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Master's Thesis

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Superoxide dismutases and glutamate-cysteine ligase subunits in obstructive lung diseases

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Superoxide dismutases (SODs) and glutamate cysteine ligase subunits (GCLC, GCLM) play an important role in protection against oxidative stress. It is known that oxidative stress is a principal driving mechanism of obstructive lung diseases. This master's thesis will focus on determining the expression of GCLC, GCLM, and SODs in patients with chronic obstructive pulmonary disease and asthma.

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2. Pierrou S., Broberg P., O'Donnell R. A., Pawłowski K., Virtala R., Lindqvist E., Richter A., Wilson S. J., Angco G., Möller S., Bergstrand H., Koopmann W., Wieslander E., Strömstedt P. E., Holgate S. T., Davies D. E., Lund J., Djukanovic R. (2007): Expression of Genes Involved in Oxidative Stress Responses in Airway Epithelial Cells of Smokers with Chronic Obstructive Pulmonary Disease. American Journal of Respiratory Critical Care Medicine 175 (6): 577–86.
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Abstract:

Although the lungs are one of the most vital organs in the human body, they are also linked with several diseases. Chronic obstructive pulmonary disease (COPD) together with asthma are heterogeneous conditions that cause serious health problems and their prevalence is increasing worldwide.

One of the driving mechanisms for the development of COPD and asthma is oxidative stress. Recent studies suggest an important role for antioxidant enzymes which could positively impact disease pathogenesis. This master's thesis aimed to determine the gene expression of antioxidants from the superoxide dismutase (SOD) family and glutamate-cysteine ligase subunits (GCLC, GCLM) in patients with chronic obstructive pulmonary disease and asthma.

Blood samples from 62 donors (patients with COPD, patients with asthma, patients with asthma before and after biological therapy, and the control group) were examined. The expression of target genes was measured by real-time PCR.

Significantly increased expression of the *SOD2* gene was observed in COPD patients compared to controls. Relative expression of the other genes did not differ significantly.

Keywords: Antioxidant enzymes, asthma, chronic obstructive pulmonary disease, gene expression, glutamate-cysteine ligase subunits, superoxide dismutases

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Abstrakt:

I když jsou plíce jedním z nejdůležitějších orgánů v lidském těle, jsou také spojeny s řadou onemocnění. Chronická obstrukční plicní nemoc (CHOPN) a astma jsou heterogenní onemocnění, která způsobují vážné zdravotní problémy a jejichž výskyt celosvětově roste.

Jedním z hnacích mechanismů rozvoje CHOPN a astmatu je oxidační stres. Nejnovější studie naznačují důležitou roli antioxidačních enzymů, které by mohly mít pozitivní vliv na patogenezi onemocnění. Cílem magisterské práce bylo stanovit genovou expresi antioxidantů z rodiny superoxiddismutáz (SOD) a podjednotek glutamát cysteinové ligázy (GCLC, GCLM) u pacientů s chronickou obstrukční plicní nemocí a astmatem.

Byly vyšetřeny vzorky krve od 62 dárců (pacienti s CHOPN, pacienti s astmatem, pacienti s astmatem před/po biologické léčbě a kontrolní skupina). Exprese cílových genů byla stanovena pomocí PCR v reálném čase.

Významně zvýšená exprese genu *SOD2* byla pozorována u pacientů s CHOPN ve srovnání s kontrolami. Relativní exprese ostatních genů se mezi testovanými skupinami významně nelišila.

Klíčová slova: Antioxidační enzymy, astma, chronická obstrukční plicní nemoc, exprese genů, podjednotky glutamát cysteinové ligázy, superoxiddismutázy

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I hereby declare that this master's thesis was written independently under the supervision of Mgr. Eva Komínková, and by using sources listed in the References section.

In Olomouc, April 26, 2024 ....

Nedavašková Adéla

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TABLE OF CONTENTS

1 INTRODUCTION 1

2 AIMS OF THE THESIS 2

3 LITERATURE REVIEW 3

3.1 Lungs and related problems 3

3.1.1 Chronic obstructive pulmonary disease 3

3.1.2 Asthma 5

3.2 Oxidants and antioxidant enzymes in the lung diseases 6

3.2.1 Superoxide dismutases 7

3.2.1.1 Superoxide dismutase 1 8

3.2.1.2 Superoxide dismutase 2 9

3.2.1.3 Superoxide dismutase 3 10

3.2.2 Glutamate-cysteine ligase 12

3.2.2.1 Glutamate-cysteine ligase catalytic subunit 13

3.2.2.2 Glutamate-cysteine ligase modifier subunit 13

4 MATERIAL AND METHODS 14

4.1 Biological material 14

4.2 List of chemicals, kits, and solutions 15

4.2.1 Chemicals 15

4.2.2 Kits 15

4.2.3 Solutions and their preparation 16

4.2.4 TaqMan assays 16

4.3 List of equipment and programs 17

4.4 Methods 18

4.4.1 Cell isolation 18

4.4.2 RNA isolation 19

4.4.3 Reverse transcription 19

4.4.4 Real-time PCR 20

4.4.5 Statistical analyses 20

5 RESULTS 21

6 DISCUSSION 26

7 CONCLUSION 32

8 REFERENCES 33

LIST OF ABBREVIATIONS

AAT	Alpha-1 antitrypsin
AOEs	Antioxidant enzymes
AP-1	Activator protein-1
ARE	AP-1 like-antioxidant response element
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
DGM	Density gradient medium
ELISA	Enzyme-linked immunosorbent assay
FEV_1	Forced expiratory volume in one second (measured by spirometry)
FVC	Forced vital capacity (measured by spirometry)
GCL	Glutamate-cysteine ligase
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
GINA	Global initiative for asthma
GOLD	Global initiative for chronic obstructive lung disease
GS	Glutathione synthetase
GSH	Glutathione
IgE	Immunoglobulin E
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
RBCs	Red blood cells
ROS	Reactive oxygen species
SOD1	Copper-zinc superoxide dismutase
SOD2	Manganese superoxide dismutase

SOD3	Extracellular superoxide dismutase
SODs	Superoxide dismutases
γ-GC	γ-glutamylcysteine

LIST OF FIGURES

Figure 1: Comparison of healthy lungs and the lung abnormalities associated with COPD. ... 4

Figure 2: Functional interrelation between the mechanism of oxidative stress and inflammation in airway diseases. 6

Figure 3: Schematic diagram of the structure of SOD enzyme isoforms. 8

Figure 4: Superoxide reactions and transformations mediated by SODs. 11

Figure 5: Glutathione synthesis. 12

Figure 6: Layer distribution of the blood sample after centrifugation. 18

LIST OF GRAPHS

Graph 1: Relative expression of *SOD1* in patients (COPD, asthma) and controls. 21

Graph 2: Relative expression of *GCLC* in patients (COPD, asthma) and controls. 21

Graph 3: Relative expression of *GCLM* in patients (COPD, asthma) and controls. 22

Graph 4: Relative expression of *SOD2* in patients (COPD, asthma) and controls. 22

Graph 5: Relative expression of *SOD1* in asthmatic patients before and after biological therapy. 23

Graph 6: Relative expression of *SOD2* in asthmatic patients before and after biological therapy. 24

Graph 7: Relative expression of *GCLC* in asthmatic patients before and after biological therapy. 24

Graph 8: Relative expression of *GCLM* in asthmatic patients before and after biological therapy. 25

Graph 9: Undetectable expression of *SOD3* in all studied subjects. 25

LIST OF TABLES

Table 1: Clinical characteristics of studied subjects. 14

Table 2: TaqMan assays used for real-time PCR. 16

Table 3: Reaction components for cDNA synthesis. 19

Table 4: Conditions for cDNA synthesis. 19

Table 5: Components of the real-time PCR reaction mix. 20

Table 6: Conditions for real-time PCR. 20

Table 7: Information from relevant studies regarding the relative expression of candidate genes. 28

1 INTRODUCTION

Lungs, a vital part of the respiratory system in humans and other animals, are directly exposed to higher oxygen concentrations than most other organs. Besides harmless molecules, the outer environment consists of pollutants and oxidants generating oxidative stress in the lung epithelial cells. The final effect is the disruption of respiratory tissue which typically leads to a variety of lung diseases.

Chronic obstructive pulmonary disease (COPD) and asthma are the most common conditions creating major health problems worldwide. The central aspect of these conditions is airflow limitation associated with chronic inflammation. Some studies have revealed that antioxidant enzymes are an appealing therapy option for individuals affected by COPD or asthma.

Superoxide dismutases (SODs) and glutamate-cysteine ligase subunits (GCLC, GCLM) have evolved special mechanisms to protect lung epithelium. SODs are the only ones converting superoxide radicals to hydrogen peroxide and oxygen. Three SOD isoforms found in mammals vary significantly in terms of expression, enzyme composition, and cell-specific localization. Glutamate-cysteine ligase (GCL) is a heterodimer consisting of a catalytic subunit (GCLC) and a modifier subunit (GCLM). The production of glutathione (GSH), a key antioxidant that shields airway epithelium from oxidative damage, is modulated by GCL to a significant degree.

Getting information on the expression of these antioxidants could help us understand their significance in COPD and asthma.

2 AIMS OF THE THESIS

- Studying available literary sources on the topic of the master's thesis and elaborating literature review.
- Gathering and analyzing human blood samples from subjects with specific clinical characteristics.
- Determining the expression of *SOD*s, *GCLC*, and *GCLM* genes in patients with chronic obstructive pulmonary disease and asthma.
- Searching different articles dealing with the same issue and comparing the results.

3 LITERATURE REVIEW

3.1 Lungs and related problems

The lungs, as the main part of the respiratory system, are paired organs lying in the thoracic cavity. This cavity is lined by pleura – a thin double-layered serous membrane, which passes into the lungs and covers them. The lungs are divided into lobes, the bigger right lung has three, and the left lung has two lobes. These lobes are further divided into pyramidal-shaped bronchopulmonary segments which have their own air and blood supply. Inside the lungs is a complex structure of branched airways and blood vessels that unite at the alveoli (Effros, 2006).

The primary functions of the lungs are respiration and air conditioning. Respiration is a process, in which gases are exchanged between blood and air. When air enters the alveoli, oxygen is taken up, and carbon dioxide diffuses out of the lungs with an exhale. Air conditioning means adjusting the temperature of the inhaled air to the body temperature, adding humidity, and eliminating foreign particles from the air (Walker *et* Wells, 1961; Effros, 2006).

Unlike most organs, lung tissue is directly exposed to ambient air. As a result, lung epithelial cells experience enhanced oxidative stress caused by environmental contaminants and irritating gasses. Foreign particles that are not removed from the lungs can, along with other factors, lead to the development of pathological conditions (Kinnula *et* Crapo, 2003).

According to the pulmonary function profile, lung pathologies can be classified into two groups – restrictive and obstructive (Hansell, 2013). The next part of this chapter will be devoted only to obstructive lung diseases.

3.1.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a heterogeneous inflammatory condition that causes persistent, often progressive, airflow obstruction from the lungs. It is one of the leading causes of morbidity and mortality. In fact, COPD is the third leading cause of death worldwide (Albano *et al.*, 2022; GOLD, 2024).

It is characterized by chronic respiratory symptoms, which usually appear due to abnormalities of the airways (bronchitis) or alveoli (emphysema), see Figure 1, and may include chest tightness, dyspnea, and cough with associated sputum production (GOLD, 2024).

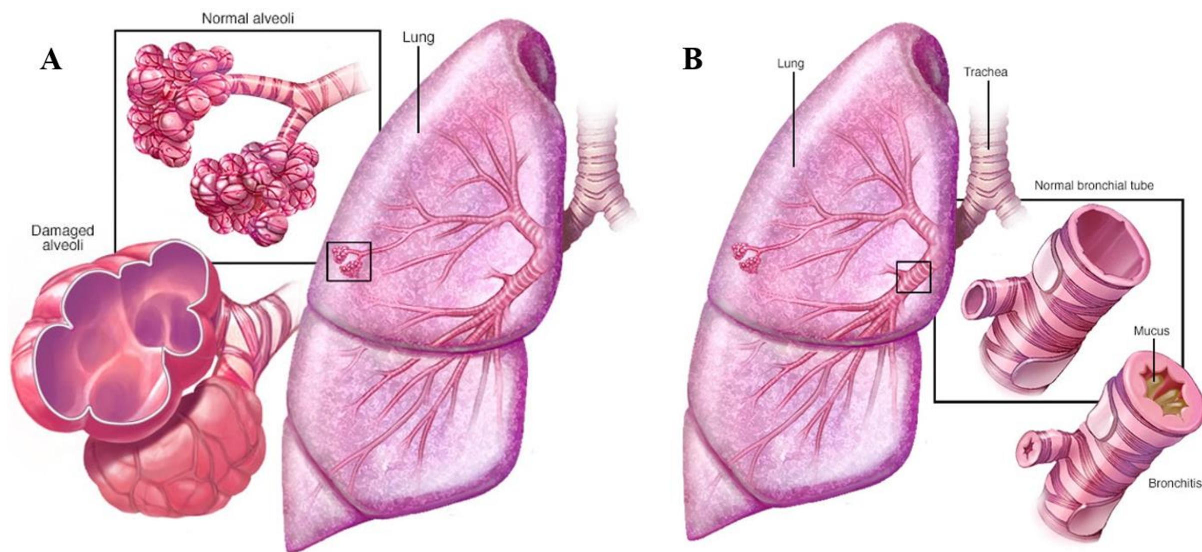


Figure 1: Comparison of healthy lungs and the lung abnormalities associated with COPD.

(A) Emphysema causes dilatation of the alveoli with subsequent destruction of their walls.
(B) Bronchitis is characterized by restricted tubes and excessive mucus production, which can further clog them.
(Modified from https://www.mayoclinic.org/diseases-conditions/copd/symptoms-causes/syc-20353679) [visited April 3, 2024].

The cause of COPD is associated with various environmental exposures, host factors, and a range of genetic variations. To fully understand the pathophysiology of COPD, a comprehensive investigation of gene-environment interactions over the lifespan of the individual is necessary (Agustí *et al*., 2022).

Still regarded as a major environmental factor contributing to chronic inflammation is cigarette smoking with inhalation of combustion products. The outer sources for pulmonary disease development are much wider and the lung is particularly exposed to injury due to the environment as the tissue is the first anatomic site exposed to it (Albano *et al*., 2022).

However, different genetic variants have also been associated with COPD-related traits (Cho *et al*., 2022). Alpha-1 antitrypsin (AAT) deficiency, resulting from mutations in the *SERPINA1* gene, is the primary hereditary risk factor. AAT is a highly potent serine protease inhibitor that targets the proteolytic enzyme neutrophil elastase. The reduced amount of AAT enzyme causes the protease-antiprotease imbalance, increasing the risk of emphysema (Silverman *et* Sandhaus, 2009).

Although COPD is a preventable, and treatable disease, many individuals do not receive treatment or receive the wrong kind of treatment because of widespread underdiagnosis and misdiagnosis. Identifying people who are at risk of developing COPD and periodically monitoring their lung function is crucial as the disease is typically asymptomatic in its early stages (van Schayck *et al*., 2003; Halpin *et* Miravitlles, 2006).

Spirometry, a non-invasive test that measures the amount of air expelled from fully expanded lungs over time, is used for that purpose. It allows an objective assessment of lung function. The two key parameters determined by spirometry are: forced vital capacity (FVC) which represents a measure of lung capacity and forced expiratory volume in one second (FEV_1), the measure of airway patency and lung elasticity (van Schayck *et al*., 2003).

GOLD guidelines provide a practical system for staging chronic obstructive pulmonary disease based on FEV_1 values after using a bronchodilator, as follows: GOLD 1 (mild COPD) to GOLD 4 (very severe COPD) (GOLD, 2024).

3.1.2 Asthma

Asthma is one of the most common and heterogeneous diseases characterized by chronic inflammation. The incidence of this condition is still increasing. An estimated 262 million individuals worldwide suffered from asthma in 2019 (GINA, 2023; WHO, 2023).

It is defined by a history of respiratory symptoms, such as chest tightness, shortness of breath wheeze, and cough, that change over time and in intensity, together with variable expiratory airflow limitation (GINA, 2023). These variations of symptoms are often triggered by complex interactions between hereditary predispositions and environmental exposures (Kleniewska *et* Pawliczak, 2017).

Asthma is primarily induced by allergic reactions to allergens that activate IgE-dependent pathways. Inflammation begins when an antigen binds to IgE on mast cells and basophils. This is followed by the release of mediators and pro-inflammatory cytokines that cause the chemotaxis reaction (Holt *et al*., 1999). Apart from allergens, other risk factors for asthma are viral infections or imbalance in the oxidation-reduction processes that favor a more oxidative state (Comhair *et* Erzurum, 2010; Busse *et al*., 2010).

To help people with asthma live a normal, active life, inhaled corticosteroids to control the symptoms are being used (GINA, 2023; WHO, 2023).

3.2 Oxidants and antioxidant enzymes in the lung diseases

Oxidants or reactive oxygen species (ROS) are a group of short-lived molecules with one or more unpaired electron(s) in their outer molecular orbitals. Their high reactivity emerges from possible interactions with oxidizable substrates via oxidation-reduction reactions (Mut-Salud *et al*., 2016). ROS can come from two sources: endogenous and exogenous. Exposure to any of these sources might alter the body's physiology (Sahiner *et al*., 2011).

Inflammatory cells, including active eosinophils, neutrophils, monocytes, and macrophages have a varied innate capacity to produce endogenous ROS upon stimulation. However, they are usually formed as intermediates or byproducts of cellular metabolism catalyzed by enzymes localized in distinct organelles, particularly mitochondria, peroxisomes, and endoplasmic reticulum (Sahiner *et al*., 2011; Kleniewska *et* Pawliczak, 2017).

Exogenous factors or substances found in the air like biomass fuel smoke or ozone are mostly a consequence of industrial and environmental pollution. Cigarette smoke, for instance, is one of the most significant exogenous oxidant generators which contains epoxides, peroxides, nitrogen dioxide, etc. (Fischer *et al*., 2015; Kleniewska *et* Pawliczak, 2017).

If the number of oxidants exceeds the amount of antioxidant defenses, oxidative stress occurs, resulting in pulmonary inflammation. These processes may determine tissue destruction and lead to the development of long-term respiratory conditions, see Figure 2 (Albano *et al*., 2022).

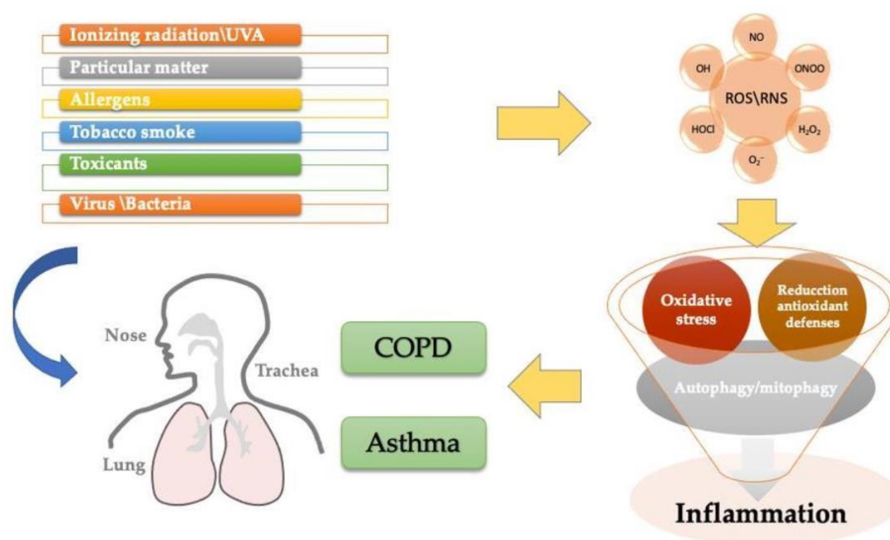


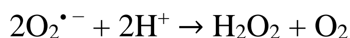
Figure 2: Functional interrelation between the mechanism of oxidative stress and inflammation in airway diseases (Albano *et al*., 2022).

Antioxidant enzymes (AOEs) are special molecules that eukaryotes have evolved to fight directly or indirectly against ROS and their derivatives. These substances can play a central role as inhibitors of reactive species production or AOE defense regulators and prevent oxidants from damaging the cells (MacNee, 2000; Mut-Salud *et al*., 2016).

High concentrations of antioxidant resources are present in the lungs and thanks to their competence, the interest in the use of AOEs as a pharmacological treatment for respiratory disorders has gained popularity in recent years (Albano *et al*., 2022). This master's thesis focused on the family of superoxide dismutases and glutamate-cysteine ligase subunits.

3.2.1 Superoxide dismutases

Superoxide dismutases (SODs) [EC 1.15.1.1] belong to the antioxidant enzyme family that participates in maintaining cell homeostasis and provides a healthy cell environment by converting the superoxide free radical anions into hydrogen peroxide and a molecule of oxygen (Shull *et al*., 1991; Comhair *et al*., 2005).



$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

SODs are well-designed to react with O_2 at speeds close to diffusion limits (Culotta, 2001). Catalysis is mediated by a transition metal ion bound to the active site of the enzyme. Copper (Cu^{2+}), manganese (Mn^{2+}), iron (Fe^{2+}), and nickel (Ni^{2+}) are the four metals used in this capacity (Araujo Eleutherio *et al*., 2021).

Copper enzymes also contain a zinc ion (Zn^{2+}), which does not directly participate in the reaction but helps with other things, for instance, to accelerate the dissociation of the hydrogen peroxide product of dismutation (Culotta, 2001). Toxic H_2O_2 is further detoxified to H_2O by catalase, peroxiredoxin, and glutathione peroxidase (Nguyen *et al*., 2020).

All of these enzymatic reactions are particularly important in lung tissue where they protect against the progression of oxidant-related diseases (Kinnula *et* Crapo, 2003; Nguyen *et al*., 2020).

Until now, three striking isoforms of SOD enzymes have been detected in mammals: copper-zinc SOD (Cu, Zn-SOD/SOD1), manganese SOD (Mn-SOD/SOD2), and extracellular SOD (EC-SOD/SOD3), see Figure 3 (Kinnula *et* Crapo, 2003).

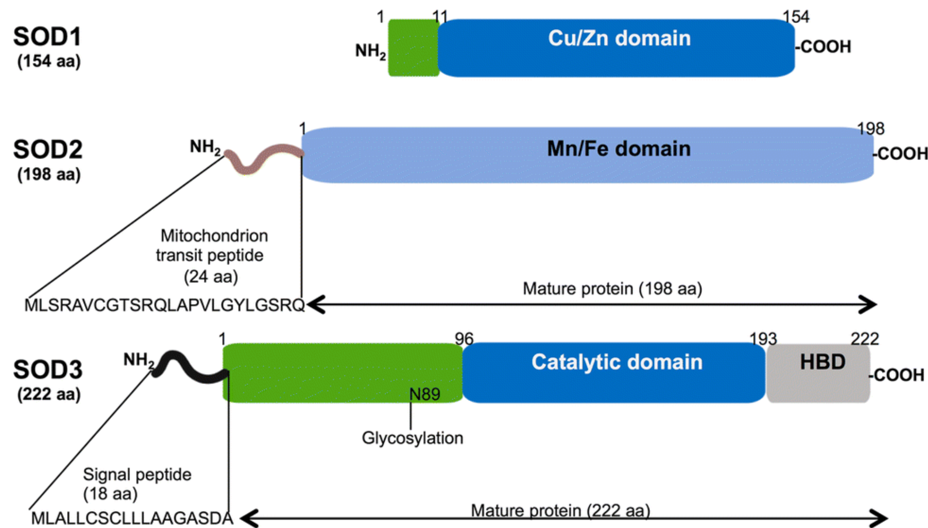


Figure 3: Schematic diagram of the structure of SOD enzyme isoforms (modified from Nguyen *et al.*, 2020).

3.2.1.1 Superoxide dismutase 1

SOD1 or Cu, Zn-superoxide dismutase, regulates ROS levels arising from mitochondrial intermembrane space, inflammatory cells, and the outer environment. In addition to being an antioxidant enzyme, it has been shown to have other roles such as activating nuclear gene transcription, regulating RNA metabolism, etc. (Kinnula *et* Crapo, 2003; Araujo Eleutherio *et al.*, 2021).

Its activity was first recognized in 1969 through the research of McCord and Fridovich, who hypothesized that the enzyme might protect the organism against the bad influence of oxidants (McCord *et* Fridovich, 1969). Before that, scientists thought it was a copper-storage protein (Mann *et* Keilin, 1938).

The *SOD1* gene has been found also in other mammals than humans, showing some similarities in the genomic organization. For instance, the gene consists of five exons interrupted by four introns or a similar pattern of localization of TATA and CCAAT boxes in the proximal promoter region (Zelko *et al.*, 2002).

In humans, the *SOD1* gene is located on a segment enclosing the distal portion of the long arm on chromosome 21. A mature enzyme with a molecular weight of 32 kDa consists of two subunits that are stabilized by the formation of the disulfide bond between cysteine amino acids. Two different ions are selectively bound to each SOD1 monomer: copper (Cu^{2+}) for its catalytic activity and zinc (Zn^{2+}) for its structure and to increase the stability of the native enzyme (Araujo Eleutherio *et al*., 2021). Reduction of the disulfide bond or depletion of metal ions could cause misfolding and toxic conformations in SOD1. The binding of small molecules between these antioxidant enzyme monomers could solve these problems, but several studies have demonstrated just a little effect on its stability (Ray *et al*., 2005; Wright *et al*., 2013).

SOD1 is extensively dispersed throughout many different tissues with the lung being the most important one. The bronchial epithelium of the human lung is the primary site of its expression (Zelko *et al*., 2002; Harju *et al*., 2004).

3.2.1.2 Superoxide dismutase 2

The second member of the SOD family, known as SOD2 or Mn-superoxide dismutase, is also responsible for mediating cellular antioxidant defense by removing toxic oxygen radicals (Shull *et al*., 1991). Researchers demonstrated its essential role in mammalian life when disruption of the *SOD2* gene proved fatal to mice by causing neurodegeneration and heart damage (Lebovitz *et al*., 1996).

SOD2 was first isolated from chicken liver mitochondria in 1973. Thanks to cyanide-sensitivity separation, an enzyme similar to superoxide dismutase was found predominantly in the mitochondrial matrix (Weisiger *et* Fridovich, 1973).

The *SOD2* gene is localized on chromosome 6 in humans and encodes a homotetramer with each of the four subunits carrying an active site with manganese ion (Mn^{2+}) as a cofactor (Zelko *et al*., 2002; Bonetta Valentino, 2022). The amino acids that interact directly with the metal are known as inner sphere residues. The following layer in the enzyme's active site is referred to as the outer sphere residues and is crucial for performing the dismutation reaction (Bonetta Valentino, 2022).

SOD2 is expressed at relatively high levels in numerous cell types and tissues, in the lungs it is the central bronchial epithelium and alveolar macrophages. It is also extensively controlled by several intracellular and environmental signals (Wan *et al*., 1994; Harju *et al*., 2004).

3.2.1.3 Superoxide dismutase 3

SOD3, also known as EC-superoxide dismutase, plays a promising role in the SOD family as a biopharmaceutical candidate for the treatment of inflammatory diseases. It is able to reduce the inflammatory response not only by lowering ROS levels but also by altering cellular signaling (Kwon *et al*., 2012).

In 1982, an enzyme that differed from previously known superoxide dismutases was isolated from human lung tissue. It had a molecular weight of approximately 135 kDa and was made up of four identical noncovalently bound subunits, each with four copper atoms. Because this unique SOD was first found in extracellular fluids, it was tentatively named EC-SOD (Marklund, 1982).

Human SOD3 homotetramer is encoded by the *SOD3* gene, which contains three exons and is located on chromosome 4. The mature form of SOD3 is composed of 222 amino acids, metal ions (Cu^{2+} and Zn^{2+}), and a signal peptide that transports the enzyme into the extracellular area. Interactions with proteoglycan and collagen bind the SOD3 to the cellular surface and the extracellular matrix (Zelko *et al*., 2002; Pereira *et al*., 2018).

In contrast to other SODs, which exhibit ubiquitous expression, SOD3 expression is more limited to certain cell types and tissues. This antioxidant enzyme is highly expressed in the cardiovascular endothelium and placenta, as well as in the extracellular matrix and vessels of the lungs (Harju *et al*., 2004; Pereira *et al*., 2018).

Figure 4 summarizes the location of SOD isoforms in different-sized compartments and their functions.

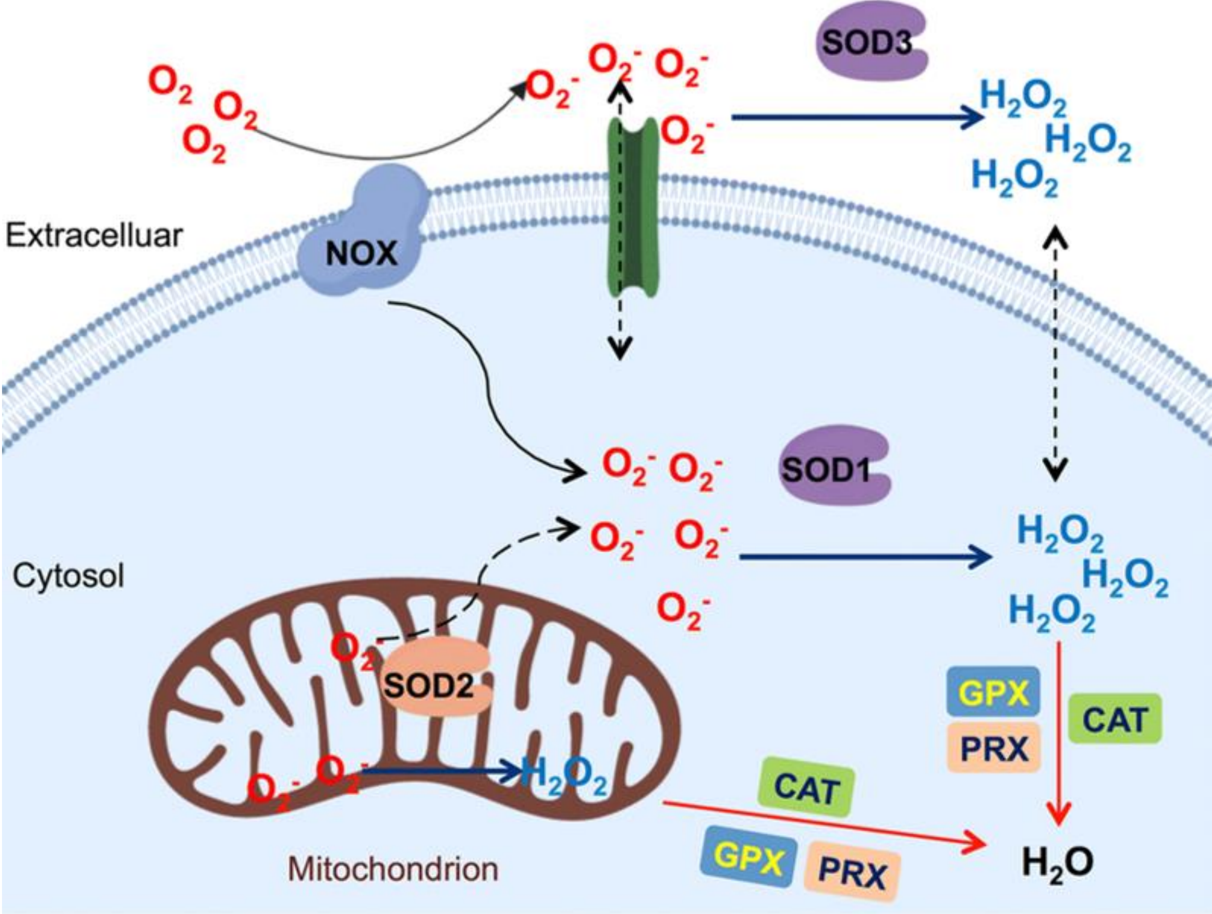


Figure 4: Superoxide reactions and transformations mediated by SODs (modified from Nguyen *et al*., 2020).

3.2.2 Glutamate-cysteine ligase

Glutamate-cysteine ligase (GCL) [EC 6.3.2.2] is a cytosolic, rate-limiting enzyme in the synthesis of glutathione (GSH), a key antioxidant that protects cells from oxidative damage (Sierra-Rivera *et al*., 1995; Yang *et al*., 2007).

GCL mediates the first of two ATP-dependent steps in GSH synthesis, the formation of γ-glutamylcysteine (γ-GC) from glutamate and cysteine. The second step is the binding of glycine to γ-GC by glutathione synthetase (GS), to form GSH, see Figure 5 (Griffith, 1999).

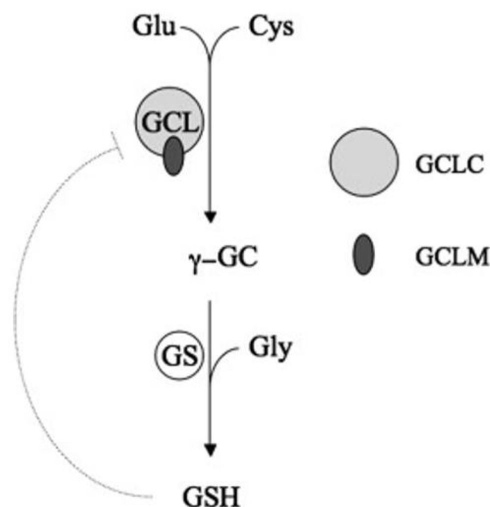


Figure 5: Glutathione synthesis (modified from Franklin *et al*., 2009).

A large amount of the GCL enzyme is inactive under normal conditions due to GSH binding. A decrease in GSH causes the release of its binding to GCL, resulting in greater amounts of active GCL and thus increased GSH production (Rahman, 2005).

GCL enzymes are often heterodimeric complexes consisting of two different gene products: the catalytic subunit (GCLC) and the modifier subunit (GCLM) (Yang *et al*., 2007). The promoter region of the human *GCLC* and *GCLM* genes contains a putative activator protein-1 (AP-1) and AP-1 like-antioxidant response element (ARE), which are required for GCL production in response to various stimuli. These stimuli can be oxidants, growth factors, or inflammatory and anti-inflammatory substances (Rahman, 2005).

3.2.2.1 Glutamate-cysteine ligase catalytic subunit

The catalytic subunit (GCLC) is the larger and heavier of the two subunits with a molecular weight of approximately 73 kDa (Franklin *et al*., 2009).

In humans, the gene encoding this subunit is located on chromosome 6 and is composed of sixteen exons (Sierra-Rivera *et al*., 1995; Franklin *et al*., 2009). Due to relatively small differences in amino acid sequence among mammalian species, the coding region of the *GCLC* gene is highly conserved across eukaryotes (Franklin *et al*., 2009).

As a monomer, GCLC possesses all the catalytic activity of the isolated enzyme (Lu, 2009). It has the active site where the ATP-dependent bond between the glutamate γ-carboxyl group and the amino group of cysteine is formed during the synthesis of glutathione (Franklin *et al*., 2009).

The *GCLC* gene is up-regulated in response to oxidative stress. This could lead to an increase in the GCL protein and GSH synthesis, protecting airway cells from oxidative stress (Rahman, 2005; Liu *et al*., 2007). On the other hand, a polymorphism in the *GCLC* gene promoter region might result in a different reaction to oxidants and decreased production of GSH. Airway cells could have a reduced capacity for antioxidants, making COPD patients more vulnerable to oxidative damage (Liu *et al*., 2007).

3.2.2.2 Glutamate-cysteine ligase modifier subunit

The modifier subunit (GCLM) is smaller and lighter, with a molecular weight of approximately 31 kDa (Franklin *et al*., 2009).

This subunit is expressed from a separate gene on a different chromosome than GCLC. There are seven exons in the human *GCLM* gene, which is found on chromosome 1 (Tsuchiya *et al*., 1995; Franklin *et al*., 2009). Its amino acid sequence is also very similar to sequences in other mammalian species (Franklin *et al*., 2009).

Even though GCLM is enzymatically inactive, it increases the catalytic efficiency of GCLC by direct interaction (Franklin *et al*., 2009). Its regulatory function is lowering the K_m of GCL for glutamate and ATP and raising the K_i for GSH feedback inhibition (Lu, 2009).

4 MATERIAL AND METHODS

4.1 Biological material

62 human blood samples used for the study were obtained from donors classified into 5 groups. These groups were created based on their clinical characteristics – patients with COPD, patients with asthma, patients with asthma before and after biological therapy, and subjects without respiratory disease enrolled as the control group (Table 1). The result of asthma treatment with omalizumab or benralizumab was assessed after 6 months to detect eventual changes in patients.

Table 1: Clinical characteristics of studied subjects.

	Control	COPD	Asthma	Asthma before biological therapy	Asthma after biological therapy
n	8	31	15	4	4
Mean age (min, max)	66 (51, 78)	74 (55, 88)	54 (27, 73)	69 (62, 75)	69 (62, 75)
Males	2	16	3	2	2
Females	6	15	12	2	2

Abbreviations: COPD, chronic obstructive pulmonary disease.

All the patients were diagnosed in the Department of Respiratory Medicine, University Hospital in Olomouc. COPD patients were stated according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines and asthmatics were stated according to Global Initiative for Asthma (GINA) guidelines.

This study was approved by the Ethics Committee of the Faculty of Medicine Palacký University and University Hospital in Olomouc. Informed consent was obtained from all the subjects before blood sample collection.

4.2 List of chemicals, kits, and solutions

4.2.1 Chemicals

- 1× PBS Buffer
- 10× Trypan Blue
- Acid-Phenol:Chloroform (Invitrogen™, Cat. No. AM9720)
- Ethanol absolute (Penta chemicals, Cat. No. 71250-11002)
- Leucosep Tube (Greiner Bio-One, Cat. No. 163288)
- Nuclease-Free Water (Sigma-Aldrich, Cat. No. W4502)
- RNA*later*™ Stabilization Solution (Invitrogen™, Cat. No. AM7021)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™, Cat. No. 10777019)
- TaqMan™ Fast Advanced Master Mix for qPCR (Applied Biosystems™, Cat. No. 4444557)
- TaqMan™ Gene Expression Assay (Applied Biosystems™, Cat. No. 4331182)

4.2.2 Kits

- *mir*Vana™ miRNA Isolation Kit (Invitrogen™, Cat. No. AM1561)
- SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen™, Cat. No. 11754050)

4.2.3 Solutions and their preparation

1× PBS Buffer

- For 1 l of buffer, start with 800 ml of distilled water in a bottle. Measure out and add 8 g of NaCl, 2.16 g of $Na_2HPO_4 \cdot 7H_2O$, 0.2 g of KCl and 0.2 g of KH_2PO_4. If necessary, adjust the pH to 7.4 using HCl and refill with distilled water to 1 l.

10× Trypan Blue

- Add 100 µl of Trypan Blue stock solution to 900 µl Nuclease-Free Water and mix well.

Wash Solutions (*mir*Vana™ miRNA Isolation Kit)

- Before first use, add 21 ml of 100% ethanol to miRNA Wash Solution 1 bottle and mix well. Store at 4 °C for longer periods.
- For Wash Solution 2/3, add 40 ml of 100% ethanol to the bottle and mix well. Store at 4 °C for longer periods.

4.2.4 TaqMan assays

For real-time PCR were used highly sophisticated, predesigned oligonucleotide assays – TaqMan™ Gene Expression Assays by Applied Biosystems™ (Table 2). These TaqMan assays were chosen from a catalog of options for human.

Table 2: TaqMan assays used for real-time PCR.

Gene	Assay ID
SOD1	Hs00533490_m1
SOD2	Hs00167309_m1
SOD3	Hs00162090_m1
GCLC	Hs00155249_m1
GCLM	Hs00978072_m1

4.3 List of equipment and programs

- Accublock™ Mini Digital Dry Bath (Labnet International, Inc.)
- Centrifuge Z 300 K with cooling system (Hermle LaborTechnik)
- GraphPad Prism version 10.0.0 (GraphPad Software, Inc.)
- Laminar Flow Box MSC 12 (Jouan, Inc.)
- Mastercycler® nexus (Eppendorf)
- Microlitre Centrifuge Z 233 MK-2 with cooling system (Hermle LaborTechnik)
- Microscope (Carl Zeiss Jena Nr. 695861)
- Microsoft Excel (Microsoft)
- NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.)
- NanoDrop™ 2000/2000c software (Thermo Fisher Scientific, Inc.)
- Realist DX Real-Time PCR analyzer (GeneTiCA s.r.o.)
- Realist DX software version 8.1.6.0 (GeneTiCA s.r.o.)
- Refrigerator (Beko)
- Spectrafuge™ Mini-centrifuge (Sigma-Aldrich)
- Ultra-low Temperature Freezer Platilab 340 (Angelantoni Industrie)
- Vortex Mixer (Labnet International, Inc.)

4.4 Methods

4.4.1 Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from human whole blood through density gradient centrifugation using Leucosep tubes with a separation medium.

Samples were processed within 1 hour after the collection.

Firstly, 4 ml of the blood sample was diluted with 1× PBS and poured carefully from the sampling tube into the Leucosep tube. The mixture was centrifuged for 15 min at 1250 × g at 8 °C. After centrifugation, the layer of enriched cell fraction was created, see Figure 6. PBMCs were harvested using a Pasteur pipette, washed with 15 ml of 1× PBS, and subsequently centrifuged for 10 min at 3000 rpm at 8 °C. The washing step was repeated twice. Then, the precipitate was resuspended with 3 ml of 1× PBS. The mixture was divided into 3 tubes, each containing 1 ml. PBMCs were counted and samples were centrifuged for 10 min at 3000 rpm at 8 °C. The precipitates were resuspended with 200 µl of RNA*later*. PBMC samples were stored at 4 °C until the next day and then, for long-term storage, transferred into a –20 °C freezer.

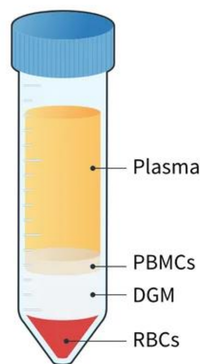


Figure 6: Layer distribution of the blood sample after centrifugation.

Plasma contains nutrients, proteins, platelets, and waste products.
PBMC is a fraction enriched by T and B cells, monocytes, dendritic cells, etc.
DGM layer includes granulocytes and morphonuclear cells.
RBCs (erythrocytes) are the main cellular component of the blood.
(From https://www.reprocell.com/blog/cls/protocol-isolating-pbmcs-whole-blood) [visited April 14, 2024].

4.4.2 RNA isolation

RNA isolation was performed on 62 PBMC samples stored in RNA*later*. The samples were defrosted, mixed with 700 µl of 1× PBS, and centrifuged for 60 min at 4000 × g at 4 °C. After the centrifugation, the supernatant was carefully removed. The next steps were proceeded by *mir*Vana™ miRNA Isolation Kit protocol. In this way, 100 µl of RNA was isolated from each sample, and the concentration of this sample was measured by a spectrophotometer NanoDrop™ 2000. Subsequently, the samples were stored at –80 °C.

4.4.3 Reverse transcription

For cDNA synthesis, RNA samples with a concentration up to 2.5 $\mu g \cdot \mu l^{-1}$ and components of SuperScript™ VILO™ cDNA Synthesis Kit were defrosted on ice and gently mixed. After that, a reaction mix without RNA was prepared in a tube on ice (Table 3). The tube content was mixed again, 6 µl of this reaction mix was pipetted into 0,2ml tubes on ice and 14 µl of RNA template was added into each tube. cDNA synthesis was performed using conditions stated in Table 4. At the end of the reaction, samples were placed immediately on ice and diluted to a final concentration of 7 $ng \cdot \mu l^{-1}$ for real-time PCR. All samples were stored at –20 °C.

Table 3: Reaction components for cDNA synthesis.

Component	Volume
5× VILO™ Reaction Mix	4 µl
10× SuperScript™ Enzyme Mix	2 µl
RNA template	14 µl
Total volume	20 µl

Table 4: Conditions for cDNA synthesis.

Temperature	Time
25 °C	10 min
42 °C	60 min
85 °C	5 min

4.4.4 Real-time PCR

For gene expression measurement by real-time PCR was used TaqMan method. Firstly, diluted cDNA samples and TaqMan™ Fast Advanced Master Mix were defrosted on ice and gently mixed. 20× TaqMan™ Gene Expression Assay was covered in tin foil and placed into the refrigerator. After that, a reaction mix without diluted cDNA was prepared in a covered tube on ice (Table 5). The tube content was mixed again, 18 µl of this reaction mix was placed into 0,1ml tubes on ice and 2 µl of diluted cDNA template was added into each tube. The samples were then covered in tin foil, transferred to Realist DX Real-Time PCR analyzer and the reaction was performed using conditions stated in Table 6. Results were shown by Realist DX software.

Table 5: Components of the real-time PCR reaction mix.

Component	Volume
20× TaqMan™ Gene Expression Assay	1 µl
TaqMan™ Fast Advanced Master Mix	10 µl
Nuclease-Free Water	7 µl
cDNA template	2 µl
Total volume	20 µl

Table 6: Conditions for real-time PCR.

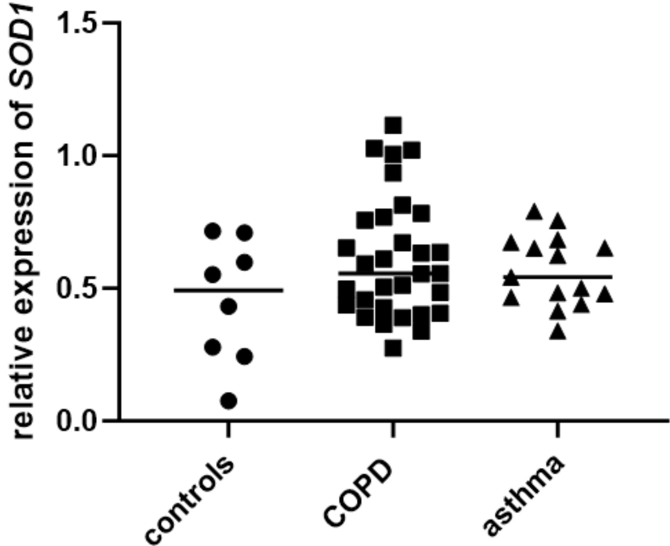
Temperature	Time	Stage
50 °C	2 min	Hold
95 °C	10 min	Hold
95 °C	15 sec	Cycle (40 cycles)
60 °C	1 min	

4.4.5 Statistical analyses

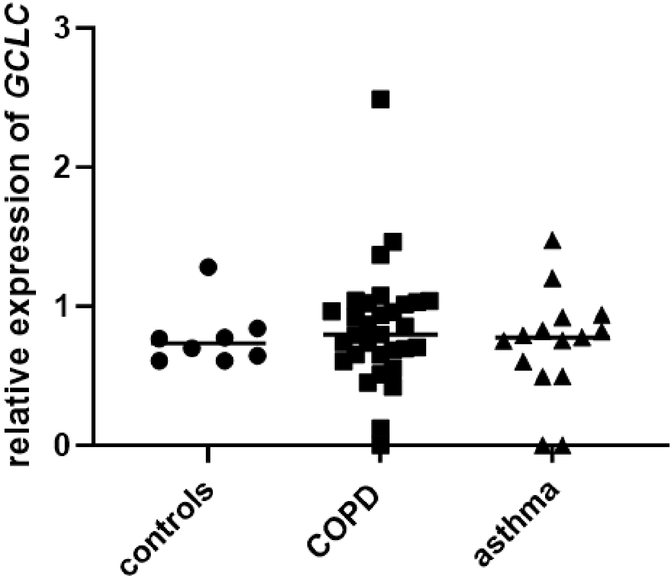
The statistical analyses were performed in the GraphPad Prism version 10.0.0 (GraphPad Software, Inc.) using the Mann-Whitney *U*-test and Wilcoxon matched-pairs signed-rank test. $P < 0.05$ was considered statistically significant.

5 RESULTS

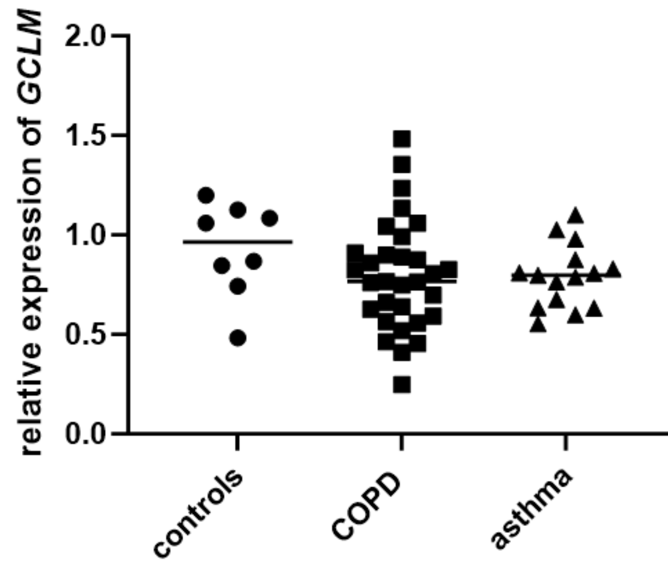
The outcome analysis of *SOD1*, *GCLC*, and *GCLM* expression revealed no significant difference among controls, COPD patients, and asthma patients [$p > 0.05$]. The results are shown in Graphs 1–3.



Graph 1: Relative expression of *SOD1* in patients (COPD, asthma) and controls.

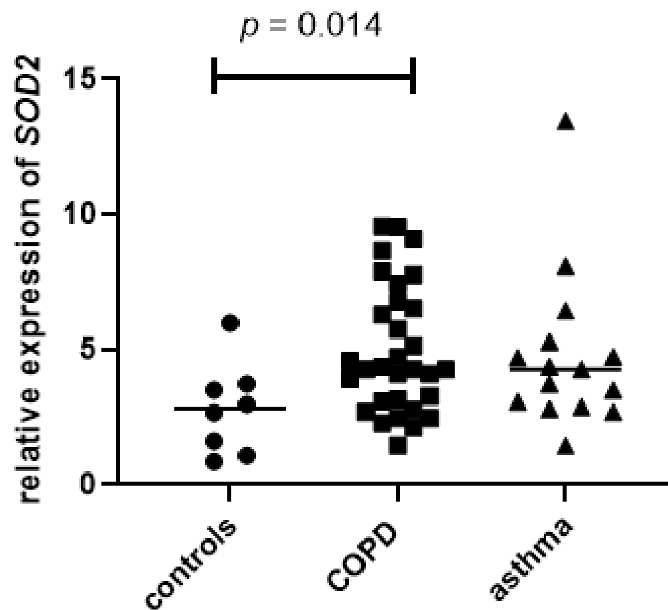


Graph 2: Relative expression of *GCLC* in patients (COPD, asthma) and controls.



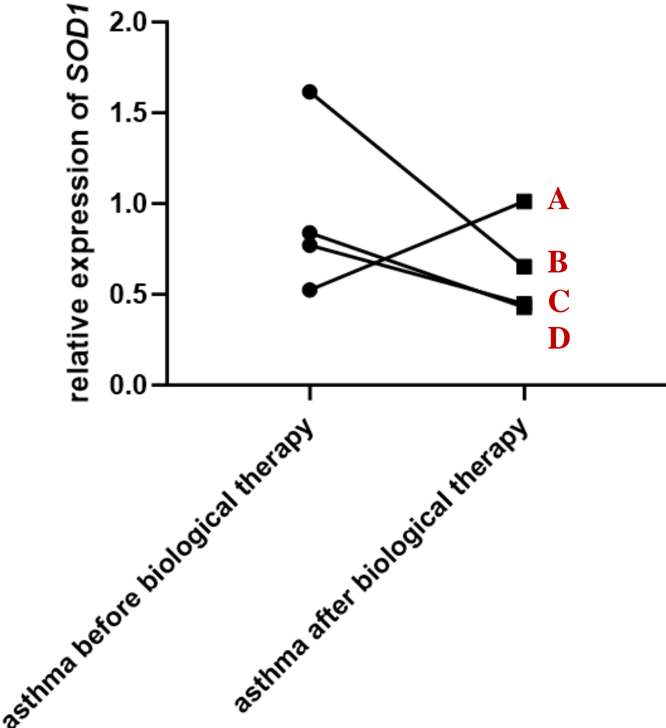
Graph 3: Relative expression of *GCLM* in patients (COPD, asthma) and controls.

The expression of *SOD2* was significantly up-regulated in COPD patients compared to controls [$p = 0.014$]. The result is demonstrated in Graph 4.

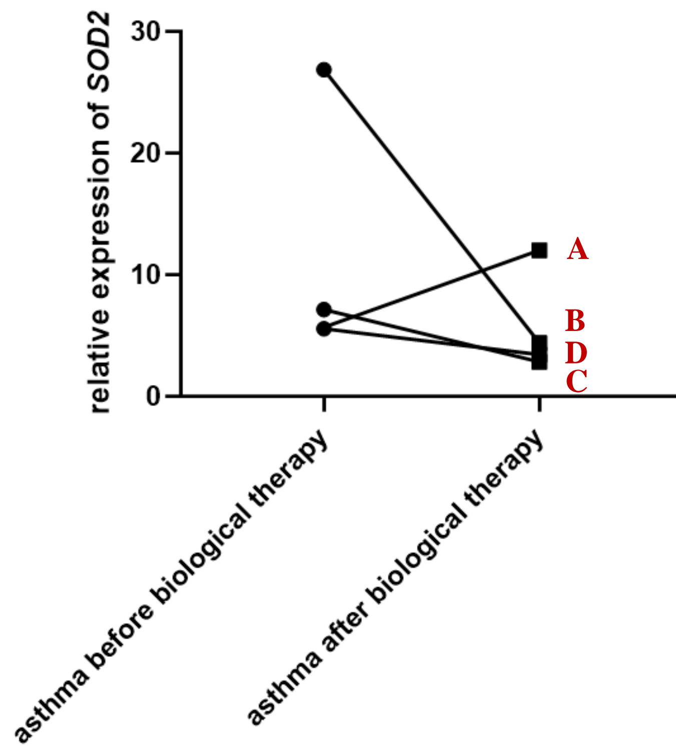


Graph 4: Relative expression of *SOD2* in patients (COPD, asthma) and controls.

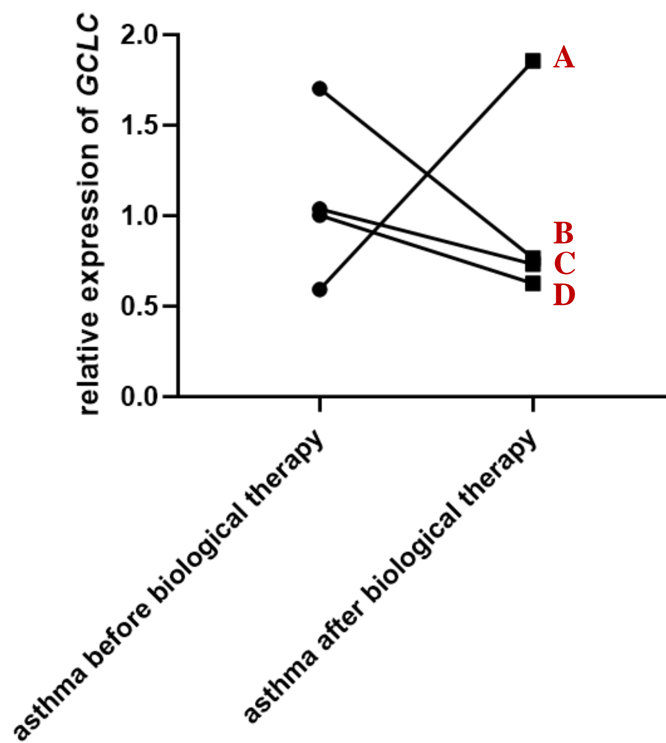
Additionally, this master's thesis assessed the differences in the expression of asthmatic patients before and after they received biological therapy with omalizumab or benralizumab. The *SOD1*, *SOD2*, *GCLC*, and *GCLM* genes were also utilized for this part, however, there was no significant change in their expression [$p > 0.05$ for all]. The results are shown in Graphs 5–8, patients are marked with letters A to D.



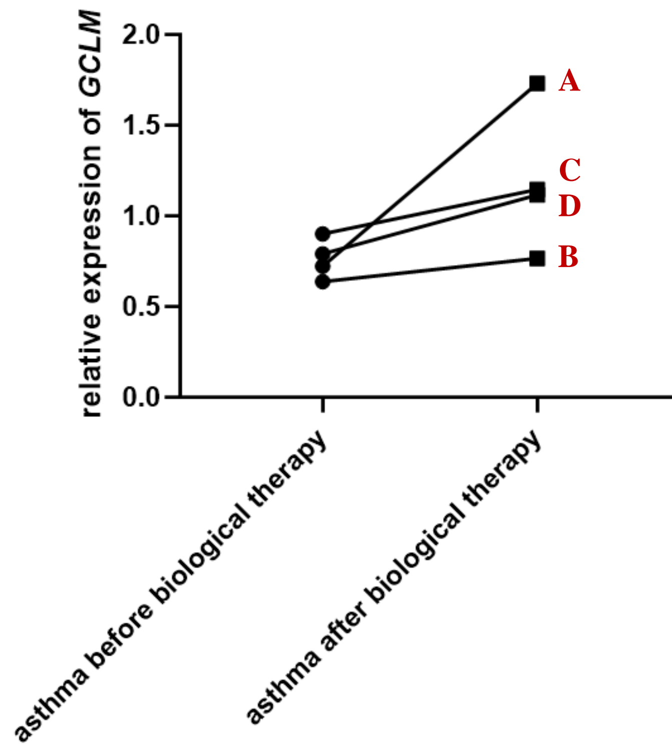
Graph 5: Relative expression of *SOD1* in asthmatic patients before and after biological therapy.



Graph 6: Relative expression of *SOD2* in asthmatic patients before and after biological therapy.

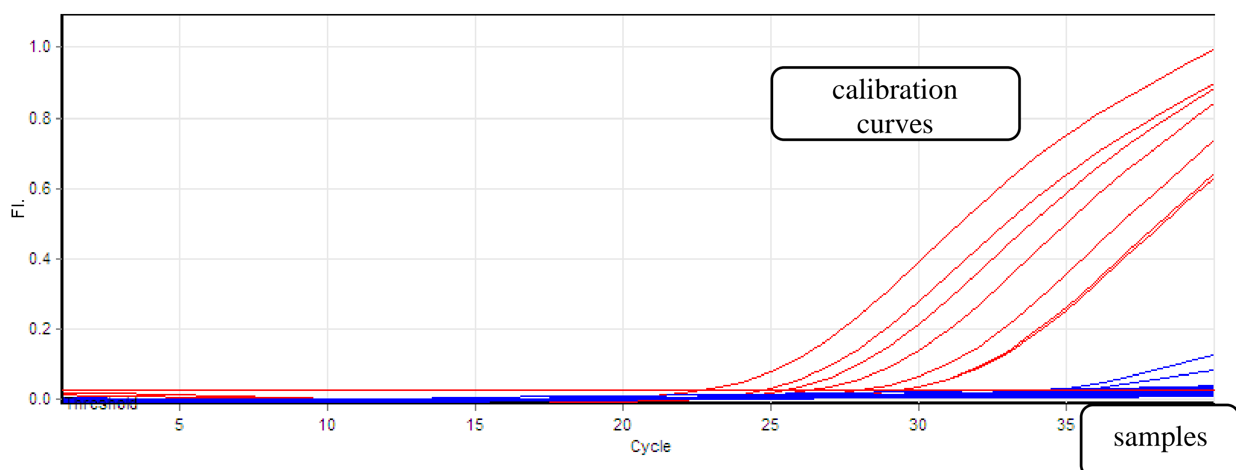


Graph 7: Relative expression of *GCLC* in asthmatic patients before and after biological therapy.



Graph 8: Relative expression of *GCLM* in asthmatic patients before and after biological therapy.

Despite being the last member of the SOD antioxidant enzyme family, the *SOD3* gene was not included in any of the analyses since real-time PCR was unable to identify its expression in the whole blood sample. The result is demonstrated in Graph 9.



Graph 9: Undetectable expression of *SOD3* in all studied subjects.

6 DISCUSSION

We found significantly increased expression of gene *SOD2* in COPD patients compared to the control group [$p = 0.014$]. There was no major difference in the other measured genes.

Relevant studies have confirmed our results on the mRNA expression of *SOD2*. Pierrou *et al*. (2007) showed a significant up-regulation of the *SOD2* gene in smokers with COPD compared to healthy smokers. Its expression level was also higher in the central bronchial epithelium of smokers with COPD and in the alveolar epithelium of smokers with/without COPD than in non-smokers (Harju *et al*., 2004). On the other hand, a different result was reported by Tomaki *et al*. (2007) where the expression of *SOD2* did not change significantly.

In terms of *SOD1*, *GCLC*, and *GCLM* gene expression, the subsequent studies have demonstrated results that differ from ours in most cases. However, two of them observed the same result.

Harju *et al*. (2004) and Tomaki *et al*. (2007) found no significant differences in *SOD1* gene expression among all tested subjects. Only Di Stefano *et al*. (2018) showed its significantly decreased expression in both COPD patients and COPD patients with prevalent emphysema compared to control non-smokers.

The expression of the *GCLC* gene was reported to be both significantly up-regulated (Pierrou *et al*., 2007) and significantly down-regulated (Cheng *et al*., 2016) in COPD patients. Fratta Pasini *et al*. (2020) conducted a longitudinal study in which they also included *GCLC* expression. When compared to controls, they observed significantly higher baseline expression in PBMCs of COPD patients. However, at follow-up, the expression was significantly decreased.

For gene *GCLM*, a significant up-regulation of its expression was demonstrated in smokers with COPD compared to smoking and non-smoking controls (Pierrou *et al*., 2007).

Various results in these studies could be related to various conditions, for example in the diversity of used biological material or methodology (Table 7).

Regarding the expression before and after the biological therapy, patient A showed an increase in the expression for all genes, but it did not reach a significant value. In the remaining patients (B, C, and D patients) we observed a trend towards decreased expression after the biological therapy for the following genes: *SOD1*, *SOD2*, and *GCLC*. On the other hand, a trend towards increased expression after the biological therapy could be seen in all patients for the *GCLM* gene [$p > 0.05$ for all]. All genes have the same biological function, they are antioxidants. Thus, we expected a positive result of this treatment, which is increased expression of the antioxidant genes. More detailed clinical characteristics of the patients (e.g. lung function, eosinophil counts) would be needed to better understand these results.

In a search for candidate gene data in asthma, there was a small body of literature that additionally addressed the measurement of its activity, not the mRNA expression. The other limiting factor was, that they studied SODs in general because some methods usually fail to accurately detect SOD isoforms. However, the results of most studies, such as Comhair *et al.* (2005) or De Raeve *et al.* (1997) studies, were consistent with ours. The remaining studies observed significant down-regulation of SODs and SOD3 activity (Table 7) (De Raeve *et al.*, 1997; Comhair *et al.*, 2000; Comhair *et al.*, 2005; Yang *et al.*, 2011).

A promising candidate and the last member of the SOD family, extracellular SOD3, should have been included in the expression evaluation. Unfortunately, we did not observe any expression for it and thus no further statistical analyses were performed with it. However, different studies have been done on this topic in the last few years and García-Valero *et al.* (2022) independently with Regan *et al.* (2011) demonstrated significant down-regulation of SOD3 at the protein level. At the mRNA level, there was no significant change in the *SOD3* expression among all studied groups (Table 7) (Tomaki *et al.*, 2007; García-Valero *et al.*, 2022).

Considering this information with the knowledge that different biological material was used, we initially hypothesized it was the possible cause of the undetectable expression. However, we gave up on this theory because data show that the *SOD3* gene is highly expressed in blood vessels and is also present in plasma (Strålin *et al.*, 1995). Human error in sample processing together with inadequate amounts of isolated cells could be another issue.

Further analysis is needed to determine the gene expression level in patients with COPD and asthma compared to controls. Conflicting results can be related to several factors, suggesting various amounts of studied subjects and the use of distinct biological materials or methods.

Table 7: Information from relevant studies regarding the relative expression of candidate genes.

Gene	Disease	n	Control	Biological material	Method	Expression	Reference
*SOD*s	asthma	13 non-smokers	11 non-smokers	bronchial epithelial cells	SOD-525 assay	↓	De Raeve *et al*., 1997
		114 non-smokers	20 non-smokers	serum	spectrophotometric assay	↓	Comhair *et al*., 2005
		50	50 non-smokers	erythrocytes	spectrophotometry	↓	Yang *et al*., 2011
SOD1	COPD	3 smokers, 11 ex-smokers	4 smokers, 5 ex-smokers, 10 non-smokers	peripheral lung tissue	real-time PCR	-	Tomaki *et al*., 2007
		22 smokers	20 smokers, 13 non-smokers	central bronchial epithelium, peripheral bronchiolar epithelium, alveolar epithelium, alveolar macrophages	immunohistochemistry	-	Harju *et al*., 2004

Table 7: Information from relevant studies regarding the relative expression of candidate genes – continued.

Gene	Disease	n	Control	Biological material	Method	Expression	Reference
SOD1	COPD	8 smokers, 37 ex-smokers	7 smokers, 2 ex-smokers, 18 non-smokers	blood samples	immunochemistry	↓	Di Stefano *et al.*, 2018
	asthma	13 non-smokers	11 non-smokers	bronchial epithelial cells	slot-blot technique and hybridization with [^{32}P]cDNA probe	-	De Raeve *et al.*, 1997
		13 non-smokers	11 non-smokers	bronchial epithelial cells	ELISA	-	De Raeve *et al.*, 1997
		114 non-smokers	20 non-smokers	serum	ELISA	-	Comhair *et al.*, 2005
SOD2	COPD	38 smokers	18 smokers	bronchial epithelial cells	microarray analysis	↑	Pierrou *et al.*, 2007
		3 smokers, 11 ex-smokers	4 smokers, 5 ex-smokers, 10 non-smokers	peripheral lung tissue	real-time PCR	-	Tomaki *et al.*, 2007
		22 smokers	20 smokers, 13 non-smokers	central bronchial and alveolar epithelium	immunohistochemistry	↑	Harju *et al.*, 2004

Table 7: Information from relevant studies regarding the relative expression of candidate genes – continued.

Gene	Disease	n	Control	Biological material	Method	Expression	Reference
SOD2	asthma	114 non-smokers	20 non-smokers	serum	ELISA	-	Comhair *et al*., 2005
SOD3	COPD	15 smokers	11 smokers, 13 non-smokers	peripheral bronchioles, alveolar macrophages	immunohistochemistry	-	Harju *et al*., 2004
		3 smokers, 11 ex-smokers	4 smokers, 5 ex-smokers, 10 non-smokers	peripheral lung tissue	real-time PCR	-	Tomaki *et al*., 2007
		20 smokers	10 smokers, 9 non-smokers	alveolar, bronchiolar and arteriolar walls	immunohistochemistry	↓	García-Valero *et al*., 2022
		20 smokers	10 smokers, 9 non-smokers	lung tissue	real-time PCR	-	García-Valero *et al*., 2022
		12 smokers	6 smokers	alveolar interstitium	immunohistochemistry	↓	Regan *et al*., 2011

Table 7: Information from relevant studies regarding the relative expression of candidate genes – continued.

Gene	Disease	n	Control	Biological material	Method	Expression	Reference
SOD3	asthma	7 with atopic asthma	5 non-atopic	BAL	ELISA	↓	Comhair *et al.*, 2000
GCLC	COPD	38 smokers	18 smokers, 14 non-smokers	bronchial epithelial cells	microarray analysis	↑	Pierrou *et al.*, 2007
		30 smokers, 28 ex-smokers	17 smokers, 19 ex-smokers, 28 non-smokers	lung tissue	real-time PCR	↓	Cheng *et al.*, 2016
		4 smokers, 26 ex-smokers, 3 non-smokers	2 smokers, 14 ex-smokers, 21 non-smokers	blood samples (PBMCs)	real-time PCR	↑/↓	Fratta Pasini *et al.*, 2020
GCLM	COPD	38 smokers	18 smokers, 14 non-smokers	bronchial epithelial cells	microarray analysis	↑	Pierrou *et al.*, 2007

Abbreviations: ↑, up-regulation; ↓, down-regulation; -, no significant difference; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; PBMCs, peripheral blood mononuclear cells; SODs, superoxide dismutases in general; SOD1, copper-zinc superoxide dismutase; SOD2, manganese superoxide dismutase; SOD3, extracellular superoxide dismutase.

7 CONCLUSION

Despite being a serious health problem affecting people throughout the world, chronic obstructive pulmonary disease (COPD) and asthma are still not given enough attention. The theoretical part of this thesis was written to introduce the basic characteristics of these diseases, but it also dealt with their treatment. The superoxide dismutase (SOD) family, which is growing in popularity, as well as the catalytic and modifier subunits of glutamate-cysteine ligase (GCLC, GCLM), were discussed.

This master's thesis suggests that elevated levels of these antioxidants could be beneficial in the pathophysiology of COPD and asthma. The major goal of the practical part was to determine the expression of *SOD*s, *GCLC*, and *GCLM* genes in studied subjects. In comparison to controls, the final results showed that *SOD2* expression was significantly increased in COPD patients. There was no significant change in the expression of the other genes. Regarding the assessment of expression differences in asthmatic patients before and after the biological therapy, the above-mentioned genes have also been used. However, no major changes in expression were observed as well. SOD3, as the final member of the family, was supposed to be a part of the analyses, however, real-time PCR was unable to detect its expression.

Larger studies are needed to evaluate the gene expression of our measured antioxidant genes. These findings could reveal the true potential of antioxidants in COPD and asthma.

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