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Proteasome composition in peripheral blood cells of COPD patients

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Andrea Urmann

(geb. Schneider)

aus

Goslar

Jahr

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Mit Genehmigung der Medizinischen Fakultät

der Universität München

Berichterstatter:	Prof. Dr. Silke Meiners
Mitberichterstatter:	PD Dr. Kathrin Kahnert
	Prof. Dr. Matthias Mann
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. Ilona Kammerl
Dekan:	Prof. Dr. med. Thomas Gudermann
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Zusammenfassung

Die chronisch obstruktive Lungenerkrankung (COPD) ist eine der führenden Todesursachen weltweit (Vogelmeier et al., 2017). Diese Erkrankung ist durch eine irreversible Abnahme der Lungenfunktion und chronisch entzündliche Prozesse gekennzeichnet (Pauwels et al., 2001). Das Proteasom spielt eine wichtige Rolle für viele Zellfunktionen wie zum Beispiel den Abbau geschädigter und fehlgefalteter Proteine, Apoptose und das Immunsystem (Coux et al., 1996; Meiners et al., 2014). In Immunzellen und als Antwort auf inflammatorische Zytokine wird das Standardproteasom durch das Immunoproteasom ersetzt. Es wird vermutet, dass Störungen im Zusammenhang mit dem Proteasom bei der Pathogenese von COPD eine Rolle spielen (Min et al., 2011; Yamada et al., 2015).

In einer Studie von Kammerl et al. wurde kürzlich gezeigt, dass die Expression des Immunoproteasoms in BAL-Zellen von COPD-Patienten vermindert ist (Kammerl et al., 2016). Das Ziel der vorliegenden Arbeit war es, diesen Zusammenhang in Immunzellen des periphervenösen Blutes von COPD-Patienten zu untersuchen. Dadurch könnte ein neuer diagnostischer Marker für COPD gewonnen werden.

Dafür wurden die mRNA-Expression, Proteinmenge und Aktivität mehrerer Proteasomuntereinheiten in PBMCs (peripheral blood mononuclear cells) von 32 COPD-Patienten und 24 gesunden Probanden verglichen. Interessanterweise war die Expression und Aktivität des Immunoproteasoms bei COPD-Patienten nicht vermindert, sondern erhöht.

Da ein Zusammenhang zwischen Proteasomaktivität und COPD gezeigt werden konnte, ist eine Verwendung als Biomarker vorstellbar. Um die Umsetzung im klinischen Alltag zu ermöglichen, müssten weitere Studien mit größeren Probandenzahlen durchgeführt werden, um die statistische Aussagekraft zu verstärken.

Summary

COPD is one of the leading causes of death worldwide (Vogelmeier et al., 2017). It is characterized by chronic inflammation and irreversible lung function decline (Pauwels et al., 2001). The proteasome is essential for many cellular functions including protein quality control, apoptosis, cell signaling and immune response (Coux et al., 1996; Meiners et al., 2014). In immune cells and upon induction by inflammatory cytokines, the standard proteasome is replaced by the immunoproteasome. Dysfunction of the proteasome system is suspected to play a role in the pathogenesis of COPD and emphysema (Min et al., 2011; Yamada et al., 2015).

Kammerl et al. recently analyzed the influence of cigarette smoke and COPD on the immunoproteasome in the lung. They found that immunoproteasome mRNA expression was decreased in bronchioalveolar lavage (BAL) cells and explanted lung tissue from COPD patients (Kammerl et al., 2016). This thesis aimed to determine proteasome expression and activity in peripheral blood mononuclear cells (PBMCs) of COPD patients and thereby evaluate proteasome function as a potential diagnostic marker for COPD.

PBMCs of 32 COPD patients and 24 healthy control subjects were analysed regarding mRNA expression, protein levels and activity of proteasome and immunoproteasome subunits. Of note, both immunoproteasome subunit expression and activity were increased in peripheral blood immune cells of COPD patients. These data suggest that systemic immune cells show distinct functional alterations in COPD patients that are different from lung resident cells.

Since this study showed an increased activity of the proteasome in COPD PBMCs, a use as a diagnostic biomarker seems feasible. Further investigations in a larger population could provide the statistical power necessary for potential clinical implementation.

1 Introduction

1.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (Pauwels et al., 2001). Clinical symptoms such as cough, sputum production and dyspnea can be early signs of COPD, however, the diagnosis is confirmed by spirometry. A post-bronchodilator forced expiratory volume in 1 second (FEV_1) of less than 80 % of the predicted value and a FEV_1/FVC (forced vital capacity) quotient under 0.7 confirm the diagnosis (Herold, 2016; Pauwels et al., 2001; Vogelmeier et al., 2017). In the past, the severity of COPD was classified based by FEV_1 reduction into GOLD stages I-IV (Herold, 2016; Pauwels et al., 2001; Vogelmeier et al., 2018, 2017). This classification, however, does not seem to adequately correlate with the subjective limitations in everyday life of COPD patients or their prognosis. Because of this, in the 2011 GOLD report a new classification was introduced which also takes into account severity of symptoms and exacerbation frequency. This was further developed in the 2017 GOLD report (Herold, 2016; Vestbo et al., 2013; Vogelmeier et al., 2018, 2017). In this new classification, COPD stages A-D are used (Figure 1).

A

GOLD Stage	FEV_1 (% of predicted value)
I	≥80
II	50-79
III	30-49
IV	<30

B

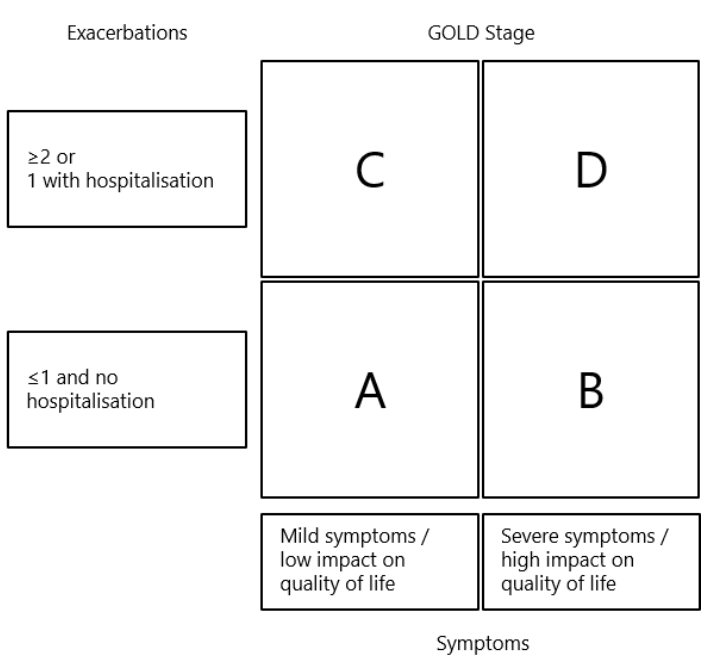

Figure 1 Classification of COPD. (A) In the past, FEV_1 reduction was used to classify COPD severity into Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages I-IV. **(B)** In the 2011 GOLD report, a new classification that uses exacerbation frequency and severity of symptoms to determine the stage of the disease (GOLD A-D) was introduced and improved by the 2017 GOLD report. Figure adapted from (Vogelmeier et al., 2018).

1.1.1 Pathogenesis and pathology

COPD is characterized by a chronic inflammation of airways and lung parenchyma (Barnes, 2017; Decramer et al., 2012; Macnee, 2007; Pauwels et al., 2001; Stockley et al., 2009; Vogelmeier et al., 2017). Inflammatory infiltrates are found predominantly in peripheral airways. As a sign of this inflammation, elevated numbers of neutrophils are found in the lungs of COPD patients (Barnes, 2017; Barnes et al., 2003; Macnee, 2007; Stockley et al., 2009). The chronic inflammation in small airways causes scar tissue formation and fibrotic remodeling of lung parenchyma resulting in an irreversible reduction of lung compliance (Barnes, 2017; Macnee, 2007; Pauwels et al., 2001; Vogelmeier et al., 2017). An additional factor contributing to airway obstruction is mucociliary dysfunction. Mucus hypersecretion and increased number of goblet cells are found in COPD patients' bronchi resulting in symptoms such as cough and sputum production (Barnes, 2017; Barnes et al., 2003; Macnee, 2007; Pauwels et al., 2001). Another characteristic of COPD is the destruction of lung parenchyma resulting in

emphysema (Barnes, 2017; Barnes et al., 2003; Decramer et al., 2012; Macnee, 2007; Pauwels et al., 2001; Stockley et al., 2009; Vogelmeier et al., 2017). Actually, this is often an early symptom of COPD and presents even before FEV1 reduction (Stockley et al., 2009). Finally, changes in pulmonary vasculature involving the thickening of vessel walls and an increased number of smooth muscle cells result in an impairment of gas exchange (Pauwels et al., 2001).

Although COPD has also been described in non-smokers, cigarette smoke remains the main risk factor for development of COPD in developed countries (Pauwels et al., 2001; Vogelmeier et al., 2017). In the past, the prevalence of COPD was shown to be higher in men than in women, but recently it was found that the number of male and female COPD patients was almost the same in developed countries, which is most likely due to more similar smoking habits between men and women (Pauwels et al., 2001; Salvi and Barnes, 2009). Cigarette smoke alone has been shown to increase granulocyte production, possibly via activation of granulocyte colony stimulating factor (G-CSF) (Barnes, 2017; Barnes et al., 2003; Macnee, 2007). Furthermore, smoking results in oxidative stress which is an important disease driving mechanism for COPD. Even in ex-smokers, oxidative stress levels remain elevated and COPD patients also have reduced expression levels of antioxidants (Barnes, 2017). Conclusive with this, COPD patients who never smoked suffer from less severe symptoms compared to COPD Patients who smoked (Thomsen et al., 2013; Vogelmeier et al., 2017).

Especially in developing countries where wood or other biomass products are used for indoor cooking and heating, exposure to biomass smoke is another major risk factor for COPD, and in these countries, the prevalence of COPD is higher in women (Olloquequi et al., 2018; Pauwels et al., 2001; Salvi and Barnes, 2009).

Though smoking is the main risk factor for COPD in developed countries, not all smokers develop COPD (Pauwels et al., 2001; Vogelmeier et al., 2017). The risk of a smoker developing CODP has been estimated at 15-20 % in the past but some studies

indicate that it might be as high as 50 % (Lundbäck et al., 2003). Additionally, genetic predispositions have an influence on the risk for COPD development. An especially well established genetic risk factor is the deficiency of α1-antitrypsin (AAT) (Pauwels et al., 2001; Stockley et al., 2009) but other COPD associated genes have been identified, for example ADAM33 or SOX5 (Martinez, 2016; Postma et al., 2015). Finally, factors that influence lung development early in life such as smoke exposure during pregnancy or early childhood and respiratory infections are important risk factors for the development of COPD (Martinez, 2016; Postma et al., 2015).

1.1.2 Exacerbations, infections

The most common symptoms of COPD are cough, sputum production and dyspnea. COPD is frequently associated with acute exacerbations of these symptoms. Severe exacerbations often require hospital admittance and significantly reduce quality of life of patients (Decramer et al., 2012; Halpin et al., 2017; Hurst et al., 2010; Vogelmeier et al., 2017; Wedzicha and Seemungal, 2007). As no causal treatment is available for COPD, reduction of exacerbation numbers is a main goal in the treatment of COPD (Decramer et al., 2012; Hurst et al., 2010). Frequent exacerbations are associated with accelerated decline in pulmonary function and increased mortality, and some patients never fully recover their lung function level from before the exacerbation (Decramer et al., 2012; Watz et al., 2018; Wedzicha and Seemungal, 2007) (Figure 2).

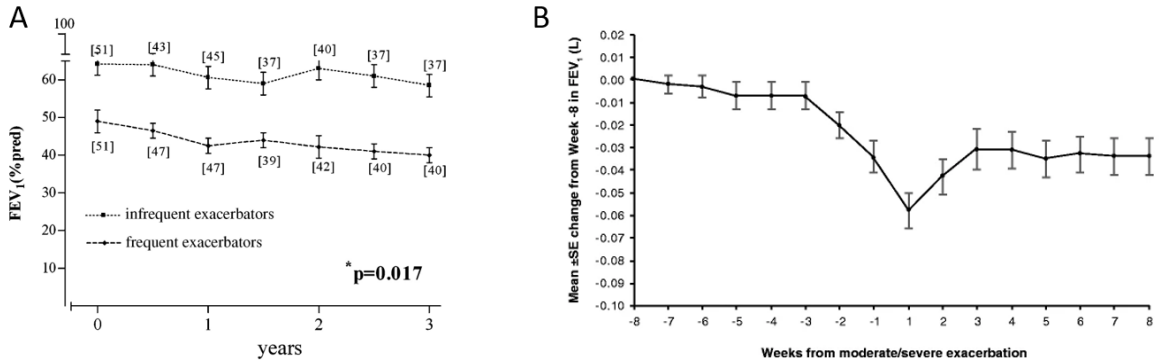

Figure 2 Exacerbations cause accelerated lung function decline. (A) COPD patients with frequent exacerbations have a lower FEV_1 than those with fewer exacerbations. Figure adapted from (Makris et al., 2007) **(B)** The lung function decline during an exacerbation is not fully reversible. Figure adapted from (Watz et al., 2018)

Exacerbations can be caused by a variety of stress factors, for example heart failure, pneumonia, pulmonary embolism or inhalation of irritants. However, the most frequent cause are bacterial and viral infections, often upper respiratory tract infections caused by common viruses such as rhinovirus, coronavirus or influenza virus (Decramer et al., 2012; Wedzicha and Seemungal, 2007). Exacerbations caused by infections are more severe than those of other causes, with greater decline in lung function and longer hospital stays (Decramer et al., 2012). Lung inflammation can be found in all smokers to some extent, but in COPD, there seems to be an abnormal inflammatory response to triggers. This goes beyond the normal protective immune response and induces lung damage (Macnee, 2007).

Airway viral infections are usually self-limited and resolve quickly in patients without chronic respiratory diseases. In COPD, however, elimination of viruses does not take place optimally. Latent viral infections may be related to persistent airway inflammation in COPD, and rhinoviral proliferation is increased in cells from the lungs of COPD patients (Matsumoto and Inoue, 2014). Viral infections can be aggravated by bacterial superinfections that are associated with longer hospital stays and worse prognosis (Decramer et al., 2012; Seemungal et al., 2001; Wedzicha and Seemungal, 2007).

The adaptive immune response to virus-infected cells is mediated by cytotoxic $CD8^+$ T-cells. They recognize viral antigens bound to major histocompatibility complex (MHC) class I molecules on the cell surface and kill the infected cells (Falk and

Rötzschke, 1993; Rock et al., 1994). Increased numbers of T-lymphocytes have been found in the lungs of COPD patients with a greater increase in $CD8^+$ cells than $CD4^+$ cells, and T-cell numbers correlate with the amount of destruction in the lung and severity of disease (Hogg et al., 2004).

1.1.3 Therapy

The changes in lung structure and the resulting decline of lung function in COPD is irreversible and to this point, there is no causal treatment available. Since it is challenging to repair existing damage caused by COPD the main goal of any therapy must be to slow down the progression of the disease and improve quality of life. For this, it is most important to reduce the frequency and severity of exacerbations which cause accelerated decline in lung function and significantly reduce patients' quality of life (Herold, 2016; Vogelmeier et al., 2017; Wedzicha and Seemungal, 2007).

The most important part of COPD therapy is smoking cessation. Cigarette smoking is not only the main risk factor for the development of COPD but also causes accelerated decline in lung function in patients already suffering from COPD (figure 3). Also, inhalation of noxious particles (like cigarette smoke) can be a trigger for exacerbation (Fletcher and Peto, 1977; Herold, 2016; Vogelmeier et al., 2018, 2017; Wedzicha and Seemungal, 2007).

In the pharmacotherapy of COPD, bronchodilators can be used to control symptoms. The most relevant bronchodilators are β-agonists such as salbutamol or formoterol and anticholinergic drugs such as tiotropium. They lead to a relaxation of airway smooth muscle cells and are used especially for acute dyspnea. Bronchodilators should be applied locally by inhalation to minimize systemic effects. In long term treatment, long-acting substances should be preferred (Vogelmeier et al., 2018). Although bronchodilators have been shown to reduce exacerbation frequency and severity and significantly improve quality of life, no long-term effect on lung function decline and

mortality could be observed (Barr et al., 2005; Calverley et al., 2007; Cazzola et al., 1995; Cheyne et al., 2015).

The same is true for inhaled or systemic corticosteroids. They can improve symptoms and reduce exacerbation frequency, but do not provide a significant improvement of long-term prognosis (Yang et al., 2012). Because of severe side effects, long term corticosteroid therapy is not recommended and treatment with corticosteroids should be reserved for specific indications. For example, corticosteroids can be used during acute exacerbation to reduce inflammatory symptoms (Vogelmeier et al., 2018).

Opioids can be used to reduce dyspnea if bronchodilator therapy alone is not enough. This treatment is used in patients with severe COPD and otherwise uncontrollable dyspnea. They do not improve long term prognosis and side effects are frequent (Vogelmeier et al., 2018).

A long-term oxygen therapy can be necessary in severe cases with continuously low arterial oxygen concentrations and can reduce mortality (Nocturnal Oxygen Therapy Trial Group, 1980; Stoller et al., 2010).

Since exacerbations are often caused by respiratory tract infections, vaccinations for influenza and pneumococcus are recommended for COPD patients (Vogelmeier et al., 2018).

1.2 The Proteasome System

The Ubiquitin-Proteasome-System degrades the majority of cellular proteins (Rock et al., 1994). The resulting peptide fragments are used for amino acid recycling and MHC class I antigen presentation (Kincaid et al., 2011; Rock et al., 1994). This protein degradation by the proteasome is essential for many cellular functions including protein quality control, apoptosis, cell signaling and immune responses (Coux et al., 1996; Meiners et al., 2014).

The proteasome consists of a cylindrical 20S catalytic core particle that is associated with several regulators that differ in structure and function. The 20S core is composed of two outer α and two inner β rings that contain seven subunits each (Figure 3). The three β-subunits β_1, β_2 and β_5 contain proteolytically active sites with caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Coux et al., 1996; Kincaid et al., 2011; Schmidt and Finley, 2014; Stadtmueller and Hill, 2011). In immune cells and upon induction by the inflammatory cytokines IFNγ and TNFα, these subunits are replaced by low molecular weight protein 2 (LMP2), Multicatalytic Endopeptidase Complex-Like 1 (MECL1) and low molecular weight protein 7 (LMP7), respectively, to form the immunoproteasome (Aki et al., 1994; Groettrup et al., 2001; Huber et al., 2012; Schmidt and Finley, 2014; Stadtmueller and Hill, 2011). These immuno-subunits have altered cleavage properties and provide peptides that are better suited for presentation on MHC class I molecules: the chymotrypsin-like activity is amplified in immunoproteasomes while the caspase-like activity is decreased, causing increased cleavage after basic and hydrophobic residues and a decrease of cleavage after acidic residues. This improves antigen presentation as most peptides that are presented on MHC I molecules have hydrophobic or basic C-termini (Groettrup et al., 2001; Huber et al., 2012; Kincaid et al., 2011; Schmidt and Finley, 2014; Stadtmueller and Hill, 2011).

Proteasomes play an important role in the adaptive immune response. They provide antigenic peptides for MHC I presentation which are recognized by $CD8^+$ cells that mediate the response to viral infections and eliminate virus infected cells (Rammensee et al., 1993; Rock et al., 1994). Immunoproteasomes are also thought to play a role in cytokine production, B cell maturation, dendritic cell function and T cell differentiation as well as regulation of transcription factors (e.g. NF-κB) (Beling and Kespohl, 2018; Groettrup et al., 2010; Kammerl and Meiners, 2016). The proteasome also influences the activity of toll like receptors (TLR).(Beling and Kespohl, 2018) Proteasome inhibition has been shown to reduce inflammatory and immune response and increase the susceptibility to viral infections (Basler et al., 2009; Kincaid et al., 2011; Schmidt and

Finley, 2014). Proteasome inhibitors are used in the treatment of malignant diseases such as multiple myeloma, and the use of specific immunoproteasome inhibitors has been proposed as a possible treatment for autoimmune and chronic inflammatory conditions (Groettrup et al., 2010; Kammerl et al., 2021a).

Proteasomal activators (PAs) bind to the 20S proteasome, opening a channel that is formed by the outer alpha rings of the 20S proteasome into the lumen of the proteasome (Schmidt and Finley, 2014; Stadtmueller and Hill, 2011). Although 20S proteasomes alone are capable of protein degradation of unfolded proteins at low but detectable levels *in vitro*, PAs are needed for sufficient cellular function (McCarthy and Weinberg, 2015; Schmidt and Finley, 2014). Three different families of PAs have been described (Stadtmueller and Hill, 2011). The 19S activator is involved in ATP dependent degradation of polyubiquitinated proteins. It can bind to either one or two ends of the 20S proteasome forming the 26S or 30S proteasome, respectively (McCarthy and Weinberg, 2015; Meiners et al., 2014; Schmidt and Finley, 2014; Stadtmueller and Hill, 2011; Wang et al., 2020b). The 11S activators PA28α, β and γ are important for generating peptide fragments especially suited for T cell receptor recognition (Dick et al., 1996).The third one is PA200. It is thought to play a role in various functions including proteasome assembly and DNA repair (Stadtmueller and Hill, 2011).

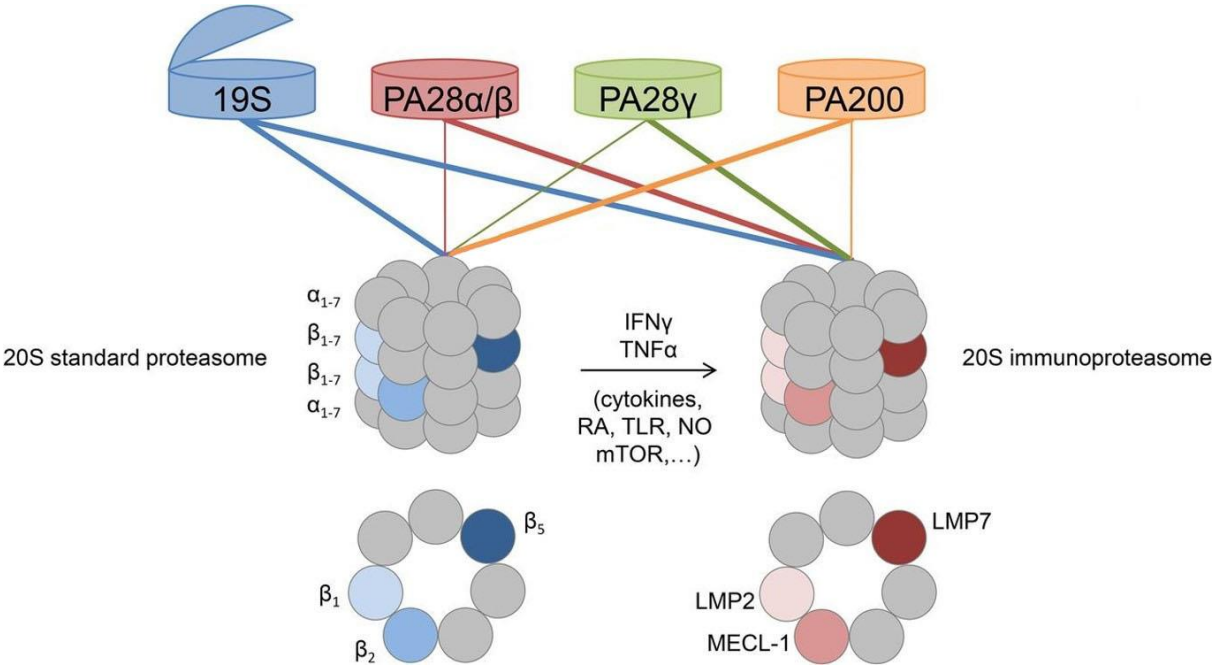

Figure 3 Proteasome complexes. The 20S core is composed of two outer alpha and two inner beta rings that contain seven subunits each. In immune cells and upon induction by inflammatory cytokines such as IFNγ, the three β-subunits β1, β2 and β5 are replaced by LMP2, MECL1 and LMP7, respectively, to form the immunoproteasome. Proteasomal activators bind to the 20S standard or immunoproteasome to form a variety of proteasome complexes. Figure adapted from (Kammerl and Meiners, 2016)

1.2.1 Role of proteasome dysfunction in COPD

Cigarette smoke has been shown to decrease protein degradation in bronchial epithelial cells (van Rijt et al., 2012; Somborac-Bacura et al., 2013) and lead to accumulation of oxidatively modified and polyubiquitinated proteins (Min et al., 2011; van Rijt et al., 2012; Somborac-Bacura et al., 2013). In the lungs of COPD Patients, accumulation of ubiquitinated proteins correlates with severity of emphysema (Min et al., 2011). One possible explanation for this decreased protein degradation could be smoke induced malfunction of the proteasome. Indeed, it was shown that cigarette smoke decreases proteasome activity but not expression in A549 human bronchial epithelial cells and mice (Kammerl et al., 2019; van Rijt et al., 2012; Somborac-Bacura et al., 2013). Kammerl et al. recently analyzed the influence of cigarette smoking and COPD on the immunoproteasome in the lung (Kammerl et al., 2016). They found that immunoproteasome mRNA expression was decreased in bronchioalveolar lavage (BAL) cells from COPD patients. For *in vivo* analysis, they analyzed explanted lung tissues

from COPD patients and organ donors. They found that RNA and protein expression of proteasome subunits was not altered in COPD patients' lungs but a decrease of overall proteasome activity was seen in the lungs of COPD patients (Baker et al., 2014; Kammerl et al., 2016).

Proteostasis-imbalance is suspected to play a causal role in the pathogenesis of COPD and emphysema. For instance, Yamada et al. showed that proteasome dysfunction increases cigarette smoke induced emphysema: Transgenic mice with decreased proteasomal chymotrypsin-like function develop increased airspace enlargement, inflammation and cell death in response to cigarette smoke compared to wildtype mice (Yamada et al., 2015). Also, mouse and human lung fibroblast cells showed increased cell death when exposed to cigarette smoke extract and a proteasome inhibitor together than each individually (Yamada et al., 2015).

Since there seems to be a correlation between proteasome function and COPD, the proteasome might provide an interesting target for innovative therapy approaches.

1.3 Aim of the Study

The objective of this work was to determine if a decrease in proteasome and especially immunoproteasome function, i.e. expression and activity of the proteasome, can be observed in the peripheral blood of COPD patients validating the analysis of proteasome function as a potential biomarker approach for progressive COPD patients.

2 Materials and Methods

2.1 Materials

2.1.1 Consumables

Material	Cat. Number	Manufacturer
15 ml Polypropylene Conical Tube	352096	Corning / Falcon
5 ml Polystyrene round-Bottom tube	352054	Corning / Falcon
50 ml Polypropylene Conical Tube	352070	Corning / Falcon
Cellulose swabs Askina Brauncel	9051015	Braun
Chromatography paper	3030690	GE Healthcare
Fuji medical X-ray film	4741019289	Fujifilm
Microplate, 96 well	655101	Greiner bio-one
Multifly-Set	81.1638.035	Sarstedt
NuPAGE Novex 3-8% Tris-Acetate Gel 1.5 mm (15 well)	EA03785BOX	Thermo Fisher Scientific
Optical seal foil	GK480-OS	Kisker
Polyvinylidene difluoride (PVDF) membrane 02 µm	1620177	BioRad
qPCR plates	GK480K-BC-100	Kisker
Quali-PCR tubes	G003-SF	Kisker
SafeSeal tubes	72.706	Sarstedt
Sepmate 50 ml	86450	Stemcell
S-Monovette K-EDTA	02.1066.001	Sarstedt

2.1.2 Reagents and Chemicals

Reagent	Cat. Number	Manufacturer
5 x First Strand Buffer	18057018	Invitrogen
Acetic acid	A3686,1000	PanReac AppliChem
Acrylamide	3029.1	Roth
Adenosine triphosphate (ATP)	BP413-25	Fischer Scientific
Ammonium persulfate (APS)	A0834,0250	AppliChem
ATP	BP413-25	Fisher Bioreagents

Reagent	Cat. Number	Manufacturer
Biocoll Separating Solution	L6115	Biochrom
Boric acid	A2940,1000	AppliChem
Bromophenol blue	A2331,0025	AppliChem
Bromphenol blue	A2331,0025	AppliChem
cOmpleteTM protease inhibitor cocktail	11697498001	Roche
di-sodium hydrogen sulfate (Na2HPO4)	A1046,0500	PanReac AppliChem
Dithiothreitol (DTT)	A2948,0025	AppliChem
DNAse	12-1091-01	PEQLAB
dNTPs	C1141	Promega
EDTA	A5097,0250	AppliChem
Ethanol	A3678,1000	PanReac AppliChem
Ethanol denatured	K928.4	Roth
Fetal bovine serum (FBS)	S 0615	Biochrom
Flow count fluorospheres	7547035	Beckman Coulter
Formic acid	F0507	Sigma-Aldrich
Glycerol	A3739,1000	PanReac AppliChem
Glycine	A1067,1000	PanReac AppliChem
Hydrochloric acid (HCl)	4625.1	Roth
Light Cycler 480 SYBR Green I Master mix	04887352001	Roche
LuminataTM classico	WBLUC0100	Millipore
LuminataTM forte	WBLUF0500	Millipore
Magnesium chloride (MgCl2)	A1036,0500	AppliChem
Magnesium chloride (MgCl2)	A1036,0500	AppliChem
Methanol	1600.2500	NeoLab
NP40	85124	Thermo Fisher
Nuclease-free water	AM9937	Ambion
PageBlueTM	24620	Thermo Scientific
Penicillin Streptomycin (Pen/Strep)	15140-122	gibco® by Life technologies
Potassium chloride (KCl)	A3582,0500	AppliChem
Potassium dihydrogen phosphate (KH2PO4)	A2946,1000	PanReac AppliChem
Random hexamers primer	48190011	Invitrogen
Reverse transcriptase M-MVL	M1427-40KU	Sigma

Reagent	Cat. Number	Manufacturer
RNAsin plus	N2615	Promega
Roti block	A151.2	Roth
RPMI-1640 Medium	R8758-500ML	Sigma-Aldrich
Sodium carbonate (Na2CO3)	A3900,0500	AppliChem
Sodium chloride (NaCl)	A2942,5000	PanReac AppliChem
Sodium dodecyl sulfate (SDS)	L5750	Sigma-Aldrich
Sodium hydroxide (NaOH)	A0991,1000	PanReac AppliChem
Sodium sulfate (Na2SO4)	238597	Sigma-Aldrich
Sodiumdesoxycholate (DOC)	D6750	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	A1148	PanReac AppliChem
Trizma® Base	T1503	Sigma
Tween® 20	A4974,1000	PanReac AppliChem

2.1.3 Kits

Kit	Cat. Number	Manufacturer
Pierce™ BCA Protein Assay Kit	23225	Thermo Scientific
Roti-Quick-Kit	A979.1	Roth

2.1.4 FACS Antibodies

Antibody	Fluorophore	Cat. Number	Clone	Manufacturer	Dilution
CD3*	Pacific Blue	558117	UCHT1	BD Pharmingen	01:50
IgG1	Pacific Blue	558120	MOPC-21	BD Pharmingen	01:50
CD3 °	FITC	IM1281	UCHT1	Beckman Coulter	01:20
IgG1	FITC	A07795	679.1Mc7	Beckman Coulter	01:20
CD4 °	V450	560345	RPA-T4	BD Pharmingen	01:50

Antibody	Fluorophore	Cat. Number	Clone	Manufacturer	Dilution
IgG1	V450	560373	MOPC-21	BD Pharmingen	01:50
CD8 °	PECy5	555368	RPA-T8	BD Pharmingen	01:50
IgG1	PECy5	555750	MOPC-21	BD Pharmingen	01:50
CD14*	APC	IM2580	RMO52	Beckman Coulter	01:50
IgG2a	APC	A12693	7T4-1F5	Beckman Coulter	01:50
CD15*	FITC	562370	W6D3	BD Pharmingen	01:20
IgG1	FITC	555748	MOPC-21	BD Pharmingen	01:20
CD16*	PE	A07766	3G8	Beckman Coulter	01:50
IgG1	PE	A07796	679.1Mc7	Beckman Coulter	01:50
CD19*	PECy5	555414	HIB19	BD Pharmingen	01:50
IgG1	PECy5	555750	MOPC-21	BD Pharmingen	01:50
CD56*	PECy7	557747	B159	BD Pharmingen	01:20
IgG1	PECy7	557872	MOPC-21	BD Pharmingen	01:20

*part of the standard panel; °part of the T-Cell panel

2.1.5 Primers for qPCR

Primer	Sequence Forward (5'-3')	Sequence Reverse (5'-3')
PSMB5	TCAGTGATGGTCTGAGCCTG	CCATGGTGCCTAGCAGGTAT
PSMB6	CAGAACAACCACTGGGTCCT	CCCGGTATCGGTAACACATC
PSMB7	TCGCTGGGGTGGTCTATAAG	TCCCAGCACCACAACAATAA
PSMB8	GTTCCAGCATGGAGTGATTG	TTGTTCACCCGTAAGGCACT
PSMB9	ATGCTGACTCGACAGCCTTT	GCAATAGCGTCTGTGGTGAA
PSMB10	AGCCCGTGAAGAGGTCTGG	CATAGCCTGCACAGTTTCCTCC
PSMA3	AGATGGTGTTGTCTTTGGGG	AACGAGCATCTGCCAACAA
PSMC3	GTGAAGGCCATGGAGGTAGA	GTTGGATCCCCAAGTTCTCA
PSME1	CAAGGTGGATGTGTTTCGTG	TGCTCAAGTTGGCTTCATTG
PSME2	GCAAACAGGTGGAGGTCTTC	GTCAGCCACATTGAGGGAGT
PSME3	TAGCCATGATGGACTGGATGG	CCTTGGTTCCTTGGAAGGCT
PSME4	CCAACAGGAAAAGAATGCCGA	CCAGGGCAGGTTTCTTTGCT
PSMF1	AAAGCTCCTTGTGAAAGCCA	CCCACTGCTCATGGATAGGT
PSMD11	GCTCAACACCCCAGAAGATGT	AGCCTGAGCCACGCATTTTA
IFNγ	TGACCAGAGCATCCAAAAGA	CATGTATTGCTTTGCGTTGG
HPRT	TGAAGGAGATGGGAGGCCA	AATCCAGCAGGTCAGCAAAGAA
RPL19	TGTACCTGAAGGTGAAGGGG	GCGTGCTTCCTTGGTCTTAG

All qPCR primers were obtained from Eurofins Genomics.

2.1.6 Western Blot Antibodies

Antibody	Host	Clonality	Cat. Number	Manufacturer
LMP2	Rabbit	Polyclonal	ab3328	Abcam
LMP7	Rabbit	Polyclonal	ab3329	Abcam
β1	Rabbit	FL-241	sc-67345	Santa Cruz Biotechnology
PSMA4	Rabbit	EPR5831(2)	ab191403	Abcam
PSME1	Rabbit	monoclonal	ab155091	Abcam
Actin	Mouse	AC-15	A3854	Sigma
2nd antibody anti rabbit IgG (coupled to HRP)	Goat	polyclonal	7074S	Cell signaling Technology
2nd antibody anti mouse IgG (coupled to HRP)	Horse	polyclonal	7076S	Cell signaling Technology

2.1.7 Machines

Machine	Manufacturer
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, 4-gel	BioRad
Chemidoc XRS+	BioRad
Curix 60	Agfa
Easy One™ world spirometer	ndd Medizintechnik AG
Eco-Maxi System EB Combs 30 well	AnalytikJena
Geldoc	Intas Science Imaging
Lightcycler	Roche
LSRII	BD
Mastercycler gradient	Eppendorf
Mikro 200R centrifuge	Hettich
Nano drop	Thermo Scientific
Q-Prep Workstation	Beckman Coulter
Rotina 420R centrifuge	Hettich
Sunrise™	Tecan
Thermomixer	Eppendorf
Typhoon	GE Healthcare

2.2 Methods

2.2.1 Blood Samples and Workflow

Blood samples from 32 COPD patients from the LMU Klinikum Großhadern were used. For the control group, blood was collected from 24 healthy control subjects. The control subjects were recruited from the Helmholtz Zentrum Munich. All study participants gave written consent. The study was approved by the Ethics Committee of the Medical Faculty of the LMU (study number 382-10). Inclusion criteria for the control group were age over 45 years. Exclusion criteria were known lung diseases (e.g. asthma, COPD), pulmonary obstruction (FEV_1 / FVC < 0.7), current medication with corticosteroids, and malignant diseases. In control subjects, lung function was measured using the EasyOne^{TM} spirometer.

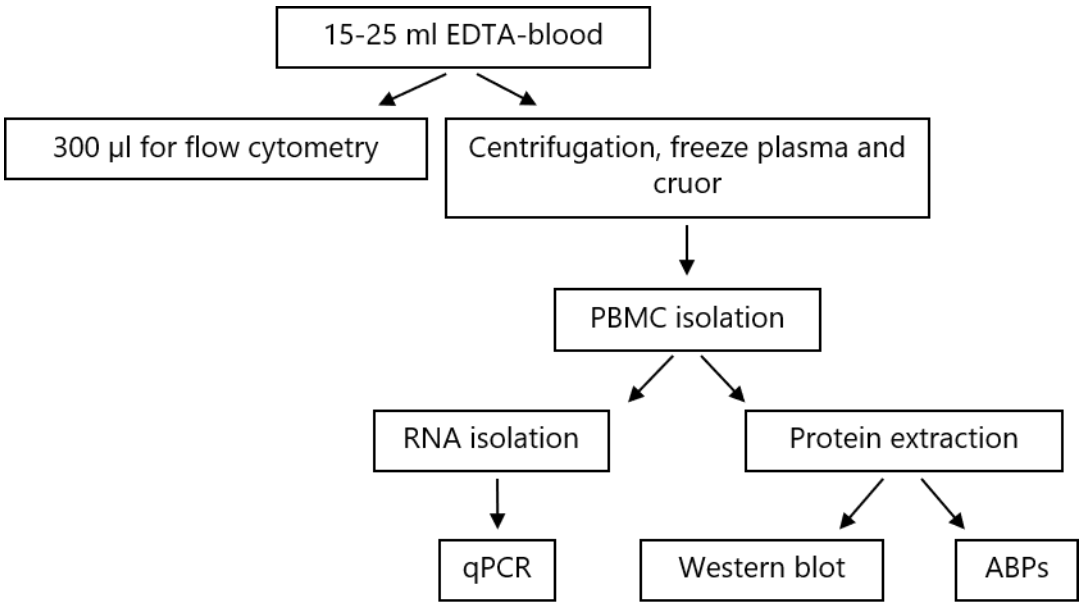

Figure 4 Study design. Of the 15-25 ml EDTA blood from each patient, 300 µl were used for flow cytometry. The rest was centrifuged and ca. 3 ml plasma and 2 ml cruor were frozen. The rest of the blood was mixed again and used for PBMC isolation. RMA and protein were isolated from the PBMCs; the RNA was used for qPCR and the protein for Western blot or ABP analysis.

From each patient, 15-25 ml EDTA-blood was collected. From this, 300 µl were used for flow cytometry analysis, the rest was centrifuged at 2200 rpm for 10 minutes, ca. 3 ml plasma and 2 ml cruor were frozen according to standard procedures of the CPC-M bioArchive. The remaining blood was carefully mixed again and used for PBMC

isolation. PBMCs were frozen and stored at -80 °C and used for protein and mRNA isolation as described below (Figure 4).

2.2.2 Flow Cytometry

Solution A for Q-Prep Workstation:

500 ml H_2O
600 µl formic acid

Solution B for Q-Prep Workstation:

500 ml H_2O
3 g Na_2CO_3
7.5 g NaCl
16.65 g Na_2SO_4
Autoclave before use

Flow cytometry was used to quantify the different leukocyte populations in the blood of COPD patients and controls.

To validate the specificity of the antibodies, each stained sample was compared to an isotype control with the same fluorophore from the same company.

100 µl of EDTA-blood was mixed with FACS-antibodies (2.1.4, *part of the standard panel; °part of the T-Cell panel) in FACS tubes and then incubated at 4 °C for 20 minutes in the dark together with an unstained control. After incubation, erythrocytes were lysed using the Q-Prep Workstation. 100 µl flow count fluorospheres with known concentration were added to the samples right before measurement.

Gating strategy:

In the forward scatter/side scatter analysis, the three main leukocyte populations can be identified:

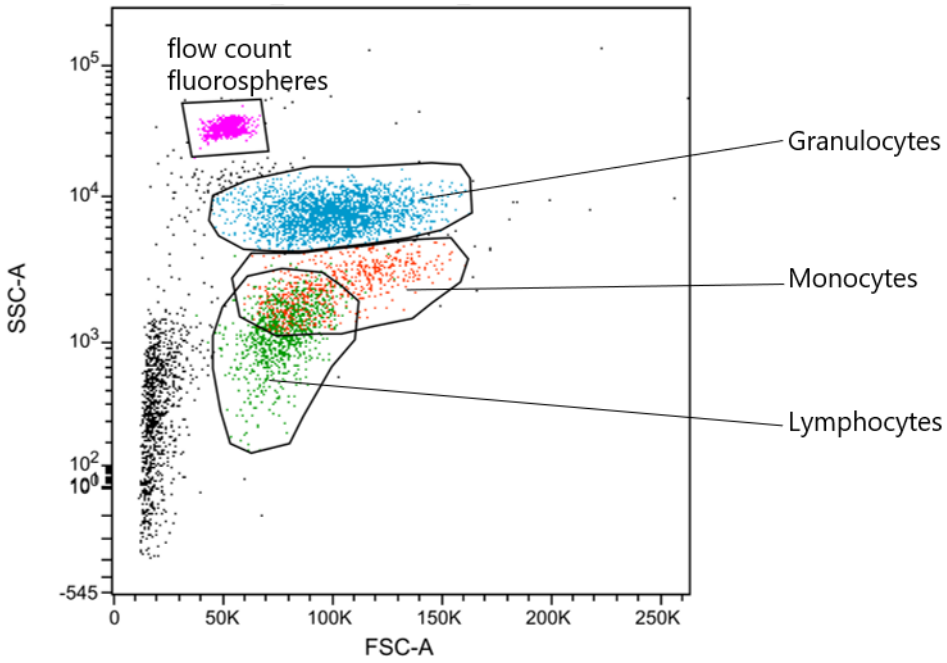

Figure 5 Gating strategy: In the forwardscatter / sidescatter analysis the three main leukocyte populations were identified: Granulocytes, Monocytes and Lymphocytes.

To specify the cell types, the following antigens were used:

Cell type	Gate	Antigens
neutrophilic granulocytes	Granulocytes	CD15, CD16
eosinophilic granulocytes	Granulocytes	CD15
classical monocytes	Monocytes	CD14
nonclassical monocytes	Monocytes	CD14, CD16
T cells	Lymphocytes	CD3
T helper cells	T cells	CD3, CD4
cytotoxic T cells	T cells	CD3, CD8
natural killer cells	T cells	CD16, CD56
B cells	Lymphocytes	CD 19

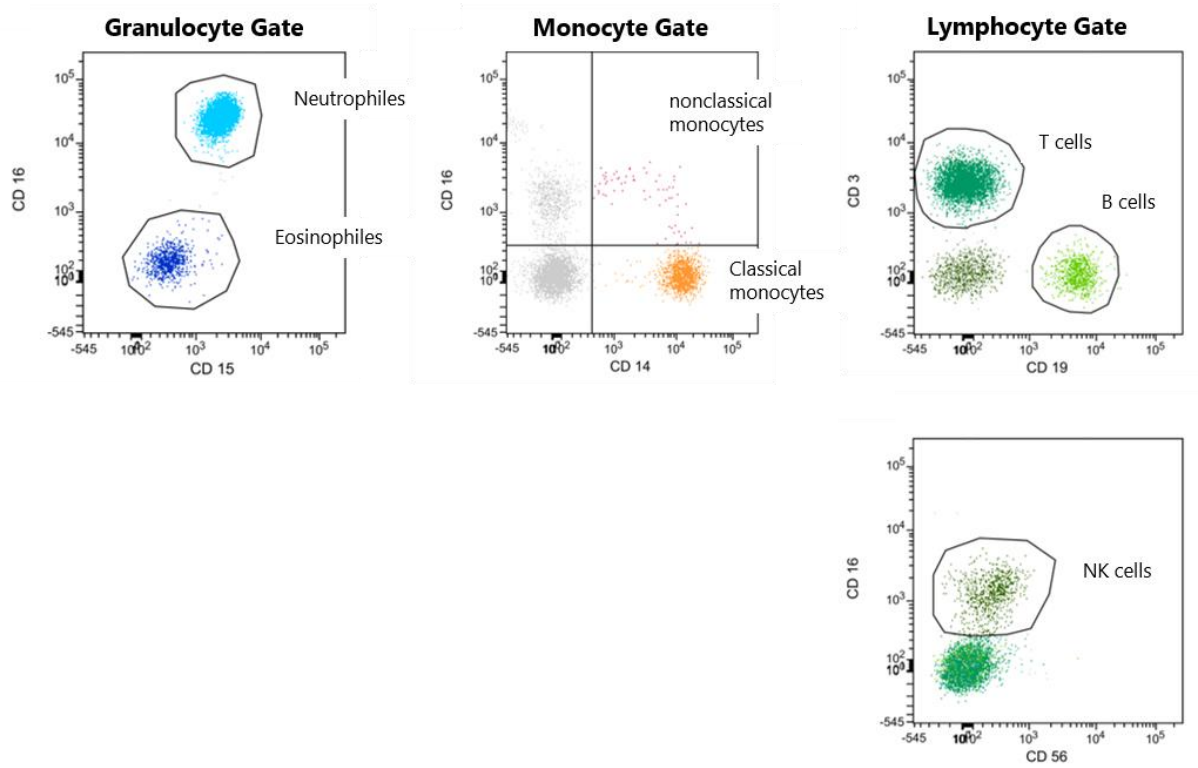

Figure 6 Gating strategy: In the granulocyte gate, CD15 and CD16 were used to discriminate between neutrophils ($CD15^{+}CD16^{+}$) and eosinophils ($CD15^{-}CD16^{-}$). In the monocyte gate, classical monocytes ($CD14^{+}CD16^{-}$) and nonclassical monocytes ($CD14^{+}CD16^{+}$) were identified. In the lymphocyte gate, CD3 was used as a T cell marker and CD19 as a B cell marker. NK cells were identified in the Lymphocyte gate using CD16 and CD56.

The T-lymphocyte population was further specified using a T-Cell Panel (CD3, CD4, CD8):

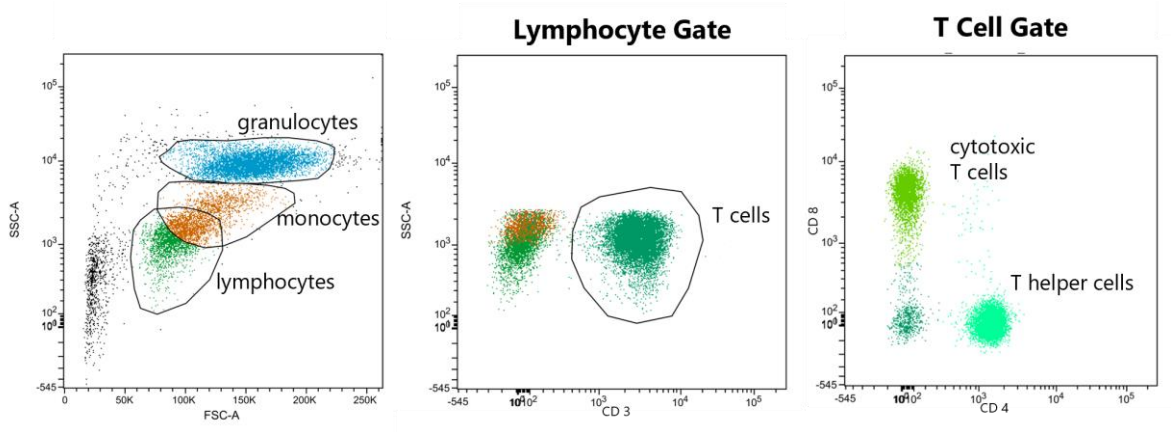

Figure 7 Gating strategy: In the forward scatter / side scatter analysis the three main leukocyte populations were identified. T cells were identified from the lymphocyte gate using CD3. In the T cell gate, CD4 and CD8 were used to differentiate T helper cells and cytotoxic T cells.

Cell numbers were calculated using the known concentration of flow count fluorospheres: $cell\,number/\mu l = \frac{fluorospheres/\mu l}{counted\ fluorospheres} \times counted\ cells$

2.2.3 PBMC-Isolation

Phosphate buffered saline (PBS):

1 l water
NaCl 160 g
Na_2HPO_4 23 g
KCl 4 g
KH_2PO_4 4 g
pH to 7.4 with HCl and NaOH
add water to 2 l

RNA and protein analyses were performed in peripheral blood mononuclear cells (PBMCs). PBMCs are composed of lymphocytes, monocytes and dendritic cells (Kleiveland, 2015). The blood (ca. 15-25 ml) was mixed 1:1 with PBS in a 50 ml tube. 15 ml Biocoll was pipetted through the opening into the lower part of the SepMate™ tube. The blood-PBS mix was pipetted carefully to the side of the SepMate™ tube. The tube was centrifuged for 10 minutes at 1200 x *g* at room temperature with the brake on. The top layer was poured into a 50 ml Falcon and PBS was added to a total volume of 50 ml. This Falcon was centrifuged for 15 minutes at 300 x *g* at room temperature, the supernatant was removed, and the pellet was resuspended in PBS and PBMCs were counted. The PBMCs were divided into portions of about 4 Mio cells in SafeSeal tubes, centrifuged for 10 minutes at 800 x *g* and the supernatant was removed. The pellets were stored at -80 °C.

2.2.4 RNA Isolation

Total RNA was isolated using the Roti-Quick-Kit which uses phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Cell pellets were resuspended in 500 µl solution 1; 650 µl solution 2 was added. Samples were vortexed and incubated on ice

for 10 minutes, then centrifuged for 15 min at 10,000 rpm at 4 °C. The upper phase was carefully transferred into a new tube, the lower phase was discarded. At this point the fluid should be clear. If this was not the case, the sample was centrifuged again at 10.000 rpm for 5 minutes and contaminations were taken out from the bottom of the tube. 500 µl solution 3 was added and mixed. Samples were incubated for at least 40 minutes at -20 °C, then centrifuged for 20 minutes at 4 °C at 13,000 rpm. The supernatant was removed and 500 µl 70 % ethanol was added. After centrifugation for 5 minutes at 13,000 rpm at 4 °C, ethanol was removed and 300 µl fresh ethanol was added. Samples were centrifuged again for 5 minutes at 13,000 rpm at 4 °C and the ethanol was removed. Pellets were left open to dry for 20 minutes, then resuspended in nuclease-free water (amount depending on pellet size, usually between 15 and 50 µl). The RNA concentration was measured with the Nanodrop spectrophotometer.

2.2.5 Reverse Transcription

Master mix (per sample):

4 µl	First strand buffer
2 µl	DTT (0,1 M)
1 µl	dNTPs (10 mM)
0.5 µl	RNAsin
1 µl	RT (200 U/µl)

The RNA was transcribed to cDNA, which is more stable during qPCR. 1 µg RNA was mixed with nuclease-free water to a total volume of 9.5 µl. 2 µl random primers were added. Samples were incubated at 70 °C for 10 minutes in the MasterCycler. The master mix was added to the samples (8.5 µl per sample) and samples were incubated in the MasterCycler: Annealing for 5 minutes at 25 °C, elongation for 60 minutes at 37 °C. 0.5 µl DNAse was added, and samples were incubated at 37 °C for 15 minutes, then heat-inactivated at 75 °C for 10 minutes. The resulting cDNA was diluted 1:5 with water to a total volume of 100 µl.

2.2.6 qPCR

cDNA and Light Cycler 480 SYBR Green I Master mix were mixed 1:2. Forward and reverse primers with a concentration of 100 µmol/l were diluted 1:50 in water together. In a 96 well plate 2.5 µl diluted primer and 7.5 µl of the cDNA mix were mixed. A no-template control of water/ Light Cycler 480 SYBR Green I Master mix for each primer was always included. The cover foil was put on the plates and after centrifugation the plates were measured in the light cycler. Mean mRNA expression of two housekeeping genes, RPL19 and HPRT, was used to obtain relative expression levels of proteasomal genes in the different samples.

2.2.7 Native Protein Lysis

Native lysis buffer:

50 mM Tris HCl, pH 7.5
2 mM DTT
5 mM MgCl2
10 % Glycerol
2 mM ATP
0.05 % digitonin

Native lysis buffer and cOmplete™ protease inhibitor were mixed 25:1 and kept on ice. Cell pellets were resuspended in 60 µl of this mix and incubated on ice for 20 minutes. The samples were centrifuged for 20 minutes at 4 °C and maximal speed. The supernatant was transferred into new tubes, the pellets were discarded. Protein concentration was determined using the BCA assay (see below) and samples were stored at -80 °C.

2.2.8 Radioimmunoprecipitation Assay Buffer (RIPA) Protein Lysis

RIPA lysis buffer:

50 mM Tris HCl, pH 7,5
150 mM NaCl
1 % NP40
0.5 % Sodiumdeoxycholate
0.1 % SDS

RIPA lysis buffer and cOmplete™ were mixed 25:1 and kept on ice. Cell pellets were resuspended in 60 µl of this mix and incubated on ice for 20 minutes. The samples were centrifuged for 20 minutes at 4 °C and maximal speed. The supernatant was transferred into new tubes, the pellets were discarded. Protein concentration was determined using the BCA assay (see below) and samples were stored at -80 °C.

2.2.9 BCA Assay

BCA assay was used to determine protein concentration in the lysates. In a 96 well plate, 10 µl standard albumin concentrations from 0.025 µg/µl to 2 µg/µl and was pipetted to 2 wells each. 2 µl protein lysate was mixed with 8 µl PBS in three wells each. The mixture of Native lysis buffer and cOmplete™ was used as a control. BCA kit solution A and B were mixed 50:1. 200 µl of this mix was added to each well. The plate was incubated at 37 °C for 30 minutes, then measured at 562 nm with the Sunrise™ absorbance reader.

2.2.10 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) - Gels

4 x stacking buffer:

6.05 g Trizma® Base
to 40 ml H2O
Adjust pH to 6.8 with HCl 36 %
to 100 ml H2O
Filter 0.45 µm
0.4 g SDS

4 x resolving buffer:

36.4 g Trizma® Base
to 150 ml H2O
Adjust pH to 8.8 with HCl 36 %
to 200 ml H_2O
Filter 0.45 µm
0.48 g SDS

stacking gel		**resolving gel**	
4 x stacking buffer	1 ml	4 x resolving buffer	2 ml
30 % acrylamide	480 µl	30 % acrylamide	4 ml
H_2O	2,52 ml	H_2O	2 ml
10 % APS	50 µl	10 % APS	100 µl
TEMED	12 µl	TEMED	12 µl

For Western blots and ABPs, 15 % gels were used. For the resolving gel, water, resolving buffer, acrylamide and APS were mixed. TEMED was added, the mix was vortexed and then quickly poured between the glass panes. Isopropanol was poured on top carefully and the resolving gel was left to polymerize for 20 minutes. The isopropanol was poured off and the gel was washed with water 4-5 times. For the stacking gel, water, stacking buffer, acrylamide and APS were mixed. TEMED was added, the mix was vortexed and then quickly poured onto the resolving gel. Combs were put into the gel.

The gel was left to polymerize for another 30 minutes and stored in wet paper towels at 4 °C until use (max. one week).

2.2.11 Western Blot

6 x Laemmli buffer:

3 ml 1 M Trizma® Base
HCl to pH 6.8
1.5 ml Glycerol
0.6 g SDS
0.5 g DTT
1 mg Bromophenol blue
to 10 ml H2O
Filter 0.45 µm

10 x running buffer:

60.6 g Trizma® Base
288.2 g Glycine
20 g SDS
to 2 l H2O

10 x transfer buffer:

30.3 g Trizma® Base
144.1 g Glycine
to 1 l H2O

1 x PBST:

999 ml PBS 1X
1 ml Tween 20

Protein lysates containing 10 µg of protein were mixed with water to a total volume of 25 µl. 5 µl of 6x Laemmli-buffer was added. Two 15 % SDS PAGE gels were placed in the electrophoresis tank and ca. 1 l of 1 x running buffer was poured into the tank and between the gels. The combs were removed and 3 µl protein marker or 12 µl sample was loaded into each well. A voltage of 100-120 V was applied until the samples had

reached the lower end of the gel (ca. 90 minutes). A PVDF membrane was placed in methanol for 1 minute. The clear side of the western blot cassette was placed in transfer buffer, the sponge and three filter papers were placed on it. The gel was carefully removed from the glass pane and placed on the filter paper. The membrane was soaked in transfer buffer for 30 seconds and placed on the gel, three filter papers and sponge were added. The cassette was closed and placed in the Western blot tank, the clear side of the cassette facing the black part of the tank. The tank was placed on ice and a current of 200 mA was applied for 90 minutes. The membrane was blocked with Roti block for 1 hour, then left in the 1st antibody diluted in Roti block overnight at 4 °C. After washing away the 1st antibody with PBST for 30 minutes, the membrane was left in the 2nd antibody for 1 hour at room temperature, then washed with PBST again.

To image the blots, 500 µl Luminata^{TM} classico was pipetted on the blot and spread evenly. In the dark, an X-ray film was placed on the blot for one or three minutes. The film was developed with the Curix 60. If the signal was too strong, a new X-ray film was placed on the blot for a shorter time; if it was too weak, imaging was repeated with Luminata^{TM} forte.

Actin was used as a loading control. The blot was left in β-actin antibody diluted in Roti block for one hour at room temperature, then washed in PBST for 30 minutes. 500 µl Luminata^{TM} classico was spread on the blot and it was imaged using the Chemidoc XRS+. The Chemidoc XRS+ could also be used for other antibodies if the signal was too strong to image the blot on film. Degraded protein samples were excluded from the analysis.

2.2.12 Proteomics

Proteomics analyses were performed by Dr. Juliane Merl-Pham of the Helmholtz Proteomics core facility according to standard protocols.

2.2.13 Activity Based Probes (ABPs)

ABP buffer:

50 mM Trizma® Base
HCl to pH 7.5
2 mM DTT
5 mM $MgCl_2$
10 % Glycerol
2 mM ATP

Proteasome activity was measured with Activity Based Probes (ABPs). ABPs are fluorescently-labeled proteasome inhibitors that bind covalently to the active sites of the proteasome, allowing the specific staining of active proteasome subunits (Cravatt et al., 2008; Li et al., 2013). MV151 was used to measure MECL1/β_2 activity and total proteasome activity, LW124 was used for LMP2/β_1 activity and MVB127 for LMP7/β_5 activity. LW124 and MVB127 have different fluorophores attached to them and can be used on the same samples and imaged on the same gel using different fluorescence channels.

ABP	specificity	channel
MV151	all proteasome subunits	Cy3 / TAMRA
LW124	β1, LMP2	Cy2
MVB127	β5, LMP7	Cy3 / TAMRA

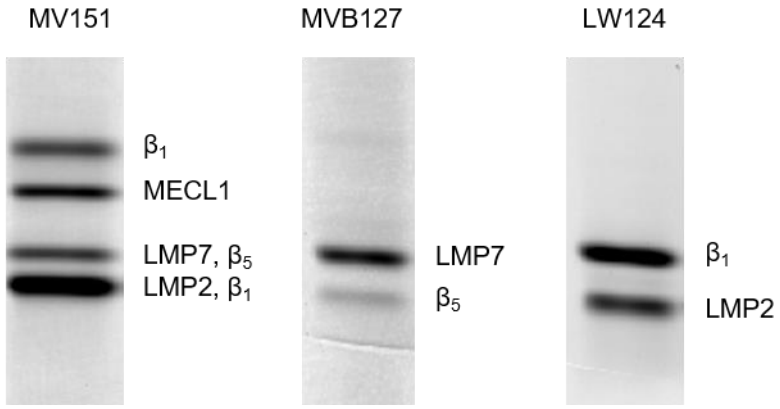

Figure 8 ABPs from THP1 cells. The ABP MV151 binds to all proteasome subunits and allows detection of overall proteasome activity but also comparison between β_2 and MECL1 subunits. MVB127 was used to compare β_5 and LMP7, and LW124 for β_1 and LMP2.

Protein lysates containing 10 µg of protein were mixed with ABP-buffer to a total volume of 25 µl. The ABPs were mixed with ABP-buffer (1:20) and 6.25 µl of this mixture was added to the sample which was then incubated for 1 hour in a Thermomixer at 37 °C and 650 rpm in the dark. After incubation, 6.25 µl Laemmli-buffer was added. Two 15 % SDS PAGE gels were placed in the electrophoresis tank and ca. 1 l of 1x running buffer was poured into the tank and between the gels. The combs were removed and 3 µl protein marker or 12 µl sample was loaded into each well. A voltage of 100-120 V was applied until the green marker had run 2/3 of the gel (ca. 2 hours) in the dark. The gels were washed in water, then fixed with 30 % ethanol and 10 % acetic acid for 30 minutes. After washing in water again for 15 minutes the gels were imaged with the Typhoon-scanner at 450 PTM and 50 µm pixel resolution. For MV151 and MVB127, the Cy3/TAMRA channel was used, LW124 was imaged with the Cy2 channel.

PageBlue™ staining was used as a loading control. The gels were left shaking in the PageBlue™ solution overnight at room temperature, then washed in water overnight. The PageBlue™ stained gels were imaged with the ChemidocXRS+. Degraded protein samples were not included in the analysis.

2.2.14 Native Gel Electrophoresis and Activity Assay

1 x Tris-Borate-EDTA (TBE) buffer:

90 mM Tris
80 mM boric acid
0.1 mM EDTA

5 x Native gel loading buffer:

2.5 µl 0.5 M tris
2.5 ml Glycerol
0.5 mg bromphenolblue

Native gel running buffer:

800 ml 1 x TBE buffer
0.2 g ATP
0.5 mM DTT
2 mM $MgCl_2$

In native gel analysis, the proteins are not denatured for electrophoresis so the relative amounts of intact 20S, 26S and 30S proteasome complexes can be evaluated.

Protein lysates containing 15 µg of Protein were mixed with ABP buffer to a total volume of 15 µl. 3.75 µl 5 x native gel loading buffer was added. Two 3-8 % native gradient gels were placed in the running chamber and native gel running buffer was poured between and around the gels. The combs were removed and 16 µl sample was loaded into each well. A voltage of 150 V was applied for 4 hours at 4 °C. The gels were placed in the substrate for 30 minutes at 37 °C in the dark. Then, the gels were imaged with the GelDoc. After imaging the gels were blotted onto PVDF membranes as described above.

Native gel electrophoresis was also used for ABP labeled samples. For this, protein lysates containing 10 µg of protein were mixed with ABP buffer to a total volume of

10 µl. The ABPs were mixed with ABP-buffer (1:20) and 3.3 µl of this mixture was added to the samples which were then incubated for 1 hour in the Thermomixer at 37 °C and 650 rpm in the dark. After incubation, 3.3 µl native gel lading buffer was added. Two 3-8 % native gradient gels were placed in the running chamber and native gel running buffer was poured between and around the gels. The combs were removed and 16 µl sample was loaded into each well. A voltage of 150 V was applied for 4 hours at 4 °C in the dark. The gels were washed with water and imaged with the Typhoon scanner as described above. After imaging the gels were blotted onto PVDF Membranes for immunoblot analysis.

2.2.15 Statistics

Statistical analysis was performed using GraphPad PRISM version 7. This software was also used for graphical representation of the results. Specific statistical methods used for each data set are included in the respective results part.

3 Results

In this study, blood samples from 32 COPD patients and 24 healthy control subjects were compared regarding differential cell counts and proteasome expression and activity. White blood cell numbers were evaluated to identify the composition of the blood sample. PBMCs were isolated to quantify mRNA levels using qPCR and protein levels of single proteasome subunits using Western blot and mass spectrometry. Proteasome assembly and activity were measured both by native gel activity substrate overlay and by labelling the active sites of the catalytic β-subunits using activity based probes (ABPs).

3.1 Study participants

Blood samples were obtained from patients of the LMU Klinikum Großhadern that had been diagnosed with COPD. The healthy control subjects were recruited from the Helmholtz Centre Munich. Inclusion criteria for the control group were age over 45 years. Exclusion criteria were known lung diseases (e.g. asthma, COPD), current medication with corticosteroids and malignant diseases. The study groups had the following characteristics:

	Control	**COPD**	**p-value**
Male subjects [%]	33.33	46.67	n.s.
Age [years; median (range)]	56.00 (47.00; 64.00)	60.00 (47.00; 84.00)	0.002
Subjects with comorbidities [%]	25.00	26.67	n.s.
Subjects taking immunosuppressive medication [%]	0.00	42.86	<0.001
Pack years [n; median (range)]	0.00 (0.00; 35.00)	40.00 (0.00; 80.00)	<0.001
Body mass index (BMI) [kg/m^2; median (range)]	24.22 (20.07; 33.43)	22.45 (17.18; 32.00)	n.s.
FEV1/FEV [%; median (range)]	78.00 (68.00; 85.00)	44.50 (29.00; 70.00)	<0,001

Table 1: study population. Differences between groups were tested using Fisher's exact test for categorical variables and Wilcoxon rank sum test for continuous variables. n.s. not significant

3.2 White Blood Cell Count Is Increased in COPD Patients

White blood cell numbers were compared in COPD and control patients to determine if a different leukocyte composition might be a confounding variable in the proteasome analysis. For this, flow cytometry was used to determine white blood cell numbers in COPD and control blood samples (Table 2). In this study, COPD patients had an average total white blood cell count of 8753 cells/µl (1401-17770cells/µl) compared to 5345 cells/µl in healthy controls (3125-11500 cells/µl; $p<0.0001$) (Figure 9 A). This increase in leukocyte numbers was primarily due to an increase in granulocytes and monocytes. Granulocyte numbers were 6655 (221-14650) cells/µl in COPD patients vs. 3307 (1373-8342) cells/µl in controls ($p<0.0001$) (Figure 9 C), monocyte numbers were increased from 413 (164-940) cells/µl in controls to 657 (315-1284) cells/µl in COPD patients ($p=0.0003$) (Figure 9 B). Lymphocyte numbers were not significantly changed (Figure 9 D).

	COPD	**Control**	**p-value**
Leukocytes	8753 (1401-17770)	5345 (3125-11500)	<0.0001
Granulocytes	6655 (221-14650)	3307 (1373-8342)	<0.0001
Neutrophils	6083 (1674-14290)	3272 (1369-8250)	0.001
Eosinophils	571 (8-5739)	35 (4-97)	<0.0001
Lymphocytes	1441 (201-4595)	1509 (789-2308)	n.s.
B cells	139 (1-649)	194 (26-752)	0.0126
T cells	1261 (375-4233)	1111 (623-1640)	n.s.
NK cells	209 (27-575)	109 (43-423)	n.s.
Monocytes	657 (315-1284)	413 (164-940)	0.0003

Table 2 White blood cell numbers in COPD and control PBMCs. Mean and range (minimal-maximal) are shown. Student´s T test was used to determine p values.

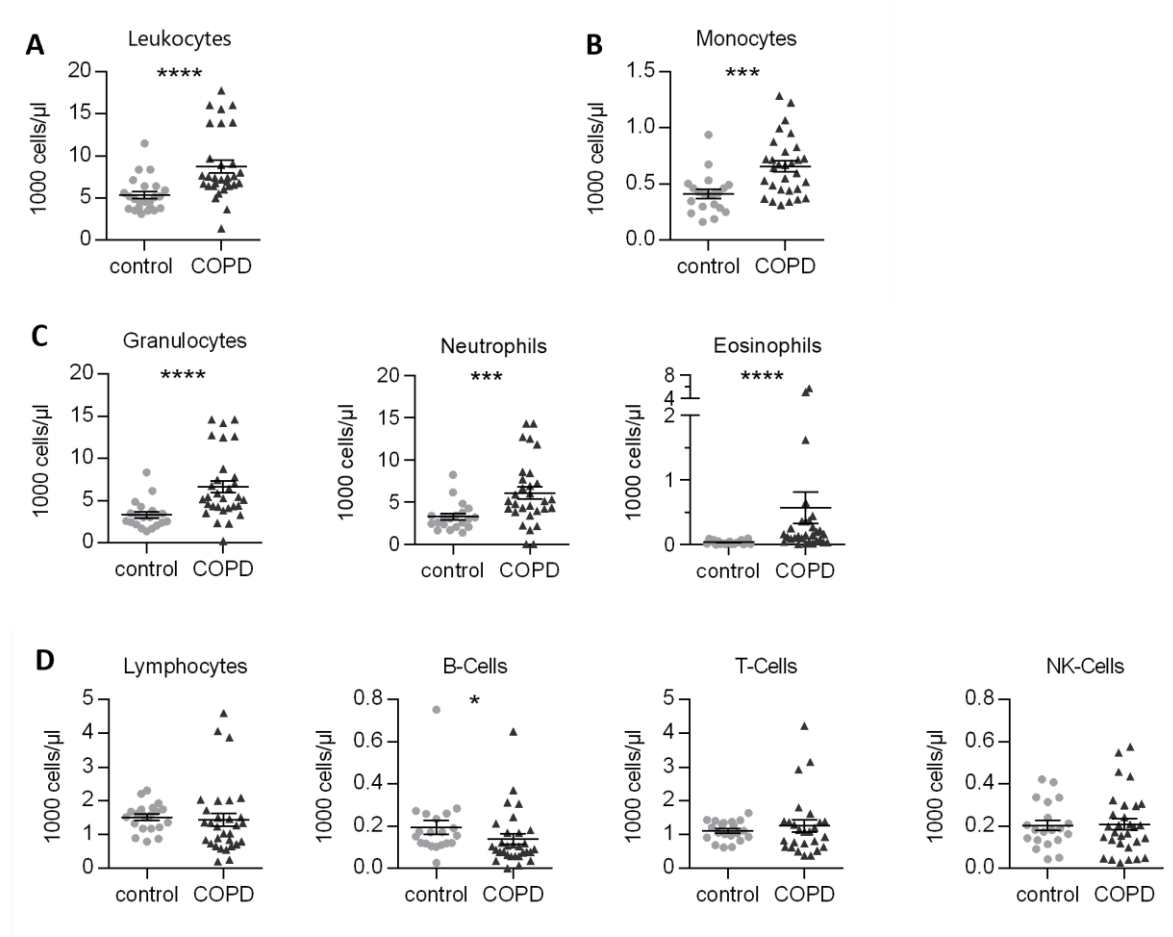

Figure 9 White blood cell numbers were increased in COPD patients. Flow cytometric analysis of cell numbers in whole blood of COPD patients (n=30) and healthy control subjects (n=23). **(A)** Total leukocyte numbers. COPD patients had a higher total white blood cell count compared to healthy controls. **(B)** Monocyte numbers. Monocyte numbers were increased in COPD patients. **(C)** Granulocyte numbers. Granulocyte numbers were higher in COPD patients. The granulocyte population is made up mainly of neutrophil granulocytes and eosinophil granulocytes in both populations. **(D)** Lymphocyte numbers. Lymphocyte numbers were not changed significantly in COPD patients compared to healthy controls. Lymphocytes can be further specified in T cells, B cells and NK cells. Student's T test was used to determine p-values; mean + SEM is shown. *: $p<0.05$; **: $p<0.005$; ***: $p<0.001$; ****: $p<0.0001$

The changes in absolute leukocyte numbers also resulted in an altered blood cell composition: in healthy control subjects, granulocytes made up 61.3 % (43.1-76.5 %) of all white blood cells, lymphocytes made up 30.6 % (16.6-50.0 %) and monocytes 8.1 % (3.6-14.2 %). In COPD patients, the distribution was 71.8 % (15.8-91.5 %) granulocytes, 19.4 % (2.6-60.5 %) lymphocytes and 8.8 % (2.5-31.5 %) monocytes (Figure 10 B). In lymphocytes, the B cell / T cell ratio was decreased in COPD Patients: B cell / T cell ratio in controls was 0.17 (0.04-0.58) in the control group, in COPD patients it was 0.12 (0.002-0.39; p=0.03) (Figure 10 C). The CD4 / CD8 ratio was not significantly altered (Figure 10 D).

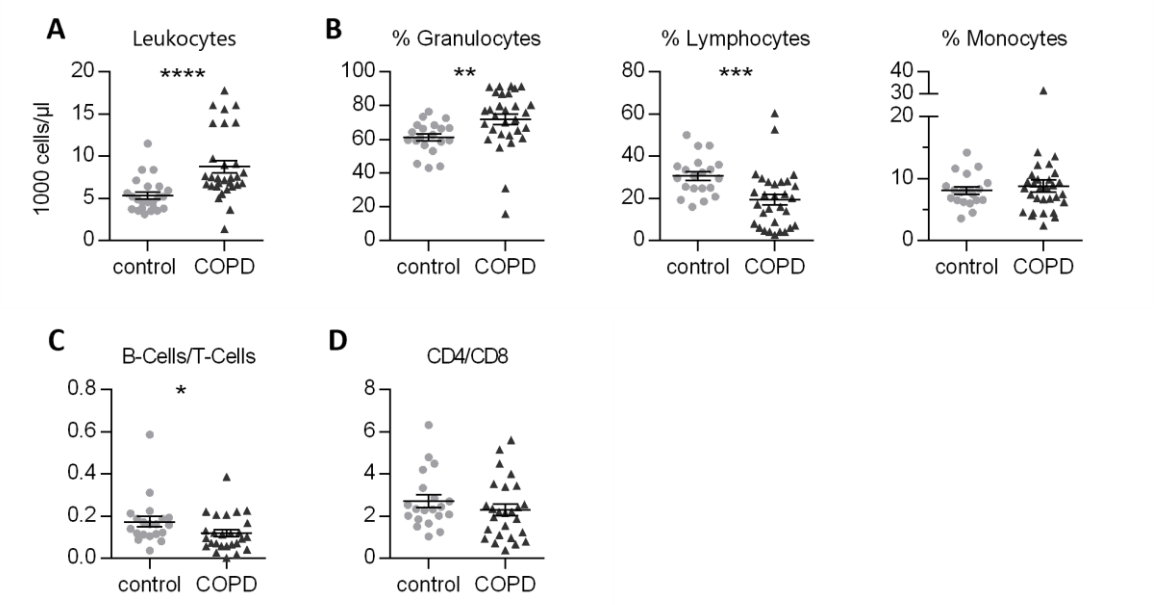

Figure 10 Distribution of white blood cells was altered in COPD patients. Flow cytometry was used to measure white blood cell numbers in whole blood of COPD patients (n=30) and healthy control subjects (n=23). **(A)** Total white blood cell count in COPD patients and controls. The total number of white blood cells was increased in COPD patients. **(B)** Percentage of the main white blood cell types in COPD patients and controls. COPD Patients had a higher percentage of granulocytes and a lower percentage of lymphocytes compared to healthy controls. **(C)** B cell – T cell ratio in COPD patients and controls. B cell / T cell ratio was decreased in COPD Patients. **(D)** Ratio of CD4 and CD8 positive T cells of COPD patients and controls. The CD4 / CD8 T cell ratio was not changed significantly in COPD patients compared to healthy controls. T test was used to determine p values; mean + SEM is shown. *: $p<0.05$; **: $p<0.005$; ***: $p<0.001$; ****: $p<0.0001$

For proteasome analysis, PBMCs were isolated from the blood of COPD patients and healthy controls. These include mainly lymphocytes and monocytes. Overall PBMC numbers were not changed significantly in COPD patients compared to healthy controls. However, there was a difference in the composition of PBMCs. In COPD patients, lymphocytes made up 50.3 % (48.1-52.5 %) of all PBMCs and monocytes made up 49.7 % (47.5-52.9 %). Healthy controls had 56.1% (53.5-58.8 %) lymphocytes and 43.9 % (41.2-46.5 %) monocytes (Figure 11).

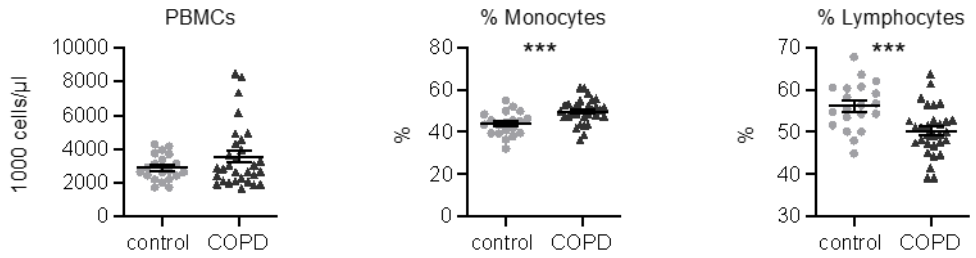

Figure 11 Composition of PBMCs was altered in COPD patients. Flow cytometry was used to measure white blood cell numbers in whole blood of COPD patients (n=30) and healthy control subjects (n=23). There was no significant change in PBMC numbers in COPD patients compared to healthy controls. However, PBMCs of COPD patients had a higher percentage of monocytes and a lower percentage of lymphocytes compared to controls. T test was used to determine p values; mean + SEM is shown. *: p<0.05; **: p<0.005; ***: p<0.001; ****: p<0.0001

3.3 mRNA Expression of Immunoproteasome Subunits was Increased in Peripheral Mononuclear Cells of COPD Patients

To check for differences in the expression of proteasome subunits and proteasome regulators, the quantity of corresponding mRNAs was measured using RTqPCR. Mean mRNA expression of two housekeeping genes, RPL19 and HPRT, was used to obtain relative expression levels of proteasomal genes in the different samples to minimize interexperimental variation.

Standard proteasome subunits PSMB5, PSMB6 and PSMB7 were expressed at the same level in PBMCs isolated from COPD patients and control subjects (Figure 12 A), but immunoproteasome subunit mRNA expression was increased in COPD compared to control PBMCs: expression of the PSMB8 gene encoding immunoproteasome subunit LMP7 was increased 1.3-fold (p=0.09), expression of the PSMB9 gene encoding LMP2 was increased 1.3-fold (p=0.08) both of these subunits were not changed significantly but a clear trend was visible. The expression of the PSMB10 gene encoding for MECL1 was increased significantly 1.4-fold (p=0.008) in PBMCs of COPD patients compared to healthy controls (Figure 12 B). PSMA3 encoding for an alpha subunit that is both part of the standard proteasome and the immunoproteasome, was not changed significantly (Figure 12 C).

Further analysis of expression levels of various proteasome regulators revealed that mRNA expression of PSMD11 was also significantly increased in COPD PBMCs (Figure 12 E). PSMD11 encodes RPN6, a subunit of the 19S regulator that is essential for the assembly of 26S and 30S proteasomes (Pathare et al., 2012). Expression of PSME3 encoding the proteasome activator PA28γ was also increased significantly in PBMCs of COPD patients (Figure 12 F). All other analysed proteasomal subunits showed no significant changes in mRNA expression (Figure 12). There was no significant difference in the expression of IFNγ in COPD compared to control PBMCs (Figure 12 G).

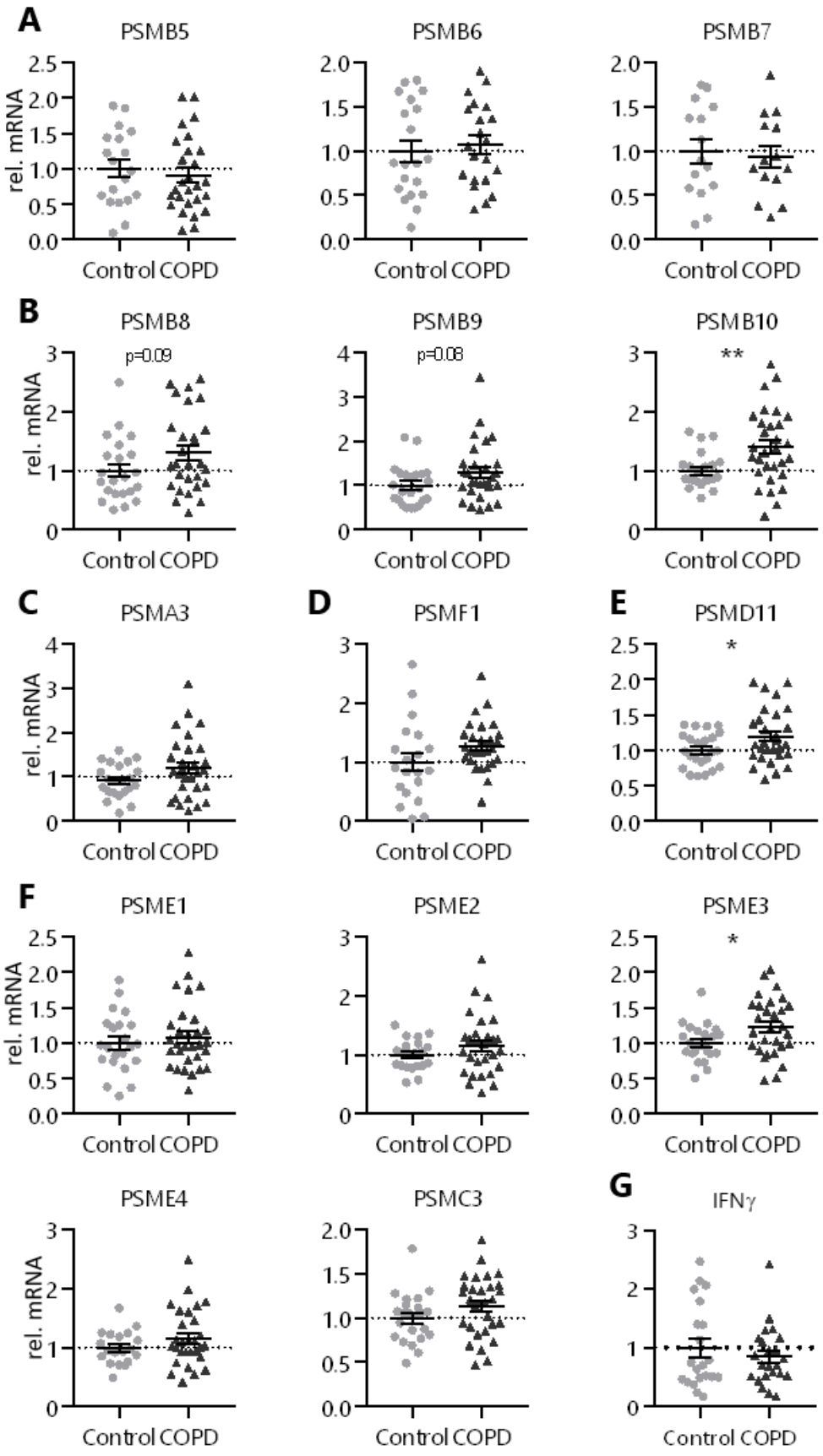
A
PSMB5
PSMB6
PSMB7
rel. mRNA
Control COPD
B
PSMB8
p=0.09
PSMB9
p=0.08
PSMB10
**
C
PSMA3
D
PSMF1
E
PSMD11
*
F
PSME1
PSME2
PSME3
*
PSME4
PSMC3
G
IFNγ

Figure 12 Immunoproteasome gene expression was increased in COPD. RTqPCR was used to measure mRNA expression in PBMCs of COPD patients (n=14-30) and healthy control subjects (n=15-23). Two housekeeping genes, RPL19 and HPRT, were used. The genes of interest were normalized to the mean of these two housekeepers. Fold change over control is shown. **(A)** Standard proteasome subunit expression. PSMB5, 6 and 7 genes encode the standard proteasome subunits β5, β1 and β2, respectively. **(B)** Immunoproteasome subunit expression. PSMB8, 9 and 10 genes encode immunoproteasome subunits LMP7, LMP2 and MECL1, respectively. **(C)** PSMA3 expression. The PSMA3 gene encodes an α subunit of the 20S proteasome. **(D)** PSMF1 expression. The PSMF1 gene encodes a proteasome inhibitor (PI) 31 subunit **(E)** Expression of PSMD11 which encodes RPN6, a 19S subunit that is essential for the assembly of the 26S and 30S proteasome. **(F)** Proteasome activator expression. PSME1, 2 and 3 genes encode PA28α, β and γ, respectively; PSME4 encodes PA200. PSMC3 encodes Rpt5, an essential subunit of the 19S regulator. **(G)** IFNγ expression. Student´s T test was used to determine p values; mean + SEM is shown. *: $p<0.05$, **: $p<0.01$

3.4 Protein Expression of Immuno- and Standard Proteasome was Only Slightly Altered in the Peripheral Blood of COPD Patients

To investigate if changes in mRNA levels of proteasomal subunits result in altered protein levels, protein expression of proteasomal subunits was analysed using Western blot and mass spectrometry based proteomics.

3.4.1 Western Blot Analysis of Selected Proteasomal Subunits

For Western blot analysis, total cellular protein of COPD and control PBMCs was isolated using a protocol for native protein lysis (see 2.2.7). Representative catalytically active standard (β_1) and immunoproteasome (LMP2, LMP7) subunits were analysed. The α_{1-7} antibody was used to measure total proteasome abundance. PA28α was analysed as an example for a proteasome activator.

Measurements were normalized to the total amount of protein in each sample using AmidoBlack staining. THP1 cell lysates were used as a control to compare samples run on different gels.

No significant changes in any of the analysed proteasomal subunits were detectable in COPD compared to control PBMCs using western blot.

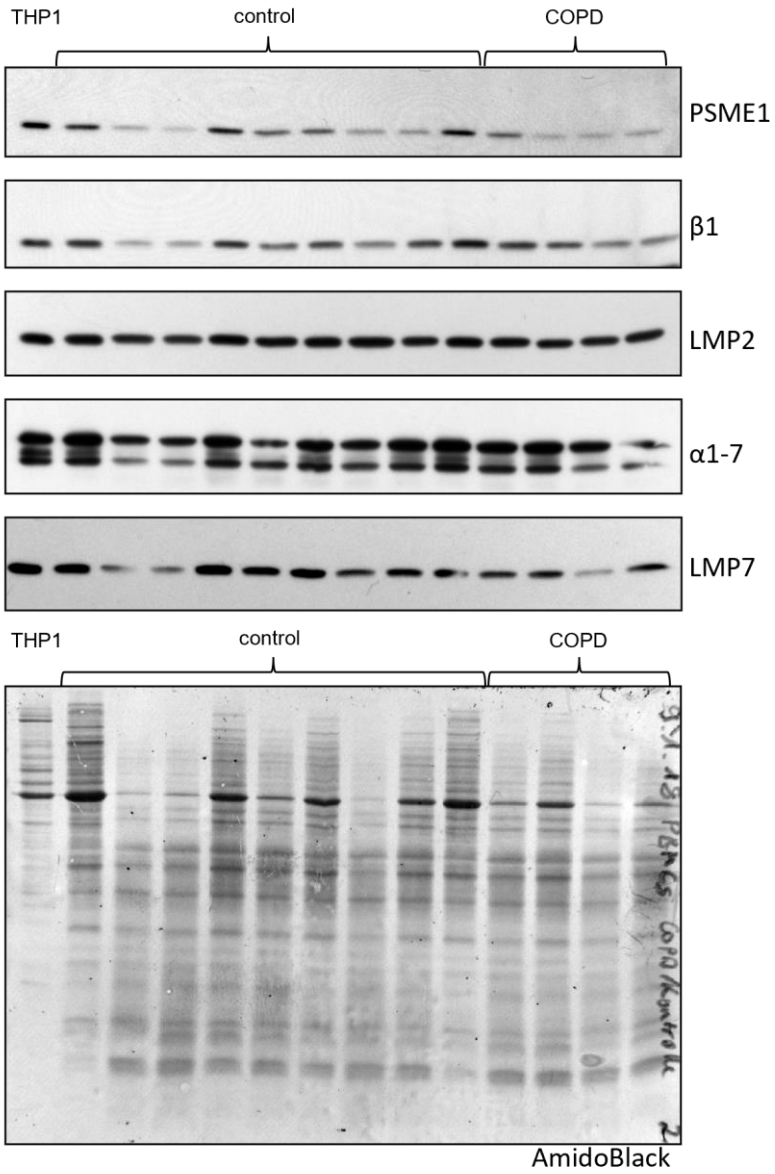

Figure 13 Example of a Western blot analyzing proteasomal protein expression in COPD and control PBMCs. Protein expression was measured using Western blot analysis of native protein lysates from isolated PBMCs of COPD patients and healthy controls. Measurements were normalized to AmidoBlack staining. THP1 cell lysates were used as a control to compare samples run on different gels.

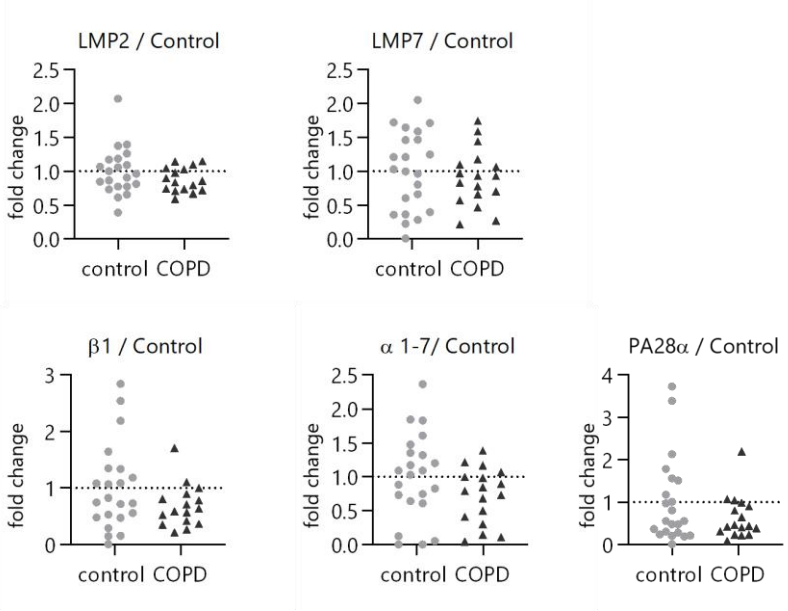

Figure 14 Protein expression of proteasome subunits is not changed in COPD compared to control PBMCs in Western blot. Protein expression was measured using Western blot of PBMCs of COPD patients (n=16-18) and healthy controls (n=21-22). Measurements were normalized to AmidoBlack staining. THP1 cell lysates were used as a control to compare samples run on different gels. Fold change over mean of control is shown. Mann-Whitney test was used to determine p values.

3.4.2 Proteomics Analysis of Proteasomal Subunits

In addition to Western blot analysis, mass spectrometry was also used to analyse protein lysates of PBMCs of COPD and control samples. In contrast to Western blot, mass spectrometry has a much higher accuracy and provides a quantitative analysis of a greater number of proteins.

For this, RIPA protein lysates from blood samples of 10 COPD patients and 10 healthy control subjects were used. With this method, a total number of 42 proteasomal subunits were analysed. Eleven of these proteins showed a significant ($p<0.05$) change in COPD vs control patients (Table 3).

The amount of two non-catalytically active 20S core subunits α_1 and α_2 was significantly increased in COPD compared to control PBMCs. The amount of β_2, a catalytically active 20S core subunit with trypsin-like activity, was also increased. RPT1 and RPT5 are subunits of the 19S PA and part of the base forming ATPases, that are responsible for the unfolding of proteins that are then degraded by the proteasome (Bard et al., 2018).

The amount of RPT1 was decreased while RPT5 was increased in COPD PBMCs. RPN3, RPN5, RPN6, RPN8, RPN9 and RPN11 are subunits of the 19S PA. Specifically, RPN6 is essential for the assembly of the 26S proteasome complex (Pathare et al., 2012; Vilchez et al., 2012). RPN11 is the deubiquitinase of the 26S proteasome complex which is a critical step in de degradation of proteins by the proteasome (Verma et al., 2002). RPN11 was decreased in COPD compared to control PBMCs while the other 19S subunits were increased (Figure 15, Table 3).

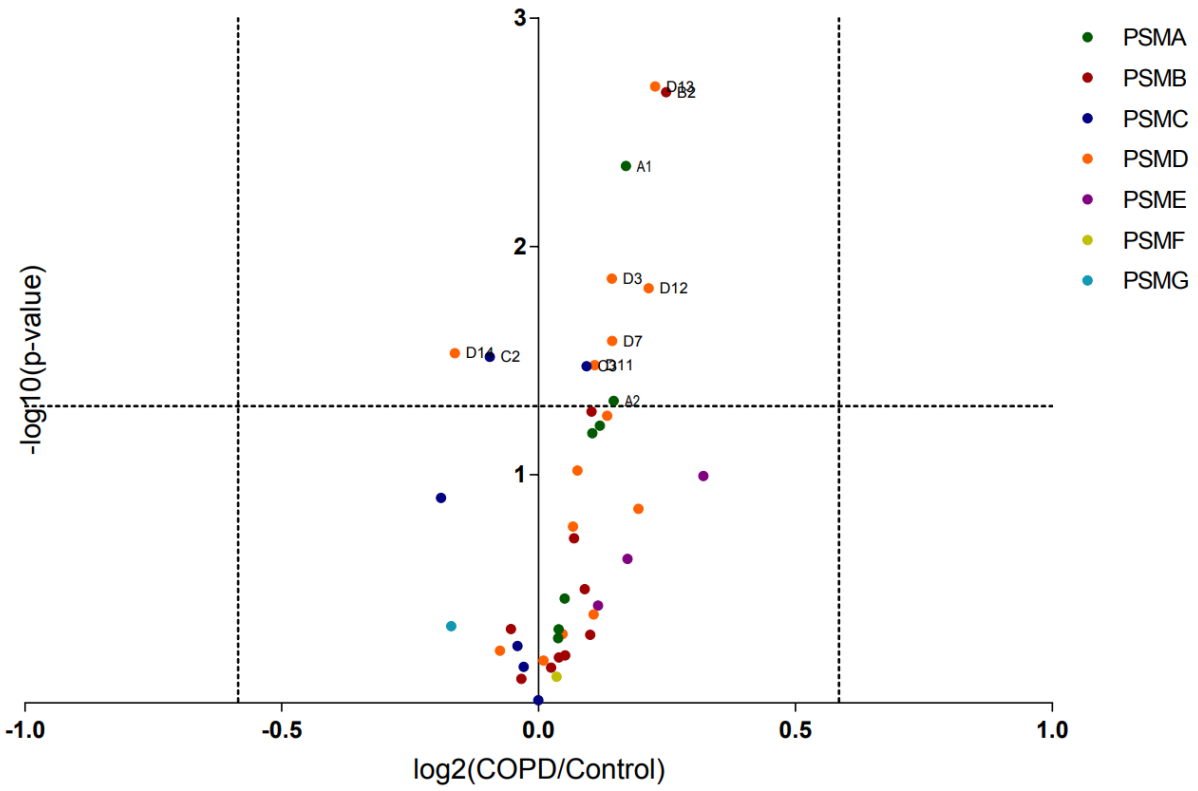

Figure 15 Protein expression of proteasome related proteins. Mass spectrometry was used to determine differences in protein expression in COPD patients compared to healthy controls. A p value of p≤0.05 was defined as significant (horizontal dotted line). T test was used to determine p values.

Protein	(gene)	function	fold change	p-value
α_1	(PSMA1)	Core subunit	1.13	0.0044
α_2	(PSMA2)	Core subunit	1.11	0.0475
β_2	(PSMB2)	Core subunit	1.19	0.0021
RPT1	(PSMC2)	ATPase subunit	0.94	0.0304
RPT5	(PSMC3)	ATPase subunit	1.07	0.0335
RPN3	(PSMD3)	19S subunit	1.10	0.0138
RPN8	(PSMD7)	19S subunit	1.10	0.0260
RPN6	(PSMD11)	19S subunit	1.08	0.0331
RPN5	(PSMD12)	19S subunit	1.16	0.0152
RPN9	(PSMD13)	19S subunit	1.17	0.0020
RPN11	(PSMD14)	19S subunit	0.89	0.0293

Table 3 Proteomic analysis of proteasome related proteins in COPD compared to control PBMCs. α_1 and α_2 are non-catalytically active subunits of the 20S proteasome. β_2 is a catalytically active subunit of the 20S core with trypsin-like activity. RPT1 and RPT5 are subunits of the AAA ATPases which form the base part of the 19S PA that associates with the 20S to form the 26S proteasome. RPN3,5,6,8,9,11 are subunits of the lid part of the 19S activator. Student´s T test was used to determine p values.

3.5 Proteasome and Immunoproteasome Activities in PBMCs of COPD Patients

Activity based probes (ABPs) were used to determine proteasome activity in native protein extracts of PBMC samples. ABPs are irreversible proteasome inhibitors that bind covalently to the active sites of the proteasome and are tagged with fluorescent markers. Thus it is possible to measure the amount of proteolytically active proteasome subunits (Cravatt et al., 2008; Li et al., 2013). The measurements were normalized to total protein loading using PageBlue staining. THP1 cell lysates were used as a control to compare samples run on different gels.

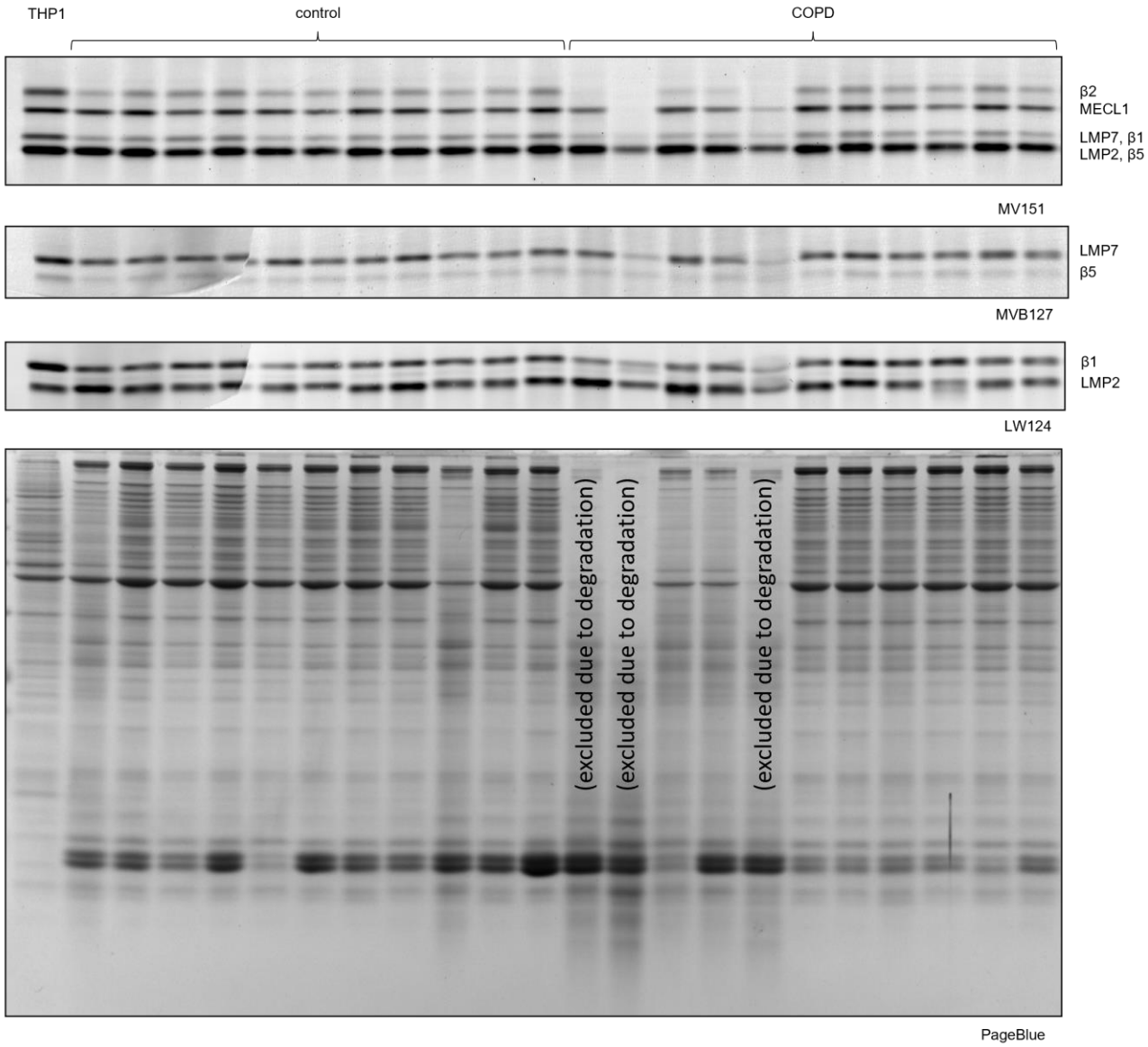

Figure 16 Example of an ABP staining of COPD and control PBMCs. Proteasome and immunoproteasome subunit activities were measured in PBMCs of COPD patients and healthy controls using ABPs. Measurements were normalized to total amount of protein in each sample using PageBlue staining. THP1 cell lysates were used as a control to compare samples run on different gels. Degraded protein samples were excluded from analysis (lanes 13, 14 and 17).

Analysis of proteasome subunits revealed that β_1 subunit activity was significantly increased in COPD patients (Figure 17 B). No significant difference was detected in other catalytic subunits. Total proteasome activity was not changed (Figure 17). We also calculated the ratio of the respective immuno- and standard catalytic subunits. The ratio of LMP2/β_1 was 0.91 in controls which indicates that 52% of all proteasome complexes in PBMCs have the immunosubunit LMP2 incorporated. The MECL1/β_2 ratio was 5.90 and the LMP7/β5 ratio was 7.27 meaning that 15% of proteasome complexes

include MECL1 and 12% include LMP7. The fact that these ratios are not the same for the different immunoproteasome subunits indicates that mixed proteasome types exist. The existence of such intermediate type proteasomes has been observed in multiple cell types (Dahlmann et al., 2000; Klare et al., 2007)

The MECL1/β_2 ratio was increased in COPD compared to control PBMCs while other ratios of immunoproteasome subunits and their respective standard subunits were not altered (Figure 18).

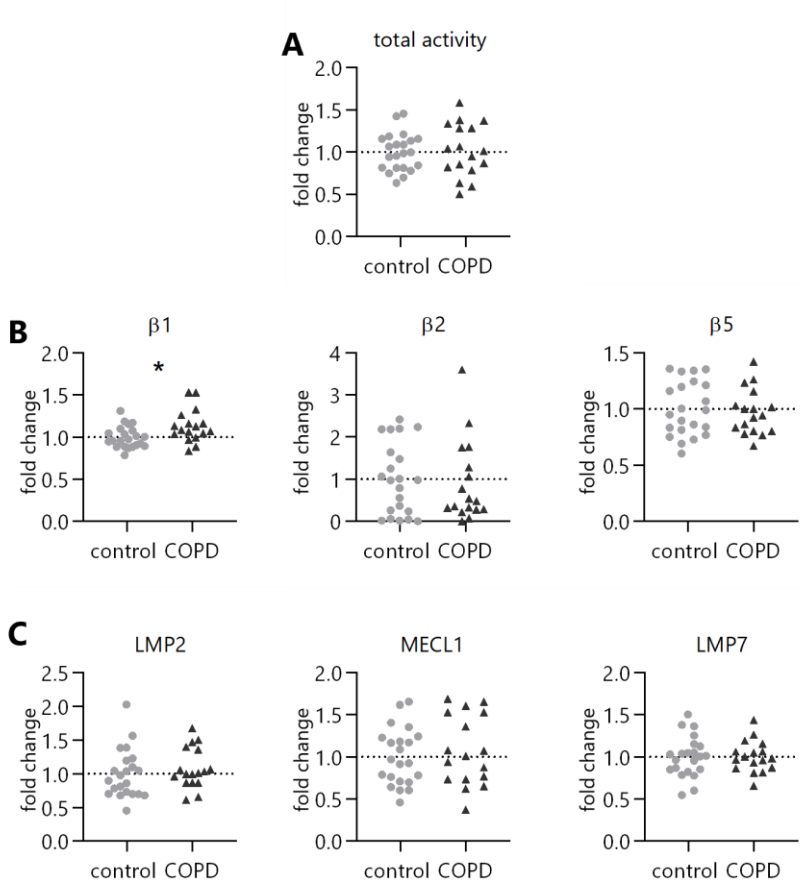

Figure 17 Immunoproteasome activity was not altered in COPD compared to control PBMCs. Activity based probes were used to measure proteasome activity in PBMCs of COPD patients (n=17) and healthy control subjects (n=22). Activities were normalized to a THP1 standard sample lysate to compare ABPs on different gels. Degraded protein samples were not included in the analysis. Fold change over mean of control samples is shown. **(A)** Total proteasome activity. Total proteasome activity was not changed significantly in COPD compared to control PBMCs. **(B)** Standard proteasome subunit activity. β1 subunit activity was slightly increased in COPD patients, in other catalytic subunits there was no significant difference. **(C)** Immunoproteasome subunit activity. There were no significant changes in immunoproteasome subunit activity. Mann-Whitney test was used to determine p values. *: $p<0.05$

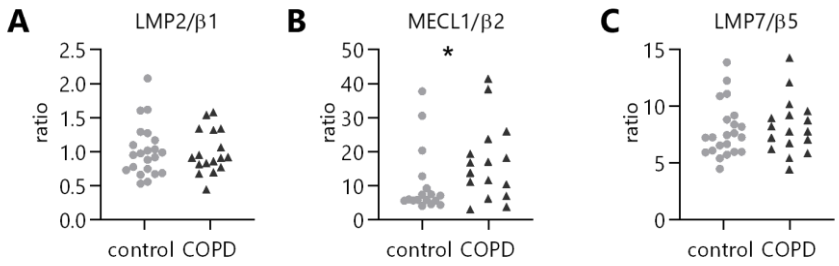

Figure 18 Ratio of respective immuno- / standard proteasome subunit activities in COPD compared to control PBMCs. Activity based probes were used to measure proteasome activity in PBMCs of COPD patients (n=17) and healthy control subjects (n=22). The activity was normalized to a THP1 standard sample lysate to compare ABPs on different gels. **(A)** LMP2/β1 ratio. **(B)** MECL1/β2 ratio. **(C)** LMP7/β5 ratio. Mann-Whitney test was used to determine p values. *: $p<0.05$

3.6 Amount of Active 30S Proteasome Was Increased in PBMCs of COPD Patients

To check if proteasome assembly is altered in COPD patients, native gels were analyzed. In native gel analysis, the proteins are not denatured for electrophoresis so the relative amounts of 20S, 26S and 30S proteasomes can be evaluated. The 26S proteasome complex is formed by binding of one 19S PA to the 20S core and the 30S by binding of two 19S PAs to either side of the core. For this, protein samples were labelled with the MV151 ABP which binds to the active sites of all proteolytic proteasome subunits allowing the detection of all active proteasome complexes. The measurements were normalized to THP1 cell lysates used as standards to compare samples run on different gels.

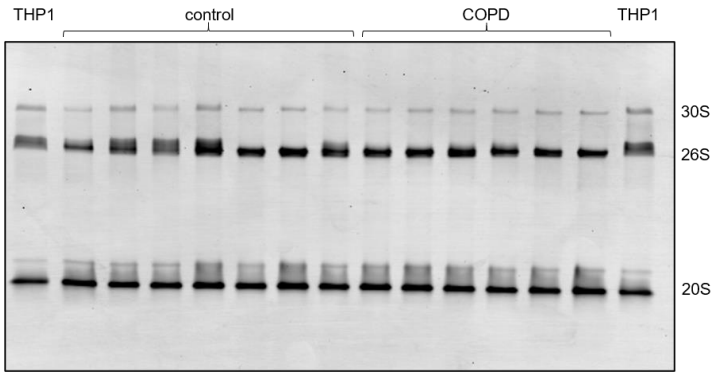

Figure 19 Example of a native gel analysis of control and COPD PBMCs. Native gel analysis was used to measure the amount of 20S, 26S and 30S proteasome complexes. Protein samples were labelled with the MV151 ABP. The measurements were normalized to THP1 cell lysates.

The activity of 20S and 26S proteasomes was not changed in COPD compared to control PBMCs (Figure 20 A, B). 30S proteasome activity was increased in the PBMCs of COPD patients (Figure 20 C). The presence of multiple bands for the 20S proteasome can be explained by the existence of different proteasome subtypes (i.e. immuno, standard and intermediate types).

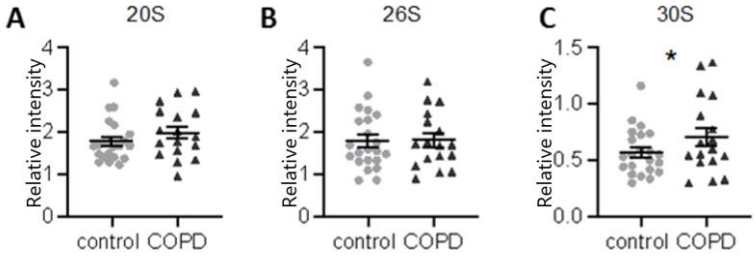

Figure 20 30S proteasome activity is increased in COPD. Native gel analysis of cell lysates from PBMCs of COPD patients and healthy controls. Measurements were normalized to THP1 cell lysates. **(A)** 20S proteasome activity. **(B)** 26S proteasome activity. **(C)** 30S proteasome activity. Student´s T test was used to determine p-values; mean +SEM is shown. *: $p<0.05$.

4 Discussion

4.1 White Blood Cell Numbers are Increased in COPD

Flow cytometry was used to determine white blood cell count in COPD and control blood. The reference value for leukocyte count in peripheral blood is 3,800 - 10,500 cells/µl (Herold, 2016). In the present study, most subjects from both control and COPD group were within this range, but COPD patients had a significantly higher white blood cell count compared to healthy controls (Figure 9). The distribution of the leukocyte populations was also within the expected range in most controls and COPD patients, but with a higher percentage of neutrophils in COPD blood (Figure 10). This confirms previous findings about blood cell counts in COPD. It has been shown that COPD patients have higher total white blood cell numbers as well as an increased neutrophil to lymphocyte ratio (Günay et al., 2014). Elevated neutrophil numbers are a rather unspecific sign for inflammation and can be found in numerous inflammatory conditions such as autoimmune diseases, acute infections or malignant diseases (Hasselbalch et al., 2018; Herold, 2016; Huang et al., 2020; Templeton et al., 2014; Wang et al., 2020a). Cigarette smoke alone has been shown to increase granulocyte production, possibly via activation of granulocyte colony stimulating factor (G-CSF) (Barnes et al., 2003; Macnee, 2007). In summary, the study population used in this thesis showed a similar cell composition as found in previous studies indicating that it is representative for this disease.

Peripheral blood mononuclear cells (PBMCs) can easily be isolated from the blood for protein and mRNA analysis. This cell population consists mainly of lymphocytes and monocytes (Kleiveland, 2015). The increased total white blood cell count in COPD patients was mainly due to an increase in the number of granulocytes. These cells are removed during the PBMC isolation process and are not included in the analysed PMBCs. This was important to rule out different white blood cell composition as a confounding variable.

However, COPD patients also showed increased monocyte numbers compared to healthy controls. This resulted in an increased monocyte to lymphocyte ratio in COPD compared to control PBMCs (Figure 11). A study by Rieckmann et al. showed that the expression levels of proteasome related proteins in lymphocytes and monocytes are similar. They analysed the cellular proteome of human hematopoietic cells using mass spectrometry for over 10,000 proteins (Rieckmann et al., 2017). Our analysis of this data for 37 proteasome related proteins showed that these proteins were similarly abundant in the cell types present in PBMC (Kammerl et al., 2021a). Thus, the monocyte to lymphocyte ratio should not have an influence on the protein content in the analysed PBMCs. An advantage of using PBMCs is that they are easily isolated potentially making a clinical implementation more feasible. Still, the increased monocyte to lymphocyte ratio should be kept in mind as it might require future subpopulation analysis to formally prove that this ratio does not affect proteasome content by itself.

4.2 Activation of The Proteasome in Immune Cells in COPD

PBMCs of COPD patients showed increased proteasome and immunoproteasome subunit expression compared to healthy controls (Figure 12 B, Table 3). This is likely due to chronic inflammation in COPD patients. The elevated white blood cell numbers in COPD patients are a sign of this inflammatory state. It has been shown that the immunoproteasome is upregulated by inflammation and oxidative stress (Aki et al., 1994; Groettrup et al., 2001; Petersen and Zetterberg, 2016; Schmidt and Finley, 2014). PSMD11 mRNA expression was also increased in PBMCs of COPD patients (Figure 12 E). This gene encodes for RPN6 which is a subunit of the 19S proteasome activator and is essential for the assembly and stability of 26S and 30S proteasome complexes (Pathare et al., 2012). Lack of RPN6 results in an arrested cell cycle demonstrating the importance of this proteasomal subunit for cellular function (Santamaría et al., 2003). Proteomics analysis revealed that RPN6 and other proteasome subunits were also increased on protein level (Table 3). This could be a sign for an increased assembly of active proteasome complexes. This is supported by results of native gel analysis which

showed an increased amount of active 30S proteasome complexes in COPD patients compared to healthy controls (Figure 20).

This increase in proteasome expression could not be verified in Western blot analysis. The changes in mRNA expression were, while significant, only small, and western blot is known to have a lower accuracy. It is likely that the small changes in mRNA expression could not be demonstrated in Western blot. Because of this, Proteomics analysis was performed using mass spectrometry. This method has a much higher accuracy compared to western blot and can detect even small changes in protein expression.

Based on these experiments, Kammerl et al. performed further experiments regarding proteasome complex abundance and activity. Using native gel analysis and a specific overlay activity assay that uses a fluorescently labelled substrate for the chymotrypsin-like activity of the proteasome, they found that both activity and abundance of proteasome complexes was increased in COPD patients. This supports the results presented in this thesis. However, the specific activity of 26S proteasome complexes, calculated from the measured activity relative to the abundance of proteasome complexes, was decreased in COPD patients compared to healthy controls. From this, they hypothesised that in COPD more proteasome complexes are assembled to compensate for the reduced specific activity of these complexes (Kammerl et al., 2021a). This is in line with the results presented in the present study showing an increase in the expression of RPN6 which is essential for proteasome complex assembly (Figure 12, Table 3) and an increased amount of assembled 30S proteasome complexes in native gel analysis (Figure 20).

4.3 Proteasome Function is not Decreased in Peripheral Blood Mononuclear Cells of COPD Patients

Kammerl et al. recently analyzed the influence of cigarette smoking and COPD on the immunoproteasome in the lung (Kammerl et al., 2016). They found that immunoproteasome mRNA expression was decreased in bronchioalveolar lavage (BAL) cells from COPD patients. For *in vivo* analysis, they analyzed explanted lung tissues from COPD patients and organ donors. They found that although RNA and protein expression of proteasome subunits was not altered in COPD patients' lungs, overall proteasome activity was decreased in the lungs of COPD patients (Kammerl et al., 2016). This finding was the rationale to analyze whether proteasome expression and activity is similarly decreased in peripheral blood cells of COPD patients, which would potentially allow the use of proteasome activity profiling for stratification of COPD patients and disease progression. In contrast to the findings on diminished immunoproteasome expression in lung-derived immune cells in COPD BAL samples, we here observed that immunoproteasome mRNA expression was increased in PBMCs of COPD patients.

One might speculate that the discrepancy between blood and lung immune cells results from the circumstance that in the lung, immune cells are directly influenced by the noxious effects of cigarette smoke resulting in the reduction of proteasome activity. In the peripheral blood, systemic inflammation might be one of the factors leading to an increased expression of the proteasome. The different lifetime of the cells used in either study may contribute to this effect: The majority of BAL cells are alveolar macrophages (Gillissen, 2020). These cells are known to have a very long life span while monocytes and lymphocytes are only present in the peripheral blood for a few days (Eguíluz-Gracia et al., 2016; Murphy et al., 2008). There might be an accumulation of damage over time in the long living BAL cells that would not take place in PBMCs due

to their shorter life span. Because of this, an acute adaptation to the inflammatory state in COPD patients that may take place in PBMCs might not be possible in BAL cells.

4.4 The Proteasome as a Potential Biomarker for COPD

Proteasome mRNA and protein expression was increased in peripheral blood mononuclear cells of COPD patients compared to healthy controls. These findings were supported by further experiments using native gel analysis. Thus, the expression and activity of the proteasome might provide a potential biomarker for COPD. In contrast to BAL cells or lung biopsies, peripheral blood can be obtained minimally invasive, and PBMCs are easily isolated from the blood, making a potential clinical application more feasible. This would allow for frequent monitoring of disease progression.

The data we have collected in this study was also used as a pilot study for examining PBMC samples of over 800 study participants from the KORA-FF4 cohort. The first results of this analysis were recently published (Kammerl et al., 2021b). ABPs were used to study differences in immunoproteasome activity in different subpopulations. The MECL1/β_2 ratio was found to be increased in COPD patients compared to control subjects, confirming the findings presented in the present study (see 3.5). Additionally, they found sex specific differences in immunoproteasome activity. The activities of the immunoproteasome subunits LMP2 and MECL1 were significantly increased in men compared to women. In our study, no significant difference of immunoproteasome activity in male compared to female subjects was observed. However, when analyzing the data, the observed trend is consistent with the results of Kammerl et al. (data not shown). The lack of significance in this work is most likely due to the much smaller number of study participants.

To implement increased proteasome activity as a biomarker for COPD, more detailed analyses would be necessary. To identify a clear cut off for the diagnosis, a large number of samples would have to be analysed. It would also be interesting to see how

the potential biomarkers react to a progression or exacerbation of the disease in the same patient. This could provide a possibility to predict these events and adjust treatment earlier. For this, data obtained from the COSYCONET study population could be promising. This study population provides longitudinal data from COPD patients that are observed over a period of 4.5 to 6 years. An ongoing study will analyse data obtained from 450 COPD patients from the COSYCONET cohort and compare that to 300 healthy controls from the KORA FF4 cohort. Proteasome and immunoproteasome expression and activity will be analysed for a correlation to disease severity, exacerbations, and lung function decline (Silke Meiners, personal communication). In addition, this large study population will allow to better understand the role of comorbidities such as diabetes, obesity, cardiovascular disease, or asthma. This study may help establish proteasome and immunoproteasome expression and activity as a diagnostic and prognostic biomarker for COPD.

5 Citations

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6 Appendix

6.1 List of Abbreviations

AAT	Alpha 1 Antitrypsin
ABP	Activity based probe
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCA	bicinchoninic acid
BMI	Body mass index
CD	Cluster of differentiation
cDNA	Complementary Desoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACS	fluorescent activated cell sorting
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IFNγ	Interferon γ
LMP	Low molecular mass protein
MECL	Multicatalytic endopeptidase complex like
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NK	Natural killer

PA	Proteasome activator
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + 0,1% Tween
PSM	Proteasome subunit
PVDF	polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
RPL19	60S ribosomal protein L19
RPN	26S proteasome non-ATPase regulatory subunit
RPT	26S proteasome AAA-ATPase subunit
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBE buffer	Tris/Borate/EDTA buffer
TEMED	Tetramethylethylenediamine
TNFα	Tumor necrosis factor

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6.3 Affidavit

Eidesstattliche Versicherung

Urmann, Andrea Franziska
Name, Vorname

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