



# The nitroxide/antioxidant 3-carbamoyl proxyl attenuates disease severity in murine models of severe asthma

Miri Assayag<sup>a</sup>, Sara Goldstein<sup>b,\*</sup>, Amram Samuni<sup>c</sup>, Alexander Kaufman<sup>a</sup>, Neville Berkman<sup>a</sup>

<sup>a</sup> Institute of Pulmonary Medicine, Hadassah-Hebrew University Medical Center, Jerusalem, 91120, Israel

<sup>b</sup> Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

<sup>c</sup> Institute of Medical Research, Israel-Canada Medical School, The Hebrew University of Jerusalem, Jerusalem, 91120, Israel

## ARTICLE INFO

### Keywords:

Nitroxide  
Chronic asthma  
3-CP  
Antioxidant  
Oxidative stress  
Oxidative injury  
Collagen content  
Protein nitration  
TGF- $\beta$

## ABSTRACT

Asthma is characterized by airway inflammation, hyper-responsiveness, symptoms of dyspnea, wheezing and coughing. In most patients, asthma is well controlled using inhaled corticosteroids and bronchodilators. A minority of patients with asthma develop severe disease, which is frequently only partially responsive or even resistant to treatment with corticosteroids. Severe refractory asthma is associated with structural changes in the airways, termed “airway remodeling”, and/or with neutrophilic rather than eosinophilic airway inflammation. While oxidative stress plays an important role in the pathophysiology of asthma, cyclic nitroxide stable radicals, which are unique and efficient catalytic antioxidants, effectively protect against oxidative injury. We have demonstrated that the nitroxide 3-carbamoyl proxyl (3-CP) attenuates airway inflammation and hyper-responsiveness in allergic asthma as well as bleomycin-induced fibrosis both using murine models, most probably through modulation of oxidative stress. The present study evaluates the effect of 3-CP on airway inflammation and remodeling using two murine models of severe asthma where mice are sensitized and challenged either by ovalbumin (OVA) or by house dust mite (HDM). 3-CP was orally administered during the entire period of the experiment or during the challenge period alone where its effect was compared to that of dexamethasone. The induced increase by OVA and by HDM of BALf cell counts, airway hyperresponsiveness, fibrosis, transforming growth factor-beta (TGF- $\beta$ ) levels in BALf and protein nitration levels of the lung tissue was significantly reduced by 3-CP. The effect of 3-CP, using two different murine models of severe asthma, is associated at least partially with attenuation of oxidative stress and with TGF- $\beta$  expression in the lungs. The results of this study suggest a potential use of 3-CP as a novel therapeutic agent in different forms of severe asthma.

## 1. Introduction

Asthma affects over 300 million people worldwide, and disease prevalence in some Western countries is greater than 20%. Asthma is a chronic disease characterized by airway hyper-responsiveness (AHR), reversible airflow obstruction, airway inflammation and development of structural changes in the airways. Although mostly responsive to treatment with bronchodilators and corticosteroids [1–3], 5–10% of patients develop severe disease, characterized by the development of refractoriness to therapy, frequent hospitalization and increased risk of asthma-related death [4–7]. Severe refractory asthma is thought to be related to development of airway remodeling and/or neutrophilic

airway inflammation. In recent years several biological agents have become available for the treatment of severe refractory asthma, but these drugs only target T2-high asthma, characterized by eosinophilia and/or atopy [8,9]. However, many patients remain inadequately controlled despite optimal treatment [10]. The development of novel drugs that target both chronic inflammation and remodeling is an important unmet need in severe asthma management.

Our understanding of the disease mechanisms underlying severe asthma remains incomplete [6]. Oxidative and nitrosative stress, are a central pathophysiological component in several respiratory diseases including asthma [11–13], idiopathic pulmonary fibrosis (IPF) [14,15] and chronic obstructive pulmonary disease (COPD) [16]. This revelation

**Abbreviations:** AHR, airway hyperresponsiveness; BALf, bronchoalveolar lavage fluid; 3-CP, 3-carbamoyl-proxyl; Dex, dexamethasone; HDM, house dust mite; H&E, hematoxylin and eosin; IP, intraperitoneal; IV, intravenous; MCH, methacholine; 3-NT, 3-nitrotyrosine; OVA, ovalbumin; TGF, transforming growth factor.

\* Corresponding author.

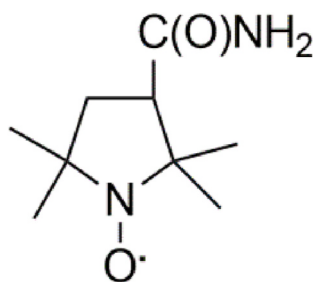
E-mail address: [sara.goldstein1@mail.huji.ac.il](mailto:sara.goldstein1@mail.huji.ac.il) (S. Goldstein).

<https://doi.org/10.1016/j.freeradbiomed.2021.10.021>

Received 9 September 2021; Received in revised form 14 October 2021; Accepted 18 October 2021

Available online 19 October 2021

0891-5849/© 2021 Elsevier Inc. All rights reserved.



Scheme 1. Structure of 3-CP.

has resulted in evaluation of a variety of antioxidant therapies [13,17,18], however, to date, the therapeutic efficacy of common antioxidants has been disappointing [19,20].

We have demonstrated that the 5-membered ring nitroxide 3-carbamoyl-proxyl (3-CP, Scheme 1) markedly reduces airway inflammation, airway hyperresponsiveness, and protein nitration of the lung tissue in a mouse model of ovalbumin-induced allergic asthma [21].

Recently, 3-CP was found to reduce the severity of bleomycin-induced lung fibrosis in mice, providing protection when administered either before or after the onset of lung injury [22]. These results led us to investigate, using mouse models, whether 3-CP may be of value for the treatment of severe asthma too.

## 2. Methods

### 2.1. Materials

3-CP, Grade V chicken ovalbumin (OVA) and dexamethasone (Dex) were purchased from Sigma-Aldrich, Methacholine from MP Biomedicals, Santa Ana, CA, House dust mite (HDM) from Greer laboratories INC. (NC, USA). Mouse chow supplemented with nitroxide was prepared by soaking normal food pellets in a minimal volume of water containing nitroxide (1% w/w) and commercial chicken soup powder (1% w/w), and drying it at 37 °C while preparing new chow pellets. Animals were reluctant to eat nitroxide-containing chow unless chicken soup powder was added. The weight of the mice was monitored daily throughout the experiment, and during a period of 3–5 weeks they gained weight. EPR spectroscopy was used to assay the residual levels of 3-CP and its reduced form as previously described [21].

### 2.2. Mouse models

Male C57Bl/6 and Balb/c mice were purchased from Envigo and held in a specific pathogen-free animal facility at Hadassah-Hebrew University Medical Center with unlimited access to sterile chow and water. The Hebrew University–Hadassah Medical School Animal Ethics Committee approved all experimental protocols.

(i) “Chronic” OVA model with airway remodeling. Mice aged 8–10 weeks were sensitized and challenged with OVA. The mice received IP injections of 10 µg OVA and 1 mg Al (OH)<sub>3</sub> in 0.5 mL saline at Days 1 and 11. Subsequently, the mice were challenged with OVA aerosol (2% in saline) for 20 min (3 times a week for 4 weeks) on Days 17–19, 24–26, 30–32 and 37–39 while animals of the control group were challenged by saline alone. The experiment ended on Day 40 (Scheme 2). We have previously found that remodeling changes are already present at 4 weeks and are not significantly greater when inhalations are performed for up to ten weeks. Mice were fed *ad-libitum* with either standard or with nitroxide-containing standard mouse chow (1% (w/w)). The effect of 3-CP was investigated by feeding the mice *ad libitum* diet of nitroxide-containing chow either during OVA-induced sensitization and challenge stages (Days –2 –40) or during the challenge stage alone (Days 17–40) (Scheme 2). Dex, as positive control, was administered *via* IP injections (1 mg/kg) 0.5 h prior to each challenge by OVA aerosol. Each group

contained 8–10 mice excluding the Saline-CP group, which included 5 mice.

(ii) HDM model. Balb/C mice aged 8–10 weeks were sensitized and challenged with HDM, which was delivered into the airways by intranasal administration (IN) of 60 µL saline solution containing 100 µg allergens. The mice were sensitized on Days 1–4 and challenged on Days 10, 11, 14 and 15 while animals of the control group were sensitized and challenged by saline alone. The experiment ended on Day 16. The effect of 3-CP was investigated by feeding the mice *ad libitum* diet of nitroxide-containing chow (1% (w/w)) during both HDM-induced sensitization and challenge stages (Days 1–16) or during the challenge period alone (Days 8–16). Each group contained 8–10 mice excluding the Saline group, which included 5 mice.

### 2.3. Assessment of airway hyperresponsiveness (AHR)

At the end of the experiment, mice were anesthetized using ketamine/xylazine, tracheostomized, and ventilated with a FlexiVent (SCIREQ, Montreal, Quebec, Canada). After baseline determination of airway resistance, mice were challenged by methacholine (0–64 mg/mL) nebulized directly into the ventilatory circuit using an AeroNebLab nebulizer (SCIREQ). Two models of respiratory mechanics were used to assess lung resistance (*R*), lung elastance (*E*) and lung compliance (*C*), namely the linear first-order single compartment model and the constant-phase model. All data points were collected using FlexiVent software (SCIREQ). Results were expressed as relative change in *R/C/E* over baseline values.

### 2.4. Analysis of the bronchoalveolar lavage fluid (BALf)

BALf was centrifuged at 1000 rpm for 10 min and the supernatants were stored at –20 °C for further examination. Cell pellets were resuspended in 1 mL saline and aliquots of 100 µL were sampled for cytospin preparations using a Shandon cytospin (Thermo Fisher Scientific, Waltham, MA, USA), and stained by Diffquik stain (Dade Diagnostica, Munich, Germany). A total of 200 cells were counted and classified for each slide. Total BALf cells were counted using a Newbauer Chamber (Marienfeld, Lauda-Königshofen, Germany). Results are expressed as number of cells per mL of BALf.

### 2.5. Lung collagen levels

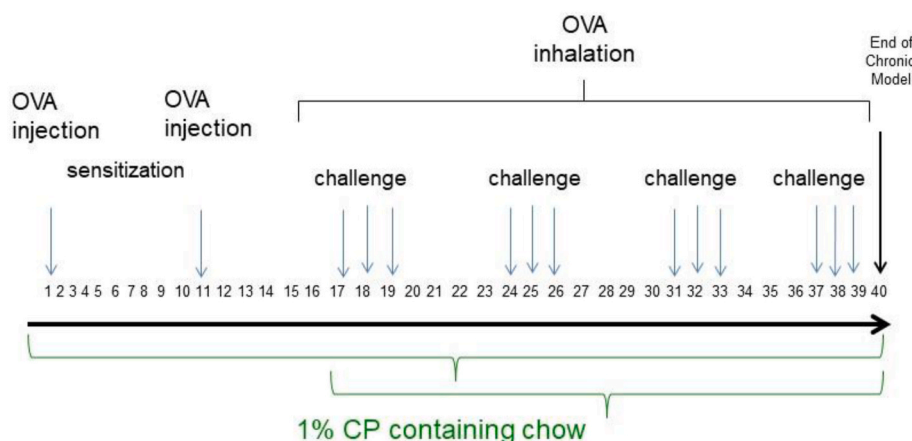
Soluble collagen content was determined using commercially available Sircol collagen assay kit (Biocolor, Ireland). Briefly, the lung tissue was processed overnight with pepsin, collagen precipitated and the results are presented as µg collagen per 50 mg tissue.

### 2.6. TGF-β and protein nitration

TGF-β levels in BALf and in lung tissue were measured by ELISA using commercially available kits (R&D Systems, Minneapolis, MN). Protein nitration assay in lung tissue was carried out using a commercially available kit (Abcam, USA) according to manufacturer recommendations. Lung tissue was homogenized in ELISA buffer, centrifuged and supernatant was used for examination. BALf measurements were performed without any additional modification.

### 2.7. Histology

The left lung lobe was fixed by intrabronchial infusion with 4% formaldehyde. Cross-sections were prepared from the lobe of the lung, and were stained with hematoxylin & eosin (H&E) and Periodic acid-Schiff to evaluate inflammation and mucus producing cells, respectively. For Masson’s Trichrome staining, the sections were stained with Masson’s Trichrome staining Kit (ScyTek Laboratories, Inc.) according to the manufacturer’s instructions. The degree of inflammation and



**Scheme 2.** Schematic overview of 3-CP administration protocols.

amount of mucus producing epithelial cells were graded on a numeric scale of 0–5.

## 2.8. Data analysis

Means and standard error of mean (SEM) values are given for each group. Either one-way or two-way ANOVA followed by Bonferroni posttest were used to compare groups.  $P < 0.05$  was considered statistically significant.

## 3. Results

All classes of nitroxides and most of their respective hydroxylamines possess antioxidant properties [23,24]. In our study of allergic asthma, 3-CP demonstrated a better protective efficacy than TEMPOL [21] and 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl (unpublished results), and therefore our research was narrowed down to 3-CP in the present chronic asthma model.

### 3.1. “Chronic” asthma with airway remodeling

Sensitization and subsequently challenge of the mice by OVA induce hallmark features of allergic asthma, including production of antigen-specific IgE, mucous hypersecretion, airway hyperresponsiveness (AHR) and eosinophilic infiltration. In long-term exposure models, prolonged OVA challenge also induces features of airway wall remodeling (e.g. thickening of the basement membrane, fibrosis and epithelial cell hyperplasia and hypertrophy) [25]. Two sets of experiments were carried out where (i) 3-CP was administered orally during the entire period (S&C) and (ii) only during the challenge period where the effect of 3-CP was compared to that of Dexamethasone.

#### 3.1.1. BALf cell counts

BALf and bronchial submucosal inflammation are characteristic features of asthma. OVA inhalation markedly increased total cell and eosinophil counts compared with those in mice treated by aerosol of saline alone, i.e.,  $(3.99 \pm 0.25) \times 10^5$  vs.  $(2.30 \pm 0.19) \times 10^4$  cells/mL and  $(2.05 \pm 0.23) \times 10^5$  vs. zero cells/mL, respectively (Fig. 1). Oral administration of 3-CP attenuated the increase in the total inflammatory cell count when delivered during the sensitization and challenge  $((1.38 \pm 0.11) \times 10^5$  cells/mL) or during the challenge phase alone  $((2.12 \pm 0.27) \times 10^5$  cells/mL) and specifically eosinophil count in BALf, i.e.,  $(3.11 \pm 0.66) \times 10^4$  and  $(1.22 \pm 0.25) \times 10^5$  cells/mL, respectively (Fig. 1). The efficacy of 3-CP delivered during the entire period (S&C) was significantly higher than that achieved by 3-CP delivered during the challenge stage alone (C). Dexamethasone led to partial attenuation of the increase in inflammatory cell counts in BALf in a manner similar to that seen

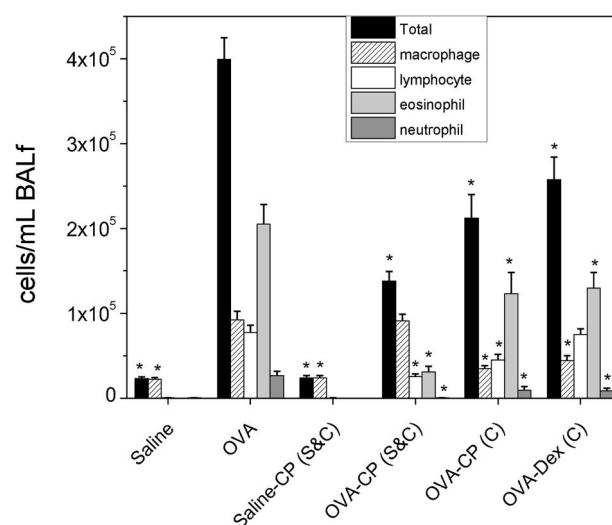


**Fig. 1.** Inflammatory cell counts in mice sensitized and challenged by OVA without and with 3-CP administered orally (1% w/w) either during the sensitization and challenge periods (S&C) or during the challenge stage alone (C). Dex was delivered via IP injection (1 mg/kg) 0.5 h prior to each challenge by OVA. Saline represents untreated mice and Saline-CP represents mice that underwent inhalation of saline together with orally delivered drug. Values presented are mean  $\pm$  SEM. \* $p < 0.05$  compared to OVA group.

when 3-CP was delivered during the challenge state alone  $((2.57 \pm 0.27) \times 10^5$  total cells/mL,  $(1.30 \pm 0.18) \times 10^5$  eosinophil cells/mL) (Fig. 1).

#### 3.1.2. Effect of 3-CP on lung histopathology

Upon histopathological examination, intense inflammation accompanied with infiltrating eosinophils and mononuclear cells surrounding the bronchi was evident in the lungs of asthmatic mice, and this was greatly diminished by the oral administration of 3-CP delivered through the entire experiment period (Fig. 2). There was no significant effect on periodic acid-schiff for mice treated with 3-CP and Dex (results not shown).

#### 3.1.3. Pulmonary function tests

OVA-CP mice showed a statistically significant improvement vs. OVA mice for measures of lung resistance, lung compliance and lung elastance in response to methacholine (Fig. 3).

#### 3.1.4. Biochemical assessment of soluble collagen content

Airway remodeling constitutes subepithelial fibrosis, deposition of

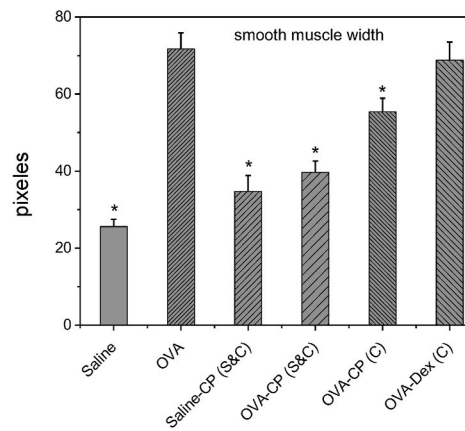
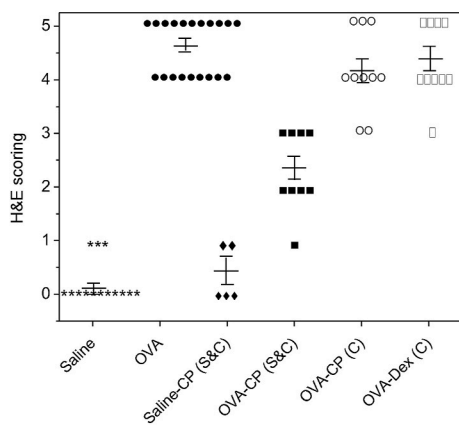


**Fig. 2.** Effect of 3-CP on H&E staining and smooth muscle width (tri-chrome) of lung tissue in mice sensitized and challenged by OVA without and with 3-CP administered orally (1% w/w) either during the challenge stage (C) or during both sensitization and challenge stages (S&C). Dex was delivered via IP injection (1 mg/kg) 0.5 h prior to each challenge by OVA. Saline represents untreated mice and Saline-CP represents mice that received inhaled saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p$  < 0.05 compared to OVA group.

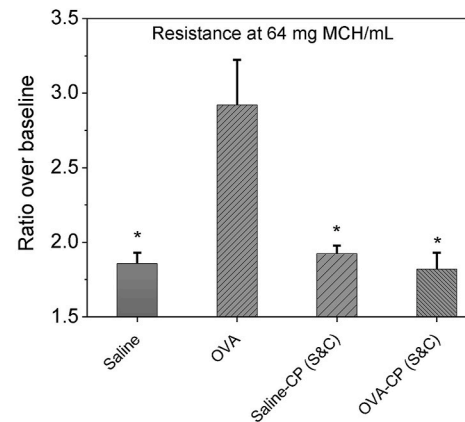
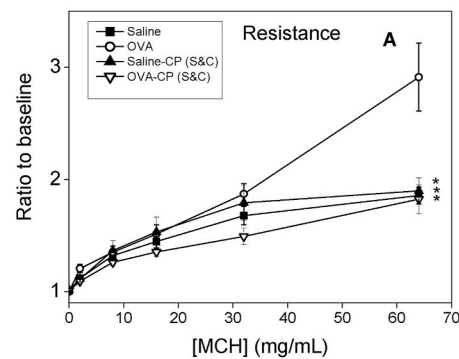
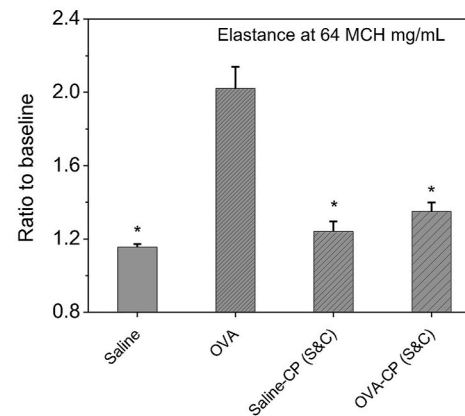
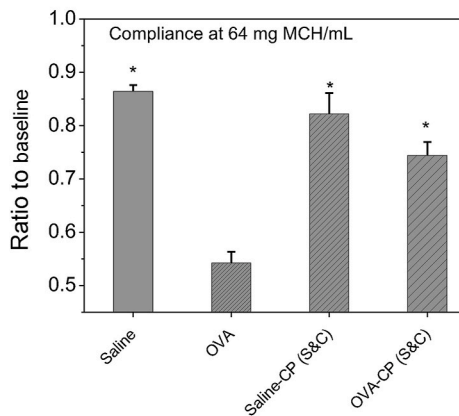


**Fig. 3.** Baseline Pulmonary function tests. Resistance in response to methacholine (MCH) (A), resistance in response to 64 mg MCH/mL (B), elastance in response to 64 mg MCH/mL (C), compliance in response to 64 mg MCH/mL (D) all measured in mice sensitized and challenged by OVA with and without 3-CP administered orally (1% w/w) during the entire period (S&C). Saline represents untreated mice and saline-CP represents mice that underwent inhalation of saline together with oral 3-CP drug for baseline drug effect. Values are mean  $\pm$  SEM. \* $p$  < 0.05 compared to OVA groups.



extracellular matrix protein, goblet cell hyperplasia, mucus gland and smooth muscle hypertrophy, and epithelial damage where inflammatory pathways regulate all such features [26]. Airway remodeling is associated with the accumulation of extracellular matrix proteins, such as collagens. Lung collagen content was lower in mice receiving 3-CP compared to OVA group when 3-CP was delivered either during the challenge stage or during both sensitization and challenge stages (Fig. 4).

### 3.1.5. TGF- $\beta$ levels

TGF- $\beta$ , which is an important mediator involved in fibrogenesis and tissue remodeling within the asthmatic lung, also plays a central role in the complex relationship between the propagation and dampening of inflammation. The latter occurs *via* multiple mechanisms, including

enhanced suppressor T cell immune function. The possibility of targeting TGF- $\beta$  as a therapeutic option remains controversial [27]. TGF- $\beta$  levels in BALf were significantly lower in OVA-CP mice compared to those in Saline and Saline-CP groups (Fig. 5). The effect of 3-CP delivered during the entire period was significantly greater than that of 3-CP delivered during the challenge stage alone, which was similar to that of Dex. OVA increased somewhat the TGF- $\beta$  levels in lung tissue but had no significant effect on TGF- $\beta$  levels among all treated mice groups.

### 3.1.6. Protein nitration levels

Lung homogenates were assayed for 3-nitrotyrosine (3-NT) levels. OVA challenge significantly increased pulmonary levels of 3-NT compared to those in mice of saline control group without and with 3-CP. The drug administered during the entire experiment (S&C) or

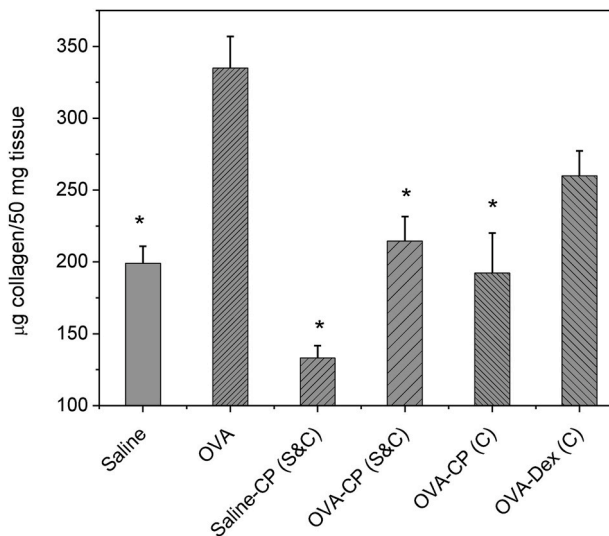


**Fig. 4. Collagen in Lung tissue.** Collagen content in lung tissue of mice sensitized and challenged by OVA without and with 3-CP administered orally (1% w/w) either during the sensitization and challenge periods (S&C) or during the challenge stage alone (C). Dex was delivered via IP injection (1 mg/kg) 0.5 h prior to each challenge by OVA. Saline represents untreated mice and Saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to OVA group.

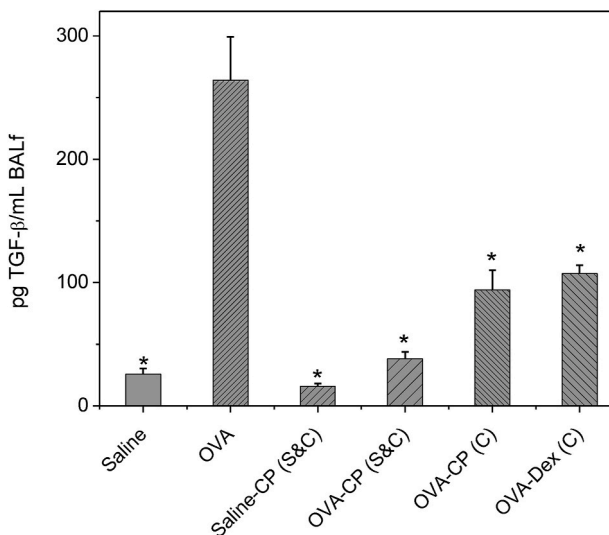


**Fig. 5. TGF-β levels in BALF** in mice sensitized and challenged by OVA without and with 3-CP administered orally (1% w/w) either during the sensitization and challenge periods (S&C) or during the challenge stage alone (C). Dex was delivered via IP injection (1 mg/kg) 0.5 h prior to each challenge by OVA. Saline represents untreated mice and Saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to OVA group.

during the challenge phase alone (C) markedly suppressed 3-NT levels compared to those in mice of the control groups (Fig. 6). Surprisingly, treatment with Dex also reduced the levels of 3-NT (Fig. 6), although in previous studies, it had no significant effect in mouse model of allergic asthma [21].

### 3.2. HDM model

Murine models of HDM-induced asthma more closely approximate the allergic response seen in human asthma than do models using OVA.

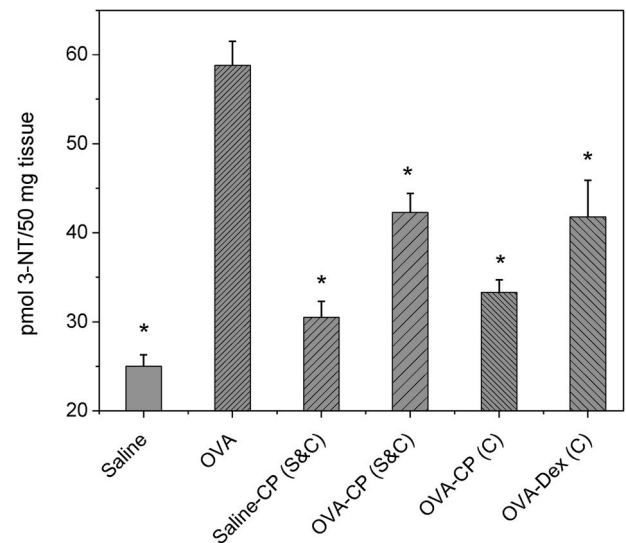


**Fig. 6. 3-NT levels in lung tissue** in mice sensitized and challenged by OVA without and with 3-CP administered orally (1% w/w) either during the sensitization and challenge periods (S&C) or during the challenge stage alone (C). Dex was delivered via IP injection (1 mg/kg) 0.5 h prior to each challenge by OVA. Saline represents untreated mice and Saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to OVA group.

The HDM mouse model is also associated with a mixed eosinophilic and neutrophilic inflammatory response seen in patients with severe asthma. Using this model, we have tested the effect of oral administration of 3-CP on BALF cell counts (Fig. 7). Total cell counts were significantly increased in response to HDM as compared to the saline group ( $(8.31 \pm 1.30) \times 10^5$  vs.  $(2.64 \pm 0.51) \times 10^4$  cells/mL). Treatment with 3-CP both during sensitization and challenge with HDM ( $(3.80 \pm 0.48) \times 10^5$  cells/mL) or during the challenge phase ( $(4.97 \pm 1.22) \times 10^5$  cells/mL), reduced total cell counts relative to that induced by HDM alone. Absolute number of lymphocytes were also significantly reduced in mice treated by HDM plus 3-CP delivered during the challenge stage alone ( $(4.42 \pm 0.47) \times 10^4$  vs.  $(2.17 \pm 0.80) \times 10^4$  cells/mL). Eosinophil counts

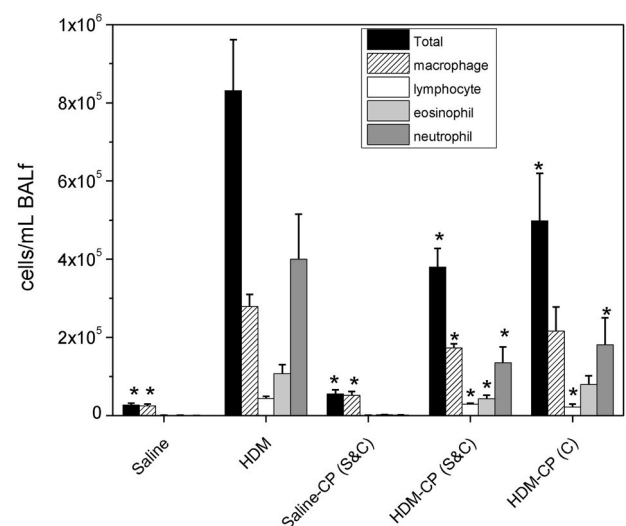


**Fig. 7. Inflammatory cell counts** in mice sensitized and challenged by HDM without and with 3-CP administered orally (1% w/w) either during the challenge period (C) or during both sensitization and challenge periods (S&C). Saline represents untreated mice and Saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to HDM group.

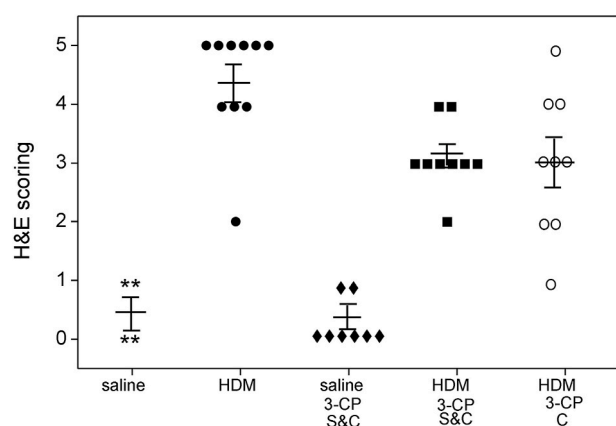


**Fig. 8.** Effect of 3-CP on H&E staining of lung tissue in mice sensitized and challenged by HDM without and with 3-CP administered orally (1% w/w) either during the challenge stage alone (C) or during both sensitization and challenge stages (S&C). Saline represents untreated mice and saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP.  $P < 0.05$  for all groups vs. HDM.

were significantly reduced in mice treated by HDM plus 3-CP administered during sensitization and challenge groups relative to treatment by HDM alone ( $(3.80 \pm 0.48) \times 10^5$  vs.  $(4.25 \pm 0.96) \times 10^4$  cells/mL). Although not statistically significant, eosinophil counts were also reduced in the HDM-CP during challenge-only group ( $(1.07 \pm 0.23) \times 10^5$  vs.  $(7.92 \pm 0.22) \times 10^4$ ). Neutrophil counts were significantly increased in response to HDM as compared to saline groups ( $4.00 \pm 1.14) \times 10^5$  vs.  $(1.41 \pm 1.39) \times 10^2$  cells/mL). Treatment with 3-CP both during sensitization and challenge phase ( $(1.35 \pm 0.40) \times 10^5$  cells/mL) or during the

challenge phase only ( $(1.81 \pm 0.69) \times 10^5$  cells/mL), reduced neutrophil counts relative to treatment by HDM alone.

Histological changes evident upon H&E staining of lung tissue are shown in Fig. 8. Peribronchial inflammation was significantly reduced in mice treated with 3-CP for both sensitization and challenge and during the challenge phase alone compared to mice treated by HDM alone.

The effect of 3-CP on various parameters of AHR are shown in Fig. 9. AHR is increased more dramatically in the HDM model than in the chronic OVA model due to the mouse strain used as well as the allergen. AHR was reduced to levels seen with saline when 3-CP was delivered during the entire period or during the challenge phase only.

TGF- $\beta$  levels in BALf were increased in response to HDM but increased less in groups receiving 3-CP (Fig. 10). HDM did not induce significant increase of TGF- $\beta$  levels in lung tissue and there was no significance difference in TGF- $\beta$  levels within lung tissue among all mice experimental groups (data not shown).

The results demonstrate that the efficacy of 3-CP as a preventive drug (delivered during the sensitization and challenge stages) or as a therapeutic drug (delivered during the challenge stage alone) are similar.

#### 4. Discussion

The results of the present study using two murine models of “severe asthma” demonstrate that 3-CP reduces inflammation, AHR, parameters of airway remodeling and protein nitration in lung tissue.

Severe asthma is defined as asthma, which requires treatment with guidelines suggested medications for GINA (Global initiative for Asthma) steps 4–5 asthma (high dose ICS and second controller) for the previous year or systemic corticosteroids for 50% of the previous year to prevent it from becoming “uncontrolled” or which remains uncontrolled despite this therapy [28]. There have been significant advances

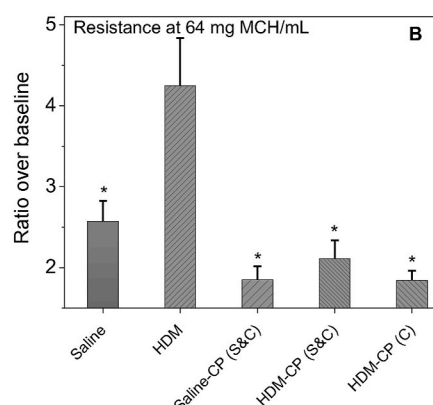
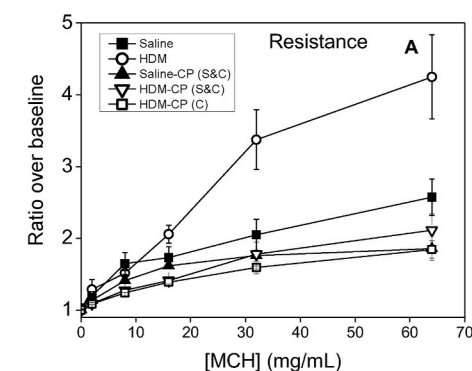
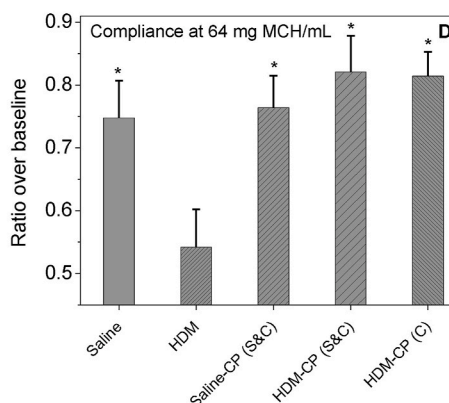
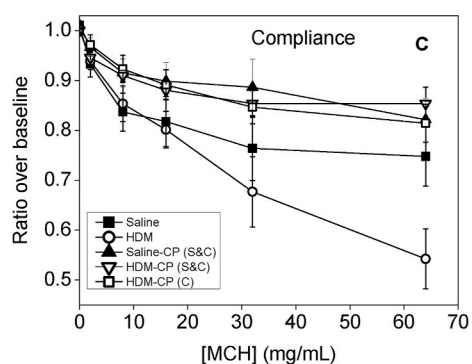


**Fig. 9.** Baseline Pulmonary function tests. Resistance (A) and compliance (C) in response to methacholine (MCH), resistance in response to 64 mg MCH/mL (B) and compliance in response to 64 mg MCH/mL (D) all measured in mice sensitized and challenged by HDM with and without 3-CP administered orally (1% w/w) either during the challenge stage (C) or during both sensitization and challenge stages (S&C). Saline represents untreated mice and saline-CP represents mice that received inhaled saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to HDM group.



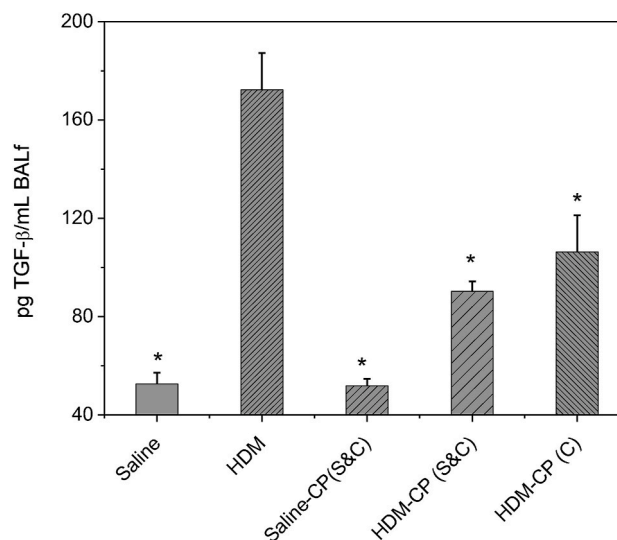


**Fig. 10.** TGF- $\beta$  levels in BALF in mice sensitized and challenged by HDM with and without 3-CP administered orally (1% w/w) either during the challenge stage (C) or during both sensitization and challenge stages (S&C). Saline represents untreated mice and saline-CP represents mice that underwent inhalation of saline together with orally delivered 3-CP. Values are mean  $\pm$  SEM. \* $p < 0.05$  compared to HDM group.

in the understanding of the molecular mechanisms of severe asthma, and, in particular, in the characterization of different “endotypes” of severe asthma patients. The “T2-high” asthma endotype is characterized by elevated T2 inflammatory markers (eosinophilia, elevated exhaled nitric oxide, markers of atopy, elevated IL-4, IL-5, IL-13). There are five monoclonal antibody therapies now approved by the FDA for the treatment of severe T2-high asthma patients [29]. “T2-low” asthma is much more poorly characterized and no biological therapy has been approved for this condition. T2-low asthma is a heterogeneous entity and includes patients with “neutrophilic” asthma and “pauci-cellular” asthma, in which airway remodeling may be the predominant feature [30]. “T2-low” asthmatics may often be refractory to corticosteroids and the development of novel and effective therapeutic options for patients who suffer from this condition remains a major unmet medical need [31–33].

Despite justified reservations regarding the relevance of animal models of asthma to human disease, the use of murine models has been key to understanding the molecular biology of asthma and to development of new therapies [34–36]. The most widely used model is the OVA model, in which eosinophilic inflammation is the predominant feature. We have previously shown that 3-CP attenuates airway inflammation and AHR in this model [21]. Several models of severe/refractory asthma are in use. Prolonged exposure to OVA induces airway remodeling changes including increased airway smooth muscle and extracellular matrix deposition. The HDM asthma model is characterized by a mixed neutrophilic and eosinophilic inflammatory response, considered less responsive to corticosteroid therapy. 3-CP was effective for both models, raising the possibility that this drug may be effective in patients with both pauci-cellular and neutrophilic asthma. We have previously shown that 3-CP attenuates pulmonary fibrosis in a bleomycin mouse model of lung fibrosis. The protective effect was at least as great as that provided by nintedanib, a drug approved by the FDA for the treatment of pulmonary fibrosis [21]. The efficacy of 3-CP in the prolonged OVA exposure murine model may be via attenuation of airway remodeling and extra cellular matrix deposition in the airways.

As regards the HDM model, treatment with 3-CP markedly reduced the induced increase in number of both eosinophils and neutrophils in BALF. While treatment with corticosteroids is extremely effective in reducing eosinophilic inflammation, they are ineffective in reducing

neutrophilic inflammation. The mechanism whereby 3-CP reduces neutrophilic inflammation is not known but suggests that 3-CP may be a better option for the treatment of neutrophilic asthma.

There is extensive evidence that reactive oxygen and nitrogen species play a role in multiple pathophysiological features of asthma, including enhanced airway inflammation, cell proliferation, enhanced smooth muscle contraction and airway AHR [37]. Oxidative stress is a major contributing factor to resistance to steroid therapy [38].

TGF- $\beta$  is a key modulator in the pathogenesis of pulmonary fibrosis in IPF and of airway remodeling in asthma. This cytokine is produced by several cell types in the airways and lungs, including eosinophils, fibroblasts, smooth muscle and airway epithelial cells. TGF- $\beta$  expression is increased in response to oxidative stress, this interaction is bidirectional and has been described in multiple cell types and disease states [39]. In the present study, 3-CP reduced expression of TGF- $\beta$  in both asthma models, and in a previous study, we found a similar reduction in TGF- $\beta$  by 3-CP in bleomycin-induced pulmonary fibrosis [40–42].

Identification of the specific molecular targets, proteins or lipids, that are protected from oxidative or nitrative damage by treatment with 3-CP is the focus of further investigation that will help clarify which molecules are most relevant in the pathogenesis of severe asthma.

Evidently, 3-CP is effective not only when given with allergen sensitization, but even when given after sensitization, at the time of allergen challenge. This makes 3-CP a more realistic and promising drug option for asthmatic patients, in whom treatment is obviously initiated only when disease is already established.

The present study shows that 3-CP is effective in attenuating disease in two different models of “severe asthma” characterized by both airway remodeling and neutrophilic airway inflammation. This observation extends and strengthens our previous findings showing that 3-CP is effective in a classic OVA murine asthma model, and in a bleomycin-induced pulmonary fibrosis model [21,22]. This agent thus combines a broad anti-inflammatory effect together with anti-fibrotic activity suggesting that 3-CP may be a promising therapeutic option for patients with severe asthma, regardless of molecular endotype.

## References

- [1] J. Bousquet, P.K. Jeffery, W.W. Busse, M. Johnson, Asthma - from bronchoconstriction to airways inflammation and remodeling, *Am. J. Respir. Crit. Care Med.* 161 (1990) 1720–1745.
- [2] R.M. Locksley, Asthma and allergic inflammation, *Cell* 140 (2010) 777–783.
- [3] H.Y.D. Kim, R. H. D.T. Umetsu, The many paths to asthma: phenotype shaped by innate and adaptive immunity, *Nat. Immun.* 11 (2010) 577–584.
- [4] M. Hew, K.F. Chung, Corticosteroid insensitivity in severe asthma: significance, mechanisms and aetiology, *Intern. Med. J.* 40 (2010) 323–334.
- [5] D. Bagnasco, M. Ferrando, G. Varricchi, F. Puggioni, G. Passalacqua, G. W. Canonica, Anti-interleukin 5 (IL-5) and IL-5Ra biological drugs: efficacy, safety, and future perspectives in severe eosinophilic asthma, *Front. Med.* 4 (135) (2017).
- [6] P.M. Hansbro, R.Y. Kim, M.R. Starkey, C. Donovan, K. Dua, J.R. Mayall, G. Liu, N. G. Hansbro, J.L. Simpson, L.G. Wood, J.A. Hirota, D.A. Knight, P.S. Foster, J. C. Horvat, Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma, *Immunol. Rev.* 278 (2017) 41–62.
- [7] E. Kostakou, E. Kaniaris, E. Filiou, I. Vasileiadis, P. Katsaounou, E. Tzortzaki, N. Koulouris, A. Koutsoukou, N. Rovina, Acute severe asthma in adolescent and adult patients: current perspectives on assessment and management, *J. Clin. Med.* 8 (2019) 1283.
- [8] P. Rogliani, L. Calzetta, M.G. Matera, R. Laitano, B.L. Ritondo, N.A. Hanania, M. Cazzola, Severe asthma and biological therapy: when, which, and for whom, *Pulmonary Therapy* 6 (2020) 47–66.
- [9] G. Hillas, E. Fouka, A.I. Papaioannou, Antibodies targeting the interleukin-5 signaling pathway used as add-on therapy for patients with severe eosinophilic asthma: a review of the mechanism of action, efficacy, and safety of the subcutaneously administered agents, mepolizumab and benralizumab, *Expert Rev. Respir. Med.* 14 (2020) 353–365.
- [10] M. Caminati, B. Polk, L.J. Rosenwasser, What have recent advances in therapy taught us about severe asthma disease mechanisms? *Expert Rev. Clin. Immunol.* 15 (2019) 1145–1153.
- [11] H. Sugiura, M. Ichinose, Oxidative and nitrative stress in bronchial asthma, *Antioxidants Redox Signal.* 10 (2008) 785–798.
- [12] L. Zuoa, N.P. Otenbakera, B.A. Rosea, K.S. Salisburaya, Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma, *Mol. Immunol.* 56 (2013) 57–63.

- [13] V. Mishra, J. Banga, P. Silveyra, Oxidative stress and cellular pathways of asthma and inflammation: therapeutic strategies and pharmacological targets, *Pharmacol. Ther.* 181 (2018) 169–182.
- [14] V.L. Kinnula, C.L. Fattman, R.J. Tan, T.D. Oury, Oxidative stress in pulmonary fibrosis, *Am. J. Respir. Crit. Care Med.* 172 (2005) 417–422.
- [15] A.G. Fois, P. Paliogiannis, S. Sotgia, A.A. Mangoni, E. Zinellu, P. Pirina, C. Carru, A. Zinellu, Evaluation of oxidative stress biomarkers in idiopathic pulmonary fibrosis and therapeutic applications: a systematic review, *Respir. Res.* 19 (2018) 51–64.
- [16] A. Di Stefano, M. Maniscalco, B. Balbi, F.L.M. Ricciardolo, Oxidative and nitrosative stress in the pathogenesis of obstructive lung diseases of increasing severity, *Curr. Med. Chem.* 27 (2020) 7149–7158.
- [17] I. Rahman, Oxidative Stress and Gene Transcription in asthma and chronic obstructive pulmonary disease: antioxidant therapeutic targets, current drug targets, *Inflammation & Allergy* 1 (2002) 291–315.
- [18] P. Kirkham, I. Rahman, Investigating antioxidant therapy for steroid-resistant asthma, *Eur. Respir. J.* 48 (suppl 60) (2016) PA570.
- [19] J. Pinkerton, R. Kim, A.-T. Essilfie, B. Rae, J. Mayall, M.K. Ali, M. Starkey, L. Wood, S. Biswal, J. Horvat, P. Hansbro, Investigating antioxidant therapy for steroid-resistant asthma, *Eur. Respir. J.* 48 (suppl 60) (2016) PA570.
- [20] T. Victoni, E. Barreto, V. Lagente, V.F. Carvalho, Oxidative imbalance as a crucial factor in inflammatory lung diseases: could antioxidant treatment constitute a new therapeutic strategy? *Oxidative Med. Cell. Longev.* 2021 (2021) 11.
- [21] M. Assayag, S. Goldstein, A. Samuni, N. Berkman, Cyclic nitroxide radicals attenuate inflammation and Hyper-responsiveness in a mouse model of allergic asthma, *Free Radic. Biol. Med.* 87 (2015) 148–156.
- [22] M. Assayag, S. Goldstein, A. Samuni, N. Berkman, 3-Carbamoyl-proxyl nitroxide radicals attenuate bleomycin-induced pulmonary fibrosis in mice, *Free Radic. Biol. Med.* 171 (2021) 135–142.
- [23] B.P. Soule, F. Hyodo, K.-i. Matsumoto, N.L. Simone, J.A. Cook, M.C. Krishna, J. B. Mitchell, The chemistry and biology of nitroxide compounds, *Free Radic. Biol. Med.* 42 (2007) 1632–1650.
- [24] A. Samuni, S. Goldstein, Hydroxylamines inhibit tyrosine oxidation and nitration: the role of their respective nitroxide radicals, *Free Radic. Biol. Med.* 160 (2020) 837–844.
- [25] S. Maltby, H.L. Tay, M. Yang, P.S. Foster, Mouse models of severe asthma: understanding the mechanisms of steroid resistance, tissue remodelling and disease exacerbation, *Respirology* 22 (2017) 874–885.
- [26] D.H. Broide, Immunologic and inflammatory mechanisms that drive asthma progression to remodeling, *J. Allergy Clin. Immunol.* 121 (2008) 560–570.
- [27] M. Al-Alawi, T. Hassan, S.H. Chotirmall, Transforming growth factor beta and severe asthma: a perfect storm, *Respir. Med.* 108 (2014) 1409–1423.
- [28] K.F. Chung, S.E. Wenzel, J.L. Brozek, A. Bush, M. Castro, P.J. Sterk, I.M. Adcock, E. D. Bateman, E.H. Bel, E.R. Bleecker, L.-P. Boulet, C. Brightling, P. Chaney, S.-E. Dahlen, R. Djukanovic, U. Frey, M. Gaga, P. Gibson, Q. Hamid, N.N. Jajour, T. Mauad, R.L. Sorkness, W.G. Teague, International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma, *Eur. Respir. J.* 43 (2014) 343–373.
- [29] M.C. McGregor, J.G. Krings, P. Nair, M. Castro, Role of biologics in asthma, *Am. J. Respir. Crit. Care Med.* 199 (2019) 433–445.
- [30] J.L. Simpson, R. Scott, M.J. Boyle, P.G. Gibson, Inflammatory subtypes in asthma: assessment and identification using induced sputum, *Respirology* 11 (2006) 54–61.
- [31] E. Sze, A. Bhalla, P. Nair, Mechanisms and therapeutic strategies for non-T2 asthma, *Allergy* 75 (2020) 311–325.
- [32] O. Kalchiem-Dekel, X. Yao, S.J. Levine, Meeting the challenge of identifying new treatments for type 2-low neutrophilic asthma, *Chest* 157 (2020) 26–33.
- [33] C. Kyriakopoulos, A. Gogali, K. Bartzikos, K. Kostikas, Identification and treatment of T2-low asthma in the era of biologics, *ERJ Open Res* 7 (2021), 00309-2020.
- [34] P.C. Fulkerson, M.E. Rothenberg, S.P. Hogan, Building a better mouse model: experimental models of chronic asthma, *Clin. Exp. Allergy* 35 (2005) 1251–1253.
- [35] J.H. Bates, M. Rincon, C.G. Irvin, Animal models of asthma, *Am. J. Physiol. Lung Cell Mol. Physiol.* 297 (2009) L401–L410.
- [36] S. Sagar, H. Akbarshahi, L. Uller, Translational value of animal models of asthma: challenges and promises, *Eur. J. Pharmacol.* 759 (2015) 272–277.
- [37] L.H.C. Vasconcelos, S.R.D. Ferreira, M.d.C.C. Silva, P.B. Ferreira, I.L.L. de Souza, F. d.A. Cavalcante, B.A. da Silva, Uncovering the role of oxidative imbalance in the development and progression of bronchial asthma, *Oxidative Med. Cell. Longev.* (2021) 6692110.
- [38] P.J. Barnes, Corticosteroid resistance in airway disease, *Proc. Am. Thorac. Soc.* 1 (2004) 264–268.
- [39] Y. Cui, J. Robertson, S. Maharaj, L. Waldhauser, J. Niu, J. Wang, L. Farkas, M. Kolb, J. Gauldie, Oxidative stress contributes to the induction and persistence of TGF- $\beta$ 1 induced pulmonary fibrosis, *Int. J. Biochem. Cell Biol.* 43 (2011) 1122–1133.
- [40] R.M. Liu, K.A. Gaston Pravia, Oxidative stress and glutathione in TGF- $\beta$ -mediated fibrogenesis, *Free Radic. Biol. Med.* 48 (2010) 1–15.
- [41] I. Montorfano, A. Becerra, R. Cerro, C. Echeverría, E. Sáez, M.G. Morales, R. Fernández, C. Cabello-Verrugio, F. Simon, Oxidative stress mediates the conversion of endothelial cells into myofibroblasts via a TGF- $\beta$ 1 and TGF- $\beta$ 2-dependent pathway, *Lab. Invest.* 94 (2014) 1068–1082.
- [42] H.Y. Chen, H.C. Chou, Y.J. Ho, S.J. Chang, E.C. Liao, Y.S. Wei, M.W. Lin, Y. S. Wang, Y.A. Chien, X.R. Yu, H.Y. Kung, C.C. Yang, J.Y. Chen, H.L. Chan, M.L. Ko, Characterization of TGF- $\beta$  by induced oxidative stress in human trabecular meshwork cells, *Antioxidants* 10 (2021) 107.