Philip O. Scumpia, Drake M. LaFace, Paul G. Heyworth, Philip A. Efron, and Lyle L. Moldawer. 2011. “A Paradoxical Role for Myeloid-Derived Suppressor Cells in Sepsis and Trauma.” *Molecular Medicine* 17 (3–4): 281–92. https://doi.org/10.2119/molmed.2010.00178.

Deitch, Edwin A., Preya Ananthakrishnan, David B. Cohen, Da Zhong Xu, Eleonora Feketeova, and Carl J. Hauser. 2006. “Neutrophil Activation Is Modulated by Sex Hormones after Trauma-Hemorrhagic Shock and Burn Injuries.” *American Journal of Physiology-Heart and Circulatory Physiology* 291 (3): H1456–65. https://doi.org/10.1152/ajpheart.00694.2005.

Delano, Matthew J., Philip O. Scumpia, Jason S. Weinstein, Dominique Coco, Srinivas Nagaraj, Kindra M. Kelly-Scumpia, Kerri A. O’Malley, et al. 2007. “MyD88-Dependent Expansion of an Immature GR-1 +CD11b+ Population

Induces T Cell Suppression and Th2 Polarization in Sepsis." *Journal of Experimental Medicine* 204 (6): 1463–74. https://doi.org/10.1084/jem.20062602.

Dong, Tao, Simão Santos, Zhaochu Yang, Shuai Yang, and Niels E. Kirkhus. 2020. "Sputum and Salivary Protein Biomarkers and Point-of-Care Biosensors for the Management of COPD." *The Analyst* 145 (5): 1583–1604. https://doi.org/10.1039/C9AN01704F.

Duffy, Brendan K., Hitinder S. Gurm, Vivek Rajagopal, Ritesh Gupta, Stephen G. Ellis, and Deepak L. Bhatt. 2006. "Usefulness of an Elevated Neutrophil to Lymphocyte Ratio in Predicting Long-Term Mortality after Percutaneous Coronary Intervention." *American Journal of Cardiology* 97 (7): 993–96. https://doi.org/10.1016/j.amjcard.2005.10.034.

El-Shimy, Wafaa S, Ayman S El-Dib, Hala M Nagy, and Wael Sabry. 2014. "A Study of IL-6, IL-8, and TNF-α as Inflammatory Markers in COPD Patients." *Egyptian Journal of Bronchology* 8 (July): 91–99. https://doi.org/10.4103/1687-8426.145698.

Ellingsen, Jens, Hans Hedenström, Marieann Högman, Karin Lisspers, Björn Ställberg, Janson Christer, Malinovschi Andrei, et al. 2016. "The Relationship between Blood Neutrophils, Blood Eosinophils and Exacerbations of COPD – Results from the TIE-Study." In *European Respiratory Journal*, 48:PA1013. European Respiratory Society (ERS). https://doi.org/10.1183/13993003.congress-2016.pa1013.

Eriksson Ström, Jonas, Jamshid Pourazar, Robert Linder, Anders Blomberg, Anne Lindberg, Anders Bucht, and Annelie F. Behndig. 2018. "Cytotoxic Lymphocytes in COPD Airways: Increased NK Cells Associated with Disease, INKT and NKT-like Cells with Current Smoking." *RESPIRATORY RESEARCH* 19 (1): 244. https://doi.org/10.1186/s12931-018-0940-7.

Estenne, Marc, Janet R. Maurer, Annette Boehler, James J. Egan, Adaani Frost, Marshall Hertz, George B. Mallory, Gregory I. Snell, and Samuel Yousem. 2002. "Bronchiolitis Obliterans Syndrome 2001: An Update of the Diagnostic Criteria." *The Journal of Heart and Lung Transplantation* 21 (3): 297–310. https://doi.org/10.1016/S1053-2498(02)00398-4.

Fan, Hongye, Guanjun Dong, Guangfeng Zhao, Fei Liu, Genghong Yao, Yichao Zhu, and Yayi Hou. 2014. "Gender Differences of B Cell Signature in Healthy Subjects Underlie Disparities in Incidence and Course of SLE Related to Estrogen." *Journal of Immunology Research* 2014: ID 814598. https://doi.org/10.1155/2014/814598.

Faner, Rosa, Jarrett D. Morrow, Sandra Casas-Recasens, Suzanne M. Cloonan, Guillaume Noell, Alejandra López-Giraldo, Ruth Tal-Singer, et al. 2019. "Do Sputum or Circulating Blood Samples Reflect the Pulmonary

Transcriptomic Differences of COPD Patients? A Multi-Tissue Transcriptomic Network META-Analysis." *Respiratory Research* 20 (1): 5. https://doi.org/10.1186/s12931-018-0965-y.

Fratta Pasini, Anna Maria, Marcello Ferrari, Chiara Stranieri, Paola Vallerio, Chiara Mozzini, Ulisse Garbin, Giorgia Zambon, and Luciano Cominacini. 2016. "Nrf2 Expression Is Increased in Peripheral Blood Mononuclear Cells Derived from Mild–Moderate Ex-Smoker COPD Patients with Persistent Oxidative Stress." *International Journal of COPD* 11 (1): 1733–43. https://doi.org/10.2147/COPD.S102218.

Fuller-Thomson, Esme, Rachel S. Chisholm, and Sarah Brennenstuhl. 2016. "COPD in a Population-Based Sample of Never-Smokers: Interactions among Sex, Gender, and Race." *International Journal of Chronic Diseases* 2016: ID 5862026. https://doi.org/10.1155/2016/5862026.

Furutate, Ryuko, Takeo Ishii, Takashi Motegi, Kumiko Hattori, Yuji Kusunoki, Akihiko Gemma, and Kozui Kida. 2016. "The Neutrophil to Lymphocyte Ratio Is Related to Disease Severity and Exacerbation in Patients with Chronic Obstructive Pulmonary Disease." *Internal Medicine* 55 (3): 223–29. https://doi.org/10.2169/internalmedicine.55.5772.

Gan, W. Q. 2004. "Association between Chronic Obstructive Pulmonary Disease and Systemic Inflammation: A Systematic Review and a Meta-Analysis." *Thorax* 59 (7): 574–80. https://doi.org/10.1136/thx.2003.019588.

Gan, Wen Qi, SF Paul Man, Dirkje S. Postma, Patricia Camp, and Don D. Sin. 2006. "Female Smokers beyond the Perimenopausal Period Are at Increased Risk of Chronic Obstructive Pulmonary Disease: A Systematic Review and Meta-Analysis." *Respiratory Research* 7 (1): 52. https://doi.org/10.1186/1465-9921-7-52.

Gao, Shupei, Jinqing Chen, Jungang Xie, and Jianmiao Wang. 2020. "The Effects of BAFF on T Lymphocytes in Chronic Obstructive Pulmonary Disease." *Respiratory Research* 21 (1): 66. https://doi.org/10.1186/s12931-020-01333-z.

Grumelli, Sandra, David B. Corry, Li Zhen Song, Ling Song, Linda Green, Joseph Huh, Joan Hacken, et al. 2004. "An Immune Basis for Lung Parenchymal Destruction in Chronic Obstructive Pulmonary Disease and Emphysema." *PLoS Medicine* 1 (1): 075–083. https://doi.org/10.1371/journal.pmed.0010008.

Hadfield, Ruth, ed. 2017. *Pocket Guide to COPD Diagnostics, Management, and Prevention*. *Global Initiative for Chronic Obstructive Lung Disease*. Global Initiative for Chronic Obstructive Lung Disease.

Han, MeiLan K, Dirkje Postma, David M. Mannino, Nicholas D. Giardino, Sonia Buist, Jeffrey L. Curtis, and Fernando J. Martinez. 2007. "Gender and Chronic Obstructive Pulmonary Disease." *American Journal of Respiratory and Critical Care Medicine* 176 (12): 1179–84. https://doi.org/10.1164/rccm.200704-553CC.

Hayashi, Yumeko, Yoshiki Ishii, Mitsumi Hata-Suzuki, Ryo Arai, Kazuyuki Chibana, Akihiro Takemasa, and Takeshi Fukuda. 2013. "Comparative Analysis of Circulating Dendritic Cell Subsets in Patients with Atopic Diseases and Sarcoidosis." *Respiratory Research* 14 (1): 29. https://doi.org/10.1186/1465-9921-14-29.

Hill, D. A., M. C. Siracusa, K. R. Ruymann, E. D. Tait Wojno, David Artis, and Jonathan M. Spergel. 2014. "Omalizumab Therapy Is Associated with Reduced Circulating Basophil Populations in Asthmatic Children." *Allergy: European Journal of Allergy and Clinical Immunology* 69 (5): 674–77. https://doi.org/10.1111/all.12375.

Hobbs, Brian D, and Craig P Hersh. 2014. "Integrative Genomics of Chronic Obstructive Pulmonary Disease." *Biochemical and Biophysical Research Communications* 452 (2): 276–86. https://doi.org/10.1016/j.bbrc.2014.07.086.

Hogg, James C., Fanny Chu, Soraya Utokaparch, Ryan Woods, W. Mark Elliott, Liliana Buzatu, Ruben M. Cherniack, et al. 2004. "The Nature of Small-Airway Obstruction in Chronic Obstructive Pulmonary Disease." *New England Journal of Medicine* 350 (26): 2645–53. https://doi.org/10.1056/NEJMoa032158.

Hou, Jia, Yongchang Sun, Yu Hao, Jie Zhuo, Xiaofang Liu, Peng Bai, Junyan Han, Xiwei Zheng, and Hui Zeng. 2013. "Imbalance between Subpopulations of Regulatory T Cells in COPD." *Thorax* 68 (12): 1131–39. https://doi.org/10.1136/thoraxjnl-2012-201956.

Huertas, Alice, and Paolo Palange. 2011. "COPD: A Multifactorial Systemic Disease." *Therapeutic Advances in Respiratory Disease* 5 (3): 217–24. https://doi.org/10.1177/1753465811400490.

İn, Erdal, Mutlu Kuluöztürk, Önsel Öner, and Figen Deveci. 2016. "The Importance of Neutrophil-to-Lymphocyte Ratio in Chronic Obstructive Pulmonary Disease." *Turk Toraks Dergisi* 17 (2): 41–46. https://doi.org/10.5578/ttj.17.2.009.

Ivetic, Aleksandar, Hannah Louise Hoskins Green, and Samuel James Hart. 2019. "L-Selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling." *Frontiers in Immunology* 10 (May): 1068. https://doi.org/10.3389/fimmu.2019.01068.

Jaroenpool, Jiraporn, Kovit Pattanapanyasat, Naiyana Noonin, and Issara Prachongsai. 2016. “Aberrant Neutrophil Function among Heavy Smokers and Chronic Obstructive Pulmonary Disease Patients.” *Asian Pacific Journal of Allergy and Immunology* 34 (4): 278–83. https://doi.org/10.12932/AP0724.

Kalathil, Suresh Gopi, Amit Anand Lugade, Vandana Pradhan, Austin Miller, Ganapathi Iyer Parameswaran, Sanjay Sethi, and Yasmin Thanavala. 2014. “T-Regulatory Cells and Programmed Death 1+ T Cells Contribute to Effector T-Cell Dysfunction in Patients with Chronic Obstructive Pulmonary Disease.” *American Journal of Respiratory and Critical Care Medicine* 190 (1): 40–50. https://doi.org/10.1164/rccm.201312-2293OC.

Kawa, Miłosz Piotr, Katarzyna Grymuła, Edyta Paczkowska, Magdalena Baśkiewicz-Masiuk, Elzbieta Dąbkowska, Monika Koziołek, Maciej Tarnowski, et al. 2010. “Clinical Relevance of Thyroid Dysfunction in Human Haematopoiesis: Biochemical and Molecular Studies.” *European Journal of Endocrinology* 162 (2): 295–305. https://doi.org/10.1530/EJE-09-0875.

Kim, Daehwan, Joseph M. Paggi, Chanhee Park, Christopher Bennett, and Steven L. Salzberg. 2019. “Graph-Based Genome Alignment and Genotyping with HISAT2 and HISAT-Genotype.” *Nature Biotechnology* 37 (8): 907–15. https://doi.org/10.1038/s41587-019-0201-4.

Koch, A, M Gaczkowski, G Sturton, P Staib, T Schinköthe, E Klein, A Rubbert, K Bacon, K Wassermann, and E Erdmann. 2007. “Modification of Surface Antigens in Blood CD8+ T-Lymphocytes in COPD: Effects of Smoking.” *The European Respiratory Journal* 29 (1): 42–50. https://doi.org/10.1183/09031936.00133205.

Koo, Hyeon-kyoung, Hyung Koo Kang, Pamela Song, Hye Kyeong Park, Sung-soon Lee, and Hoon Jung. 2017. “Systemic White Blood Cell Count as a Biomarker Associated with Severity of Chronic Obstructive Lung Disease.” *Tuberculosis and Respiratory Diseases* 80 (3): 304–10. https://doi.org/10.4046/trd.2017.80.3.304.

Kowal, K., M. Moniuszko, M. Dabrowska, and A. Bodzenta-Lukaszyk. 2012. “Allergen Challenge Differentially Affects the Number of Circulating Monocyte Subsets.” *Scandinavian Journal of Immunology* 75 (5): 531–39. https://doi.org/10.1111/j.1365-3083.2012.02685.x.

Krieger, Bruce P. 2009. “Hyperinflation and Intrinsic Positive End-Expiratory Pressure: Less Room to Breathe.” *Respiration* 77 (3): 344–50. https://doi.org/10.1159/000192790.

Kuijpers, TW, AT Tool, CE van der Schoot, LA Ginsel, JJ Onderwater, D Roos, and AJ Verhoeven. 1991. “Membrane Surface Antigen Expression on

Neutrophils: A Reappraisal of the Use of Surface Markers for Neutrophil Activation." *Blood* 78 (4): 1105–11. https://doi.org/10.1182/blood.v78.4.1105.1105.

Lee, Heock, Soo-Jung Um, Yun Seong Kim, Deog Kyeom Kim, An Soo Jang, Hye Sook Choi, Yee Hyung Kim, Tae Eun Kim, Kwang Ha Yoo, and Ki-Suck Jung. 2016. "Association of the Neutrophil-to-Lymphocyte Ratio with Lung Function and Exacerbations in Patients with Chronic Obstructive Pulmonary Disease." Edited by Stelios Loukides. *PLOS ONE* 11 (6): e0156511. https://doi.org/10.1371/journal.pone.0156511.

Li, Jian, Qingyu Chen, Xiaohong Luo, Jun Hong, Kunyi Pan, Xianghua Lin, Xiaoqiang Liu, et al. 2015. "Neutrophil-to-Lymphocyte Ratio Positively Correlates to Age in Healthy Population." *Journal of Clinical Laboratory Analysis* 29 (6): 437–43. https://doi.org/10.1002/jcla.21791.

Løkke, A, P Lange, H Scharling, P Fabricius, and J Vestbo. 2006. "Developing COPD: A 25 Year Follow up Study of the General Population." *Thorax* 61 (11): 935–39. https://doi.org/10.1136/thx.2006.062802.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2." *Genome Biology* 15 (12): 550. https://doi.org/10.1186/s13059-014-0550-8.

Lutfi, Mohamed Faisal. 2017. "The Physiological Basis and Clinical Significance of Lung Volume Measurements." *Multidisciplinary Respiratory Medicine*. BioMed Central Ltd. https://doi.org/10.1186/s40248-017-0084-5.

Mandal, Chandi Charan, Jatin Mehta, and Vijay K. Prajapati. 2018. "Programmed Death 1 (PD1)-Mediated T-Cell Apoptosis and Cancer Immunotherapy." In *Apoptosis and Beyond: The Many Ways Cells Die*, 695–722. Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/9781119432463.ch29.

Marco, Roberto de, Giancarlo Pesce, Alessandro Marcon, Simone Accordini, Leonardo Antonicelli, Massimiliano Bugiani, Lucio Casali, et al. 2013. "The Coexistence of Asthma and Chronic Obstructive Pulmonary Disease (COPD): Prevalence and Risk Factors in Young, Middle-Aged and Elderly People from the General Population." Edited by Antony Bayer. *PLoS ONE* 8 (5): e62985. https://doi.org/10.1371/journal.pone.0062985.

Marini, Olivia, Sara Costa, Dalila Bevilacqua, Federica Calzetti, Nicola Tamassia, Cecilia Spina, Donata De Sabata, et al. 2017. "Mature CD10+ and Immature CD10- Neutrophils Present in G-CSF-Treated Donors Display Opposite Effects on T Cells." *Blood* 129 (10): 1343–56. https://doi.org/10.1182/blood-2016-04-713206.

Martinez, Fernando J., Jeffrey L. Curtis, Frank Sciurba, Jeanette Mumford, Nicholas D. Giardino, Gail Weinmann, Ella Kazerooni, et al. 2007. "Sex Differences in Severe Pulmonary Emphysema." *American Journal of Respiratory and Critical Care Medicine* 176 (3): 243–52. https://doi.org/10.1164/rccm.200606-828OC.

Mazzoni, Alessio, Lorenzo Salvati, Laura Maggi, Manuela Capone, Anna Vanni, Michele Spinicci, Jessica Mencarini, et al. 2020. "Impaired Immune Cell Cytotoxicity in Severe COVID-19 Is IL-6 Dependent." *Journal of Clinical Investigation* 130 (9): 4694–4703. https://doi.org/10.1172/JCI138554.

McCormick, Barry, Julia Y. Chu, and Sonja Vermeren. 2019. "Cross-Talk between Rho GTPases and PI3K in the Neutrophil." *Small GTPases* 10 (3): 187–95. https://doi.org/10.1080/21541248.2017.1304855.

Mhetre, Manisha, Yogesh Patil, and H. K. Abhyankar. 2018. "Design and Evaluation of Turbine Spirometer: A New Approach." *Innovations in Electronics and Communication Engineering. Lecture Notes in Networks and Systems* 7: 587–94. https://doi.org/10.1007/978-981-10-3812-9_60.

Milara, Javier, Gustavo Juan, Teresa Peiró, Adela Serrano, and Julio Cortijo. 2012. "Neutrophil Activation in Severe, Early-Onset COPD Patients versus Healthy Non-Smoker Subjects in Vitro: Effects of Antioxidant Therapy." *Respiration* 83 (2): 147–58. https://doi.org/10.1159/000332834.

Moayyedkazemi, Alireza, and Mohammad Hossein Rahimirad. 2018. "Evaluating Serum C-Reactive Protein Level in Patients with Chronic Obstructive Pulmonary Disease and Its Correlation with Disease Severity." *Biomedical Research and Therapy* 5 (11): 2784–88. https://doi.org/10.15419/bmrat.v5i11.494.

Moorlag, Simone J.C.F.M., Yessica Alina Rodriguez-Rosales, Joshua Gillard, Stephanie Fanucchi, Kate Theunissen, Boris Novakovic, Cynthia M. de Bont, et al. 2020. "BCG Vaccination Induces Long-Term Functional Reprogramming of Human Neutrophils." *Cell Reports* 33 (7): 108387. https://doi.org/10.1016/j.celrep.2020.108387.

Núñez, Belén, Jaume Sauleda, Josep Maria Antó, Maria Rosa Julià, Mauricio Orozco, Eduard Monsó, Aina Noguera, Federico P. Gómez, Judith Garcia-Aymerich, and Alvar Agustí. 2011. "Anti-Tissue Antibodies Are Related to Lung Function in Chronic Obstructive Pulmonary Disease." *American Journal of Respiratory and Critical Care Medicine* 183 (8): 1025–31. https://doi.org/10.1164/rccm.201001-0029OC.

O'Donnell, R. 2006. "Inflammatory Cells in the Airways in COPD." *Thorax* 61 (5): 448–54. https://doi.org/10.1136/thx.2004.024463.

Oudijk, E. J.D., E. H.J. Nijhuis, M. D. Zwank, E. A. Van De Graaf, H. J. Mager, P. J.

Coffer, J. W.J. Lammers, and L. Koenderman. 2005. “Systemic Inflammation in COPD Visualised by Gene Profiling in Peripheral Blood Neutrophils.” *Thorax* 60 (7): 538–44. https://doi.org/10.1136/thx.2004.034009.

Pascual-González, Yuliana, Marta López-Sánchez, Jordi Dorca, and Salud Santos. 2018. “Defining the Role of Neutrophil-to-Lymphocyte Ratio in COPD: A Systematic Literature Review.” *International Journal of Chronic Obstructive Pulmonary Disease* 13 (November): 3651–62. https://doi.org/10.2147/COPD.S178068.

Pettersson, Ulrika Sofia, Gustaf Christoffersson, Sara Massena, David Ahl, Leif Jansson, Johanna Henriksnäs, and Mia Phillipson. 2011. “Increased Recruitment but Impaired Function of Leukocytes during Inflammation in Mouse Models of Type 1 and Type 2 Diabetes.” *PLoS ONE* 6 (7): e22480. https://doi.org/10.1371/journal.pone.0022480.

Picelli, Simone, Omid R. Faridani, Åsa K. Björklund, Gösta Winberg, Sven Sagasser, and Rickard Sandberg. 2014. “Full-Length RNA-Seq from Single Cells Using Smart-Seq2.” *Nature Protocols* 9: 171–81. https://doi.org/https://doi.org/10.1038/nprot.2014.006.

Polverino, Francesca, Leen J. M. Seys, Ken R. Bracke, and Caroline A. Owen. 2016. “B Cells in Chronic Obstructive Pulmonary Disease: Moving to Center Stage.” *American Journal of Physiology-Lung Cellular and Molecular Physiology* 311 (4): L687–95. https://doi.org/10.1152/ajplung.00304.2016.

Ravi, Arjun K, Jonathan Plumb, Rosemary Gaskell, Sarah Mason, Caroline S Broome, George Booth, Matthew Catley, Jørgen Vestbo, and Dave Singh. 2017. “COPD Monocytes Demonstrate Impaired Migratory Ability.” *Respiratory Research* 18 (1): 90. https://doi.org/10.1186/s12931-017-0569-y.

Roberts, Mustimbo E. P., Brandon W. Higgs, Philip Brohawn, Fernanda Pilataxi, Xiang Guo, Michael Kuziora, Russell P. Bowler, and Wendy I. White. 2015. “CD4+ T-Cell Profiles and Peripheral Blood Ex-Vivo Responses to T-Cell Directed Stimulation Delineate COPD Phenotypes.” *Chronic Obstructive Pulmonary Diseases: Journal of the COPD Foundation* 2 (4): 268–80. https://doi.org/10.15326/jcopdf.2.4.2015.0131.

Rumora, Lada, Lara Milevoj, Sanja Popović-Grle, Karmela Barišić, Ivana Čepelak, and Tihana Žanić Grubišić. 2008. “Levels Changes of Blood Leukocytes and Intracellular Signalling Pathways in COPD Patients with Respect to Smoking Attitude.” *Clinical Biochemistry* 41 (6): 387–94. https://doi.org/10.1016/J.CLINBIOCHEM.2007.12.013.

Scapini, Patrizia, Olivia Marini, Cristina Tecchio, and Marco A. Cassatella. 2016. “Human Neutrophils in the Saga of Cellular Heterogeneity: Insights and

Open Questions." *Immunological Reviews* 273 (1): 48–60. https://doi.org/10.1111/imr.12448.

Schulze-Koops, Hendrik. 2004. "Lymphopenia and Autoimmune Diseases." *Arthritis Research and Therapy* 6 (4): 178–80. https://doi.org/10.1186/ar1208.

Scrimini, Sergio, Jaume Pons, Alvar Agustí, Joan B. Soriano, Borja G. Cosio, Juan A. Torrecilla, Belén Núñez, et al. 2013. "Differential Effects of Smoking and COPD upon Circulating Myeloid Derived Suppressor Cells." *Respiratory Medicine* 107 (12): 1895–1903. https://doi.org/10.1016/J.RMED.2013.08.002.

Seddiki, Nabila, Brigitte Santner-Nanan, Jeff Martinson, John Zaunders, Sarah Sasson, Alan Landay, Michael Solomon, et al. 2006. "Expression of Interleukin (IL)-2 and IL-7 Receptors Discriminates between Human Regulatory and Activated T Cells." *Journal of Experimental Medicine* 203 (7): 1693–1700. https://doi.org/10.1084/jem.20060468.

Segal, Leopoldo N, and Fernando J Martinez. 2018. "Chronic Obstructive Pulmonary Disease Subpopulations and Phenotyping." *Journal of Allergy and Clinical Immunology* 141 (6): 1961–71. https://doi.org/10.1016/j.jaci.2018.02.035.

Sevenoaks, Martin J., and Robert A. Stockley. 2006. "Chronic Obstructive Pulmonary Disease, Inflammation and Co-Morbidity – a Common Inflammatory Phenotype?" *Respiratory Research* 7 (1): 70. https://doi.org/10.1186/1465-9921-7-70.

Shen, Chong, Philippe Maerten, Karel Geboes, Gert Van Assche, Paul Rutgeerts, and Jan L. Ceuppens. 2005. "Infliximab Induces Apoptosis of Monocytes and T Lymphocytes in a Human-Mouse Chimeric Model." *Clinical Immunology* 115 (3): 250–59. https://doi.org/10.1016/j.clim.2005.01.007.

Shin, Tae Rim, Yeon Mok Oh, Joo Hun Park, Keu Sung Lee, Sunghee Oh, Dae Ryoung Kang, Seungsoo Sheen, et al. 2015. "The Prognostic Value of Residual Volume/Total Lung Capacity in Patients with Chronic Obstructive Pulmonary Disease." *Journal of Korean Medical Science* 30 (10): 1459–65. https://doi.org/10.3346/jkms.2015.30.10.1459.

Silverman, Edwin K., Scott T. Weiss, Jeffrey M. Drazen, Harold A. Chapman, Vincent Carey, Edward J. Campbell, Paul Denish, et al. 2000. "Gender-Related Differences in Severe, Early-Onset Chronic Obstructive Pulmonary Disease." *American Journal of Respiratory and Critical Care Medicine* 162 (6): 2152–58. https://doi.org/10.1164/ajrccm.162.6.2003112.

Staveren, Selma van, Twan ten Haaf, Margot Klöpping, Bart Hilvering, Gerjen

H. Tinnevelt, Karin de Ruiter, Maria F. Piacentini, et al. 2018. “Multi-Dimensional Flow Cytometry Analysis Reveals Increasing Changes in the Systemic Neutrophil Compartment during Seven Consecutive Days of Endurance Exercise.” Edited by Amit Gaggar. *PLOS ONE* 13 (10): e0206175. https://doi.org/10.1371/journal.pone.0206175.

Stoll, Paul, Martin Ulrich, Kai Bratke, Katharina Garbe, J Christian Virchow, and Marek Lommatzsch. 2015. “Imbalance of Dendritic Cell Co-Stimulation in COPD.” *Respiratory Research* 16 (1): 19. https://doi.org/10.1186/s12931-015-0174-x.

Stoller, James K., and Loutfi S. Aboussouan. 2012. “A Review of A1-Antitrypsin Deficiency.” *American Journal of Respiratory and Critical Care Medicine* 185 (3): 246–59. https://doi.org/10.1164/rccm.201108-1428CI.

Sun, J., T. Liu, Y. Yan, K. Huo, W. Zhang, H. Liu, and Z. Shi. 2018. “The Role of Th1/Th2 Cytokines Played in Regulation of Specific CD4+ Th1 Cell Conversion and Activation during Inflammatory Reaction of Chronic Obstructive Pulmonary Disease.” *Scandinavian Journal of Immunology* 88 (1): e12674. https://doi.org/10.1111/sji.12674.

Supek, Fran, Matko Bošnjak, Nives Škunca, and Tomislav Šmuc. 2011. “REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms.” Edited by Cynthia Gibas. *PLoS ONE* 6 (7): e21800. https://doi.org/10.1371/journal.pone.0021800.

Szabó, Mariann, Veronika Sárosi, Zoltán Balikó, Kornélia Bodó, Nelli Farkas, Tímea Berki, and Péter Engelmann. 2017. “Deficiency of Innate-like T Lymphocytes in Chronic Obstructive Pulmonary Disease.” *Respiratory Research* 18 (1): 197. https://doi.org/10.1186/s12931-017-0671-1.

Tackenberg, Heidi, Sonja Möller, Marie-Dominique Filippi, and Tamás Laskay. 2020. “The Small GTPase Cdc42 Is a Major Regulator of Neutrophil Effector Functions.” *Frontiers in Immunology* 11 (June): 1197. https://doi.org/10.3389/fimmu.2020.01197.

Tang, Ying, Xiaodan Li, Man Wang, Qi Zou, Shasha Zhao, Bowen Sun, Lijun Xu, and Yanfang Jiang. 2013. “Increased Numbers of NK Cells, NKT-Like Cells, and NK Inhibitory Receptors in Peripheral Blood of Patients with Chronic Obstructive Pulmonary Disease.” *Clinical and Developmental Immunology* 2013: ID 721782. https://doi.org/10.1155/2013/721782.

Taraseviciene-Stewart, Laimute, Robertas Scerbavicius, Kang Hyeon Choe, Melissa Moore, Andrew Sullivan, Mark R. Nicolls, Andrew P. Fontenot, Rubin M. Tuder, and Norbert F. Voelkel. 2005. “An Animal Model of Autoimmune Emphysema.” *American Journal of Respiratory and Critical Care Medicine* 171 (7): 734–42. https://doi.org/10.1164/rccm.200409-1275OC.

Tavakolpour, Soheil, Taha Rakhshandehroo, Erin X. Wei, and Mohammad Rashidian. 2020. “Lymphopenia during the COVID-19 Infection: What It Shows and What Can Be Learned.” *Immunology Letters* 225 (September): 31–32. https://doi.org/10.1016/j.imlet.2020.06.013.

Taylan, Mahsuk, Melike Demir, Halide Kaya, Hadice Selimoglu Sen, Ozlem Abakay, Ali İhsan Carkanat, Abdurrrahman Abakay, Abdullah Cetin Tanrikulu, and Cengizhan Sezgi. 2017. “Alterations of the Neutrophil-Lymphocyte Ratio during the Period of Stable and Acute Exacerbation of Chronic Obstructive Pulmonary Disease Patients.” *The Clinical Respiratory Journal* 11 (3): 311–17. https://doi.org/10.1111/crj.12336.

Teresa, Rashmi, and Smitha Bhat. 2013. “Peripheral Blood T-Cell Populations in COPD, Asymptomatic Smokers and Healthy Non-Smokers in Indian Subpopulation-A Pilot Study.” *Journal of Clinical and Diagnostic Research* 7 (6): 1109–13. https://doi.org/10.7860/JCDR/2013/5977.3094.

Tomita, Katsuyuki, Takahiko Tanigawa, Hiroki Yajima, Kouji Fukutani, Yukio Matsumoto, Yoshinori Tanaka, and Takao Sasaki. 1995. “Identification and Characterization of Monocyte Subpopulations from Patients with Bronchial Asthma.” *The Journal of Allergy and Clinical Immunology* 96 (2): 230–38. https://doi.org/10.1016/S0091-6749(95)70012-9.

Urbanowicz, Richard A., Jonathan R. Lamb, Ian Todd, Jonathan M. Corne, and Lucy C. Fairclough. 2009. “Altered Effector Function of Peripheral Cytotoxic Cells in COPD.” *Respiratory Research* 10 (1): 53. https://doi.org/10.1186/1465-9921-10-53.

Veglia, Filippo, Vladimir A. Tyurin, Maria Blasi, Alessandra De Leo, Andrew V. Kossenkov, Laxminarasimha Donthireddy, Tsun Ki Jerrick To, et al. 2019. “Fatty Acid Transport Protein 2 Reprograms Neutrophils in Cancer.” *Nature* 569 (7754): 73–78. https://doi.org/10.1038/s41586-019-1118-2.

Venet, Fabienne, Fanny Davin, Caroline Guignant, Audrey Larue, Marie Angélique Cazalis, Romain Darbon, Caroline Allombert, et al. 2010. “Early Assessment of Leukocyte Alterations at Diagnosis of Septic Shock.” *Shock* 34 (4): 358–63. https://doi.org/10.1097/SHK.0b013e3181dc0977.

Watson, Louise, Jorgen Vestbo, Dirkje S. Postma, Marc Decramer, Stephen Rennard, Victor A. Kiri, Paul A. Vermeire, and Joan B. Soriano. 2004. “Gender Differences in the Management and Experience of Chronic Obstructive Pulmonary Disease.” *Respiratory Medicine* 98 (12): 1207–13. https://doi.org/10.1016/j.rmed.2004.05.004.

Wheaton, Anne G., Roy A. Pleasants, Janet B. Croft, Jill A. Ohar, Khosrow Heidari, David M. Mannino, Yong Liu, and Charlie Strange. 2016. “Gender and Asthma-Chronic Obstructive Pulmonary Disease Overlap Syndrome.” *Journal of Asthma* 53 (7): 720–31.

https://doi.org/10.3109/02770903.2016.1154072.

Willinger, Tim, Tom Freeman, Hitoshi Hasegawa, Andrew J. McMichael, and Margaret F. C. Callan. 2005. "Molecular Signatures Distinguish Human Central Memory from Effector Memory CD8 T Cell Subsets." *The Journal of Immunology* 175 (9): 5895–5903. https://doi.org/10.4049/jimmunol.175.9.5895.

Xiong, Wei, Mei Xu, Yunfeng Zhao, Xueling Wu, Bigyan Pudasaini, and Jin-Ming Liu. 2017. "Can We Predict the Prognosis of COPD with a Routine Blood Test?" *International Journal of Chronic Obstructive Pulmonary Disease* 12: 615–25. https://doi.org/10.2147/COPD.S124041.

Yasuda, N, K Gotoh, S Minatoguchi, K Asano, K Nishigaki, M Nomura, A. Ohno, et al. 1998. "An Increase of Soluble Fas, an Inhibitor of Apoptosis, Associated with Progression of COPD." *Respiratory Medicine* 92 (8): 993–99. https://doi.org/10.1016/S0954-6111(98)90343-2.

Yoshikawa, Takahiro, Gordon Dent, Jon Ward, Gilbert Angco, Guangmin Nong, Naho Nomura, Kazuto Hirata, and Ratko Djukanovic. 2007. "Impaired Neutrophil Chemotaxis in Chronic Obstructive Pulmonary Disease." *American Journal of Respiratory and Critical Care Medicine* 175 (5): 473–79. https://doi.org/10.1164/rccm.200507-1152OC.

Zhang, Jisong, Jian He, Jingwen Xia, Zhen Chen, and Xiaodong Chen. 2012. "Delayed Apoptosis by Neutrophils from COPD Patients Is Associated with Altered Bak, Bcl-Xl, and Mcl-1 MRNA Expression." *Diagnostic Pathology* 7 (1): 65. https://doi.org/10.1186/1746-1596-7-65.