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To my children Hugo, Heily and Haidi

As long as there's breath in our lungs,
our story is still being written.
Bart Millart

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–III):

I. Oit-Wiscombe I, Virag L, Soomets U, et al. Increased DNA damage in progression of COPD: a response by poly(ADP-ribose) polymerase-1. PLoS One. 2013;8(7):e70333.
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III. Oit-Wiscombe I, Soomets U, Altraja A. Antioxidant Glutathione Analogues UPF1 and UPF17 Modulate the Expression of Enzymes Involved in the Pathophysiology of Chronic Obstructive Pulmonary Disease. Curr Issues Mol Biol. 2024 Mar 12;46(3):2343–2354.

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ABREVIATIONS

5-LO	5-lipoxygenase
AA	Arachidonic acid
bio-NAD^+	6-biotin-17-nicotinamide-adenine-dinucleotide
BMI	Body-mass index
CAT	Chronic obstructive pulmonary disease Assessment Test
CI	Confidence intervals
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CS	Cigarette smoke
DL_{CO}	Diffusing capacity of the lungs for carbon monoxide
DMSO	Dimethyl sulfoxide
DPP4	Dipeptidyl peptidase 4
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GCL	Glutamyl-cysteine ligase
GCLC	Catalytic subunit of glutamyl-cysteine ligase
GCLM	Modulatory subunits of glutamyl-cysteine ligase
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPx	Glutathione peroxidase
GSH	Glutathione
GSR	Glutathione reductase
GSS	Glutathione synthetase
HDAC	Histone deacetylase
HPRT-1	Hypoxanthine phosphoribosyl- transferase-1
K_{CO}	Carbon monoxide transfer coefficient
LTA_4H	Leukotriene A_4 hydrolase
LTB_4	Leukotriene B_4
mMRC	Modified British Medical Research Council Questionnaire
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OR	Odds ratios
OS	Oxidative stress
PARP-1	Poly(ADP-ribose) polymerase-1
PBMC	Peripheral blood mononuclear cells
PEF	Peak expiratory flow
PLS-DA	Partial least squares discriminant analysis
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD1	Superoxide dismutase 1
TLC	Total lung capacity

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive condition characterized by persistent respiratory symptoms and airflow limitation (*GOLD*, 2023). COPD represents a major global health challenge due to its high prevalence, increasing burden, and significant impact on individuals and healthcare systems worldwide. A primary contributor to the healthcare strain linked to COPD is acute exacerbations (AE-COPD). COPD produces remarkable systemic consequences that arise from triggering inflammation and oxidative stress (OS), that in turn cause oxidative damage to important biomolecules, including DNA, in distant organs liaised by the circulation. It is important to note that smoking and air pollution are major contributors to this process. These environmental factors significantly increase inflammation and OS in the body, exacerbating the systemic effects of COPD (Austin *et al.*, 2016; Pauwels & Rabe, 2004). COPD is underrecognized and under-diagnosed and currently available treatments have minimal impact on disease progression. Therefore, a strategy to more properly diagnose and prevent the development of COPD is a critical priority

Investigating DNA damage, poly(ADP-ribose) polymerase (PARP) activity, and mRNA expression levels of antioxidant, pro- and anti-inflammatory enzymes in peripheral blood mononuclear cells (PBMC) of non-smoking individuals, non-obstructive smokers, patients with COPD of all stages and those with AE-COPD, could provide insight into the complex interplay between OS, inflammation, and DNA damage repair mechanisms in COPD progression.

PARP is rapidly activated by OS-induced DNA lesions (Pandey & Black, 2021; Robu *et al.*, 2013). Pro-inflammatory enzymes cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LO), dipeptidyl peptidase 4 (DPP4), leukotriene A_4 hydrolase (LTA_4H) and anti-inflammatory enzymes histone deacetylase 2 (HDAC2) and poly(ADP-ribose) polymerase-1 (PARP-1) have been demonstrated to be connected to COPD and underlying systemic inflammation (Hoxha *et al.*, 2017; Ito *et al.*, 2005; Robu *et al.*, 2013; Seys *et al.*, 2018). Glutathione (GSH) is predominantly known as an antioxidant (Liu *et al.*, 2014). Therefore, it is important to understand the relationships of systemic inflammation and GSH metabolism pathway enzymes [superoxide dismutase 1 (SOD1), GSH synthetase (GSS), catalytic and modulatory subunits of glutamyl-cysteine ligase (GCLC and GCLM), GSH reductase (GSR) and GSH peroxidase (GPx)] with smoking and lung function, as well as the presence of exacerbations. Additionally, it is crucial to examine these relationships in the context of the Global Initiative for COPD (GOLD) classification categories.

The response by blood cells in COPD is less well understood, as there is little to no data on its modulation by antioxidants. Targeting OS and systemic inflammation could prove to be an effective way to improve survival and quality of life in these patients (Austin *et al.*, 2016; Pauwels & Rabe, 2004). Increasing the level of intracellular GSH, through GSH metabolism pathway enzymes, could be useful in different clinical modes. Thanks to the diverse characteristics of GSH,

many different GSH analogues with different properties have already been synthesized (Lucente *et al.*, 1998). Tetrapeptide analogues of GSH, named UPF1 and UPF17, could potentially have an effect on the expression of antioxidative and pro- and anti-inflammatory enzymes. UPF1 and UPF17 are more hydrophobic than glutathione and can more strongly interact with plasma membrane and/or with hydrophobic binding sites of different proteins. It has been previously shown that UPF1 has 60-fold higher antioxidative capacity and UPF17 has 3000-fold higher antioxidative capacity compared to glutathione. Both of them are non-toxic to primary neuronal cultures (Ehrlich *et al.*, 2007; Kairane *et al.*, 2012; Mahlapuu *et al.*, 2006). We hypothesized that UPF1 and UPF17 have an impact on mRNA expression levels of enzymes connected with OS and have an opposite effect on inflammatory enzymes providing protective effect on the development of COPD in PBMC.

2. REVIEW OF THE LITERATURE

2.1 Chronic obstructive pulmonary disease (COPD)

COPD is characterized by persistent respiratory symptoms and airflow limitation that is caused by a mixture of small airways disease and parenchymal destruction (emphysema) (*GOLD*, 2023). In earlier years, COPD was described as a combination of emphysema and chronic bronchitis, but chronic respiratory symptoms also exist in individuals with normal spirometry and a significant number of smokers without airflow limitation have evidence of lung disease manifested by the varying presence of emphysema, gas trapping, and chronic bronchitis (Regan *et al.*, 2015; Woodruff *et al.*, 2016). Major COPD risk factors are tobacco smoking and outdoor, occupational, and indoor air pollution, but also genetics, airway hyper-responsiveness and poor lung growth during childhood play a role in COPD development and progression (Eisner *et al.*, 2010; *GOLD*, 2023).

COPD is now one of the three leading causes of death worldwide and is a major cause of chronic morbidity and mortality throughout the world (*Centers for Disease Control and Prevention*, 2024; *GOLD*, 2023). According to WHO, more than 3 million people died of COPD in 2019 (*World Health Organization (WHO)*, 2023). COPD burden is projected to increase in coming decades because of continued exposure to COPD risk factors and aging of the population (Mathers & Loncar, 2006).

2.1.1 Diagnosis of COPD

COPD does not develop suddenly and sometimes progresses imperceptibly to higher stages of the disease. By the time patients seek for help, they have often already developed extensive airway damage. Due to that, COPD should be considered in any patient who has dyspnea, chronic cough or sputum production, and/or a history of exposure to risk factors for the disease (*GOLD*, 2023; Rabe *et al.*, 2007). The most characteristic symptom of COPD is usually chronic and progressive dyspnea. Cough and sputum production are present in about 30% of the patients. To diagnose COPD, spirometry is required. Presence of a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) below the normal limit confirms the presence of persistent airflow limitation and thus of COPD in patients with risk factors (*GOLD*, 2023).

2.1.2 Classification of COPD

COPD assessment and classification are implemented to determine the level of airflow limitation, its impact on the patient's health status with symptoms, and the risk of future exacerbations, to best guide their therapy. Over time, COPD classification has changed. Until 2011, COPD was classified purely by the severity of airflow limitation GOLD 1–4 (Table 1). Due to COPD being a complex disease, this classification did not reflect the actual progression of the

disease. In 2011, GOLD proposed to move to a combined assessment strategy based on the level of symptom instead of the simple spirometric grading system. New multidimensional classification was developed, GOLD A-D (Table 2), which includes exacerbation history, the severity of airflow limitation and questionnaires Modified British Medical Research Council Questionnaire (mMRC) (Table 3) or COPD Assessment Test (CAT) (Table 4) (*GOLD*, 2023). From 2022 again a new GOLD A-D classification was proposed, where severity of airflow limitation was excluded as one of the classifying factors (Table 5). From 2023, GOLD ABE classification was proposed, where compared to the previous classification, groups C and D were combined into group E to highlight the clinical relevance of exacerbations (Table 6).

Table 1. The classification of chronic obstructive pulmonary disease (COPD) by airflow limitation severity [Global Initiative for COPD (GOLD) 2011] (*GOLD*, 2011). No substantial changes have occurred until present.

Classification of airflow limitation severity in COPD (based on post-bronchodilator FEV_1)		
In patients with $FEV_1/FVC < 0.70$:		
GOLD 1	Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4	Very severe	$FEV_1 < 30\%$ predicted

FEV_1 – forced expiratory volume in 1 second, FVC – forced vital capacity, GOLD – Global Initiative for Chronic Obstructive Lung Disease.

Table 2. Combined chronic obstructive pulmonary disease (COPD) ABCD assessment used from 2011 through 2017 [Global Initiative for COPD (GOLD) 2011] (*GOLD*, 2011).

The ABCD assessment tool		
	Airflow limitation and moderate and severe exacerbation history	Symptoms
GOLD A	$FEV_1 \geq 50\%$ 0 or 1 (not leading to hospital admission)	mMRC 0–1 CAT < 10
GOLD B	$FEV_1 \geq 50\%$ 0 or 1 (not leading to hospital admission)	mMRC ≥ 2 CAT ≥ 10
GOLD C	$FEV_1 < 50\%$ predicted ≥ 2 or ≥ 1 leading to hospital admission	mMRC 0–1 CAT < 10
GOLD D	$FEV_1 < 50\%$ predicted ≥ 2 or ≥ 1 leading to hospital admission	mMRC ≥ 2 CAT ≥ 10

FEV_1 – forced expiratory volume in 1 second, GOLD – Global Initiative for Chronic Obstructive Lung Disease, CAT – Chronic obstructive pulmonary disease Assessment Test, mMRC – Modified British Medical Research Council Questionnaire.

Table 3. Modified British Medical Research Council (mMRC) questionnaire (*GOLD*, 2023).

MRC Dyspnea Scale	
Description	Grade
I only get breathless with strenuous exercise	0
I get short of breath when hurrying on level ground or walking up a slight hill	1
On level ground, I walk slower than people of my age because of breathlessness, or I have to stop for breath when walking at my own pace on the level	2
I stop for breath after walking about 100 yards or after a few minutes on level ground	3
I am too breathless to leave the house or I am breathless when dressing/undressing	4

MRC – Medical Research Council

Table 4. Chronic obstructive pulmonary disease (COPD) Assessment Test (CAT) questionnaire (*GOLD*, 2023).

							SCORE
I never cough	1	2	3	4	5	I cough all the time	
I have no phlegm (mucus) in my chest at all	1	2	3	4	5	My chest is completely full of phlegm (mucus)	
My chest does not feel tight at all	1	2	3	4	5	My chest feels very tight	
When I walk up a hill or one flight of stairs, I am not breathless	1	2	3	4	5	When I walk up a hill or one flight of stairs, I am very breathless	
I am not limited doing any activities at home	1	2	3	4	5	I am very limited doing activities at home	
I am confident leaving my home despite my lung condition	1	2	3	4	5	I am not at all confident leaving my home because of my lung condition	
I sleep soundly	1	2	3	4	5	I don't sleep soundly because of my lung condition	
I have lots of energy	1	2	3	4	5	I have no energy at all	
						TOTAL SCORE	

Table 5. Combined chronic obstructive pulmonary disease (COPD) ABCD assessment used from 2017 through 2023 [Global Initiative for COPD (GOLD) 2022] (*GOLD*, 2022).

	The refined ABCD assessment tool	
	Moderate and severe exacerbation history	Symptoms
GOLD A	0 or 1 (not leading to hospital admission)	mMRC 0–1 CAT < 10
GOLD B	0 or 1 (not leading to hospital admission)	mMRC ≥ 2 CAT ≥ 10
GOLD C	≥ 2 or ≥ 1 leading to hospital admission	mMRC 0–1 CAT < 10
GOLD D	≥ 2 or ≥ 1 leading to hospital admission	mMRC ≥ 2 CAT < 10

CAT – Chronic obstructive pulmonary disease (COPD) Assessment Test, GOLD – Global Initiative for COPD, mMRC – Modified British Medical Research Council Questionnaire.

Table 6. Combined chronic obstructive pulmonary disease (COPD) ABE assessment used from 2023 onward [Global Initiative for COPD (GOLD) 2023] (*GOLD*, 2023).

	The refined ABE assessment tool	
	Moderate and severe exacerbation history	Symptoms
GOLD A	0 or 1 (not leading to hospital admission)	mMRC 0–1 CAT < 10
GOLD B	0 or 1 (not leading to hospital admission)	mMRC ≥ 2 CAT ≥ 10
GOLD E	≥ 2 or ≥ 1 leading to hospital admission	mMRC 0–4 CAT 0–40

CAT – Chronic obstructive pulmonary disease (COPD) Assessment Test, GOLD – Global Initiative for COPD, mMRC – Modified British Medical Research Council Questionnaire.

In 2012, Lange *et al.* proposed a subclassification, where classes C and D, that consist of individuals with quite variable lung function and a variable history of exacerbations, were subdivided depending on which of these scenarios or both were causing the individuals to occur in the categories C or D, to better prognose the risk of exacerbations and mortality (Table 7) (Lange *et al.*, 2012). While this subclassification should not be used for clinical decision-making, it can give researchers a valuable approach in studying the significance of genetic variants and can provide additional context to clinicians when considering further testing or follow-up.

Table 7. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2011 C1–D3 subgroups (Lange *et al.*, 2012).

	C subgroups of GOLD 2011			D subgroups of GOLD 2011		
	C1	C2	C3	D1	D2	D3
FEV_1	< 50% predicted	≥ 50% predicted	< 50% predicted	< 50% predicted	≥ 50% predicted	< 50% predicted
Exacerbations	< 2	≥ 2	≥ 2	< 2	≥ 2	≥ 2

FEV_1 – forced expiratory volume in 1 second, GOLD – Global Initiative for Chronic Obstructive Lung Disease.

2.1.3 Acute exacerbation of COPD (AE-COPD)

AE-COPD is an event characterized by dyspnea, sputum purulence, and sputum volume that worsen over less than two weeks (*GOLD*, 2023; Ritchie & Wedzicha, 2020). Patients may also experience tachypnea and tachycardia that involve heightened local and systemic inflammation (*GOLD*, 2023; Ritchie & Wedzicha, 2020). Various triggers can spark an exacerbation, including respiratory infections, exposure to air pollutants, congestive heart failure, weather changes, etc. AE-COPD account for the largest share of the COPD burden on the healthcare system (*American Lung Association*, 2024; *GOLD*, 2023). AE-COPD also plays a key role in the management of COPD, as it accelerates the disease progression, increases morbidity and mortality, impairs quality of life, and requires specific preventive and therapeutic measures (Austin *et al.*, 2016; *GOLD*, 2023). Once AE-COPD occurs, there is an increasing likelihood of a subsequent event occurring (Hurst *et al.*, 2010).

2.1.4 Treatment of COPD

Multidimensional classification provides not only information regarding severity of airflow limitation, but also about symptom burden and risk of exacerbation, that can be used in making initial COPD management decisions (*GOLD*, 2023). Following initial therapy, patients should be reassessed for attainment of treatment goals and then therapy should be adjusted if needed (Agusti *et al.*, 2023).

Pharmacological therapy for COPD is used to reduce symptoms, reduce the frequency and severity of exacerbations, and improve exercise tolerance and health status (*GOLD*, 2023).

The current main pharmacological approaches include:

1. Antimuscarinic drugs are used to block the bronchoconstrictor effects in airway muscle, but used alone, they provide small benefits in terms of lung function (Appleton *et al.*, 2006; Melani, 2015).
2. Inhaled $beta_2$-agonists alter the airway smooth muscle tone and widen the airways improving FEV_1 and/or other spirometric variables (*GOLD*, 2023; Vathenen *et al.*, 1988).
3. Methylxanthines may act as non-selective phosphodiesterase inhibitors and show only a modest bronchodilator effect in stable COPD (*GOLD*, 2023; Ram *et al.*, 2002). The use of methylxanthines in the treatment of COPD has been largely marginalized in modern clinical practice due to their unfavorable side effect profile and relatively weak therapeutic efficacy compared to other available interventions (*GOLD*, 2023; Ram *et al.*, 2002).
4. The combination of inhaled long-acting antimuscarinergic agents and long-acting $beta_2$-agonists increases the effect and decreases the side-effects compared to increasing the dose of a single bronchodilator (Ray *et al.*, 2019).
5. Inhaled corticosteroids are beneficial for both current and ex-smokers with COPD in terms of lung function and exacerbation rates, though the benefits are less pronounced in current smokers compared to ex-smokers (Sonnex *et*

al., 2020). Regular treatment with inhaled corticosteroids, which are never used as monotherapy, does not modify the long-term decline in FEV_1 nor mortality in patients with COPD (*Global Strategy for Asthma Management and Prevention*, 2024; Yang *et al.*, 2012).

6. Triple therapy with long-acting antimuscarinic drugs, long-acting β_2-agonists, and inhaled corticosteroids in one single inhaler improves lung function, patient-reported outcomes and reduces exacerbation (*GOLD*, 2023). Triple combination of antimuscarinic drugs, long-acting β_2-agonists, and inhaled corticosteroids shows the greatest improvement in quality of life amongst patients with a greater baseline symptom burden, but there is a significantly higher risk of pneumonia in those treated with bronchodilators and inhaled corticosteroids (*GOLD*, 2023; Suissa *et al.*, 2019).
7. Other pharmacological therapies, like phosphodiesterase-4 inhibitors, macrolide antibiotics, mucolytic and antioxidant agents are also in use in selected circumstances (*GOLD*, 2023). Oral corticosteroids are not in use to treat stable COPD, but are considered effective in AE-COPD (*GOLD*, 2023). Vaccination against influenza, respiratory syncytial virus (RSV), pneumococcus and pertussis play a crucial role in managing COPD (*Global Strategy for Asthma Management and Prevention*, 2024; Ji *et al.*, 2022; Simon *et al.*, 2023).

The main non-pharmacological therapies include:

1. Smoking cessation, which reduces overall mortality rate,
2. Pulmonary rehabilitation,
3. Long term oxygen therapy,
4. Non-invasive positive pressure ventilation,
5. Lung transplant and lung volume reduction surgery in selected patients (*GOLD*, 2023).

Although there are many pharmacological and non-pharmacological treatments available, they have limited effectiveness and fail to alter the long-term progression of COPD, its exacerbations, its comorbidities and its mortality (Austin *et al.*, 2016).

2.1.5 Risk factors of COPD

Cigarette smoking is the main factor of COPD, but not all COPD patients are smokers (Fabbri & Rabe, 2007; *U.S. Department of Health and Human Services*, 2024). Even for heavy-smokers, fewer than 50% develop COPD during their lifetime (*GOLD*, 2023). It has been shown that never smokers with COPD have fewer symptoms, milder disease and lower burden of systemic inflammation (Thomsen *et al.*, 2013). Cigarette smoke (CS) prompts inflammation and OS both in the airways and systemically involving NF-κB activation (Bhalla *et al.*, 2009). CS contains up to $10^{15} – 10^{17}$ free radicals in one puff (Bernardo *et al.*, 2015; Pryor & Stone, 1993). These radicals are capable of initiating oxidation of proteins, DNA, and lipids, that may cause direct tissue injury or instigate a variety

of cellular responses (Kirkham & Rahman, 2006). In addition, smokers have been found to have significantly higher levels of iron in their lungs that increase the potential reactive oxygen species (ROS) burden, that in turn can cause cell and tissue damage (Kirkham & Rahman, 2006). Smoking cessation continues to be the best way to influence the natural history of COPD (*GOLD*, 2023).

Outdoor air pollution, passive smoking, occupational dust, early-life events (premature birth, low birth weight, nutritional problems in early childhood, childhood asthma, respiratory infections in childhood etc.), chemicals, asthma, airway hyper-activity, genetic predisposition (like α-1-antitrypsin deficiency) and biomass fuel exposure have previously been suggested as additional risk factors for COPD (Carraro *et al.*, 2014; Filippone & Baraldi, 2011; *GOLD*, 2023; Han & Martinez, 2020; Savran & Ulrik, 2018; Thomsen *et al.*, 2013; Yigla *et al.*, 2007). In addition to that, age, gender, lung growth and maldevelopment, previous infections and low socioeconomic status can be risk factors of COPD (*GOLD*, 2023). In addition, there is growing evidence that exposure to modern and traditional fuels used during cooking can make you more prone to develop COPD (Zhou *et al.*, 2014).

Studies suggest that females could have a higher biological vulnerability to the detrimental impacts of tobacco smoke compared to their male counterparts (Ben-Zaken Cohen *et al.*, 2007; Chung *et al.*, 2023). A study conducted in Poland showed a growing prevalence of COPD-related hospitalizations among female patients (Bochenek *et al.*, 2024). Not much is understood about the mechanisms driving this epidemic, but could be linked to the smaller lung size in women, compared to men (Ben-Zaken Cohen *et al.*, 2007; Chung *et al.*, 2023). Research conducted on animals suggests that significant gender-based variations may exist in how certain components of CS are processed by the body. These differences could potentially result in females producing higher levels of cancer-causing and respiratory system-damaging substances (Ben-Zaken Cohen *et al.*, 2007; Van Winkle *et al.*, 2002). Historically, women have had lower smoking rates, which means that non-smoking factors play a more prominent role in the development of COPD among females. Furthermore, research suggests that women may have an increased vulnerability to COPD, not just from tobacco use, but also from various other environmental and physiological factors (Gut-Gobert *et al.*, 2019; Sama *et al.*, 2017). This heightened susceptibility extends beyond smoking-related causes, encompassing a broader range of potential triggers for COPD in women (Gut-Gobert *et al.*, 2019; Kim & Lee, 2017).

2.1.6 Pathophysiology of COPD

Pathological changes characteristics of COPD, like chronic inflammation, and structural changes resulting from repeated injury and repair, are found in the airways, lung parenchyma, and pulmonary vasculature (*GOLD*, 2023; Hogg & Timens, 2009). Systemic inflammation has been shown to be present and could play a role in the multiple comorbid conditions found in patients with COPD (Agusti *et al.*, 2012). In COPD patients, the inflammatory response to chronic

irritants like CS, appears to be amplified compared to normal inflammatory response (Colarusso *et al.*, 2017; *GOLD*, 2023). Although some non-smoking patients develop COPD, the nature of the inflammatory response in these patients is not yet thoroughly understood. Inflammation is further modified by OS and an excess of proteinases in the lung, that can lead to the characteristic pathological changes in COPD, like increase in air flow limitation, FEV_1 decrease, decreased gas transfer etc. (*GOLD*, 2023).

During AE-COPD, sudden increase in resistance in airways occurs, decreasing the expiratory flow (O'Donnell & Parker, 2006). Increased airway and systemic inflammation, increased gas trapping and hyperinflation with reduced expiratory flow occur during these exacerbations and are associated with increased local and systemic inflammation caused by airway infection, pollution, or other insults to the lungs (*GOLD*, 2023; Parker *et al.*, 2005). Treatment for AE-COPD has not evolved significantly and due to that, prevention of AE-COPD is a leading therapeutic goal in COPD management (Mador & Sethi, 2013). For proper preventative care, identifying patients with a risk of AE-COPD is vital. One way to potentially identify future or reoccurring exacerbations is to explore the relationship of pro- and anti-inflammatory enzymes, and GSH metabolism pathway enzymes and AE-COPD and the progression of the disease.

2.1.7 Systemic and extra-pulmonary manifestation of COPD

While COPD primarily affects the lungs, it is also associated with several systemic and extra-pulmonary manifestations that extend beyond the lungs. These manifestations can significantly impact patients' overall health, quality of life, and prognosis. These widespread consequences can include unexpected weight loss, skeletal muscle dysfunction, heightened risk of cardiovascular disease (like coronary artery disease, heart failure, arrhythmias, and pulmonary hypertension), osteoporosis, and mental health issues such as depression, among various other complications (Agusti & Soriano, 2008; Barnes & Celli, 2009). It has been believed that systemic inflammation in mild-moderate degree, may increase cardiovascular morbidity and mortality in patients with COPD (Sin & Man, 2003). Osteoporosis is potentially caused due to systemic inflammation, use of corticosteroids, and reduced physical activity, which in turn can cause muscle weakness, which consecutively lessens physical activity (Agusti *et al.*, 2003; Incalzi *et al.*, 2000; Xiang & Luo, 2024). In addition, the presence of airflow limitation increases the likelihood that patients with COPD may develop lung cancer over time (Barnes & Celli, 2009). Anemia, polycythemia, gastroesophageal reflux disease (GERD), endocrine abnormalities, tissue hypoxia are among other comorbidities of COPD (Ferrari *et al.*, 2015; Xiang & Luo, 2024). The process by which anemia emerges in individuals with COPD may be similar to that of other chronic diseases, where inflammatory response mediators contribute to the development and progression of anemia (John *et al.*, 2005). GERD is primarily caused due to changes in chest pressure, airway irritation and damage due to reflux that is caused by prescribed receptor agonists, bronchoconstriction due to

recurrent coughing, and bacterial reflux and even bacterial colonization due to aspiration (Broers *et al.*, 2018; Huang *et al.*, 2020; Lee & Goldstein, 2015; Xiang & Luo, 2024) Mental health disorders are the most common diseases associated with COPD, caused by recurrent illnesses and reduced social engagement (Xiang & Luo, 2024). This in turn can increase the risk of AE-COPD (Montserrat-Capdevila *et al.*, 2017).

The inflammatory response in COPD is mediated by inhaled oxidants or oxidants that are released by activated immune cells, specifically neutrophils, alveolar macrophages, eosinophils and epithelial cells leading to production of ROS (Albano *et al.*, 2022; Rahman & Adcock, 2006). ROS, in turn, will interact with cellular components, leading to lipid peroxidation, DNA and protein damage, contributing to the ongoing inflammatory cascade and tissue injury that is characteristic of COPD (Rahman & Adcock, 2006; Zinellu *et al.*, 2016). In patients with COPD, it remains an ongoing debate if the intense inflammatory response in the lungs of COPD patients spills over into the systemic circulation, meaning the lungs are the primary source of the inflammation, or rather there is an increased production of inflammatory mediators in multiple organs and tissues beyond the lungs, suggesting that the systemic inflammation is not solely derived from the pulmonary compartment, but rather represents a more widespread inflammatory response throughout the body (King, 2015; Oudijk *et al.*, 2003; Tkacova, 2010). In either case, both mechanisms may contribute to the overall systemic inflammatory state observed in COPD patients, and the complex relationships between local and systemic inflammation in this disease are still not well understood.

2.1.8 Premature aging in COPD

COPD is characterized by the early onset of age-related changes in both pulmonary and extrapulmonary systems (Karametos *et al.*, 2019). Aging is defined as a gradual decline in physiological integrity, leading to decreased performance and a higher susceptibility to various health issues and mortality (Lopez-Otin *et al.*, 2013). It happens when anti-inflammatory and antioxidant mechanisms become overwhelmed, leaving the body vulnerable to harm caused by chronic low-grade inflammation and excess ROS (Easter *et al.*, 2020). Research shows that people with COPD aged 55 to 65 have similar levels of density and coexisting health conditions as those without COPD who are 10 to 20 years older (Divo *et al.*, 2018). Sex and growth hormones alike are biological markers of age and were found to be significantly reduced in patients with COPD, indicating increased aging amongst COPD patients (Karametos *et al.*, 2019). This abnormal aging is caused by several mechanisms, like increased cellular senescence, DNA damage to lung cells, stem cell depletion, abnormal extracellular matrix remodeling, and premature senescence of the immune system (Brandsma *et al.*, 2017; Easter *et al.*, 2020). It is known that COPD causes lung function decline and structural changes, but it also causes decline in effectiveness of pulmonary defense mechanisms, leading to higher susceptibility to respiratory infections and a diminished

ability to defend against oxidative damage and inflammatory responses (Brandsma *et al.*, 2017; Easter *et al.*, 2020). The premature aging associated with COPD has significant clinical implications, for example, they tend to develop diseases characteristic of the elderly at younger ages compared to those without COPD. COPD patients also have higher mortality rates and tend to die at younger ages than those without this disease (Divo *et al.*, 2018).

2.2 Systemic oxidative stress (OS) and COPD

ROS, like superoxide anion radical ($O_2 \cdot^-$), singlet oxygen ($^1O_2$), hydroxyl radical ($\cdot OH$), perhydroxyl radical ($HO_2 \cdot$) and hydrogen peroxide (H_2O_2) are generated by CS and other inhaled particulates, and released from activated inflammatory cells such as macrophages and neutrophils (Ahsan *et al.*, 2003; *GOLD*, 2023; Richter *et al.*, 1995). Although at low levels superoxide anion, hydroxyl radicals, hydrogen peroxide, and molecular oxygen take part in normal cellular processes including cell proliferation, apoptosis, immune responses, and cell differentiation, overproduction or inadequate removal of these oxidants can lead to OS, altered metabolism, dysregulated signal transduction events, and biomolecular damage that in turn can cause DNA damage (Trouba *et al.*, 2002).

In chronic inflammatory conditions, such as COPD, OS primarily results from the increased production of ROS from CS, infections and continuous activation of endogenous enzymes (Bernardo *et al.*, 2015). OS itself in turn plays a key role in the development and progression of COPD (Brassington *et al.*, 2019). There is also a decrease in the levels of circulating antioxidants in smokers and COPD patients, which accounts for the increased circulating contents of ROS (Brassington *et al.*, 2019). OS is a major mechanism in the pathophysiology of COPD and is particularly increased in COPD patients during AE-COPD (Barnes, 2020). In addition, activated neutrophils in the lung and peripheral blood in patients with COPD release increased amount of ROS, especially during exacerbations, increasing local and systemic OS (Barnes, 2020; Noguera *et al.*, 2001).

Due to the location, anatomy, and function, the lungs are exceptionally susceptible to oxidative damage caused by ROS. In order for the lungs to protect themselves from oxidant-induced tissue damage, they contain many antioxidant defenses (Kirkham & Rahman, 2006).

2.2.1 Glutathione (GSH)

GSH plays an important role in many critical cellular processes, including DNA synthesis and repair, protein synthesis, amino acid transport, enhancement of immune function, and enzyme activation, but most importantly, it protects cells from the toxic effects of ROS (Lomaestro & Malone, 1995; Maher, 2005). High levels of GSH have been related with good health during aging and decreased GSH status with various diseases including COPD (Bains & Shaw, 1997; Julius *et al.*, 1994).

GSH is synthesized from glutamate, cysteine and glycine by glutamyl-cysteine ligase (GCL) and GSS. GSH eliminates H_2O_2 in a reaction catalyzed by GPx. H_2O_2 is produced from O_2^- by SOD. The GSH disulfide (GSSG) produced in this reaction can be converted back to GSH through the action of GSR (Figure 1) (Maher, 2005; Meister, 1995). Antioxidant enzymes GPx and superoxide dismutase (SOD) are the first-line defendants against ROS and their by-products (Gutteridge & Halliwell, 2000; Pizent *et al.*, 2020).

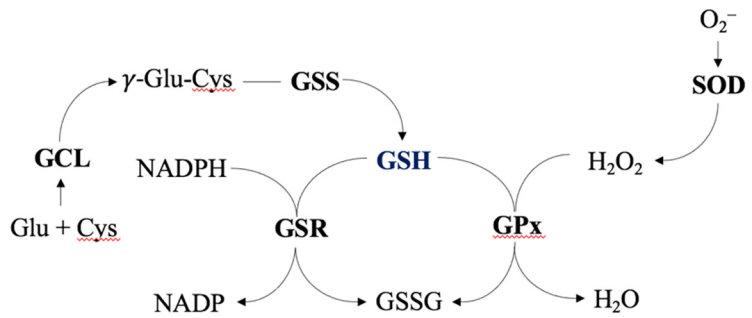

Figure 1. Outline of the metabolism of glutathione (GSH). GSH is composed of glutamate (Glu), cysteine (Cys), and glycine (Gly) by glutamyl-cysteine ligase (GCL) and GSH synthase (GSS). GSH eliminates H_2O_2 in a reaction catalyzed by GSH peroxidase (GPx). The GSH disulfide (GSSG) produced in this reaction can be converted back to GSH through the action of GSH reductase (GSR). NADP – nicotinamide adenine dinucleotide phosphate, SOD – superoxide dismutase.

2.3 Systemic inflammation and COPD

The mechanism of amplified inflammation in patients, who develop COPD, is not completely understood (*GOLD*, 2023). The structural changes, like narrowing of the small airways and destruction of the lung parenchyma, is associated with chronic pulmonary and systemic inflammatory responses (Pelgrim *et al.*, 2019). These changes lead to the loss of alveolar attachments to the small airways, decrease lung elastic recoil, and diminish the ability of the airways to remain open during expiration (McDonough *et al.*, 2011; *World Health Organization (WHO)*, 2023).

Inflammation and OS are closely connected, but their exact relationships are variable and are still poorly described. Many inflammatory cells are involved in the pathophysiology of COPD, including macrophages, neutrophils and T-cells. In response to CS, these cells release many mediators, including ROS and leukotriene B_4, which in turn, increases the systemic OS and inflammatory burden. This constant repeated cycles of injury and repair can cause or worsen the chronic systemic inflammation via overflow of the pro-inflammatory mediators, cytokines, enzymes, and inflammatory cells into the systemic circulation and triggering inflammatory reactions in distant extra-pulmonary organs (Abdulkhaleq

et al., 2018; Austin *et al.*, 2016; Furman *et al.*, 2019; *GOLD*, 2023; Schett & Neurath, 2018).

2.3.1 Pro-inflammatory enzymes

Pro-inflammatory enzymes play a significant role in the pathogenesis and progression of COPD, as they contribute to tissue destruction, airway remodeling, and continuous inflammation. Studying the complex interplay between these enzymes and the progression of COPD could give insights to the disease mechanism, serve as potential biomarkers for COPD, and lead to the development of targeted therapies.

2.3.1.1 Dipeptidyl peptidase 4 (DPP4)

DPP4 is a cell surface protein in various tissues and plays a role in immunology, tumorigenesis, glucose homeostasis, and infectious pathophysiology. DPP4 also acts as a cofactor in intracellular T-cell proliferation and T-cell activation signaling pathways. Therefore, DPP4 has been studied as a biomarker in various malignancies, immune/inflammatory diseases, metabolic disorders, and as a potential therapeutic target (Nadkarni *et al.*, 2014; Ohnuma *et al.*, 2008; Pan *et al.*, 2021; Shao *et al.*, 2020). The enzyme DPP4 could be a key player in both normal lung function and disease processes (Hua *et al.*, 2023). DPP4 exhibits versatile dipeptidase activity, acting on a wide range of substrates. These include various cytokines, growth factors, neuropeptides, and enterokinetic hormones. Through its cleavage mechanism, DPP4 can activate, inactivate, or modify the receptor specificity of these substrates playing significant roles in multiple physiological processes, such as intracellular signaling, management of OS, lipid metabolism, programmed cell death (apoptosis), immune system activation, insulin resistance, and inflammatory responses (Cheng *et al.*, 2018; Chitadze *et al.*, 2021; Hua *et al.*, 2023). Therefore, DPP4 could be potential biomarker for COPD, and further, inhibiting DPP4 could be effective in decreasing the mortality of COPD (Shao *et al.*, 2020; Zhang *et al.*, 2021).

2.3.1.2 Leukotriene A_4 hydrolase (LTA_4H) and 5-lipoxygenase (5-LO)

Leukotrienes are signaling molecules that have an important role in the mediation of airway inflammation. They can be divided into proinflammatory leukotriene B_4 (LTB_4) and the spasmogenic leukotrienes C_4, D_4, and E_4. LTB_4 is one of the strongest chemotactic and activating agents for neutrophils known to date and is an important component in the development and maintenance of inflammation (Haeggstrom, 2000; Santus *et al.*, 2005).

In the biosynthesis of leukotrienes, free arachidonic acid (AA) interacts with 5-LO and 5-LO activating protein (FLAP) to produce the unstable intermediate leukotriene A_4 (LTA_4), which in turn is hydrolyzed to proinflammatory compound LTB_4 by LTA_4H or conjugated with reduced GSH by GSH-S-transferase to produce LTC_4 (Figure 2) (Haeggstrom, 2000; Zhang *et al.*, 2006).

5-LO is mainly expressed in various leukocytes, including polymorphonuclear cells. Activity of 5-LO in a cell is dependent on the oxidant-antioxidant balance of a given cell (Santus *et al.*, 2005). 5-LO inhibitors are used for the treatment of many chronic diseases, like asthma, COPD and atherosclerosis (Steinhilber & Hofmann, 2014).

LTA_4H is found in many different cell types, like epithelial cells, endothelial cells, fibroblasts, keratinocytes, and erythrocytes and has been detected in almost all mammalian tissues and organs (Haeggstrom, 1999; Numao *et al.*, 2017). LTA_4H has two main functions: 1) converting LTA_4 to LTB_4, a potent proinflammatory mediator, and 2) degrading the neutrophil chemoattractant tripeptide proline-glycine-proline (PGP) (Snelgrove *et al.*, 2010; Szul *et al.*, 2016). PGP recruits and activates neutrophils, whereas LTA_4H degrades PGP into proline-glycine (PG) and free proline, and through that, it decreases neutrophil influx (Szul *et al.*, 2016). CS selectively inhibits LTA_4H's aminopeptidase activity leading to the accumulation of PGP and persistent neutrophil recruitment (Snelgrove *et al.*, 2010). It has been demonstrated that patients with COPD exhibit decreased LTA_4H aminopeptidase activity in sputum, resulting in elevated levels of PGP (Wells *et al.*, 2014). LTA_4H inhibitors, to prevent the LTB_4 biosynthesis, have been widely used as anti-inflammatory drugs (Numao *et al.*, 2017). Inhibitors have exhibited their ability to reduce neutrophilic inflammation and mitigate inflammatory exacerbations in clinical settings, but have failed to show any significant effect in terms of improving lung function (Rohn *et al.*, 2021). Due to LTA_4H's dual function, inhibiting LTA_4H can have complex effects. Besides inhibiting inflammation, it can potentially increase inflammation through activating PGP, affect cancer progression, and modulate immune response (Audat *et al.*, 2020; Lee *et al.*, 2019; Zhao *et al.*, 2019).

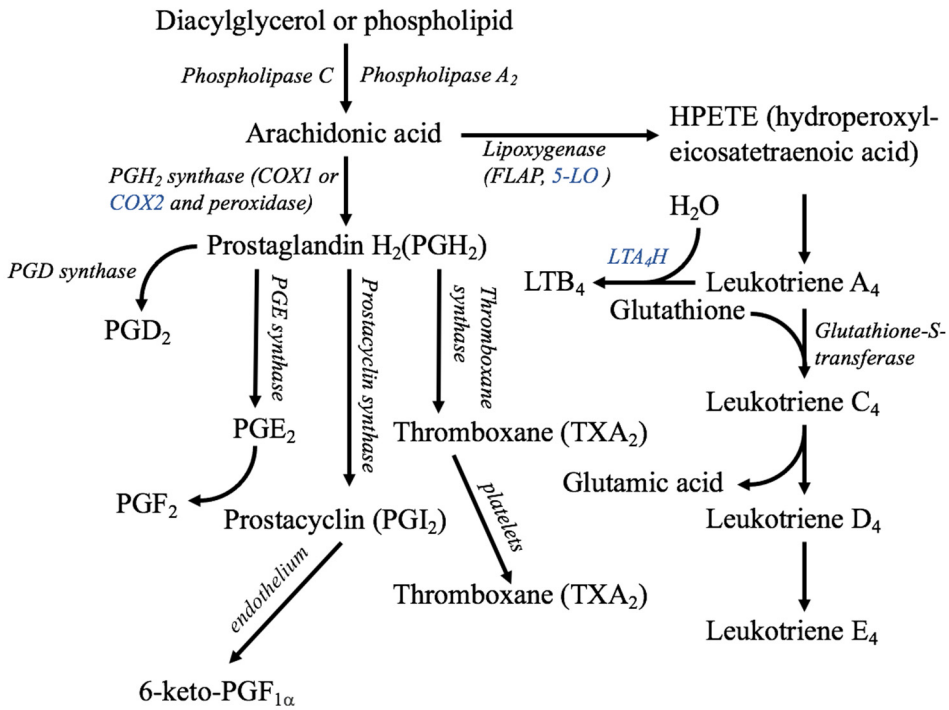

Figure 2. Eicosanoid synthesis. The biosynthesis of eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes, is facilitated by the enzymes cyclooxygenases and lipoxygenases. mRNA expression of the currently studied enzymes is marked with blue. 5-LO – 5-lipoxygenase, COX-1 – cyclooxygenase 1, COX-2 – cyclooxygenase 2, FLAP – 5-lipoxygenase activating protein, LTA_4H – leukotriene A_4 hydrolase, LTB_4 – leukotriene B_4, PGD – prostaglandin D, PGE – prostaglandin E, PGF – prostaglandin F, PGH – prostaglandin H.

2.3.1.3 Cyclooxygenase 2 (COX-2)

When AA interacts with COX-1, a cyclooxygenase (COX) constitutive isoform, many different processes are activated, such as thromboxane synthesis in platelets, prostacyclin (PGI_2) production in vascular endothelium, and prostanoid generation at gastric mucosal level. When AA interacts with COX-2, a COX inducible isoform, proinflammatory stimuli is expressed, causing an increased synthesis of prostanoids (Figure 2) (Hoxha *et al.*, 2017). COX-2 is expressed in circulating inflammatory cells and is more important source of prostanoid formation in inflammation than COX-1 (Smyth *et al.*, 2009). Elevated levels of COX-2 expression have been found in lung tissue from COPD patients compared to smokers without COPD and non-smokers (Shi *et al.*, 2017; Xaubet *et al.*, 2004). Higher levels of prostaglandin E2, a product of COX-2, in the airways are associated with increased respiratory symptoms and exacerbations in COPD patients (Tejwani *et al.*, 2023). Inhibition of COX-2 has shown suppressive

effects on lung inflammation in animal models of COPD (Lebedeva *et al.*, 2017). Although COX inhibitors have been used as antipyretic drugs, and to relieve pain and inflammation, inhibiting COX-2 may increase the risk of myocardial infarction and stroke (Grosser *et al.*, 2006; Smyth *et al.*, 2009). Inhibiting COX-2 has also shown an exaggerated inflammatory airway response and hyperresponsiveness in mice (Card *et al.*, 2006).

2.3.2 Anti-inflammatory enzymes

Anti-inflammatory enzymes are involved in perpetuating inflammation and OS in COPD, thus, targeting these enzymes and the related pathways represent a promising therapeutic strategy.

2.3.2.1 Poly(ADP-ribose) polymerase-1 (PARP-1)

OS caused by direct effects of smoking and persistent inflammation, causes oxidative damage to important biomolecules, including DNA (Ceylan *et al.*, 2006). ROS induced DNA damage in turn is considered to be a trigger for apoptosis and might suggest a role of the nuclear enzyme PARP-1 in the pathophysiology of COPD (Virag, 2005). PARP-1, a member of the PARP superfamily of 18 enzymes, is responsible for more than 90% of the cellular poly(ADP-ribosyl)ation capacity (Virag, 2005). Besides playing an important role in DNA repair, over activation of PARP-1 leads to depletion of its substrate NAD^+ and thus causing necrosis and increasing inflammation further on (Dharwal & Naura, 2018; Ha & Snyder, 1999). Recent studies highlight PARP-1 as a key player in the complex processes leading to COPD (Dharwal & Naura, 2018). Hageman *et al*. has previously shown increase in PARP-1 activity in peripheral blood lymphocytes in patients with COPD (Hageman *et al.*, 2003). It has also been shown that inhibiting PARP-1 offers substantial defense against elastase induced inflammation and emphysema in mouse models (Dharwal & Naura, 2018). As a key mediator of inflammation and tissue damage, PARP-1 is emerging as a valuable therapeutic prospect in the ongoing efforts to manage COPD more effectively.

2.3.2.2 Histone deacetylase 2 (HDAC2)

CS-mediated OS activates NF-κB-dependent transcription of pro-inflammatory mediators. NF-κB-dependent gene expression, at least in part, is regulated by changes in histone deacetylases (HDACs) (Rajendrasozhan *et al.*, 2008). HDAC2 is well characterized and reported to play a role in the regulation of inflammation and has been implicated in the dysregulation in smokers and patients with COPD (Barnes *et al.*, 2004; Ito *et al.*, 2001). Changes in levels of HDAC2, causes disruption in the acetylation/deacetylation balance, which may lead to a chronic inflammatory response (Rajendrasozhan *et al.*, 2008). It has been shown, that HDAC2 expression and activity is reduced in COPD patients compared to smokers without COPD and non-smokers (Tan *et al.*, 2016). The reduction of HDAC2 in COPD can lead to amplified inflammatory response, due to increased

expression of multiple inflammatory genes; corticosteroid resistance, due to HDAC2 being a crucial step in the anti-inflammatory action of corticosteroids; and skeletal muscle weakness, due to acetylation and activation of NF-κB (Barnes, 2009; Liao *et al.*, 2020; To *et al.*, 2017). In addition, oxidants from CS can also reduce glucocorticoid sensitivity by attenuating HDAC2 activity and expression, which may account for the glucocorticoid insensitivity in patients with COPD (Barnes *et al.*, 2004).

2.4 Glutathione analogues UPF1 and UPF17

Due to OS playing a role in the pathogenesis of COPD and the possibility of antioxidant deficiency being a risk factor for a decline in lung function (Deslee *et al.*, 2009; Lin *et al.*, 2010), new GSH analogues were synthesized in hopes to increase the level of systemic antioxidant protection. Two tetrapeptide analogues of GSH, named UPF1 (O-methoxy-L-tyrosinyl-γ- L-glutamyl-L-cysteinyl-glycine) and UPF17 (4-methoxy-L-tyrosinyl-α-L-glutamyl-L-cysteinyl-glycine), were chosen to this study to reveal their effect on the expression of antioxidative and proinflammatory enzymes (Figure 3). UPF1 and UPF17 are more hydrophobic than GSH and can therefore more strongly interact with plasma membrane and/or with hydrophobic binding sites of different proteins (Ehrlich *et al.*, 2007; Kairane *et al.*, 2012). It has been previously shown that UPF1 has a 60-fold higher antioxidative capacity and UPF17 has a 3000-fold higher antioxidative capacity compared to GSH (Ehrlich *et al.*, 2007; Kairane *et al.*, 2012). Both of them are nontoxic to primary neuronal cultures (Ehrlich *et al.*, 2007). Hansen *et al.* showed that UPF1 increased the level of intracellular GSH via up-regulating the transcription factor Nrf2 (Hansen *et al.*, 2019). The Nrf2 regulates multiple antioxidant genes and seems to be lowered in lung tissue and in alveolar macrophages of COPD patients (Barnes, 2020; Suzuki *et al.*, 2008; Zhao *et al.*, 2017). Due to that, UPF-s could potentially increase Nrf2 levels in cells, and through that increase cellular antioxidant defense leading to possible intervention or prevention of oxidative-induced COPD progression.

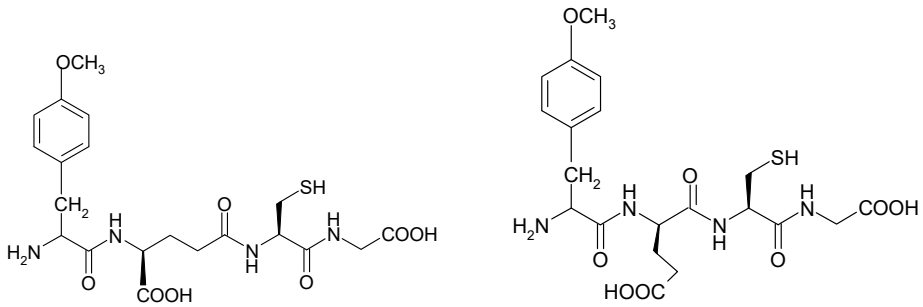

Figure 3. The chemical formulas of UPF1 and UPF17.

2.5 DNA damage measurement

Measuring DNA damage in patients with COPD can give valuable insights into disease mechanisms, severity, and potential treatment strategies. The Comet Assay, or a Single Cell Gel Electrophoresis, offers a simple way of measuring single and double-strand breaks in the DNA chain in cellular level in a wide array of different cell types (de Lapuente *et al.*, 2015; Lu *et al.*, 2017). The concept of the assay is that under an electric field, fragmented DNA in fixed cells migrates out of the nucleoid body (also known as the "comet head") and forms a DNA smear in the agarose gel (also known as the "comet tail"). With fluorescent nucleic acid staining, the extent of DNA damage can be quantified by analyzing "comets" formed by this single cell electrophoresis (Lu *et al.*, 2017). There are many software's to help measure and calculate different output parameters. Most common parameters are the tail length (shows the length of the comet tail, but can't be used with high levels of DNA damage, as the tail does not tend to change once the tail is established), the percentage of DNA in the tail (shows the ratio of DNA in the tail compared to the head and is linearly related to the breaking frequency), and the tail moment (combines tail length and tail intensity in one single value) (Lu *et al.*, 2017) (Figure 4). It has been shown that COPD patients have higher DNA damage (Ceylan *et al.*, 2006; Xie *et al.*, 2005), but it has not been previously shown how the DNA damage differentiates between all the stages of the severity of the disease and AE-COPD.

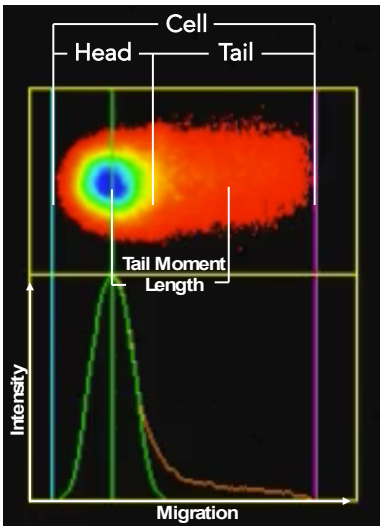

Figure 4. Fluorescence microscopy visualization of DNA damage using the Comet Assay IV software (Perceptive Instruments Ltd., Suffolk, UK). The intensity and migration of the DNA from the head (green line) to the tail (red line), as well as the areas of head, tail and tail moment length are indicated.

2.6 PARP activity measurement

As a response to DNA damage, activated PARP enzyme catalyzes the conversion of NAD^+ to nicotinamide and poly-ADP-ribose (PAR) (Bakondi *et al.*, 2002; de Murcia & Menissier de Murcia, 1994; Lee *et al.*, 2022). Although PARP is critical in DNA repair, during for example inflammation and free radical/oxidant-induced DNA single-strand break induced overactivation of PARP can cause NAD^+ depletion (Bakondi *et al.*, 2002; Lee *et al.*, 2022). To detect PARP activation in cells, 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD^+) was used (Bakondi *et al.*, 2002). From PARP family, under conditions of OS, it has been shown that PARP-1 is responsible for bio-NAD^+-incorporating activity (Bakondi *et al.*, 2002). Bio-NAD metabolized by PARP can be detected in nuclei using streptavidin-peroxidase nuclear staining. After staining, intensity of the color shows the level of PARP activity (Figure 5). Previously, increased activity of PARP-1 has been observed in peripheral blood lymphocytes in patients with COPD (Hageman *et al.*, 2003). The direct PARP activity in different stages of COPD has not been measured before.

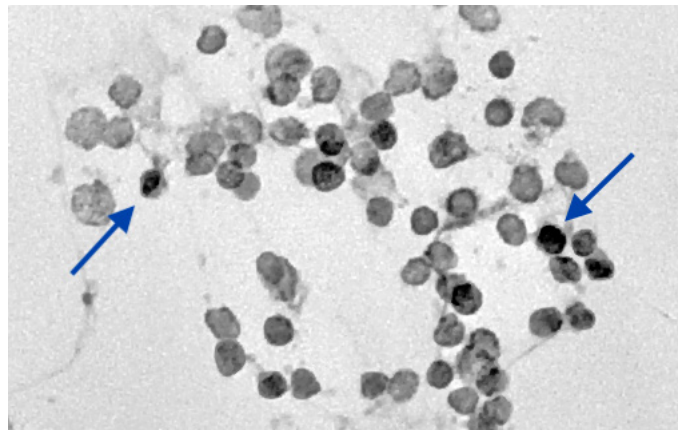

Figure 5. Cells stained with the 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD^+) method reflecting increased poly-ADP ribosylation activity (arrows) in peripheral blood mononuclear cells.

2.7 Rationale of the studies

COPD is a major health concern worldwide (*World Health Organization (WHO)*, 2023). Many cases, especially early stages of COPD remain undiagnosed (Barrecheguren *et al.*, 2018). COPD, even in early stages, has significant effects beyond the lungs, impacting multiple organs throughout the body connected by the circulatory system (van Eeden & Sin, 2013). The two key mechanisms of COPD are OS and systemic inflammation (Agusti *et al.*, 2003; van Eeden & Sin, 2013). The inflammatory process in COPD is triggered by inhaled oxidants and oxidants generated by activated immune cells. Those oxidants contribute to the production of ROS, that in turn trigger lipid peroxidation, DNA and protein damage (Rahman & Adcock, 2006; Zinellu *et al.*, 2016). Measuring DNA damage with Comet Assay, a method that measures single and double-strand breaks in the DNA chain in cellular level, could help elucidate the molecular mechanisms underlying COPD, provide insights into disease progression, and help identify patients at higher risk of exacerbations. PARP-1, an enzyme that plays an important role in DNA repair, could serve as a valuable therapeutic prospect in the ongoing efforts to manage COPD and its progression. Examining the relationship of pro-inflammatory enzymes COX-2, 5-LO, DPP4, LTA4H and anti-inflammatory enzymes HDAC2 and PARP-1 and GSH metabolism pathway enzymes (SOD1, GSS, GCLC, GCLM, GSR, GPx) mRNA expression levels with smoking data and lung function parameters could offer important understanding of the mechanisms of smoking-related lung damage and potential therapeutic targets for smoking-related diseases like COPD. Investigating the complex interplay between OS, inflammation, and DNA damage and its repair mechanism could give us insight of the COPD progression, while helping to give us a better strategy to more properly diagnose and prevent the development of COPD.

Furthermore, AE-COPD contributes heavily to the overall healthcare challenges posed by COPD (Criner & Han, 2018). Finding a pathway to better diagnose an upcoming AE-COPD could help prevent severe respiratory deterioration, reduce hospitalizations, and improve overall quality of life.

In addition of COPD being under-recognized and under-diagnosed, currently available treatments have minimal impact on disease progression. Interventions designed to combat OS and systemic inflammation could emerge as powerful tools for increasing survival and quality of life in patients with COPD. One of the pathways to decrease OS and systemic inflammation could be through enhancing intracellular GSH, through GSH metabolism pathway enzymes. UPF1 and UPF17, synthetic GSH analogues, could potentially influence the mRNA levels of enzymes associated with OS and inflammation, particularly in the context of COPD.

3. AIMS OF THE STUDY

Overall aim

To evaluate the severity of COPD through the degree of systemic DNA damage, PARP activity, and mRNA expression levels of enzymes involved in systemic inflammation and OS in PBMC in patients with an AE-COPD, as well as those with a stable COPD of all categories, in comparison to non-obstructive non-smokers and non-obstructive smokers.

Specific aims

1. To evaluate the degree of systemic DNA damage and PARP activity along with the different evolutional stages of COPD in order to shed light onto the molecular mechanisms behind the pathogenesis and progression of the disease.
2. To explore the relationship of pro-inflammatory enzymes COX-2, 5-LO, DPP4, LTA_4H and anti-inflammatory enzymes HDAC2 and PARP-1 and GSH metabolism pathway enzymes (SOD1, GSS, GCLC, GCLM, GSR, GPx) with smoking data and lung function parameters, as well as the presence of exacerbations and GOLD classification of COPD.
3. To assess the protective effect of UPF1 and UPF17 on mRNA expression levels of enzymes connected with OS and inflammation in PBMC in non-obstructive non-smokers, non-obstructive smokers, patients with an AE-COPD, as well as those with a stable COPD of all categories.

4. SUBJECTS AND METHODS

The study protocol was approved by the Ethics Committee on Human Research of the University of Tartu (protocol nr: 170/T-22) and all procedures were conducted accordingly to the ethical standards of the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (*World Medical Association, 2023*). All participants were recruited from the Lung Clinic of the Tartu University Hospital, Tartu, Estonia, and informed consent was obtained in written form from all individuals before entering the study.

4.1 Study subjects

A total of 156 subjects were recruited. Patients diagnosed with severe AE-COPD, defined as those requiring hospitalization according to criteria that now fully correspond to the GOLD consensus document 2023 (*GOLD*, 2023), and patients diagnosed as having stable COPD of all categories and degrees of severity that meet the GOLD 2023 criteria were included in the study over the course of 3 years (*GOLD*, 2023). All patients with COPD had their post-bronchodilator FEV_1/forced vital capacity (FVC) ratio below 0.7. The patients with COPD were classified and managed according to the GOLD assessment tool (*GOLD*, 2023). Therefore, different classification was used for different studies (Figure 6). For DNA damage by Comet Assay IV and PARP activity in PBMC (I) GOLD severity of airflow obstruction categorization according to the post-bronchodilator FEV_1 was used (*GOLD*, 2023), except AE-COPD patients were grouped separately independent on their airflow obstruction severity. Multidimensional GOLD A–D (*GOLD*, 2022) (II and III) and GOLD 1–4 (*GOLD*, 2023) (II) stratification was used to assess the changes according to the GOLD 2023 (*GOLD*, 2023). When comparing AE-COPD, subgroups of the GOLD categories C1–3 and D1–3 were used (Lange *et al.*, 2012) (II). Exclusion criteria for patients with both stable COPD and AE-COPD included mechanical ventilation, hospitalization into an intensive care unit, unstable coronary artery disease, and presence of active cancer. Healthy non-smokers and smokers with their lung function within normal range were incorporated for reference. A current smoker was defined as a person, who currently smoked ≥ 1 cigarette per day and a non-smoker was defined as the one, who had never smoked or who had quit smoking for at least 6 months prior to the study. Smoking cessation was calculated as the time point, from which onward the individual has remained abstinent of tobacco use. Exposure of the burning-biomass smoke was unlikely to influence the course of COPD pathogenesis and due to that, was not included into this study. The study criteria specified that participants should be free from respiratory infections affecting both the upper and lower respiratory tract (including acute bronchitis, bronchiolitis and pneumonia) for a minimum of 4 weeks prior to and throughout the study period, with the exception of patients with AE-COPD in whom respiratory infections other than pneumonia were permitted, provided that the treating pulmo-

nary physician determined these infections were directly related to the current COPD exacerbation. Spirometry (*GOLD*, 2023) and lung diffusing capacity (*GOLD*, 2023) measurements were performed in concordance with the American Thoracic Society/European Respiratory Society standards (*American Thoracic Society, 2023*; *European Respiratory Society, 2023*), whereas multi-ethnic and Finnish reference values were used for spirometry (*GOLD*, 2023) and diffusing capacity (Piirila *et al.*, 2007) indices, respectively.

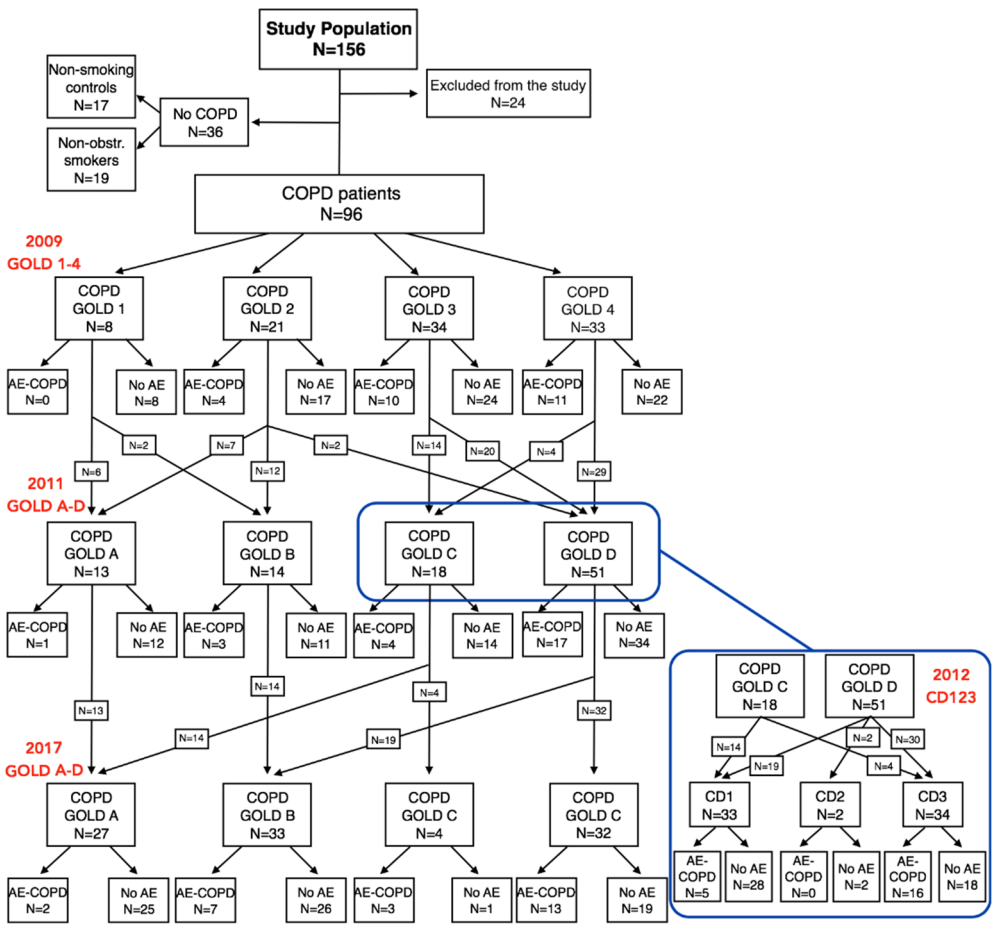

Figure 6: Classification of the study subjects into different groups. In the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2009 1–4 classification, forced expiratory volume in one second (FEV_1) % predicted defines the severity of chronic obstructive pulmonary disease (COPD) (GOLD 1–4) identifying population at risk of hospitalizations, intubation, and mortality. In the GOLD 2011 A-D classification, symptoms, exacerbation history, and FEV_1 % predicted was used for grading A-D. In the GOLD 2017 A-D classification, symptoms and exacerbation history was used for grading A-D without FEV_1 % predicted. Some patients from previous C and D were therefore

reclassified as A and B, respectively (concerns those, who were previously classified to C and D due to their FEV_1 being below 50% predicted only). In Lange 2012 CD123 classification, categories C and D were subdivided into subgroups C_1, C_2, C_3, D_1, D_2 and D_3 through different scenarios. The scenario C_1 and D_1 included patients with FEV_1 < 50% predicted and fewer than two exacerbations in the previous year (i.e. the low-lung-function-only category). In scenario C_2 and D_2, patients with $FEV_1 \geq 50\%$ predicted and two or more exacerbations in the previous year (i.e. the frequent exacerbations-only category). The scenario C_3 and D_3 included patients with both $FEV_1 < 50\%$ predicted and two or more exacerbations in the previous year (Lange *et al.*, 2012).

4.2 Methods

4.2.1 Extraction of PBMC

PBMC were separated from blood using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), in which they were centrifuged at 1500 g for 21 min at 20°C. Isolated PBMC were washed twice with 10 mL phosphate-buffered saline and centrifuged at 300 g for 15 min and 10 minutes at 20°C. 200 µL of PBMC suspension (6 x 10^6 cells/mL) were frozen down in 10% dimethyl sulfoxide (DMSO) and RPMI-1640 [including 10% fetal calf serum (FCS) and 1% penicillin/streptomycin] for the Comet Assay and measurement of PARP activity. For mRNA expression studies, mononuclear cells were divided into three even amounts. One of them was cultivated in the presence of 0.5 mM UPF1, the second was cultivated in the presence of 0.5 mM UPF17 and the third portion was cultivated without UPF peptides, using RPMI-1640 medium (includes 10% FCS and 1% penicillin⁄streptomycin). The incubation time was 12 h.

4.2.2 Comet Assay

PBMC were embedded with 0.5% low melting point agarose (Gibco by Life Technologies, Carlsbad, CA, USA) in distilled water and the mixture was added to a microscope slide, pre-coated with 1% of normal melting point agarose in distilled water, and covered with a coverslip. The slide was placed briefly to 4˚C for the agarose to achieve solidify. The slides were then immersed in lysis solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris, pH 10.0] containing freshly added 1% triton X-100 and 10% DMSO for at least 1 h at 4˚C. Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 40 min for DNA unwinding and electrophoresed in the same buffer. The conditions for electrophoresis were 30 min at 300 mA and 25 V. Following electrophoresis, the slides were neutralized with PBS, washed in distilled water and 70% ethanol and dried overnight. The gels were stained for DNA with 1 µg/mL 4',6- diamidino-2-phenylindole dihydrochloride solution in distilled water and air-dried. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were taken with Zeiss Axiolab microscope (Carl Zeiss AG, Oberkochen, Germany) and

analyzed using Comet Assay IV software (Perceptive Instruments Ltd., Suffolk, UK). All standard parameters originally provided by the Comet Assay IV were used. In addition, a set of novel parameters were tested for their ability to more precisely characterize the DNA damage by the progression of COPD: the tail moment length, extent tail moment, cell length, tail length/cell length ratio and tail migration/cell length ratio. The tail moment length shows the distance from the center of the head to the center of the tail, extent tail moment was calculated by dividing DNA % in the tail by the tail length and cell length shows the distance from the beginning of the head until the end of the tail.

4.2.3 Detection of PARP Activity

PARP activity was measured using bio-NAD^+, as previously described (Bakondi *et al.*, 2002). PBMC were cultured on poly-L-lysine-coated coverslips in RPMI medium, supplemented with 10% FCS. After 20 min incubation allowing cells to attach to slides, the medium was removed and replaced with PARP reaction buffer [56 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, pH 8.0, complemented with 0.01% digitonin and 250 μM biotinylated nicotinamide adenine dinucleotide (NAD^+) immediately before use]. After 60-min incubation at 37˚C, the cells were fixed at -20˚C in 95% ethanol followed by 10% trichloroacetic acid. The coverslips were rinsed in PBS and endogenous peroxidase was blocked by 0.5% H_2O_2/ methanol for 15 min. After rinses with PBS, the coverslips were blocked in 1% bovine serum albumin/PBS for 30 min, followed by rinses in PBS–Triton X-100 (0.1%). Incorporated biotin was detected by streptavidin–peroxidase (diluted 1:100 in PBS–Triton X-100) for 30 min. Coverslips were washed with PBS–Triton X-100 and color was developed with cobalt-enhanced nickel-3,3'- diaminobenzidine substrate. The coverslips were mounted with glycerol on slides and viewed under a Zeiss Axiolab microscope (Carl Zeiss). The pictures were taken with a Zeiss Axiocam digital camera (Carl Zeiss) and analyzed using AlphaView Software (ProteinSimple, Santa Clara, CA, USA).

4.2.4 RNA extraction from PBMC and cDNA synthesis

RNA was extracted from the mononuclear cells with the Trizol method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and stored at -80°C until cDNA synthesis. cDNA was synthesized with reverse transcriptase reaction, from total RNA (250 ng) using the SuperScript III enzyme (Invitrogen) according to the manufacturers' instructions. The conditions for reverse transcriptase reaction were: incubation at 65°C for 5 min, after that incubation at 0°C for 1 min and then synthesis at 50°C for 90 min, followed by inactivation at 75°C for 15 min; cDNA was stored at –80°C until qRT-PCR.

4.2.5 Measurement of mRNA expression

The gene expression levels were detected using the TaqMan-qRT-PCR method (ABI Prism 7900HT Sequence Detection System, Applied Biosystems by Life Technologies, Waltham, MA, USA) using Hs00166575 for SOD1, Hs00829989_gH for GPx, Hs00609286_m1 for GSS, Hs00167317_m1 for GSR, Hs00155249_m1 for GCLC, Hs00157694_m1 for GCLM, Hs00175218 for DPP4, Hs00167536_m1 for LTA_4H, Hs00167536_m1 for 5-LO, Hs00153133_m1 for COX-2, Hs00242302_m1 for PARP-1, Hs00231032_m1 for HDAC2, and hypoxanthine phosphoribosyl- transferase-1 (HPRT-1) as a house keeper (both from Applied Biosystems by Life Technologies). All reactions were carried out in quadruplicates. The comparative Ct method (ΔCt value) was used for quantification of mRNA, where the amount of target transcript was normalized to the level of endogenous HPRT-1.

4.2.6 Statistical analysis

In the final study dataset, missing enzyme expression level data (12 variables) was populated by multiple imputation with random numbers within the range of measured data leading to 5 new datasets. In all studies (I–III), the equality of the baseline data across the study groups was evaluated with Kruskal-Wallis test in numeric variables and Pearson's chi-square test in nominal variables. All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS, version 17.0, SPSS Inc., Chicago, IL, USA) software. *p*-values below 0.05 were considered to designate statistical significance.

4.2.6.1 Statistical analysis of the Comet Assay and PARP activity data (I)

Associations of the Comet Assay parameters, as well as PARP activity, with the presumably increasing degree of impairment from healthy never-smokers through non-obstructive smokers, stable COPD patients of GOLD stages I-IV to patients with AE-COPD, were assessed with ordinal logistic regression analysis with calculating the odds ratios (OR) and their 95% confidence intervals (95% CI). Because of the very high collinearity between the Comet Assay indices due to their derivation, the comet parameters were not simultaneously included into the multivariate model. Instead, the ability of each of the Comet Assay parameters to indicate the increasing degree of DNA impairment and PARP activity throughout the study groups was assessed separately, though each of them was adjusted for age, gender, body mass index (BMI), lung function (FEV_1 % predicted), pack-years of smoking and status of a current smoker.

4.2.6.2 Statistical analysis of the mRNA expression data (II)

Logistic regression analysis was used to determine whether the transcription level of enzymes associated with COPD presence, exacerbations or smoking history. General linear model that was used to predict continuous variables based on the transcription level of enzymes. Multinomial logistic regression was performed in order to identify whether any of the genes is differentially expressed between the GOLD A-D or 1–4 stratifications. Partial least squares discriminant analysis (PLS-DA) was used to address the grouping ability of the enzymes. All regression analyses were adjusted to age, gender, BMI, smoking habits, presence of current exacerbation, and lung function data. PLS-DA is a widely-used multivariate machine learning algorithm used for classifying and interpreting metabolomics data, especially applicable when the number of metabolites (independent variables) is much larger than the number of data points (samples or study individuals) (Mendez *et al.*, 2020). In this context, the advantage of PLS-DA includes handling data sets with collinear variables along with noisy and/or missing values (Mendez *et al.*, 2020). For collinearity diagnostics in the general linear model, as well as in the multinomial logistic regression analysis, calculation of the variance inflation factor (VIF) was used and explanatory variables with a VIF greater than 10 as indicative of significant multicollinearity were removed. All statistical analyses were performed with the R version 4.2.1 software (R Foundation for Statistical Computing, Vienna, Austria). Additionally to the basic R functions, the following packages were used: mitools, broom, dplyr, effects, ggplot2, ggstance, grDevices, mixOmics and nnet. Regression models were in the form of $Y_i = \beta_{i1} \times BMI + \beta_{i2} \times Age + \beta_{i3} \times gender + \beta_{i4} \times enzyme + e_i$. The described models were applied to the study groups in two settings: all participants and COPD patients only. Unless stated otherwise, the results are from the analyses involving all participants. All regression analyses were adjusted to participants' age, gender and BMI. Results obtained with logistic regression results are presented as OR and its 95% confidence intervals (95% CI). All other results are presented as regression coefficients and their 95% CI.

4.2.6.3 Statistical analysis of the effect of the GSH analogues data (I and III)

The effect of the GSH analogues on the expression of the enzymes was analyzed with linear mixed model for repeated measures using IBM SPSS Statistics (version 29, Chicago, IL, USA). The data were adjusted for age, gender, pack-years of smoking, the GOLD A-D group (*GOLD*, 2022), and COPD exacerbation status. Mann-Whitney U-Test was performed to determine the difference between the COPD patients and non-COPD individuals for the effect of UPF17 on PARP-1 mRNA expression level.

5. RESULTS

5.1 Study population

Out of the 156 subjects, different subpopulations were used for different studies (Tables 10–12). For the DNA damage and PARP activity study (I), 43 subjects and for the mRNA expression and UPF effect studies (II and III, respectively), 116 subjects were included. A total of 24 patients were excluded from the whole study due to various reasons, ranging from missing data and missing lung function parameters to incorrectly handled samples.

5.2 DNA Damage and PARP Activity (I)

GOLD severity of airflow obstruction categorization according to the post-bronchodilator FEV_1 was used (*GOLD*, 2023), except AE-COPD patients were grouped separately independent on their airflow obstruction severity (Table 10). Amongst the patients, who were chosen for DNA damage and PARP activity analysis, a notable variability in smoking history was observed amongst patients with AE-COPD, due to one patient reporting an extensive tobacco use of 116 pack-years.

Table 10. Characteristics of the individuals involved in the measurement of DNA damage by Comet Assay IV and poly(ADP-ribose) polymerase (PARP) activity in peripheral blood mononuclear cells. Patients had been diagnosed as having stable chronic obstructive pulmonary disease (COPD) grouped by the airflow limitation severity according to the Global Initiative for COPD consensus document 2023 (GOLD 2023) (*GOLD*, 2023).

			Airflow limitation severity					
Characteristics	**Non-smoking controls (n = 7)**	**Non-obstructive smokers (n = 7)**	**GOLD 1 (n = 5)**	**GOLD 2 (n = 6)**	**GOLD 3 (n = 7)**	**GOLD 4 (n = 5)**	**AE-COPD (n = 6)**	***p*-values****
Age (years)	63.3 ± 2.4	58.4 ± 5.2	64.3 ± 3.6	63.4 ± 4.1	70.2 ± 4.5	64.5 ± 6.3	73.4 ± 3.0	0.16
Male	7 (100%)	7 (100%)	4 (80%)	5 (83%)	7 (100%)	5 (100%)	5 (83%)	0.71
BMI ($kg \times m^{-2}$)	32.3 ± 2.1	28.7 ± 1.5	24.8 ± 2.3	22.0 ± 1.9	23.6 ± 1.0	27.4 ± 2.8	22.6 ± 0.8	0.017
Smoking (pack-years)	-	27.8 ± 7.7	31.1 ± 3.9	34.5 ± 6.9	32.7 ± 4.3	36.1 ± 9.1	53.5 ± 13.5	0.70
Current smoker	-	5 (71%)	3 (60%)	4 (67%)	0 (0%)	3 (60%)	4 (67%)	0.14
PEF % predicted	98.1 ± 9.5	94.8 ± 5.5	81.1 ± 9.9	61.7 ± 4.9	32.7 ± 5.1	24.8 ± 3.1	24.5 ± 1.7	< 0.001
FEV_1 % predicted	95.9 ± 4.7	96.4 ± 4.5	81.8 ± 4.7	86.3 ± 3.4	36.1 ± 3.0	23.8 ± 2.1	30.2 ± 5.6	< 0.001
Absolute decline in FEV_1 % over years ($\%\times year^{-1}$)*	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	1.6 ± 0.2	2.1 ± 0.3	1.5 ± 0.2	< 0.001
FVC % predicted	90.1 ± 4.4	94.4 ± 2.8	102.2 ± 5.9	82.7 ± 4.8	57.4 ± 4.4	40.2 ± 3.0	49.3 ± 7.5	< 0.001
FEV_1 % of FVC	83.5 ± 2.2	80.5 ± 2.8	63.8 ± 2.1	66.8 ± 1.1	52.0 ± 3.6	49.2 ± 6.1	49.4 ± 2.3	< 0.001

Data are presented as mean ± SEM or n (%).

* Annual change in FEV_1 % predicted after the age of 25, assuming that FEV_1 % predicted was 100% at the age of 25 years.

**To test the equality of the data across the study groups, Kruskal-Wallis test and Pearson's chi-square test was applied for numeric and nominal variables, respectively. In case of smoking-related variables, non-smoking controls were omitted from the analysis. In case of current acute exacerbation of COPD, non-smoking control individuals and non-obstructive controls were omitted from the analysis. BMI – body mass index, FEV_1 – forced expiratory volume, FVC – forced vital capacity, PEF – peak expiratory flow.

After adjusting for variables such as age, gender, BMI, smoking history (pack-years), current smoking status and FEV_1 % predicted, of the Comet IV standard parameters, olive tail moment, DNA % in the tail, head length, tail length and tail migration increased significantly (OR > 1.0, $p < 0.05$). Conversely, DNA % in the head, as well as mean grey level and total intensity decreased significantly (OR > 1.0, $p < 0.05$). These changes were observed progressively from healthy control individuals through non-obstructive smokers, patients with increasing stages of stable COPD (1–4), and were most pronounced in individuals with AE-COPD. Tail moment length, extent tail moment, and cell length increased significantly, indicating a progression to the next group in our study (OR > 1.0, $p < 0.05$). From the novel parameters, tail length/cell length and tail migration/cell length ratios had the highest OR. One unit increase in tail length/cell length ratio and tail migration/cell length ratio was associated with a 7.88-fold (95% CI 4.26 – 14.57, $p < 0.001$) and a 3.91-fold (95% CI 2.69 – 5.66, $p < 0.001$) higher probability, respectively, of progressing into the next group of individuals (Figure 7).

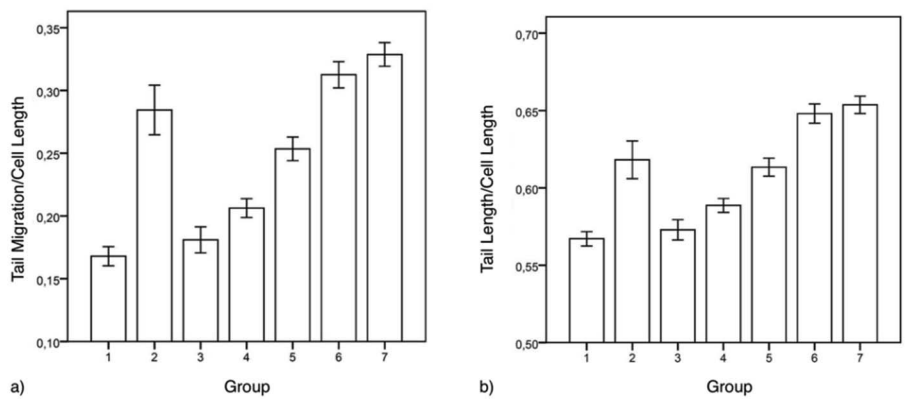

Figure 7. The level of damage to DNA in peripheral blood mononuclear cells analyzed by ordinal logistic regression analysis (I). a) Tail migration/cell length ratio measured with Comet Assay IV. b) Tail length/cell length ratio measured with Comet Assay IV. The study groups are designated as follows: (1) non-smoking, non-obstructive control individuals; (2) non-obstructive smokers; (3) patients with mild chronic obstructive pulmonary disease (COPD); (4) patients with moderate COPD; (5) patients with severe COPD; (6) patients with very severe COPD; (7) patients with COPD exacerbation requiring hospitalization. Presented as mean ± SEM.

PARP activity was a significant factor associated with escalation into the next airway obstruction 1–4 group in our study (OR = 1.014, 95% CI 1.006 – 1.023, $p < 0.001$) (Figure 8).

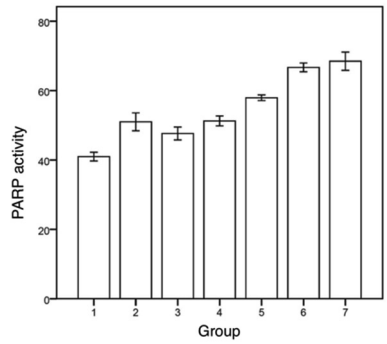

Figure 8. Poly(ADP-ribose) polymerase (PARP) activity in peripheral blood mononuclear cells using biotinylated-NAD incorporation assay analyzed by ordinal logistic regression analysis (I). (1) non-smoking, non-obstructive control individuals; (2) non-obstructive smokers; (3) patients with mild chronic obstructive pulmonary disease (COPD); (4) patients with moderate COPD; (5) patients with severe COPD; (6) patients with very severe COPD; (7) patients with COPD exacerbation requiring hospitalization. Presented as mean ± SEM.

5.3 mRNA expression (II)

For measurement of the mRNA expression of enzymes implicated in systemic inflammation and GSH metabolism, a total of 116 subjects were included into the study. The relationship of the enzymes was explored between airflow limitation COPD GOLD 1–4 (*GOLD*, 2011) (Table 11) and COPD GOLD A-D 2022 (*GOLD*, 2023) (Table 12), as well as the presence of AE-COPD (Lange *et al.*, 2012) (Table 13).

Table 11. Characteristics of the individuals included to the measurement of the mRNA expression of enzymes implicated in systemic inflammation and glutathione (GSH) metabolism in peripheral blood mononuclear cells. Patients had been diagnosed as having stable chronic obstructive pulmonary disease (COPD) grouped by the airflow limitation severity according to the Global Initiative for COPD consensus document 2023 (GOLD 2023) (*GOLD*, 2023).

	Non-smoking controls	**Non-obstructive smokers**	**Severity of airflow limitation**				
Characteristics	**(n = 10)**	**(n = 15)**	**GOLD 1 (n = 4)**	**GOLD 2 (n = 20)**	**GOLD 3 (n = 34)**	**GOLD 4 (n = 33)**	***p*-values****
Age (years)	64.0 ± 3.4	61.1 ± 2.9	76.8 ± 3.0	67.9 ± 2.1	70.1 ± 2.0	70.0 ± 2.0	0.029
Male	7 (70%)	9 (60%)	4 (100%)	19 (95%)	32 (94%)	30 (91%)	0.010
BMI ($kg \times m^{-2}$)	25.7 ± 1.6	27.0 ± 1.6	23.4 ± 1.7	23.8 ± 1.1	25.5 ± 0.7	23.2 ± 0.9	0.093
Smoking (pack-years)	-	35.7 ± 2.6	56.3 ± 10.3	41.7 ± 3.1	38.2 ± 3.5	42.8 ± 4.4	0.236
Current smoker	-	10 (67%)	2 (50%)	13 (65%)	15 (44%)	16 (48%)	0.471
Smoking cessation amongst ex-smokers (years ago)	-	6.8 ± 3.5	5.0 ± 1.0	8.0 ± 3.8	13.1 ± 2.2	10.4 ± 2.5	0.093
Current exacerbation	-	-	0 (0%)	4 (20%)	10 (29%)	11 (33%)	0.439
PEF % predicted	99.5 ± 6.8	80.6 ± 4.1	71.0 ± 14.4	51.4 ± 3.6	32.6 ± 1.5	22.4 ± 0.9	< 0.001
FEV_1 % predicted	97.6 ± 4.5	82 ± 4.5	94.5 ± 9.0	60.7 ± 2.1	38.7 ± 1.1	23.4 ± 0.8	< 0.001
Absolute decline in FEV_1 % over years ($\%\times year^{-1}$)*	0.1 ± 0.1	0.6 ± 0.1	0.1 ± 0.2	1.0 ± 0.1	1.7 ± 0.3	1.9 ± 0.1	< 0.001
FVC % predicted	96.8 ± 4.7	79.9 ± 4.7	110.3 ± 7.7	80.0 ± 2.6	57.7 ± 1.7	38.0 ± 1.4	< 0.001
FEV_1 % of FVC	81.9 ± 1.2	82.3 ± 1.6	64.7 ± 2.5	60.7 ± 1.3	54.6 ± 1.5	51.8 ± 2.2	< 0.001

Data are presented as mean ± SEM or n (%).

*Annual change in FEV_1 % predicted after the age of 25, assuming that FEV_1 % predicted was 100% at the age of 25 years.

**To test the equality of the data across the study groups, Kruskal-Wallis test and Pearson's chi-square test was applied for numeric and nominal variables, respectively. In case of smoking-related variables, non-smoking controls were omitted from the analysis. In case of current acute exacerbation of COPD, non-smoking control individuals and non-obstructive controls were omitted from the analysis. BMI – body mass index, FEV_1 – forced expiratory volume, FVC – forced vital capacity, PEF – peak expiratory flow.

Table 12. Characteristics of the individuals included to the measurement of the mRNA expression of enzymes implicated in systemic inflammation and glutathione (GSH) metabolism in peripheral blood mononuclear cells. Patients had been diagnosed with exacerbation of chronic obstructive pulmonary disease (COPD) and patients diagnosed as having stable COPD of all categories and degrees of severity of airflow obstruction in accordance with the Global Initiative for COPD (GOLD) consensus document 2022 (GOLD 2022) (*GOLD*, 2023).

Characteristics	Non-smoking controls (n = 10)	Non-obstructive smokers (n = 15)	COPD				*p*-values**
			GOLD A (n = 25)	GOLD B (n = 30)	GOLD C (n = 4)	GOLD D (n = 32)	
Age (years)	64.0 ± 3.4	61.1 ± 2.9	69.0 ± 2.5	67.1 ± 2.0	80.0 ± 2.7	71.7 ± 1.6	0.008
Male	7 (70%)	9 (60%)	21 (84%)	29 (97%)	4 (100%)	31 (97%)	0.004
BMI ($kg \times m^{-2}$)	25.7 ± 1.6	27.0 ± 1.6	25.8 ± 1.0	23.4 ± 0.8	22.1 ± 0.6	24.0 ± 0.9	0.138
Smoking (pack-years)	-	35.7 ± 2.6	40.4 ± 3.8	39.5 ± 3.6	45.0 ± 5.0	43.5 ± 4.4	0.799
Current Smoker	-	10 (67%)	17 (68%)	16 (53%)	0 (0%)	13 (41%)	0.038
Smoking cessation amongst ex-smokers (years ago)	-	6.8 ± 3.5	12.1 ± 3.4	9.0 ± 2.5	7.6 ± 3.1	7.9 ± 1.9	0.061
FEV_1 % predicted	97.6 ± 4.5	82 ± 4.5	53.6 ± 4.7	41.1 ± 3.4	34.8 ± 3.0	30.0 ± 1.8	< 0.001
Absolute decline in FEV_1 % over years ($\% \times year^{-1}$)*	0.1 ± 0.1	0.6 ± 0.1	1.4 ± 0.4	1.6 ± 0.2	1.2 ± 0.1	1.6 ± 0.1	< 0.001
Current exacerbation	-	-	2 (8%)	7 (23%)	3 (75%)	13 (41%)	0.006
PEF % predicted	99.5 ± 6.8	80.6 ± 4.1	43.7 ± 4.3	35.1 ± 2.9	27.8 ± 5.5	27.3 ± 1.6	< 0.001
FVC % predicted	96.8 ± 4.7	79.9 ± 4.7	70.8 ± 4.8	59.2 ± 3.8	53.8 ± 7.8	46.7 ± 2.7	< 0.001
FEV_1 % of FVC	81.9 ± 1.2	82.3 ± 1.6	62.7 ± 1.9	60.9 ± 1.6	55.8 ± 2.6	52.5 ± 1.5	< 0.001
K_{CO} ($mmol \times min^{-1} \times kPa^{-1} \times L^{-1}$)	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.020
K_{CO} % predicted	93.4 ± 4.8	80.3 ± 8.9	76.4 ± 7.5	56.6 ± 5.6	48.8 ± 0.0	40.5 ± 9.5	0.005
DL_{CO} ($mmol \times min^{-1} \times kPa^{-1}$)	6.3 ± 0.6	5.6 ± 0.8	4.8 ± 0.6	3.5 ± 0.4	2.8 ± 0.0	2.8 ± 0.6	0.014

Characteristics	Non-smoking controls (n = 10)	Non-obstructive smokers (n = 15)	COPD				*p*-values**
			GOLD A (n = 25)	GOLD B (n = 30)	GOLD C (n = 4)	GOLD D (n = 32)	
DL_{CO} % predicted	77.3 ± 9.0	61.6 ± 7.4	54.4 ± 5.3	38.7 ± 3.8	30 ± 0.0	31.3 ± 6.1	0.002
TLC (L)	5.3 ± 0.3	4.82 ± 0.3	4.8 ± 0.3	4.6 ± 0.2	4.8 ± 0.0	4.6 ± 0.3	0.716
TLC % predicted	84.9 ± 5.8	79.2 ± 3.4	73.9 ± 2.1	68.0 ±2.5	63.4 ± 0.0	67.4 ± 3.2	0.021

Data are presented as mean ± SEM or n (%).

* Annual change in FEV_1 % predicted after the age of 25, assuming that FEV_1 % predicted was 100% at the age of 25 years.

**To test the equality of the data across the study groups, Kruskal-Wallis test and Pearson's chi-square test was applied for numeric and nominal variables, respectively. In case of smoking-related variables, non-smoking controls were omitted from the analysis. In case of current acute exacerbation of COPD, non-smoking control individuals and non-obstructive controls were omitted from the analysis. BMI – body mass index, D_{LCO} – diffusing capacity of the lungs for carbon monoxide, FEV_1 – forced expiratory volume, FVC – forced vital capacity, K_{CO} – carbon monoxide transfer coefficient, PEF – peak expiratory flow, TLC – total lung capacity.

Table 13. Characteristics of the individuals involved in the measurement of enzymes involved in systemic inflammation and glutathione (GSH) metabolism mRNA expression in peripheral blood mononuclear cells. Patients diagnosed as having chronic obstructive pulmonary disease (COPD) divided into 3 groups, patients with forced expiratory flow (FEV_1) < 50% predicted and fewer than two exacerbations in the previous year, patients with $FEV_1 \geq 50\%$ predicted and two or more exacerbations in the previous year, and patients with FEV_1 <50% predicted and two or more exacerbations in the previous year according to the Global Initiative for COPD consensus document 2023 (GOLD 2023) (*GOLD*, 2023).

	CD-123			
Characteristics	**1 (n = 33)**	**2 (n = 2)**	**3 (n = 34)**	***p*-values**[**]
Age (years)	67.1 ± 2.2	72.0 ± 0.2	70.1 ± 2.0	0.222
Male	29 (88%)	2 (100%)	33 (97%)	0.310
BMI ($kg \times m^{-2}$)	25.4 ± 0.9	30.2 ± 5.2	23.4 ± 0.7	0.052
Smoking (pack-years)	37.6 ± 3.7	52.0 ± 2.0	43.2 ± 4.2	0.353
Current smoker	19 (58%)	1 (50%)	12 (35%)	0.178
Smoking cessation amongst ex-smokers (years ago)	11.4 ± 2.5	4.0 ± 0.0	12.2 ± 2.3	0.322
PEF % predicted	29.1 ± 1.7	48.5 ± 2.5	26.1 ± 1.4	0.039
FEV_1 % predicted	32.8 ± 1.7	50.5 ± 0.5	29.6 ± 1.5	0.025
Absolute decline in FEV_1 % over years ($\%\times year^{-1}$)[*]	2.0 ± 0.3	1.1 ± 0.0	1.6 ± 0.1	0.107
FVC % predicted	49.9 ± 2.2	71.0 ± 7.0	46.1 ± 2.5	0.064
FEV_1 % of FVC	53.4 ± 1.8	56.9 ± 4.0	53.0 ± 2.0	0.745

Data are presented as mean ± SEM or n (%).

[*] Annual change in FEV_1 % predicted after the age of 25, assuming that FEV_1 % predicted was 100% at the age of 25 years.

[**]To test the equality of the data across the study groups, Kruskal-Wallis test and Pearson's chi-square test was applied for numeric and nominal variables, respectively. BMI – body mass index, FEV_1 – forced expiratory volume, FVC – forced vital capacity, PEF – peak expiratory flow.

In contrast to stable COPD, ongoing AE-COPD showed association with increased expression of PARP-1 (OR = 27.3; 95% CI: 1.2 – 648.5, $p = 0.042$). A significantly reduced expression of GPx was observed in individuals with a history of smoking, when compared to those, who had never smoked (OR = 0.994, 95% CI: 0.9887 – 0.9997, $p = 0.038$). Versus never-smokers and ex-smokers together, current smoking significantly decreased the expression of GSR (OR = 1.031, 95% CI: 1.001 – 1.062, $p = 0.044$).

The longer the time from smoking cessation, the lower the expression levels of GPx ($\beta = -0.02$; 95% CI: -0.037 – -0.004, $p = 0.013$) (Figure 9a) and GSR ($\beta = -0.27$; 95% CI: -0.505 – -0.033, $p = 0.026$) (Figure 9b) were. Conversely, individuals with higher numbers of pack-years showed significantly higher expression of GPx ($\beta = 0.02$; 95% CI: 0.002 – 0.038, $p = 0.03$) (Figure 9c).

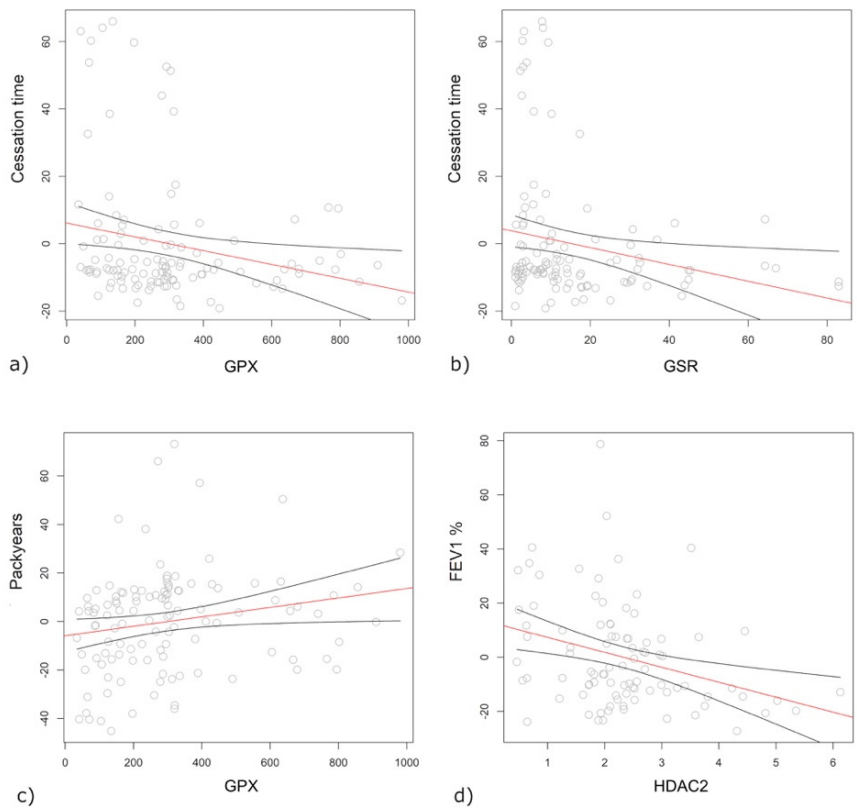

Figure 9. The transcription levels of enzymes associated with lung function parameters analyzed by general linear model (II). Residual errors after correction to age, gender and body-mass index (BMI) vs. the mRNA expression levels of the enzymes and estimated regression line with 95% confidence intervals are shown. a) Time from smoking cessation (months) vs. glutathione peroxidase (GPx) b) Time from smoking cessation (months) vs. glutathione reductase (GSR) c) Number of smoking pack-years vs. GPx d) Forced expiratory volume in one second (FEV_1) vs. histone deacetylase 2 (HDAC2).

Among COPD patients, lower lung function parameters, FEV_1 % predicted (β = -5.42; 95% CI: -8.854 – -1.977, p = 0.002) (Figure 9d), FEV_1 decline over years (β = 0.2; 95% CI: 0.042 – 0.359, p = 0.013) (Figure 10a), PEF % predicted (β = -3.73; 95% CI: -6.724 – -0.731, p = 0.015), and FVC % predicted (β = -6.09; 95% CI: -10.124 – -2.063, p = 0.003) were significantly associated with higher HDAC2 levels. An inverse relationship was observed between FEV_1/FVC % predicted and SOD1 expression levels (β = -0.13; 95% CI: -0.225 – -0.036, p = 0.007) (Figure 10b).

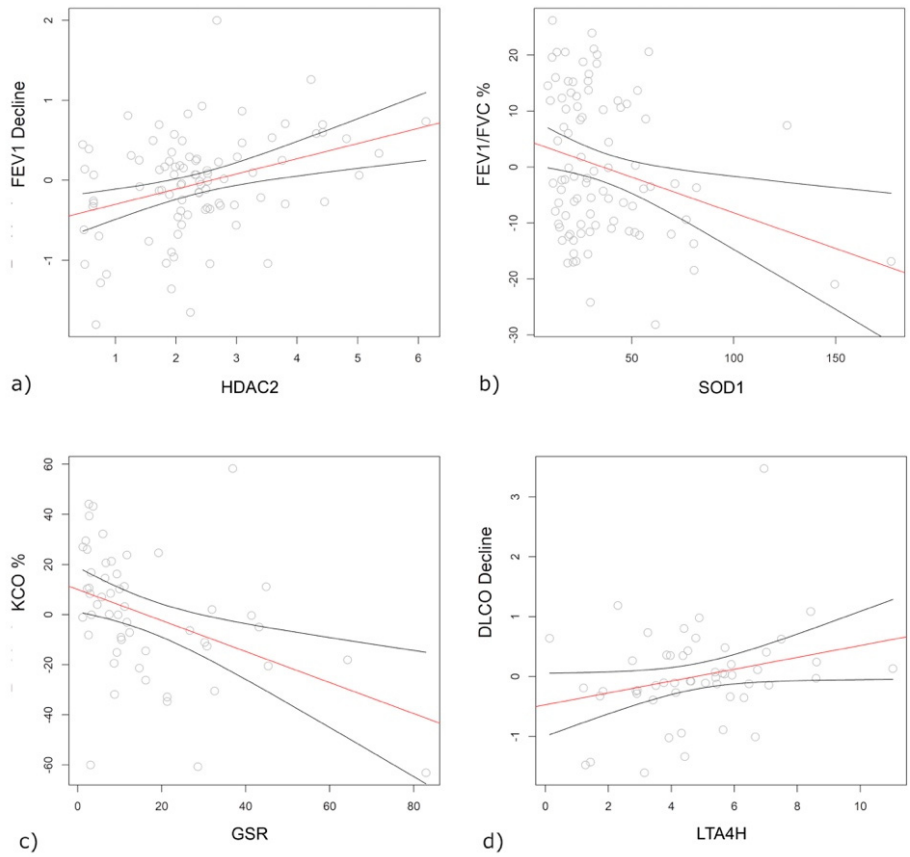

Figure 10. The transcription levels of enzymes associated with lung function parameters analyzed by general linear model (II). Residual errors after correction to age, gender and body-mass index (BMI) vs. the mRNA expression levels of the enzymes and estimated regression line with 95% confidence intervals are shown. a) Forced expiratory volume in one second (FEV_1) decline over the years vs. histone deacetylase 2 (HDAC2); b) FEV_1/forced vital capacity (FVC) % vs. superoxide dismutase (SOD1); c) carbon monoxide transfer coefficient (K_{CO}) % vs glutathione reductase (GSR); d) Diffusing capacity of carbon monoxide (D_{LCO}) decline vs. leukotriene A_4 hydrolase (LTA_4H).

Among the transfer test parameters, K_{CO} % predicted, but not K_{CO} itself, showed a significant decrease along with the increase in GSR expression (β = -0.642; 95% CI: -1.051 – -0.232, p = 0.002) (Figure 10c). Reduced levels of LTA_4H expression correlated with a decrease in DL_{CO} (β = 0.103, 95% CI: 0.001 – 0.205, p = 0.048) (Figure 10d).

The expression of HDAC2 was reduced amongst patients with COPD GOLD airflow obstruction severity grades 1–3, but increased amongst individuals with more severe obstruction (GOLD airflow obstruction severity grade 4). PARP-1 levels were elevated in GOLD grades 1–3 and GCLM was higher in GOLD grades 1–2 and 4, but GCLC levels were reduced in GOLD grades 3–4 and DPP4 was lower in GOLD grade 4, compared to non-obstructive controls regardless of their smoking history (Figure 11a). If non-obstructive smokers were categorized as a separate class, the expression of PARP-1, GSR, GCLM and GCLC was different between COPD GOLD grades 1–4 and non-obstructive non-smokers, where PARP-1 was upregulated in cases of milder stages of airflow obstruction (GOLD airflow obstruction severity grades 1–3), while it was downregulated in patients with more severe obstruction (GOLD airflow obstruction severity grade 4). DPP4 and LTA_4H levels were lower in COPD GOLD grade 4 than in non-obstructive non-smokers. In comparison to non-obstructive non-smokers, the expression of HDAC2 was decreased amongst COPD GOLD grades 1–2 and upregulated in GOLD 4. Non-obstructive non-smokers and non-obstructive smokers showed differences solely in the expression levels of GSR and LTA_4H, with smokers exhibiting higher levels of GSR and lower levels of LTA_4H (Figure 11b).

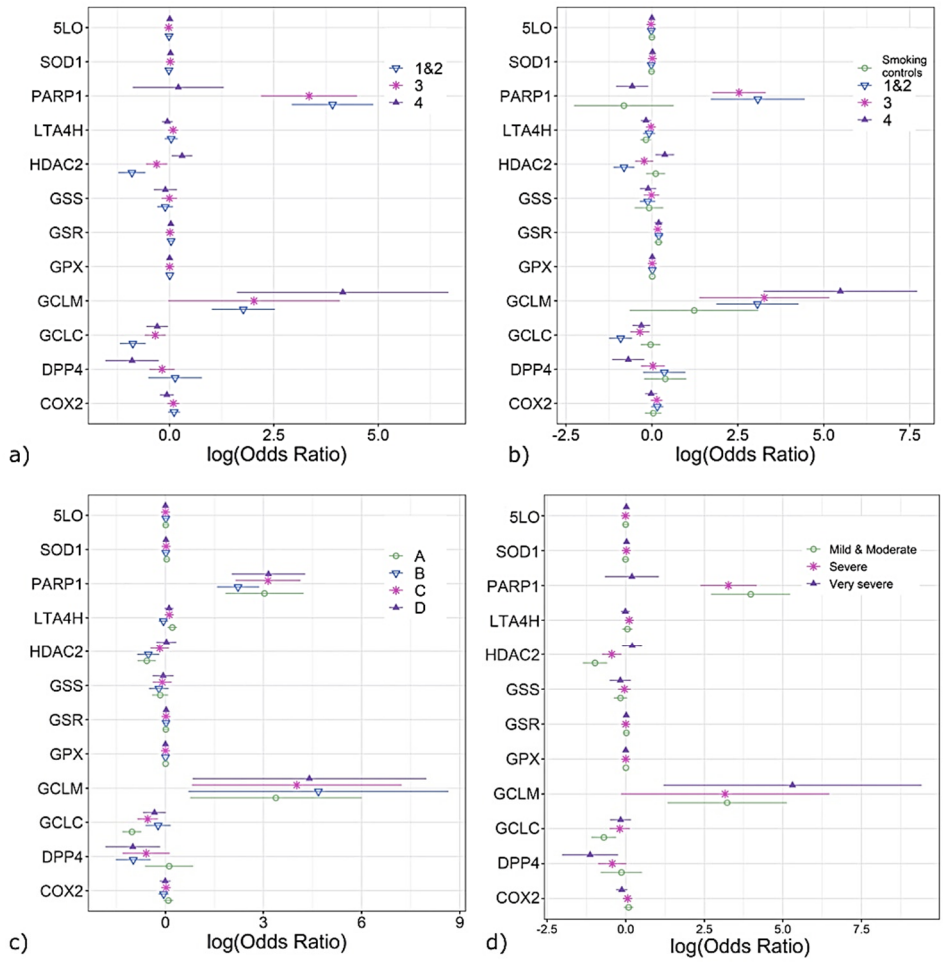

Figure 11. Differences in the levels of expression of mRNA of enzymes involved in glutathione (GSH) metabolism and inflammation analyzed by multinominal logistic regression (II). Natural logarithms of odds ratios vs. comparators with 95% confidence intervals for each enzyme are shown. The data were adjusted to age, gender and body-mass index (BMI). a) Patients with chronic obstructive pulmonary disease (COPD) by airflow limitation severity according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification 1–4 (*GOLD*, 2023) compared to all non-obstructive controls irrespective of their smoking history. b) Non-obstructive smokers and patients with COPD by airflow limitation severity according to GOLD classification 1–4 (*GOLD*, 2023) compared to non-obstructive non-smokers. c) Patients with COPD according to the GOLD classes A-D (*GOLD*, 2023) compared to pooled non-obstructive controls irrespective of their smoking history. d) Patients with a history of at least two moderate or at least one severe exacerbation within the last year compared to all individuals, who have not experienced an exacerbation (non-obstructive non-smokers, non-obstructive smokers and patients with COPD by airflow limitation severity according to GOLD classification 1–4 (*GOLD*, 2023) without an acute exacerbation of COPD).

When the patients were categorized based on the GOLD A-D classification (*GOLD*, 2022), the four COPD groups exhibited similar differences in expression when compared to non-obstructive individuals, regardless of their smoking history. Specifically, when compared to non-obstructive individuals, the levels of PARP-1 and GCLM were elevated across all GOLD groups A-D, but the increase in expression of LTA_4H was limited to GOLD group A. Distinctly, the HDAC2 levels were reduced amongst GOLD groups A and B, DPP4 levels amongst GOLD groups B and D, and GCLC amongst GOLD groups A and C (Figure 11c). Using the A-D stratification (*GOLD*, 2022), the results were independent of whether the comparator group included only non-obstructive non-smokers or a combination of non-obstructive non-smokers and non-obstructive smokers.

In patients with AE-COPD history of ≥ 2 moderate or ≥ 1 severe exacerbation within the last year compared to patients without AE-COPD within the last year and non-obstructive controls irrespective of their smoking history, there was a notable increase in PARP-1 expression across GOLD grades 1–3 of airflow obstruction. This increase was also observed in GCLM in GOLD grades 1–2 and 4. Conversely, the expression of HDAC2 was decreased in GOLD grades 1–3, while that of DDP4 was reduced in GOLD grade 4 and GCLC in GOLD grades 1–2 (Figure 11d). HDAC2 exhibited a trend towards increase in its expression amongst patients with a history of ≥ 2 moderate or ≥ 1 severe AE-COPD within the past year.

Investigating the enzymes' capacity to group COPD patients based on the severity of the disease did not uncover any significant classifying properties amongst any combination of the enzymes studied. When PARP-1 and HDAC2 were analyzed together, they exhibited a tendency to be associated with the presence of AE-COPD.

5.4 UPF1 and UPF17 influence on mRNA expression (I and III)

To study the effect of the GSH analogues on the expression of the enzymes, a total of 116 subjects were recruited and COPD GOLD A-D 2022 classification was used (*GOLD*, 2023) (Table 8).

Both, UPF1 and UPF17 notably elevated HDAC2 expression amongst all participants ($p = 0.001$) (Figure 12a, left panel) (III). However, UPF17 reduced the expression of HDAC2 in patients with ≥ 2 moderate or ≥ 1 severe AE-COPD within the last year ($p = 0.01$). Among the individuals, who had never smoked, the effect of UPF1 was the opposite between males and females, with a decrease in males, while increasing in females ($p = 0.009$) (Figure 12a, right panel) (III).

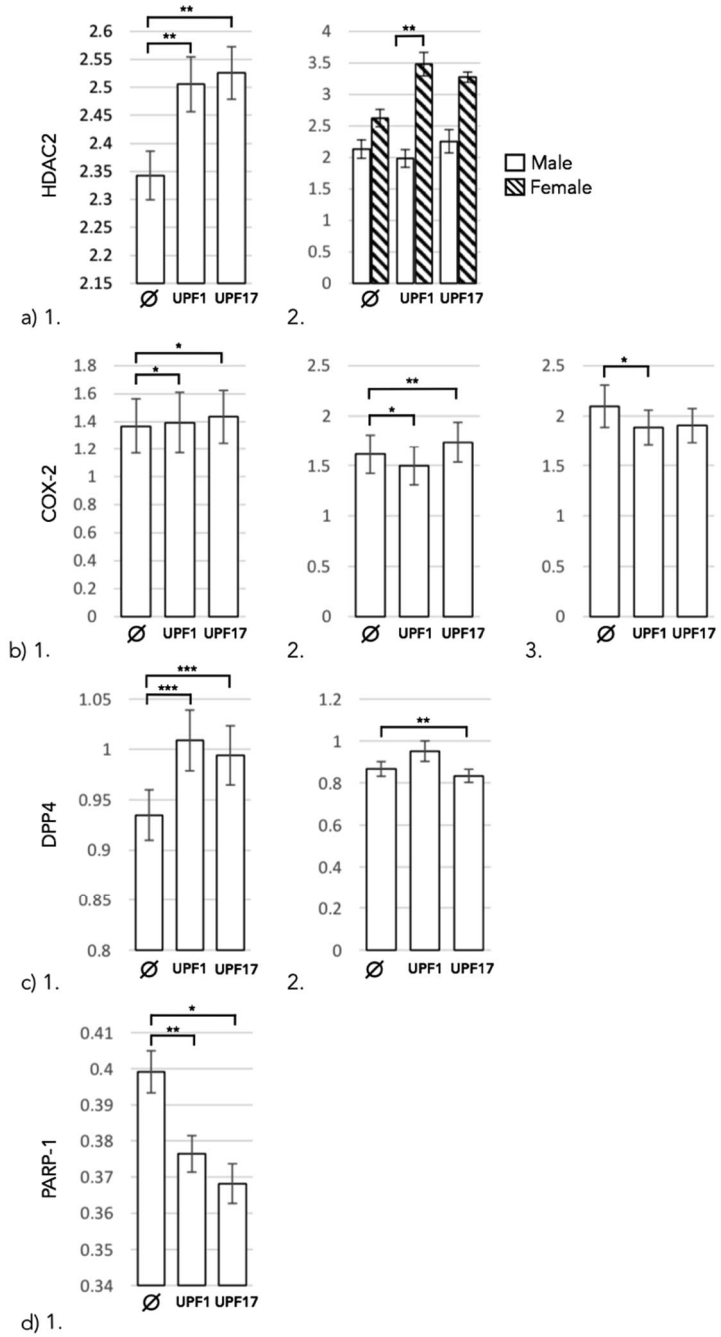

Figure 12. The effect of glutathione (GSH) analogues UPF1 and UPF17 on the levels of expression of the mRNA of the enzymes involved in inflammation analyzed using a linear mixed model for repeated measures (III). Data are adjusted for age, gender, and body mass index (BMI). a) UPF1 and UPF17 effect on the mRNA expression level of histone deacetylase-2 (HDAC2) (1) in all individuals and (2) in patients who had never smoked,

using both male and female subjects. b) The effect of UPF1 and UPF17 on the mRNA expression level of cyclooxygenase-2 (COX-2) (1) in patients with ongoing acute exacerbation of chronic obstructive pulmonary disease (COPD), (2) in patients with a history of at least two moderate or at least one severe exacerbation within the last year, and (3) in COPD patients who had quit smoking at least 6 months prior to this study. c) The effect of UPF1 and UPF17 on the mRNA expression level of dipeptidyl peptidase 4 (DPP4) (1) in all individuals and (2) in non-obstructive individuals and COPD patients who had quit smoking at least 6 months prior to this study. d) The effect of UPF1 and UPF17 on the mRNA expression level of poly(ADP-ribose) polymerase-1 (PARP-1) (1) in all individuals analyzed together. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. baseline (∅).

In patients experiencing AE-COPD, UPF1 and UPF17 significantly increased the expression of COX-2 ($p = 0.027$ and $p = 0.049$, respectively) (Figure 12b, left panel) (III). UPF17 increased ($p = 0.007$) but UPF1 decreased ($p = 0.018$) the expression of COX-2 amongst patients with a history of ≥ 2 moderate or ≥ 1 severe AE-COPD within the last year (Figure 12b, middle panel). Furthermore, UPF1 reduced the expression of COX-2 in COPD patients, who had quit smoking at least 6 months ago ($p = 0.049$) (Figure 12b, right panel). A comparable reduction in the expression of COX-2 was observed when analyzing both COPD patients and non-obstructive individuals who had never smoked or had quit smoking at least six months prior, together as a pooled group ($p = 0.037$) (III).

Both UPF1 and UPF17 enhanced the expression of DPP4 in all participants ($p < 0.001$ and $p < 0.001$, respectively) (Figure 12c, left panel). Conversely, UPF17 reduced the expression of DPP4 amongst ex-smokers, who had quit smoking at least 6 months ago ($p = 0.006$) (Figure 12c, right panel) (III).

UPF1 and UPF17 reduced the expression of PARP-1 amongst all participants ($p = 0.001$ and $p = 0.039$, respectively) (Figure 12d) (III). UPF17 demonstrated a more significant reduction in PARP-1 mRNA expression levels among patients with COPD compared to non-obstructive individuals, which included both non-obstructive non-smokers and non-obstructive smokers ($p = 0.040$) (Figure 13) (I).

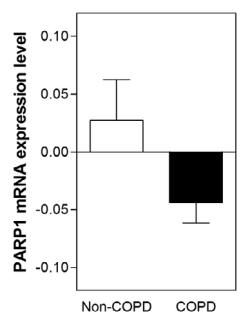

Figure 13. Mann-Whitney U-Test showing a significant down-regulation of the poly(ADP- ribose) polymerase-1 (PARP-1) mRNA expression in peripheral blood mononuclear cells (PBMC) from patients with chronic obstructive pulmonary disease (COPD) by an antioxidant tetrapeptide UPF17 in comparison with that in non-obstructive individuals ($p = 0.040$) (I).

The expression of SOD1 was influenced solely by UPF17 in a gender- and AE-COPD-dependent manner as follows (III). UPF17 reduced the expression of SOD1 in males, while increasing it in females, when all study individuals were included ($p = 0.022$) (Figure 14a, right panel). Among the current smokers, the expression of SOD1 was elevated by UPF17 amongst the patients with AE-COPD, while it was reduced in those without it ($p = 0.001$) (Figure 14a, right panel). In contrast to SOD1, the expression of GSR was solely affected by UPF1 and showed a gender-dependent effect: UPF1 elevated GSR mRNA levels in females while reducing them in males, when all study individuals were included ($p = 0.046$) (Figure 14b).

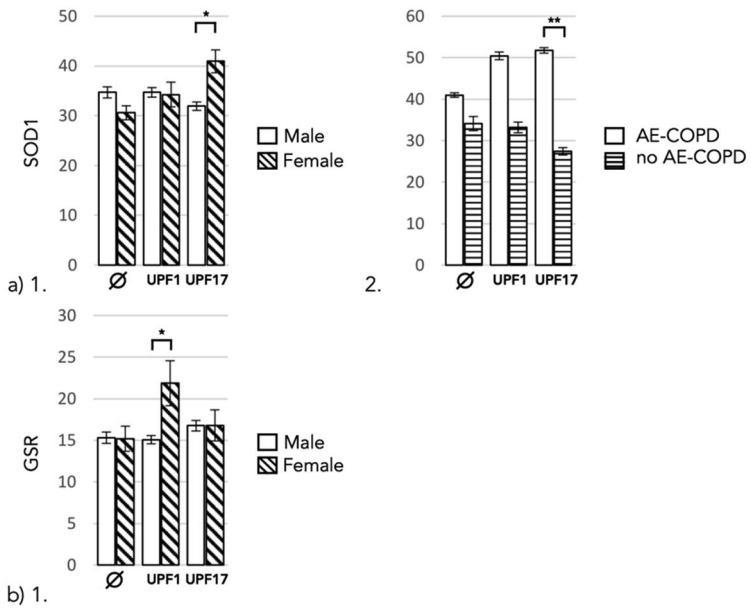

Figure 14. The effect of GSH analogues UPF1 and UPF17 on the levels of expression of the mRNA of the enzymes involved in GSH metabolism analyzed using a linear mixed model for repeated measures (III). Data are adjusted for age, gender, and body mass index (BMI). a) The effect of UPF1 and UPF17 on the mRNA expression level of superoxide dismutase-1 (SOD1) (1) with the inclusion of all individuals and comparing males and females and (2) with the inclusion of all current smokers, comparing patients with current acute exacerbation of chronic obstructive pulmonary disease (AE-COPD) and those without AE-COPD. b) The effect of UPF1 and UPF17 on the mRNA expression level of glutathione reductase (GSR) (1) with the inclusion of all individuals and comparing males and females. * $p \leq 0.05$, ** $p \leq 0.01$ vs. baseline (Ø).

Solely UPF17 was able to affect the expression of GCLC, which exhibited an increase, when all participants were analyzed together ($p = 0.012$) (III).

6. DISCUSSION

6.1 The degree of systemic DNA damage and PARP activity along with the evolution of COPD (I)

The common Comet Assay IV parameters indicative of DNA damage, such as tail length, tail migration, olive tail moment, and DNA % in the tail, demonstrated a notable meaningful incremental association with progression across the whole spectrum from healthy non-smoking individuals to those with AE-COPD (I). The variables that indicate DNA damage were adjusted for demographic and clinical parameters like age, gender, BMI, smoking habits, and lung function. These adjustments indicate that the increase in DNA damage associates with the progression of OS and inflammation from non-smoking controls up to AE-COPD, rather than being attributed solely to age, a decline in FEV_1 %, or CS alone. There are numerous studies to assess the association between the severity of COPD and DNA strand breaks. Some studies, like ours (I), include patients from several severity groups (Maluf *et al.*, 2007), whereas others have only included patients with moderate or severe COPD (Ceylan *et al.*, 2006; Lin *et al.*, 2010). Compared to our study (I), Maluf *et al*. did not detect a correlation between the increased DNA damage in PBMC and COPD severity (Maluf *et al.*, 2007). Similarly to our finds, Ceylan *et al*. showed an increased DNA damage in peripheral blood mononuclear lymphocytes in patients with COPD (Ceylan *et al.*, 2006). The major difference between the current study and those conducted by Ceylan *et al*. and Maluf *et al*. was that in our study, we employed specialized software (Comet Assay IV) to analyze a minimum of 10 different variables of the Comet Assay, moving further beyond just visual scoring of the tail intensity (I) (Ceylan *et al.*, 2006; Maluf *et al.*, 2007). Lin *et al*. and Dos Santos *et al.* used Comet Assay IV software and showed that endogenous white blood cells (Lin *et al.*, 2010) and peripheral blood lymphocytes (Dos Santos *et al.*, 2022), respectively, DNA damage in COPD patients was significantly greater than that in the healthy controls (Dos Santos *et al.*, 2022; Lin *et al.*, 2010). In our study we showed DNA injury progressing in 7 groups from non-smoking controls and non-obstructive smokers through the four COPD stages to AE-COPD (I). COPD is often referred to as a disease of accelerated lung ageing, whereas DNA damage is regarded as one type of damage that has been implicated in ageing (Aoshiba *et al.*, 2013). There was also a slight increase in DNA damage amongst non-obstructive smokers compared to non-obstructive non-smokers. Similarly, Tang *et al*. showed that CS extract-induced airway inflammation is associated with ROS overproduction, increased DNA damage, and inappropriate airway epithelial cell apoptosis (Tang *et al.*, 2023). CS exposure is the primary factor responsible for the development and progression of COPD (Punturieri *et al.*, 2009). CS-mediated DNA damage leads to cellular senescence, apoptosis, inflammation, and mutagenesis, all of which have been associated with the development of COPD (Adcock *et al.*, 2018; Barnes, 2017; Rodier *et al.*, 2009).

In addition, alongside the variables offered by the Comet Assay IV, 5 new parameters were introduced, which were shown to more effectively illustrate the connection between DNA damage and disease progression. Out of our newly introduced parameters (I), the tail length/cell length ratio and tail migration/cell length ratio were found to show the highest ORs with the development and progression of COPD up to AE-COPD. A one unit increase in tail length/cell length ratio and tail migration/cell length ratio was associated with a 7.88-fold or 3.91-fold higher probability, respectively, for the process of progressing to the next stage and therefore, these parameters can appear most suitable ones to describe the DNA damage in PBMC over the evolution of COPD. To the best of our knowledge, this is the first report to demonstrate that these parameters are the most effective indicators for monitoring the progression of COPD and to suggest the tail length/cell length ratio and the tail migration/cell length ratio as new precision tools for the autonomous evaluation of DNA damage in smokers and in patients with COPD (I).

The present study also revealed a strong relationship between the evolution of COPD and PARP activity in PBMC, indicating an intensified DNA repair occurring in PBMC of patients with higher COPD stages and provides a helpful supplement to the knowledge of COPD's systemic aspect (I). PARP activity is upregulated in response to DNA damage to maintain DNA stability, integrity, and repair (Bai, 2015). PARP activation in stable COPD patients compared to control individuals in peripheral blood lymphocytes has been previously demonstrated (Hageman *et al.*, 2003). This study showed increase in PARP activity in 7 groups from non-smoking controls and non-obstructive smokers through the four COPD stages to severe AE-COPD (I). Gender differences appear to play a significant role, as studies have found that PBMC PARP activity levels are notably lower in female compared to male subjects (Zaremba *et al.*, 2011). Additionally, studies using rodent (Mabley *et al.*, 2005) and porcine (Hauser *et al.*, 2006) models have indicated that the regulation of PARP is more pronounced in males than in females. The primary aim of PARP-1 is to maintain the genome integrity, its overactivation under extensive and persistent DNA damaging can result in extensive inflammation via interacting with various cellular proteins and transcription factors, and energy crisis due to depletion of their substrate, i.e., NAD^+, thus leading to necrosis (Ha & Snyder, 1999; Sethi *et al.*, 2017).

In addition, PARP activity was heightened amongst the non-obstructive smokers (I). Previously, *in-vitro* studies have shown that CS enhances PARP activity (Kovacs *et al.*, 2012). This suggests that although PARP is a very important player in the DNA repair, it does not represent the only pathway to prevent smokers from developing COPD.

6.2 The relationship of pro- and anti-inflammatory enzymes with COPD (II)

Our results revealed that AE-COPD was associated with PARP-1 expression, indicating an enhanced DNA repair process taking place in PBMC during AE-COPD (II). In addition, PARP-1 was highly expressed among GOLD classes of airflow obstruction 1–3, compared to non-obstructive controls. However, patients with high risk of AE-COPD (GOLD groups C and D) failed to distinguish from A and B. In patients with milder airflow obstruction, PARP-1 expression was higher in patients with a history of AE-COPDs, compared to those having no exacerbations within the last year. As we showed before, DNA damage increases with the advancement of COPD (II). PARP-1 binds to damaged DNA and thereby promotes the cellular response to DNA single- and double strand breaks (Yao *et al.*, 2013). Our find indicates that AE-COPDs may lead to potentially long-lasting systemic enzymatic changes already in relatively less advanced COPD. While PARP-1 is not the sole mechanism to protect patients from AE-COPDs, it could potentially be utilized as a predictive marker for AE-COPD.

The expression of HDAC2 was notably elevated in PBMC concurrently with the decline in lung function, implying an intensification of the systemic inflammatory response (II). Importantly, the decline in FEV_1 % over years co-occurred with increased HDAC2 expression (II). Ito *et al.* showed a significant reduction in HDAC activity and expression in peripheral lung tissue, alveolar macrophages and bronchial-biopsy specimen from patients with COPD compared to non-smoking controls (Ito *et al.*, 2005). When COPD patients with lower degree of airflow limitation were grouped together, but excluding AE-COPD patients and patients with more advanced obstruction, we showed similar results (II). CS contains benzo[*a*]pyrene, a polycyclic aromatic hydrocarbon that has been shown to increase the activity of HDAC2, explaining this phenomenon (Bukowska & Sicinska, 2021). In addition, HDAC2 is recruited to DNA break sites when DNA damage occurs (Miller *et al.*, 2010). HDACs have important roles in the complex cellular network that detects the damaged lesion in DNA, signals the presence of the lesion to the cell to activate the appropriate response, and ultimately repairs the lesion to maintain the correct DNA sequence (Alseksek *et al.*, 2022; Robert & Rassool, 2012; Stengel & Hiebert, 2015). Increased HDAC2 level could help protect cells from damage and increased inflammation.

PARP-1 and HDAC2 are overexpressed in various cancers, playing significant roles in tumor progression by participating in DNA repair, transcriptional regulation, and preserving genomic integrity (Kruglov *et al.*, 2020). Lung carcinoma and COPD have a shared risk factor in CS. This common exposure suggests they may also share similar underlying disease mechanisms at the molecular level (Caramori *et al.*, 2011; Durham & Adcock, 2015). As a result, CS may be the underlying cause of the increase in HDAC2, rather than inflammation itself. Under normal circumstances, elevated levels of HDAC2 would lead to a reduction in the acetylation of PARP-1. However, in COPD patients, the influence of CS might be the reason of increased HDAC2 and PARP-1. Lung cancer is up to

five times more prevalent in smokers who have airflow obstruction compared to those with normal lung function (Young & Hopkins, 2010). Additionally, lung cancer ranks as one of the most common causes of death among patients with COPD (Caramori *et al.*, 2011). Increased HDAC2 can contribute to increased risk of developing lung carcinoma in patients with COPD (Czarnecka-Chrebelska *et al.*, 2023; Lawless *et al.*, 2009).

Similarly to HDAC, COX-2 has been shown to possibly be involved in the association between COPD and cancer (Roca-Ferrer *et al.*, 2011). The expression of COX-2 is generally increased in inflammation and cancer (Roca-Ferrer *et al.*, 2011), but this did not occur in our study (II). COX-2 appears to have a dual role in the inflammatory process, initially contributing to the onset of inflammation and later helping to resolve the process (Ricciotti & FitzGerald, 2011). Shi *et al*. showed an elevated expression of COX-2 in the vascular endothelial cells of COPD patients after an exposure to CS extract. This find suggests that CS plays an important part in the apoptosis, and consequently, COX-2 might have a protective role in the progression of tissue damage in the lungs (Shi *et al.*, 2017). Given that COX-2 plays a crucial role in both the initiation and resolution of inflammation, its expression levels in PBMCs at a single time point may not be as indicative of disease severity in our study context. The dynamic nature of the involvement of COX-2 throughout the inflammatory process suggests that a more comprehensive, longitudinal analysis might be necessary to fully elucidate its significance in our research setting.

The expression of DPP4 was downregulated in COPD patients with more severe airflow obstruction and AE-COPD history (II). Decreased level of DPP4 could cause an accumulation of inflammatory cells and factors in the airway tract due to the decrease in the inactivation of inflammatory chemokines (Chang *et al.*, 2016). This could lead to airway wall thickening, small airway obstruction and changes in the structure of the airways leading to worsening of clinical symptoms, decreased exercise tolerance and reduced quality of life (Chang *et al.*, 2016). Interestingly, Says *et al*. showed that DPP4 levels correlated with COPD stage in alveolar epithelial cells (Seys *et al.*, 2018). Chang *et al*. showed lower serum DPP4 concentrations in COPD patients and in patients with AE-COPD compared to healthy controls (Chang *et al.*, 2016). Likewise, Somborac-Bačura *et al.* found a reduction in serum DDP4 activity in COPD patients (Somborac-Bacura *et al.*, 2012). Similarly to our findings, Chang *et al*. also demonstrated that there was no correlation between serum levels of DPP4 and smoking history, age and sex (Chang *et al.*, 2016). Systemic DPP4 might be linked to the T-lymphocyte-related immune response and could serve as a potential biomarker of COPD (Chang *et al.*, 2016).

Increased LTA_4H, a downstream leukotriene metabolizing enzyme, activity is pivotal in the inflammatory process of COPD (Szul *et al.*, 2016). Wells *et al*. showed decreased LTA_4H activity amongst COPD patients (Wells *et al.*, 2014). Similarly, we showed a decrease in LTA_4H mRNA expression level amongst patients with higher stages of airflow limitation and AE-COPD, as well as association with the decline in DL_{CO} (II). LTA_4H has both pro- and anti-inflammatory functions, but Snelgrove *et al*. suggested that CS shifts its emphasis more towards

pro-inflammatory phenotype (Snelgrove *et al.*, 2010). Decreased LTA_4H levels could then explain the constant increase of inflammation amongst patients with COPD. In contrast to LTA_4H, 5-LO did not have any changes in its expression in our study. LTA_4H is found in numerous cell types that do not exhibit substantial 5-LO activity. Additionally, blood cells can transport LTA_4H to regions where it is required. As a result, LTA_4H levels in PBMCs may more accurately reflect changes in disease severity compared to 5-LO (Haeggstrom, 2000).

6.3 The relationship of GSH metabolism pathway enzymes with COPD (II)

Our findings demonstrated an increase in the expression of enzymes involved in GSH metabolism in patients with COPD (II). GSH plays an important role in fighting against OS, with SOD1 and GPx serving as the primary defenses against ROS and their by-products (Liu *et al.*, 2014; Pizent *et al.*, 2020). In our study, the expression of GSR and SOD1 increased along with the decline in lung function, and that of GPx increased in connection with increasing smoking history (II). Similarly, Pannuru *et al*. showed an increased GPx activity in erythrocytes of smokers (Pannuru *et al.*, 2011). The positive association of GPx expression with having ever smoked, smoking pack-years and smoking during the last 6-month, as well as the negative association with longer time passed from smoking cessation refer to increased CS-induced systemic OS stress and either overwhelmed or depleted GSH response to the OS stress in longer-time or heavier smokers. GPx metabolizes H_2O_2 to water, thereby protecting cells against OS (Sogut *et al.*, 2003), suggesting protective role of defense enzymes against oxidative damage induced by CS.

The negative association between FEV_1/FVC % and the expression of SOD1, and the increased GSR expression amongst COPD patients and non-obstructive smokers compared to non-obstructive non-smokers in our study (II), supports the hypothesis that increased availability of GSH in individuals with a significant oxidant burden may protect against lung damage (Bentley *et al.*, 2008). In line with this, the expression of GSR declined, when time interval from smoking cessation increased and increased, when K_{CO} % declined (II). An increase in GSR levels has been observed in airway epithelial cells in patients with COPD, compared to non-obstructive individuals (Bentley *et al.*, 2008; Hackett *et al.*, 2003). Similarly, it has been shown that SOD1 mRNA expression in alveolar macrophages was increased in patients with COPD (Li *et al.*, 2022). We demonstrated in PBMC the shifts in expression of both GSR and SOD1 along with increased airflow obstruction, suggesting that CS can lead to significant systemic OS rather than just localized OS (II).

Our results on PBMC showed increased GCLM and decreased GCLC in parallel with the decline in lung function and increase in COPD severity by GOLD A-D (II). It has been previously shown that CS-induced lung oxidative damage upregulates several cytoprotective genes, including GCLM (Rangasamy *et al.*,

2004). It has been demonstrated that GCLC mRNA is downregulated amongst COPD patients and smokers compared to non-obstructive non-smokers, suggesting hypermethylation of the GCLC promoter region (Cheng *et al.*, 2016). In contrary to our results (II), Malhotra *et al*. showed significant decline in GCLM levels in lung epithelial cells of COPD patients (Malhotra *et al.*, 2008). They debated if the decline suggests that GCLM and its associated pathways may be compromised in individuals with COPD (Malhotra *et al.*, 2008). GCLM has no enzymatic activity on its own, but increases the catalytic efficiency of the GCLC. Increase in the level GCLM would have a much greater effect on GCL activity and GSH production *in vivo* than an increase in that of GCLC (Lee *et al.*, 2006).

NADPH is crucial for the metabolism of GSH, where it regenerates GSSG to GSH by GSR, producing $NADP^+$, the oxidized form of NADPH (Chen *et al.*, 2019; Xiao *et al.*, 2018). $NADP^+$ itself can bind to the catalytic sites of PARP enzymes and inhibit their activity by competing with NAD^+, which is the substrate for PARP-mediated ADP-ribosylation (Bian *et al.*, 2019; Xiao *et al.*, 2018). In our study, GSR was increased along with the decline in lung function (II). Similarly, we showed that PARP-1 was highly expressed among COPD patients (II). Increased GSH production should inhibit the expression of PARP-1, but we showed an increase in both of them, supporting the hypothesis that inflammation and OS are not the only underlying causes of COPD and further research is needed to find new knowledge of what exact genes and pathways may be involved in AE-COPD and COPD progression. The connection between the enzymes and the disease are schematically summarized in Figure 15.

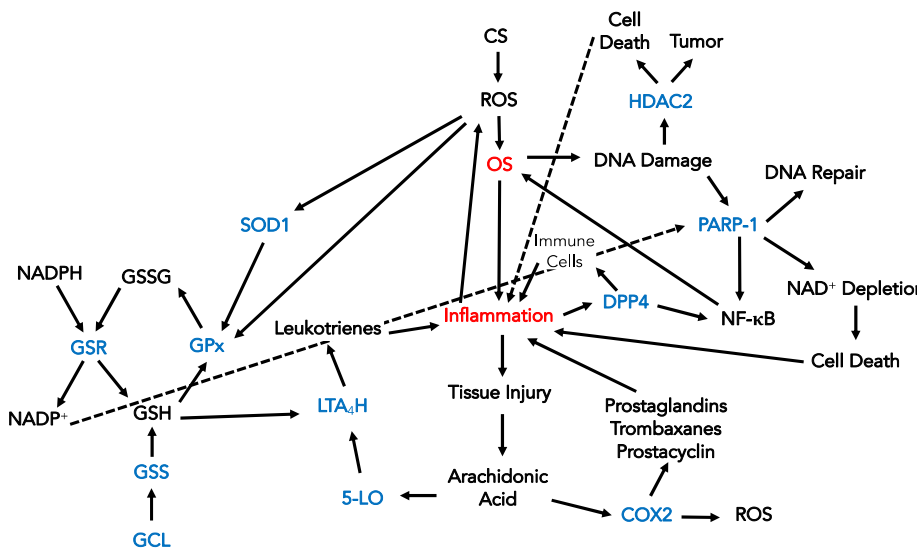

Figure 15. A schematic diagram explaining how the enzymes involved in inflammation and glutathione (GSH) pathways, whose mRNA expression was currently studied (marked with blue), are linked to oxidative stress (OS) and inflammation caused by ROS from environmental agents, primarily from cigarette smoke (CS), as well as from cellular

responses. Solid and dashed lines are used alternately to provide a visual differentiation between adjacent elements, yet both types represent identical information. 5-LO – 5-lipoxygenase, CS – cigarette smoke, COX-2 – cyclooxygenase- 2, DPP4 – dipeptidyl peptidase 4, DNA – deoxyribonucleic acid, GCL – glutamyl-cysteine ligase, GPx – GSH peroxidase, GSH – glutathione, GSR – GSH reductase, GSS – GSH synthetase, GSSG – GSH disulfide, HDAC2 – histone deacetylase, LTA_4H – leukotriene A4 hydrolase, NAD^+ – nicotinamide adenine dinucleotide, NADP – nicotinamide adenine dinucleotide phosphate, NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells, OS – oxidative stress, PARP-1 – poly(ADP-ribose) polymerase-1, ROS – reactive oxygen species, SOD1 – superoxide dismutase 1.

6.4 The effect of UPF1 and UPF17 on mRNA expression levels of enzymes connected with OS and inflammation in COPD (III)

Both GSH analogues, UPF1 and UPF17, had an effect on the expression of enzymes connected with OS and inflammation in COPD. Both of them decreased the mRNA expression level of PARP-1 amongst all individuals (III). UPF17 had a significant inhibitory effect on PARP-1 mRNA expression in patients with COPD, compared to that in non-obstructive individuals (I). The modifying effect of UPF17 on PARP-1 mRNA expression in COPD is of great interest creating a new hypothesis for testing UPF17 as a novel disease-sensitive PARP inhibitor to lower the augmented inflammation and to possibly slow down the development of COPD. It has been demonstrated that inhibiting PARP-1 protects against emphysema and elastase-induced inflammation (Dharwal & Naura, 2018) and can minimize acute lung injury (Neudecker *et al.*, 2017). Amongst COPD patients with milder airflow obstruction, the expression of PARP-1, which is generally responsible for more than 90% of the cellular poly(ADP-ribosyl)ation capacity, expression was increased to protect the cells from OS-related DNA damage (II) (Virag, 2005). In conditions of overexpression, modest inhibition of PARP-1 expression can have anti-inflammatory effects, but inhibiting PARP-1 in a greater degree in return might reduce cellular DNA repair and can thus be deleterious in most circumstances (Curtin & Szabo, 2013). PARP plays a role in almost all acute and chronic inflammatory and immune-mediated diseases. PARP inhibitors are currently in use for the treatment of several cancer types such as breast and ovarian cancer (Weil & Chen, 2011). PARP-1 inhibitors have appeared as new drug target candidates, since they play a critical role in elastase-induced lung inflammation and emphysema (Dharwal & Naura, 2018). PARP-1 inhibitors have also been shown to suppress inflammation and promote recovery after ischemic injury (Hamby *et al.*, 2007; Kauppinen *et al.*, 2009), as well as reduce the levels of transcription factors such as NF-κB and inflammatory prostaglandins such as COX-2 (Gutierrez-Quintana *et al.*, 2022; Koh *et al.*, 2005; Koh *et al.*, 2004). PARP-1 inhibitors, like nicotinamide and 1,5-dihydroxyisoquinoline, have also been shown to be beneficial to be used in inflammatory bowel disease in rats (Sanchez-Fidalgo *et al.*, 2007), as it is the case with olaparib in lung injury, sepsis,

liver disease, and asthma models in mice (Gariani *et al.*, 2017; Ghonim *et al.*, 2015; Kapoor *et al.*, 2015; Mukhopadhyay *et al.*, 2017). More research on the therapeutic potential and clinical safety of PARP-1 inhibitors is needed.

Both UPF peptides increased the expression level of DPP4 amongst all patients (III). As the expression of DPP4 is downregulated in PBMC in COPD patients with more severe airflow obstruction and AE-COPD history (II), increasing systemic expression of DPP4 may help patients with COPD to mobilize systemic inflammatory response. DPP4 has been proposed as a target for augmentation therapy in COPD (Dey *et al.*, 2018). Studies suggest that recombinant DPP4 may exert anti-inflammatory effects by reducing neutrophil infiltration in lung inflammation models (Herlihy *et al.*, 2013). Interestingly, UPF17 decreased DPP4 expression amongst ex-smokers, who had quit at least 6 months prior to the study (III). This could be explained by inhibiting systemic inflammation through DPP4, when removing immense CS-induced systemic OS. Although DPP4 inhibitors may offer some benefits, studies indicate that even when there is a modest risk reduction in severe exacerbations (Pradhan *et al.*, 2022), it has been formerly shown that the use of DPP4 inhibitors can lead to worsening of heart failure in patients with established cardiovascular disease or metabolic abnormalities (Packer, 2018). DPP4 inhibitors that have been used to treat diseases like diabetes, have been reported to be causing breathing difficulties and cough, raising caution about using DPP4 inhibitors amongst COPD patients (Lauster *et al.*, 2007).

UPF1, as well as UPF17, elevated the expression of COX-2 in patients with AE-COPD (III). The dual nature of COX-2, exhibiting both pro- and anti-inflammatory properties, presents a challenge in its potential as a novel drug target, as the effectiveness may depend on the specific inflammatory context within the lungs, necessitating a nuanced approach to drug development and administration (Effros & Casaburi, 2005; Park & Christman, 2006). An elevated COX-2 expression has been associated with several chronic inflammatory diseases including COPD (Cao *et al.*, 2007). Since COX-2 is the rate-limiting enzyme in prostanoid synthesis, its upregulation may lead to increased production of downstream prostanoid products. This relationship suggests that excessive COX-2 stimulation might not produce the anticipated outcome in terms of inflammation (Alqarni *et al.*, 2022). Eicosanoids are potent inflammatory mediators that are produced from AA by cyclooxygenases and lipoxygenase (LO) (Costa *et al.*, 2019). LTA_4H is a major enzyme of the 5-LO pathway (Haeggstrom, 2000). Although there was a significant decrease in LTA_4H mRNA expression in patients with higher stages of airflow limitation and AE-COPD (II), neither UPF1 nor UPF17 had any influence on LTA_4H and 5-LO expression (III).

The two GSH analogues UPF1 and UPF17 increased HDAC2 expression amongst all patients (III). HDAC2 is necessary to deacetylate histones, resulting in suppression of several activated inflammatory genes (Roffel *et al.*, 2020). Reduced HDAC2 has been linked to increased OS in the lungs of COPD patients (Barnes, 2009). Our study found that the FEV_1 % is negatively associated with HDAC2 expression (II). Decreased HDAC2 can lead to unwinding of chromatin

and activation of several proinflammatory cytokines and chemokines, increasing inflammation as a result (Roffel *et al.*, 2020). Increasing HDAC2 expression or activity can restore the failure to suppress OS-induced pro-inflammatory cytokine production and it may be effective in promoting anti-inflammatory activities of corticosteroids (Barnes, 2005; Liao *et al.*, 2020; Song *et al.*, 2015). Interestingly, when COPD patients and non-obstructive smokers were analyzed separately as a subgroup, UPF1 increased the HDAC2 expression level in female individuals but decreased it in males (III). Aging and gender represent two of the prime biological variables that influence human health and disease progression (Gilbert *et al.*, 2019) Gender-specific HDAC expression differences in human brain have been shown before by Gilbert *et al.* who showed higher HDAC expression in females compared to males (Gilbert *et al.*, 2019). It has also been previously demonstrated that epigenetic factors related to HDAC expression, at least in part, regulate liver recovery rates that are dependent on gender in mice, which might be caused by an initially lower expression of HDAC1 and HDAC2 in female mice (Wang *et al.*, 2013). UPF1's ability to differently influence the HDAC2 expression in human PBMCs in female and male individuals may be related to different regulation of the anti-inflammatory genes. UPF17 decreased the HDAC2 mRNA expression level in patients with a history of ≥ 2 moderate or ≥ 1 severe exacerbation within the last year (III). COPD and AE-COPD are mediated by the increased expression of multiple inflammatory genes, many of which are regulated by the acetylation of core histones around which DNA is wound. Conversely, these activated genes are switched off by deacetylation of these histones, resulting in suppression of the inflammatory gene expression by HDACs (Barnes, 2006). The smoking-induced OS, that causes damage to the lung matrix and death of structural cells, is not only associated with an accumulation of ROS but also with a depletion of GSH, which together inhibit HDACs' activity (Druz *et al.*, 2012; Roffel *et al.*, 2020). This in turn leads to increased acetylation of histones and thus DNA uncoiling which can cause increased expression of pro-inflammatory genes (Druz *et al.*, 2012). Due to that inhibition of HDAC2 in patients with COPD would not be clinically favorable. However, increasing HDAC2 expression could suppress inflammatory gene expression and, through that, balance out chronic inflammation (Barnes, 2009).

Out of the enzymes involved in the GSH metabolism pathway, amongst current smokers, UPF17 increased SOD1 mRNA expression in patients with AE-COPD and decreased it in patents without it (III). SOD1 is an antioxidant enzyme that plays a critical role in protecting cells against ROS-induced OS, and therefore provides anti-inflammatory effect in COPD (Shuvaev *et al.*, 2013). Foronjy *et al.* showed that SOD1 can prevent smoke-induced lung inflammation, oxidant injury, protease expression, and emphysema formation in transgender mice, suggesting that superoxide is a key mediator of the pathophysiologic responses that lead to the development of emphysema (Foronjy *et al.*, 2006). Given the role of SOD1 in OS, it has been suggested that increasing its activity or expression could be a therapeutic strategy in managing COPD. Increasing SOD1 expression in alveolar macrophages in COPD patients have shown a promise to potentially

offer a pathway for therapeutic intervention (Li *et al.*, 2022). On the contrary, inhibiting SOD1 could increase ROS levels, which in turn could increase the damage to DNA and, consequently, activate PARP-1 (Haq *et al.*, 2023). PARP-1 is often upregulated in response to DNA damage, which can occur due to presence of cancer, chemotherapy or inflammation (I) (Murnyak *et al.*, 2017; Wang *et al.*, 2021). Similarly to that of SOD1, the expression of GCLC, was increased by UPF17 amongst all patients (III). GCL, which is composed of a catalytic (GCLC) and a modifier (GCLM) subunit, catalyzes the GSH rate-limiting reaction (Lu, 2013; Shang *et al.*, 2016). Hansen *et al*. showed that UPF1 increases intracellular GSH levels by increasing Nrf2-mediated GCLC mRNA expression levels in the K562 human cell line (Hansen *et al.*, 2019). Altraja *et al*. showed that UPF1 is able to entirely restore the intracellular GSH levels in human bronchial epithelial cells under OS caused by CS condensate (Altraja *et al.*, 2013). Increasing antioxidant enzyme expression could provide a potential therapy for COPD, while decreasing antioxidants increases ROS-induced OS and pro-inflammatory immunoinflammatory responses (Hwang *et al.*, 2020).

In a gender-dependent manner, UPF17 decreased SOD1 expression in males and increased it in females (III). Likewise, UPF1 decreased the GSR mRNA expression level in males and increased it in female individuals (III). Frutiger *et al*. showed a specific gender-dependent alteration of SOD1 concentration in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis (Frutiger *et al.*, 2008). Why antioxidants SOD1 and GSR are differently influenced in PBMCs in male and female subjects needs to be further studied. Therapies to increase the GSH levels have been used in the treatment of many lung disorders, including COPD and chronic bronchitis (Papi *et al.*, 2024). Previous studies have shown that increasing GSH has shown efficacy in the prevention of acute exacerbations and improved the overall symptoms of COPD (Papi *et al.*, 2024).

6.5 Limitations and strengths of the studies

The limitations of this study include small cohort sizes in certain subgroups that could reduce the magnitude of the differences found (I–III). However, due to Estonia's relatively small population size, patient recruitment presents significant challenges. Secondly, this was a single-center study and the involvement of a wider population might have increased or decreased the significance of our results (I–III). Collaborating with multiple centers would have provided us with more diverse population coverage and increased generalizability (I-III). As there were no preliminary data, no sample size calculations were performed before this study (I–III). In addition, future studies using whole-genome microarray analysis could provide new knowledge of what other genes may be involved in AE-COPD and COPD progression (II). Further, protein expression-level studies could offer valuable insights into identifying biomarkers for the disease (II). The treatment with UPF's part of our study was performed *ex vivo*, and due to this, it might not fully replicate the exact physiological conditions (III). Future studies *in vivo*

could help to better understand the clinical relevance and potential therapeutic efficacy of UPF1 and UPF17 (III). The gene expression data should be confirmed by functional proteomics or enzymatic analysis to elucidate the levels of the cytokines involved in the respective cascades or clarify the ability to scavenge ROS (III). To counterbalance this, the strengths of our current research include the large number of enzymes measured: six inflammatory enzymes and six anti-OS enzymes, the prospective design, and adequacy of the statistics used.

7. CONCLUSION

1. The DNA damage and PARP activation in PBMC are related both to the progression of COPD and to its exacerbation (AE-COPD). DNA damage and PARP activity both increased in PBMC along with the progression of the disease all the way to AE-COPD adding further support to the fact that COPD is a systemic disease. The newly derived Comet Assay parameters (tail length/cell length ratio and tail migration/cell length ratio) demonstrate superior sensitivity in capturing the progressive changes associated with COPD evolution compared to the conventional comet parameters that have been utilized thus far.
2. Furthermore, GSH metabolism and proinflammatory gene expression potentially play a role in the lung tissue damage that is characteristic of the long-term progression of COPD. They also highlight the importance of a network of genes in the context of lungs' response to OS and systemic inflammation. Importantly, as airway obstruction worsens, the expression of some genes remains unchanged, while others fluctuate. Anti-inflammatory enzymes, like PARP-1 and HDAC2, the key players in the complex processes leading to COPD, were elevated amongst patients with AE-COPD and with the decline in lung function, respectively. Anti-inflammatory enzymes like DPP4 and LTA_4H were under-expressed amongst COPD patients. The key enzymes in GSH metabolism pathway, SOD1, GPX, and GSR were increased along with the decline in lung function and declined, when time interval from smoking cessation increased. This observation raises intriguing questions for future studies regarding the potential factors linked to declining lung function or the onset of COPD. Our results also support that severity of systemic inflammation and OS does not consistently align with the GOLD A-D COPD classification. As GOLD A-D COPD classification does reflect the biological process of the progression of the disease, it is expected that the enzymatic changes that happen during the progression of the disease do not necessarily correspond to the man-made clinical GOLD classifications. Further integration of association and expression studies to determine the nature of the biological relationships may reveal crucial relationships that enhance our comprehension of the condition, potentially leading to improved strategies for disease management and control.
3. Moreover, our research demonstrated that while the experimental UPF peptides exhibited antioxidant capabilities, they unexpectedly led to an upregulation of specific enzymes associated with inflammation. While these peptides are not suitable for direct use in their current form, they hold promise as starting points for developing targeted treatments to combat the excessive OS associated with COPD.

8. POINTS OF PERSPECTIVE

1. The newly derived Comet Assay parameters (tail length/cell length ratio and tail migration/cell length ratio) are more sensitive describing the degree of DNA damage and could lead to more accurate diagnostic and monitoring tools. This, however, needs to be validated in further studies.
2. Due to the strong relationship between DNA damage, PARP activity and COPD progression and AE-COPD, PARP inhibitors should be explored further as one of the treatment strategies.
3. Identified patterns of GSH metabolism and pro- and anti-inflammatory enzyme expression that change with COPD progression, airflow limitation, and AE-COPD, could be developed into molecular signatures for disease staging and predicting exacerbations. Further research is needed to reveal crucial biological relationships in COPD, to better understand COPD pathophysiology and to identify new therapeutic targets.
4. GSH analogue peptides UPF1 and UPF17 show promise as a starting point for new COPD therapies. While they are not suitable for direct use, they demonstrate antioxidant capabilities. Further research is needed to determine their possibilities of being a starting point for a possible COPD treatment.

SUMMARY IN ESTONIAN

Ensümaatika geneetilised markerid kroonilise obstruktiivse kopsuhaiguse kui süsteemse haiguse patogeneesis ja antioksüdantsete peptiidide mõju

Kirjanduse ülevaade

Krooniline obstruktiivne kopsuhaigus (KOK) on peamiselt sigaretisuitsu kroonilisest sissehingamisest tingitud pidevalt progresseeruv, kopsufunktsiooni ja füüsilist sooritusvõimet kahjustav ning surmaga lõppev haigus, millele iseloomulikult kaasneb organismi üldhaigestumine. KOK-i tekkemehhanismideks on kahjustavate ainete, peamiselt sigaretisuitsu kroonilisest sissehingamisest tingitud oksüdatiivne stress (OS) ja põletiku ebanormaalne võimendumine. KOK-ile on omane süsteemne, organismi kõiki elundeid ja kudesid haarav põletik ja OS, mis omakorda võimendab KOK-i progresseerumist ning ägenemiste teket.

KOK-ist on tänapäevaks saanud üks juhtivamaid haigestumise ja suremuse põhjustajaid üle maailma. KOK manifesteerub enamasti keskeas (alates 45 eluaastast) suitsetavate meeste seas. KOK põhjustab üle maailma umbkaudselt 3 miljonit surmajuhtu aastas ja kujundab seega endast tõsist koormat tervishoiusüsteemile ja sotsiaalsfäärile. Tänu rahvastiku vananemisele ja jätkuvale KOK-i riskiteguritega kokkupuutele oletatakse, et KOK-i levimus ajas ainult suureneb.

KOK ei teki äkki, pigem võib tema areng toimuda märkamatult. Ajaks, mil patsiendid hakkavad abi otsima, on neil sageli juba tekkinud ulatuslik hingamisteede kahjustus. Seetõttu tuleks kahtlustada KOK-i olemasolu kõigil, kellel on KOK-ile iseloomulikud tunnused, nagu õhupuudus, krooniline köha, rögaeritus, koormustaluvuse langus, pingsustunne rindkeres või vilistav hingamine ning taustal on pidev kokkupuude haiguse riskiteguritega.

KOK-i klassifikatsioon on ajaga teinud läbi mitu suurt muudatust. Kuni aastani 2011 grupeeriti KOK-i haiged puhtalt kopsufunktsiooni, täpsemalt forsseeritud esimese sekundi ekspiratoorse mahu (FEV_1) % alusel eeldatavast väärtusest, kuid selline klassifitseerimine ei peegeldanud haiguse tegelikku olemust ning kulgu. Aastast 2011 tuldi välja uue, multidimensionaalse klassifikatsioonisüsteemiga, kus KOK-i haiged grupeeriti vastavalt kopsufunktsiooni, ägenemiste ajaloo ja KOK-ile omaste sümptomite alusel. Aastast 2022 muudeti klassifikatsiooni taas ja grupeerivateks teguriteks jäid vaid ägenemiste anamnees ja sümptomite olemasolu.

KOK-i ägenemine on äkiline sümptomite süvenemine, mis toimub vähema kui 2 nädala jooksul. Ägenemised mängivad KOK-i ravis võtmerolli, mõjutades negatiivselt haiguse kulgu, elukvaliteeti ja suremust. Kui ägenemine on juba korra esinenud, tekib järjest suurenev tõenäosus ägenemiste kordumiseks. Kuna ägenemiste ravi ei ole ajas põhimõttelisi muutusi läbi teinud, on spetsiifiliste ja täpsemate ennetusmeetodite leidmine üks juhtivaid teemasid KOK-i ravimeetodite arendamisel.

Sigaretisuitsetamine on juhtiv KOK-i riskifaktor. Sigaretisuits on väga rikas oksüdantide ja vabade radikaalide allikas. Samuti sisaldab sigaretisuits rauaioone, mis kopsu akumuleerides soodustab vabade radikaalide teket. Reaktiivsed hapniku radikaalid (ROS) satuvad kopsu peamiselt suitsetamise tagajärjel, kuid ka rakkude normaalse metabolismi käigus. ROS-ide üleproduktsioon või nende ebapiisav kahjutustamine võib põhjustada OS-i, mis omakorda toob kaasa põletiku aktivatsiooni, metabolismi häirumise ja signaali ülekande regulatsiooni muutuse. Põletikuprotsessid ja OS on omavahel väga tihedalt seotud, kuid nende täpne suhe on praeguseks üksnes pinnapealselt selgitatud.

Glutatioon (GSH) mängib olulist rolli paljudes bioloogilistes protsessides, sealhulgas DNA ja valkude sünteesis, aminohapete transpordis ning ensüümide aktivatsioonis, kuid tema olulisim funktsioon on kaitsta rakke ROS-i toksiliste mõjude eest. GSH-i metabolismis osalevad mitmed olulised ROS-i elimineerivad ensüümid, nagu superoksiidi dismutaas 1 (SOD1), GSH peroksüdaas (GPx), GSH reduktaas (GSR), GSH süntetaas (GSS) ning olulised GSH sünteesi ensüümid nagu glutamaadi-tsüsteiini ligaas (GCL).

Suurenenud ROS-i produktsioon viib valkude, DNA ja lipiidide oksüdatsioonile, mis omakorda võib põhjustada kahjustusi nii süsteemselt kui ka kopsudes. Põletikupiirkonna fagotsüütide produtseeritud ROS on peamised raku- ja koekahjustuse tekitajad, mis omakorda on seotud mitmete krooniliste põletikuliste haigustega, nagu KOK, astma, idiopaatiline kopsufibroos jne.

KOK-i põletikuprotsessis osalevad paljud pro- ja anti-inflammatoorsed ensüümid. Histooni deatsetülaas 2 (HDAC2) mängib rolli põletiku regulatsioonis. HDAC2 ekspressiooni või aktiivsuse vähenemine korreleerub põletikku toetavate geenide ekspressiooni tõusu, KOK-i ägenemiste ja kortikosteroididele allumise vähenemisega. Polü(ADP-riboosi) polümeraas-1 (PARP-1) on oluline DNA kahjustuste elimineerija. PARP-1 aktiveeritakse ROS-i põhjustatud DNA kahjustuse tagajärjel, et tagada DNA stabiilsus ja terviklikkus. Ulatusliku ja püsiva OS-i ja põletiku tõttu tekkiv DNA kahjustus võib põhjustada PARP-1 üleaktiveerimise ning üle selle energiakriisi rakkudes, mis omakorda võib viia rakkude apoptoosini ja seeläbi koekahjustuseni. Lipoksügenaas-5 (5-LO), leukotrieeni A_4 hüdrolaas (LTA_4H) ja tsüklooksügenaas-2 (COX-2) on olulisemaid ensüüme eikosanoidide sünteesirajas. Dipeptidüülpeptidaas 4 (DPP4) võib muuta erinevate kemokiinide ja tsütokiinide funktsioone ja retseptorite spetsiifilisust, kuid on ka leitud seoseid DPP4 ja T-lümfotsüütide aktiveerimisega seotud signaaliradade vahel, viidates võimalikule terapeutilisele sihtmärgile.

Kuna OS ja võimalik antioksüdantse kaitse vähenemine mängib olulist rolli KOK-i patogeneesis, on GSH-i analoogid üheks võimalikuks terapeutiliseks lähenemiseks KOK-i ravis. Käesolevas töös valiti testimiseks GSH-i sünteetilised analoogid UPF1 ja UPF17, kus UPF1 on ~60 korda ning UPF17 ~3000 korda parem hüdroksüülradikaalide elimineerija kui GSH. Mõlemad GSH-i analoogid on primaarsetele rakukultuuridele mittetoksilised.

Uurimustöö eesmärgid

Antud töö eesmärk oli hinnata KOK-i raskusastet süsteemse DNA kahjustuse, PARP-i aktiivsuse ja KOK-i puhuse süsteemse põletiku ning OS-i tekke eest vastutavate ensüümide mRNA ekspressiooni taseme kaudu perifeerse vere mononukleaarsetes rakkudes (PBMC) nii KOK-i ägenemisega patsientidel kui KOK-i erinevates haigusastmetes patsientidel võrreldes mitteobstruktiivsete suitsetajate ja mittesuitsetajatega.

Uurimustöö täpsed eesmärgid

1. Hinnata süsteemse DNA kahjustuse astet ja PARP aktiivsust KOK-i progresseerumises ja ägenemises, et heita valgust haiguse patogeneesi ja progresseerumise taga olevatele molekulaarsetele mehhanismidele.
2. Selgitada välja pro-ja anti-inflammatoorsete ensüümide ja antioksüdantsete ensüümide mRNA ekspressioonide seoseid KOK-i raskusastmete, suitsetamise staatuse, kopsufunktsiooni parameetrite ja KOK-i ägenemistega.
3. Hinnata GSH-i analoogide UPF1 ja UPF17 võimet taastada KOK-i haigetel esinevad ja/või suitsetamisega seotud nihked uuritavate pro-ja anti-inflammatoorsete ensüümide ja antioksüdantsete ensüümide mRNA ekspressioonis.

Uuritavad ja meetodid

Kokku võeti uuringusse 156 patsienti, kes kõik koguti Tartu Ülikooli Kopsukliinikust. Uuringus osalesid hospitaliseerimist vajavad KOK-i ägenemisega patsiendid, KOK-i haiged erinevates haiguse raskusastmetes ja jaotusklassides ning kontrollgrupid, kelle moodustasid ealt ja soolt vastavad mitte-obstruktiivsed suitsetajad ja mittesuitsetajad. Kõikide KOK-i haigete FEV_1/forsseeritud vitaalkapatsiteedi (FVC) suhe oli alla 0.7.

Uuringu väljaarvamiskriteeriumiteks olid mehhaanilise ventilatsiooni rakendamine, intensiivraviosakonda hospitaliseerimine, ebastabiilsed südame-veresoonkonna haigused ning aktiivse pahaloomulise kasvaja olemasolu uuringu ajal. Lisaks ei tohtinud uuritavad põdeda ülemiste ja alumiste hingamisteede infektsioone (ägedat bronhiiti, bronhioliiti ja kopsupõletikku) viimase 4 nädala jooksul enne uuringut. KOK-i ägenemisega patsiendid ei tohtinud põdeda kopsupõletikku viimase 4 nädala jooksul enne uuringut.

Patsientide PBMC-s mõõdeti DNA kahjustust üksikute rakkude geelelektroforeesi abil, PARP-i aktiivsust inkorporeeritud biotiini abil ning KOK-i puhuse süsteemse põletiku ja OS-i tekke eest vastutavate ensüümide mRNA ekspressiooni taset qRT-PCR meetodil. Uuringuandmestikus täideti puuduvad ensüümide ekspressioonitaseme andmed (12 muutujat) mitmekordse imputatsiooniga. Kõigis uuringutes (I–III) hinnati lähteandmete võrdsust uuringurühmade vahel numbriliste muutujate puhul Kruskal-Wallis'e testiga ja nominaalsete muutujate puhul Pearsoni χ^2 testiga. DNA kahjustuse taset ja PARP-i aktiivsust hinnati

ordinaalse logistilise regressioonianalüüsiga, arvutades šansside suhted (OR) ja nende 95% usaldusvahemikud (95% CI). Tulemused kohandati vanusele, soole, kehamassiindeksile (KMI), kopsufunktsioonile (FEV_1 % eeldatavast), suitsetatud pakkaastatele ja jätkuva suitsetaja staatusele. Logistilist regressioonianalüüsi kasutati, et määrata, kas ensüümide transkriptsioonitase on seotud KOK-i esinemise, ägenemiste või suitsetamisajalooga. Üldist lineaarset mudelit kasutati pidevate muutujate ennustamiseks ensüümide transkriptsioonitaseme põhjal. Multinomiaalset logistilist regressiooni kasutati tegemaks kindlaks, kas mõni geen on statistiliselt oluliselt erinevalt ekspresseerunud GOLD A-D või 1–4 alagruppide vahel. Osalist vähimruutude diskriminantanalüüsi (PLS-DA) kasutati ensüümide grupeerimisvõime hindamiseks. Kõik regressioonianalüüsid kohandati vanusele, soole ja KMI-le. GSH-i analoogide mõju ensüümide ekspressioonile analüüsiti korduvmõõtmiste lineaarse segamudeli abil. Andmed kohandati vanusele, soole, suitsetamise pakkaastatele, GOLD A-D alagruppidele ja KOK-i ägenemise staatusele.

Uurimustöö tulemused

1. Uuring näitas, et DNA kahjustus ja PARP-i aktivatsioon PBMC-s on seotud KOK haiguse kulu, kuid ka KOK-i ägenemisega. Mida raskem oli hingamisteede ahenemine, seda suurem oli DNA kahjustus ja PARP-i aktivatsioon PBMC-s. Lisaks tavapärastele DNA kahjustuse parameetritele töötasime välja uued parameetrid (saba pikkuse/raku pikkuse ja saba rände/raku pikkuse suhted), mis veel tugevamalt kajastavad hingamisteede ahenemise raskusastet. Selgus, et huvitaval kombel on mitteobstruktiivsete suitsetajate DNA kahjustus raskem ja PARP-i aktiivsus kõrgem mittesuitsetajate omast.
2. Lisaks eelnevale näitas uuring, et KOK-iga seotud süsteemse põletiku ja OS-i tekke eest vastutavate ensüümide mRNA ekspressiooni tasemed muutuvad kopsufunktsiooni languse või KOK haiguse süvenemisega. Oluliseks leiuks on kõrgem PARP-1 ekspressioon KOK-i ägenemise korral ning patsientidel, kellel on eelnevalt esinenud KOK-i ägenemisi. Kui PARP-1 ekspressioon suurenes, siis HDAC2 ekspressioon vähenes KOK-i ägenemisega patsientidel. GCL modifitseeriva alaühiku (GCLM) ekspressiooni tase oli tõusnud ning GCL katalüütilise alaühiku (GCLC) ning DPP4 tase oli langenud KOK-iga patsientidel võrreldes mitteobstruktiivsete suitsetajate ja mittesuitsetajatega. LTA_4H ekspressiooni tase oli tõusnud KOK-iga patsientidel vastavalt haiguse raskusastmele võrreldes mitteobstruktiivsete isikutega. GPx-i ekspressiooni tase aga suurenes mitteobstruktiivsetel suitsetajatel ja KOK-iga patsientidel suitsetatud pakiaastate arvu suurenemisega. Oluline on veel ära märkida, et mitte kõikide geenide ekspressioonid tõusid või langesid koos kopsufunktsiooni languse või KOK-i haiguse süvenemisega. Niisuguste nähtuste põhjused vajavad selgitamist edasiste uuringutega.
3. Sünteetilised GSH-i analoogid UPF1 ja UPF17 tõstsid GSH metabolismis osalevate antioksüdantsete ensüümide ekspressiooni. Mõlemad GSH analoogid

tõstsid universaalselt SOD1 ja GCLC taset KOK-i ägenejate ja stabiilse KOK-iga patsientidel, aga ka mitteobstruktiivsetel suitsetajatel ja mittesuitsetajatel. Sarnaselt suurendasid nii UPF1 kui ka UPF17 põletikuliste ensüümide PARP-1, DPP4 ja COX-2 ekspressiooni. Huvitavaks leiuks oli tõsiasi, et mõlemad UPF analoogid toimisid soospetsiifiliselt suurendades teatud põletikuvastaste (HDAC2) ja GSH-i metabolismi rajaga (SOD1 ning GSR) seotud ensüümide ekspressiooni naistel ja vähendades samade geenide ekspressiooni meestel.

Arutelu

Meie tulemused toetavad veelgi tugevamini seisukohta, mille kohaselt on KOK süsteemne, kogu organismi hõlmav haigus. Näitasime, et DNA kahjustus ja PARP-i aktivatsioon PBMC-s on seotud KOK-i haiguse progresseerumise, kuid oluliselt ka KOK-i ägenemisega. KOK-i korral esinev krooniline põletik ja OS võivad põhjustada DNA kahjustusi. Näitasime oma töödes suuremat DNA kahjustust ja PARP-i aktivatsiooni hingamisteede ahenemise süvenemisel. PARP-i kompleksi ensüümid, eriti PARP-1, mängivad olulist rolli DNA kahjustusele reageerimisel. PARP-1 tuvastab DNA ahela katkemisi, aktiveerub ning osaleb DNA parandamises. PARP-1 aktiveerumine DNA kahjustuse korral aitab kaasa raku kaitsemehhanismide käivitamisele. PARP-i aktiveerumine aitab DNA kahjustusi parandada, kuid liigne aktivatsioon võib viia rakkude energiavarude ammendumiseni. Lisaks tavapärastele DNA kahjustuse mõõtmise parameetritele comet-meetodil töötasime välja uued parameetrid, mis veel tugevamalt kajastavad hingamisteede ahenemise raskusastet. Antud tulemus näitab, et DNA kahjustus tõenäoliselt suureneb vastavalt OS-i ja põletiku suurenemisega ega ole sõltuv ainuüksi ainult east, soost, FEV_1 (% eeldatavast) vähenemisest ega eksposítsioonist sigaretisuitsule. Huvitaval kombel oli mitteobstruktiivsete suitsetajate DNA kahjustus ja PARP-i aktiivsus kõrgem mittesuitsetajate omast, mis viitab sigaretisuitsust põhjustatud ROS-i üleproduktsioonile, DNA kahjustusele ning lõpuks ka aktiivsele DNA reparatsioonile.

Näitasime, et KOK-iga seotud süsteemse põletiku ja OS-i tekke eest vastutavate ensüümide mRNA ekspressiooni taseme muutused võivad kaasa aidata KOK-ile iseloomulikule kroonilisele kopsukahjustusele. Samuti rõhutavad tulemused OS-i ja süsteemse põletiku vastu võitlevate geenide võrgustiku olulisusele kopsus ja tegelikult kogu organismis süsteemselt. Oluline on veel ära märkida, et mitte kõikide geenide ekspressioonid tõusid või langesid paralleelselt KOK-i süvenemisega tõstatades küsimuse selle kohta, millised võiksid olla kopsufuktsiooni languses või KOK-i arengus olulisemad ensüümid. Tähtsaks leiuks võib pidada siin PARP-1 seost KOK-i ägenemisega, mis näitab, et KOK-i ägenejate seas leiab aset oluliselt tugevnenud DNA reparatsioon. PARP-1 ekspressioon oli kõrgem ka nende patsientide seas, kellel on eelnevalt esinenud KOK-i ägenemisi viidates sellele, et ägenemised põhjustavad potentsiaalseid pikaajalisi ensümaatilisi muutusi juba madalamates KOK-i raskus- või arenguastmetes. Kuigi PARP-1 ei ole ainus ensüüm kaitsmaks patsiente ägenemiste eest, võib ta olla

potentsiaalne märklaud ägenemiste ennustamiseks. Lisaks sellele, näitasid meie tulemused suurenenud GCLM-i ja vähenenud GCLC taset paralleelselt kopsufunktsiooni languse ja KOK-i raskusastme GOLD A–D suurenemisega. GCLM-il ei ole iseenesest ensümaatilist aktiivsust, kuid ta suurendab GCLC katalüütilist efektiivsust. GCLM-i taseme tõus mõjutaks oluliselt GCL aktiivsust ja GSH tootmist *in vivo*. HDAC2 ekspressioon tõusis märkimisväärselt paralleelselt kopsufunktsiooni langusega, mis viitab süsteemse põletikulise vastuse võimendamisele. Langenud DPP4 tase KOK-iga haigetel võib põhjustada põletikuliste kemokiinide inaktiveerimise vähenemise tõttu põletikuliste rakkude kuhjumist hingamisteedesse. See võib omakorda selgitada hingamisteede seinte paksenemist, väikest hingamisteede obstruktsiooni jt. muutusi hingamisteede struktuuris, mille püsitulemuseks on kliiniliste sümptomite halvenemine koormustaluvuse ja elukvaliteedi halvenemine jt. probleemid. LTA_4H on proinflammatoorne ensüüm, mille ekspressiooni langus võib seletada põletiku pidevat suurenemist KOK-iga patsientide seas. SOD1 ja GPx on rakusisesed esmased kaitsvad ensüümid, mis lõppkokkuvõttes reguleerivad ROS-i kontsentratsiooni. Nende ensüümide üleekspressioon viitab suurenenud süsteemsele OS-ile KOK-iga haigetel.

GSH-i analoogid UPF1 ja UPF17 demonstreerisid väga tugevaid antioksüdatiivseid omadusi aidates rakkudel võidelda efektiivsemalt ROS-i kahjustava toime vastu ning vähendada seeläbi süsteemset OS-i ja omakorda vähendada ka KOK-ile omast süsteemset põletikku. Lisaks eelnimetatule tõstsid UPF1 ja UPF17 ka teatud proinflammatoorsete ensüümide DPP4 ja COX-2 taset. Antioksüdantsete ensüümide ekspressiooni suurendamine pakub potentsiaalselt uusi väljavaateid KOK-i raviks, kuid paralleelne proinflammatoorsete ensüümide ekspressioonitaseme tõstmine võib süvendada OS-i ja seeläbi ka süsteemset põletikku. Seetõttu ei saaks antud GSH analooge otseselt ravimina kasutada, kuid nad võiksid olla aluseks KOK-i tulevikuravimite kavandamisel.

Järeldused

Kokku võttes toetavad meie tulemused oma uute aspektidega endisest veelgi tugevamini seda, et KOK on süsteemne haigus ning DNA kahjustus ja PARP aktivatsioon PBMC-s on seotud nii KOK-i progresseerumisega kui ka selle ägenemistega. Lisaks, GSH-i metabolismi ja põletikuga seotud ensüümide geeniekspressioonid aitavad kaasa KOK-i puhusele süsteemsele kahjustusele tekitades samal ajal küsimusi edasisteks uuringuteks geenikspressiooni muutuste, KOK-i progresseerumise ja ägenemiste vaheliste seoste selgitamiseks. Sünteetilised UPF-peptiidid suurendavad GSH metabolismis osalevate ensüümide ekspressiooni, kuid tõstavad samas ka teatud põletikuga seotud ensüümide ekspressiooni. Seetõttu ei saa neid peptiide tõenäoliselt otseselt meditsiinis kasutada, kuid nad võiksid olla prototüübiks potentsiaalsete KOK-i ravimite kavandamisel võitlemaks ülemäärase OS-i vastu.

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Publications

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1. Ehrlich, K.; Ida, K.; Mahlapuu, R.; Kairane, C.; Oit, I.; Zilmer, M.; Soomets, U. Characterization of UPF peptides, members of the glutathione analogues library, on the basis of their effects on oxidative stress-related enzymes. Free Radic. Res. 2009, 43, 572–580
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