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Oxidative stress and anti-oxidant response in allergen, virus, and corticosteroids withdrawal-induced asthma exacerbation.

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By

Caterina Folisi

Supervisor: Prof. G. U. Di Maria

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Preface

The experiments included in the current thesis have been performed during my appointment as PhD student in Molecular Medicine from November 2010 to November 2014 at:

- Dipartimento di Bio-medicina Clinica e Molecolare Sezione Malattie Respiratorie Università di Catania Ospedale Garibaldi-Nesima, Catania, Italia,
- Dept. of Respiratory Medicine, Academic Medical Centre (AMC), University of Amsterdam, Amsterdam, the Netherlands;
- Centre for Proteomic Research, Life Sciences Building, University of Southampton, United Kingdom.

Under supervision of:

- Professor Giuseppe Di Maria (Dipt. di Bio-medicina Clinica e Molecolare, University of Catania) and Professor Riccardo Polosa (Dipt. di Medicina Interna e Immunologia Clinica, University of Catania),
- Professor Peter Sterk and Dr. Rene Lutter (Dept. of Respiratory Medicine, Univeristy of Amsterdam);
- Professor Ratko Djukanovic and Dr. Paul Skipp (Dept. of Respiratory Medicine and Proteomics Centre, University of Southampton).

This thesis is based on the following manuscripts, which are under revision:

- *"Enhanced oxidative stress and reduced anti-oxidative capacity of airway macrophages during rhinovirus 16-induced asthma exacerbation*." Caterina Folisi, Suzanne M. Bal, Marianne van de Pol, Annemiek Dijkhuis, Koen F. van der Sluijs, Giuseppe Di Maria, Peter J. Sterk and René Lutter.

- *"Local and systemic increased oxidative stress and reduced anti-oxidant capacity In House Dust Mite-induced asthma exacerbations."* Caterina Folisi, Marianne van de Pol, Barbara S. Dierdorp, Jaring van der Zee, Guiseppe U. Di Maria, Peter J. Sterk and René Lutter.

- *"Susceptibility to allergies is associated with inadequate cellular anti-oxidant responses".* Lara U.M. Gouveia, Caterina Folisi, J.H. Akkerdaas, Adrian Logiantar, Marianne A. van de Po, Jaring van der Zee, Esmeralda J.M. Krop, René Lutter, Ronald van Ree, and Leonie van Rijt.

- *"Systemic increased oxidative stress and reduced anti-oxidant capacity In corticosteroids withdrawal-induced asthma exacerbations."* Caterina Folisi, Marianne van de Pol, Barbara S. Dierdorp, Guiseppe U. Di Maria, Peter J. Sterk and René Lutter.

I declare that the work presented in this thesis is my own, and that no part has been submitted for a degree or comparable award of this or any other university or institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Acknowledgements

I have been able to complete this research program with the support and active co-operation of several persons who, now here, I wish to sincerely thank.

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Finally and above all, I would like to express gratitude to my family and friends for their emotional support, care and love throughout these years. I am truly blessed for their invaluable presence in my life.

Abstract

It is now estimated that over than 300 million people of all ages and races, suffer from asthma. The burden of this disease for governments, families, and patients is increasing globally. Asthma is a heterogeneous and complex condition caused by a combination of genetic and environmental factors that result in recurrent, reversible bronchial obstruction. Asthma is characterized by recurrent cough, wheeze, chest tightness, and responsive to bronchodilators. [1] Airway hyper-responsiveness, chronic airway inflammation, remodelling, and mucus hyper-secretion are important features of asthma. Oxidative stress is thought to play a central role in asthma. It occurs when the production of oxidative species overcomes the ability of the biological systems to readily detoxify them or repair the resulting cellular damage. Oxidative stress and a disturbed anti-oxidant status are well established in asthmatics. However, no systematic examination of protein oxidation and anti-oxidant defenses in asthmatics has been performed.

This thesis has been focused on the evaluation of oxidative stress and anti-oxidant response in asthma and during its exacerbation (worsening of symptoms). Specifically, the current thesis was aimed to assess the oxidative consequences of an asthma exacerbation on cellular proteins and to identify anti-oxidant pathways mainly involved in the protective response. The thesis also has had as object the relation between oxidative stress, anti-oxidant status, and asthma symptoms in adult patients. A comprehensive bio-chemical evaluation of oxidative status and anti-oxidant defenses is needed to identify the nature and extent of any possible anti-oxidant deficience or oxidative abnormality during asthma and its exacerbation. A full understanding of the redox control of asthma exacerbation could support the development of safe and effective therapeutic interventions. The current thesis also aims to highlight gaps in knowledge and potential avenues for further investigation.

The population that participated in the studies included in this thesis consists of 4 groups of asthmatics. The first group of nine mild asthmatics was challenged with Rhinovirus-16 in order to cause a virus-induced asthma exacerbation. The second group included twenty allergic asthmatics exposed to House Dust Mite (HDM) in order to provoke an allergen-induced asthma. The third group was composed of thirty-seven

laboratory animal workers exposed to occupational allergens from rodents over a period of two years; some of them did become allergic. The fourth group included twenty-three asthmatics under corticosteroids treatment whose withdrawal caused the asthma exacrbation on-set. In vivo, ex-vivo, and in-vitro experiments have been performed in different settings and with different purposes in order to elucidate the relation of oxidative status and asthma exacerbation. Protein oxidation has been evaluated as stable bio-marker of oxidative stress and the expression level was measured for several anti-oxidant and cyto-protective proteins in plasma and induced sputum from asthmatics. Pro-inflammatory mediator production has been also determined.

Patients during asthma exacerbation, as expected, showed higher level of oxidative stress. Interestingly, patients during an exacerbation were also more susceptible to oxidative protein damage; this was associated with a reduced anti-oxidant capacity, reduced nuclear translocation of the main anti-oxidant transcription factor, and enhanced pro-inflammatory mediator production. Furthermore, baseline levels of oxidative stress were able to predict which patients were more prone to develop exacerbation symptoms. Taken together these results suggest that enhancing local anti-oxidant mechanisms in asthmatics may attenuate airway inflammation and the exacerbation.

Keywords: Oxidative stress; Anti-oxidant response; Virus-induced asthma exacerbation; Allergen induced asthma exacerbation; Corticosteroids-induced asthma exacerbation; Induced sputum; Allergy; Sensitization; Rhinovirus-16; House Dust Mites (HDM);.Sirtuins; Carbonylation; Lipid peroxidation; Heat Shock Proteins; Acetylation.

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Chapter 1

1. Asthma

Asthma is one of the most prevalent chronic diseases worldwide. This condition is characterized by a complex inter-relation of airflow obstruction, bronchial hyperresponsiveness and airway inflammation.[1] Several aetiological risk factors have been identified for this disease, including genetic and environmental causes. Due to the rapid increase in the prevalence of asthma observed over the past three decades, it can be hypothesized that biological, life style and environmental factors play a role in the susceptibility of individuals. In this first chapter the main pathophysiological and aetiological factors thought to play a role in asthma will be described, as well as its epidemiological, diagnosis and prognosis.

1.1.History

The term Asthma comes from the Greek verb *aazein*, meaning to pant, exhale with open mouth, and sharp breath. Asthma has been already known from ancient Egyptian times. Indeed, the Georg Ebers Papyrus encompasses prescriptions for over 700 remedies for asthma as to heat a mixture of herbs and inhale their fumes.

Hundred years ago it was common in China to treat a person with asthma using herbs containing ephedrine.

It was in the Iliad, a Greek epic poem attributed to Homer, that the expression asthma appeared for the first time. However, the Corpus Hippocraticum is the first manuscript where the term is used as a medical term. Hippocrates assumed that spasms associated to asthma were more expected to occur amongst anglers, tailors and metal-workers. Aretaeus of Cappadocia (100 AD) composed a clinical description of asthma.

Galen (130-200 AD) defined asthma as bronchial obstructions and treated it with owl's blood in wine. Moses Maimonides (1135-1204 AD), the philosopher from Andalucia (Spain), wrote Treatise of Asthma for Prince Al-Afdal. Maimonides showed that his

patient's symptoms often started as a common cold. Eventually the patient gasped for air and coughed until mucus was expelled. Maimonides recommended avoidance of strong medication, plenty of rest, fluids, moderation of sexual activity, and warm soups.

Jean Baptiste Van Helmont (1579-1644 AD), a physician from Belgium, assumed that asthma initiates in the pipes of the lungs. Bernardino Ramazzini (1633-1714 AD), the predecessor of sports medicine, identified a link between asthma and organic dust. He, moreover, recognized and defined the exercise-induced asthma.

At the beginning of the 20th century asthma was considered as a psychosomatic illness with management frequently involving psychoanalysis and 'talking cures'. This psychiatric model was disproved and asthma became recognized as a physical condition. Asthma, as an inflammatory disease, was not really accepted until the 1960s.

1.2. Pathophysiology

Asthma can be considered the result of chronic inflammation of the airways which causes an increase in the contractibility of the surrounding smooth muscles and narrowing of the airway. The constriction is normally reversible. Changes in the airways include an increase in eosinophils and thickening of the lamina reticularis. The airways' smooth muscle can increase in size together with an increase of mucous glands. Cell types involved include: T lymphocytes, macrophages, and neutrophils. There is also the contribution of cytokines, chemokines, histamine, and leukotrienes .[2]The next paragraphs will describe in detail the mechanisms of inflammation, cellular and soluble mediators involved in the pathophysiology of asthma.

1.2.1. Inflammation

The pathophysiology of asthma is characterized by airway inflammation. Indeed, patients with acute asthma have extensively inflamed airways often reddened and swollen. The lumen is obstructed by mucus composed of proteins exuded from airway vessels and secreted from epithelial cells. The airway wall is infiltrated with inflammatory cells, mainly eosinophils and lymphocytes. Broncho Alveolar Lavage

(BAL) from asthmatics has shown an increase in lymphocytes, mast cells, eosinophils and activated macrophages. Biopsies have shown augmented stimulated mast cells, macrophages, eosinophils and T-lymphocytes.[3] These changes are found even in mild asthma. The inflammation in allergic asthma is determined by exposure to allergens through immunoglobulin E (IgE)-dependent mechanisms and is mainly characterized by eosinophils infiltration. Acute inflammatory response is converted into a chronic inflammation which structural consequences. The degree of inflammation is related to airway hyper-responsiveness (AHR), as measured by histamine or methacholine challenge. The severity of AHR in turn is related to asthma symptoms and to the necessity for treatment. Inflammation may increase AHR by stimulation of airway sensory nerve endings (**Fig. 1.1**).

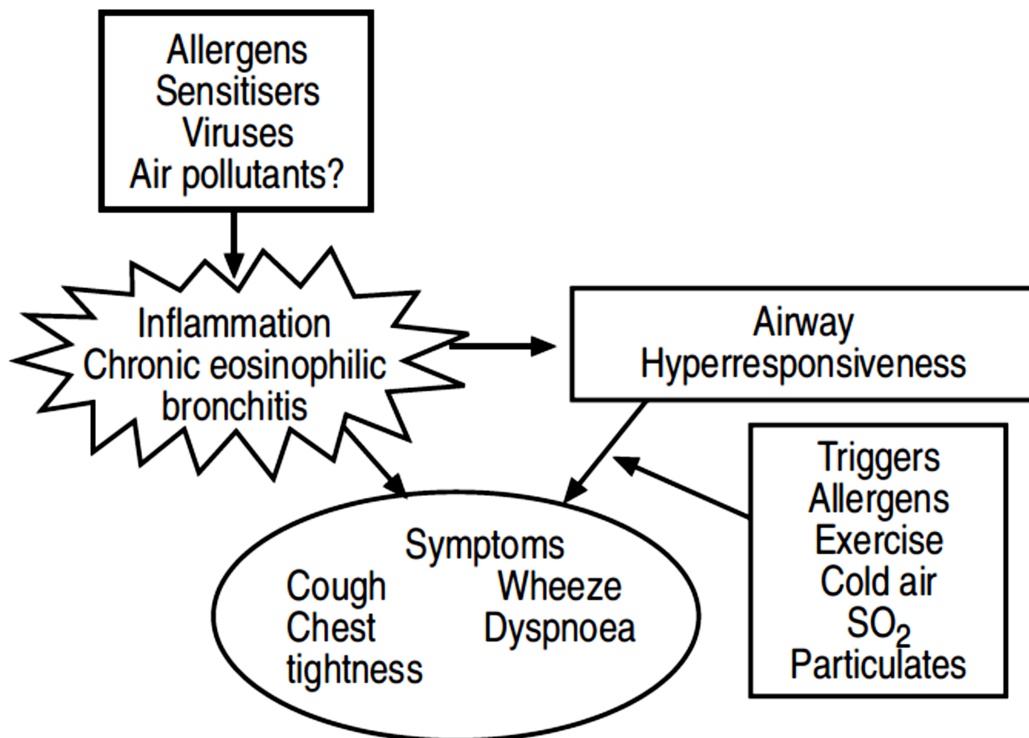


Fig. 1.1 *Inflammation in the airways of asthmatic patients leads to airway hyperresponsiveness and symptoms.* Th2: T-helper 2 cells; SO_2: sulphur dioxide. Source: Pathophysiology of asthma P.J. Barnes.

1.2.1.1. Inflammatory cells involved in asthma

In the inflammatory pathophysiology of asthma are involved different cell types among which the most important are: mast cells, airway T cells, CD (+) (T helper) cells, basophils, macrophages, and eosinophils. In the next paragraphs the role of these cells in asthma pathophysiology will be briefly described.

Mast cells are derived from the myeloid stem cells and contain granules rich in histamine and heparin.[4] Mast cells are important in initiating the acute broncho-constrictor responses to allergens, exercise, hyperventilation, etc. These cells release neurotrophins, pro-inflammatory cytokines, chemokines and growth factors.[5]Asthmatics are characterized by a marked increase in mast cells in airway smooth muscle (ASM).[6] Treatment with prednisone results in a decrease in mast cells.[7] Furthermore, mast cells stimulate human lung fibroblast proliferation.[8] Mast cells secrete interleukin (IL)-4 and tumor necrosis factor (TNF)-α.[9] These cells are activated by an IgE-dependent mechanism. Humanized anti-IgE antibodies inhibit IgE-mediated effects.[10,11] Although this treatment shows marginal improvements in severe steroid-dependent asthma.[12,13]

Macrophages are activated by allergen via low affinity IgE receptors (FceRII).[14,15] Alveolar macrophages have a suppressive effect on lymphocyte function which appears to be reduced after allergen exposure.[16] In asthma the secretion of the anti-inflammatory protein IL-10 is reduced in alveolar macrophages.[17] Macrophages also inhibit the secretion of IL-5 but this is defective in allergic asthmatics.[18] These cells act as antigen-presenting cells to T-lymphocytes.[19] No changes in the macrophage sub-populations in induced sputum of allergic asthmatic have been identified.[20]

Dendritic cells induce a T-lymphocyte mediated immune response[21] acting as antigen-presenting effectors.[22,23] Myeloid dendritic cells promote the differentiation of T-helper (Th) 2 cells[24] and eosinophilia.[25]Immature dendritic cells require cytokines such as IL-12 and TNF-α to promote the normally preponderant Th1 response.[26]

Eosinophils play a cardinal role in asthma. Indeed, allergen inhalation results in a marked increase in eosinophils and there is a correlation between blood eosinophil or

bronchial lavage and AHR. Eosinophils release basic proteins and oxygen-derived free radicals.[27,28] Activated eosinophils induce airway epithelial damage.[29]

Neutrophils are prominent in severe asthma.[30,31,32,33] High doses of corticosteroids inhibit neutrophils' apoptosis.[34,35] When neutrophils are recruited an increase of IL-8 in induced sputum occurs possibly due to the increased oxidative stress. Neutrophilia is also associated with a reduced responsiveness to corticosteroids and acute asthma.

T-lymphocytes release cytokines promoting the recruitment of eosinophils and mast cells.[36,37] The balance between Th1 cells and Th2 cells is determined by locally released cytokines. IL-12 promotes Th1 cells whereas IL-4 or IL-13 favour Th2 cells (**Fig. 1.2**). Steroids effect the balance between IL-12 and IL-13.[38,39] Regulatory T (Tr) cells suppress the immune response through the secretion of IL-10 and transforming growth factor (TGF)-β. [40,41]

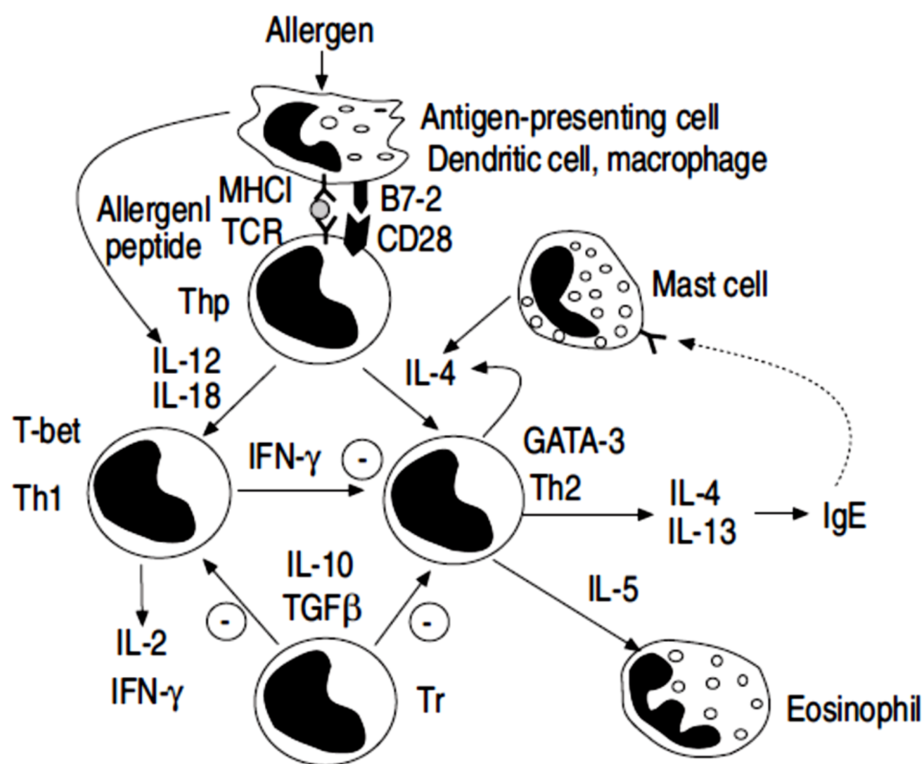


Fig. 1.2 *Asthmatic inflammation is characterised by a preponderance of T-helper (Th) 2 lymphocytes.* The transcription factors T-beta and GATA-3 may regulate the balance between Th1 and Th2 cells. Regulatory T-cells (Tr) have an inhibitory effect. Source: Pathophysiology of asthma P.J. Barnes.

B-lymphocytes secrete IgE.[42] IL-4 is crucial in switching B-cells to IgE production, and CD40 on T-cells is an important signal through interaction with CD40-ligand on B-cells.

Basophils have uncertain role in asthma.[43] An increase in basophils has been documented in the airways of asthmatics after allergen challenge.[44,45]

Platelets fall in circulating after allergen challenge with increased release of the chemokine RANTES.[46,47] Chemokines associated with Th2-mediated inflammation activate and aggregate platelets.[48]

Epithelial cells, endothelial cells, fibroblasts and airway smooth muscle cells are also an important source of inflammatory mediators.[49,50,51,52] Epithelial cells are important target of inhaled glucocorticoids (**Fig. 1.3**).

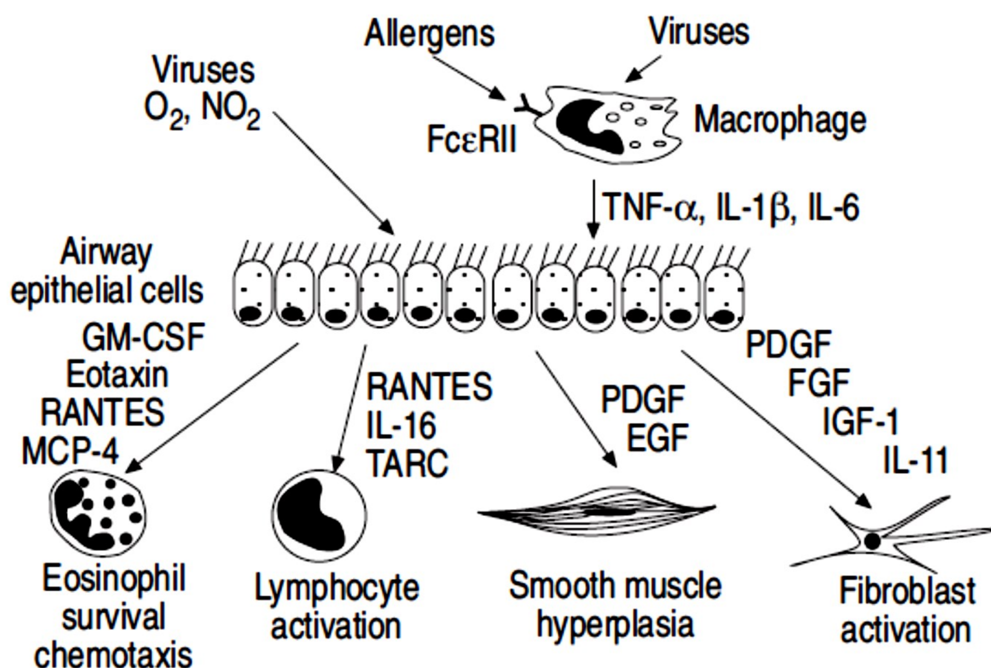


Fig. 1.3 *Airway epithelial cells and inflammatory mediators'release*. O2: oxygen; NO2: nitrogen dioxide; TNF: tumour necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MCP: monocyte chemotactic protein; TARC: thymus and activation regulated chemokine; PDGF: platelet-derived growth factor; EGF: endothelial growth factor; FGF: fibroblast growth factor; IGF: insulin-like growth factor. Source: Pathophysiology of asthma P.J. Barnes.

1.2.1.2. Inflammatory mediators

Different mediators are implicated in asthma showing a variety of effects (**Fig. 1.4**).[53]

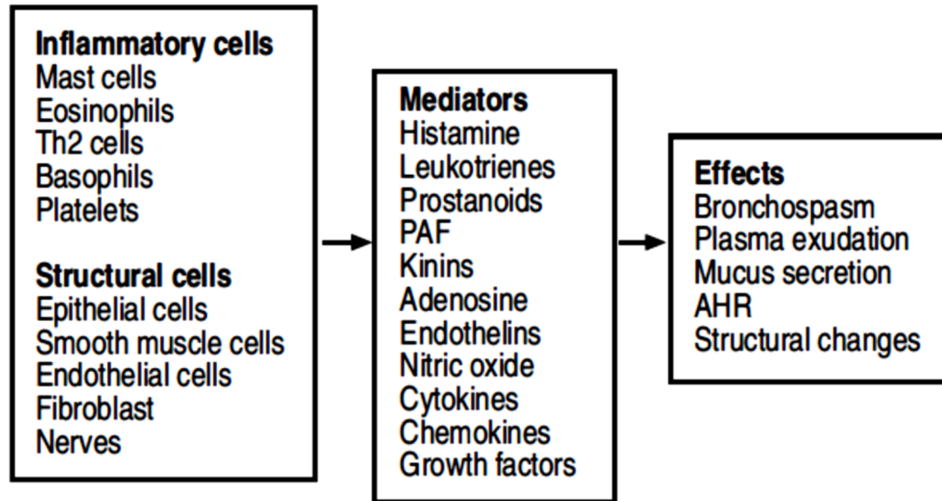


Fig. 1.4 *Cells and mediators involved in asthma.* Source: Pathophysiology of asthma P.J. Barnes.

Histamine, prostaglandine, leukotrienes and kinins contract airway smooth muscle, increase microvascular leakage and airway mucus secretion, and attract other inflammatory cells.

The cysteinyl-leukotrienes, LTC4, LTD4 and LTE4, are potent constrictors of human airways.[54] Potent LTD4 antagonists protect against exercise- and allergen-induced broncho-constriction. Chronic treatment with anti-leukotrienes improves lung function and asthma symptoms.[55] Cys-LTs increase in eosinophils in induced sputum.[56,57]

Platelet-activating factor (PAF) is a potent inflammatory mediator.[58] A genetic mutation of the PAF metabolising enzyme is associated with severe asthma.[59] However PAF antagonists, such as modipafant, do not control asthma symptoms.[60]

Prostaglandins (PG) have potent effects on airway function.[61] Nevertheless, the inhibition of their synthesis with COX inhibitors does not have any effect in most patients. Aspirin-sensitive asthmais associated with increased formation of cys-LTs.[62,63] PGD2 stimulates the chemo-attractant receptor of Th2 cells (CRTH2), which is

expressed on Th2 cells, eosinophils and basophils. Deletion of the PGD2 receptors in mice significantly inhibits inflammatory responses to allergen and AHR.[64]

Cytokines play a critical role in orchestrating the inflammatory response (**Fig. 1.5**).[65]

IL-3 is important for the survival of mast cells. IL-4 is critical in switching B lymphocytes to produce IgE and for expression of VCAM-1 on endothelial cells.[66]

IL-5 is important in the differentiation, survival and priming of eosinophils.[67] The administration of an anti-IL-5 antibody (mepolizumab) is associated with a significant decrease in eosinophil.

IL-9 may play a critical role in sensitising responses to the cytokines IL-4 and IL-5.[68]

IL-1β, IL-6, TNF-α and GM-CSF are released from a variety of cells. TNF-α is increased in asthmatic airways.[69] Inhalation of TNF-α increased airway responsiveness.[70] TNF-α and IL-1β activate the pro-inflammatory transcription factors, nuclear factor-kB (NF-kB) and activator protein-1 (AP-1). Interferon (IFN)-ɤ, IL-10, IL-12 and IL-18, play a regulatory role and inhibit the allergic inflammatory process.

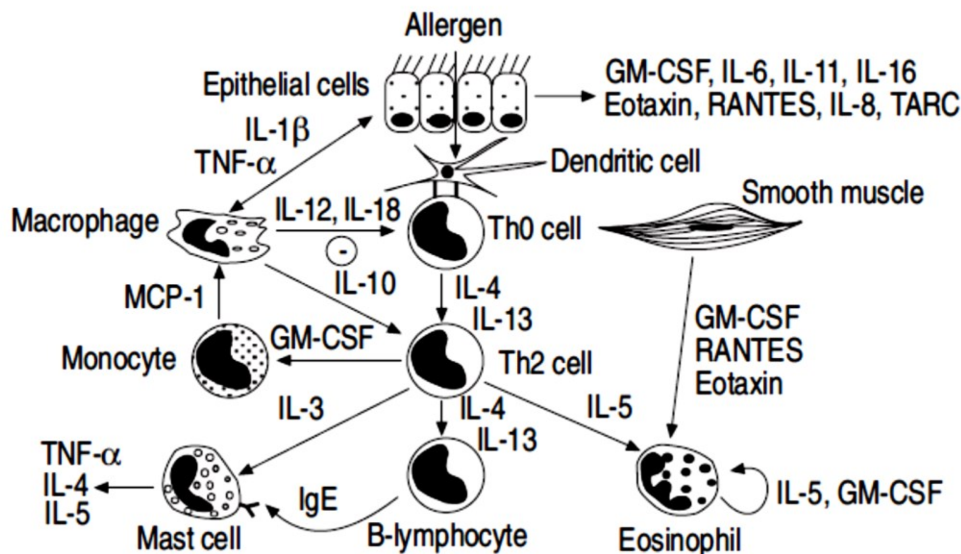


Fig. 1.5 *The cytokine network in asthma.* TNF: tumour necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MCP: monocyte chemotactic protein; TARC: thymus and activation regulated chemokine; PDGF: platelet-derived growth factor; EGF: endothelial growth factor; FGF: fibroblast growth factor; IGF: insulin-like growth factor; Th: T-helper. Source: Pathophysiology of asthma P.J. Barnes.

Chemokines are a large superfamily of mostly small, secreted chemotactic cytokines that function in leukocyte trafficking, recruitment and activation. The actions of chemokines are important for a wide range of processes such as allergic responses, infectious and autoimmune diseases. Over 50 different chemokines are now recognised.[71] There is increased expression of eotaxin, eotaxin-2, MCP- 3, and MCP-4 in the airways of asthmatics. [72,73,74] These molecules activate a common receptor on eosinophils termed CCR3. [75, 76] A neutralising antibody against eotaxin reduces eosinophil recruitment in to the lung after allergen.[77] RANTES also activates CCR3.[78] MCP-1 activates CCR2 on monocytes and T-lymphocytes. MCP-1 levels are increased in BAL fluid of asthmatics. Blocking MCP-1 results in a marked reduction of AHR.[79] CCR4 are selectively expressed on Th2 cells and are activated by the chemokines monocyte-derived chemokine (MDC) and thymus activation regulated chemokine (TARC). [80] Epithelial cells of patients with asthma express TARC. [81] Increased concentrations of TARC are found in BAL fluid of asthmatic.[82]

Endothelins are potent peptide mediators that are vaso-constrictors and broncho-constrictors.[83] Endothelin-1 levels are increased in the sputum of asthmatics depending on allergen exposure and steroid treatment. [84, 85] Endothelins induce ASM cell proliferation promoting a pro-fibrotic phenotype.

NO is produced by several cells in the airway by NO synthases.[86,87,88] The level of NO in the exhaled air of asthmatics is increased especially during an acute exacerbations.[89] [90,91] Measurement of exhaled NO in asthma is increasingly used as a noninvasive way of monitoring the inflammatory process.[92,93] Under oxidative stress the formation of the potent radical peroxynitrite may result in nitrosylation of proteins in the airways.[94]

1.2.1.3. Effects of inflammation

The acute and chronic allergic inflammatory responses have several effects (**Fig. 1.6 A and B**). The structural changes that occur in the airways are named "remodelling".[95]

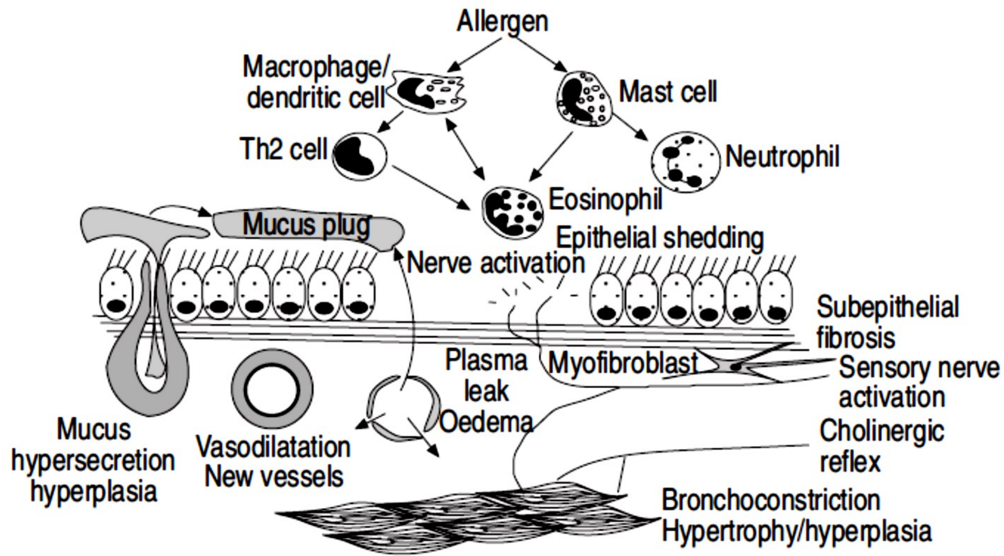


Fig. 1.6 A *Acute and chronic inflammatory effects on the airway in asthma.* Barnes. Source: Pathophysiology of asthma P.J. Barnes.

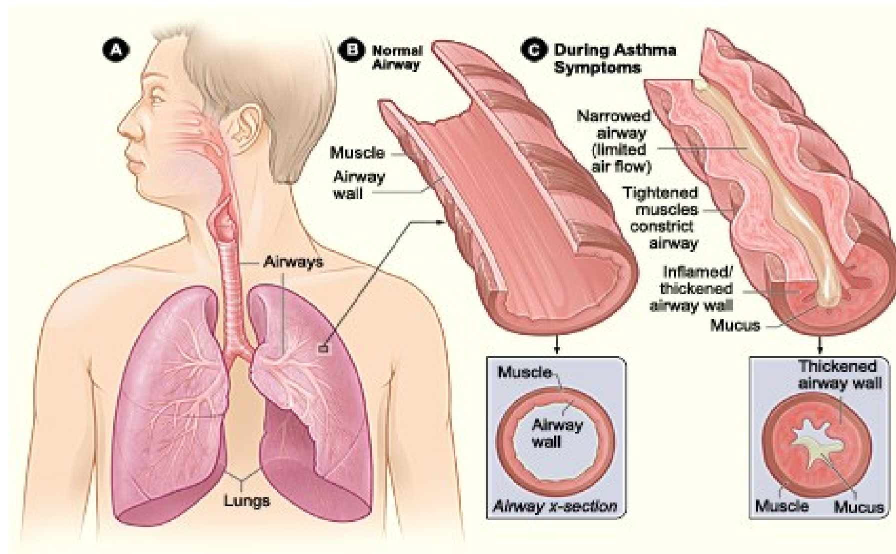


Fig. 1.6 B *Acute and chronic inflammatory effects on the airway in asthma.* A: location of lungs in the body and airways in the lungs. B: a normal, non-asthmatic airway. C: an airway during asthmatic symptoms. The airway is narrowed, limiting air flow. Tightened muscles constrict air flow, as do inflamed and thickened airways. Excess mucus clogs the airway. Image: http://www.nhlbi.nih.gov/health/dci/Diseases/Asthma/Asthma_WhatIs.html

Airway epithelial shedding is a characteristic feature of asthma. Ozone-exposure, viruses, chemicals and allergens can lead to its development as a consequence of inflammatory mediator's production. Epithelial damage results in loss of its barrier function to allow penetration of allergens, loss of enzymes which normally degrade inflammatory mediators, loss of a relaxant factor, and exposure of sensory nerves. Several inhaled allergens activate protease-activated receptor (PAR)-2, which shows increased expression in airway epithelial cells of asthmatics.[96] Epithelial cells may also release growth factors that stimulate structural changes in the airways.[97]

A thickened bronchial epithelial basement membrane has long been regarded as a histo-pathologic characteristic of asthma. Sub-epithelial fibrosis has been observed even in mild asthmatics.[98] The basement membrane appears thickened due to the deposition of Type III and V collagen.[99,100] TGF-β, platelet-derived growth factor (PDGF), and endothelin-1 can be produced by epithelial cells or macrophages in the inflamed airway.[101] There is also evidence for fibrosis in ASM.[102]

ASM contraction has a key role in the symptomatology of asthma. Many inflammatory mediators have broncho-constrictor effects.[103]. Reduced responsiveness to β-adrenergic agonists has been reported in post mortem bronchi from asthmatics.[104] Chronic exposure to inflammatory cytokines, such as IL-1β, down-regulates the response of ASM to β2-adrenergic agonists.[105,106,107] In asthmatics has been documented a characteristic hypertrophy and hyperplasia of ASM.[108,109]

Allergic inflammation has several effects on blood vessels in the respiratory tract. Recent studies have revealed an increased airway mucosal blood flow in asthma.[110] An increase in the vascular volume contributes to airway narrowing and exercise-induced asthma.[111] The increase in blood vessels in asthmatics may also be due to the release of VEGF and TNF-α.[112,113] Microvascular leakage is an essential component of the inflammatory response in asthma.[114,115]

In asthmatics has been reported hyperplasia of sub-mucosal glands.[116,117] Th2 cytokines IL-4, IL-13 and IL-9 induce mucus hypersecretion.[118] The epithelial growth factor (EGF) stimulates the expression of the mucin gene MUC5AC.[119,120] This is associated with the

expression of a specific calcium-activated chloride channel in goblet cells designated gob-5.[121]

Inflammatory products sensitize sensory nerve to become hyperalgesic. Neurotrophins, such as nerve growth factor (NGF), may be released from inflammatory and structural cells in asthmatic airways. [122,123,124] Neurotrophins cause proliferation and sensitisation of airway sensory nerves.[125] Bronchodilator nerves have been shown to be defective in asthma.[126] Lack of vasoactive intestinal peptide (VIP)-immuno-reactive nerves has been reported in severe asthma.[127] Airway nerves release also neurotransmitters which have inflammatory effects (**Fig. 1.7**).[128] An increase in SP-immuno-reactive nerves has been described in severe asthma.[129] A reduction in the activity of enzymes which degrade neuropeptides [130] and an increased gene expression of the receptors which mediate the inflammatory effects and bronchoconstrictor effects of SP have been descibed.[131]

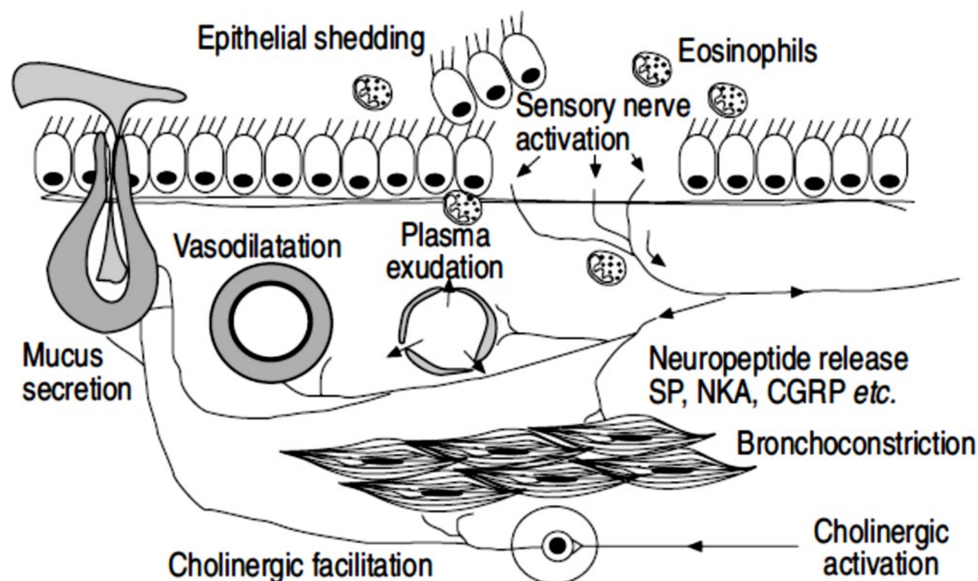


Fig. 1.7 *Possible neurogenic inflammation in asthmatic airways.* Substance P (SP) causes vasodilatation, plasma exudation and mucus secretion, whereas neurokinin A (NKA) causes bronchoconstriction and enhanced cholinergic reflexes and calcitonin generelated peptide (CGRP) vasodilatation. Source: Pathophysiology of asthma P.J. Barnes.

A number of transcription factors are involved in the regulation of the expression of inflammatory proteins in asthma (**Fig. 1.8**).[132]

NF-Kb is triggered by multiple stimuli including protein kinase C activators, oxidants and proinflammatory cytokines.[133] Activation of NF-kB has been shown increased in asthmatic airways.[134] NF-kB regulates the expression of several pro-inflammatory cytokines (IL-1β, TNF-α, GM-CSF), chemokines (RANTES, MIP-1a, eotaxin), adhesion molecules (ICAM-1, VCAM-1) and inflammatory enzymes (cyclooxygenase-2 and iNOS).

The c-Fos component of AP-1 is also activated in asthmatic airways.[135]

GATA-3 determines the differentiation of Th2 cells and is increased expression in asthmatics.[136,137] The differentiation of Th1 cells is regulated by the transcription factor T-bet.[138] In a murine model the deletion of the T-bet gene is associated with asthma-like phenotypes.[139]

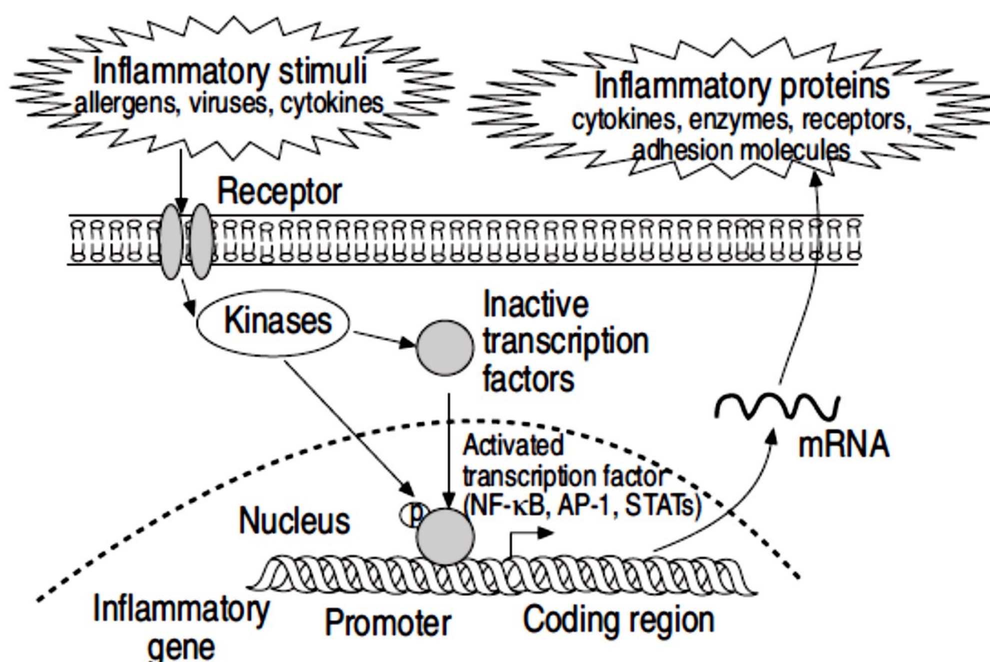


Fig. 1.8 *Transcription factors activated by inflammatory stimuli and responsible for increase the expression of multiple inflammatory genes.* Nuclear factor kappa-B (NF-kB), activator protein-1 (AP-1), signal transduction-activated transcription factors (STATs), messenger ribonucleic acid (mRNA). Source: Pathophysioloof gy of asthma P.J. Barnes.

1.2.1.4. Anti-inflammatory mechanisms

A numbr of anti-inflammatory mechanisms have been shown defective in asthma.[140]

Cortisol regulates the allergic inflammatory response. Inhibition of endogenous cortisol secretion by metyrapone results in an increase in the late response to allergen in the skin. [141] Cortisol is converted to the inactive cortisone by the enzyme 11-β-hydroxysteroid dehydrogenase. [142] This enzyme seems to function abnormally in asthma.[143]

IL-1 receptor antagonist (IL-1ra) inhibits the binding of IL-1 to its receptors and therefore has a potential anti-inflammatory potential. It is reported to be effective in an animal model of asthma.[144]

IL-12 and IFN-ɤ enhance Th1 cells and inhibit Th2 cells. IL-12 infusions in patients with asthma inhibit peripheral blood eosinophilia. [145] The IL-12 expression seems impaired in asthma.

IL-10 inhibits the expression of multiple inflammatory mediators. IL-10 secretion and gene transcription are defective in macrophages and monocytes from asthmatics (**Fig. 1.9**). [146, 147, 148] PGE2 has inhibitory effects on macrophages, epithelial cells and eosinophils. 15-hydroxyeicosatetraenoic (15-HETE) and lipoxins inhibit cysteinyl-leukotriene effects on the airways. [149] Lipoxins have also strong anti-inflammatory effects.[150]

The peptide adrenomedullin, which is expressed in high concentrations in the lung, has bronchodilator activity[151] and inhibit the secretion of cytokines from macrophages.[152] Plasma concentrations are no different in patients with asthma.[153]

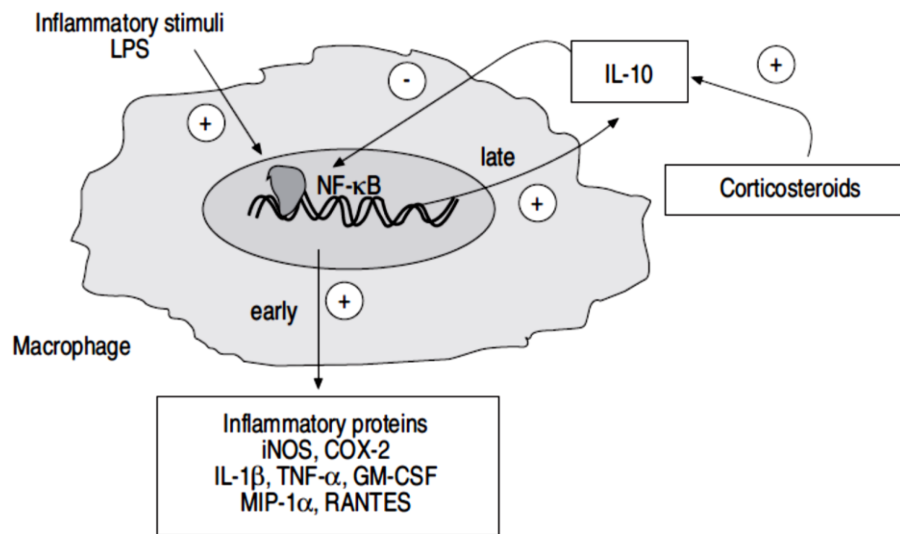


Fig. 1.9 *Transcription factors play a key role in amplifying and perpetuating the inflammatory response in asthma.* IL-10 secretion is deficient in macrophages from patients with asthma, resulting in increased release of inflammatory mediators. NF-kB: nuclear factor kappa-B; LPS: lipopolysaccharide; inducible nitric oxide synthase; COX: cyclooxygenase; TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MIP: macrophage inflammatory protein. Source: Pathophysiology of asthma P.J. Barnes.

1.3. Epidemiology of asthma

In 2011 ~235 million people worldwide were affected by asthma, and approximately 250,000 people die per year from the disease. [154] To date, most of the epidemiological evidence on the burden of asthma comes from developed populations. In the next paragraphs epidemiological data from a study on asthma prevalence in the United States from 2001 to 2010 will be shown. This study has shown an increase from 7.3% in 2001 to 8.4% in 2010 (**Fig. 1.10**).[155] In the United States in 2010, an estimated 25.7 million people had asthma: 18.7 million adults aged 18 and over, and 7.0 million children aged 0–17 years.[155]

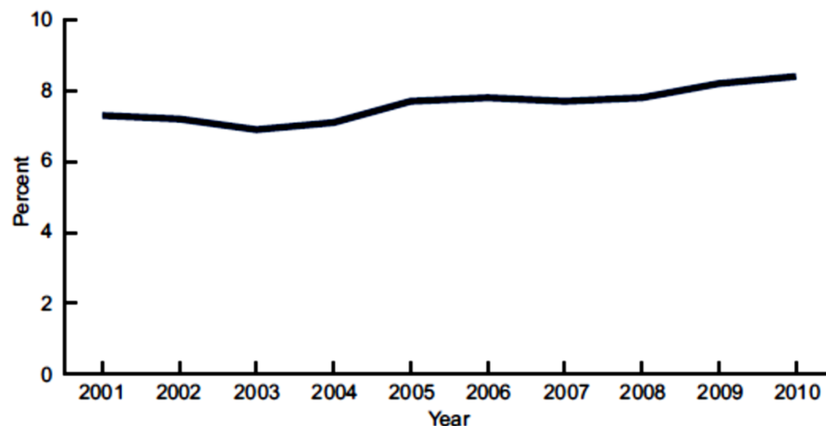


Fig. 1.10 *Asthma prevalence in the United States, 2001-2010.* Source: CDC/NCHS, National Health Interview Survey.

The study showed that children aged 0–17 years had higher asthma prevalence (9.5%) than adults aged 18 and over (7.7%) for the period 2008–2010. Females had higher asthma prevalence than males (9.2% compared with 7.0%).[155] Persons of multiple race had the highest asthma prevalence (14.1%), while Asian persons had the lowest rates (5.2%). Persons of black (11.2%) and American Indian or Alaska Native (9.4%) races had higher asthma prevalence compared with white persons (7.7%). Among Hispanic groups, asthma prevalence was higher among persons of Puerto Rican (16.1%) than Mexican (5.4%) descent (**Fig. 1.11**).[155]

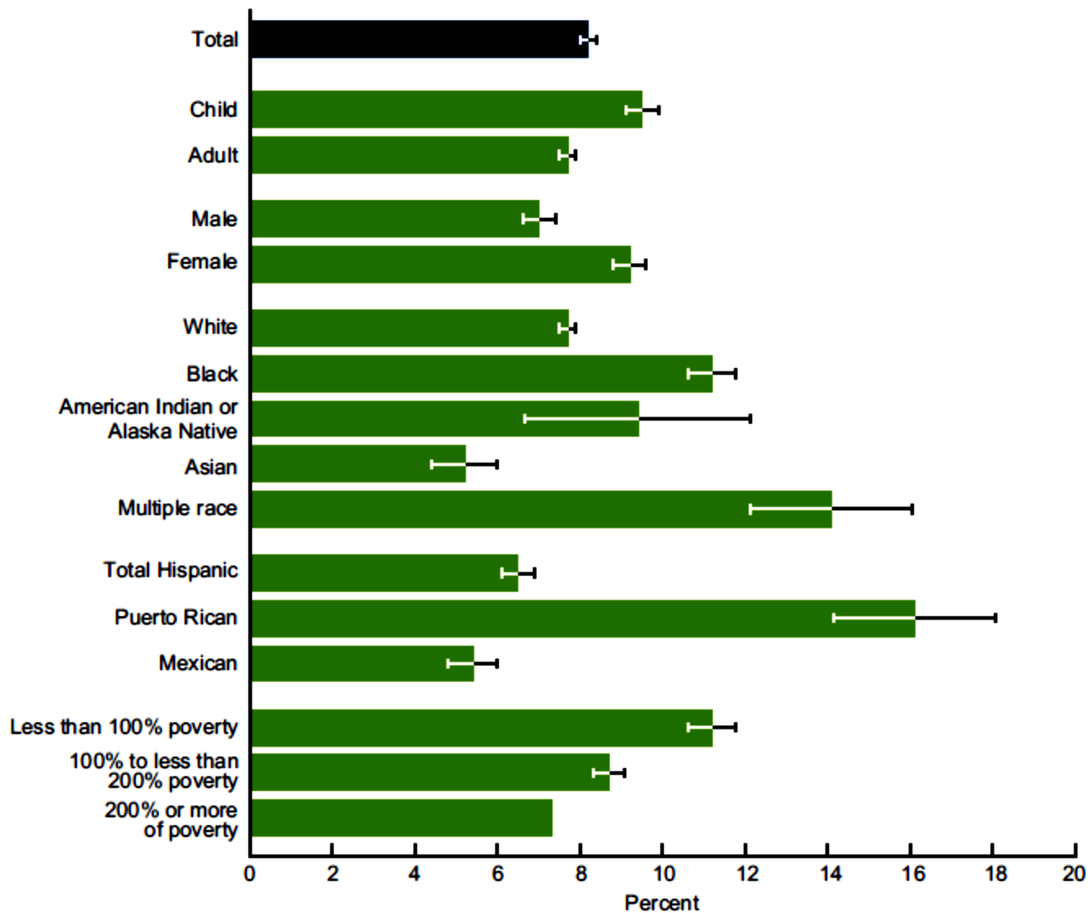


Fig. 1.11 *Asthma prevalence, by selected demographic characteristics*: Sources: CDC/NCHS, Health Data Interactive and National Health Interview Survey.[155]

In the United States asthma prevalence from 2001 to 2010 was higher for groups with lower income-to-poverty level ratios. While 11.2% of those with incomes less than 100% of the poverty level had asthma, asthma prevalence was 8.7% for persons with incomes 100% to less than 200% of the poverty level, and 7.3% for persons with incomes at least 200% of the poverty level.[155] Asthma death rates per 1,000 persons with asthma declined from 2001 to 2009 (**Fig. 1.12**).

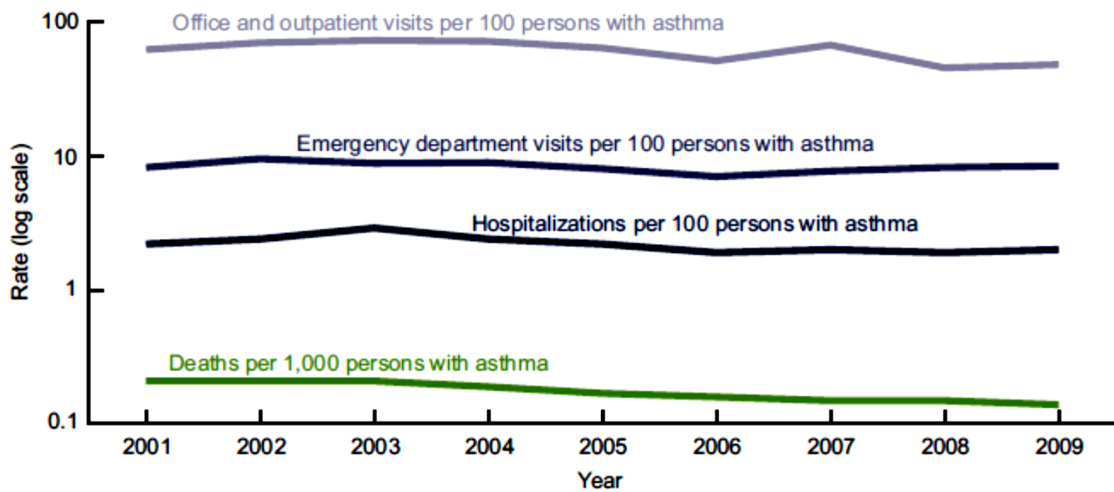


Fig. 1.12 *Asthma health care encounters per 100 persons with asthma, and asthma deaths per 1,000 persons with asthma:United States, 2001–2009*. Access at: http://www.cdc.gov/nchs/data/databriefs/db94

The rates of health care encounters per 100 persons with asthma across all health care settings (**Fig. 1.13**) were similar for males and females, and for black and white persons, but the rate for children was higher than that for adults.[155]

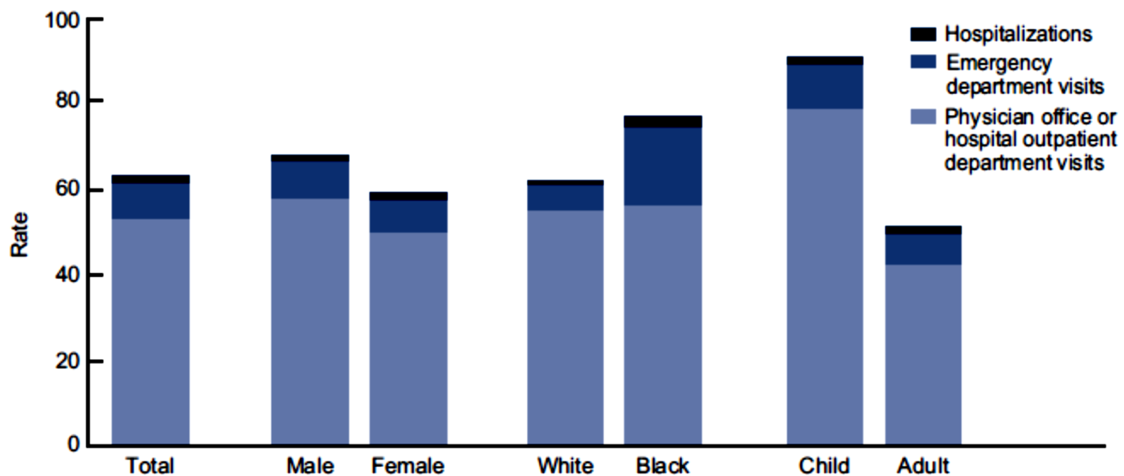


Fig. 1.13. *Asthma health care encounters per 100 persons with asthma: United States, 2001–2009*. NOTE: Access data table for at: http://www.cdc.gov/nchs/data/databriefs/db94_tables.pdf#4

Children aged 0–17 years with asthma had a higher asthma visit rate for primary care and a higher ED visit rate than adults aged 18 and over.[155]

In the United States the asthma death rate per 1,000 persons with asthma was 0.15 for the period 2007–2009. The highest rate was for adults aged 65 and over (0.58 per 1,000 persons with asthma) (**Fig. 1.14**).[155]

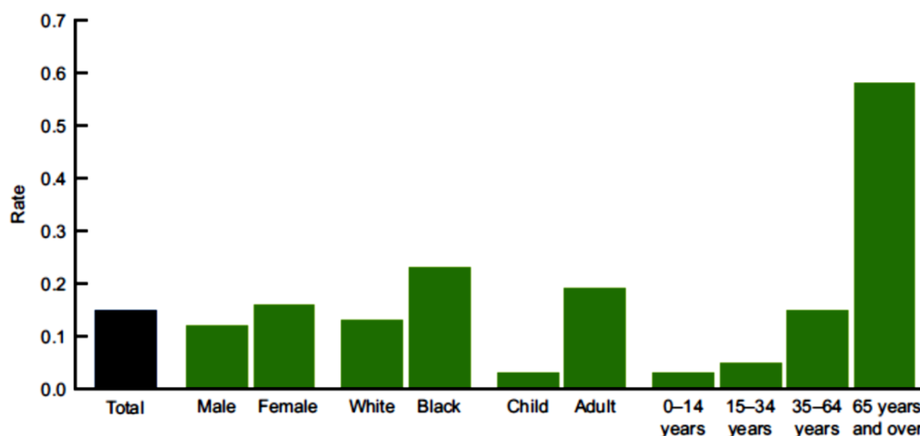


Fig. 1.14 *Asthma deaths per 1,000 persons with asthma, by selected demographic characteristics: United States, average annual 2007–2009.*[155]

Asthma prevalence also differs between populations of the same ethnicity. U.S.-born Mexican populations, for example, have higher asthma rates than non-U.S. born Mexican populations that are living in the U.S.[156]

Asthma affects approximately 5% of the United Kingdom's population.[157] In England, an estimated 261,400 people were newly diagnosed with asthma in 2005; 5.7 million people had an asthma diagnosis and were prescribed 32.6 million asthma-related prescriptions.[158]

In Italy from 1990 to 2010 the national median prevalenceof asthma and allergic rhinitis increased from 4.6% to 6.6% and from 19.4% to 25.8%, respectively.[159] Antonicelli et al in 2014 illustrated the overall costs of asthma in Italy with highest values for severe persistent asthma, **Fig. 1.15**.

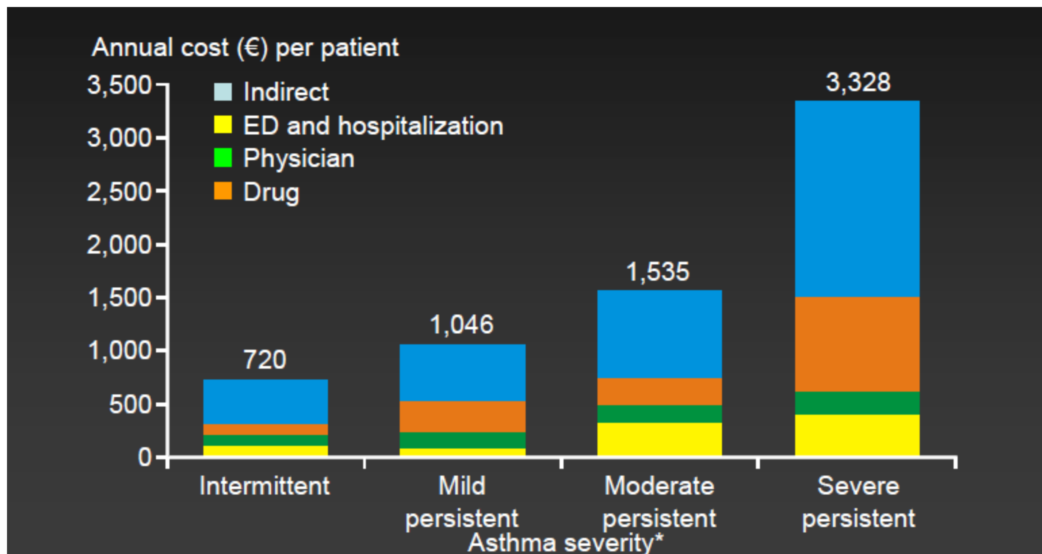


Fig. 1.15 *Overall costs of asthma Italy*. Adapted from Antonicelli L. et al. Eur Respir J 2004. GINA 2002 classification.

In spite of the epidemiological suggestion for an increase in the prevalence of asthma in several countries, the bases of the increase are still debated. While the exact cause of asthma is not known, it is thought that a variety of factors interacting with one another, early in life, result in the development of asthma. It has been hypothesized that asthmatic subjects may have a genetic predisposition to develop the disease. Elements of the pathogenesis of asthma, including the immune response and the regulation of pro-inflammatory cytokines, are also under genetic control and are activated under environmental factors in genetically predisposed subjects.

The fast increase detected in asthma prevalence cannot be explained on the basis of genetic predisposition only. Therefore, attention has been centred on a number of environmental factors. Indoor and outdoor allergens, such as domestic mites, animal allergens, pollens, fungi and molds, have been suggested to have a role in the manifestation and persistence of asthma. Environmental pollutants, mainly industrial smog and those derived from ozone and nitrogen oxides, may intensify clinical manifestations of asthmatic subjects.

As seen previously developed countries with a higher socio-economic level have the highest prevalence of asthma. It has been proposed that better hygienic conditions

derived from this affluent status may be in part related to the increase in allergic diseases. One of the underlying mechanisms hypothesized for the rise of atopy and asthma in industrialised countries, is the reduction in the incidence of early childhood infections and the consequent expansion of T helper type 2 lymphocytes, which would lead to an imbalance in the regulatory mechanisms of the inflammatory response later on in life.

Children with siblings are more likely to acquire infections during their childhood and consequently they would be protected against allergic diseases later on in life. This has contributed to the hypothesis that family size and, specifically number of older siblings may be related to asthma. Nevertheless, changes in family size over the past 30 years do not explain the growth in asthma observed in the same period in the United Kingdom or New Zealand, two of the countries with highest prevalence.

1.4. Diagnosis

The diagnosis of asthma typically is based on family history, the pattern of symptoms and response to therapy (**Fig. 1.17**). [160] A diagnosis of asthma should be supposed if there is a history of recurrent wheezing, coughing or difficulty breathing and these symptoms worsen due to exercise, viral infections, allergens or air pollution. [161] Spirometry is used to confirm the diagnosis. [162] Spirometry measures the lung function and specifically the amount (volume) and/or speed (flow) of air that can be inhaled and exhaled. In children under the age of six the diagnosis is more difficult as they are too young for spirometry. If the Forced Expiratory Volume in 1 second **(**FEV_1) measured by this technique improves more than 12% following administration of a bronchodilator such as salbutamol, this is supportive of the diagnosis (**Fig 1.16**).[163] As caffeine is a bronchodilator its use before a lung function test may interfere with the results. [164] Diffusing capacity of the lung (D_L) measures the transfer of gas from air in the lung, to the red blood cells in lung blood vessels. Single-breath diffusing capacity helps to differentiate asthma from Chronic Obstructive Pulmonary Diseases (COPD).

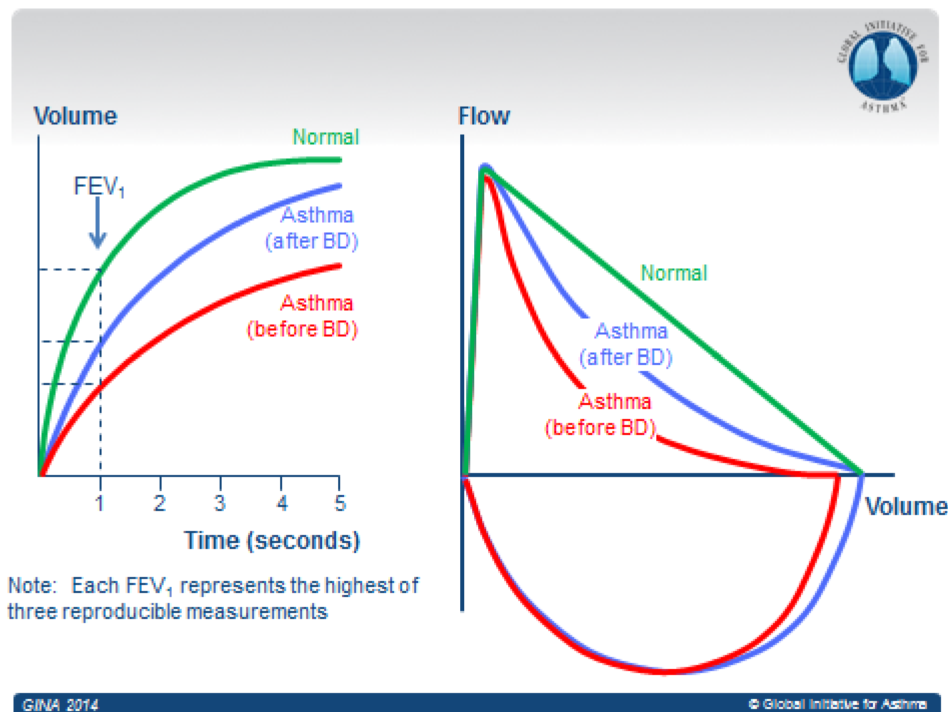


Fig. 1.16 *Typical spirometric tracings in asthma.*

Bronchial hyper-responsiveness (BHR) can be defined as the tendency for the airways of asthmatic subjects to broncho-constrict when exposed to various chemical and physical stimuli. Exposure to stimuli, such as allergens, which are specific for an individual, produce a different effect, in that the non-specific stimuli generally cause a short-lived period of broncho-constriction without inducing significant airway inflammation whilst antigenic stimuli cause more prolonged bronchoconstriction with an immediate response lasting for 1-2 hours that may follow a late response at 4-8 hours, which is characterized by inflammatory cell recruitment to the airways. Many broncho-constrictor stimuli can be used to measure the degree of BHR, including inhaled histamine or methacholine, inhaled hypertonic saline or distilled water, exercise or cold air. During obstructive processes, the reduction of FEV_1 is bigger than the reduction of FVC and the FEV_1/FVC ratio is reduced; contrariwise, in restrictive lung disease the reduction in FVC is greater than in FEV_1 and the ratio is augmented or normal. The assessmenot f BHR has been done mainly through challenge with histamine and more recently methacholine. The methacholine challenge consist of the inhalation of increasing concentrations of a methacholine that causes airway narrowing in those predisposed. If negative a person does not have asthma; if positive, however, it is not specific for the disease.

Other supportive indications for asthma includes: a ≥20% difference in peak expiratory flow (PEF) rate on at least three days in a week for at least two weeks, a ≥20% improvement of PEF following treatment with either salbutamol, inhaled corticosteroids or prednisone, or a ≥20% decrease in PEF following exposure to a trigger. Testing PEF may be useful for daily self-monitoring of asthma and in guiding treatment in those with acute exacerbations.

1.4.1. Classification

Although asthma is a chronic obstructive condition, it is not considered as a part of chronic obstructive pulmonary diseases. [165] Unlike to these diseases, the airway obstruction in asthma is usually reversible.[166] Two main factors, severity and control, determine asthma classification, which in turn affect the type of therapy initiated (depending on the severity) and how therapy should be adjusted over time (based on the

control level). Severity and control should be assessed separately. Asthma is clinically classified according to the frequency and severity of symptoms, FEV_1, and PEF rate.[167,168] Asthma may also be classified as atopic (extrinsic) or non-atopic (intrinsic), when symptoms are precipitated by allergens.[169] Based on severity level, asthma can be classified as intermittent or persistent. Patients with intermittent asthma usually have minimal asthma symptoms and no interference with normal activity, whereas patients with persistent asthma have more severe symptoms and limitations in normal activity due to reduced lung function

In acute asthma exacerbation is commonly referred to asthma attacks with worsening of the classic symptoms are shortness of breath, wheezing, and chest tightness.[170] In severe cases, air motion may be significantly impaired.[171] During an attack can occur the use of accessory muscles of respiration, a paradoxical pulse, and over-inflation of the chest. [172] A blue color of the skin and nails may occur from lack of oxygen.[173] In a mild exacerbation the peak expiratory flow rate (PEFR) is ≥200 L/min or ≥50% of the predicted best.[174] Moderate is defined as between 80 and 200 L/min or 25% and 50% of the predicted best while severe is defined as ≤ 80 L/min or ≤25% of the predicted best. Acute severe asthma is an acute exacerbation of asthma that does not respond to standard treatments. Risk factors for exacerbations include:

- Ever intubated for asthma,
- Uncontrolled asthma symptoms;
- Having ≥1 exacerbation in last 12 months;
- Low FEV_1 (measure lung function at start of treatment, at 3-6 months to assess personal best, and periodically thereafter);
- Incorrect inhaler technique and/or poor adherence;
- Smoking;
- Obesity, pregnancy, blood eosinophilia.

Brittle asthma is distinguishable by recurrent, severe attacks. Type 1 brittle asthma is a disease with wide peak flow variability, despite intense medication. Type 2 brittle asthma is background well-controlled asthma with sudden severe exacerbations.

Exercise can trigger bronchoconstriction.[175] It occurs in most people with asthma and up to 20% of people without asthma. It is more common when it is dry and cold. Inhaled β2-agonists do not improve athletic performance among those without asthma.[176] However oral doses may improve endurance and strength.[177]

Asthma when is a result of workplace exposures is commonly reported as occupational disease. It is estimated that 5–25% of asthma cases in adults are work–related. Isocyanates, grain and wood dust, colophony, soldering flux, latex, animals, and aldehydes have been implicated as most common agents.[178]

Many other conditions can cause symptoms analogous to those of asthma. In children allergic rhinitis and sinusitis should be considered as well as foreign body aspiration, tracheal stenosis, vascular rings, enlarged lymph nodes, etc. In adults, COPD, congestive heart failure, airway masses, as well as drug-induced coughing due to ACE inhibitors should be considered. COPD can coexist with asthma and can occur as a complication of chronic asthma. When older than 65 years most people with obstructive airway disease develop also asthma. A deep level of investigation is not performed due to COPD and asthma sharing similar principles.[179,180,181]

1.5.Prevention

The evidence for the effectiveness prevention of asthma is not strong.[182] Limiting smoke exposure both in utero and after delivery, breastfeeding, and increased exposure to daycare are not well supported. Early pet exposure may be useful. Dietary restrictions during pregnancy or breast feeding have not been found to be effective. Removing compounds known to sensitive people from the work place may be effective. Annual influenza vaccinations may affect the risk of exacerbations.[183] Immunization, however, is recommended by the World Health Organization.[184] Smoking prohibition is effective in decreasing exacerbations of asthma.[185]

The Global Initiative for asthma recommends:[1]

- Provide skills and support for guided asthma self-management:

This comprises self-monitoring of symptoms and/or PEF, a written asthma action plan and regular medical review

- Prescribe medications or regimen that minimize exacerbations:

ICS-containing controller medications reduce risk of exacerbations

For patients with ≥1 exacerbations in previous year, consider low-dose ICS/formoterol maintenance and reliever regimen

- Encourage avoidance of tobacco smoke:

Provide smoking cessation advice and resources at every visit

- For patients with severe asthma

Refer to a specialist center, if available, for consideration of add-on medications and/or sputum-guided treatment

- For patients with confirmed food allergy:

Appropriate food avoidance

Ensure availability of injectable epinephrine for anaphylaxis

1.6.Management and therapy

Despite the fact there is no cure for asthma, symptoms can usually be improved (**Fig. 1.17** and **1.18**). [186]

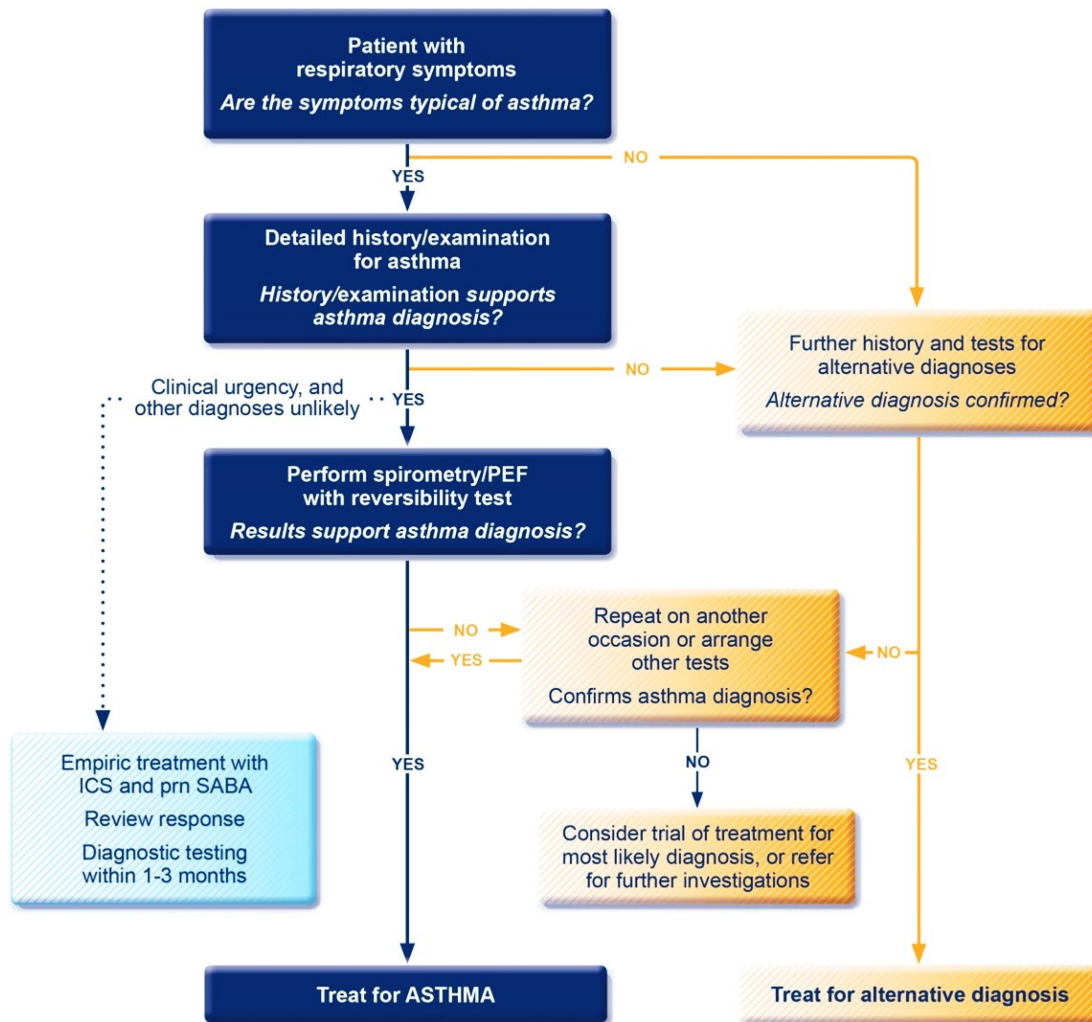


Fig. 1.17 *GINA 2014, Box 1-1*

Ineffective management of asthma significantly influences morbidity, mortality and health care utilization, resulting in increased health care costs. A precise, detailed, and customized plan for monitoring and managing of the symptoms is firmly necessary. This should comprise the reduction of exposure to allergens, testing the severity of symptoms, and the usage of medications. The treatment should be adjusted according to

changes in symptoms. The effective management for asthma should include identifying and eliminating triggers, such as cigarette smoke, pets, or aspirin.[187] Exercise is beneficial in people with stable asthma.[188] Medications are selected based on the severity of illness and the frequency of symptoms (**Fig. 1.17**).[189]

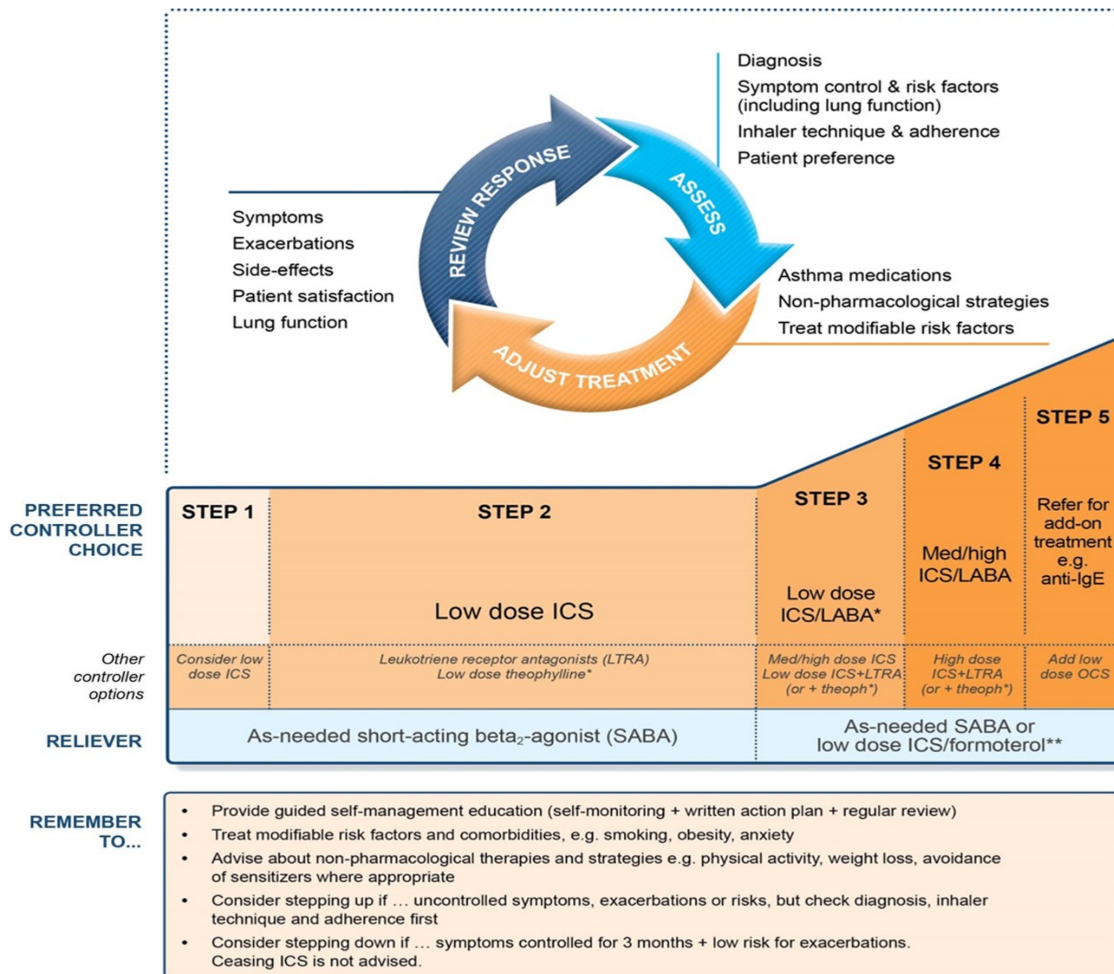


Fig.1.18 *Stepwise approach to control asthma symptoms and reduce risk.* GINA 2014, Box 3-5

Short-acting beta-2-adrenoceptor agonists (SABA), such as salbutamol (albuterol USAN) represent the the most effective agents for quick symptom relief as they rapidly reverse airflow obstruction for all patients with asthma. Bronchodilation occurs due to blocking β2-adrenergic receptors, which antagonize bronchoconstriction. The most commonly used SABAs are albuterol, levalbuterol, and pirbuterol. It is recommended using SABAs only as needed for symptom relief, but not for regular use. Tachycardia,

tremor, and anxiety are the most common dose-dependent side effects. They are recommended before exercise in those with exercise induced symptoms.[190] No-selective adrenergic agonists[191] are not recommended due to their excessive cardiac stimulation.

Anticholinergic medications, such as ipratropium bromide, provide additional benefit when used in combination with SABA in those with moderate or severe symptoms.[192] Ipratropium bromide is used to overcome acute bronchospasms by blocking muscarinic cholinergic receptors. Common side effects associated with ipratropium bromide use are dry mouth, increased wheezing, and blurred vision.

Corticosteroids are generally considered the most effective treatment available for long-term control. OCSs are used for exacerbation management. These medications reverse inflammation and decrease relapse occurrences. Systematic corticosteroids have a potent anti-inflammatory effect, but should be used with caution due to complex adverse effects such as abnormalities in glucose metabolism, fluid retention, weight gain and hypertension. Methylprednisolone, prednisolone and prednisone are oral corticosteroids used for asthma exacerbations management and severe persistent asthma Inhaled corticosterois (ICS) such as beclomethasone are generally used except in severe persistent disease, in which oral corticosteroids are required.

Long-acting beta-adrenoceptor agonists (LABA) such as salmeterol and formoterol can improve asthma control, once given in combination with inhaled corticosteroids.[193] LABAs have a bronchodilator effect, but do not affect airway inflammation. LABAs activate adenylate cyclase and produce functional antagonism of bronchoconstriction providing a bronchodilator effect When used without steroids they increase the risk of severe side-effects.[194] Available combinations of ICS/LABA inhalers are fluticasone/salmeterol, budesonide/formoterol and mometasone/formoterol. Potential life-threatening exacerbations associated with LABA use include tachycardia, skeletal muscle tremor and hypokalemia.

Leukotriene antagonists (such as montelukast and zafirlukast) are used in addition to inhaled corticosteroids, usually also in conjunction with LABA.[195] In children they appear to be of little advantage when added to ICS.[196] Leukotriene modifiers include

two groups of agents: leukotriene receptor antagonists (LTRAs) (i.e., montelukast, zafirlukast) and leukotriene synthesis inhibitors (LTSIs) (i.e., zileuton).

Mast cell stabilizers (such as cromolyn sodium) are another non-preferred alternative to corticosteroids. The mechanism of anti-inflammatory action is determined by blocking early and late reactions to allergens and by stabilizing mast cells membranes. The anti-inflammatory effect and excellent safety profile of these agents provide symptom control, along with a decrease in the number of exacerbations compared to placebo. Potential side effects are cough and throat irritation.

Emergency management of asthma includes oxygen to alleviate hypoxia.[197] Oral corticosteroids are recommended with five days of prednisone.[198] Magnesium sulfate intravenous provide a bronchodilating effect in severe acute asthma attacks.[199] Heliox, a mixture of helium and oxygen, may also be considered in severe unresponsive cases. The use of Methylxanthines (such as theophylline) in acute exacerbations is controversial. It has bronchodilator and mild anti-inflammatory effects. Theophylline provides muscle relaxation by inhibition of phosphodiesterase. It is not preferred therapy since it can lead to frequent adverse events (e.g., severe headache, tachycardia, nausea, vomiting) and it is not as effective in asthma as low dose ICSs. Theophylline is used when asthma is not well-controlled with ICS, LABAs or LTRAs

Ketamine is theoretically useful when intubation and mechanical ventilation is needed.[200] In severe not controlled and persistent asthma bronchial thermo-plasty represent an option.[201] Sublingual immuno-therapy in allergic rhinitis and asthma improve outcomes.

Many asthmatics use alternative treatments and approaches.[202,203,204] Complementary and alternative medicine (CAM) asthma treatment ranges from breathing exercises to herbal remedies. Unfortunately, a lack of well-designed clinical trials makes it difficult to assess the safety and efficacy of these treatments. There is insufficient evidence to support the use of acupuncture, osteopathic, chiropractic, physiotherapeutic and respiratory therapeutic maneuvers in asthma.[205,206,207] Air-ionisers show no evidence that they improve asthma symptoms or benefit lung function.[208]

1.7.Prognosis

The prognosis for asthma is usually good, especially for children with mild disease.[209] Mortality has decreased over the last few decades due to better recognition and improvement in therapeutic intervention.[210] Globally it causes moderate or severe disability in 19.4 million people.[211] Of asthma diagnosed during childhood, half of cases will no longer carry the diagnosis after a decade.[212] Airway remodeling is observed, but it is unknown whether these represent harmful or beneficial changes.[213] Early treatment with corticosteroids seems to prevent the decline in lung function.[214]

Chapter 2

2. Asthma and oxidative stress

2.1. Introduction

Oxidative stress is the condition characterize by an overproduction of Reactive Oxygen Species (ROS) and/or antioxidant decreases. At physiological levels, ROS function as "redox messengers" in intracellular signaling. Excess ROS induce oxidative modification of cellular macromolecules, inhibit protein function and promote cell death. The alteration of intracellular redox homeostasis, and irreversible oxidative modifications of lipid, protein or DNA accompanies a wide spectrum of clinical disorders including asthma. As described in **Chapter 1** asthma is a chronic inflammatory disorder of the airways involving interaction of cells and mediators. The increase of inflammatory processes in asthma ultimately result in high levels of reactive oxygen and nitrogen species (ROS, RNS).[215,216,217,218] In asthma the increased oxidative species and the deficiency of anti-oxidant capacity lead to modifications of proteins and alterations in their function that are biologically relevant to the initiation and maintenance of inflammation. This chapter will first explain the process of oxidative stress, then focus on the redox abnormalities in asthma and finally elucidate the consequences on molecular processes.

2.2. Oxidative stress and redox systems in the lungs

The lungs show a vast mucosal epithelial surface directly exposed to inhaled oxygen and airborne reactive pollutants and microorganisms. This makes the lungs particularly susceptible to oxidant-mediated damage. Also endogenously are generated high levels of RNS and ROS to maintain a sterile internal environmental. Altogether, endogenous RNS and ROS produce an oxidizing lung environment (**Fig. 2.1**). However, because of the abundance of antioxidant systems available to the lung the redox state in the healthy lung is reducing.[219]

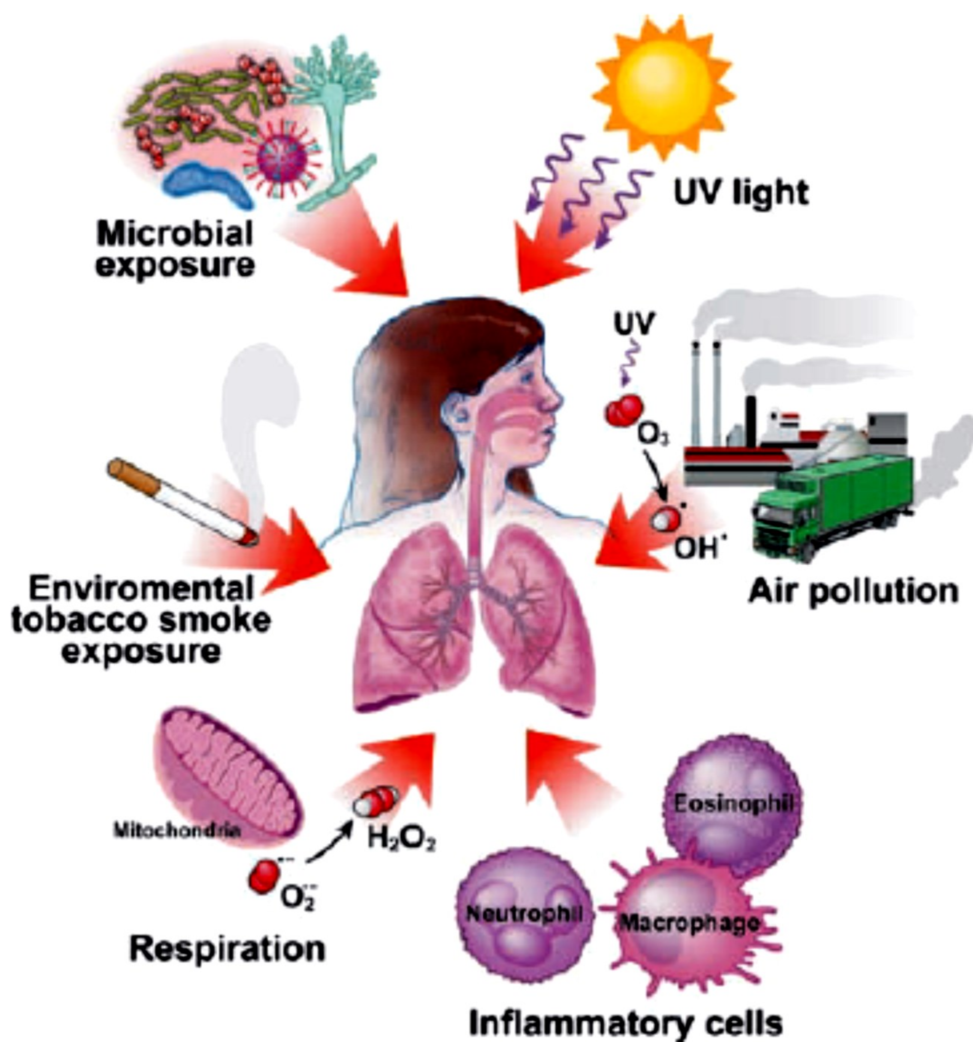
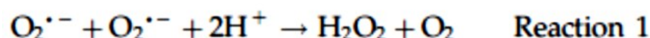


Fig. 2.1 *Sources of exogenous inhalational and endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the lung.* Environmental sources are ozone, air pollutants, particulates containing metals, and cigarette smoke. Endogenous ROS are produced as byproducts of mitochondrial respiration. Inflammatory cells can produce high levels of ROS and RNS in response to allergens and microbial infections. Source: Redox Control of Asthma: Molecular Mechanisms and Therapeutic Opportunities Suzy A.A. Comhair and Serpil C. Erzurum. Antioxidants & redox signaling volume 12, number 1, 2010

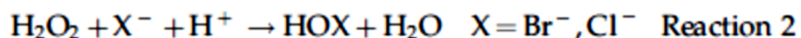
2.2.1. Endogenous reactive oxygen species.

The tetravalent reduction of oxygen during mitochondrial electron transport can result in formation of the radical superoxide ($O_2\cdot$).[220] Another source for intracellular generation of O_2 is the NADPH oxidase found in neutrophils, monocytes, and macrophages.[221,222,223,224] $O_2\cdot$ can be also produced by molybdenum hydroxylase reactions and arachidonic acid metabolism.[225] $O_2^{\cdot}$ does not easily cross cell membranes

and react with proteins that contain transitionmetal prosthetic groups, such as heme or iron/sulfur groups. [226,227,228] The main reaction of superoxide is to react with itself to produce hydrogen peroxide and oxygen (Reaction 1).[229] Superoxide dismutation can be spontaneous or can be catalyzed by the enzymes (SOD).

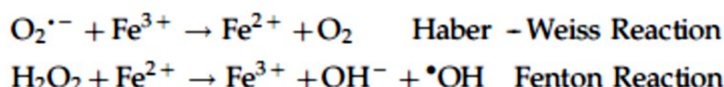


Once formed, the oxidizing potential of H_2O_2 may be amplified by eosinophil and neutrophil derived peroxidases eosinophil peroxidase (EPO) and myeloperoxidase (MPO), respectively (Reaction 2). [230,231,232,233] MPO is the most abundant protein stored in neutrophil granules, and secreted during cell activation.[234]

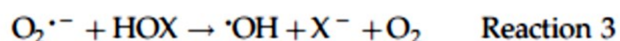


Kinnula et al. has shown that alveolar macrophages and Type II cells produce high levels of H_2O_2.[235]

Onother extremely reactive oxidizing is the hydroxyl radical ($^{\cdot}OH$).[236] The $^{\cdot}OH$ can be formed by Haber–Weiss Reaction followed by the Fenton Reaction.[237]



An alternative pathway for OH formation in vivo may involve MPO and EPO. Under physiological concentrations of halides, MPO produces hypochlorous acid (HOCl) and EPO produces hypobromous acid (HOBr). Hypohalous acids can generate $^{\cdot}OH$ after reacting with $O_2^{\cdot}$ (Reaction 3). $^{\cdot}OH$ can react with different molecules such as protein, DNA, and lipids. [238,239,240,241]



In the lung is widely produced nitric oxide (·NO) by nitric oxide synthases (NOS).[242] All NOS convert L-arginine to NO and L-citrulline. There are three forms of NOS, the inducible NOS ($_i$NOS or NOS_2), neuronal NOS (nNOS or NOS_1), and endothelial NOS (eNOS or NOS_3).[243] nNOS and eNOS are constitutively expressed in neuronal and endothelial cells.[244] In the airway NOS_3 is primarily localized in pulmonary endothelial cells, and NOS_1 in non-adrenergic, non-cholinergic inhibitory neurons.[245246] NOS_2 is continuously expressed in normal human airway epithelium.[247,248.249.250]NO is also produced by the upper respiratory tract epithelium within the nasopharynx and paranasal sinuses.[251] Epithelial NOS_2 activity is a major determinant of NO present in exhaled breath.[252] The iNOS is regulated at the level of transcription and mRNA stability, is calcium independent, and produces nanomolar levels of NO. Regulation of iNOS expression is increased by cytokines and proinflammatory factors, interferon gamma, TNF-α, and IL1-β.[253] iNOS is also regulated by availability of arginine and cofactor tetrahydrobiopterin. Conditions that decrease arginine will lead to greater superoxide formation.[254] Auto-oxidation of ·NO with O_2 results in the formation of nitrite (NO_2^-). NO_2^- is also a substrate for hemeperoxidases such as MPO and EPO. Peroxidase-catalyzed oxidation of NO_2^- results in the formation of nitrogen dioxide radical ($NO_2\cdot$).[255] NO reacts with superoxide to form peroxynitrite ($ONOO^·$). $ONOO^·$ can nitrate tyrosine residues and alter levels or function of enzymes, structural and signaling proteins.[256]

2.2.1.1. Environmental exposures.

Because the lung interfaces with the external environment, it is frequently exposed to airborne oxidants. Ozone, particulate matter and cigarette smoke represent the most common air pollution problems.

Ozone is formed from volatile hydrocarbons, halogenated organics, and oxides of nitrogen in the presence of sunlight.[257] Ambient ozone levels usually vary between 20 and 40 parts per billion (ppb).[258] High concentrations of ozone can be harmful to the lung.[259,260,261,262,263,264] Ozone reacts with unsaturated fatty acids and cell membranes to produce lipid ozonation products.[265,266]

Particulate matter pollution is one of the most serious air pollution problems in urban environments. One of the most dangerous forms of particulate matter pollution is diesel exhaust particle. Diesel exhaust particles are a polyaromatic hydrocarbon, a hydrophobic molecule that can diffuse easily through cell membranes. Diesel exhaust particles may therefore modify cell growth and differentiation.

Environmental tobacco smoke is a complex mixture of gases and particles. Cigarette smoke contains >4,000 chemicals including 50 that are known to cause cancer. Some of them are carbon monoxide, cyanide, arsenic, mercury, and NO. Furthermore, cigarette smoke generates or contains*10^{14} oxidative molecules per puff such as hydrogen peroxide and superoxide. Tobacco smoke leads to activation of phagocytes augmenting release of free radicals.[267]

2.2.2. Biological oxidative processes in the lungs and anti-oxidant

The formation of ROS and RNS is an essential for neutrophils, monocytes, macrophages, and eosinophils in order to kill bacteria. These phagocytic cells use NADPH oxidase enzymatic systems to generate $O2^{\cdot -}$.[268] They can also form HOCl through myeloperoxidase-catalyzed oxidation of the Cl^- ion by H_2O_2. $^{\cdot}NO$ is also involved in mononuclear cell-mediated killing of Mycobacterium tuberculosis and other pathogens in rodents and is toxic to tumor cell lines in vitro.[269] Cytochrome P450 also exploits the reactivity of the iron–oxygen complex to catalyze oxidation of a number of endogenous compounds and xenobiotics.[270]

The balance between physiologic functions and damage is determined by the relative rates of formation and the removal of free radicals. The lungs have developed several endogenous antioxidant systems. These systems may be divided into enzymatic and nonenzymatic groups.

2.2.2.1. Non-Enzymatic lung antioxidants

The most well-researched nonenzymatic antioxidants include lipid-soluble vitamin E (tocopherol), vitamin A, and carotenoids (including beta-carotene), and water-soluble vitamin C and glutathione (GSH).

Vitamin E is an important hydrophilic antioxidant. It protects the cell membrane from oxidation by reacting with lipid radicals, such as lipid peroxyl radicals (LOO·) that are produced during lipid peroxidation reactions.[271]

Vitamin C is a hydrophilic vitamin that can directly scavenge $O2^{\cdot-}$ and ·OH by forming the semidehydroascorbate free radical that subsequently is reduced by GSH.[272] Vitamin C, however, is usually not considered a major antioxidant because it also has pro-oxidant properties.[273]

Glutathione (GSH) is the predominant protein for maintenance of the cellular redox.[274] GSH is a cysteine-containing peptide found in most forms of aerobic life, and is present in high concentration in blood and lung.[275] Lung epithelial lining fluid contains up to 300 micromolar concentration of GSH,[276] and >90% of the GSH is maintained in the reduced form. ROS increase GSH through induction of g-glutamyl cysteine synthetase, the ratelimiting enzyme of GSH biosynthesis.[277]

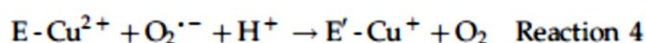
Other non-enzymatic antioxidants include β-carotene, uric acid, bilirubin, taurine, albumin, cysteine and cysteamine.

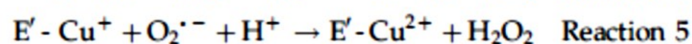
2.2.2.2. Enzymatic lung antioxidants.

The enzymatic antioxidants include superoxide dismutases (SOD), catalase, glutathione peroxidases, heme oxygenase, glutaredoxin, thioredoxin, and peroxiredoxin. These antioxidant enzymes usually require trace metal cofactors. SOD, for example, consists of proteins co-factored with copper, zinc, or manganese.[278] Iron is required as a co-factor for catalase.[279]

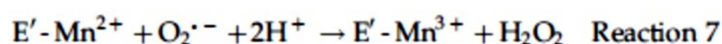
Superoxide dismutases (SOD) are ubiquitous enzymes with an essential function in protecting aerobic cells against oxidative stress. They catalyze the reaction of

superoxide radicals to hydrogen peroxide. Human lung epithelium expresses three forms of eukaryotic SODs.[280] The copper/zinc superoxide dismutase (CuZnSOD) is expressed in bronchial epithelium, alveolar epithelium, mesenchymal cells, fibroblasts, arterioles, and capillary endothelilal cells.[281] The Mn superoxide dismutase (MnSOD) is expressed in the airways, especially in the alveolar duct and arterioles.[282] Furthermore, MnSOD is also moderately or highly expressed in respiratory epithelium, alveolar type II epithelial cells, and alveolar macrophages.[283] The extracellular superoxide dismutase (EC-SOD) is found in bronchial epithelium, alveolar epithelium, epithelial cells lining intrapulmonary airways, alveolar macrophages, and endothelial cells lining both arteries and veins. The CuZnSOD is mainly found in the cytosol, although it also is present at low levels in lysosomes, peroxisomes, nucleus, and intermembrane space of the mitochondria.[284] CuZnSOD is expressed in lung cells, such as bronchial epithelial, alveolar macrophages, and capillary endothelium of the lung.[285]





The MnSOD protein constitutes up to 10% of the intracellular SOD activity and is mainly expressed in the matrix of the mitochondria.[286] Superoxide dismutation by MnSOD proceeds through the following reactions:



Oxidative stress can upregulate MnSOD gene expression[287] via Nrf-2.[288] Genetic deletion of this critical enzyme in mice is inconsistent with life.[289] The EC-SOD is the major extracellular SOD in the interstitial spaces of the lungs.[290] EC-SOD contains a heparin/matrix binding domain consisting of positively charged arginines and lysines.[291] The heparin/matrix-binding domain is sensitive to proteolysis, which can lead to release of EC-SOD from tissue matrix. EC-SOD protects the oxidative fragmentation of heparin/heparan suflate/ syndecan-1.[292] The localization of EC-SOD in the lungs is primarily within the smooth muscle region surrounding blood vessels and airways. Polymorphisms are associated with patient outcomes in COPD and lung injury.

Catalase is the principal scavenger of H_2O_2 when is present at high concentrations. Catalase is relatively limited in cellular distribution.[293] Under prolonged oxidative stress with oxidation of NADPH, catalase activity drops.[294] This enzyme is not generally inducible by oxidative stress.[295] Enzyme activity can be regulated by post-translational processes. [296,297]

The reducing capacity of glutathione peroxidase enzymes are based on high levels of GSH (L-g-glutamyl-L-cysteinylglycine). Glutathione peroxidases reduce hydrogen peroxide to water by oxidizing glutathione to oxidized/disulfide form (GSSG). The glutathione disulfide (GSSG) that is formed in the course of the reaction is subsequently reduced back to GSH by glutathione reductase. The capacity to recycle GSH makes the glutathione system crucial for the antioxidant mechanisms.

Thioredoxins (Trx-) are oxidoreductase enzymes containing a dithiol–disulfide active site (-Cys-Gly-Pro-Cys-), (see **Chapter 3**).[298] The cysteine residues reverse from a dithiol (-SH HS-) group to a disulfide bridge (-S-S-). Trxs are kept in the reduced state by flavoenzyme thioredoxin reductase, via an NADPH-dependent reaction. There are two thioredoxins, 1 and 2, with different cellular locations, and there are two thioredoxin reductases, with locations corresponding to the intracellular thioredoxins 1 and 2. Thioredoxin 1 is found in the cytoplasm and Thioredoxin 2 in the mitochondria.[299] Overall, Trxs can reduce protein disulfides and protein sulfenic acid intermediates by cysteine thiol–disulfide exchanges.[300]. Thioredoxin 1 augments gene expression of other antioxidants, such as MnSOD.[301] Specific protein disulfide targets for reduction by thioreoxin are ribonucleotide reductase, [302] protein disulfide isomerase,[303] and several transcription factors including p53, NF-kB, and AP-1.[304] Thioredoxins are expressed in bronchial epithelial cells and alveolar macrophages, metaplastic alveolar epithelial cells, and bronchial chondrocytes.[305]

Glutaredoxins (GRX) are thiol–disulfide oxidoreductases that use glutathione as a cofactor and catalyze the reversible exchange of GSH with protein thiol groups. The human cell contains four GRXs, two dithiol (GRX1 and GRX2), one multiple monothiol (GRX3), and one monothiol (GRX4).[306] The formation of protein–SG mixed disulfide (glutathionylation) by glutaredoxin through a monothiol mechanism may play

an important role in protecting against more drastic irreversible modifications of protein thiols.[307]

Peroxidredoxins is a new family of non-seleno peroxidases. Prxs modulate cytokine induced hydrogen peroxide levels. Six different types of Prxs have been characterized in human lung. The bronchial epithelium showed moderate to high expression of Prxs I, III, V, and VI, the alveolar epithelium expressed mainly Prxs V and VI, and alveolar macrophages expressed mainly Prxs I and III.

Heme oxygenases (HO-) are members of the heat-shock family of proteins that play a protective role in inflammation and oxidative stress (See **Chapter 3**). There are three forms of heme oxygenases. Heme oxygenase-1 is inducible, whereas heme oxygenase-2 and – 3 are constitutive. These enzymes catalyze the degradation of heme molecules into biliverdin, bile pigments, and generate carbon monoxide and iron. Carbon monoxide and biliverdin have been attributed antioxidant properties. HO-1 knockout mice are more susceptible to oxidative stress.[308] Furthermore, induction of HO- by administration of hemin suppresses inflammation in the airway in ovalbumin-challenged guinea pigs.[309] Heme oxygenases are expressed in lung inflammatory cells of rats exposed to hypoxia. HO-1 has been reported in human airways during asthma; levels in sputum of asthma patients are higher than in controls. Carbon monoxide concentrations are higher in exhaled breath of asthmatics as compared to healthy controls. Heme oxygenase is expressed in airway epithelial cells, alveolar macrophages, bronchial epithelial cells, and inflammatory cells of the lungs.[310]

2.2.3. Redox imbalance in asthma

Enhanced levels of oxidant production are abundantly documented in asthma. Inflammatory cells are increased in asthmatics and produce more ROS as compared to control subjects. Asthma attacks and experimental Ag challenge are both associated with immediate formation of $O2^{\cdot -}$.[311] Spontaneous and experimental allergen-induced asthma attacks lead to leukocyte (eosinophil, neutrophil) activation, during which ROS are rapidly formed. ROS production by asthmatics'neutrophils correlates with severity of reactivity of airways. Oxidative modifications are characteristics of asthma.[312] Increased levels of eosinophil peroxidase and myeloperoxidase are found at higher than

normal levels in asthmatic peripheral blood, induced sputum, and bronchoalveolar lavage fluid. Biomarkers of eosinophil activation include release of granule proteins including EPO [313]and major basic protein (MBP). 3- bromotyrosine is a unique product of EPO and eosinophils. Increased levels of 3-bromotyrosine are found in asthmatics bronchoalveolar lavage as compared to controls subjects. The levels of 3-bromotyrosine are increased further when asthmatics are exposed to antigen challenge.[314,315] The urinary 3-bromotyrosine is elevated in asthmatics as compared to healthy controls, and may increase during exacerbations. MPO-mediated oxidant modifications also contribute to the pathophysiology of severe asthma. Malondialdehyde and thiobarbituric acid reactive products have also been detected in urine, plasma, sputum, and bronchoalveolar lavage fluid that relate to the severity of asthma. 8-isoprostane, a biomarker of lipid peroxidation, is also elevated in exhaled breath condensate in adults and children with asthma. Tyrosine nitration increases following allergen exposure.[316] Eosinophils may contribute to the generation of large number of oxidant products in asthma.[318]

Ozone and diesel exhaust particles have an additive effect on airway hyperreactivity and inflammation in asthma.[317] Ozone increases hyperreactivity, induces IL-5 and granulocyte-macrophage-colony stimulating factor (GM-CSF) in bronchoalveolar lavage, which recruits and enhances the longevity of eosinophils.[318] Ozone also leads to oxidative modification of surfactant proteins.[319] Exposure of human airway epithelial cells to lipid ozonation products in vitro leads to activation of eicosanoid metabolism.

Evidence supporting increased ·NO in asthma is substantial. ·NO is increased in the lower airway and in the exhaled breath of asthmatics. Exhaled ·NO in asthmatics increases after allergen challenge during the late asthmatic response. Individuals with asthma have 3-fold higher than normal NO concentrations, and increased NOS_2 mRNA and protein in airway epithelial cells.[320] NO synthesis under oxidative and acidic conditions causes injury. Increased nitration is found during an asthma exacerbation and S-nitrosothiols concentrations are elevated in EBC in patients with asthma.

2.2.4. Antioxidant deficiency in asthma.

In asthma, SOD activity is significantly lower in epithelial lining fluid and airway epithelial cells.[321]Murine models of asthma also provide evidence of a link between antioxidants and airway hyper-responsiveness. For example, transgenic mice that overexpress SOD have decreased allergen-induced physiologic changes in the airway in comparison to controls.[322] Studies indicate that the lower SOD activity in asthma is a consequence of the increased oxidative and nitrative stress in the asthmatic airway. Oxidatively modified and nitrated MnSOD is present in epithelial cells recovered during bronchoscopy from asthmatic. Red blood cells of asthmatic children were shown to have lower catalase activity than healthy children.[323] Tyrosine oxidant modifications of catalase occur in asthma.[324,325]

In contrast extracelluluar GPx (eGPx) is present at higher than normal levels in lungs of individuals with asthma. The increase is due to induction of eGPx mRNA.[326]

Glutathione in exhaled breath of children with asthma during acute asthma exacerbation is reduced than control subjects, and the glutathione levels in exhaled breath of subjects with asthma increase after oral steroid treatment.[327] Minutes after challenge, GSH levels drop and GSSG increases in the lung epithelial lining fluid. GSH depletion in vivo and/or in vitro leads to inhibition of Th1-associated cytokine production and/or favors Th2-associated response. Thus, GSH facilitates a Th2 phenotype, and reduction in GSH levels supports the maintenance of Th2 response in asthma.[328]

Reynaert et al. demonstrate that glutaredoxin 1 is upregulated in a mouse model of asthma.

During asthma exacerbation in humans, the levels of serum Trx-1 increase and are inversely correlated with airflow.[329] This suggests that Trx-1 may have a protective effect in asthma. In vitro studies have shown that exogenous Trx-1 can prevent Th2 development by upregulating the expression of Th1-like cytokines.[330] The protective effects of Trx-1 in asthma are thought to be partly dependent on its antioxidant effect.[334]

NF-kB and activation protein-1 (AP1) are regulated by the redox status and are implicated in the transcriptional regulation of a wide range of genes involved in

oxidative stress.[331] Evidence suggest signaling pathways such as the family of mitogen-activated protein kinases (MAPKs) are also altered by redox changes.[332]Redox-sensitive molecular targets usually contain highly conserved cysteine residues, and oxidation, nitrosylation, or the formation of disulfide links are crucial events in oxidant-redox signal. There is evidence of activation of NF-kB in biopsies and sputum inflammatory cells such as macrophages and neutrophils of asthmatics.[333] Nitrosation of NF-kB subunits is an important mechanism for the redox sensing of NF-kB.[334] Activator protein-1 (AP-1) is a protein dimer, composed of a heterodimer of Fos and Jun proteins. AP1 regulates many of the inflammatory and immune genes in oxidant-mediated diseases.

Gene expression of g-GCS, the rate-limiting enzyme for the GSH synthesis, is induced by the activation of AP1.[335] Asthmatic epithelial cells have increased expression of c-Fos. Cigarette smoke increases AP-1 DNA binding in human epithelial cells in vivo. High levels of NO and hydrogen peroxide cause increases in c-fos and c-junmRNAof epithelial cells. The process of acetylation and deacetylation of histone is also influenced by redox changes (See **Chapter 9**).[336] In biopsies and peripheral blood mononuclear cells from asthmatics, there is an increase in acetylation and a reduction in deacetylation activity, which upregulates some inflammatory gene expression and downregulates others.[337] Redox changes also can activate members of the mitogen-activated protein kinase signaling (MAPK), such as extracellular signalregulated kinase (ERK), c-jun N-terminal kinase (JNK), p38 kinase, and phosphoinositol-3 kinase, all of which may ultimately promote inflammation.[338]

Binding of cytokines, including IL-4 and INF, to their specific receptors leads to transphosphorylation of tyrosine residues on Janus kinases (JAK), which then recruit and phosphorylate the signal transducers and activators of transcription (STAT) family of transcription factors on tyrosine residues and result in gene expression of pro-inflammatory genes such as NOS_2.

STAT1 and STAT3 activation is redox regulated. Although STAT3 has not been evaluated in asthma, STAT1 is activated at high levels in asthmatic airway epithelium but not in healthy controls.[339] Simon et al. showed that members of the STAT family of transcription factors, including STAT1 and STAT3, are activated in response to H_2O_2 or

GSH-depletion.Wang et al. showed that vanadium leads to STAT-1 activation.[340] Recently, the detailed redox mechanisms that regulate STAT activation by IL-4 have been identified.[341] Hom1eostatic control of cytokine-receptor activation and signal transduction occurs through ROS generation via activation of NOX enzymes.[342]

2.2.5. Genetics of redox in asthma

Genetic variability of SODs may play a role in the development of asthma. A polymorphism (R213G) of EC-SOD causes more than nine fold higher levels of EC-SOD in plasma due loss of anchoring to heparin in the interstitium.[343] Two novel polymorphisms occur in the noncoding 5' untranslated region (Exon 1) and first intron (Intron 1) of the SOD3 gene. A recent report by Dahl et al. found that ECSOD homozygous for the Exon1/Intron1 polymorphism associates with reduced lung functions in individuals with COPD. This supports a role for EC-SOD in oxidant-mediated events influencing airway diseases and lung function.[344] The deletion allele of glutathione S-transferase M1, GSTM 1, (null-genotype) has been associated with increased risk of asthma and lower lung function. Islam et al.[345], report that children with a Val105 mutation in GSTP1 variant allele may have a lower risk of asthma.

2.3.Consequences of oxidative stress in asthma

Oxidative stress can have many detrimental effects on airway function, including airway smooth muscle contraction,[346] induction of airway hyperresponsiveness,[347] mucus hypersecretion,[348] epithelial shedding[349] and vascular exudatio.[350] Furthermore, ROS can induce cytokine and chemokine production through induction of the oxidative stress-sensitive transcription of nuclear factor-kB in bronchial epithelial cells.[351]

2.4. Exacerbations of asthma and oxidative stress

Most of the studies on oxidative stress in asthma have concentrated on the oxidant/antioxidant imbalance that occurs in stable asthma. The overlaid effects of exacerbations have received much less attention. Episodic worsening of asthma is associated with increased airway inflammation. There is also indication of enhanced oxidative stress during exacerbations, both systemically and locally. However, a direct correlation between increased oxidative burden and changes in pulmonary function and/ or airway inflammation described during exacerbations remains speculative. [352] A hypothesis that relates exacerbations of asthma to dietary antioxidant deficiency has been proposed. [353] Many indirect markers of oxidative stress such as H_2O_2 and isoprostanes are increased in exhaled air, sputum, and BAL fluid during exacerbations and after allergen exposure.[354] Respiratory viruses represent the most important causes of asthma exacerbations. Rhinoviruses are the virus type most frequently identified in respiratory tract specimens during exacerbations of asthma, both in children and in adults. [355] Experimental rhinovirus infection of asthmatic patients can induce an inflammatory response in the airways associated with variable airflow obstruction and increased airway hyperresponsiveness. Rhinovirus induced airway inflammatory responses involve eosinophils and neutrophils, possibly recruited via cytokines or chemokines released by bronchial epithelial cells or T cells.[356] Rhinovirus infection of respiratory epithelial cells causes intracellular oxidant generation which is a crucial step in the activation of NF-kB and in the following production of pro-inflammatory adhesion molecules and cytokines.[357] Reducing agents inhibit both rhinovirus induced oxidant generation and inflammatory mediator production and release. These observations provide evidence of an increased oxidative burden in asthma exacerbations.

Chapter 3

3. Definition and classification of oxidative and anti-oxidative biomarkers used for the study of asthma

Direct measurement of oxidants is difficult since they are highly reactive, short-lived species. Thus, oxidative stress is often measured by observing the damage inflicted by oxygen radicals upon various biomolecules, such as lipids, proteins or deoxyribonucleic acid. Aspects of the antioxidant defence system are also often examined as an indirect marker of oxidative stress. Before moving to the experimental part of this thesis, this chapter will briefly introduce the bio-markers of oxidative stress and anti-oxidant response that have been chosen to investigate the oxidative unbalance during asthma exacerbation.

3.1.Markers of oxidative stress and protein oxidative damage

Protein oxidation is defined as the covalent modification of protein, either directly by ROS or indirectly by a reaction with secondary by-products of oxidative stress. Oxidative damages to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, protein aggregation and enhanced susceptibility to proteolysis. Protein oxidation serves as a useful marker for assessing oxidative stress. In the studies included in the current thesis 4-Hydroxy-2-nonenal and protein carbonylation have been investigated in asthma exacerbation as stable and reliable bio-markers of oxidative damage on proteins.

3.1.1. Lipid peroxidation and 4-Hydroxy-2-nonenal

4-HNE (4-Hydroxy-2-nonenal) is an α,β-unsaturated hydroxyalkenal which is formed during lipid peroxidation (LP).[358] LP is an autocatalytic process initiated by free radical attack on the unsaturated (double) bonds of membrane fatty acids. Superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide, (H_2O_2), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{\cdot}$) are most commonly involved in the initiation of LP. The major

source of superoxide in most cells is that produced in mitochondria as “by-product” of oxidative phosphorylation in the electron transport chain. Much of the superoxide is rapidly converted to hydrogen peroxide by mitochondrial superoxide dismutase (SOD2) and cytoplasmic superoxide dismutase (SOD1). Hydroxyl radical can then be produced by a process called the Fenton reaction in which Fe^{2+} or Cu^{+} interact with hydrogen peroxide. Nitric oxide is generated in response to elevations of intracellular Ca^{2+} levels; Ca^{2+} binds calmodulin, which then activates nitric oxide synthase which catalyzes the conversion of arginine to citrulline and nitric oxide. Nitric oxide may then interact with superoxide to generate peroxynitrite. Among the different ROS, hydroxyl radical and peroxynitrite are particularly aggressive inducers of LP. The process of lipid peroxidation is initiated by interaction of hydroxyl radical or peroxynitrite with unsaturated lipids which triggers chain peroxidation by abstracting allylic hydrogens. The resulting lipid radicals rapidly interact with oxygen, thereby propagating the reaction via peroxyl radical intermediates; this process simultaneously generates lipid hydroperoxides and aldehydes of various chain lengths. Lipid peroxidation can be terminated by so-called chain-breaking antioxidants such as vitamin E. 4-HNE is one specific aldehydic product of LP increasingly recognized as a particularly important mediator and marker of cellular dysfunction and degeneration in a range of disorders including asthma.

Fig. 3.1 explains how exposure to allergens, gaseous pollutants, chemicals, drugs, bacteria and viruses leads to the recruitment and activation of inflammatory cells in asthmatic airways, including mast cells, eosinophils, neutrophils, lymphocytes, macrophages and platelets. Allergen-specific reactions involving the acquired immune system are characterised by the production of IL-5 and the subsequent recruitment and activation of eosinophils. In contrast, stimuli that act via the innate immune system lead to the production of IL-8 and the subsequent recruitment and activation of neutrophils. However, both of these pathways lead to the production of ROS, primarily due to the respiratory burst of activated inflammatory cells. Activated inflammatory cells respond with a "respiratory burst", which involves the uptake of oxygen and subsequent release of ROS into surrounding cells.

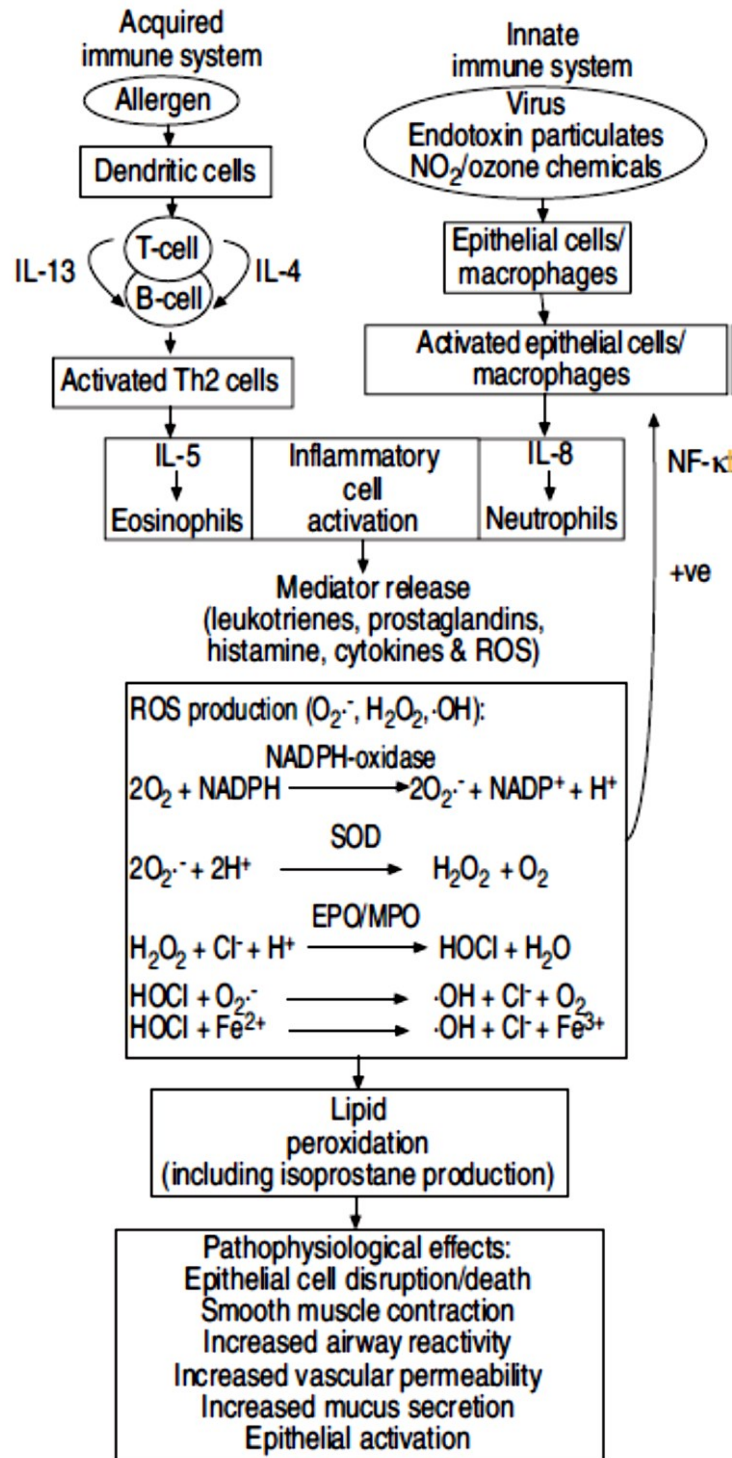


Fig. 3.1 *Mechanisms leading to lipid peroxidation in asthma.* IL: interleukin; Th2: T-helper type-2 cells; NO2: nitrogen dioxide; ROS: reactive oxygen species; O2?-: superoxide; H2O2: hydrogen peroxide; OH: hydroxyl radical; NADPH: reduced nicotinamide-adenine, dinucleotide phosphate; NADP: nicotinamide-adenine dinucleotide phosphate; SOD: superoxide dismutase; EPO: eosinophil peroxidase; MPO: myeloperoxidase; NF: nuclear transcription factor. Source: L.G. Wood, P.G. Gibson, M.L. Garg. Biomarkers of lipid peroxidation, airway inflammation and asthma. Eur Respir J 2003; 21: 177–186

HNE is a 9-carbon amphiphilic lipid formed when n-6-polyunsaturated fatty acids such as arachidonic acid and linoleic acid are attacked by peroxidative free radicals .4-HNE acts as a key mediator of oxidant-induced cell signaling and apoptosis. For his high affinity toward cysteine, histidine, and lysine groups numerous proteins have been shown to be modified by HNE **(Fig. 3.2)**. In many cases the function of the protein will be impaired. While lower levels of intacellular 4-HNE are beneficial to cells, possibly promoting cellular proliferation, higher levels can cause a toxic response in the cell and may lead to cell death. Thus 4-HNE is recognized as a particularly important marker of cellular degeneration in a range of disorders including asthma. 4-HNE has also been reported to activate GSH synthesis via induction of the glutamate cysteine ligase gene and a variety of pro-inflammatory genes, such as IL-8, monocyte chemoattractant protein (MCP)-1, cyclooxygenase-2, epidermal growth factor receptor, and of mucin 5AC. 4-HNE has been shown to induce expression of the protective antioxidant gene λ-gluta-mylcysteine synthetase (λ -GCS) mRNA in alveolar epithelial cells.

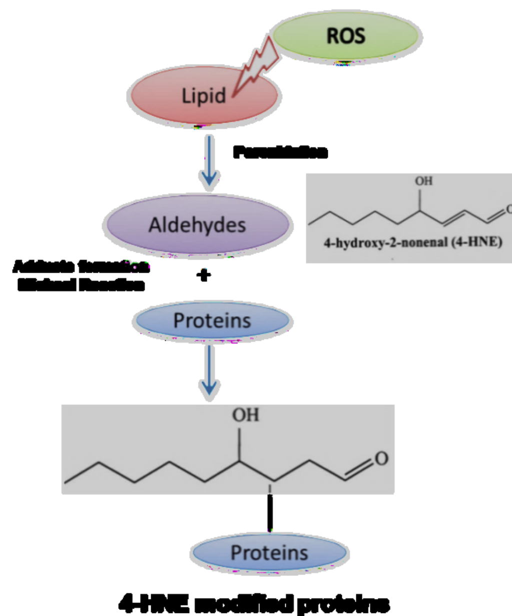


Fig. 3.2 *The process of lipid peroxidation and 4-HNE.* The process of lipid peroxidation is initiated by interaction of hydroxyl radical with unsaturated lipids. The resulting lipid radicals rapidly interact with oxygen, thereby propagating the reaction via peroxyl radical intermediates; this process simultaneously generates lipid hydroperoxides and aldehydes. One specific aldehydic product of LP called 4-hydroxynonenal (HNE) is increasingly recognized as a particularly important mediator and marker of cellular dysfunction When HNE encounters proteins, it can interact with thiol (SH) and amino (NH_2) groups of cysteine, lysine and histidine residues via a process called Michael addition resulting in a covalent bond between HNE and the amino acid. Numerous proteins have been shown to be modified by HNE including: lasma membrane ion and nutrient transporters; receptors for growth factors and neurotransmitters; mitochondrial electron transport chain proteins;protein chaperones; proteasomal proteins; and cytoskeletal proteins. C. Folisi

3.1.2. Proteins carbonylation

A common biochemical marker of oxidative stress is the formation of protein carbonyl groups (aldehydes and ketones) on protein side chains particularly of prolines, arginines, lysines and threonines.[359,367]Carbonyl groups are composed of a carbon atom double-bonded to an oxygen atom, and are formed primarily from lipid electrophiles generated under conditions of oxidative stress. Electrophile adduction and other oxidative reactions can irreversibly alter protein structure and function.

The use of the marker of severe protein oxidation (carbonylated proteins), involve derivatization of the carbonyl group with 2,4-di-nitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl hydrazone product. Carbonylation reflects the oxidation of Lys, Arg or Pro residues in proteins and is the most commonly used marker for protein oxidation in body fluids (**Fig. 3.3** and **3.4**). [360,361,362]

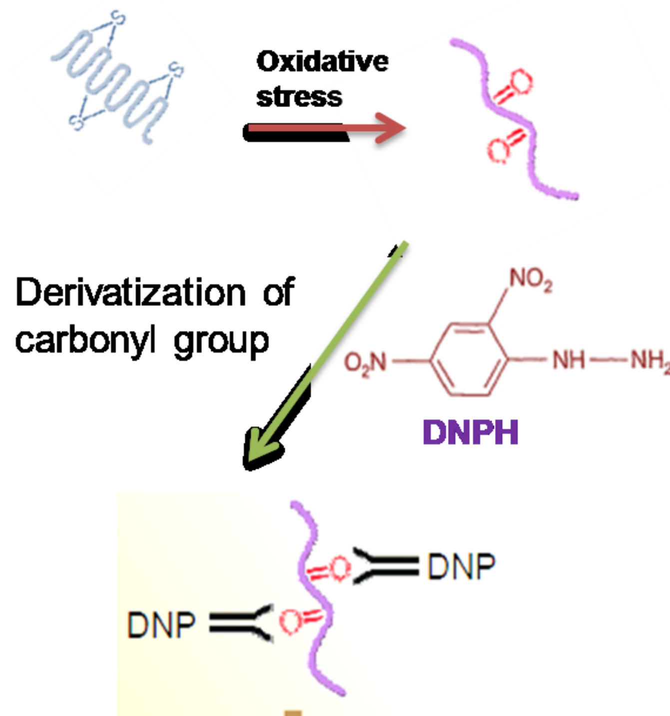


Fig. 3.3 *Carbonyls derivatization with DNPH.* Under oxidative stess carbonyls groups are formed on the side chain of proteins. Carbonylated proteins are relatively stable thereby allowing the derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) which leads to the formation of a stable dinitrophenyl (DNP) hydrazone product. Using the Western blot technology and anti-DNP antibodies allows for the rapid and highly sensitive determination of protein carbonyl formation. C. Folisi.

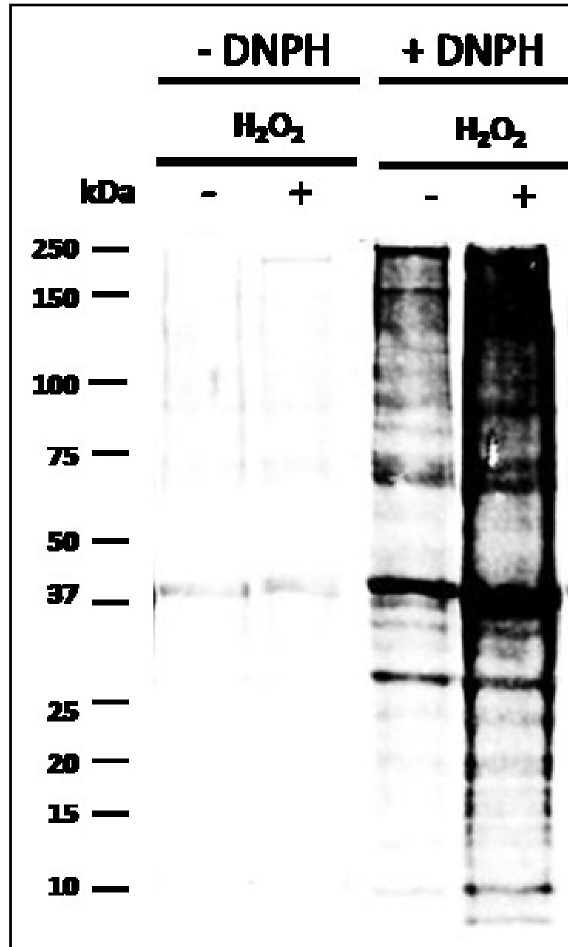


Fig. 3.4 *Representative Westen Blot for carbonylated protein without (-) and after (+) derivatization to DNP.* The DNPH derivatization allows the immuno-detection of carbonylated proteins, without derivatization was not possible to detect carbonylated proteins. Cells were untreated (-) or treated (+) with H_2O_2. The treated cells showed higher proteins carbonylation. C. Folisi.

Protein carbonylation has been studied in asthma. Nadeem et al. showed an increase in plasma protein carbonyls in asthmatics.[363] Foreman et al. found increased levels of carbonylated proteins among BALF proteins in atopic asthmatic adults 18 h after allergen challenge.[364] In asthmatic children, the number of inflammatory cells in bronchoalveolar lavage fluid (BALF) has been showed significantly correlated with the concentration of protein carbonyls.[365] By contrary, some studies have shown no increase in carbonylated proteins in sputum from patients with mild asthma or in BALF from asthmatic children respect to healthy subjects.[366]

3.2.Markers of anti-oxidant response

The balance between physiologic functions and damage is determined by the relative rates of formation and the removal of free radicals. The lungs have developed several endogenous antioxidant systems to deal with the production of free radicals. Many controlled studies suggest that there is a deficiency of antioxidants in the lungs or circulation of asthmatic subjects. Given the critical role of anti-oxidant response in the pathogenesis of asthma, we tested whether different expression of cyto-protective and anti-oxidant proteins might serve as biomarkers during evolving acute exacerbation. In the next paragraphs will be described the important markers of anti-oxiant response that have been assayed for the study i.e. Heme-oxygenase-1 (HO-1), Heat shock protein-70, Thioredoxin reductase (Trx-R), Thioredoxin-2 (Trx-2), Sirtuin-1 and -2, and NF-E2-related nuclear factor 2 (Nrf-2).

3.2.1. Sirtuins

The silent information regulator (SIR) proteins mediate transcriptional silencing.[367] Sir2p encodes an NAD-dependent histone deacetylase and is thought to mediate silencing by regulating histone acetylation[368] The SIR2 gene family is conserved from archaebacteria to eukaryotes.[369] Humans have seven proteins with homology to Sir2p, which have been named sirtuins (SIRTs). In mammals three of the seven sirtuins are associated with mitochondria.[370,371] There are several evidences which show that Sirtuins are implicated in stress resistance. In particular, Sirt-1 is a metabolic NAD+-dependent belonging to class III histone/protein deacetylases that regulates proinflammatory mediators playing an important role in stress resistance, metabolism, apoptosis, senescence, differentiation, and aging (**Fig. 3.5**). SIRT1 deacetylates the tumor suppressor p53 to inhibit its transcriptional activity, resulting in reduced apoptosis in response to various genotoxic stimuli.[372,373] On the other hand, in cultured primary cells, SIRT1 is required for the expression of the tumor suppressor p19ARF, which promotes p53 stability.[374] MEFs (mouse embryonic fibroblasts) lacking SIRT1 have an increased resistance to senescence induced by chronic oxidative stress, a phenomenon associated with decreased levels of the tumor suppressor p19ARF and thus p53 levels.

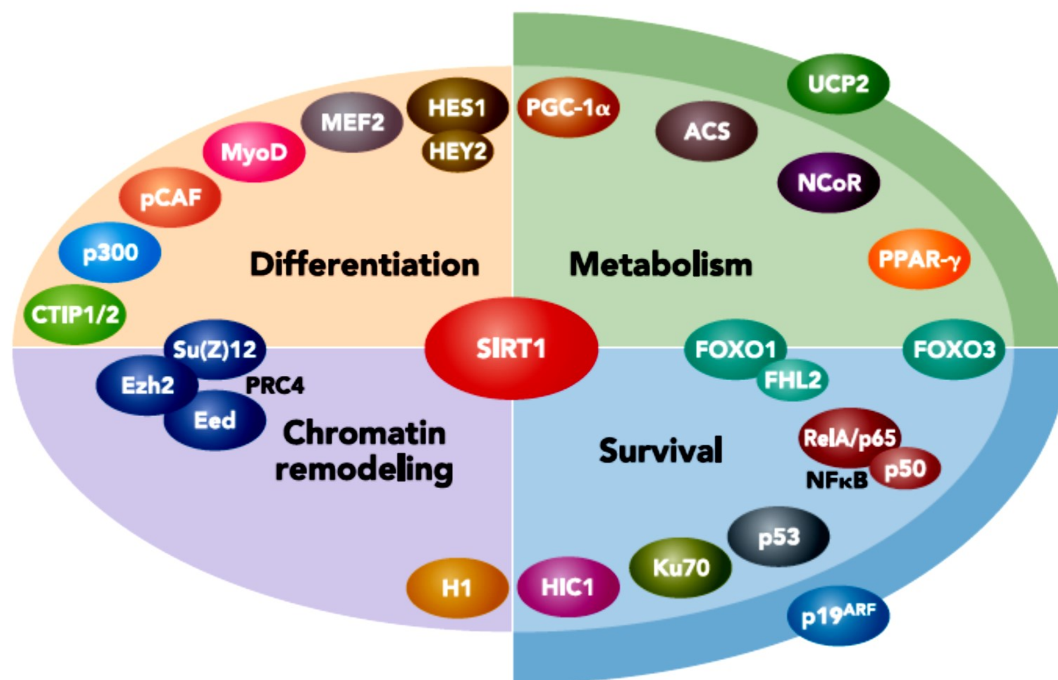


Fig. 3.5. *Interacting partners, substrates, and downstream effectors of Sirt-1*. Source: Dimitrios Anastasiou and Wilhelm Krek. *Physiology* 21:404-410, 2006. doi:10.1152/physiol.00031.2006.

The role of Sirtuins in asthma has been increasingly studied. In recent times, Yeung and colleagues demonstrated that Sirt-1 interacts with the RelA/p65 subunit of NF-kB and inhibits gene transcription by deacetylation at the lysine 310. Lung cells from COPD patients and from rats exposed to cigarette smoke display reduced expression of Sirt1 associated with increased NF-kB activity and matrix metalloproteinase-9 expressions compared with lung cells from healthy controls. Lee and collaborators reported that treatment with sirtinol, an inhibitor of Sirt-1 and Sirt-2, reduces airway inflammation and hyperreactivity in a mouse model of atopic asthma likely due to the impairment of the activation by Sirt1 of hypoxia-inducible factor (HIF)-1a. Sirt-1 represses the activity of the nuclear receptor peroxisome proliferator-activated receptor-g in dendritic cells, thereby favoring their maturation toward a pro-Th2 phenotype. Sirt-1 inhibition impairs the optimal reactivation of Th2 responses upon allergen challenge of the airways through PPAR-g–dependent mechanisms. Other studies have shown that the activity of Sirt-1 is reduced in peripheral blood mononucleocytes (PBMCs) from patients with severe asthma compared to mild asthma and healthy volunteers. In addition, treatment

of healthy PBMCs with the sirtuin inhibitor sirtinol has been shown to increased expression of the Th2 cytokines IL-4 and IL-13 (but no alteration in expression of the Th1 cytokine IFNγ. Incubation of HUT78 cells with sirtinol, followed by immunoprecipitation of GATA3, has been shown to increase lysine acetylation and a subsequent increase in activity and accumulation of Th2 cytokines.

Sirtuin type 2 (Sirt-2), is a predominantly cytoplasmic protein that colocalizes with microtubules. The microtubule network is formed by the polymerization of α and β-tubulin heterodimers and plays an important role in the regulation of cell shape, intracellular transport, cell motility, and cell division. -α and β-tubulin subunits are subject to numerous post-translational modifications, including tyrosination, phophorylation, polyglutamylation, polyglycylation, and acetylation. [375] Sirt-2 deacetylates lysine-40 of α-tubulin both in vitro and in vivo. Sirt-2 colocalizes and interacts in vivo with HDAC-6, another tubulin deacetylase.

3.2.2. Heat shock proteins

Heat shock proteins (Hsp) are a class of functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress. Thus, in response to a wide variety of stressful stimuli, there is a marked increase in total Hsp synthesis, known as the cellular stress response. The stress response is designed to enhance the ability of the cell to cope with increasing concentrations of unfolded or denatured proteins.

Of all heat shock proteins, the Hsp-70 family constitutes the most conserved and best studied class. This family consists of the constitutively expressed Hsp-70 (Hsc-70; 73 kDa), the stress inducible Hsp-70 (Hsp-70; 72 kDa), the mitochondrial Hsp-70 (Hsp75; 75 kDa), and the endoplasmic reticulum Hsp-70 (Grp78; 78 kDa). The function of Hsp70 is exquisitely related to its structure. The Hsp-70 family members all contain two major functional domains, including a N-terminal domain, also referred to as the ATPase domain which is composed of 45 kDa amino acids, and a C-terminal domain composed of a 15–18 kDa substrate-binding domain (SBD), and a 10 kDa carboxy-terminal domain of largely unknown function .

HSPs are also induced by ROS produced by granulocytes or macrophages and providing autoprotective functions in these cells. HSPs may also amplify the immune response by modulating antigen processing and/ or by acting as autoantigens.[376] After admixing eHsp-70 to APCs, specific signal transduction pathways are activated that result in the stimulation of an immune response. Early as 2-4 hours post exposure of APC to exogenous eHsp-70, there is significant release of cytokines including TNF-α, IL-1β, IL-6 and IL-12[377] and GM-CSF;[378] nitric oxide, a potent apotogenic mediator;[379] chemokines including MIP-1, MCP-1 and RANTES[380] (**Fig. 3.6**). Peptide-bearing and non-peptide-bearing eHsp-70 is capable of inducing pro-inflammatory cytokine production by APCs.[381] eHsp-72 induces the DC maturation by augmenting the surface expression of CD40, CD83, CD86 and MHC class II molecules on DC[382,383,384,385] and migration of DC[386]and NK cells (**Fig.3.6**).

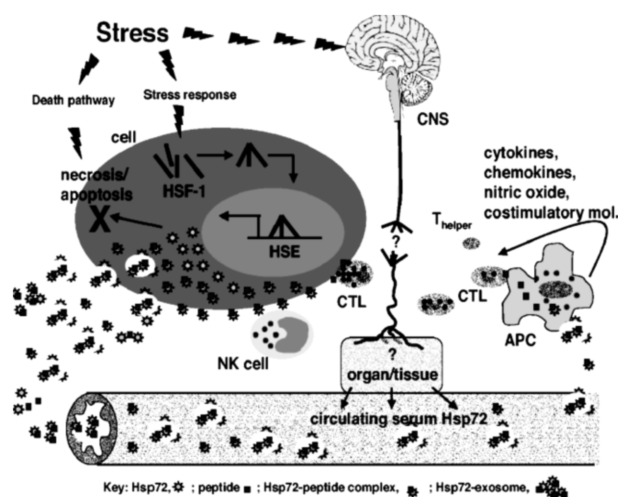


Fig.3.6 *Schematic representation of stress-induced release of eHsp-72.* Stress activates three pathways that result in the release of Hsp-72. First, the death pathway either by necrosis or apoptosis. Second, the trimerization and nuclear translocation of cytoplasmic HSF-1 (brown rods) to the heat shock element (HSE) and subsequent transcription of Hsp-72 (stars). The increased intracellular Hsp-72 chaperones peptides (Hsp-72-peptide complex) and protects the cell from cell death under certain conditions. The Hsp72-peptide complex is expressed on the cell surface and released into the extracellular milieu within exosomes; Hsp-72-exosomes. Hsp-72-peptide complexes (Hsp-72-pc) and Hsp-72-exosomes (Hsp-72-ex) make their way into the circulation. Antigen presenting cells bind and internalize Hsp72-pc and Hsp72-ex. Internalization of Hsp-72-pc and Hsp-72-ex allows the peptides to be processed and presented in the context of MHC class I to cytotoxic T lymphocytes (CTL). CTL's become activated and will recognize and destroy cells presenting the specific peptide. Circulating Hsp72-ex induces NK cells migration and the expression of Hsp72-pc on the surface of stressed cells activates NK lytic functions. Thirdly, stress in form of physical of psychological stress will stimulate the release of Hsp72-pc and Hsp72-ex into the circulation by a hitherto unknown mechanism and by a yet to be discovered tissue/organ. Alexzander Asea. Stress Proteins and Initiation of Immune Response: Chaperokine activity of Hsp72. Exerc Immunol Rev. 2005; 11: 34–45.

Extracellular Hsps are likely to act as indicators of the stress conditions. Some extracellular Hsp, are associated with export vesicles, displaying a robust activation of macrophages. For instance, during the response to stress or injury like temperature, exercise, and infection, Hsp-70 may be released from dying cells that have lysed, as well as from live cells via receptor-mediated exocytosis.

In asthmatics, differences in the expression and localization of some Hsp have been found. The percentage of alveolar macrophages expressing Hsp-70 is significantly increased in comparison with that of chronic bronchitis patients and control subjects.[387] Hsp-60 may be involved in alveolar macrophages functions in a context of allergic. In biopsies from asthmatic patients, the staining with anti-Hsp-70 mAb is intense and localized on ciliated epithelial cells, epithelial goblet cells and basal cells, mononuclear cells, smooth muscle cells, and cells of mucous glands and the basal membrane. Sputum and plasma concentrations of Hsp-70 in asthmatics patients has been shown significantly higher than that in control subjects.

3.2.3. Thioredoxin-2 and Thioredoxin Reductase

The Trx- system contains many antioxidative proteins such as Trx, mitochondrial TRX-2, their reductases (Trx-Rs), and peroxiredoxins. Thioredoxin is a 12-kD oxidoreductase enzyme containing a dithiol-disulfide active site. Thioredoxin is known to possess antioxidant activity that regulates redox-sensitive molecules such as nuclear factor-κB and glucocorticoid receptors (See **Chapter 2**, **Fig. 3.7**). Plays a role in the reversible S-nitrosylation of cysteine residues in target proteins, and thereby contributes to the response to intracellular nitric oxide. Nitrosylates the active site Cys of CASP3 in response to nitric oxide (NO), and thereby inhibits caspase-3 activity. Induces the FOS/JUN AP-1 DNA-binding activity in ionizing radiation cells through its oxidation/reduction status and stimulates AP-1 transcriptional activity. The system controls the activation of a number of transcription factors through sulphydryl transfer and, through its activity on HIF-1α, it is able to regulate vascular endothelial growth factor levels and hence angiogenesis. Trx-R is an important selenoenzyme and has been implicated in selenium metabolism and protection against oxidative stress.

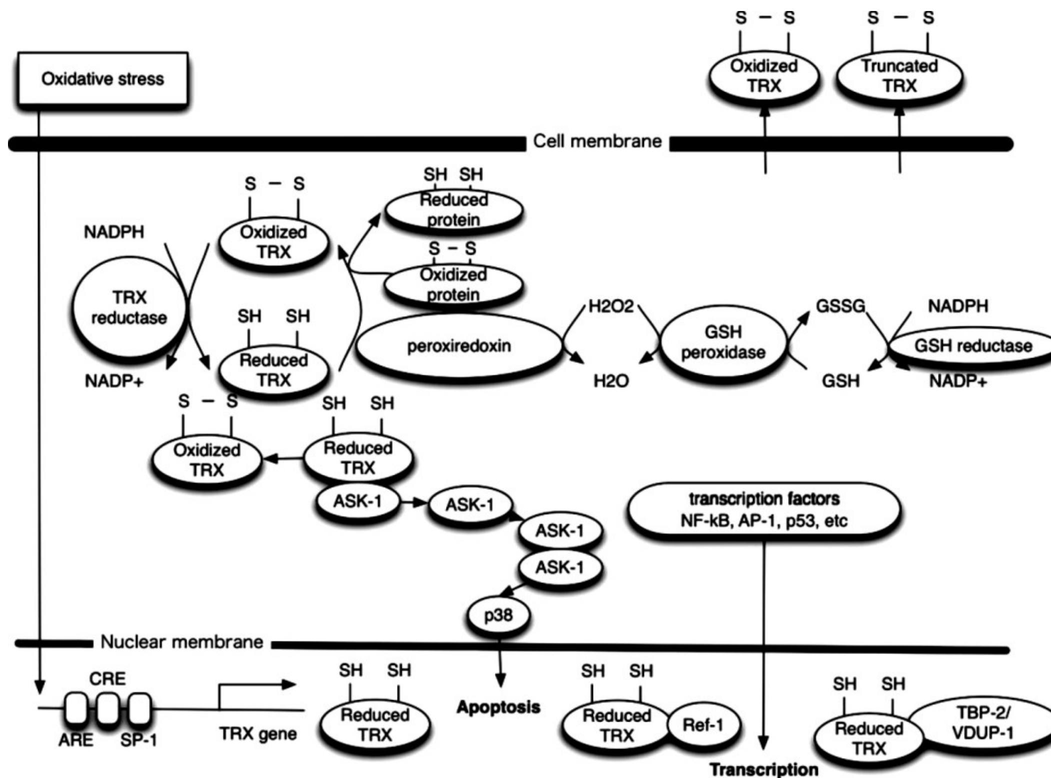


Fig. 3.7 *Biological functions of thioredoxin (TRX).* TRX is a redox-acting protein that exchanges disulfide with dithiol to maintain the reducing status of various molecules. The TRX system (TRX, TRX reductase, and NADPH) reduces peroxiredoxin or oxidized proteins. In the cytoplasm, TRX interacts with intracellular signal transduction. Oxidative stress induces TRX expression. In the nucleus, TRX has interactions with transcription factors or TRX-binding protein-2 (TBP-2)/vitamin D3-upregulated protein-1 (VDUP-1). Oxidized TRX or truncated TRX was released from cells. S, oxidized cysteine residue (S-S, disulfide bond); SH, reduced cysteine residue; ASK-1, apoptosis signal-regulating kinase 1; AP-1, activator protein-1; CRE, AMP responsive element; ARE, antioxidant responsive element; SP-1, specificity protein-1 binding site; Ref-1, redox factor-1. Source:M. Kobayashi-Miura, K. Shioji, Y. Hoshino, H. Masutani, H. Nakamura and J. Yodoi Oxygen sensing and redox signaling: the role of thioredoxin in embryonic development and cardiac diseases Am J Physiol Heart Circ Physiol 292:H2040-H2050, 2007.

Thioredoxin has been shown slightly increased in asthmatics. Yamada et al first reported that the serum levels of Trx-R positively correlated with the severity of asthma. [388]

3.2.4. Heme oxygenase

Heme oxygenase (HO-) is an enzyme that catalyzes the degradation of heme in biliverdin e iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide which inhibit both inflammation and apoptosis. The

bile pigments biliverdin IXα and bilirubin IXα have demonstrated antioxidant properties. Until now, relatively few studies have addressed the role of HO-1 in pulmonary medicine (**Fig 3.8**). Several investigators have focused on the diagnostic application of the HO-1/CO system, by measuring exhaled CO (E-CO) in various pathological pulmonary conditions, such as asthma or COPD.[389] In another experimental approach, investigators have examined the expression of HO-1 in lung tissue from healthy or diseased subjects.[390,391]

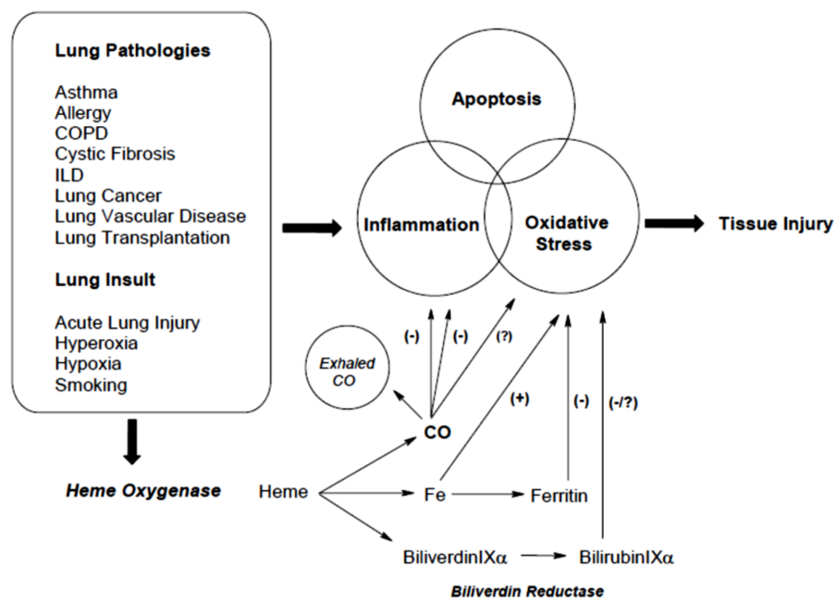


Fig. 3.8 *Role of heme oxygenase and carbon monoxide in lung diseases.* Heme oxygenase (HO-) generates biliverdin IXα, ferrous iron, and carbon monoxide (CO) from the oxidation of heme. Exhaled CO reflects active heme metabolism. Inflammation, oxidative stress, and apoptosis represent an axis of disease, against which both endogenous HO activity and exogenous CO exert protective effects. CO may inhibit both inflammation and apoptosis. The toxicological properties of CO imply increased pro-oxidant activity; however, the pro-oxidant/and antioxidant consequences of CO in the physiological range remain unclear. The bile pigments biliverdin IXα and bilirubin IXα have demonstrated antioxidant properties, though their prospective roles in modulation of inflammation and apoptosis are currently under investigation. Iron (Fe) released from HO activity returns to a transient chelatable pool, where it may potentially promote oxidative stress and apoptosis. Induction of ferritin synthesis and sequestration of the released iron into ferritin may represent one possible detoxification pathway that limits the potential of iron in pro-apoptotic and pro-oxidative processes. Source: Dirk-Jan Slebos, Stefan W Ryter and Augustine MK Choi. Heme oxygenase-1 and carbon monoxide in pulmonary medicine. Respiratory Research 2003, 4:7.

In lung tissue, HO-1 expression may occur in respiratory epithelial cells, fibroblasts, endothelial cells, and to a large extent in alveolar macrophages. HO-1 induction responds to common causes of oxidative stress to the airways, including hyperoxia, hypoxia, endotoxemia, heavy metal exposure, bleomycin, diesel exhaust particles, and

allergen exposure.[392] In a mouse model of asthma, HO-1 expression increased in lung tissue in response to ovalbumin aerosol challenge. In a similar model of aeroallergen-induced asthma in ovalbumin-sensitized mice, exposure to a CO atmosphere resulted in a marked attenuation of eosinophil content in BALF and downregulation of the proinflammatory cytokine. Recent human studies have revealed higher HO-1 expression in the alveolar macrophages and higher E-CO in untreated asthmatic patients than in healthy non-smoking controls.[393] HO-1 has been reported to be elevated in alveolar macrophages recovered from sputum of individuals with uncontrolled asthma, as compared with cells from control subjects without asthma, individuals with well controlled asthma, and individuals with asthma treated with systemic corticosteroids. Macrophages of induced sputum show prominent but transient HO-1 immunoreactivity, in untreated asthmatics, but not in asthmatics treated with corticosteroids.

3.2.5. Nuclear factor E2-related factor 2

Nuclear factor erythroid 2–related factor 2 (Nrf-2) is a central transcription factor that regulates the antioxidant defense. The Nrf-2 transcription factor is activated to counteract accumulating reactive oxygen species and electrophiles.[394] Under basal conditions, Nrf-2 is sequestered in the cytoplasm by the repressor protein Keap1 (Kelch-like ECH-associated protein 1) and targeted for proteasomal degradation (**Fig. 3.9**).[395] Exposure to pharmacological activators, such as oltipraz or CDDO-Im (2-cyano-3,12-dioxooleana-1,9-dien-28-oic imidazolide) or generation of oxidative stress, triggers Nrf-2 to translocate to the nucleus where it transactivates a battery of genes by binding to antioxidant-response elements (ARE) in upstream promoter regions.[396,397] Targets of Nrf-2 transcription include proteins involved in drug metabolism, efflux transporters (such as multidrug resistanceassociated proteins, Mrps), antioxidant enzymes, heat shock responses, and proteasomal degradation.

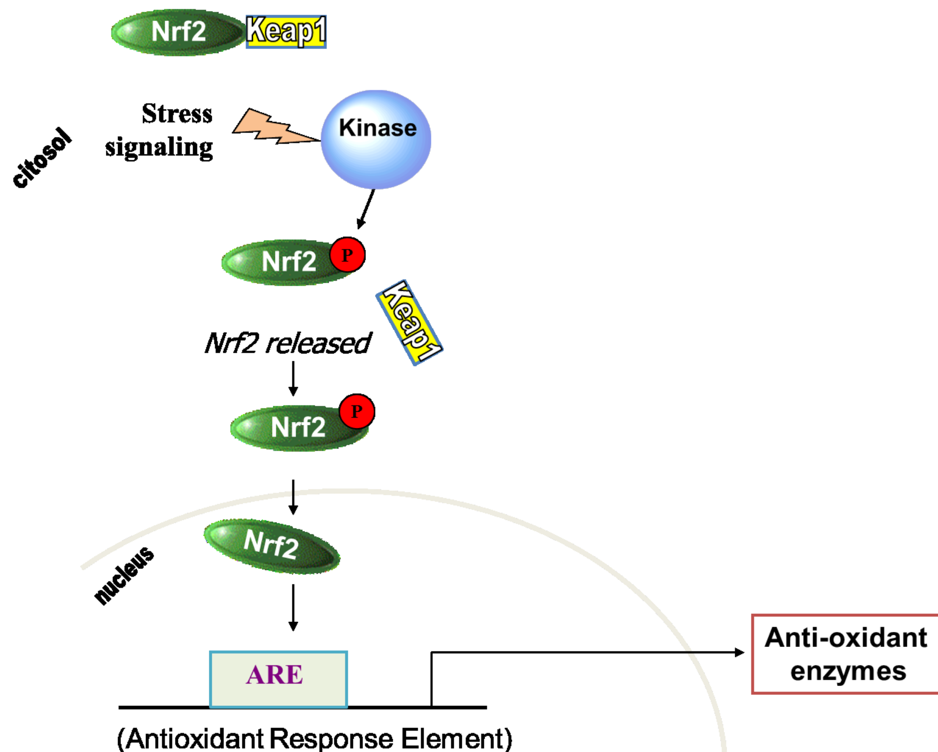


Fig. 3.9. *Nrf-2 binds to the Antioxidant Response Element (ARE) and promotes transcription of antioxidant genes.* In the activation process seems to be involved a protein kinase (an enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to specific target molecules called substrates). The transfer of a phosphate group 'activate' the molecule with subsequent release and nuclear translocation. C. Folisi.

The role of Nrf-2 has been investigated in pulmonary medicine. Nrf-2 deletion provided the first evidence of a direct link between the regulation of antioxidant genes and alveolar destruction in the cigarette smoke– induced emphysema in a murine model.[398] Nrf-2 has shown a predominant protective role in a number of lung inflammatory diseases because it increases sensitivity of Nrf-2-disrupted mice to allergen-induced asthma,[399,400] bacterial lipopolysaccharideinduced sepsis,[401] hyperoxia-induced acute injury,[402] ventilation- induced acute lung injury,[403] and diesel exhaust–induced DNA damage.[404] Recent evidence suggests that selective inactivating mutations in the Nrf-2 inhibitor, Keap-1, enhances Nrf-2 directed constitutive expression of multiple antioxidants and xenobiotic-detoxification genes that endows non–small cell lung cancer (NSCLC) tumors with selective survival advantage and chemoresistance.[405]

Experimental part

Chapter 4

4. Oxidative stress and Rhinovirus-induced asthma exacerbation

This chapter is based on the submitted manuscript: *"Rhinovirus infection in asthma attenuates the anti-oxidant capacity of airway macrophages"*

Caterina Folisi[1,2,3], Suzanne M. Bal[2,3], Marianne A. van de Pol[2,3], Annemiek Dijkhuis[3], Koen F. van der Sluijs[2,3,4], Giuseppe Di Maria[1], Peter J. Sterk[2] and René Lutter[2,3]

Author Affiliations

Dipartimento di Biomedicina Clinica e Molecolare Sezione Malattie Respiratorie, Università di Catania Ospedale, Garibaldi-Nesima 95122, Catania, Italia[1]. Depts. of Respiratory Medicine[2], Experimental Immunology[3], and Intensive Care Medicine[4], Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

Corresponding author

Caterina Folisi, Dipartimento di Biomedicina Clinica e Molecolare, Sezione Malattie Respiratorie, Università di Catania, Ospedale Garibaldi-Nesima, via Palermo 636, 95122 Catania, Italia. Telephone number: 0039 0957598742, Fax number: 0039 095472988. Email: C.Folisi@soton.ac.uk

Contributorship

CF performed the analyses, analysed and interpreted the data and wrote the manuscript, SMB and MvdP performed the clinical study and revised the manuscript, KFvdS, GDM and PJS were involved in the design of the study, RL designed the study, interpreted the data and wrote the manuscript.

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Abstract

Rhinoviruses (RVs) are responsible for 60 to 80% of all respiratory virus-induced asthma exacerbations. The mechanism that leads to the exacerbation is unknown. RVs have been able shown able to modify the oxidative cellular balance by produce superoxide and depleting anti-oxidant defence. Hence, oxidative response is thought to play a central pathogenetic role in RVs-induced airway disease.

The aim of this study was to investigate the local oxidative stress due to a rhinovirus infection in asthma patients and its effect on the anti-oxidant capacity of airway macrophages.

We purified macrophages from induced sputum (IS) and bronchoalveolar lavage (BAL) from mild asthmatics (n=9) before and a week after rhinovirus infection when patients showed exacerbation simptoms. First, we assessed the basal oxidative stress on the basis of the amount of oxidized proteins (carbonyls protein formation) and lipids-proteins adducts, 4-Hydrossyl-2-nonenal Protein Adducts (4-HNE PAs). We also evaluated the expression of Heme Oxygenase-1 (HO-1), Heat shock protein 70 (Hsp-70), Thioredoxin (Trx-2), Thioredoxin Reductase (Trx-R) and the cyto-protective proteins Sirt-1 and 2. Next, we exposed macrophages to oxidative stress by supplementing superoxide by mean of the redox system Xanthine/Xanthine Oxidase. In these stimulated macrophages the oxidative susceptibility was assayed in terms of oxidative demage on proteins whereas the anti-oxidant capacity was evaluated according the increase in cyto-protective and anti-oxidant proteins expression under oxidative exposure.

We found that after rhinovirus infection the basaline oxidative proteins damage was increased (4-HNE PAs Relative Optical Density (Rel. O.D.) shifted from 69.0±7.1 to 108.6±13.8 $p=0.02$ and that of Carbonyls proteins from 23.8±3.5 to 43.4±2.5 $p=0.0004$). In IS macrophages HO-1, Trx-2, Trx-R levels were higher after rhinovirus infection (Rel. O.D. 0.28±0.03 vs 0.05±0.02 $p<0.0001$, 0.66±0.06 vs 0.46±0.05 $p=0.02$ and 0.43±0.07 vs 0.20±0.04 $p=0.01$, respectively). Whereas, Sirt-1 and 2 levels were lower (0.50±0.06 vs 0.31±0.04 $p=0.021$; 0.43±0.05 vs 0.63±0.10 $p=0.03$ respectively). After rhinovirus infection IS macrophages exposed to superoxide showed a higher oxidative susceptibility and a reduced anti-oxidant capacity. Macrophages after rhinovirus

infection showed a reduced translocation of Nrf-2 into the nuclei and enhanced pro-inflammatory mediator production. Taken together these resuls suggest that RV16 infection is accompanied by an increased oxidative stress in macrophages, associated with a reduced activation of Nrf-2. Therefore, enhancing local anti-oxidant mechanisms may attenuate airway inflammation and an asthma exacerbation.

4.1.Introduction

Asthma is an inflammatory disorder of the airways characterized by episodic and reversible airflow limitation and airway hyperresponsiveness to endogenous or exogenous stimuli.[406] These episodes of acute worsening of asthma symptoms, referred to as exacerbations, are paralleled by more severe local inflammation. ROS such as hydroxyl radicals, superoxide and hydrogen peroxide, are generated during inflammation and so during an exacerbation more ROS are generated.[407] ROS can trigger the production of pro-inflammatory mediators and can also lead to post-translational modifications of cellular constituents that affect cell functions. The overall effect of ROS, however, depends on the capacity of cyto-protective mechanisms that scavenge ROS and repair ROS-induced post-translational modifications. Several studies have shown that during an asthma exacerbation the ROS production and the cyto-protective mechanisms are not in balance as reflected by more post-translationally modified proteins. Restoration of such an imbalance was shown to attenuate experimental exacerbations in murine studies, highlighting the relevance of ROS in driving an exacerbation. We hypothesized that the capacity to raise cyto-protective mechanisms during an exacerbation in asthma patients may be reduced.

Respiratory viral infections are a major cause of exacerbations and rhinoviruses (RVs) are responsible for about 70% of all respiratory virus-induced asthma exacerbations.[408] Therefore we employed a low dose experimental RV16 infection model to trigger a mild exacerbation in mild asthma patients.[409] We studied airway macrophages as these are the most abundant leukocytes in the airway lumen, have a wide range of immunoregulatory functions and have the capacity to produce and counteract ROS.[410,411,412,413,414,415,416] We assessed the ROS-induced carbonyl proteins and formation of lipid peroxidation protein adducts and the expression of cyto-protective proteins in macrophages collected before and one week after RV16 challenge (**Fig. 4.1**). For the latter we determined the expression of heme-oxygenase 1 (HO-1), heat shock protein 70 (Hsp-70), thioredoxin (Trx-2), thioredoxin reductase (Trx-R) and the cyto-protective proteins Sirt-1 and 2. Ex vivo, we challenged these macrophages to oxidative stress by the xanthine-xanthine oxidase system and assessed their cyto-protective response.

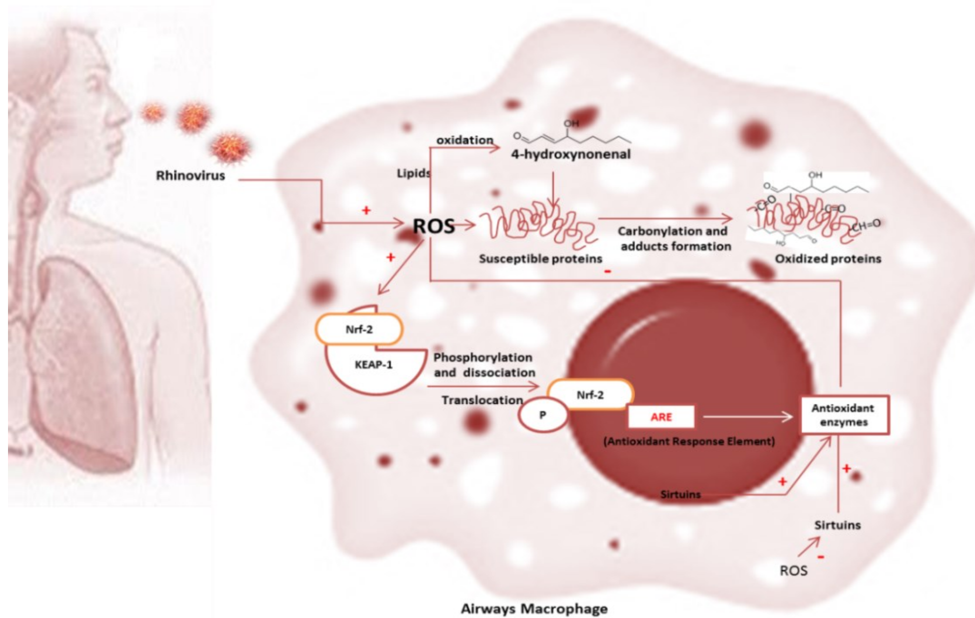


Fig. 4.1 *Study synopsis and rational.* Rhinovirus has been shown able to increase the generation of reactive oxygen species (ROS). ROS can react with susceptible proteins and lipids and generate products of oxidation as carbonylated proteins and lipid-protein conjugation as 4-HNE protein adducts. An increase in ROS and electrophilic compound s also promote the activation of the transcription factor Nrf-2 which is considered the master regulator of the anti-oxidant response. Nrf-2 after activation translocate into the nucleus where bound the antioxidant response elements inducing the expression of a set of cito-protective proteins and antioxidant enzymes. ROS down-regulate Sirtuins, a class of NAD-dependent deacetylase involved in oxidative stress resistance. C. Folisi

4.2.Material and Methods

Study population. Nine non-smoking patients with allergic asthma, using only short-acting inhaled β2-agonist on demand, participated in the study (demographic and baseline data in **Table 1**). Patients were atopic, as defined by positive skin tests in response to common airborne allergens, had a forced expiratory volume in 1 s (FEV1) of at least 80% of the predicted value and responded to a metacholine provocation dose causing 20% decrease in FEV_1 (PC_{20}) <8 mg/mL. Patients were excluded if they had a respiratory infection in the preceeding 6 weeks or had neutralising antibodies against RV16 (titre >6).[413] The study was conducted in accordance with the Declaration of Helsinki and was approved by a local ethics committee and the national regulatory agency. Written informed consent was obtained from each subject before study entry.

Study set up. Patients participated in a randomised double-blind placebo-controlled parallel trial to investigate the efficacy of mepolizumab on Rhinovirus-Induced Asthma exacerbations (MATERIAL) study (NCT01520051). Patients received a single intravenous dose of mepolizumab (750 mg) or placebo on day 0. Two weeks later the patients were infected with RV16 (dose of 10 $TCID_{50}$). Induced sputum was obtained four days before and four days after and a bronchoalveolar lavage (BAL) was performed one day before RV16 infection. With respect to the oxidative stress measurements all samples were analysed irrespective of the treatment (mepolizumab/placebo).

Asthma Control Questionnaire (ACQ). The ACQ has 7 questions (5 asthma symptoms, FEV_1% pred. and daily rescue bronchodilator use). Patients were asked to recall how their asthma has been during the previous week and to respond to the symptom on a 7-point scale (0=no impairment, 6= maximum impairment). The FEV_1% predicted on a 7-point scale was scored. The questions were equally weighted and the ACQ score was considered as the mean of the 7 questions and therefore between 0 (totally controlled) and 6 (severely uncontrolled). The ACQ has strong discriminative and evaluative properties it can detect small differences between patients with different levels of asthma control and it is very sensitive to within-patient change in asthma control over time. Patients with a score below 1.0 have adequately controlled asthma and above 1.0 not controlled. Between 0.75 and 1.25 patients are on the borderline of adequate control.

On the 7-point scale of the ACQ, a change or difference in score of 0.5 is the smallest that can be considered clinically important. Changes of 0.5 or greater would justify a change in the patient's treatment.

Wisconsin Upper Respiratory Symptom Survey (WURSS). The WURSS is an evaluative illness-specific quality of life instrument, designed to assess the negative impact of acute upper respiratory infection. Influenza-like illness symptoms of headache, body aches and fever were included on the WURSS used for this study.

*Sputum induction and bronchoalveolar lavage***.** Sputum induction was performed as described earlier.[417] In short, patients received pre-treatment with 400 μg salbutamol before undergoing three episodes of 5 minutes inhalation of aerosolised 4.5% hypertonic saline solution generated by an ultrasonic nebulizer (KLAVAmed, Bielefeld, Germany). BAL fluid was collected by a standardised bronchoscopic procedure as described previously.[418] Sputum and BAL fluid were collected in a sterile container and transported on ice to the lab for immediate processing.

*Sputum processing***.** Sputum was processed, with dithiotreitol (DTT) to liquefy the whole sample as described before but with minor modifications.[419] After addition of DTT, samples were placed on a shaker at 4°C for 15 minutes to prevent activation of cells. When necessary, remaining lumps were removed by treatment with DNase IV by shaking for 15 minutes at 4°C. BAL cells were processed as described before.[420] Differential cell counts were expressed as number and percentage of cells excluding squamous epithelial cells.

Collection and culturing of airway macrophages. Macrophages were obtained from sputum and BAL fluid (BALF) by negative selection using RosetteSep reagent (RosetteSep monocytes enrichment reagent, #15068, Stem Cell Technologies via Cell Systems). Cells were collected by centrifugation (10 min at 400g) of DTT-liquified sputum and BAL fluid and resuspended in 3 ml phosphate-buffered saline (PBS) with 2% Fetal Bovine Serum (FBS) and 1 mM EDTA. Thirty μl packed erythrocytes (obtained following centrifugation over LymphoPrep gradient) and 50 μl RosetteSep Human Monocyte Enrichment Cocktail were added to sputum or BAL cells. After 20 min incubation at room temperature, the sputum or BAL cells were layered on top of a

density gradient (3ml medium LymphoPrep) and centrifuged for 22 min at 1355g. The mononuclear layer remained at the interphase of the density gradient and was aspirated followed by 3 washes with ice-cold IMDM/1%FCS/Penicillin/Streptomycin (4 mM). Cells were counted, and viability was evaluated using 0.2% trypan blue. Purity was assessed by Quick diff staining on cytospin and was higher than 98%. Macrophages were processed for analyses by Western blotting or exposed to oxidative stress.

Exposure to oxidative stress. Macrophages were resuspended at $0.5x10^6$ cells/ml in IMDM/1%FCS/Penicillin/Streptomycin and 2 ml suspensions were transferred to 6-well plates. After overnight incubation at 37°C with 5% CO_2 under humidified conditions, macrophages were pre-treated with 20 mM N-Acetyl Cysteine for 1 h to maximize their anti-oxidant capacity of cells.[421] Subsequently, cells were exposed for 12 h to the superoxide-generating system xanthine (X) (0.2 mM) and xanthine oxidase (XO) (10mU/ml; Sigma-Aldrich, St. Louis, MO, USA). The X/XO system allows for generation of controlled superoxide production, mimicking the chemical environment of oxidants exposure in tissues. Cell viability after superoxide exposure was determined by trypan blue exclusion.

Western blot analysis. Whole-cell lysates were prepared from freshly purified macrophages. Macrophages were washed twice in PBS and subsequently lysed on ice using Laemmli buffer (4% Sodium Dodecyl Sulphate (SDS), 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue in 0.125 M Tris-HCl pH 6.8) with complete protease inhibitor cocktail (Roche, city, country). Fifty microgram of protein (see assay below) per lane of whole cell proteins were separated by 12% SDS-PAGE gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes for immunodetection. After this step, membranes were washed and reversible red ponceau (Sigma Aldrich) staining was performed to check for adequate transfer. Then, membranes were blocked for 30 min at room temperature in PBS with 3% non-fat dry milk. Membranes were then probed with 1:500 diluted polyclonal rabbit antibodies to Sirt-1, Sirt-2, Trx-R and Trx-2, and goat antibodies to HO-1 and Hsp-70 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), overnight at 4°C in 0.05% Tween-20 PBS (TPBS) with 0.5% non-fat dry milk. As a loading control we used goat anti-β-actin 1:5,000 in TPBS (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA). As positive control we used

Hela cells whole lysates for HO-1 and Hsp-70, K293 cells whole lysates for Trx-R and Trx-2 and K562 cells for Sirt-1 and 2. Parallel blots incubated with only secondary antibody were used in order to evaluate aspecific binding. Membranes were washed three times in TPBS and incubated for 60 min at room temperature with IRDye 680LT and IRDye 800CW conjugates secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) 1:15,000 diluted in TPBS with 0.5% non-fat milk. After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA). The bands of Sirt-1 (≈120 kDa), Sirt-2 (≈42 kDa), HO-1 (≈32 kDa), Hsp-70 (≈70 kDa), Trx-2 (≈12 kDa), and Trx-R (≈52 kDa) were quantified using densitometry and expressed relative to that of the corresponding β-actin (≈37kDa).

Protein Assay. The amount of protein was determined using the bicinchoninic acid (BCA) kit (Bio-Rad Laboratories Inc., Hercules, California, USA). Protein standards were obtained by dilution of a stock solution of Bovine Serum Albumin (BSA).

Carbonyls protein detection: OxyBlot Procedure. Carbonyl groups of oxidized proteins were detected after derivatization with 2,4-dinitrophenylhydrazine (DNPH) to a stable dinitrophenyl (DNP) hydrazone product using OxyBlot Protein Oxidation Detection Kit (Merck Millipore) View All ».[422] In brief, two aliquots (15-20 μg/5 μL of protein sample) of each specimen to be analyzed were prepared. Proteins were denatured by adding 5 μL of 12% SDS. One aliquot was subjected to the derivatization reaction by adding 10 μL of 1x 2,4-dinitrophenylhydrazine (DNPH) followed by 15 min of incubation at room temperature, after which 7.5 μL of Neutralization Solution provided in the kit was added. The negative control was treated in parallel but with derivatization-control solution instead of DNPH. Equal volumes of both samples were loaded onto a SDS-PAGE gel (10%) without prior heating of the samples. After electrophoresis and blotting to PVDF membranes in transfer buffer (12 mM Tris, 96 mM Glycine, 20% Methanol) reversible red ponceau staining was performed to check for adequate transfer and then blots were washed and blocked by placing the membrane into 3% non-fat dry milk for 1 h with gentle shaking. Subsequently, 15 mL of Rabbit Anti-DNP primary antibody 1:150 diluted in TPBS with 0.5% non-fat dry milk was

added and left overnight at 4ºC while shaking on an orbital shaker. The membrane was washed with multiple changes of TPBS for a total of 30 min before adding 15 ml of Goat Anti-Rabbit IgG Horse Radish Peroxidase (HRP) conjugated secondary antibody (1:300 dilution) in 0.5 non-fat dry milk TPBS and left for 1 h at room temperature on an orbital shaker. Next, the membrane was washed using multiple changes of TPBS for a total of 30 min before adding the chemiluminescent reagent (luminol and enhancer, Bio-Rad Laboratories Inc., Hercules, California, USA) according to manufacturer's specifications. The membranes were exposed for 1-3 min. Derivatized bands were quantified by measuring the optical density of the bands in comparison to the signal from the negative control using Image J 1.46r software Values were expressed relative to that of β-Actin.

Immunochemical detection of 4-Hydroxynonenal Protein adducts (HNE-Pas) in macrophages. Thirty to 50 µg of total proteins were diluted in Laemmli sample buffer till a final volume of 35 µL, and boiled for 5 minutes at 95°C before separation on 12 % SDS PAGE gels. Then, proteins were transferred onto PVDF membranes. After this step, reversible red ponceau staining was performed. Then, membranes were washed and blocked for 60 min at room temperature in 3% non-fat dry milk in PBS. Next, blots were washed and probed against the HNE moiety of proteins by using polyclonal goat anti 4-HNE (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) 1:500 diluted in 0.05% TPBS with 0.5% not-fat dry milk overnight at 4°C. Membranes were washed three times in TPBS and incubated for 60 min at room temperature with IRDye 680LT conjugates secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) 1:15,000 diluted in TPBS with 0.5% non-fat milk. Blots incubated with only secondary anti-body were used to evaluate aspecific secondary antibody binding. After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA). After stripping the blot was re-probed for β-Actin in order to normalize for variable protein loading. Optical density was obtained by using Odyssey LICOR software, in which the values are expressed relative to β-Actin.

Nuclear protein extraction. To prepare nuclear protein extracts, macrophages were washed with ice-cold PBS and then removed by scraping in detachment buffer (150 mM NaCl, 1 mM EDTA, and 40 mM Tris; pH 7.6). After centrifugation of the cell suspension at 2,000 rpm in a table centrifuge, the cell pellets were resuspended in a cold buffer containing KCl (10 mM), EDTA (0.1 mM), dithiothreitol (1 mM), and phenylmethylsulfonyl fluoride (1 mM) for 15 min. The cells were lysed by adding 10% Nonidet P-40 and then centrifuged at 6,000 rpm in a table centrifuge to obtain nuclei. The pelleted nuclei were resuspended in cold Laemmli buffer, and then vigorously agitated from time to time, followed by centrifugation. The supernatant containing the nuclear proteins was used for Western blot analysis. Equal loading of nuclear proteins was confirmed relative to that of Histone 3 (H3).

Cigarettes smoke condensate (CSC) stimulation. Macrophages were resuspended at 0.5x106 cells/ml in IMDM/1%FCS/4 mM Penicillin/ 4mM Streptomycin and 2 ml suspensions were transferred to 6-well plates. After overnight incubation at 37°C with 5% CO2 under humidified conditions, macrophages were exposed for 4, 12 and 24 h to 40 µg/mL CSC from Kentucky reference (3R4F).[423] Twenty-four hours CSC exposure was performed with and without pre-treatment with 20 mM N-Acetyl Cysteine for 1 h. Cell viability after superoxide exposure was determined by trypan blue exclusion.

Inflammatory response. The amount of a small panel of inflammatory mediators released in the supernatant by macrophages after exposure to the xanthine – xanthine oxidase system was determined by multiplex fluorescent bead assay for IL-1β, IL-6, IL-8Aand TNF-α Luminex, R&D systems, Minneapolis, MN, USA).

Statistics. Results are presented as mean ± SEM of at least two replicate experiments. Statistical analyses was performed utilizing GraphPad Prism 5. Analysis of significance was calculated by unpaired Student's *t*-test and was used to assess between- and within-study group differences. A p value <0.05 was considered significant.

4.3. Results

Subjects and RV16 infection. Demographic and baseline characteristics of participating mild asthma patients are provided in **Table 1**. All 9 patients were clinically stable before exposure to RV16 and developed common cold and asthma complaints between 2 to 6 days after RV16 infection and had increased RV16-neutralising antibodies in serum, 6 weeks after infection (**Table 1**, **Fig**. **4.2**).

TABLE 1. PATIENT CHARACTERISTICS AND THEIR RESPONSE TO RV16	
Age (years)	23.9 (19-33)
Sex (male/female)	2/7
FEV_1 (% predicted)	107 (85-114)
PC_{20} (mg/L)	1.8 (0.7-2.5)
Maximum fall FEV_1 (%)	9.6 (5-26)
Increase in ACQ	0.57 (0-1)
RV16 titer post-infection	51 (23-76)

Data are presented as mean values and range. ACQ: Asthma Control Questionnaire. FEV_1: forced expiratory volume in 1 second; %pred: percentage of predicted; PC20: dose of methacholine required to cause a 20% drop in FEV_1;

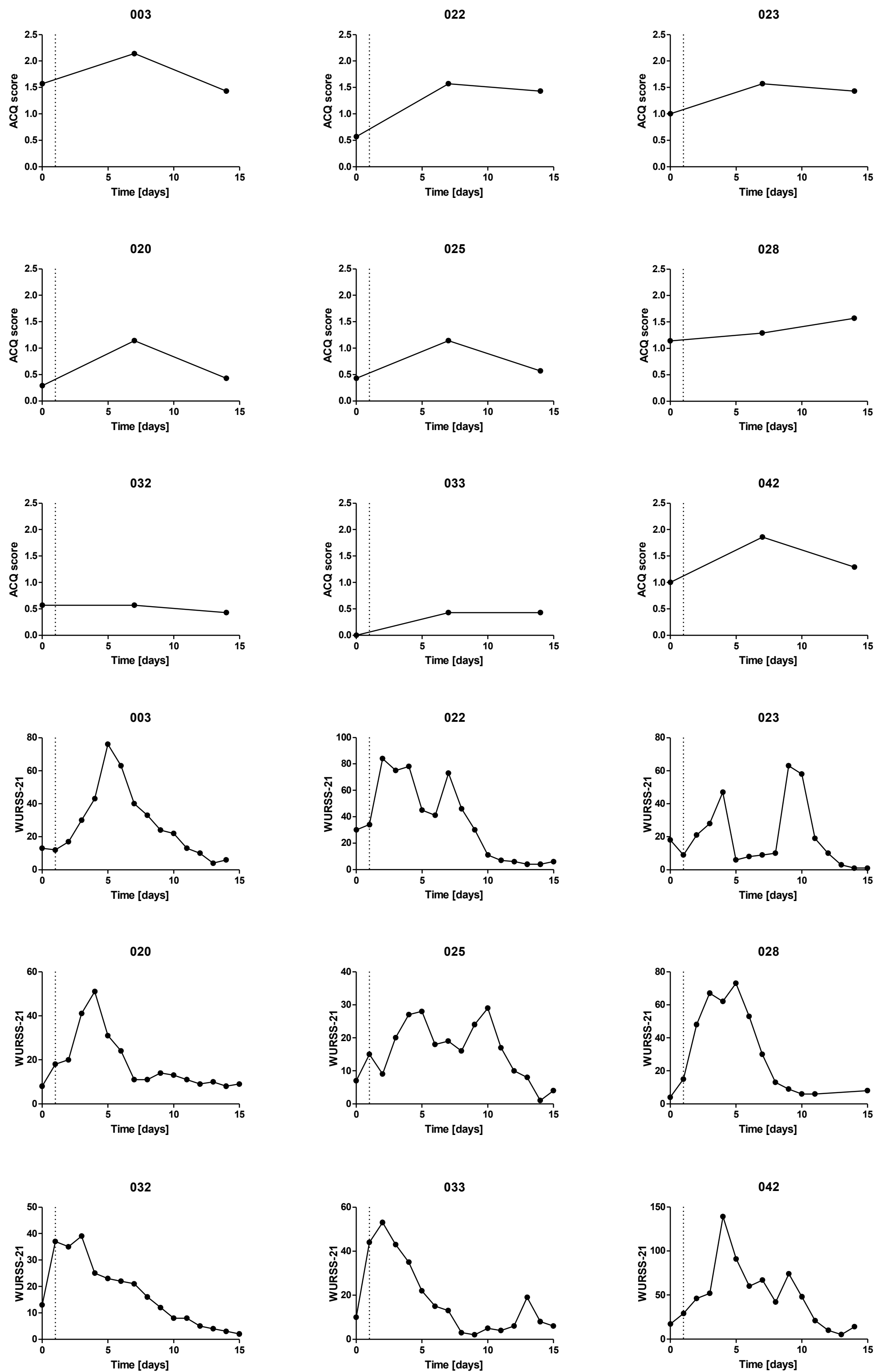


Fig. 4.2. *Average ACQ scores and total WURSS-21 scores for all nine patients over time*. Dotted lines indicate time of RV16 infection. On the middle top of the graphs is indicated the identification number per patient. ACQ: Asthma Control Questionnaire. WURSS: Wisconsin Upper Respiratory Symptom Survey.

Oxidative stress-induced modifications of proteins in sputum macrophages before and after RV16 infection. Sputum macrophages were collected before and 4 days after RV16 exposure and oxidative stress-induced post-translational modifications of proteins, carbonylation and 4-HNE protein adducts, were assessed in total whole cell lysates. **Fig. 4.3 A, B** and **E1** show typical western blots and histograms of combined data from all 9 patients, for carbonylated proteins and for 4-HNE protein adducts in sputum macrophages. Carbonylation was 23.81 ± 3.46 before and 43.43 ± 2.53 (p=0.0004) after RV16 infection and for 4-HNE protein adducts 69 ± 7.13 and 108.57 ± 13.83 (p= 0.02), respectively (**Table 2**). These increases indicate that RV16 infection results in the production of local reactive oxygen species, leading to post-translational modifications of cellular proteins.

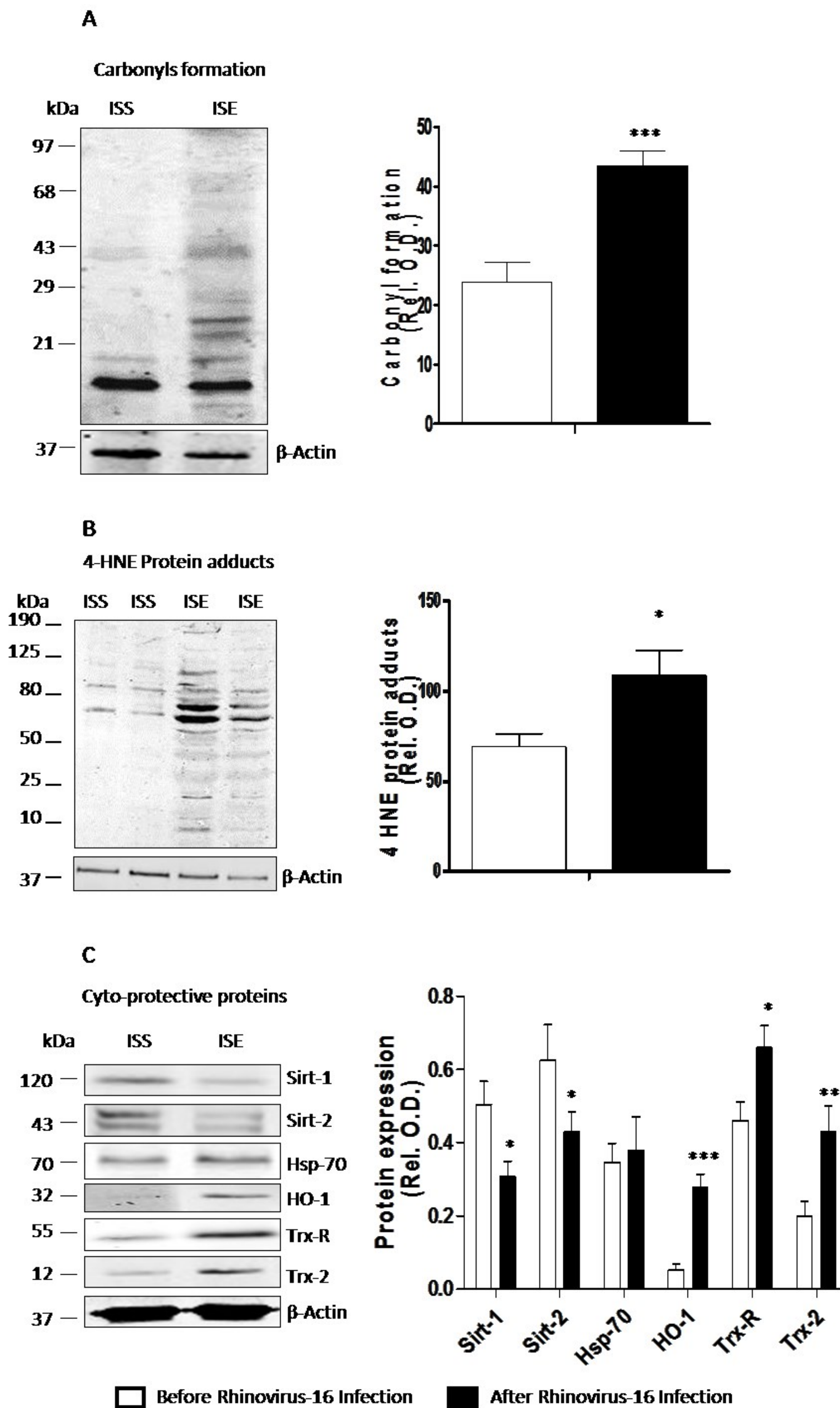


Fig. 4.3 *ROS-induced modifications of proteins and cyto-protective response in sputum macrophages obtained before and after RV16 infection*. Protein carbonyl formation (A), 4-HNE protein adducts (B) and cyto-protective protein expression (C) in induced sputum macrophages before (ISS) and after RV16 infection (ISE). Representative blots (left figures) are shown and data, expressed as mean ± SEM, is relative to β-Actin, for nine patients (right figures). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

TABLE 2. OXIDATIVE STRESS-INDUCED MODIFICATIONS OF PROTEINS AND CYTO-PROTECTIVE RESPONSE BEFORE AND AFTER RV16 INFECTION

	ISS	**ISE**	**P value**
Carbonylation	23.81±3.46	43.43±2.53	0.0004
4-HNE Pas	69.00±7.13	108.57±13.83	0.02
Sirt-1	0.50±0.01	0.31±0.04	0.02
Sirt-2	0.62±0.10	0.43±0.06	0.03
Hsp-70	0.35±0.05	0.38±0.10	0.76
HO-1	0.05±0.02	0.30±0.04	< 0.0001
Trx-R	0.46±0.05	0.66±0.06	0.02
Trx-2	0.20±0.04	0.43±0.07	0.01

Relative Optical densities are expressed as mean ± SEM. P values refer to inter-group comparison. ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection.

Cyto-protective protein response in sputum macrophages before and after RV16 infection. Next, we determined whether the RV16-induced increase in oxidative stress was paralleled by an enhanced expression of anti-oxidant and cyto-protective proteins in sputum macrophages. **Fig. 4.3 C** shows typical western blots and, in the histogram, the analyses for all 9 patients. Sirt-1 and Sirt-2 were downregulated after RV16 infections whereas the anti-oxidant proteins HO-1, Trx-R and Trx-2 were significantly upregulated. Hsp-70 remained unaffected (**Table 2**). Together these data show that sputum macrophages respond to oxidative stress generated during a RV16 challenge by enhancing the expression of major anti-oxidant proteins whereas that of the sirtuins, which can reverse the oxidative stress-induced acetylation, was reduced.

Anti-oxidative capacity of sputum macrophages before and after RV16 infection. Despite the enhanced cyto-protective response, sputum macrophages showed more oxidative stress after RV16 exposure (**Fig. 4.3 A** and **B**). To test whether sputum macrophages after RV16 exposure, as opposed to those before RV16 exposure, have a reduced capacity to counteract oxidative stress we purified macrophages from sputum and left them overnight to recuperate. Subsequently, macrophages were exposed to the superoxide-generating system xanthine/xanthine oxidase (X/XO), in the presence or absence of the anti-oxidant NAC, to control for ROS-induced changes. Carbonylation (**Fig. 4.4 A** and **Table 3**) in sputum macrophages collected before RV16 exposure increased 1.74-fold ($p=0.03$) and for macrophages collected after RV16 exposure this increased 1.88-fold ($p<0.0001$). Similarly, 4-HNE adduct formation in sputum macrophages collected before RV16 exposure increased, though non-significantly, 1.27-fold and for macrophages collected after RV16 exposure 1.61-fold ($p<0.009$; **Fig. 4.4 B**, **Table 3**). NAC treatment was able to completely block the formation of these superoxide-induced post-translational modifications. Together the relative amounts of modified proteins in sputum macrophages collected after RV16 exposure were markedly higher, both at baseline and after exposure to the xanthine oxidase system, as compared to those in sputum macrophages collected before RV16 exposure. This shows that sputum macrophages that were collected after RV16 are more vulnerable to exposure to ROS. In line herewith, sputum macrophages collected after RV16 exposure showed maximal 4-HNE adduct formation within 3 h after exposure to superoxide, whereas 4-HNE adduct formation in sputum macrophages from before the RV16 challenge were maximal after 12 h of exposure to superoxide (**Fig. 4.4** C and **Table 4**). We also purified macrophages from BALF collected before RV16 exposure to determine whether sputum (ISS) and BALF macrophages (BS) behave similarly (**Fig. 4.3 B** and **C**). The results for BS and ISS are comparable throughout the study.

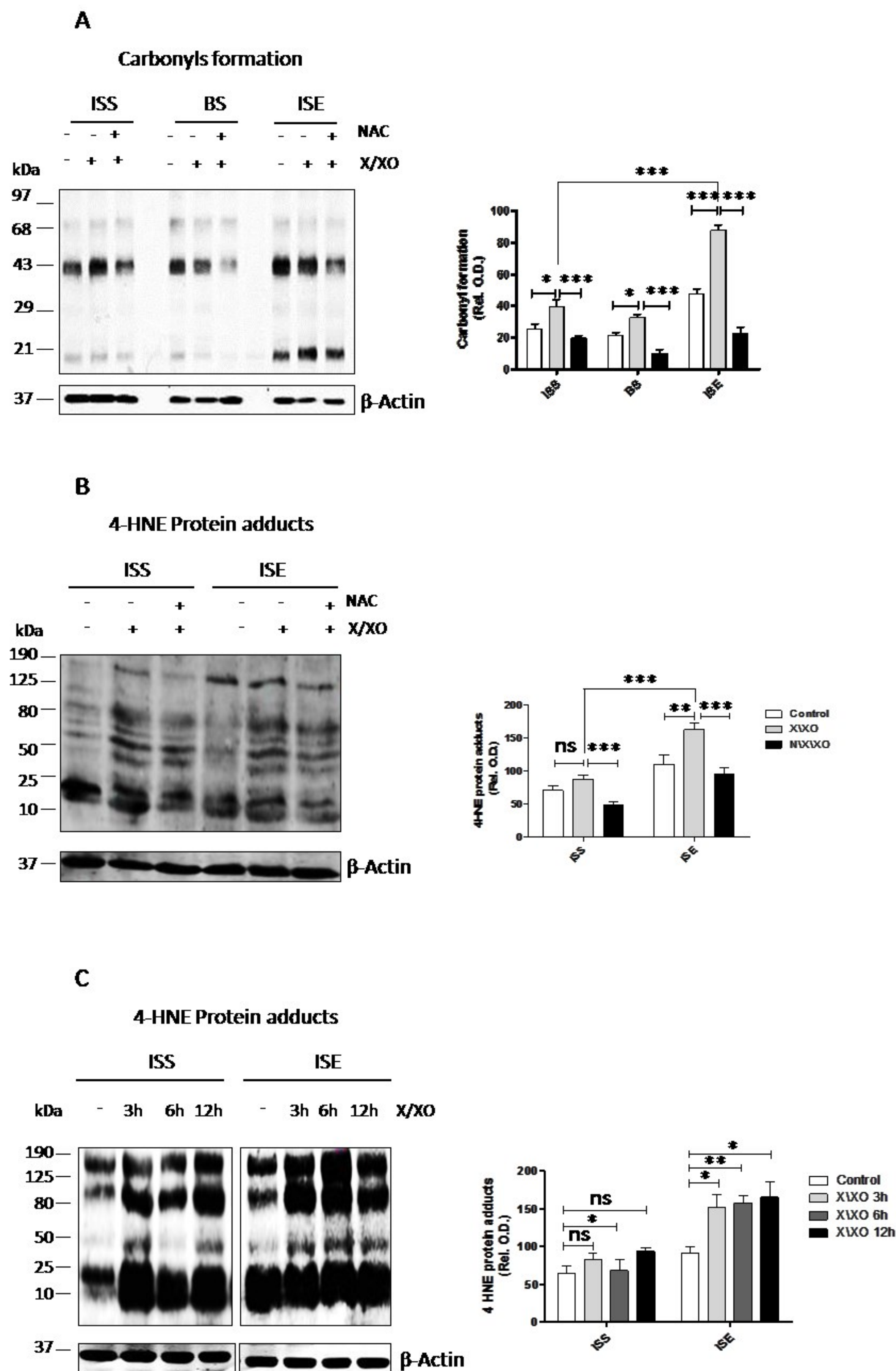


Fig. 4.4. *Anti-oxidative capacity of sputum macrophages obtained before and after RV16 infection.* Effect of the X/XO superoxide-generating system on protein carbonyl formation (A) and 4-HNE protein adducts (B) in induced sputum macrophages before and after rhinovirus infection. C. Kinetics of 4-HNE protein adducts. Representative blots (left figures) are shown and data, expressed as mean ± SEM, is relative to β-Actin, for nine patients (righ figures). Induced sputum macrophages before (ISS) and after (ISE) RV16 exposure. BALF macrophages before RV16 exposure (BS). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

TABLE 3. OXIDATIVE INDUCED PROTEIN MODIFICATIONS IN MACROPHAGES BEFORE AND AFTER RV16 INFECTION UNDER OXIDATIVE EXPOSURE

	Carbonylation			4-HNE Pas	
	ISS	**ISE**	**BS**	**ISS**	**ISE**
Control	24.93±3.88	39.43±4.35	19.43±1.82	71.13±7.12	88.15±6.61
X\XO	47.68±2.86 p=0.03	87.69±3.59 p<0.0001	22.32±4.48 p=0.03	110.70±14.7 p= 0.10	163.10±9.25 p<0.009
NAC\X\XO	21.13±2.36 p=0.22	32.45±2.05 p=0.0003	9.97±2.16 p=0.03	48.46±5.18 p=0.02	96.44±5.18 p=0.004

Relative Optical densities are expressed as mean ± SEM. P values refer to comparison to control.ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection. BS: BALF macrophages before rhinovirus infection. X\XO: Xanthine\xanthine oxidase. NAC\X\XO: N-Acetyl cysteine\Xanthine\Xanthine oxidase.

TABLE 4. KINETICS OF 4-HNE PROTEIN ADDUCTS FORMATION IN MACROPHAGES BEFORE AND AFTER RV16 INFECTION UNDER EXPOSURE

	4-HNE Pas		
	ISS	**ISE**	**P value**
Control	64.21±10.38	91.60±8.62	0.08
X\XO 3h	82.58±9.23	150.99±17.9	0.01
X\XO 6h	68.04±14.80	157.13±9.99	0.002
X\XO 12h	93.75±4.3	164.86±20.60	0.01

Relative Optical densities expressed as mean ± SEM. P values refer to inter-group comparison. ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection. X\XO: Xanthine\Xanthine oxidase.

Cyto-protective response to oxidative stress by sputum macrophages before and after RV16 challenge. Next, we assessed the X/XO system-induced expression of cyto-protective proteins in sputum macrophages collected before and after RV16 exposure (**Fig. 4.5** and **Table 5**). The top panels show typical examples of the expression of the six cyto-protective proteins before and after exposure to oxidative stress. Comparable to the data for the *ex vivo* analyses (*cf.* **Fig.4.3 C**), in particular HO-1, Trx-R and Trx-2 were markedly upregulated in sputum macrophages exposed to oxidative stress *in vitro*. Strikingly, the increase of these cyto-protective proteins in sputum macrophages isolated after the viral challenge is far less (maximally around two-fold), whereas the increase of these cyto-protective proteins in sputum and indeed BALF macrophages obtained before the viral challenge was around four-fold (**Fig. 4.5 A** and **B**). In all cases the presence of NAC eradicated the effect of superoxide. There were no differences in the expression of the sirtuins and Hsp-70 between sputum macrophages collected before or after the viral challenge. Together this indicates that macrophages after the viral infection could deal less well with oxidative stress than macrophages collected before a viral challenge. This is in line with the enhanced oxidative damage in the macrophages collected after viral challenge.

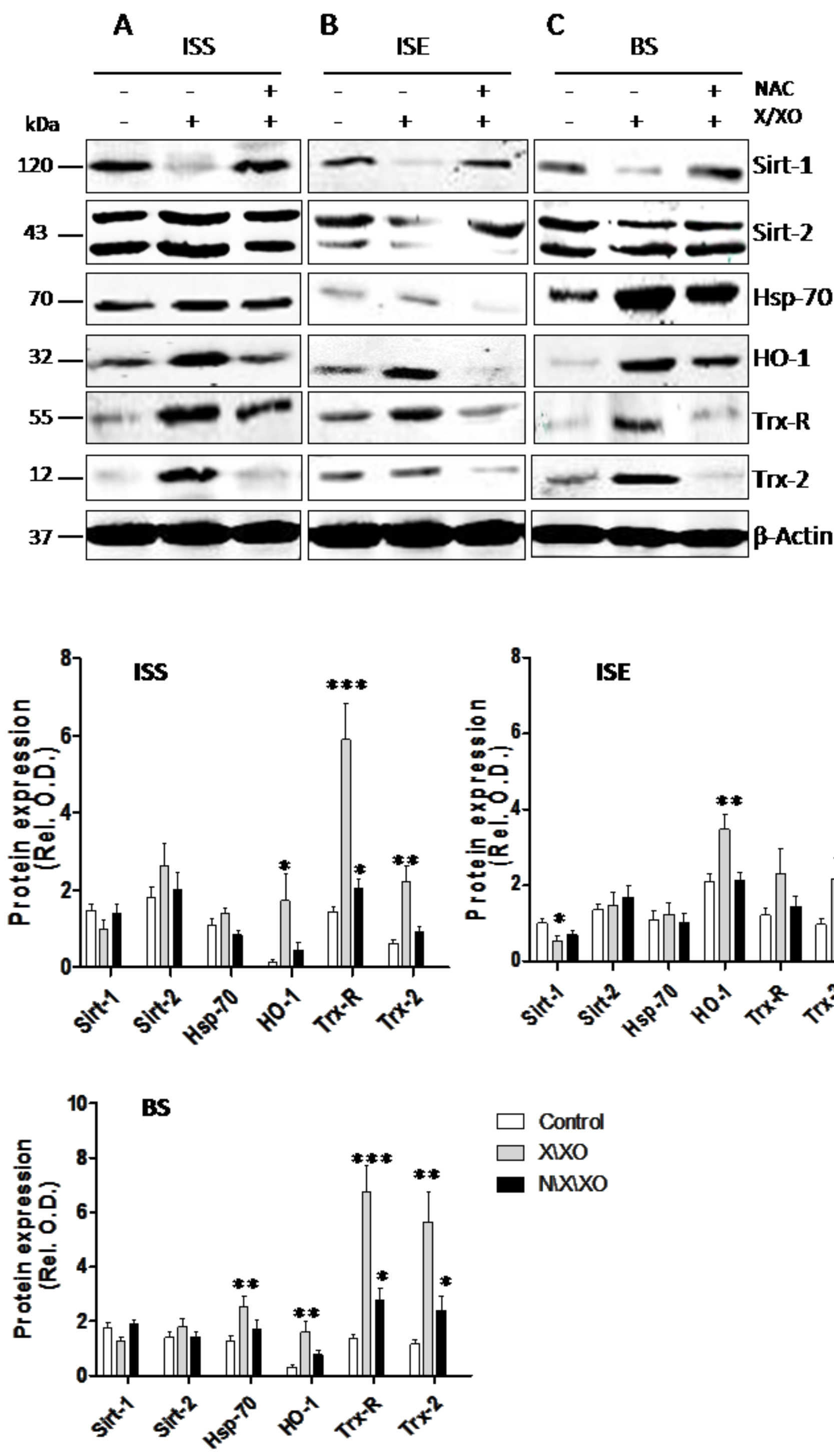


Fig. 4.5 *Cyto-protective response to oxidative stress by sputum macrophages obtained before and after RV16 challenge.* X/XO superoxide-induced expression of cyto-protective proteins in sputum macrophages collected before (**A**) and after RV16 exposure (**B**) and in BAL macrophages collected before RV16 exposure (**C**). Representative blots (top figures) are shown and data, expressed as mean ± SEM, is relative to β-Actin, for nine patients (bottom figures). Induced sputum macrophages before (ISS) and after (ISE) RV16 exposure. BALF macrophages before RV16 exposure (BS).

TABLE 5. CYTO-PROTECTIVE RESPONSE IN MACROPHAGES BEFORE AND AFTER RV16 INFECTION UNDER EX-VIVO OXIDATIVE EXPOSURE

ISS

	Control	**X\XO**	**N\X\XO**
Sirt-1	1.48±0.17	1.01±0.20 p=0.10	1.41±0.22 p=0.84
Sirt-2	1.81±0.27	2.63±0.56 p=0.20	1.88±0.24 p=0.71
Hsp-70	1.10±0.15	1.41±0.14 p=0.15	1.14±0.18 p=0.17
HO-1	0.14±0.05	1.73±0.7 p=0.02	0.21±0.07 p=0.10
Trx-R	1.43±0.15	5.90±0.93 p=0.0002	2.08±0.31 p=0.02
Trx-2	0.62±0.11	2.22±0.41 p=0.002	1.11±0.21 p=0.15

ISE

	Control	**X\XO**	**N\X\XO**
Sirt-1	1.01±0.11	0.54±0.12 p=0.01	0.70±0.10 p=0.06
Sirt-2	1.35±0.16	1.50±0.34 p=0.75	1.69±0.31 p=0.35
Hsp-70	1.10±0.25	1.24±0.28 p=0.71	1.02±0.24 p=0.82
HO-1	2.10±0.20	3.48±0.38 p=0.006	2.12±0.21 p=0.94
Trx-R	1.22±0.21	2.29±0.66 p=0.14	1.43±0.29 p=0.54
Trx-2	0.97±0.17	2.18±0.57 p=0.05	1.25±0.18 p=0.27

BS

	Control	**X\XO**	**N\X\XO**
Sirt-1	1.77±0.18	1.26±0.17 p=0.09	1.92±0.15 p=0.63
Sirt-2	1.40±0.19	1.80±0.30 p=0.28	1.41±0.18 p=0.95
Hsp-70	1.29±0.18	2.54±0.38 p=0.009	1.70±0.33 p=0.29
HO-1	0.32±0.06	1.60±0.42 p=0.008	0.77±0.15 p=0.016
Trx-R	1.35±0.18	6.75±0.94 p=0.0001	2.75±0.45 p=0.01
Trx-2	1.15±0.16	5.64±1.08 p=0.001	2.39±0.53 p=0.04

Relative Optical densities are expressed as mean ±SEM. P values refer to comparison to control. ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection. BS: Bronchoalveolar lavage fluid macrophages before rhinovirus infection. X\XO: Xanthine\xanthine oxidase. NAC\X\XO: N-Acetyl cysteine\Xanthine\Xanthine oxidase.

No Nrf-2 nuclear translocation in macrophages after RV16 infection. Nrf-2 drives protection against oxidative stress. In the absence of oxidative stress, Nrf-2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1).[424] ROS result in the release of Nrf-2 from Keap-1 and its translocation into the nucleus, facilitating transcriptional activation of anti-oxidant and cyto-protective genes. Macrophages collected before and after RV16 exposure were exposed to the X/XO system for 0, 4, 8 and 12 h, after which the nuclear and cytoplasmic fractions were collected and subjected to analyses by western blot (**Fig. 4.6** and **Table 6**). Sputum and BALF macrophages, collected before RV16 exposure responded to oxidative stress by enhancing the amount of Nrf-2 in the nucleus, whereas the amount of Nrf-2 in the cytoplasm apparently was reduced. In fact, the rapid nuclear increment of Nrf-2 fits with the prevention of oxidative stress-induced post-translational modifications of proteins in sputum macrophages collected before RV16 exposure (**Fig. 4.4 C**). Sputum macrophages collected after RV16 exposure were not able to respond with an enhanced nuclear translocation of Nrf-2 in response to oxidative stress, not even after 12 h. The enhanced nuclear localization of Nrf-2 in sputum macrophages was paralleled by an enhanced cytoplasmic expression of HO-1, which is one of the enzymes under control of Nrf-2. So, this indicates that macrophages during an RV16-induced exacerbation lose their capacity to enhance nuclear Nrf-2 thereby failing to raise expression of anti-oxidant and cyto-protective proteins to counteract the effect of ROS.

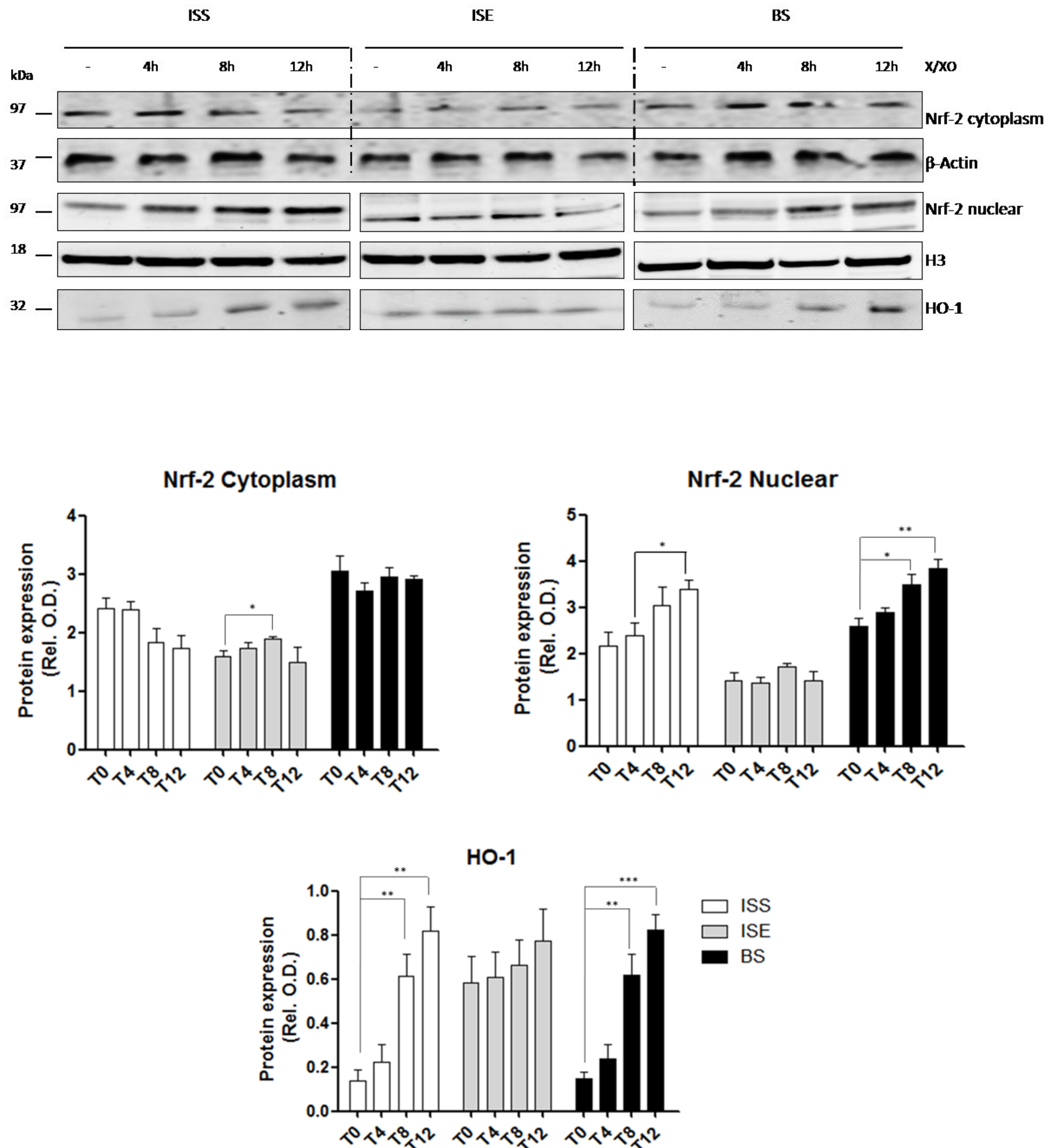


Fig. 4.6. *Reduced Nrf-2 nuclear translocation parallels the attenuated anti-oxidant capacity after RV16 infection.* X/XO-induced expression of cytoplasmic and nuclear Nrf-2, and cytoplasmic HO-1 in sputum macrophages collected before (ISS) and after RV16 exposure (ISE) and in BAL macrophages collected before RV16 exposure (BS). Representative blots (top) are shown and optical densities are relative to that of β-Actin for the cytosolic fraction and that of H3 for the nuclear fraction. Data represent the mean ± SEM of four experiments done in duplicate. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

TABLE 6. Nrf-2 NUCLEAR TRANSLOCATION AND CYTO-PROTECTIVE RESPONSE IN MACROPHAGES BEFORE AND AFTER RV16 INFECTION UNDER EX-VIVO OXIDATIVE EXPOSURE

	ISS	ISE	BS
Nrf-2 Cytoplasm			
Control	2.41±0.17	1.60±0.10	3.05±0.26
X\XO 4h	2.39±0.15 p=0.92	1.74±0.10 p=0.33	2.71±0.14 p=0.28
X\XO 8h	1.82±0.24 p=0.1	1.88±0.05 p=0.04	2.96±0.14 p=0.76
X\XO 12h	1.74±0.22 p=0.05	1.49±0.26 p=0.71	2.91±0.05 p=0.61
Nrf-2 Nuclear			
Control	2.17±0.28	1.41±0.18	2.60±0.17
X\XO 4h	2.39±0.27 p=0.6	1.37±0.11 p=0.86	2.89±0.1 p=0.21
X\XO 8h	3.05±0.39 p=0.12	1.72±0.07 p=0.16	3.48±0.23 p=0.02
X\XO 12h	3.39±0.19 p=0.01	1.43±0.18 p=0.96	3.83±0.21 p=0.004
HO-1			
Control	0.14±0.05	0.58±0.12	0.15±0.03
X\XO 4h	0.22±0.08 p=0.4	0.61±0.11 p=0.87	0.21±0.06 p=0.24
X\XO 8h	0.62±0.10 p=0.005	0.66±0.11 p=0.64	0.63±0.09 p=0.003
X\XO 12h	0.82±0.11 p=0.001	0.77±0.14 p=0.35	0.83±0.07 p=0.0001

Relative Optical densities are expressed as mean ±SEM. P values refer to comparison to baseline.ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection. BS: Bronchoalveolar lavage fluid macrophages before rhinovirus infection. X\XO: Xanthine\Xanthine oxidase.

4-HNE Protein Adducts formation and HO-1 expression after Cigarette Smoke Condensate (CSC) exposure. Instead of using the X/XO superoxide-generating system we also wanted to see whether the more patho-physiologically relevant CSC, resulted in similar findings for 4-HNE protein adduct formation.[425] Sputum macrophages collected before RV16 exposure were not affected by CSC, but macrophages collected after RV16 exposure were (**Fig. 4.7A** and **Table 7**). Interestingly, sputum macrophages collected before RV16 exposure responded by an increased expression of the cyto-protective HO-1 (**Fig. 4.7 B** and **Table 7**), whereas macrophages collected after RV16 showed a smaller response in line with the enhanced 4-HNE protein adducts seen in these cells. Also here the addition of NAC inhibited the effects of CSC. Taken together, these findings parallel those obtained with the X/XO system.

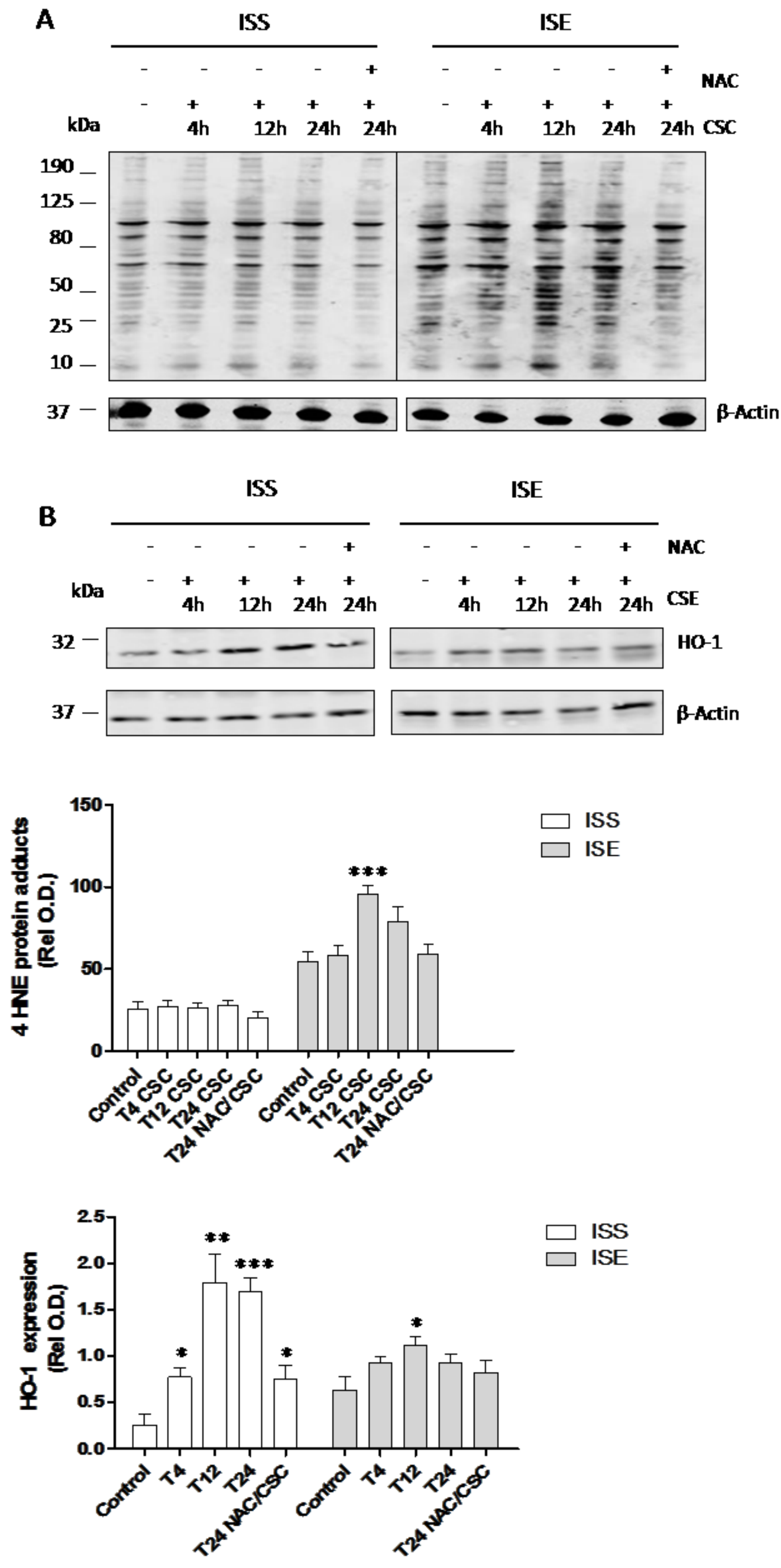


Fig. 4.7 *4-HNE protein adducts formation and HO-1 expression after Cigarette Smoke Condensate (CSC) exposure.* Effect of Cigarette Smoke Condensate on 4-HNE protein adducts formation (A) and HO-1 expression (B) in induced sputum macrophages before (ISS) and after (ISE) rhinovirus infection.

TABLE 7. 4-HNE PROTEIN ADDUCTS AND HO-1 IN MACROPHAGES BEFORE AND AFTER RV16 UNDER CIGARETTE SMOKE CONDENSATE EXPOSURE

	4-HNE Pas			**HO-1**		
	ISS	**ISE**	**P value**	**ISS**	**ISE**	**P value**
Control	25.64±4.28	54.14±6.54	0.01	0.26±0.12	0.63±0.14	0.09
CSC 4h	27.13±3.34	57.96±6.71	0.006	0.77±0.10	0.92±0.08	0.06
CSC 12h	26.54±2.99	95.52±5.34	<0.0001	1.79±0.3	1.11±0.09	0.07
CSC 24h	27.75±3.29	79.23±8.73	0.01	1.69±0.15	0.92±0.1	0.005
NAC/CSC 24h	20.25±3.99	58.99±5.95	0.002	0.75±0.15	0.82±0.13	0.71

Relative Optical densities are expressed as mean ±SEM. P values refer to inter-group comparison. ISS: Induced sputum macrophages before rhinovirus infection. ISE: Induced sputum macrophages after rhinovirus infection. CSC: Cigarette Smoke Condensate. NAC/CSC: N-Acetyl cysteine/ Cigarette Smoke Condensate

Pro-inflammatory mediator responses by macrophages subjected to oxidative stress. From 8 patients we were able to collect supernatants from sputum macrophages exposed to X/XO, NAC/X/XO and no stimulus for determining the release of inflammatory mediators. Evidently, macrophages collected after RV16 exposure responded to superoxide with an enhanced release of IL-1β and IL-8 and not significantly TNF-α and IL-6 (**Fig. 4.8**, **Table 8**). These increases were not observed with macrophages collected before RV16 exposure, indicating that macrophages become primed to respond to oxidative stress by RV16 exposure.

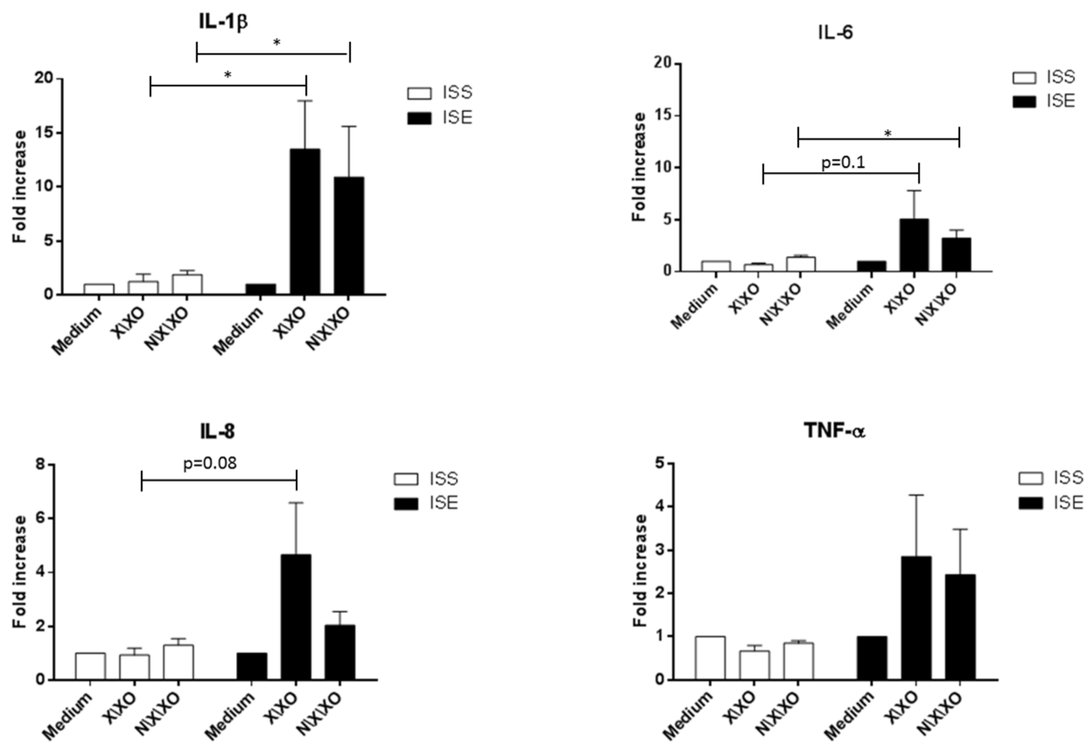


Fig. 4.8 *Enhanced pro-inflammatory mediator responses to ROS in macrophages obtained after RV16 exposure.* 24 h-culture supernatants from macrophages obtained before and after RV16 exposure from 8 patients and tested in duplicate were analysed for IL-1β, IL-6, IL-8 and TNF-α. Data are expressed as fold-increase (mean ± SEM) as compared to medium only. ISS: sputum macrophages before and (ISE) after rhinovirus infection. * $p<0.05$

TABLE 8. PRO-INFLAMMATORY MEDIATOR RESPONSES BY MACROPHAGES BEFORE AND AFTER RHINOVIRUS INFECTION SUBJECTED TO OXIDATIVE STRESS

ISS

	Control	**X\XO**	**N\X\XO**
IL-1β	3.12±1.03	3.83±1.97 p=0.74	5.74±1.51 p=0.45
IL-6	25.34±4.68	60.87±42.56 p=0.42	31.49±5.63 p=0.50
IL-8	3213.0.5±524.58	2344.29±223.4 p=0.15	3533.44±335.62 p=0.01
TNF-α	224.190±41.70	147.61±33.60 p=0.69	194.63±48.63 p=0.07

ISE

	Control	**X\XO**	**N\X\XO**
IL-1β	1.27±0.44	14.76±7.57 p=0.13	10.38±4.13 p=0.59
IL-6	12.63±4.79	21.89±4.66 p=0.20	24.65±7.34 p=0.75
IL-8	5848.75±4125.19	14304.05±9988.64 p=0.44	7750.81±5024.40 p=0.56
TNF-α	132.06±42.39	275.19±56.21 p=0.11	242.9±33.01 p=0.64

4.4.Discussion

During inflammation ROS are generated intentionally to cause damage to the evoking stimulus, but the reactivity of ROS implicates that also host components are targeted. Whether ROS cause post-translational modifications to cellular proteins, reflecting oxidative stress, depends on the capacity to counter ROS and to repair these post-translational modifications. We have shown here that a low-dose RV16 infection of mild asthma patients results in a pronounced oxidative stress in airway macrophages, which relates to a markedly reduced capacity of macrophages to raise an adequate anti-oxidant response to ROS. As a consequence of this reduced anti-oxidant capacity, macrophages in response to ROS release pro-inflammatory mediators that may drive inflammation during an exacerbation.

Although viral infections have been shown to induce ROS in epithelial cells,[426,427] this is the first *in vivo* study on ROS in virus-induced asthma exacerbations. Despite using RV16, which is considered a mild virulent rhinovirus species and causes a moderate exacerbation only, we noticed a pronounced oxidative stress in macrophages as reflected by an enhanced expression of two oxidative stress-induced post-translational modifications of proteins. Recently we showed, also with a low-dose RV16 exposure, that tryptophan catabolites generated by indoleamine 2,3-dioxygenase (IDO) were reduced in airways of asthma patients as opposed to that of healthy individuals.[16] IDO degrades tryptophan at the expense of superoxide and thus is also considered an anti-oxidant protein.[428] Therefore, both findings indicate that there is a reduced anti-oxidant capacity in the airways from asthma patients after RV16 exposure.

Cells are equipped with robust cellular anti-oxidant defence mechanisms that protect and restore damaging effects of ROS. Among these, HO-1 cleaves heme to produce the anti-oxidant biliverdin, inorganic iron and carbon monoxide (CO).[429] Hsp-70 is a chaperone of naïve, aberrantly folded, or mutated proteins involved in cyto-protection.[430] Trx-2 and its reductase (Trx-R) participate in various redox reactions through dithioldisulphide exchange reaction and have an essential role in limiting oxidative stress.[431] Sirt-1 and Sirt-2 deacetylate histone and non-histone protein substrates and thus have been implicated in protecting cells from stress.[432] Surprisingly, the expression of anti-oxidant and cyto-protective proteins HO-1, Trx-R and Trx-2 in

macrophages obtained after RV16 exposure were enhanced, which contrasts with the observed enhanced oxidative stress (**Fig. 4.3**). It is unclear whether HO-1, Trx-2 and Trx-R in macrophages purified after RV16 infection were not functional, or whether other anti-oxidant and cyto-protective proteins, like the sirtuins, failed to counter oxidative stress. That is why macrophages were obtained and subjected *ex vivo* to oxidative stress. Macrophages purified after RV16 infection were less capable of counteracting oxidative stress, despite their enhanced baseline expression of HO-1, Trx-2 and Trx-R. In addition, we showed that the kinetics of the HO-1 response in macrophages obtained before RV16 infection was rapid and this response was disabled in macrophages obtained after RV16 infection. This was also reflected by a reduced nuclear translocation of Nrf-2 in these macrophages, which is one of the master regulators of the anti-oxidant and cyto-protective response. Macrophages express, in a Nrf-2-dependent manner, several other anti-oxidant enzymes including MnSOD, ECSOD, catalase and eGRX,[433,434,435,436] and these too may have been affected after RV16 infection. Also we found that Sirt-1 was reduced which may further explain a reduced capacity to counteract oxidative stress. Together these findings indicate that an enhanced level of expression of anti-oxidant and cyto-protective proteins should not be taken as a measure of an enhanced anti-oxidant capacity. Clearly, macrophages that have been subjected to RV16-induced oxidative stress have a reduced capacity to fight oxidative stress.

These findings are important for a number of reasons. First of all it shows that oxidative stress is manifest even during a low dose RV16-induced mild asthma exacerbation. Oxidative stress has also been implicated in allergen and air pollution-induced exacerbations, and thus oxidative stress is a common denominator in exacerbations. Secondly, it shows that macrophages are more vulnerable to oxidative stress after an exacerbation. In support, we showed that CSC, a known source of ROS,[437] caused similar differential responses as shown with X/XO. It is unknown whether other local cells are also more vulnerable to oxidative stress, but given that cells employ similar mechanisms to counteract oxidative stress, we consider this likely. Thirdly, macrophages are among the most abundant leukocytes in the airways and exert a range of immune-regulatory functions, which are affected by oxidative stress.[438] Strikingly oxidative and carbonyl stress inhibits activity of the transcriptional co-repressor HDAC-

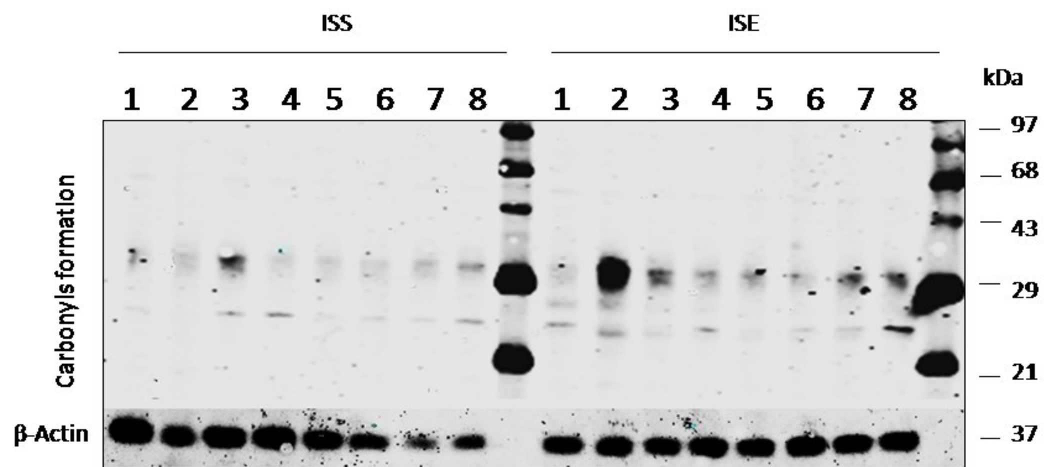
2 (histone deacetylase 2), which under normoxic conditions helps to suppress pro-inflammatory gene expression.[439] In line herewith, macrophages with a reduced capacity to counteract ROS showed a ROS-enhanced release of pro-inflammatory mediators. Other local cells like bronchial epithelial cells may display a similar enhanced pro-inflammatory response and thus also contribute to RV16-induced inflammation.

There are a couple of limitations to this study. Macrophages were obtained from patients that were either treated with placebo or anti-IL-5. We cannot exclude that anti-IL-5 has an impact on ROS production and therefore oxidative stress. The results for the macrophages obtained from the 9 patients, however, were very similar, indicating that anti-IL-5 has no impact on the oxidative stress. It is not clear whether the observed reduced capacity to counteract oxidative stress is specific for asthma, or that a similar effect occurs in healthy individuals exposed to RV16. It is known that basal oxidative stress in healthy individuals compared to that in asthma patients is low, and therefore we predict that RV16-induced ROS are better countered in healthy individuals, which however remains to be studied.

The combination of measuring oxidative stress, the anti-oxidant and cyto-protective protein responses and the anti-oxidant capacity in relation to sequential sampling, before and after RV16 infection, of macrophages obtained by negative selection, strongly support the conclusion that RV16 infection attenuates the anti-oxidant capacity of macrophages. The redox network is complex, but our findings indicate that improving the anti-oxidant capacity may attenuate an exacerbation. Whether this can be achieved by N-Acetyl cysteine or requires more advanced redox interventions remains to be determined.

Supplementary material

E1



Chapter 5

5. Oxidative stress and Allergen-induced asthma exacerbation

This chapter is based on the submitted manuscript: *"Inadequate anti-oxidant response in allergen-induced airway response"*

Caterina Folisi[1,2,3], Marianne A. van de Pol[2,3], Barbara S. Dierdorp[2,3], Jaring van der Zee[4], Peter J. Sterk[2], Giuseppe U. Di Maria[1] and René Lutter[2,3].

Author Affiliations

1. Dipartimento di Biomedicina Clinica e Molecolare Sezione Malattie Respiratorie Università di Catania Ospedale Garibaldi-Nesima 95122 Catania, Italia.

2. Dept. of Respiratory Medicine, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

3. Dept. of Experimental Immunology, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

4. Dept. of Respiratory Medicine, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands.

Corresponding author: Caterina Folisi, Dipartimento di Biomedicina Clinica e Molecolare, Sezione Malattie Respiratorie, Università di Catania, Ospedale Garibaldi-Nesima, via Palermo 636, 95122 Catania, Italia. Telephone number: 0039 0957598742; Fax number: 0039 095/472988; Email: caterina.folisi@libero.it

Contributorship

CF performed the analyses, analysed and interpreted the data and wrote the manuscript, BSD and MvdP performed the clinical study and revised the manuscript, JvdF, GDM and PJS were involved in the design of the study and revised the manuscript, RL designed the study, interpreted the data and wrote the manuscript.

Abstract

Oxidative stress has been implicated in asthma exacerbations. We hypothesized that oxidative stress determines allergen-induced fall in FEV_1 during late asthmatic response (FEV_{1-LAR}).

The aim of this study was to assess reactive oxygen species-induced post-translational modifications of proteins and anti-oxidant and cyto-protective responses in relation to fall in FEV_{1-LAR} upon inhaled house dust mite (HDM).

Induced sputum (supernatant and cells) was collected from 27 HDM-allergic asthmatics before and 6 and 24h after HDM challenge, and plasma before, 1, 6 and 24h after. Oxidative stress was assessed by expression of 4-HNE PAs and protein carbonylation, and anti-oxidant responses by HO-1, Hsp-70, Trx-R, Trx-2, Sirt-1 and Sirt-2 and Nrf-2, all by western blotting.

Strong correlations were found for baseline 4-HNE-PAs and carbonylated proteins in sputum supernatant and cells with fall in FEV_{1-LAR} ($r \geq 0.8$, $p<0.0001$). Patients with a large fall in FEV_{1-LAR} had significantly higher 4-HNE PAs and carbonylated proteins, even at baseline. 4-HNE PAs in sputum and even plasma increased more rapidly after HDM challenge in patients with a large fall in FEV_{1-LAR}. Only patients with a small fall in FEV_{1-LAR} showed significant up-regulation for Trx-2, HO-1 and Trx-R to HDM challenge, paralleled by an enhanced expression of Nrf-2.

The fall in FEV_{1-LAR} to HDM challenge in allergic asthma correlates with oxidative stress and an inadequate anti-oxidant response that is even reflected at baseline. Raising the anti-oxidant capacity in these patients may attenuate allergen-induced exacerbations.

Capsule summary: HDM allergic asthmatics were challenged with HDM to cause a 20% fall in FEV_1 during the early asthmatic response. The allergen challenge caused oxidative stress, which was positively associated with the fall in FEV_1 in the late allergic response. Even baseline oxidative stress strongly correlated with the fall in FEV_1. An enhanced oxidative stress correlated with an inadequate anti-oxidant response, indicating that the extent by which the anti-oxidant capacity can counter oxidative stress determines the clinical response to allergen in allergic asthma patients. These findings indicate that an enhanced anti-oxidant capacity may attenuate the severity of allergen-induced exacerbations.

5.1. Introduction

Asthma is an inflammatory disease of the airways characterized by recurrent and variable symptoms such as wheezing, coughing, chest tightness, and shortness of breath.[440] The episodic nature of asthma[441,442] relates to variable exposure to provocative stimuli such as allergens in allergic asthma patients, infections by respiratory viruses or exposure to air pollution. These periods with sudden worsening of asthma symptoms, i.e. exacerbations, severely impact the patient's quality of life, may contribute to an accelerated decline of lung function and in severe asthma may even be life-threatening. Prevention and control of exacerbations would be major steps forward in the management of asthma, but the mechanisms that underlie the development of an exacerbation are still far from clear.

A common underlying feature of exacerbations, irrespective of the triggering event, is enhanced airway inflammation. Inflammatory processes result in the generation of reactive oxygen species (ROS), which may lead to oxidative stress when ROS and its effects are not adequately balanced by the anti-oxidant and cyto-protective responses.[443,444] Oxidative stress is reflected among others by ROS-induced post-translational modifications of proteins, which may lose their biological function.[445] In addition, oxidative stress in itself is considered a pro-inflammatory trigger and thus may aggravate inflammation.[446]

There are several studies that have implicated ROS in asthma exacerbations, which is supported further by murine studies.[447-448] We hypothesized that oxidative stress determines the allergen-induced fall in FEV_1 in the late asthmatic response (LAR). This was addressed by detailed analyses of the kinetics of oxidative stress and that of the anti-oxidant defense in relation to the clinical response to an allergen challenge. To that end, we analyzed induced sputum and plasma that were collected over time in an earlier study, in which house dust mite (HDM)-allergic asthma patients were subjected to a HDM challenge.[449,450] The amount of HDM for the challenge was titrated so that patients had a 20% fall in FEV_1 in the early asthmatic response, suggestive of a similar mast cell-driven response in all patients. The LAR in these patients varied largely with respect to neutrophilic and eosinophilic inflammation and the fall in the FEV_1.

Oxidative stress was assessed on basis of two independent markers. ROS attack n-6-polyunsaturated fatty acids, such as arachidonic acid and linoleic acid, generating 4-hydroxyl-2-nonenal (4-HNE).[451] When 4-HNE encounters proteins, it can interact with thiol and amino groups of cysteine, lysine and histidine residues via Michael addition resulting in a covalent bond between 4-HNE and the amino acid (4-HNE protein adducts; 4-HNE PAs), and therefore is a relatively stable marker of oxidative stress.[452] ROS also generate lipid electrophiles that may lead to the formation of carbonyl groups on protein side chains (carbonylation), particularly of prolines, arginines, lysines and threonines.[453] Anti-oxidant and cyto-protective proteins to counteract oxidative stress were assessed by quantifying heme-oxygenase-1 (HO-1), Heat shock protein-70 (Hsp-70), Thioredoxin Reductase (Trx-R), Thioredoxin-2 (Trx-2) and Sirtuin-1 and -2 (Sirt-1 and -2)[454] and nuclear factor erythroid 2–related factor 2 (Nrf-2) expression. Nrf-2 is a primary master transcription factor directing anti-oxidant and cyto-protective responses.[455]

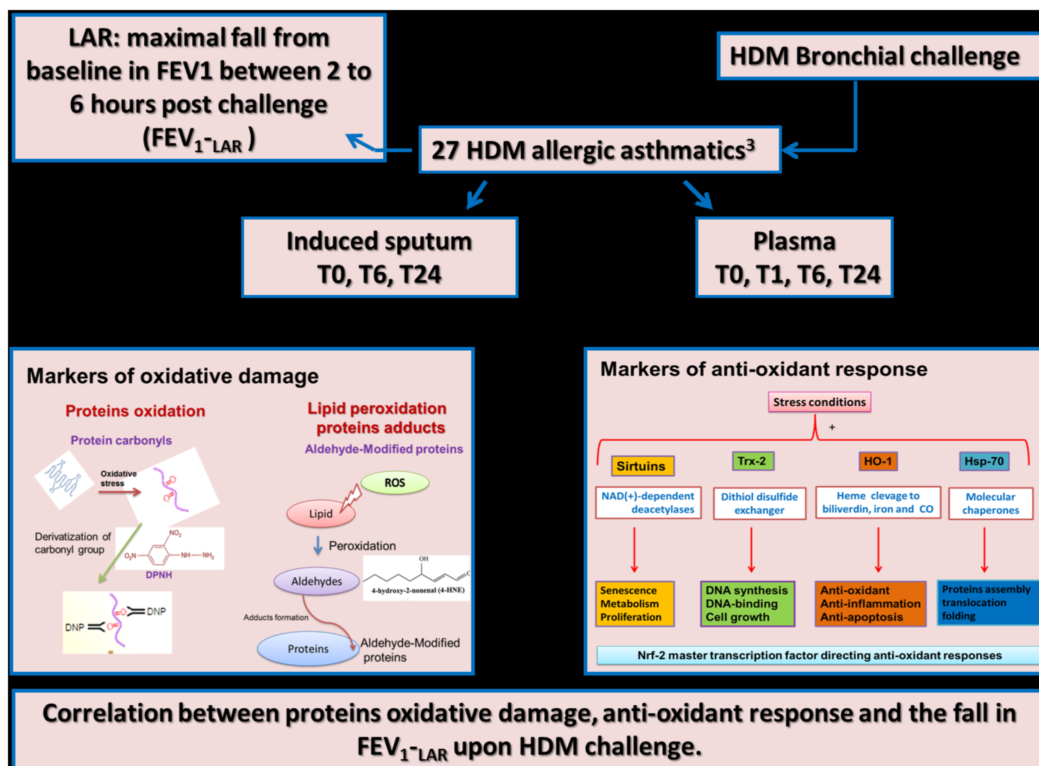


Fig. 5.1 *Study Synopsis and rational*. Induced sputum (supernatant and cells) was collected from 27 HDM-allergic asthmatics before and 6 and 24h after HDM challenge, and plasma before, 1, 6 and 24h after. Oxidative stress was assessed by expression of 4-hydroxyl-2-nonenal protein adducts (4-HNE PAs) and protein carbonylation, and the anti-oxidant response by heme-oxygenase-1 (HO-1), heat shock protein-70 (Hsp-70), thioredoxin reductase (Trx-R), thioredoxin-2 (Trx-2), sirtuin-1 and -2 (Sirt-1 and -2) and NF-E2-related nuclear factor 2 (Nrf-2) all by western blot analysis. C. Folisi.

5.2.Material and Methods

Study population and study design. The original study, the study population and related procedures have been described before in detail.[452] In short, house dust mite (HDM)-allergic patients with intermittent to mild persistent asthma were included. At baseline (T0), blood and induced sputum were collected and lung function was determined by spirometry. Subsequently patients were challenged with HDM and blood was collected at 1h (T1), 6h (T6) and 24h (T24) after challenge. Induced sputum was collected at T6 and T24. All samples were stored at -80°C in aliquots till analyses. The HDM challenge procedure has been described in detail.[10] In short, doubling doses of HDM were inhaled with 10 min interval until a fall of ≥ 20% relative to baseline FEV_1 occurred 10 or 20 min after inhalation, so that patients all had a similar early asthmatic response. After the final dose of HDM the FEV_1 was measured every 10 min up to 1 h, at 90 min and after that, to follow up the late asthmatic response (LAR), hourly until 6 h after HDM inhalation. The LAR was defined as the maximal fall from baseline in FEV_1 between 2 to 6 hours post challenge. The study was approved by the AMC Medical Ethics Committee and all patients provided written informed consent.

Measurements. 4-hydroxynonenal protein adducts (4-HNE PAs), carbonylated proteins, anti-oxidant and cyto-protective proteins were analysed by Western blotting as described in detail in the previous chapter. The protein determination is also described in the chapter 3.

Exosomes isolation and western blot analysis. Plasma and sputum supernatant were centrifuged in a table centrifuge at 300 x g for 10 minutes at 4°C to remove cells. Then samples were centrifuged at 16,500 x g for 20 minutes at 4°C to further remove cell debris and the supernatant was filtered through a 0.2 µm filter to remove particles larger than 200 nm. The filtered supernatant was ultracentrifuged at 120,000 x g for 90 minutes at 4°C to pellet extracellular vesicles such as exosomes. The low-density membrane pellets were solubilized in 5µl Laemmli sample buffer, separated on SDS/PAGE, blotted and HO-1 was detected as described in the Supplemental information.

Statistical analysis. The fall in FEV_1 during the LAR was correlated with post-translational modifications in sputum and plasma proteins for all patients. To be able to determine whether patients with a large fall in FEV_1 differ from patients with a small fall in FEV_1 with respect to oxidative stress and the anti-oxidant response, patients were subdivided in two equal-sized groups on the basis of the fall in FEV_1 during the LAR.

Results are presented as mean ± SEM of at least two replicate experiments. Statistical analyses were performed utilizing GraphPad Prism 5. Correlations between parameters were determined with Pearson's correlation coefficient with two-tailed p values and a confidence interval of 95 %. Analysis of significance was calculated by unpaired Student's *t*-test and was used to assess between- and within-study group differences. A *p* value <0.05 was considered significant.

5.3.Results

Subjects and HDM bronchial challenge. Complete sample sets were available for 27 out of 29 patients participating in the study. Demographic and clinical characteristics of the 27 mild asthma patients are provided in **Table 9**, whereas those for all 29 patients were published before. Patients were clinically stable before exposure to HDM.

TABLE 9. PATIENTS AND LUNG FUNCTION CHARACTERISTICS

	All patients	Group A	Group B	P value
Male/female	(6/21)	(3/11)	(3/10)	
Age†	28.3 (20-51)	25.3 (20-42)	31.2 (21-51)	0.1
FEV_1, baseline	3.5±0.1	3.7±0.2	3.3±0.1	0.1
FEV_1 % pred, baseline	95.4±1.9	97.6±1.8	93.1±3.4	0.3
PC_{20} methacholine, (mg/ml)††	1.9 (0.1-7.7)	2.2 (0.2-7.7)	1.6 (0.1-7.6)	0.9
Total doses allergen (BU)	574.0±91.8	490.3±103.6	657.7±152.3	0.4
PD_{20} HDM††	384.5 (15-1115)	305.5 (15-666)	458 (25-1115)	0.3
Δ FEV_1, max (%) EAR	-31.2±1.5	-32.9±2.7	-29.5±1.5	0.3
AUC EAR (ΔFEV_1 0-60min)	20.0±1.2	21.0±2.0	18.9±1.4	0.4
Δ FEV_1, max (%) LAR	-20.9±2.5	-29.4±3.5	-11.8±0.9	<0.0001
AUC LAR (Δ FEV_1 2-6hr)	43.5±4.1	54.3±5.6	31.9±4.0	0.004

Values are expressed as mean ±SEM, unless indicated otherwise. Abbrevations: FEV_1: forced expiratory volume in 1 second; %pred: percentage of predicted; PC20: dose of methacholine required to cause a 20% drop in FEV_1; BU: Biological Units; PD20: dose of HDM required to cause a 20% drop in FEV_1; EAR: early allergic response; LAR: Late allergic response. EAR and LAR are defined by maximal drop in FEV_1 (max) and area under the curve (AUC) over indicated period. † mean (Range). †† median (Range).

Oxidative damage of proteins in sputum supernatant before and after HDM bronchial challenge. 4-HNE PAs and carbonylated sputum proteins increased with time after the HDM challenge (**Fig. 5.2 A**), which was 1.3 times higher for 4-HNE PAs at 24h ($p=0.001$). Both post-translational modifications of sputum proteins correlated strongly with the fall in the FEV_1 at 6 and 24h (4-HNE PAs: t=6: $r=-0.72$ $p<0.0001$ and t=24: $r=-0.73$ $p<0.0001$; carbonylated proteins: t=6: $r=-0.79$ $p<0.0001$ and t=24: $r=-0.75$ $p<0.0001$), and interestingly even stronger at baseline (**Fig. 5.2 B**). At each time point, 4-HNE PAs and carbonylated proteins correlated significantly (**Fig. 5.2 B**). To reveal whether patients with a large fall in FEV_1 in the LAR as opposed to patients with a limited fall in FEV_1 differ with respect to the generation of 4-HNE PAs and carbonylated proteins, patients were subdivided in two equal-sized groups; group A (n=14) with a fall in FEV_1 of ≥19% and group B (n=13) with a decline of <19% (**Table 9**). Baseline characteristics for group A and B are comparable (**Table 9**) and so are sputum and blood relative cell counts and inflammatory markers, apart from increased systemic amounts of IL-5 at T6 (**Tables 10** and **11**). Patients from group A showed significantly higher levels of 4-HNE PAs and carbonylated sputum proteins at baseline (**Fig. 5.2 C** and **D**). Also, the increase in these post-translational modifications after the HDM challenge was significantly higher for group A as compared to group B (**Fig. 5.2 C** and **D**; **Table 12**). The enhanced oxidative stress in group A did not relate to exposure to more allergen. In fact, patients in group B were exposed to more allergen.

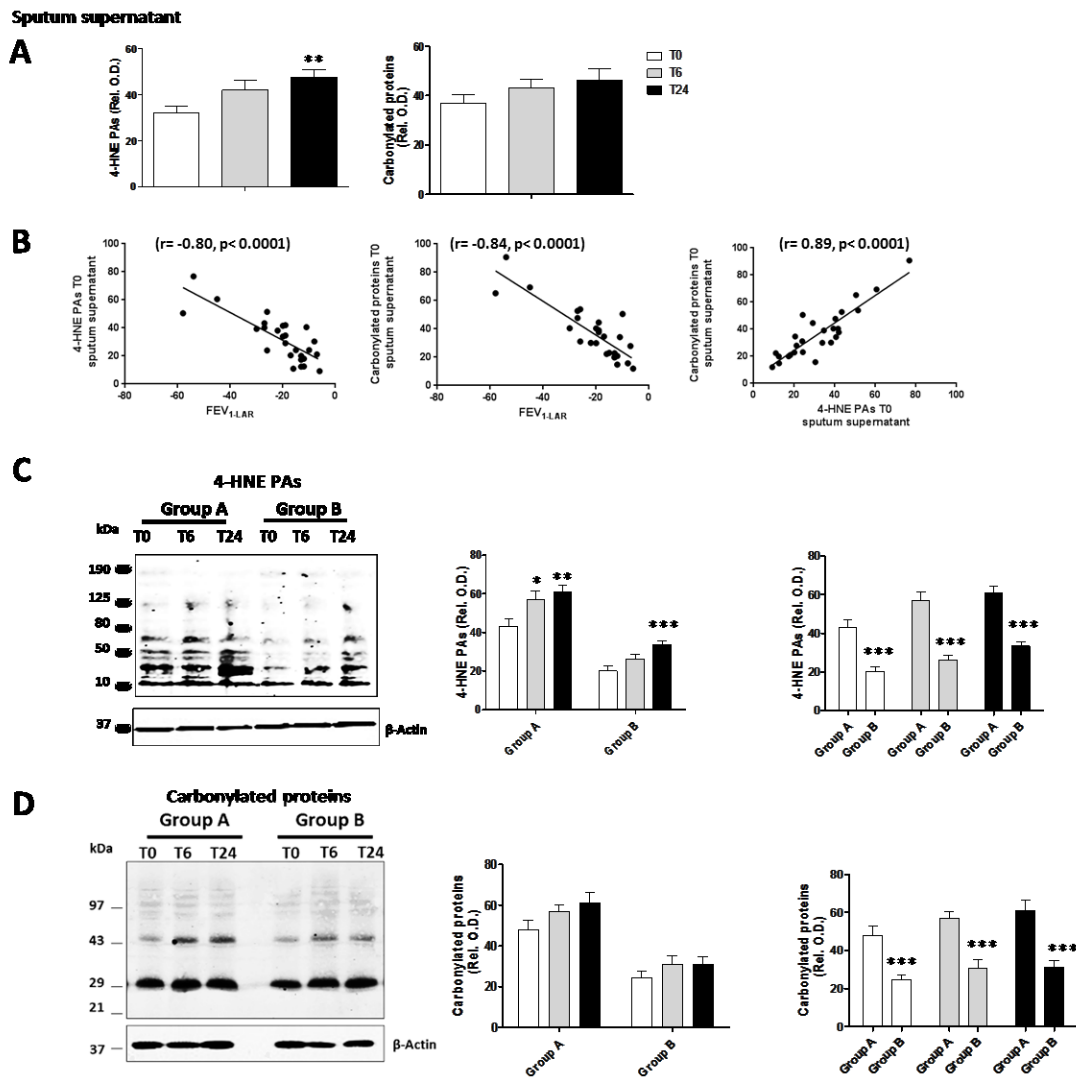


Fig. 5.2 *Oxidative damage on proteins in sputum supernatant before and after HDM bronchial challenge.* Quantitative data for 4-HNE PAs and carbonylated proteins (A); correlation between 4-HNE PAs (on the left), carbonylated proteins (center) and maximal drop in FEV_1 during the late asthmatic response; correlation between baseline 4-HNE PAs and carbonylated proteins (on the right) (B); representative western blot for 4-HNE PAs and quantitative data (on the right) (C); representative western blot for carbonylated proteins and quantitative data (on the right) (D). Group A= asthmatics showing a severe Late Asthmatic Response (FEV_1 drop ≥19%) after HDM bronchial challenge. Group B= asthmatics showing a moderate Late Asthmatic Response (FEV_1 drop <19%) after HDM bronchial challenge. T0= before HDM bronchial challenge. T1= 1 hour after HDM bronchial challenge. T6= 6 hours after HDM bronchial challenge. T24= 24 hours after HDM bronchial challenge. (*N*=27, 14 Group A, 13 Group B). *P <0.05, **P <0.01, ***P <0.001.

TABLE 10. RELATIVE SPUTUM CELL COUNTS AND INFLAMMATORY MARKERS

	All patients	Group A	Group B	P values
T0				
% neutrophils	34.70±3.79	35.20±5.58	34.15±5.31	0.89
% eosinophils	5.83±1.44	5.57±1.22	6.12±2.76	0.85
% lymphocytes	3.45±0.45	2.82±0.45	4.13±0.77	0.15
% basophils	0.14±0.07	0.05±0.03	0.25±0.15	0.18
% macrophages	55.86±3.78	56.36±5.28	55.32±5.62	0.89
% epithelial	31.74±5.20	28.36±5.07	35.38±9.48	0.51
MPO (ng/ml)	1275.33±274.54	1364.29±428.75	1179.54±351.04	0.74
ECP (ng/ml)	46.78±11.41	52.58±18.76	40.52±12.99	0.61
IL-8 (pg/ml)	765.37±227.33	713.50±350.90	821.23±297.32	0.81
T6				
% neutrophils	44.75±2.76	42.44±4.27	47.23±3.49	0.40
% eosinophils	21.93±2.89	22.07±3.81	21.78±4.53	0.96
% lymphocytes	4.79±0.42	5.14±0.56	4.40±0.63	0.38
% basophils	0.03±0.02	0.00±0.00	0.06±0.03	0.1
% macrophages	28.50±2.71	30.34±3.54	26.52±4.22	0.49
% epithelial	22.62±4.13	25.15±5.25	19.90±6.58	0.54
MPO (ng/ml)	2083.33±405.60	1344.57±241.30	2878.92±755.42	0.06
ECP (ng/ml)	565.13±244.66	313.77±135.63	835.84±485.94	0.29
IL-8 (pg/ml)	1918.63±821.35	716.21±210.70	3213.54±1647.56	0.13
T24				
% neutrophils	42.51±3.79	41.62±6.05	43.40±4.81	0.82
% eosinophils	15.62±2.12	15.15±3.02	16.08±3.09	0.81
% lymphocytes	5.04±0.54	4.85±0.82	5.24±0.71	0.72
% basophils	0.06±0.03	0.03±0.02	0.09±0.06	0.36
% macrophages	36.76±3.53	38.35±4.47	35.16±5.60	0.66
% epithelial	27.38±4.82	34.23±7.28	20.52±6.00	0.16
MPO (ng/ml)	3283.00±1105.77	1678.92±595.42	4887.08±2076.37	0.15
ECP (ng/ml)	1063.56±434.00	618.97±412.27	1508.15±762.82	0.32
IL-8 (pg/ml)	3552.69±1653.23	1276.54±498.75	5828.85±3205.62	0.17

Values presented as mean±SEM. P values refer to the comparison between Group A and B. N=27, Group A=14, Group B=13

Abbreviations: eo: eosinophil number; ECP: eosinophil cationic protein; MPO: myeloperoxidase; IL-8: interleukin-8.

TABLE 11. BLOOD CELLS AND MARKERS OF INFLAMMATION AND ALLERGY

	All patients	Group A	Group B	P values
T0				
% neutrophils	50.82±1.88	51.51±3.10	50.13±2.26	0.72
% eosinophils	6.17±0.65	7.06±1.03	5.28±0.75	0.17
% lymphocytes	35.00±1.73	33.18±2.68	36.83±2.19	0.30
% basophils	0.46±0.06	0.45±0.09	0.48±0.08	0.80
% monocytes	7.55±0.36	7.82±0.61	7.28±0.40	0.47
Total leukocytes (10E9/L)	5.87±0.25	5.72±0.41	6.01±0.31	0.58
Total eosinophils (10E6/L)	350.96±32.76	386.08±48.50	315.85±43.75	0.30
IL-5 (pg/ml)	3.86±1.24	5.43±2.25	2.29±0.96	0.21
IgE total (kU/l)	304.67±45.80	343.09±62.01	266.25±68.18	0.41
IgE HDM (kUA/l)	48.04±9.72	61.52±16.80	34.55±8.99	0.17
IgE Der p1 (kUA/l)	17.93±3.66	23.58±6.03	12.29±3.76	0.13
IgE Der p2 (kUA/l)	28.49±5.51	34.32±9.16	22.66±6.06	0.31
T1				
% neutrophils	59.50±1.77	58.69±2.83	60.32±2.20	0.65
% eosinophils	4.09±0.47	4.60±0.64	3.58±0.68	0.28
% lymphocytes	29.04±1.43	29.17±2.53	28.91±1.46	0.93
% basophils	0.46±0.06	0.36±0.05	0.56±0.11	0.11
% monocytes	6.92±0.33	7.18±0.50	6.65±0.44	0.44
Total leukocytes (10E9/L)	6.90±0.39	6.38±0.46	7.42±0.63	0.2
Total eosinophils (10E6/L)	256.27±25.72	278.31±37.61	234.23±35.50	0.40
IL-5 (pg/ml)	3.49±1.19	4.97±2.22	2.01±0.78	0.22
T6				
% neutrophils	58.65±1.55	59.97±2.65	57.34±1.66	0.41
% eosinophils	3.81±0.43	3.99±0.64	3.62±0.60	0.68
% lymphocytes	30.05±1.29	28.55±2.15	31.55±1.37	0.25
% basophils	0.35±0.04	0.33±0.06	0.38±0.07	0.60
% monocytes	7.16±0.32	7.18±0.52	7.14±0.38	0.94
Total leukocytes (10E9/L)	7.75±0.31	7.71±0.45	7.79±0.46	0.87
Total eosinophils (10E6/L)	271.85±25.16	287.08±40.15	256.62±31.41	0.56
IL-5 (pg/ml)	11.91±2.71	17.46±4.52	6.35±2.22	0.03
T24				
% neutrophils	58.10±2.17	61.06±3.71	55.37±2.26	0.2
% eosinophils	7.66±0.90	7.67±1.28	7.65±1.31	0.99
% lymphocytes	27.02±1.70	24.15±2.99	29.66±1.52	0.11
% basophils	0.48±0.07	0.43±0.10	0.52±0.09	0.48
% monocytes	6.76±0.24	6.69±0.28	6.82±0.39	0.80
Total leukocytes (10E9/L)	7.16±0.39	7.76±0.60	6.62±0.48	0.15
Total eosinophils (10E6/L)	510.20±56.69	555.58±95.89	468.31±65.38	0.45
IL-5 (pg/ml)	46.36±15.18	70.71±27.84	23.89±11.96	0.13

Values are presented as mean±SEM. P values refer to the comparison between Group A and B. N=27, Group A=14, Group B=13.

IL-5: interleukin-5

TABLE 12. MARKERS OF OXIDATIVE DAMAGE ON PROTEINS IN INDUCED SPUTUM CELLS AND SUPERNATANT BEFORE AND AFTER HDM BRONCHIAL CHALLENGE

4-HNE Pas

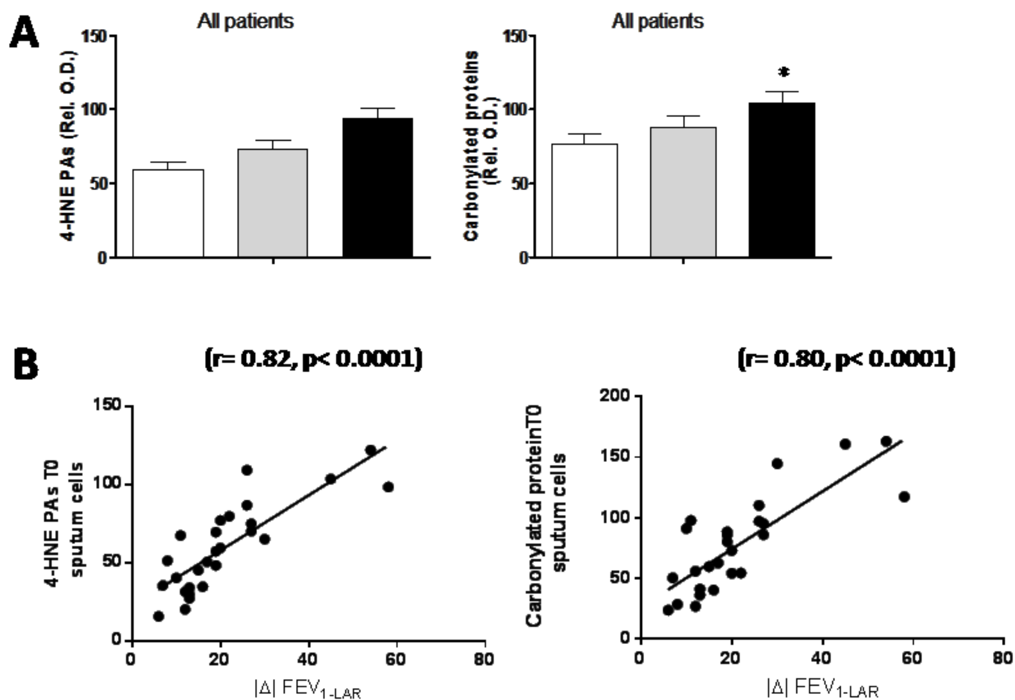
	T0	T6	T24
Sputum supernatant			
All patients	32.11±3.12	42.09±4.01 P=0.05	47.59±3.41 P=0.001
Group A	43.25±3.58	56.90±4.63 P=0.03	60.71±3.57 P=0.002
Group B	20.11±2.39	26.15±2.55 P=0.09	33.47±2.3 P=0.005
Sputum cells			
All patients	59.31±5.43	72.64±6.29 P=0.11	94.01±7.19 P=0.0003
Group A	79.98±5.73	95.99±7.7 P=0.10	121.54±7.55 P=0.0002
Group B	37.04±3.85	47.49±2.68 P=0.04	64.36±4.99 P=0.0002

Protein carbonylation

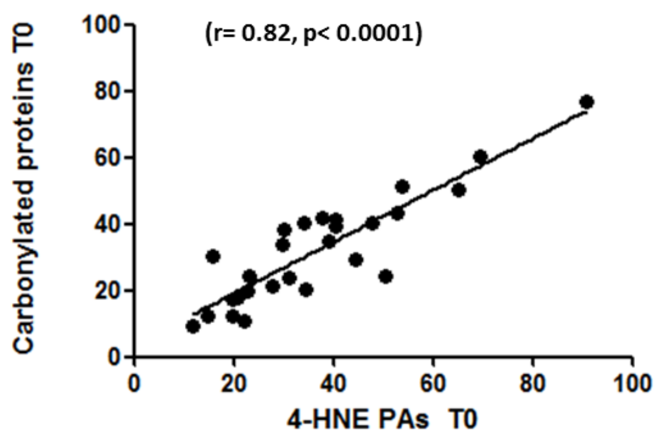
	T0	T6	T24
Sputum supernatant			
All patients	36.72±3.56	43.07±3.65 P=0.22	46.43±4.47 P=0.09
Group A	48.06±4.62	56.97±3.33 P=0.13	60.80±5.62 P=0.09
Group B	24.50±2.87	30.88±4.13 P=0.42	30.95±3.80 P=0.19
Sputum cells			
All patients	76.16±7.55	87.87±8.19 P=0.3	104.71±7.64 P=0.01
Group A	100.65±9.39	117.88±8.96 P=0.53	126.45±8.77 P=0.01
Group B	49.07±5.78	62.71±8.86 P=0.20	83.27±6.94 P=0.05

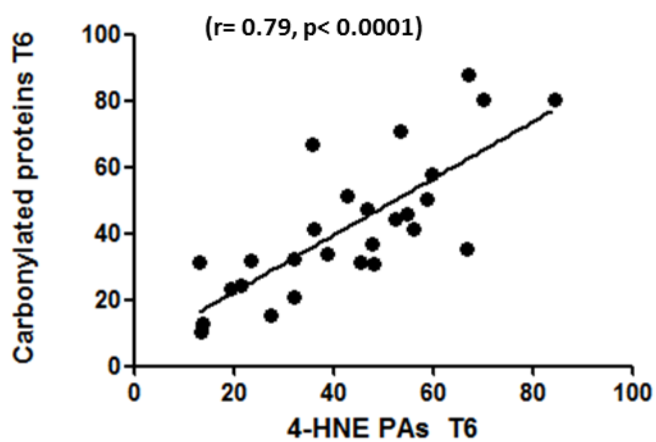
Rel. O.D. presented as mean±SEM. P values compare to T0. N=27, Group A=14, Group B=13

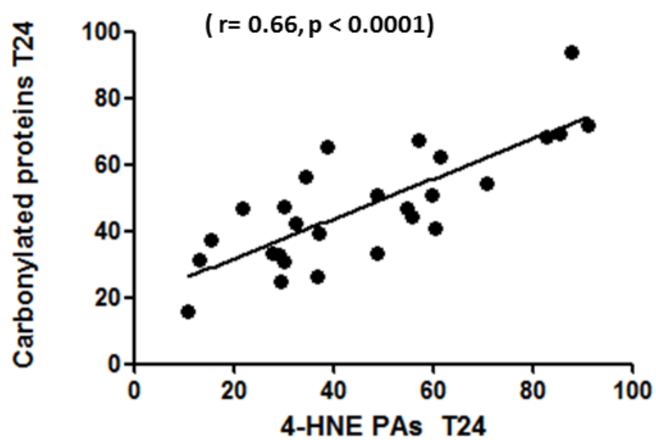
Oxidative damage on proteins in sputum cells before and after HDM bronchial challenge. Like for sputum proteins, 4-HNE PAs and carbonylated sputum cellular proteins increased with time after the HDM challenge (**Fig. 5.3 A**; **Table 12**). Strong correlations between the fall in FEV_1 during the LAR and modifications of the cellular proteins were observed at baseline (**Fig. 5.2 B**: $r \geq 0.8$, $p<0.0001$) and at 6 and 24h (**Fig. E3**: 4-HNE PAs: t=6: r=0.83 p<0.0001 and t=24: r=0.76 p<0.0001; carbonylated proteins: t=6: r=0.79 p<0.0001 and t=24: r=0.56 p<0.0001). 4-HNE PAs and carbonylated cellular proteins correlated significantly (**Fig. 5.3 C**). Following 4-HNE PAs (**Fig. 5.3 D**) and carbonylated proteins (**Fig. 5.3 E**) for group A and B over time, the highest baseline values (for both 4-HNE PAs and carbonylated proteins) and the earliest changes in 4-HNE PAs (no clear differences for carbonylated proteins) were observed in group A. Finally, 4-HNE PAs and carbonylated proteins from sputum cells and supernatant strongly correlated.

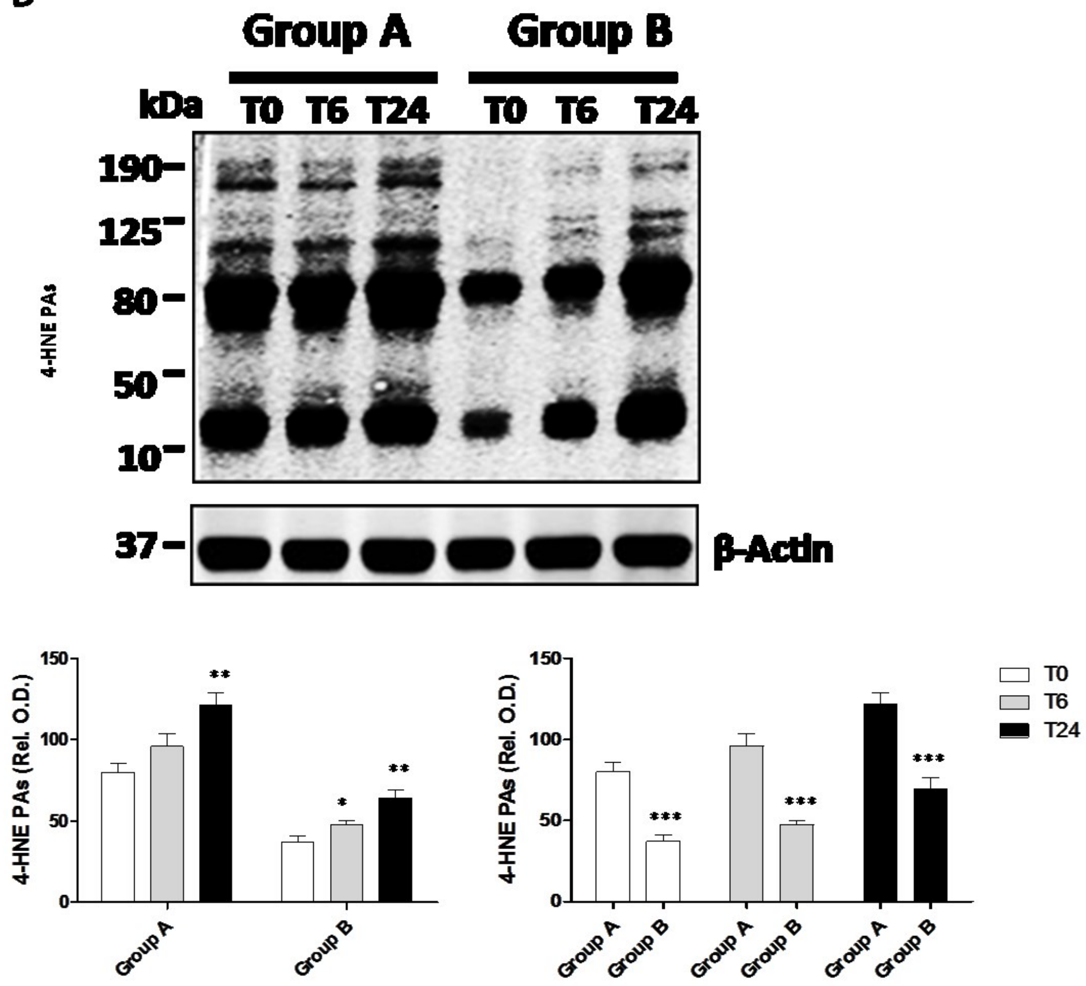


C








D
Group A
Group B
kDa
T0 T6 T24
T0 T6 T24
190
125
80
50
10
4-HNE PAs
37
β-Actin
4-HNE PAs (Rel. O.D.)
150
100
50
0
Group A
Group B
T0
T6
T24

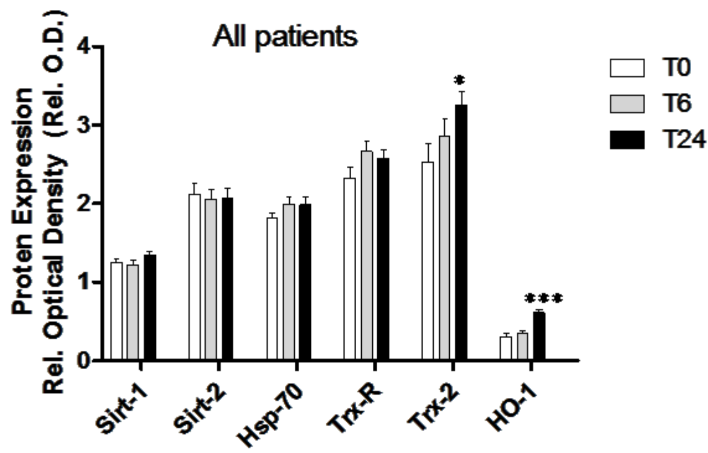
E

Fig. 5.3 *Oxidative damage on proteins in sputum cells before and after HDM bronchial challenge.* Quantitative data for 4-HNE PAs and carbonylated proteins (**A**); correlation between 4-HNE PAs, carbonylated proteins and $FEV_{1\text{-}LAR}$ (**B**); correlation between 4-HNE PAs and carbonylated proteins (**C**); representative western blot for 4-HNE PAs and quantitative data (**D**); representative western blot for carbonylated proteins and quantitative data (**E**). See legends **Fig. 5.2** for further explanation.

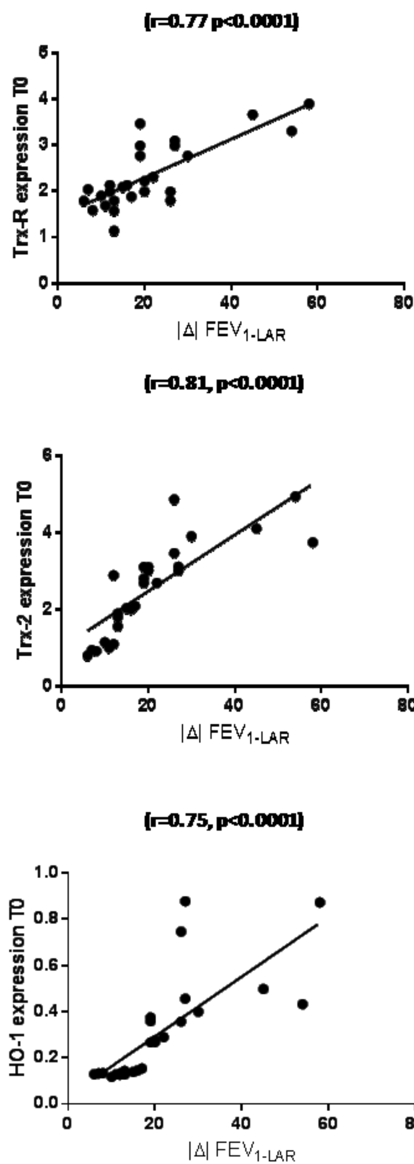
Cyto-protective and anti-oxidant proteins in sputum cells before and after HDM bronchial challenge. The expression of only Trx-2 and HO-1 in sputum cells were enhanced in response to HDM (**Fig. 5.4 A**, **E5** and **Table 13**). The expression of Trx-2, HO-1 and Trx-R (enhanced in group B, see below) were strongly correlated with the fall in FEV_1 during the LAR at baseline (**Fig. 5.4 B**) and at 6 and 24h (**Fig. E6** Trx-2: t=6: r=0.78 p<0.0001 and t=24: r=0.73 p<0.0001; Trx-R: t=6: r=0.72 p<0.0001 and t=24: r=0.76 p<0.0001; less so for HO-1: t=6: r=0.56 p=0.002 and t=24: r=-0.11 p=0.56). Comparison within group A and B, however, shows marked up-regulation for Trx-2, HO-1 and, although slightly less, for Trx-R in sputum cells from group B over time in response to the HDM challenge (**Fig. 5.4 C**). In contrast, for group A there was a slight, but significant, up-regulation for HO-1 and Hsp-70 only. These results indicate that sputum cells from group A with the largest fall in FEV_1 have a reduced capacity to enhance the expression of cyto-protective proteins. Interestingly, the baseline expression of the cyto-protective Hsp-70, Trx-R, Trx-2 and HO-1 in group A were significantly higher and that for Sirt-1 significantly lower compared to those in group B (**Fig. 5.4 C** and **E6**). Therefore, we assessed the expression of Nrf-2 in sputum cells. Strikingly the expression of Nrf-2 remained stable in both group A and B upon allergen exposure, but its expression was significantly higher in sputum cells from group B (**Fig. 5.4 D**).

Sputum cells

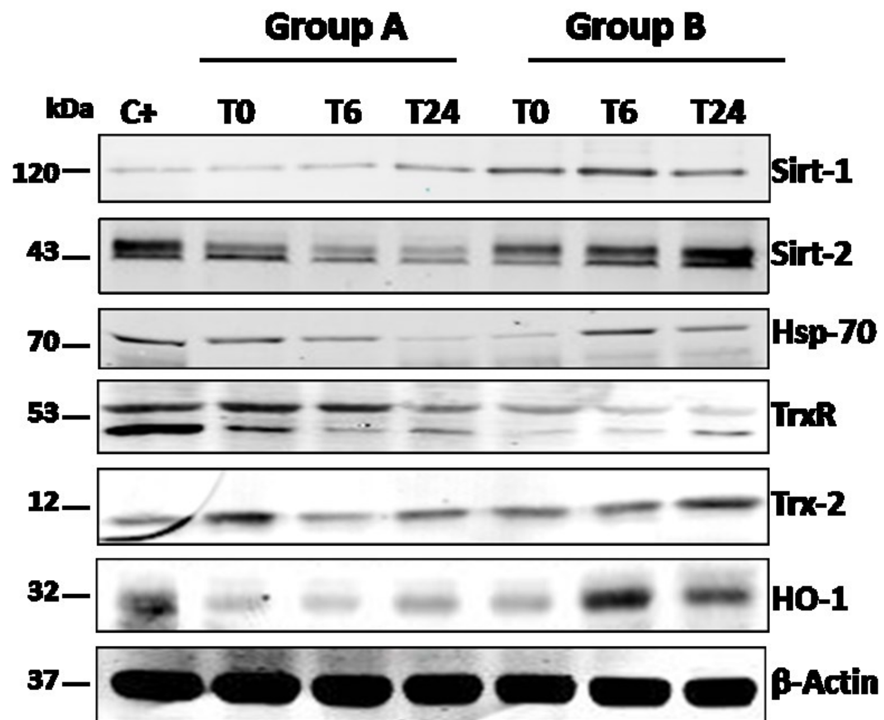
A

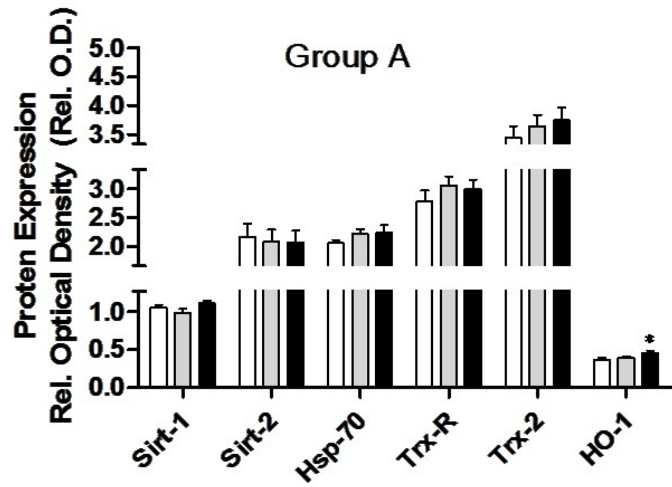


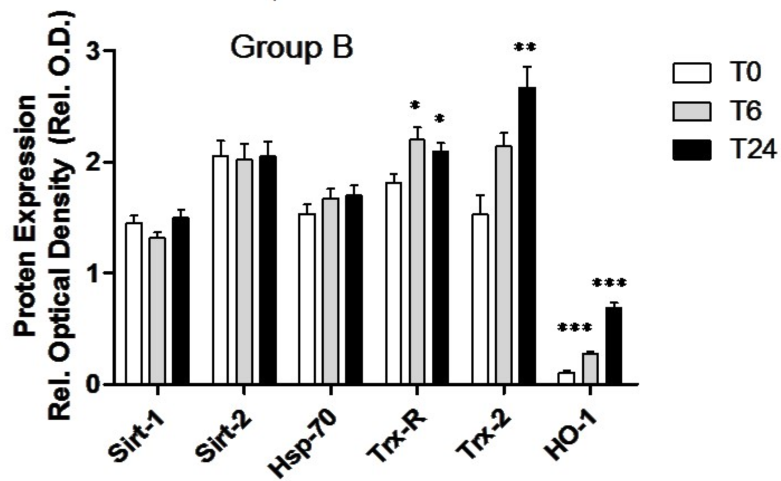
B



C







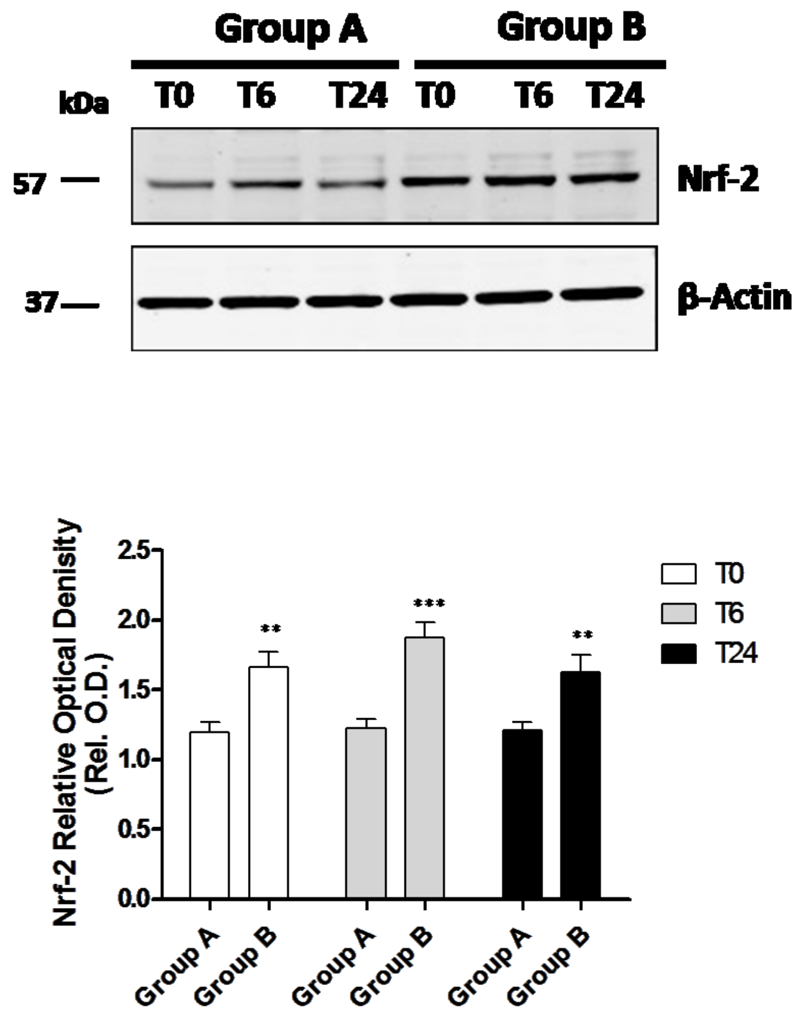


Fig. 5.4 *Cyto-protective proteins in sputum cells before and after HDM bronchial challenge*. Quantitative data for Sirt-1, Hsp-70, Sirt-2, Trx-2, Trx-R and HO-1 (**A**); correlation betweenTrx-R, Trx-2, HO-1 and $FEV_{1\text{-}LAR}$ (**B**); representative western blot for Sirt-1, Sirt-2, Hsp-70, Trx-R, Trx-2, HO-1 and quantitative data for group A and B (**C**); western blot for Nrf-2 and quantitative data (**D**); Rel. Optical Density (Rel. O.D.). See legends **Fig. 5.2** for further explanation. C+ = HeLa cells lysate used as positive control.

TABLE 13. CYTO-PROTECTIVE PROTEINS IN SPUTUM CELLS AND SUPERNATANT BEFORE AND AFTER HDM CHALLENGE

Sputum Cells

All Patients	T0	T6	T24	P value T0/T6	P value T0/T24
Sirt-1	1.25±0.05	1.22±0.06	1.34±0.05	0.77	0.21
Sirt-2	2.12±0.13	2.05±0.12	2.07±0.12	0.72	0.78
Hsp-70	1.81±0.07	1.99±0.09	1.99±0.09	0.12	0.13
Trx-R	2.32±0.14	2.66±0.13	2.57±0.12	0.07	0.20
Trx-2	2.53±0.2	2.86±0.20	3.25±0.18	0.30	0.01
HO-1	0.30±0.04	0.35±0.03	0.61±0.04	0.36	<0.0001
Group A					
Sirt-1	1.05±0.04	0.98±0.05	1.11±0,03	0.35	0.23
Sirt-2	2.17±0.22	2.09±0.2	2.07±0.20	0.79	0.74
Hsp-70	2.06±0.05	2.23±0.07	2.24±0.13	0.05	0.19
Trx-R	2.79±0.18	3.06±0.16	2.99±0.16	0.27	0.39
Trx-2	3.45±0.20	3.65±0.19	3.75±0.21	0.48	0.30
HO-1	0.37±0.02	0.39±0.02	0.46±0.03	0.56	0.03
Group B					
Sirt-1	1.45±0.06	1.32±0,05	1.50±0.07	0.107	0.66
Sirt-2	2.05±0.14	2.02±0.14	2.05±0.13	0.88	0.99
Hsp-70	1.53±0.08	1.68±0.08	1.70±0.09	0.26	0.19
Trx-R	1.81±0.08	2.98±0.11	2.09±0.08	0.01	0.02
Trx-2	1.53±0.17	2.14±0.12	2.67±0.19	0.09	0.0002
HO-1	0.11±0.01	0.28±0.02	0.69±0.04	<0.0001	<0.0001

Sputum supernatant

All Patients	T0	T6	T24	P value T0/T6	P value T0/T24
Sirt-1	0.99±0.06	1.05±0.05	0.98±0.07	0.47	0.88
Sirt-2	1.30±0.10	1.29±0.09	1.56±0.12	0.95	0.44
Hsp-70	1.63±0.08	1.63±0.09	1.68±0.08	0.98	0.71
Trx-R	2.26±0.16	2.20±0.13	2.40±0.21	0.77	0.59
Trx-2	0.86±0.06	1.01±0.06	1.27±0.19	0.05	<0.0001
HO-1	0.03±0.01	0.11±0.02	0.37±0.04	0.002	<0.0001
Group A					
Sirt-1	0.77±0.05	0.88±0.05	0.69±0.05	0.14	0.21
Sirt-2	1.15±0.16	1.17±0.14	1.03±0.15	0.90	0.57
Hsp-70	1.94±0.09	1.90±0.08	1.69±0.14	0.76	0.14
Trx-R	2.73±0.19	2.26±0.22	1.67±0.14	0.11	0.0001
Trx-2	1.03±0.08	0.93±0.10	0.64±0.04	0.45	0.004
HO-1	0.02±0.01	0.06±0.03	0.28±0.04	0.25	<0.0001
Group B					
Sirt-1	1.24±0.05	1.24±0.04	1.31±0.04	0.96	0.29
Sirt-2	1.45±0.10	1.41±0.10	1.3±0.10	0.75	0.25
Hsp-70	1.31±0.07	1.34±0.07	1.66±0.08	0.70	0.002
Trx-R	1.75±0.18	2.13±0.15	3.18±0.29	0.11	0.0003
Trx-2	0.68±0.04	1.11±0.04	1.97±0.26	<0.0001	<0.001
HO-1	0.04±0.02	0.15±0.02	0.45±0.08	0.002	<0.0001

Cyto-protective proteins in induced sputum supernatant before and after HDM bronchial challenge. Several cyto-protective proteins are released actively by cells in response to oxidative stress, either free or packed within vesicles.[19-22] Over time extracellular Hsp-70, Trx-2 and Trx-R increase in sputum supernatant from group B, whereas it was reduced in sputum supernatants from group A (**Fig. 5.5 A**, **Fig. E7**, **Table 13**). HO-1 enhances in both group A and B, although values for HO-1 are higher in group B (**Fig. 5.5 A**). In fact, baseline levels and changes over time resemble those observed for cyto-protective proteins within sputum cells (**Fig. 5.4 A** and **C**; cf. **Fig.5.5 C**) and even are relatively more pronounced. HO-1 and Trx-2 (**Fig. 5.5 B**) were contained in extracellular vesicles, but some HO-1 and Trx-2 remained in the supernatant after centrifugation.

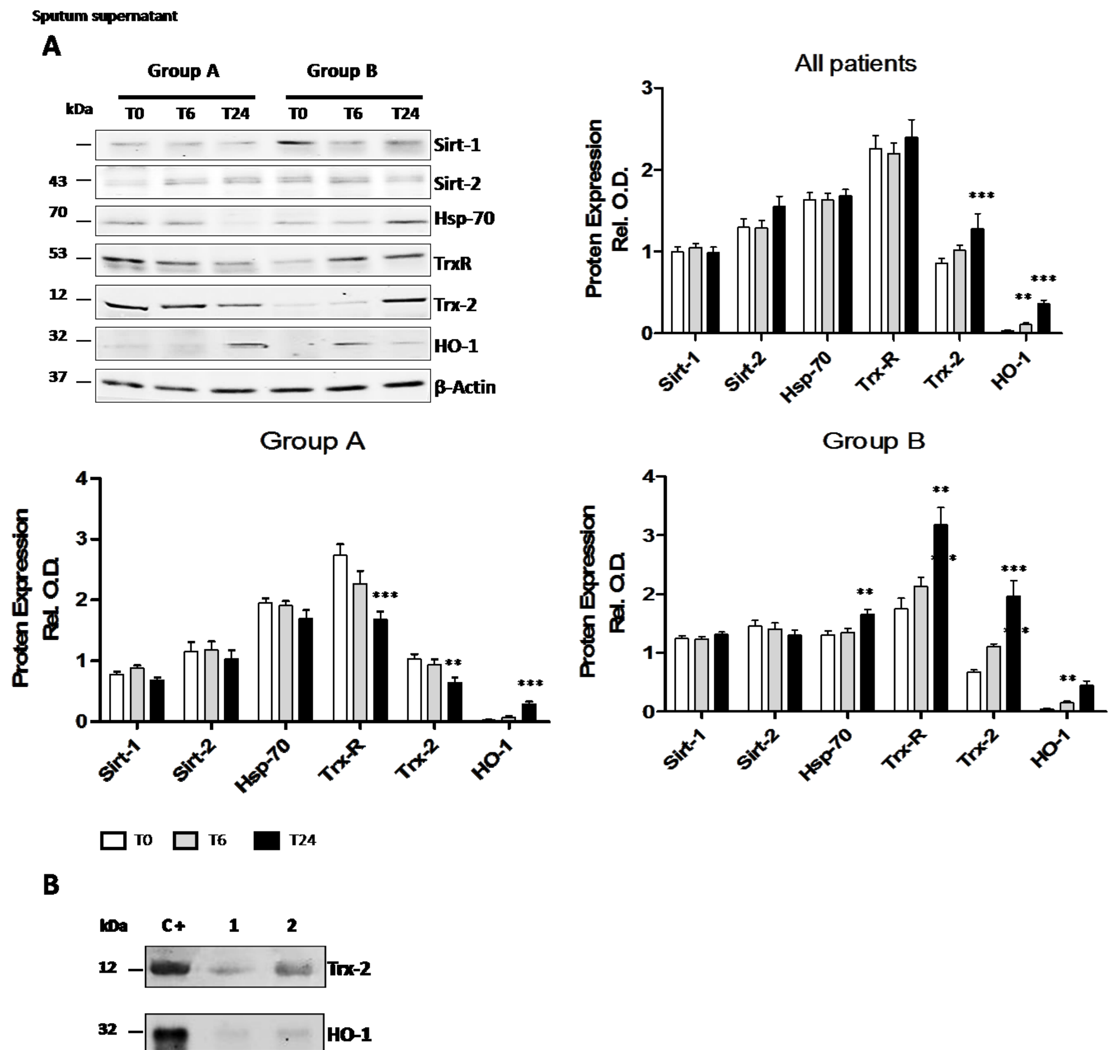


Fig. 5.5 *Cyto-protective proteins in sputum supernatant before and after HDM bronchial challenge*. Representative western blot for Sirt-1, Sirt-2, Hsp-70, Trx-R, Trx-2, HO-1 and quantitative data for all patients (on the right) and for group A and B (below) (A); western blot for Trx-2 and HO-1 in exosome-containing fraction and cleared supernatant (B); quantitative data for Sirt-1, Hsp-70, Sirt-2, Trx-2, Trx-R and HO-1 in group A and B (C). C+ = HeLa cells lysate used as positive control 1= sputum supernatant after ultracentrifugation 2= pellet after ultracentrifugation. See legends Fig. 5.2 for further explanation.

Plasma 4-HNE protein adducts before and after HDM bronchial challenge. 4-HNE PAs in plasma increased in both groups, but more in group A and already 1h after HDM challenge (**Fig. 5.6 A**, **Table 14**). Despite the fast increase in 4-HNE PAs in plasma, only at 1 and 24h we found significant correlations with the fall in FEV_1 (T1: r=0.44. p=0.03; T24: r=0.50, p=0.01). HO-1 in plasma increased in both groups, but more in group B and here too a significant difference was obtained 1h after HDM challenge (**Fig. 5.6 B, Table E5**). HO-1 and 4-HNE-PAs in plasma correlated (T24: r=-0.42, p=0.04). HO-1 plasma levels correlated with fall of FEV_1 (r=-0.53, p=0.006 at 6h; r=-0.46, p=0.02 at 24h). Interestingly, extracellular HO-1 in plasma was associated with extracellular vesicles, similar to that found for sputum.

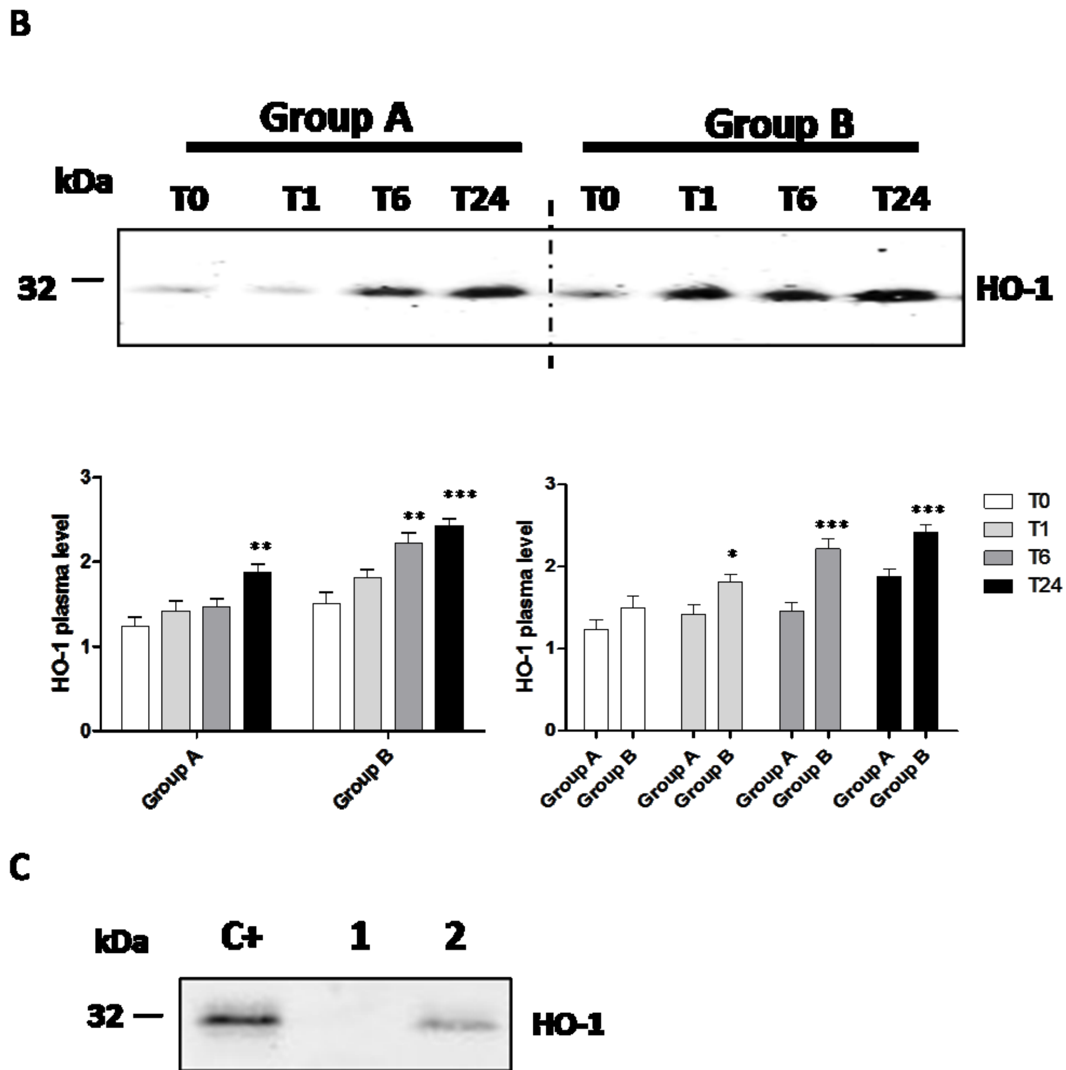


Fig. 5.6 *Plasma 4-HNE protein adducts before and after HDM bronchial challenge.* Representative western blot for HNE PAs and quantitative data (A); western blot for HO-1 and quantitative data (B); western blot for HO-1 of the exosome-containing fraction and cleared supernatant (C). C+= HeLa cells lysate used as positive control; 1= plasma supernatant after ultra-centrifugation; 2= pellet after ultra-centrifugation. See legends Fig. 5.2 for further explanation.

TABLE 14. MARKERS OF OXIDATIVE STRESS AND OXIDATIVE RESPONSE IN PLASMA BEFORE AND AFTER HDM BRONCHIAL CHALLENGE.

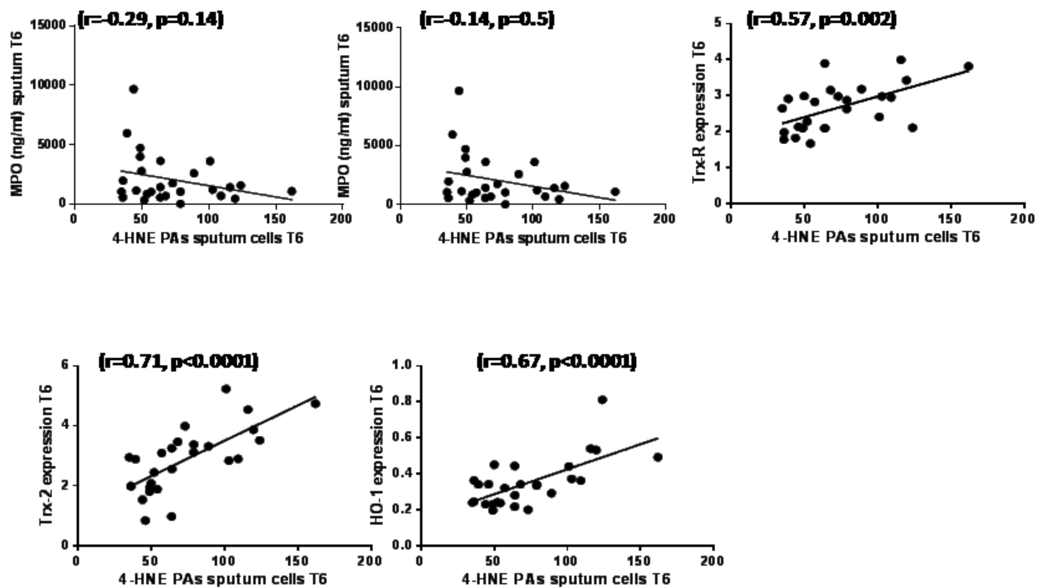
4-HNE PAs	**T0**	**T1**	**T6**	**T24**
All patients	37.10±3.60	59.78±6.38 P=0.003	61.71±4.99 P=0.0002	78.28±8.99 P< 0.0001
Group A	41.95±5.38	78.96±8.97 P= 0.22	80.29±6.11 P=0.05	104.34±14.83 P=0.004
Group B	32.62±4.67	42.08±5,83 P=0.002	39.18±4.22 P=0.0001	54.22±4.76 P=0.007
HO-1	**T0**	**T1**	**T6**	**T24**
All patients	1.38±0.09	1.62±0.08 P=0.06	1.86±0.11 P=0.002	2.16±0.08 P<0.0001
Group A	1.24±0.11	1.42±0.12 P=0.3	1.46±0.10 P=0.15	1.88±0.10 P=0.003
Group B	1.51±0.13	1.81±0.10 P=0.08	2.22±0.13 P=0.0008	2.43±0.09 P < 0.0001

Rel. O.D. are presented as mean±SEM. P values compare to T0. N=27, Group A=14, Group B=13

Correlations between oxidative stress and anti-oxidant response with cells producing reactive oxygen species. The ROS-induced post-translational modifications of proteins in response to the HDM challenge depend on the anti-oxidative response, as shown above, and the amount of ROS generated. Neutrophils and eosinophils are an important source of ROS and therefore we have taken the number and activation status of sputum neutrophils and eosinophils at 24h after the HDM challenge as an approximation of ROS production. Interestingly, we found no correlations for neutrophils and eosinophils and their activation products with carbonylated proteins and 4-HNE PAs in sputum cells and proteins (**Fig. 5.7**). In contrast, carbonylated proteins and 4-HNE PAs correlated strongly with cyto-protective proteins (**Fig. 5.7**), indicating that cyto-protective responses are key determinants in oxidative stress.

A

4-HNE PAs



B

Carbonylated proteins

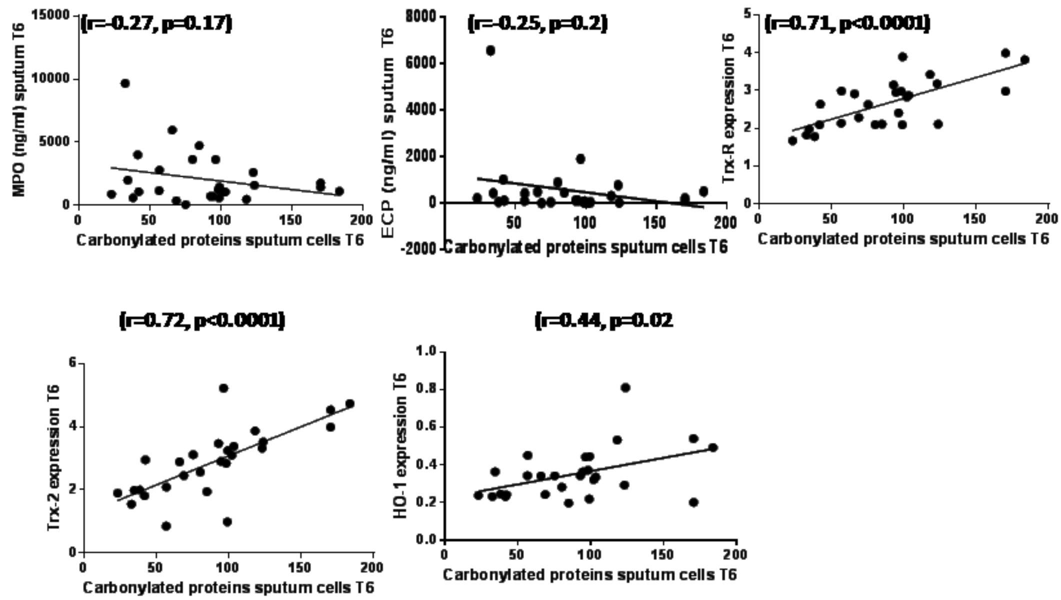


Fig. 5.7 *Oxidative damage on proteins and cyto-protective proteins in sputum cells.* Correlation between MPO, ECP, Trx-R, Trx-2, HO-1 and 4-HNE PAs (**A**), carbonylated proteins (**B**) in sputum cells 6 hours after HDM challenge.

5.4.Discussion

HDM challenges, as expected, enhanced ROS-induced post-translational modifications of proteins. In patients with a low baseline oxidative stress, however, the ROS-induced post-translational modifications resulting from the HDM challenge were delayed and remained relatively low, paralleled by a marked anti-oxidant and cyto-protective response. In patients with a high baseline oxidative stress, ROS-induced post-translational modifications increased markedly and rapidly after the HDM challenge and the anti-oxidant and cyto-protective responses were almost non-existing. The oxidative stress at t=6 and t=24 correlated strongly with the fall in FEV_1. The most striking finding in this study was the strong positive correlation between baseline oxidative stress and the fall in FEV_1. Taken together these findings indicate that the fall in FEV_1 in the late asthmatic response depends on the capacity to counter ROS. Patients with a reduced capacity to counter ROS experienced larger falls in FEV_1. Interestingly, ROS-induced post-translational modifications did not correlate with inflammatory markers, but showed strong correlations with the anti-oxidant and cyto-protective responses. This further confirms that the anti-oxidant and cyto-protective responses are predominantly influencing the ROS-induced post-translational modifications.

To the best of our knowledge this is the first study in which oxidative stress, anti-oxidant and cyto-protective responses have been analyzed in parallel in HDM-sensitized mild asthma patients, before and after allergen challenges. In addition, these parameters were followed over time and studied both locally and systemically. The provocative dose of HDM was titrated to cause a 20% drop of the FEV_1 in the early asthmatic response, which suggests that mast cell responses were similar between patients. The late asthmatic response, which reflects the recruitment and activation of inflammatory and immune cells, varied considerably between patients. Interestingly, for group B apparently higher amounts of allergen were employed (Table 9) and more activation of inflammatory cells (**Table E1**) was found, which indicates that more ROS may have been generated. Nevertheless, the ROS-induced post-translational modifications were less, in accordance with a potent anti-oxidant capacity in these patients.

It is not clear how the reduced anti-oxidant capacity and thus enhanced oxidative stress for group A is linked to marked fall of the FEV_1 during the late asthmatic response. Recent reports suggest that ROS-induced damage leads to an enhanced contractility of airway smooth muscle cells.[456,457] Therefore, we propose that the ROS-induced damage in sputum supernatant and cells, may also reflect that of airway smooth muscle cells and relate to the fall of the FEV_1 during the late asthmatic response.

There are multiple stressors of cells and proteins. Since both 4-HNE-PAs and carbonyl groups were strongly correlated and paralleled by anti-oxidant and cyto-protective responses, it is likely that ROS and no other stress drove the HDM-induced responses. The most marked anti-oxidant and cyto-protective responses were found for Trx-2, Trx-R, HO-1 and Nrf-2. Trx-2 is a 12-kD oxidoreductase containing a dithiol-disulfide active site with anti-oxidant activity, although it also reverses nitrosylation of cysteine residues.[458] Trx-2 is kept functionally active by Trx reductase (Trx-R) and NADPH. Serum Trx-2 is increased in asthmatics and correlates positively with the severity of asthma. HO-1 catalyzes the degradation of heme in biliverdin, carbon monoxide and free iron, by which it exerts anti-oxidant and cyto-protective properties.[459] HO-1 expression in alveolar macrophages and exhaled CO are higher in untreated asthmatics than in healthy controls and well-controlled asthma patients.[460,461] Nrf-2 is sequestered in the cytoplasm by the repressor protein Keap 1 (Kelch-like ECH-associated protein 1), but when exposed to oxidative stress, Nrf-2 goes to the nucleus where it triggers the transcription of anti-oxidative genes. It has been reported that disruption of the Nrf-2 gene leads to severe allergen-driven airway inflammation and hyper-responsiveness in mice.[462] These anti-oxidant responses were found predominantly in sputum proteins, in proteins from sputum cells and, to a lesser extent, in proteins in the circulation, which is in line with allergen-induced airway inflammation as the source of ROS.

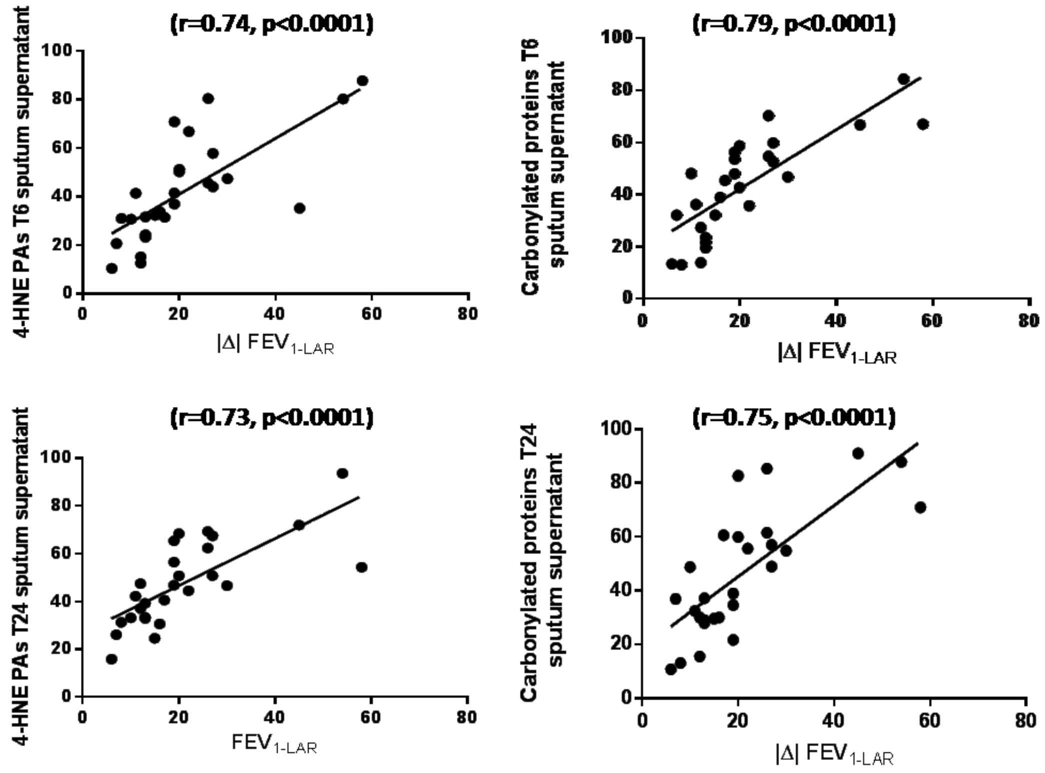
This study also provides novel insight into several other aspects. First, the high expression of most anti-oxidant and cyto-protective proteins in patients with high basal oxidative stress suggest a high anti-oxidative capacity, but from the response to HDM it is clear that this was not the case. Hence, this indicates that the level of expression of anti-oxidant and cyto-protective proteins does not necessarily reflect the anti-oxidative capacity. Secondly, we found that HO-1 and Trx-2 were released in vesicular fractions,

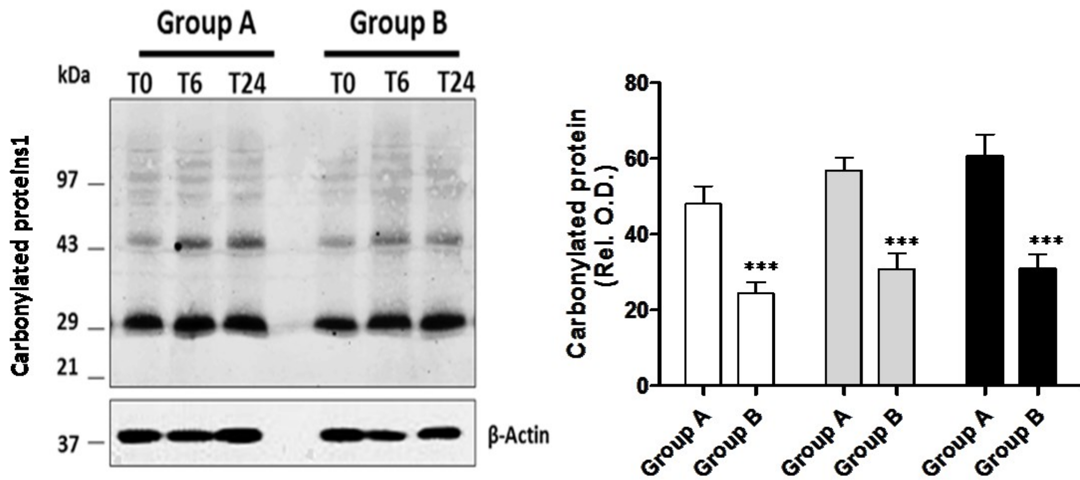
likely to be exosomes, in both sputum and circulation and particularly by patients who responded well to oxidative stress. Lasser et al. showed that exosomes derived from cells grown under oxidative stress can mediate tolerance to oxidative stress in recipient cells.[463] Therefore, these vesicular fractions may reflect a means to counter oxidative stress over a larger area. There are no other reports showing vesicular fractions containing these enzymes. Kim et al.[464], however, showed an association of HO-1 with plasma membrane caveolae in endothelial cells, which could lead to exosome formation. Further characterization of these bodies that contain HO-1 and Trx-2 is warranted.

There are a couple of limitations to this study. The storage conditions prevented us from assessing the actual anti-oxidative capacity of sputum cells ex vivo. Nevertheless, the analyses of sequential samples of both the ROS-induced post-translational modifications and the anti-oxidant and cyto-protective responses provide a clear insight into the responses to the HDM challenge over time. Furthermore, this is an observational study and therefore a causal relationship between ROS-induced post-translational modifications, the anti-oxidant capacity and the drop in FEV_1 to an HDM challenge is not proven. This awaits an intervention study for example by treating allergic patients with N-Acetyl Cysteine or similar anti-oxidant agents.

Asthma exacerbations continue to be a major cause of morbidity, disability and healthcare costs. The current findings indicate that baseline oxidative stress may identify patients at risk of a marked fall in lung function upon exposure to the relevant allergen. To verify this, a prospective study has to be carried out. Furthermore, our study indicates that enhancing the anti-oxidant and cyto-protective capacity may attenuate the allergen-induced late drop in FEV_1. Whether similar considerations apply to other triggers of asthma exacerbations, such as respiratory virus infections and air pollution is not unlikely, but remains to be determined.

5.5. Supplementary material





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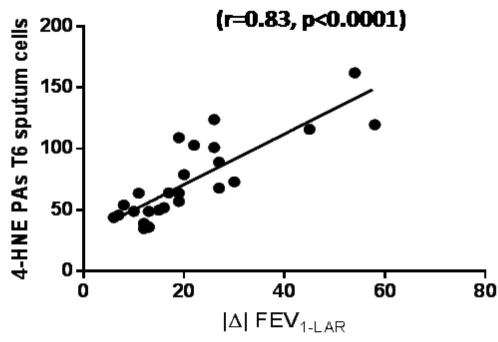

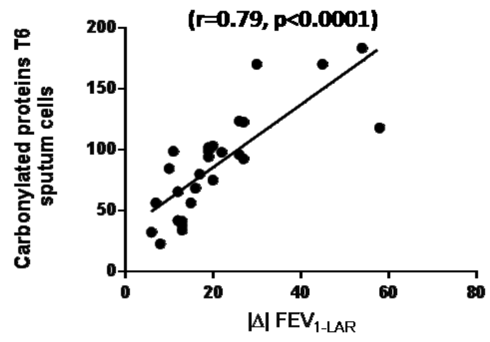

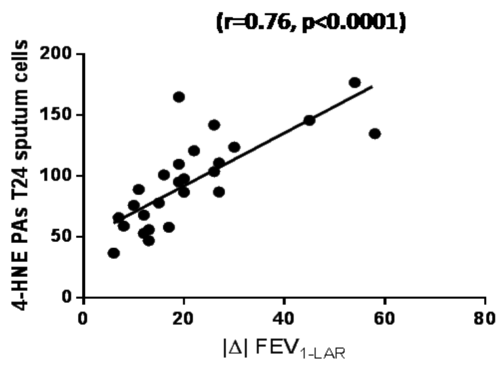

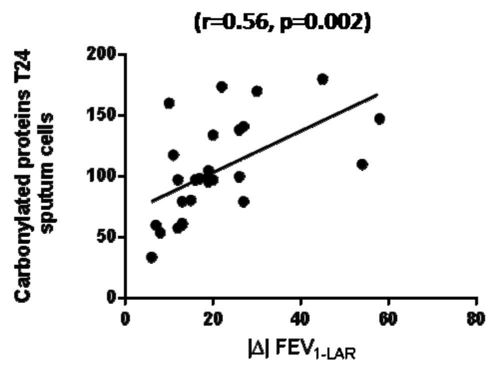

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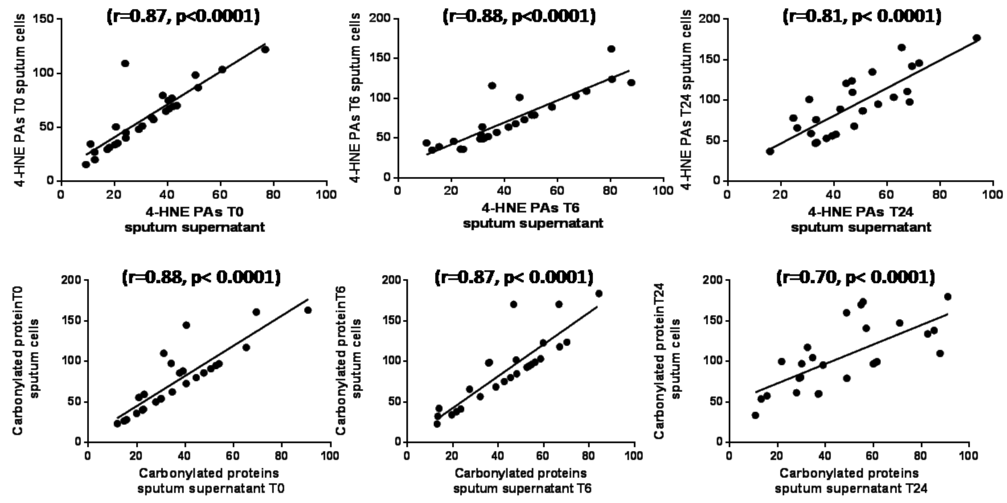

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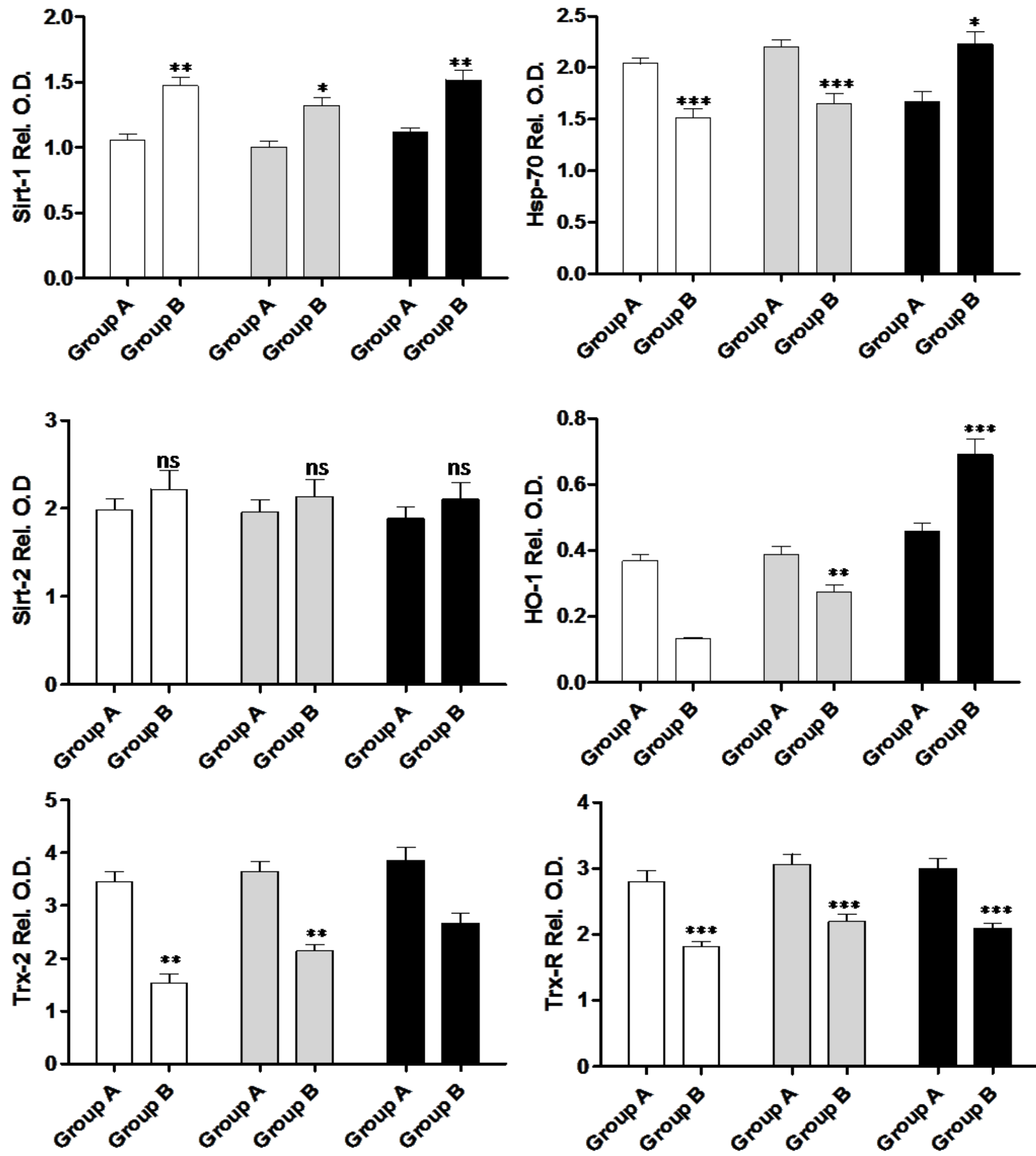

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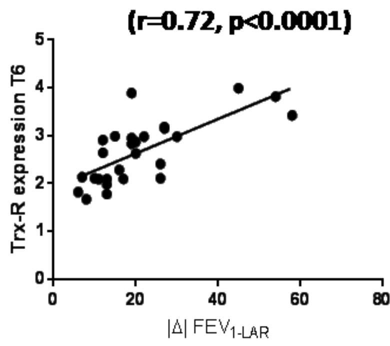
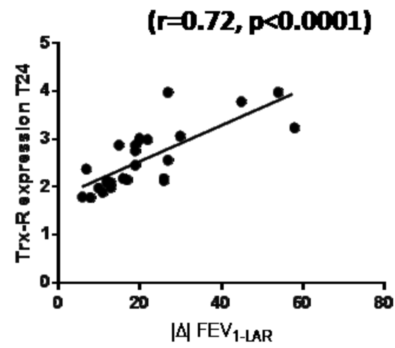
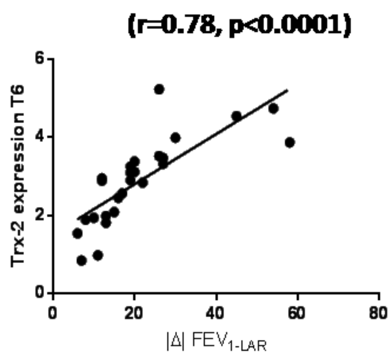
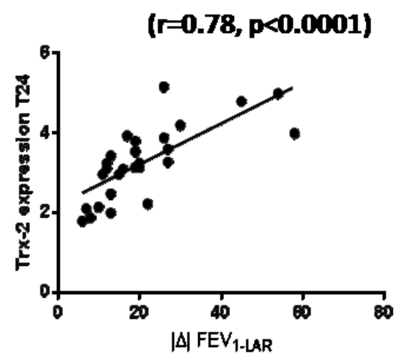
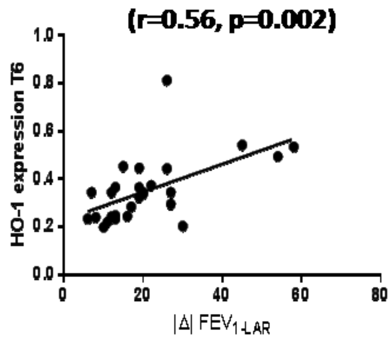
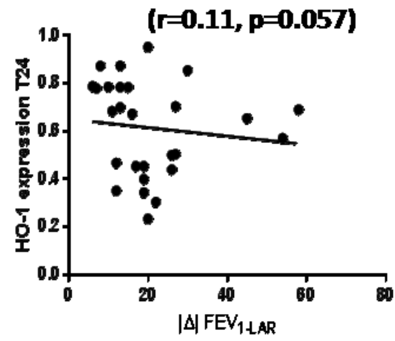












Chapter 6

6. Oxidative stress and allergic sensitization

This chapter is based on the submitted manuscript: *"Inadequate anti-oxidant responses promote allergic sensitization"*

Lara U.M. Gouveia, MSc[1], C. Folisi MSc[1,3], J.H. Akkerdaas, PhD[1], Adrian Logiantara, BSc[1], Marianne A. van de Pol, PhD[1,3], Jaring S. van der Zee MD, PhD[4], Esmeralda J.M. Krop, PhD[5], René Lutter, PhD[1,3,#], Ronald van Ree, PhD[1,2,#], Leonie van Rijt, PhD[1]

[1]Depts. of Experimental Immunology,[2]Otorhinolaryngology, [3]Respiratory Medicine, Academic Medical Center/University of Amsterdam, Amsterdam, the Netherlands, [4]Dept. Resp. Med., OLVG, Amsterdam, The Netherlands, [5]Institute for Risk Assessment Sciences, Utrecht University, The Netherlands. [#] These authors contributed equally to the study.

Correspondence should be addressed to:

E: **lara.utsch@gmail.com**, **caterina.folisi@libero.it**

Abstract

Allergies are aberrant Th2 responses to allergens. The processes initiating allergic Th2 responses remain elusive. Mite allergens have proteolytic activity which can induce oxidative stress *in vivo.* We showed that by reducing mite protease capacity to generate oxidative stress, allergic sensitization and inflammation were also reduced. Susceptibility to mite allergy was highly dependent on host genetic background. Comparing two strains of mice, high baseline oxidative stress and poor anti-oxidant responses were prerequisites for allergic sensitization. We showed that in human subjects, the same condition was a decisive host factor for the development of occupational allergy to rodent urinary proteins. Our results indicate that oxidative stress generated by inadequate anti-oxidant responses, determines allergic sensitization and provides an opportunity for prevention.

6.1. Introduction

Allergies are mediated by IL-4, IL-5, IL-13 and IgE, and arise from aberrant immune responses by T helper type 2 cells to allergens.[465] The processes that underlie these aberrant responses and that lead to allergic sensitization largely have remained elusive. Pattern recognition receptors (PRRs) like Toll-like receptors, expressed by antigen-presenting cells and non-immune cells like epithelial cells, are important regulators of immune response to microbial components such as bacterial lipopolysaccharides (LPS).[466] TLR4 triggering by LPS has been found crucial for the initiation of allergen-specific Th2 responses to HDM in mice.[467,468] However, this is disputed as Th2 responses to inhaled HDM can also be induced in the absence of TLR4 signalling.[469,470] These contradictory findings argue strongly for an additional mechanism independent of the LPS-TLR4 pathway.

LPS is a major contaminant of HDM and is able to induce oxidative stress.[471] HDM however, also contains various allergens which are proteins with distinct biological functions among which proteases (Der p 1, Der p 3, Der p 6).[472] Proteases can induce oxidative stress *in vivo*[473] and therefore we hypothesized that sensitization to HDM can also depend on oxidative stress induced by its proteolytic activity. Oxidative stress condition happens when reactive oxygen species (ROS) from local and/or environmental sources can overwhelm anti-oxidant responses. ROS, during oxidative stress condition, can activate the immune system[474] and in the context of allergen exposure, may facilitate allergic sensitization. This has not been clarified, although several studies are in support for a role of oxidative stress in sensitization to HDM. Murine studies show that, birch pollen-induced oxidative stress mediated IL-4 and IgE production;[475] and ROS generation by papain was responsible for its adjuvant effect in an ovalbumin-induced allergic sensitization. In human experimental studies, allergic sensitization was induced when intranasal exposure to allergens was accompanied by exposure to diesel exhaust particles (an oxidizing agent).[476]

We set off to determine whether sensitization to HDM depends on its capacity to induce oxidative stress. We addressed our hypothesis in a murine model of HDM-induced allergic inflammation. We showed that the proteolytic activity in HDM in itself is

sufficient to promote sensitization to HDM. Interestingly, mice with a marked anti-oxidant response after inhalation of HDM low in endotoxin were resistant to allergic sensitization. In contrast, mice having a small anti-oxidant response and in parallel higher baseline oxidative stress were susceptible to allergic sensitization. To extend our murine data, we analysed serum and PBMC from a cohort of allergic individuals, who were or were not *de novo* sensitized to murine and rat urinary proteins upon occupational exposure. The individuals that became allergic sensitized displayed high baseline oxidative stress and low anti-oxidant protein expression. Contrary, non-sensitized subjects, displayed low baseline oxidative stress and high anti-oxidant protein expression. Our study indicates that an adequate anti-oxidant response is the major denominator in preventing allergic sensitization.

6.2.Methods

Reagents. HDM (HT): Greer; HDM (LT): LoTox (Lot no. 33019), INDOOR biotechnologies; Periodic Acid Schiff's, *N*-acetyl-L-cystein (NAC), Xanthine, Xanthine oxidase and Propidium iodide, Sigma-Aldrich; rGM-CSF, Thermo scientific; MHCII-FITC, CD11c-APC, CD86-PE, CD80-PE, CD40-PE, Rat $IgG2_a$, Ham IgG antibodies and ELISA kit Ready-set-go! IL4, IL5, IL13 and IFNγ, eBioscience Inc; Antibody to FcRγII/III 2.4G2, provided by Louis Boon, Bioceros, Utrecht, The Netherlands; Bicinchoninic acid (BCA) kit, Bio-Rad Laboratories Inc.; BlueSepharose, Amersham; Antibodies to 4-HNE, Nrf-2, HO-1, Santa Cruz; Antibody to β-actin, GeneTex; IgE, IgG_1 and $IgG2_a$, ELISA kit Opteia, BD; Trizol, Invitrogen; First strand cDNA Synthesis Kit, Fermentas; SYBR Green PCR Master Mix, Applied Biosystems.

6.2.1. Murine studies

Mice. Female C3H/HeJ, -HeN from (Harlan, Bicester Oxon, UK) and Balb/c (Harlan, Horst, The Netherlands) mice, were housed under specific pathogen-free conditions at AMC animal facility. All experiments were approved by the AMC animal ethics committee, The Netherlands.

Sensitization. Mice were anaesthetized with isoflurane and 30 µ of HDM extracts (1 µg Der p 1 per mice) or Phosphate Buffered Saline (PBS) were administrated as described in [39]. Briefly, mice were exposed intranasally for 3 cycles of five consecutive days and two days' rest. Four weeks after the last cycle, mice were re -challenged three times and sacrificed two days later.

Bronchoalveolar lavage fluid. Cells from the airway lumen were obtained by three subsequent washes with 1 ml PBS 0.1 mM EDTA, after intratracheal cannulation. Cell differentiation was determined by FACS as described elsewhere.[477]

Lymph node restimulation. Cells were plated in 96-well round bottom plates at $2x10^5$ cells per well and restimulated for 4 days with 100 µg ml^{-1} HDM extract. Cytokines in supernatants were analysed by ELISA.

Immunoglobulins. Serum total or HDM-specific IgE, IgG_1 and $IgG2_a$ were analysed by ELISA. Standard curve of murine immunoglobulin were used as qualitative reference.

Histology. Frozen lung sections (6 µm) were stained with Periodic Acid Schiff's. Inflammation and mucus-producing goblet cells were semi-quantified as described in[478].

Bone-Marrow derived dendritic cells (BMDCs). BMDCs from C3H/HeJ were obtained as described in [42]. On day 9 of culture, cells were incubated with or without NAC 5 mM at 37 ^{o}C in 5% CO_2 prior to incubation with LT HDM extract (1 µg Der p 1/ml). At day 10, CD40, CD80 and CD86 expression were determined by FACS. Propidium iodide was used for cell viability.

Western blots (WB). Proteins from lung homogenates and BMDCs were extracted with Laemmli buffer: 20% wt/vol sodium dodecyl sulfate (SDS), 30% vol/vol glycerol, 30% vol/vol deionized water in 1 M Tris-base pH 6.8. Proteins were next diluted in 4% wt/vol SDS, 10% vol/vol 2-mercaptoethanol, 20% vol/vol glycerol, and 0.004% vol/vol bromophenol blue in 125 mM Tris-HCl pH 6.8 and separated on 13% SDS/PAGE. After transferred to polyvinylidene difluoride membranes and blocked with 5% wt/vol skim milk, blots were incubated with primary antibodies to Nrf-2 or 4-HNE. Subsequently, they were incubated with IRDye 680LT-conjugated secondary antibodies. Blots were visualized using infrared fluorescence detection Odyssey Imager and software (LI-COR Biosciences). Loading was normalized per β-actin.

Real-time PCR. Total lung RNA was extracted with Trizol according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using First strand cDNA Synthesis Kit. PCR was performed in a 10 ml reaction volume including 5 ml of SYBR Green, 200 nM of each FW and RV primers, 2 µl of cDNA and nuclease free water. For each gene, reaction was performed in duplicates. Duplicate standard curves were constructed by serial dilution (1:5) from a concentrated pool of cDNA. All reactions were performed in optical 96-well reaction plates using the ABI Prism 7500 system (Applied Biosystems). mRNA concentrations was calculated based on the standard curve method [479] and normalized to the housekeeping gene HPRT.

Cysteine, Trypsin, and Chymotrypsin activity assay. Heated LT HDM was serially diluted (1:2) in reaction buffer (0.2 M sodium phosphate, 1 mM EDTA, pH 7.0) and mixed with 20% vol/vol 20 mM cysteine substrate OR reaction buffer (50 mM TRIS, 20 mM $CaCl_2$, pH 8.2) and mixed with 50% vol/vol 2 mM trypsin substrate OR reaction buffer (0.1 M TRIS, 0.96 M NaCl, 10 mM $CaCl_2$, pH 8) and mixed with 50% vol/vol chymotrypsin substrate in 96-well NUNC plates. For the standard curves, enzymes were serially diluted (1:2) and mixed with respective substrates. Starting enzymes dilutions: papain, 700 µg ml^{-1}; trypsin, 2 µg ml^{-1}, and chymotrypsin, 200 µg ml^{-1}. Absorbance was measured at 415 nm after the development of colour.

Cap-inhibition assay. ImmunoCAP component (ThermoFisher Scientific) was performed according the manufacturer's instructions. Prior to incubation in ImmunoCAP, human serum was inhibited for 1 hour with heated LT HDM. After inhibition, residual IgE binding was measured using CAPs coated with mite extract, Der p 1 or Der p 2. Results were expressed in % inhibition.

6.2.2. Human studies

Study design. Study population consisted of 37 temporary laboratory animal workers from a previous study[17]. Briefly, participants were followed for 2 years and occupational allergic sensitization to rodent urinary proteins was monitored. They were seen at the start of their application as animal workers (T0), after 4 months (T4), 1 year (T12) and 2 years (T24) for blood collection and clinical evaluations. Herein, we compared 21 workers who did not develop sensitization to rodents with 16 animal workers who did. 4-HNE modified proteins and HO-1 were accessed in serum and Nrf-2 was accessed in Peripheral Blood Mononuclear Cells (PBMC) by WB.

Western blots. Serum samples were treated with BlueSepharose 6B CL to reduce the albumin content. Proteins were treated and blotted as described in the murine section. Antibodies to Nrf-2, HO-1 or 4-HNE were used. Total protein was determined by BCA. Samples were normalized per 50 µg of protein.

PBMC. Cells were cultured overnight with Xanthine (0.5 mM): Xanthine oxidase (50 mU). Nrf-2 protein expression was analysed by WB in total cell lysate.

Statistical analysis. Statistical significance was tested with Mann-Whitney U test. Experiments were repeated at least twice unless stated otherwise in **Fig.** legends. For correlation analysis, Pearson correlation coefficient was calculated. Significance was established at $P < 0.05$.

6.3. Results

Low endotoxin HDM (LT) extract induced a full-blown allergic Th2-type response in C3H but not in Balb/c mice. To determine whether inhaled HDM was able to induce allergic sensitization and inflammation in a TLR4-independent manner, TLR4-mutant C3H/HeJ mice and as controls Balb/c mice were intranasally exposed to two HDM extracts that differed in endotoxin level: a crushed whole body HDM extract referred to as high endotoxin (HT; 153 EU mg^{-1}) and a mite spent medium extract referred to as low endotoxin (LT; < 3 EU mg^{-1}). HT HDM inhalation by Balb/c mice resulted in a strong Th2-type inflammatory response, as reflected by eosinophil recruitment in the airway lumen, local Th2 cytokine production, peri-bronchial inflammatory infiltrates, goblet cell hyperplasia, total IgE and specific IgG_1. In addition, IFNγ and HDM-specific IgG_{2a} were also increased (**Fig. 6.1 *a-e***). LT HDM failed to induce any significant immune response in Balb/c mice, except for a small increase in mucus production (**Fig. 6.1 *e***). Despite the non-functional TLR4 in C3H/HeJ mice, HT HDM exposure induced all hallmarks of a robust Th2 inflammation, similar to that in Balb/c mice, although the magnitude of the influx of eosinophils was less pronounced. Also here, IFNγ and IgG_{2a} were increased. However, in contrast to Balb/c mice, C3H/HeJ mice also developed a full-blown Th2-type immune response upon intranasal exposure to LT HDM (**Fig. 6.2 *a-e***). This was not accompanied by an increase in IFNγ and IgG_{2a}.

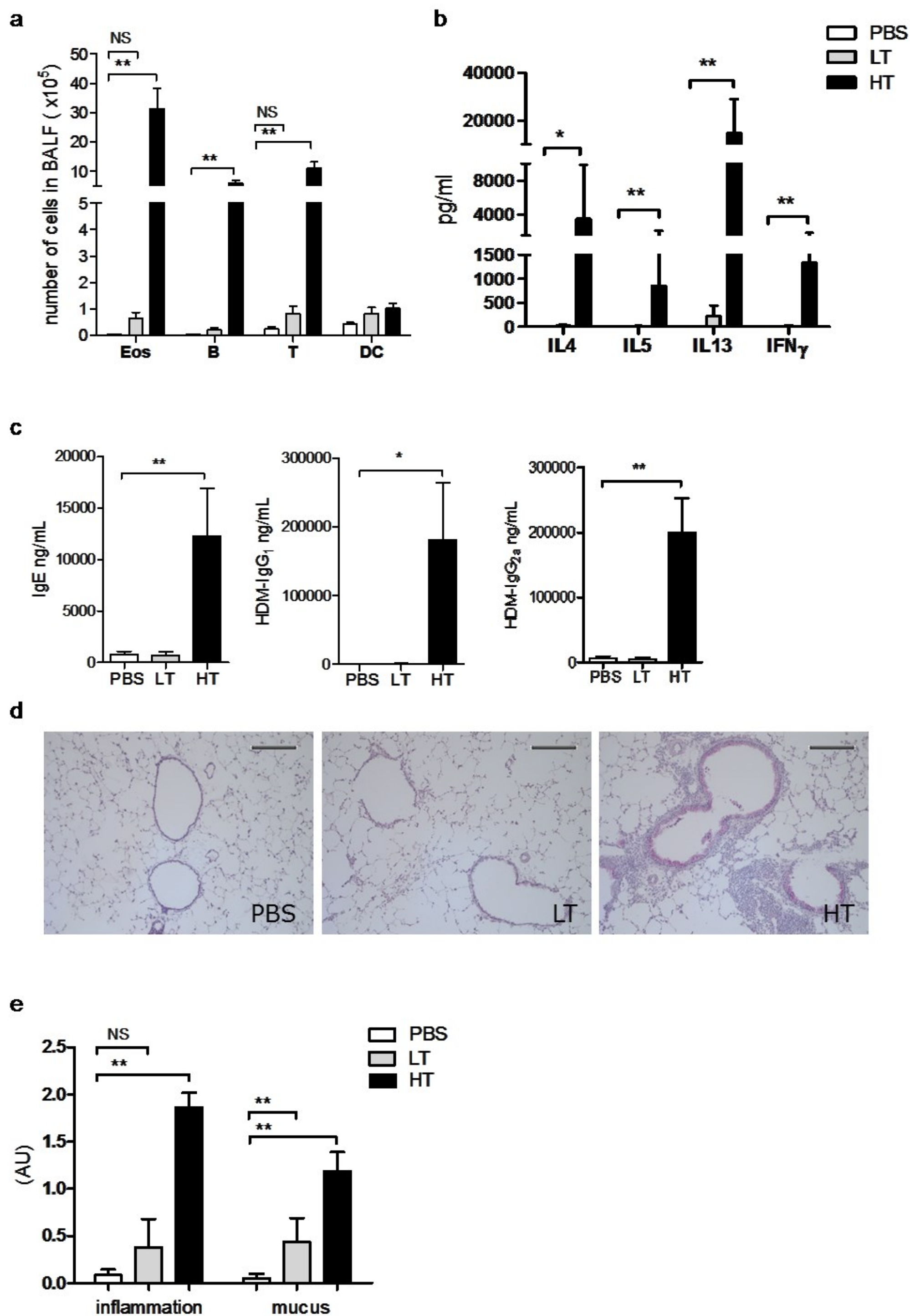


Fig. 6.1 *LT HDM extract does not trigger immune response in Balb/c*. Balb/c mice (five mice per group) were intranasally exposed to LT HDM or HT HDM or PBS as a control as described in methods. (**a**) Absolut number of inflammatory cells in bronchoalveolar lavage (BALF). (**b**) Production of Th2 cytokines IL4, IL5, IL13 and IFNγ in supernatants of *ex-vivo* HDM restimulated lung draining lymph node cells. (**c**) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production in lung slides stained with PAS and (**e**) quantification of peri-bronchial infiltrates and mucus production. Scale bars in d represents 200 µm. Data are presented as means ± SD, *$P<0.05$.

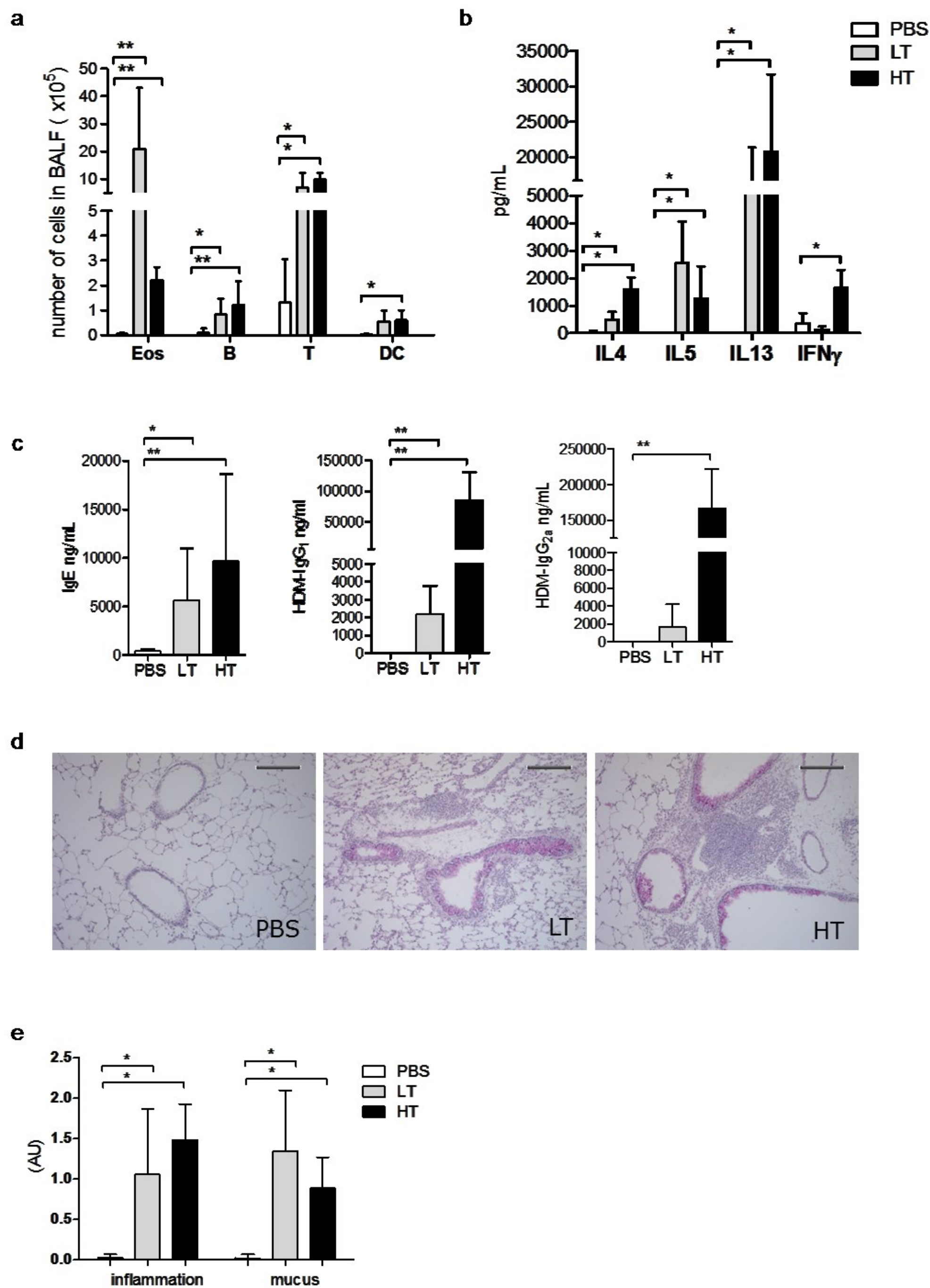


Fig. 6.2 *LT and HT HDM extracts triggers inflammation in TLR4-mutant mice*. C3H/HeJ mice (five mice per group) were intranasally exposed to LT HDM or HT HDM or PBS as a control as described in methods. (**a**) Absolut number of inflammatory cells in bronchoalveolar lavage (BALF). (**b**) Production of Th2 cytokines IL4, IL5, IL13 and IFNγ in supernatants of ex-vivo HDM restimulated lung draining lymph node cells. (**c**) Total IgE, HDM-IgG1 and HDM-IgG2a in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production in lung slides stained with PAS and (**e**) quantification of peri-bronchial infiltrates and mucus production. Scale bars in d represents 200 µm. Data are presented as means ± SD, *P<0.05.

To clarify whether the differential responsiveness to LT-HDM between C3H/HeJ and Balb/c mice was due the different genetic background rather than to the mutation, we studied C3H/HeN mice with the same background as C3H/HeJ mice, but with a functional tlr4 gene. Similar to the mutant C3H/HeJ mice, C3H/HeN developed a full-blown Th2 mediated airway inflammation in response to LT-HDM without the induction of IFNγ and IgG_{2a} (**Fig. 6.3 *a-e***). HT HDM extract induced an inflammatory response in HeN mice, which was accompanied by the induction of IFNγ and IgG_{2a} similarly to C3H/HeJ mice. Together this indicates that sensitization to HDM depends on the genetic background of the mice and is not strictly dependent on TLR4.

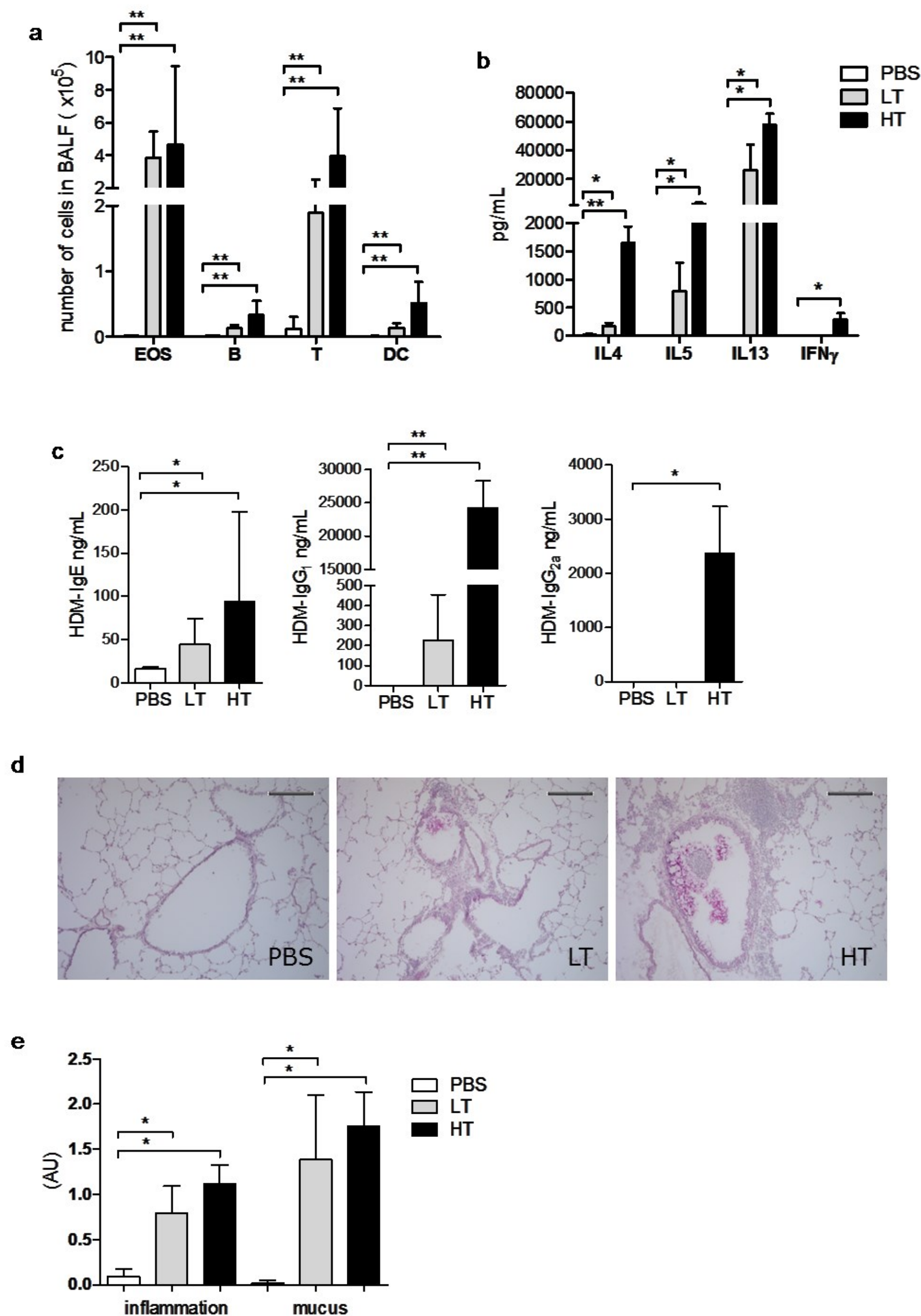
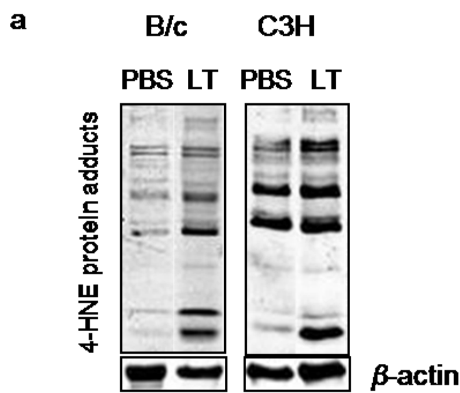


Fig. 6.3 *LT and HT HDM extracts triggers inflammation in non-mutant mice.* C3H/HeN mice were intranasally exposed to LT HDM or HT HDM or PBS as a control as described in methods. (**a**) Absolut number of inflammatory cells in bronchoalveolar lavage (BALF). (**b**) Production of Th2 cytokines IL4, IL5, IL13 and IFNγ in supernatants of *ex-vivo* HDM restimulated lung draining lymph node cells. (**c**) HDM-IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production in lung slides stained with PAS and (**e**) quantification of peri-bronchial infiltrates and mucus production. Scale bars in **d** represents 200 µm. Experiment performed once with five mice per group. Data are presented as means ± SD, *P<0.05.

HDM-induced oxidative stress was followed by rapid up-regulation of anti-oxidant proteins in Balb/c but not in C3H/HeJ mice. To evaluate the possible contribution of oxidative stress in sensitization to HDM and the related Th2 inflammation, we compared the level of oxidative stress at baseline and after a single exposure to LT HDM in Balb/c and C3H/HeJ mice, resistant and susceptible to LT HDM sensitization, respectively. At baseline, the concentration 4-HNE modified proteins, a marker for oxidative stress [13], was markedly lower in lungs of Balb/c mice than in those of C3H/HeJ mice. LT HDM induced an increase of 4-HNE-modified proteins in both Balb/c and C3H/HeJ mice. This increase was significant in Balb/c but not in C3H/HeJ mice, which may relate to its high baseline level (**Fig. 6.4 *a*, *b***). Levels of mRNA for anti-oxidant enzymes GPx-1 and HO-1 were increased in response to LT HDM in lungs of Balb/c mice, but not in those of C3H/HeJ mice (**Fig. 6.4 *c***). The levels of 4-HNE modified proteins inversely correlated with the levels of HO-1 mRNA (**Fig. 6.4 *d***).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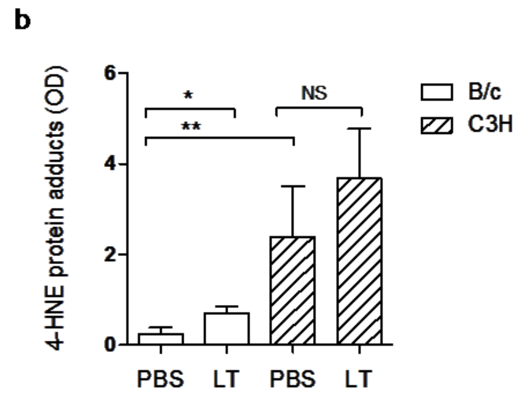
B/c
C3H
PBS
LT
PBS
LT
4-HNE protein adducts
β-actin


b
4-HNE protein adducts (OD)
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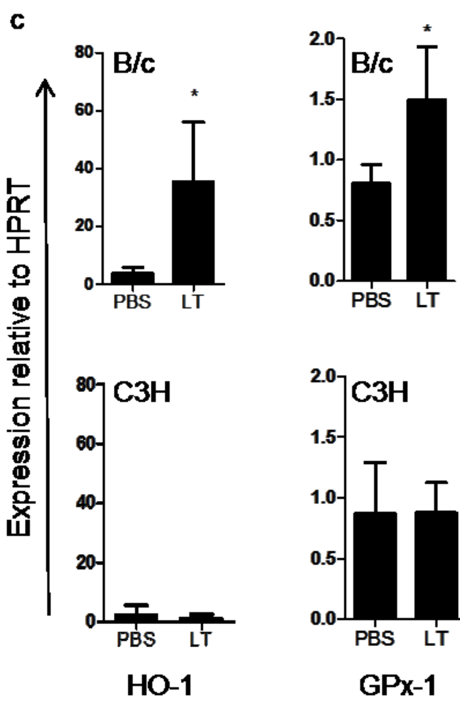
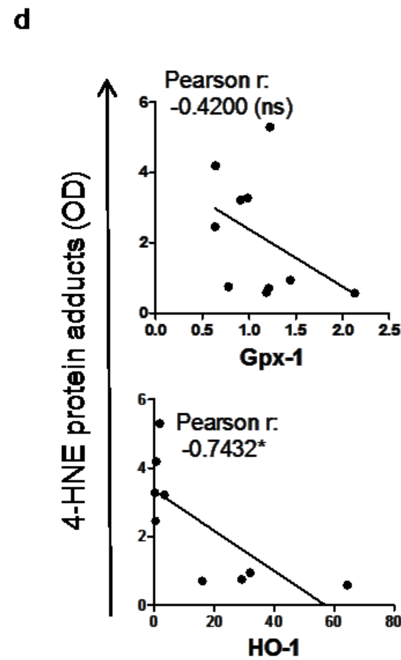
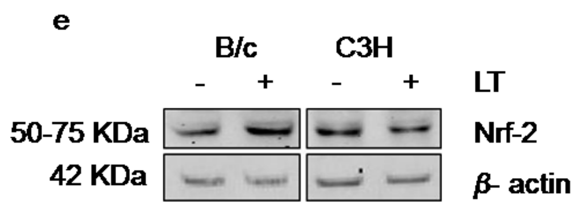
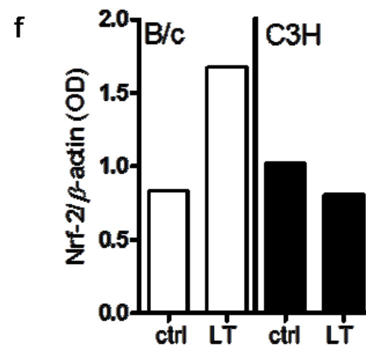
**
NS
B/c
C3H
PBS
LT
PBS
LT


c
Expression relative to HPRT
B/c
C3H
PBS
LT
HO-1
GPx-1


d
4-HNE protein adducts (OD)
Pearson r:
-0.4200 (ns)
Gpx-1
Pearson r:
-0.7432*
HO-1


e
B/c
C3H
LT
50-75 KDa
42 KDa
Nrf-2
β- actin


f
Nrf-2/β-actin (OD)
B/c
C3H
ctrl
LT
ctrl
LT

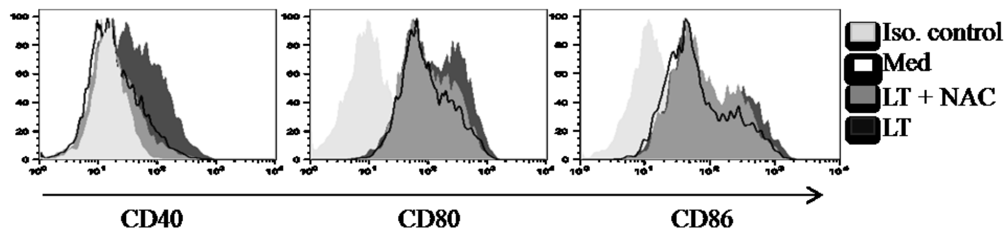


Fig. 6.4 *LT-induced oxidative stress is followed by up-regulation of anti-oxidant proteins in resistant mice.* Balb/c and C3H/HeJ (five mice per group) were intranasally exposed to LT HDM or PBS as a control and lungs were removed 24h later for analysis. (**a**) Immunoblotting of 4-HNE modified proteins in lung homogenate and (**b**) quantification of protein expression. (**c**) mRNA expression of anti-oxidant enzymes Hemoxigenase-1 (HO-1) and Glutathione Peroxidase-1 (GPx-1) determined by qPCR in lung tissue. **(d)** Correlation of 4-HNE modified proteins with GPx-1 and HO-1 mRNA expression. (**e**) Immunoblotting of Nrf-2 in total bone-marrow derived dendritic cell lysates (representative of two independent experiments) and (**f**) quantification of protein expression (ctrl= medium control). (**g**) Expression of co-stimulatory molecules CD40, CD80 and CD86 on bone-marrow derived dendritic cells from C3H/HeJ mice in the absence or presence of ROS scavenger *N*-acetyl-L-cysteine (NAC). Data are presented as means ± SD, $^*P<0.05$

In this study we compared the capacity to up-regulate nuclear factor erythroid 2-related factor 2 (Nrf-2), a master regulator of the anti-oxidant response among which that of HO-1,[480] in response to LT HDM in bone marrow-derived dendritic cells (BMDCs) from C3H/HeJ and Balb/c mice. As early as 2 h after LT HDM exposure, Nrf-2 expression was increased in Balb/c but not in C3H/HeJ mice (**Fig. 6.5** ***e*****,** ***f***). Taken together this shows that LT HDM induces oxidative stress and that C3H/HeJ mice are unable to respond adequately to LT HDM-induced oxidative stress whereas Balb/c mice can.

As DC activation and subsequent migration to lymph nodes is an important step in the initiation of an adaptive response [481] and subsequent induction of specific-allergen Type 2 cell differentiation,**[482]** we examined whether LT HDM-induced activation of DC from C3H/HeJ mice was dependent on oxidative stress. In LT HDM-exposed bone marrow-derived DCs (BMDCs), CD40, CD80 and CD86 were up-regulated compared to unexposed cells. Treatment of DCs with anti-oxidant N-acetyl-L-cysteine (NAC), a potent ROS scavenger, inhibited up-regulation of these molecules (**Fig. 6.5** ***g***).

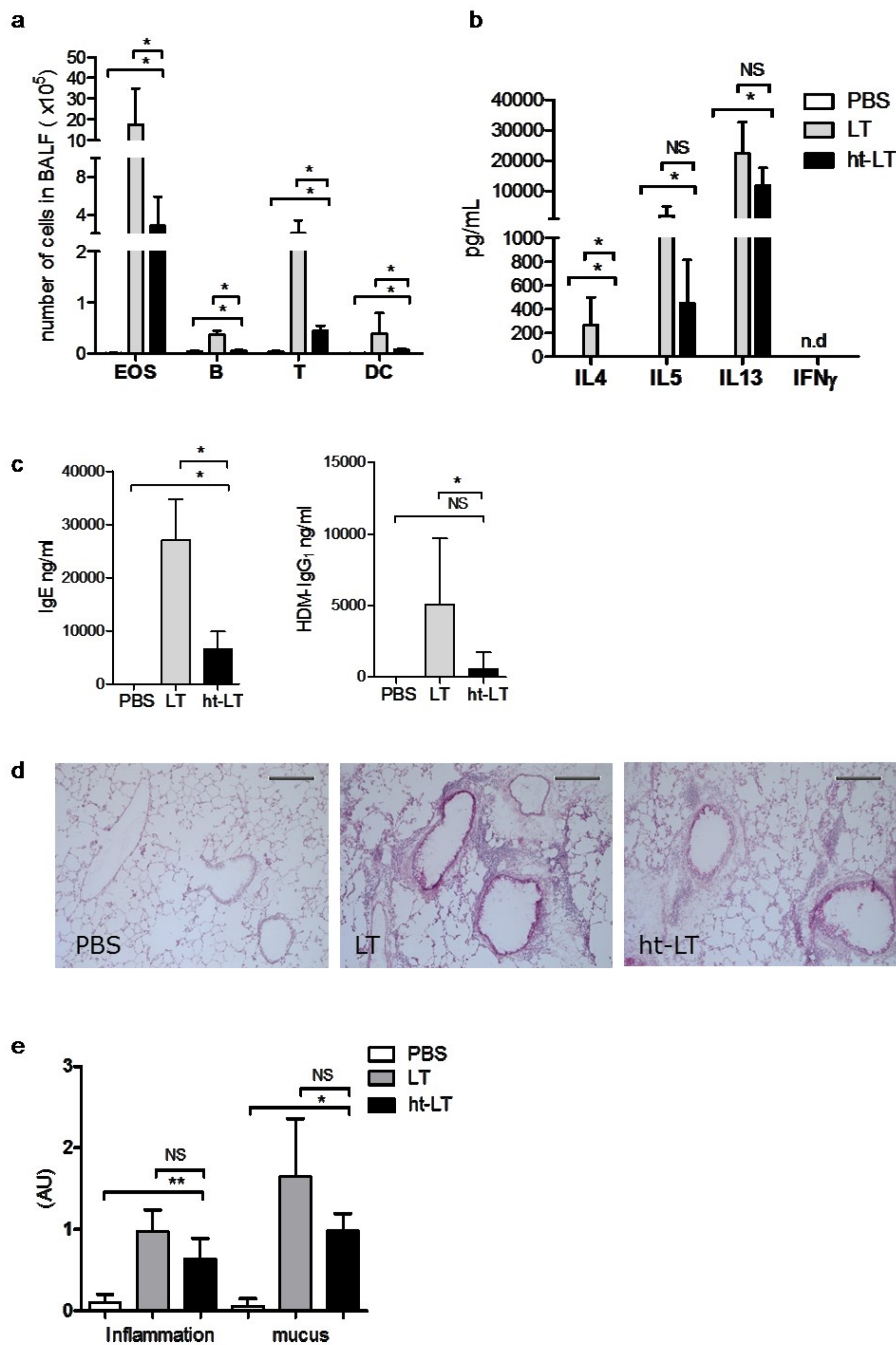


Fig. 6.5 *Inhibition of protease activity decreases HDM capacity to induce allergic inflammation.* C3H/HeJ mice were intranasally exposed to LT HDM or Heat-Treated LT HDM (ht-LT) or PBS as a control as described in methods. (**a**) Absolut number of inflammatory cells in bronchoalveolar lavage (BALF). (**b**) Production of Th2 cytokines IL4, IL5, IL13 and IFNγ in supernatants of *ex-vivo* HDM restimulated lung draining lymph node cells. (**c**) Total IgE and HDM-IgG_1 in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production in lung slides stained with PAS and (**e**) quantification of peri-bronchial infiltrates and mucus production. Scale bars in **d** represents 200 µm. Experiment performed once with five mice per group. Data are presented as means ± SD, *P<0.05.

Heat-inactivation of HDM proteases decreased oxidative stress and Th2 inflammatory response in C3H/HeJ mice. Next, we verified whether the induction of oxidative stress was dependent on the protease activity in LT HDM. The protease activity was attenuated by moderate heating (30 minutes at 65° C; ht LT HDM). In ht-LT HDM there was significant inhibition of its trypsin and chymo-trypsin protease activity while its cysteine protease activity was not affected (**Supplementary Fig. F1**). Importantly, moderate heating did not compromise the allergenic potency as expressed by unaltered IgE recognition of heated mite allergens (**Supplementary Fig. F2** and **3**). Ht-LT HDM induced markedly less 4-HNE-modified proteins in C3H/HeJ BMDCs, compared to control LT HDM (**Supplementary Fig. F4**). Next, we examined the effect of the reduced oxidative capacity of ht-LT HDM *in-vivo*. Allergic characteristics were significantly decreased in mice exposed to ht LT HDM in comparison to LT HDM. Heat-treatment significantly reduced the recruitment of eosinophils, dendritic cells, and T and B lymphocytes to the airways, IL4, total IgE and HDM-specific IgG_1 production (**Fig. 6.5 *a-e***). Although peri-bronchial inflammatory infiltrates, goblet cell hyperplasia, IL5 and IL13 showed a small decrease that however, did not reach statistical significance. Thus even the protease activity in an HDM extract is sufficient to promote sensitization via oxidative stress.

Increased oxidative stress markers and decreased anti-oxidant proteins expression are associated with allergic sensitization in humans. Sensitization to HDM depends on its ability to induced oxidative stress in recipients. Whether oxidative stress is induced depends on the amount of reactive oxygen species generated and the anti-oxidant capacity. LT HDM caused sensitization in C3H mice with a reduced anti-oxidant capacity, but failed to do so in Balb/c mice with a high anti-oxidant capacity. Previously we have determined sensitization to murine and rat urinary proteins in a cohort of atopic individuals up to 2 years after de novo occupational exposure.[483] To extend the murine data and clarify whether an insufficient capacity to cope with oxidative stress also correlates to allergic sensitization in humans, we analysed PBMC and serum from this cohort of animal workers. Sixteen out of 37 atopic individuals became sensitized to murine proteins during this period as determined by allergic symptoms, the development of allergen-specific IgE and allergen-induced IL4 production. We accessed 4-HNE-modified proteins in serum collected before exposure (T0) and after four

months (T4), one year (T12) and two years (T24). Those who became sensitized to laboratory animals showed significantly higher levels of 4-HNE-modified proteins in serum at baseline (T0), similar to what we found in mice, and all subsequent time points (**Fig. 6.6** ***a*****,** ***b***). Expression of HO-1 in serum was significantly lower in these individuals, indicative of a reduced anti-oxidant capacity (**Fig 6.6** ***c*****,** ***d***). At T0, T4 and T12, a significant inverse correlation was observed between 4-HNE modified proteins and HO-1 expression (**Fig. 6.6** ***e***), although surprisingly this correlation attenuated over time and was lost after two years. In order to analyse the capacity to respond to acute oxidative stress, we evaluated PBMCs collected at T0 from individuals who became sensitized (n=5) and from individuals who did not (n=4), for the up-regulation of Nrf-2 upon exposure to xanthine/xanthine oxidase, which induces the ROS superoxide. Nrf-2 expression in PBMCs from individuals who developed sensitization (S) was lower than in those who did not (NS). Although this did not reach significance due to limited number of available PBMC samples (**Fig. 6.6** ***f*****,** ***g***) the relevance of the differences in Nrf-2 expression between both sensitized and non-sensitized subjects was reflected by the strong negative correlation with the level of oxidative stress (4-HNE modified proteins) (**Fig. 6.6** ***h***). Overall, these human data are in support of the concept established with our murine studies in which an inadequate anti-oxidant response predisposes to allergic sensitization.

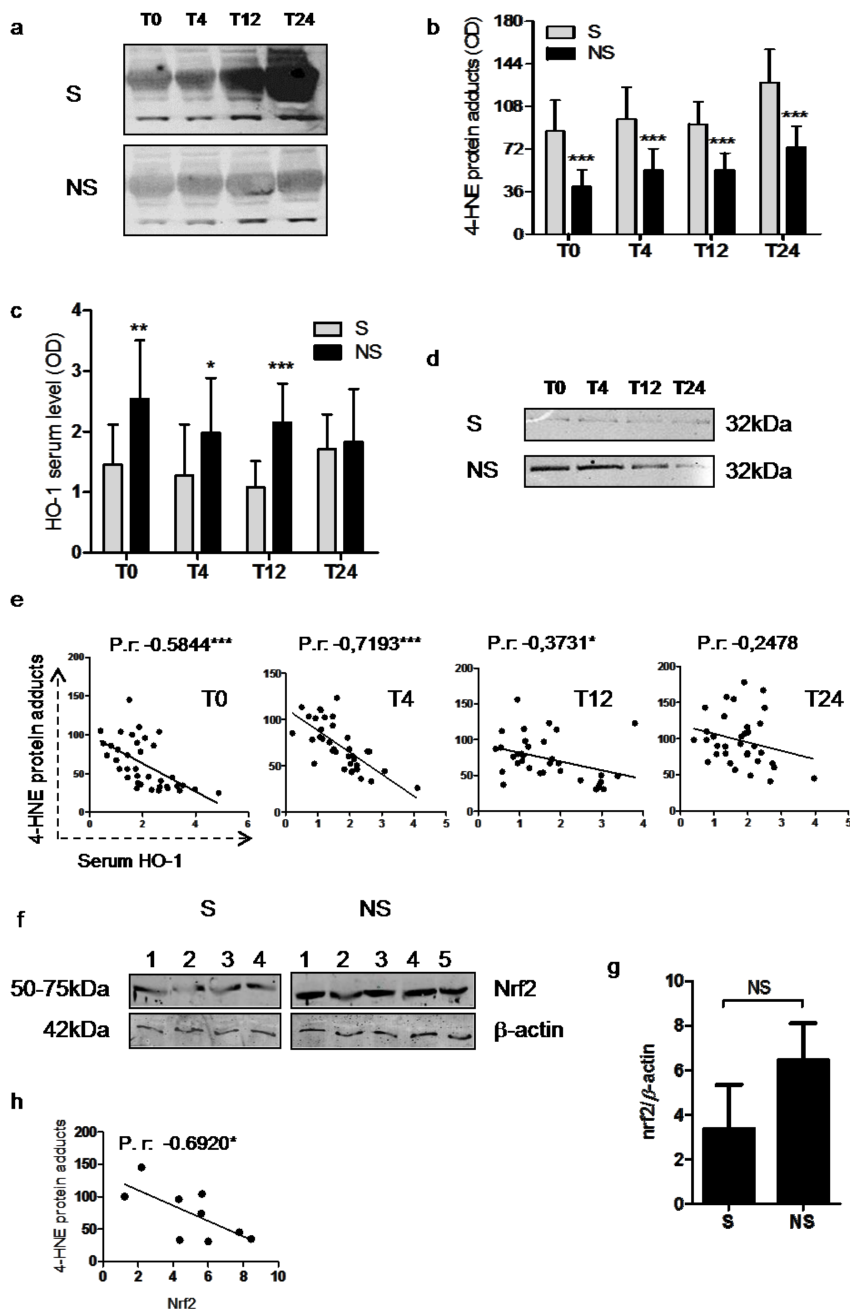


Fig. 6.6 *Increased oxidative stress and decreased anti-oxidant proteins expression in de novo sensitized subjects.* Human serum samples from 16 *de novo* sensitized (S) and 21 non-*de novo* sensitized (NS) individuals were analyzed for 4-HNE modified proteins and Hemoxigenase-1 expression in different time points (T0=before occupational exposure and T4, T12 and T24=4, 12 and 24 months respectively after occupational exposure). (**a**) Representative immunoblotting of 4-HNE modified proteins from one individual per group and (**b**) quantification of protein expression. (**c**) Immunoblotting of Hemoxigenase-1 and (**d**) quantification of protein expression. (**e**) Correlation of 4-HNE modified proteins and Hemoxigenase-1 expression in different time points (Pearson r: T0=-0.5844***, T4= -0.7193***, T12= -0.3731* and T24=-0.2478). (**f**) Immunoblotting of Nrf-2 in PBMCs from S (n=4) and NS (n=5) individuals and (**g**) quantification of protein expression. (**h**) Correlation of 4-HNE modified proteins and Nrf-2 expression (Pearson r: -0.6920*). Data are presented as means ± SD, *P<0.05.

6.4. Discussion

What makes individuals more susceptible than others to allergic sensitization is considered a multifactorial process that involves genetic predisposition, gene-environment interactions, lifestyle, environmental exposures and types of allergens. In the present study we show that inadequate anti-oxidant responses are strongly associated with sensitization to allergens.

Recently, it was elegantly demonstrated by Hammad *et al.* that the process of sensitization to HDM is TLR4-dependent and requires endotoxin. In concordance, intranasal exposure to HDM extract low in endotoxin (LT HDM) was indeed not sufficient to sensitize Balb/c mice, whereas exposure to HDM extract high in endotoxin (HT HDM) did. Nevertheless, the fact that C3H/HeJ and –HeN mice developed allergic inflammation to LT HDM extract indicated that an alternative TLR4 independent pathway related to host genetic background was playing a role. We showed that LT HDM caused HDM sensitization via the induction of oxidative stress in the lungs of both resistant Balb/c and susceptible C3H/HeJ mice. However, adequate anti-oxidant responses were induced only in Balb/c mice, limiting oxidative stress and preventing allergic sensitization. As a reflection of its inadequate anti-oxidant responses, in C3H/HeJ mice the baseline level of oxidative stress was already higher than in Balb/c mice. Oxidative stress is known to lead to the induction of type 2 cytokines (IL4, IL13) in $CD4^+$ T cells and not that of type 1 cytokines (IFNγ).[484] In line herewith, LT HDM induced a polarized type 2 response in both HeJ and HeN mice, but when LPS is present, such as in the HT HDM extract, this results in mixed Th1/Th2 responses. Taken together this indicates that oxidative stress promotes susceptibility to allergic sensitization.

To address whether protease activity of HDM allergens was involved in the induction of oxidative stress and allergic inflammation we modified HDM allergens rather than systemic use of anti-oxidants or the addition of protease inhibitors to HDM. This has the advantage of specific intervention whereas systemic use of anti-oxidants and protease inhibitors can affect processes other than that induced by allergen. We chose controlled mild heating of HDM, reducing proteolytic activity and leaving the IgE-binding potency

intact. This led to a clear inhibition of oxidative stress in dendritic cells in-vitro and significant attenuation of allergen sensitization and Th2 inflammation in-vivo. This suggests that the proteolytic activity in HDM extract is in itself sufficient to induce HDM sensitization such as in C3H/HeJ mice. When however the anti-oxidant capacity is sufficient to counter that induced by the HDM proteases this will not lead to sensitization such as in Balb/c mice. Contamination of the HDM extract with LPS may be sufficient to out-compete the anti-oxidant capacity in Balb/c mice resulting in HDM sensitization.

Occupational sensitization provides an unique opportunity to study de-novo sensitization in man. To study whether a reduced anti-oxidant capacity is associated with allergic sensitization to murine urinary proteins in human subjects, we further analysed a prospective occupational cohort study. Murine urinary proteins are potent allergens to humans.[485,486] and the mechanisms by which these proteins exert its immunogenicity is not completely known. We found that, anti-oxidant capacity strongly correlated with oxidative stress and allergic sensitization to urinary proteins in humans. This together with the findings from the murine studies indicate that exposure to allergenic proteins combined with host inadequate anti-oxidant response, dramatically increases the likelihood for the development of allergic sensitization. The inverse correlation between oxidative stress and anti-oxidant responses indicate that the anti-oxidant response is the major denominator in preventing allergic sensitization.

There is increasing evidence indicating that a deficient anti-oxidant system may contribute to allergy development. Nrf-2 and HO-1 deficiency predisposes mice to more severe allergic inflammatory responses.[487,488,489] In humans, polymorphisms in genes coding for enzymes that play a role in scavenging ROS have been associated with an increased risk for the development of atopic disorders.[490,491] Anti-oxidant proteins not only provide protection against oxidant injury but are also involved in immune modulation. For example, HO-1 suppresses T cell function and proliferation in-vitro and in-vivo[492] and its expression in DCs is involved in the induction of $CD4^{+}CD25^{+}$ T regulatory cells.[493] Antioxidant mechanisms are crucial in the regulation of cellular redox homeostasis. Deficiency of key antioxidant components (such as Nrf-2) perturbs

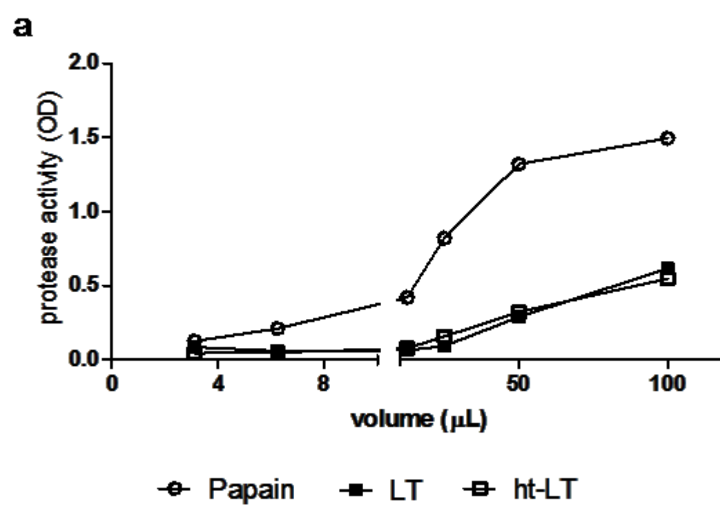
intracellular redox status, increasing the basal levels of intracellular ROS[494] affecting cell phenotype and function.[495,496]

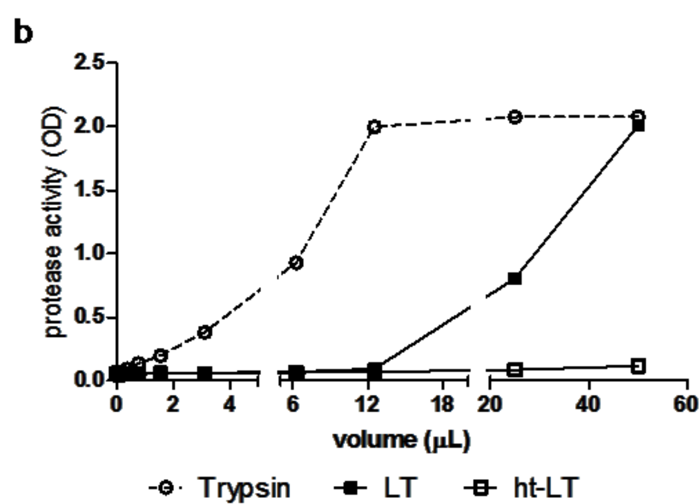
The exact mechanism by which ROS, in the context of oxidative stress, can initiate adaptive immune responses to an allergen is not known. ROS at relatively low concentration serve as essential second messenger mediating cellular responses to many physiological stimuli for example, by regulating the redox status of transcription factors.[497] However, excessive ROS production can contribute to an enhanced immune response.[498] ROS are known stimulate Th2 like responses and to induce maturation of DCs, which is an important step in the initiation of adaptive immunity including allergy. ROS can also facilitate adaptive responses by enhancing the antigen-presentation capacity of DCs,[499] and decreasing the triggering thresholds of CD28 activation on T cells enhancing IL-2 and IL-2R expression.[500,501] ROS can affect DCs directly but also indirectly. Under oxidative stress condition, damaged, dead or activated structural cells, can release Danger-Associated Molecular Patterns (DAMPs) and cytokines, able to promote DC maturation.[502,503,504]

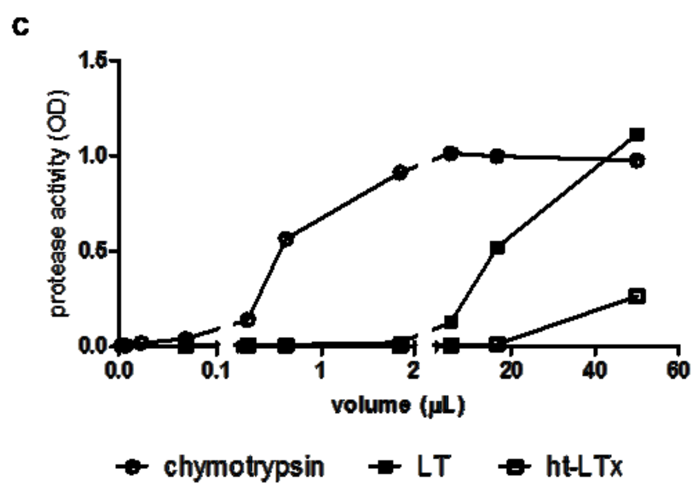
In conclusion, although TLR4 activation by LPS has been held responsible for HDM induced allergic inflammation, our study suggests an alternative (complementary) mechanism for the initiation of Th2 allergic responses mediated by oxidative stress. The inability to cope with oxidative stress determines allergic sensitization and provides an opportunity for prevention.

Supplementary Material

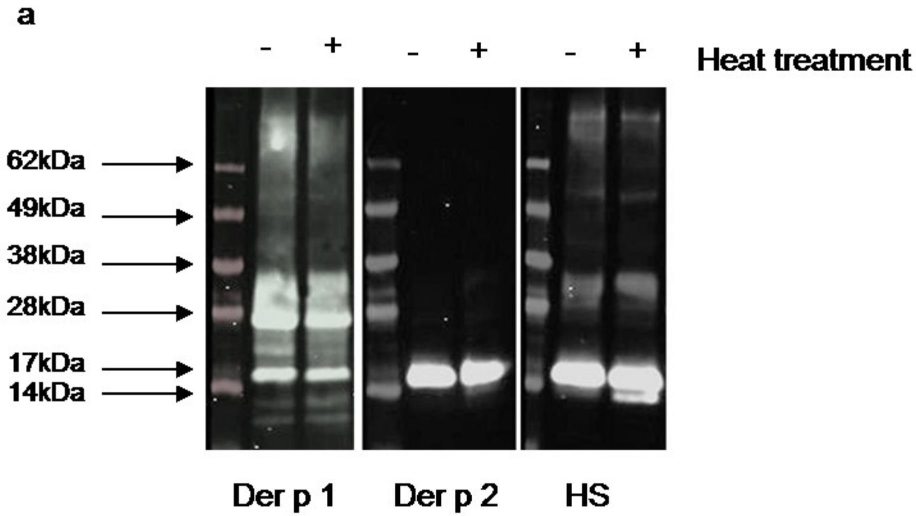
Supplementary Figure F1

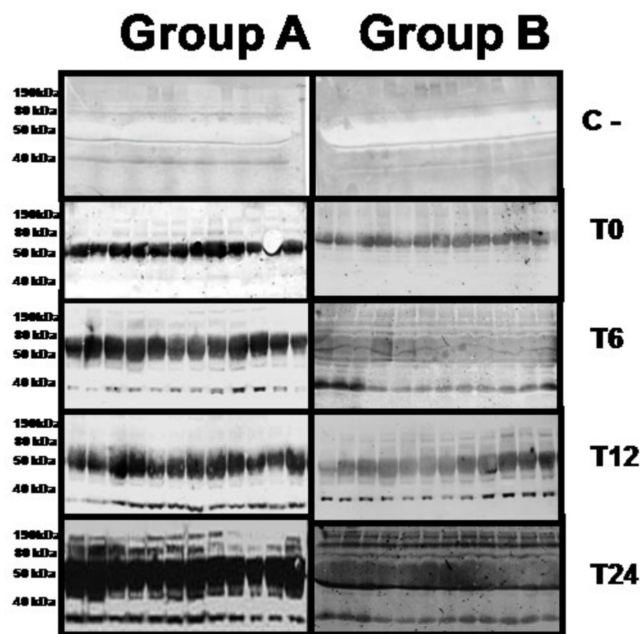






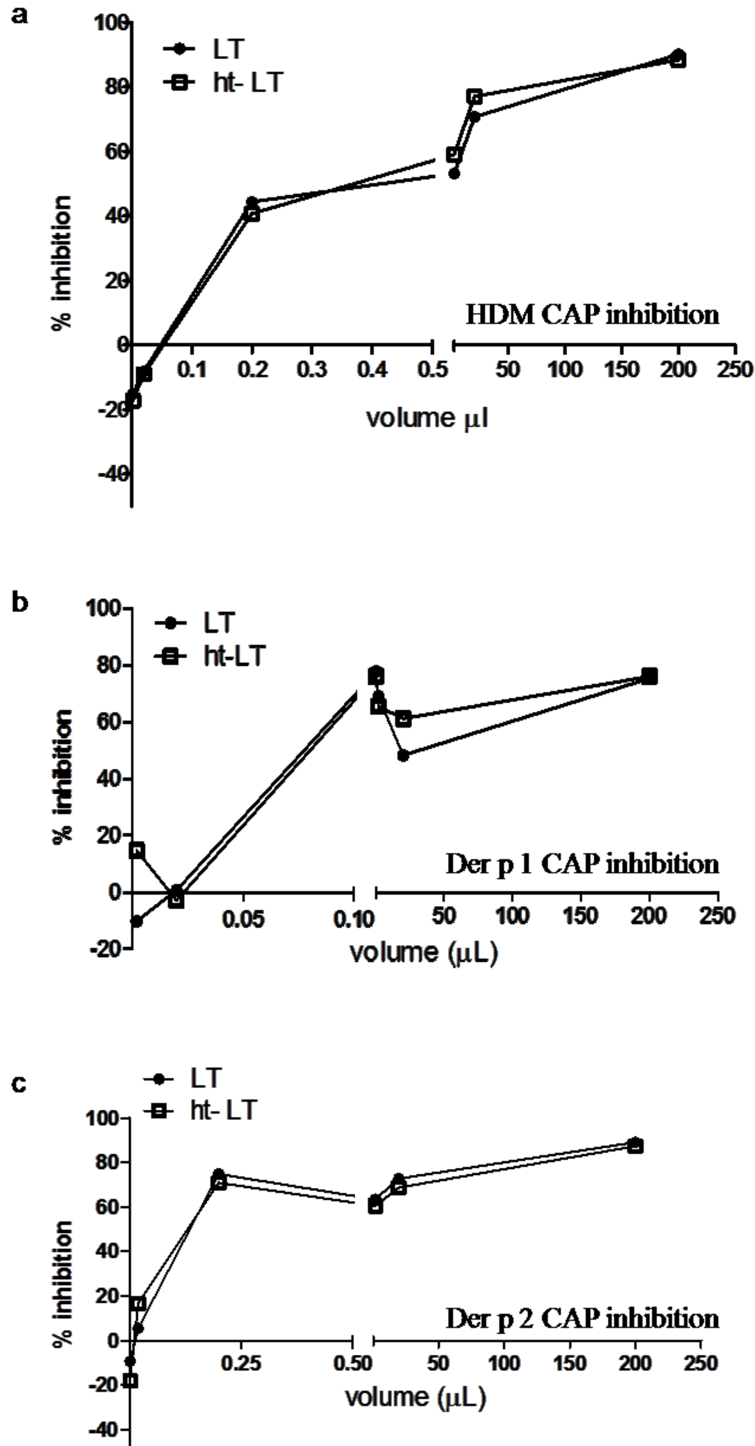
Supplementary Figure F2



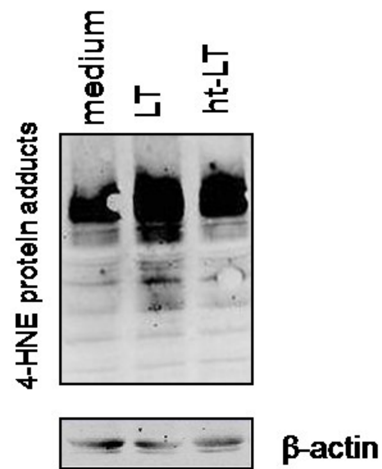


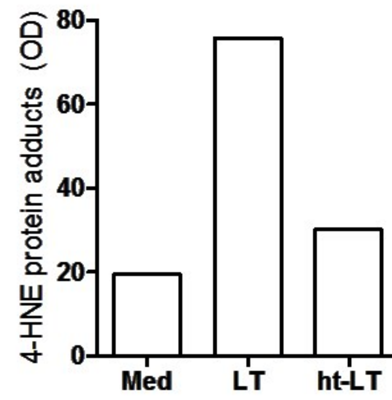
Western blots for 4HNE-PAs in plasma samples from Group A subjects (n = 14) and Group B (n = 14). Legends: C- = Only secondary antibody. Group A= allergics who did become allergic to rodents (rats/mice). Group B= allergics who did not become allergic to rodents. T0= before occupational exposure to rodents. T6= 6 months after occupational exposure to rodents. T12= 12 months after occupational exposure to rodents.T24= 24 months after occupational exposure to rodents .

Supplementary Figure F3



Supplementary Figure F4





Chapter 7

7. Oxidative stress and corticosteroids withdrawal-induced exacerbation

This chapter is based on the study: *"Systemic increased oxidative stress and reduced anti-oxidant capacity in corticosteroids withdrawal-induced asthma exacerbations."*

Caterina Folisi[1,2,3], Marianne A. van de Pol[2,3], Annemiek Dijkhuis[3], Giuseppe Di Maria[1], Peter J. Sterk[2] and René Lutter[2,3]

Author Affiliations

Dipartimento di Biomedicina Clinica e Molecolare Sezione Malattie Respiratorie, Università di Catania Ospedale, Garibaldi-Nesima 95122, Catania, Italia[1]. Depts. of Respiratory Medicine[2], and Experimental Immunology[3], Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

Corresponding author

Caterina Folisi, Dipartimento di Biomedicina Clinica e Molecolare, Sezione Malattie Respiratorie, Università di Catania, Ospedale Garibaldi-Nesima, via Palermo 636, 95122 Catania, Italia. Telephone number: 0039 0957598742, Fax number: 0039 095472988. Email: C.Folisi@soton.ac.uk

Contributorship

CF performed the analyses, analysed and interpreted the data and wrote the manuscript, SMB and MvdP performed the clinical study and revised the manuscript, GDM and PJS were involved in the design of the study and revised the manuscript, RL designed the study, interpreted the data and wrote the manuscript.

Abstract

Oxiditively modified proteins levels are raised in asthma as a result of an increase of oxidative stress. The relationship between oxidized proteins formation and airway inflammation has not been extensively investigated in asthmatic patients treated with corticosteroids.

The aim of this study was to determine the role of the oxidative damage on proteins and anti-oxidant response in corticosteroids withdrawal-induced asthma exacerbation.

Twenty-three patients with asthma were admitted at the study. Lung function test, collection of blood and sputum were performed when patients were in stable condition under corticosteroids treatment (stable), after corticosteroids withdrawal when they showed clear symptoms of exacerbation (exacerbation) and when they did recovery from the exacerbation (recovery). 4-HNE PAs, carbonylated proteins were assessed as bio-marker of oxidative stress where as Sirt-1, Trx-2, and HO-1 levels were measured as bio-markers of anti-oxidant response in serum by western blot analysis after albumin removal.

We found higher levels of 4-HNE PAs, carbonylated proteins, HO-1 and Trx-2 during a corticosteroids withdrawal-induced exacerbation. Whereas the level of Sirt-1 was lower.

Oxidative damage and anti-oxidant capacity are two important features of corticosteroids withdrawal-induced asthma exacerbation.

7.1.Introduction

Asthma is the most common chronic disease in westernized countries. Patients with asthma have an underlying chronic inflammation of the airways characterized by activated mast cells, eosinophils, and T-helper 2 lymphocytes. This chronic inflammation underlies the typical symptoms of asthma, which include intermittent wheezing, coughing, shortness of breath, and chest tightness.

Corticosteroids are the most effective treatment for asthma, and inhaled corticosteroids have become first-line treatment for children and adults with persistent symptoms. Corticosteroids suppress the chronic airway inflammation in patients with asthma. Ad described earlier inflammation in asthma is characterized by the increased expression of multiple inflammatory genes, including those encoding for cytokines, chemokines, adhesion molecules, and inflammatory enzymes and receptors. Increased expression of inflammatory genes is regulated by proinflammatory transcription factors, such as nuclear factor-B and activator protein-1. These bind to and activate coactivator molecules, which then acetylate core histones and switch on gene transcription. Corticosteroids suppress the multiple inflammatory genes that are activated in asthmatic airways by reversing histone acetylation. This mechanism acts by binding of the activated glucocorticoid receptors to coactivators and recruitment of histone deacetylases to the activated transcription complex. Understanding how corticosteroids work in asthma may help in designing corticosteroids with less systemic effects, as well as novel anti-inflammatory approaches. The decrease of suspension of the dose can cause exacerbation of asthma symptoms which can occurs with still unknown mechanisms.

Earlier studies in adults have indicated that increased oxidative stress may occur in the circulation and airways of asthmatic subjects.[505,506] So far studies to evaluate the oxidative status during corticosteroids treatment in animal or humans have used BALF[507,508,509,510] In certain patient groups, such as asthmatic children, it would be best to develop protocols to monitor the oxidative stress before and during corticosteroids treatment using peripheral blood samples rather than collecting BALF or tissues, or to analyze exhaled breath condensate.[511,512,513]

Here, we analized the relation between cortisteroids treatment and change in oxidant status by measuring 4HNE PAs, carbonylated proteins, HO-1, Trx-2 and Sirt-1 in serum from asthmatic who had been undergone to corticosteroids withdrawal.

7.2. Material and Methods

*Study population and set up***.** We included 23 adult asthamatics with mild persistent asthma, according to American Thoracic Society criteria.[514] Subjects were recruited via advertisement in the Academic Medical Center (AMC) in Amsterdam and gave written informed consent. The study was approved by the AMC Medical Ethics Committee. The study started with a baseline visit: on day one, blood samples were collected and baseline lung function was determined by spirometry (Stable); then corticosteroids were withdrawal and blood was collected when patients showed exacerbation symptoms (Exacerbation) and when they did recovery from the exacerbation (Recovery).

Lung function. Spirometry, and bronchial allergen challenge were performed according to standardized procedures.[515] Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were measured with a Vmax 22 spirometer (SensorMedics) and with a portable spirometer (Micromedical diarycard, Sensor Medics).

Processing and analysis of blood. Total and differential leukocyte counts were determined in EDTA-blood. Serum samples were stored at -80°C until analysis.

Carbonyls protein detection OxyBlot Procedure. In serum samples the total amount of proteins was determined using the bicinchoninic acid (BCA) kit (Bio-Rad Laboratories Inc., Hercules, California, USA). After BCA, carbonyl groups of oxidized proteins were detected after derivatization with 2,4-dinitrophenylhydrazine (DNPH) to a stable dinitrophenyl (DNP) hydrazone product using OxyBlot Protein Oxidation Detection Kit (Merk Millepore). In brief, two aliquots (15-20 µg/µL of protein sample) of each specimen to be analyzed were prepared. Proteins were denatured by adding 5 µL of 12% Sodium Dodecyl Sulphate (SDS). One aliquot was subjected to the derivatization reaction by adding 10 µl of 1x 2,4-dinitrophenylhydrazine (DNPH) followed by 15 min of incubation at room temperature, after which 7.5 µL of Neutralization Solution provided in the kit was added. The negative control was treated in parallel but with

derivatization-control solution instead of DNPH. Equal volumes of both samples were loaded onto a SDS-PAGE gel (10%) without prior heating of the samples. After electrophoresis and blotting to polyvinylidene difluoride (PVDF) membranes for 90 minutes at 125 V in transfer buffer (12 mM Tris, 96 mM Glycine, 20% Methanol), blots were blocked by placing the membrane into 5% non-fat dry milk for 1 hour with gentle shaking. Subsequently, 15 ml of rabbit anti-DNP primary antibody 1:150 diluted in 0.05% Tween 20 Phosphate Buffer (TPBS) with 0.5% non-fat dry milk was added and left overnight at 4ºC while shaking on an orbital shaker. The membrane was washed with multiple changes of TPBS for a total of 30 min before adding 15 ml of goat anti-rabbit IgG HorseRadish Peroxidase (HRP) conjugated secondary antibody (1:300 dilution) in 0.5% non-fat dry milk TPBS and left for 1hr at room temperature on an orbital shaker. Next, the membrane was washed using multiple changes of TPBS for a total of 30 min before adding the chemiluminescent reagent (luminol and enhancer) according to manufacturer's specifications. Blots were developed by using a chemiluminescence detection system under the same conditions. Densitometry was performed on scanned gels by using the ImageJ software. Derivatized bands were quantified by measuring the optical density of the bands in comparison to the signal form the negative control.

Serum immunochemical detection of 4-hydroxy-2-nonenal Protein Adducts (4-HNE PAs). After BCA, for the estimation of HNE-PAs, 50 µg of total proteins were diluted Laemmli sample (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue in 0.125 M Tris-HCl) till a final volume of 35µL, and boiled for 5 minutes at 95°C before to be separated on 13% SDS PAGE gels. Then, proteins were transferred onto PVDF membranes. After this step, membranes were washed and reversible red ponceau staining was used as protein loading control. Following, membranes were blocked for 60 min at room temperature in 5% non-fat dry milk in Phosphate Buffer Saline (PBS). Next, blots were washed and probed against the HNE moiety of proteins by using goat anti 4-HNE (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:500 diluted in 0.05% TPBS with 0.5% not-fat dry milk overnight at 4°C. Membranes were washed three times in TPBS and incubated for 60 min at room temperature with IRDye 680LT conjugates secondary antibodies (1:15.000 diluted in TPBS with 0.5% non-fat milk). Blots incubated with only secondary antibody were

used as negative control. After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA).

Serum immunochemical detection of Heme oxygenase-1 (HO-1), Situin-1(Sirt-1) and Thioredoxin-2 Trx-2). Western blot analysis. Serum samples were treated with BlueSepharose 6B CL in order to reduce the albumin content which could interfere with the proteins detection. In brief, to 0.3 mL of serum were added 0.3 mg of dry BlueSepharose, and left incubated for 30 minutes at 4°C under soft shaking. After 10 minutes of centrifugation at 3.200 rpm the supernatant was collected and the amount of total proteins was determined using BCA kit. Next, 50 µg/lane of proteins were separated by 13% SDS-PAGE gel electrophoresis, and transferred to PVDF membranes for the immuno-detection. All samples were normalized per protein (50µg). Membranes were blocked for 60 min at room temperature in PBS with and 5% non-fat dry milk. After this step, membranes were washed and reversible red ponceau staining was used as protein loading control. Membranes were then washed and probed with 1:200 diluted polyclonal goat anti HO-1 antibody, rabbit anti Sirt-1 and Trx-2 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) in TPBS with 0.5% non-fat dry milk overnight at 4°C. As positive control we used Hela cells whole lysates for HO-1, 293T cells whole lysates for Trx-2 and K562 cells for Sirt-1. Parallel blots incubated with only secondary antibody where used as negative control. Membranes were washed three times in TPBS and incubated for 60 min at room temperature with IRDye 680LT and IRDye 800CW conjugates secondary antibodies 1:15.000 diluted in TPBS with 0.5% non-fat milk. After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA).

Exosomes isolation and western blot analysis. Serum was centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was transferred into a ultracentrifuge tube. Volumes were adjusted by adding PBS. Then samples were centrifuged at 17 000 x g for 15 minutes at 4°C to further remove cell debris and the supernatant was filtered through a 0.2 µm filter to remove particles larger than 200 nm. The filtered supernatant was ultracentrifuged at 200 000 x g for 120 minutes at 4°C to obtain a low-density

membrane pellet. The low-density membrane pellets were solubilized in 5µl Laemmli sample buffer to a final concentration of 1.5% SDS, 6% glycerol, and 10 mM Tris·HCl (pH 6.8) and the proteins separated by SDS/PAGE separated with 10% polyacrylamide and transferred to PVDF membranes. The blot was probed against HO-1 (goat anti HO-1), Trx-2 (rabbit anti Trx-2) and, Sirt-1 (rabbit anti Sirt-1) 1:200 diluted in TPBS with 0.5 not fat dry milk. Membranes were washed three times in TPBS and then incubated for 60 min at room temperature with IRDye conjugates secondary antibodies (1:15.000 in TPBS with 0.5% non-fat milk). After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA). Hela cells whole lysate was used as positive control for HO-1, 293T cells whole lysates for Trx-2 and K562 cells for Sirt.1.

Statistical analysis. Results are presented as mean±SEM of at least two replicate experiments. Statistical analysis was performed by utilizing GraphPad prisma 5. Analysis of significance was calculated by unpaired Student's *t*-test was used to assess between- and within-study group differences. A p value <0.05 was considered.

7.3. Discussion of results

Oxidative damage on proteins in serum before, during and after corticosteroids withdrawal-induced asthma exacerbation. Generation of adducts with lipids and carbonyls formation are considered reliable markers ox oxidative damage on protein. In our study we collected serum samples before, during and after corticosteroids withdrawal in asthmatics. Serum samples were analyzed for the detection of 4-HNE APs and carbonylated protein. Firstly, we found a significant increase in 4-HNE PAs detention of 2.2 times during corticosteroids withdrawal induced asthma exacerbation ($P<0.0001$) if compared to stable conditions(**Fig 7.1**). Whereas 4-HNE PAs detention does not seem to change significantly during the recovery phase if compare to stable condition (**Fig. 7.1 A, Table 15**). Notably, we observed similar findings by analyzing the carbonylated proteins levels. In fact, we found a significant increase of 2 times in carbonylated proteins during exacerbation ($P<0.0001$) and no significant change during the recovery ($P=0.11$) (**Fig. 7.1 B, Table 15**). Furthermore, we found a strong positive correlation between 4-HNE proteins adducts and carbonylated proteins in serum in each condition of the patients (**Fig. 7.1 C**). Being 4-HNE PAs and carbonylated protein two reliable markers of oxidative stress, our results might indicate that an increase in oxidative damage on proteins is associated with a corticosteroids withdrawal asthma induced exacerbation.

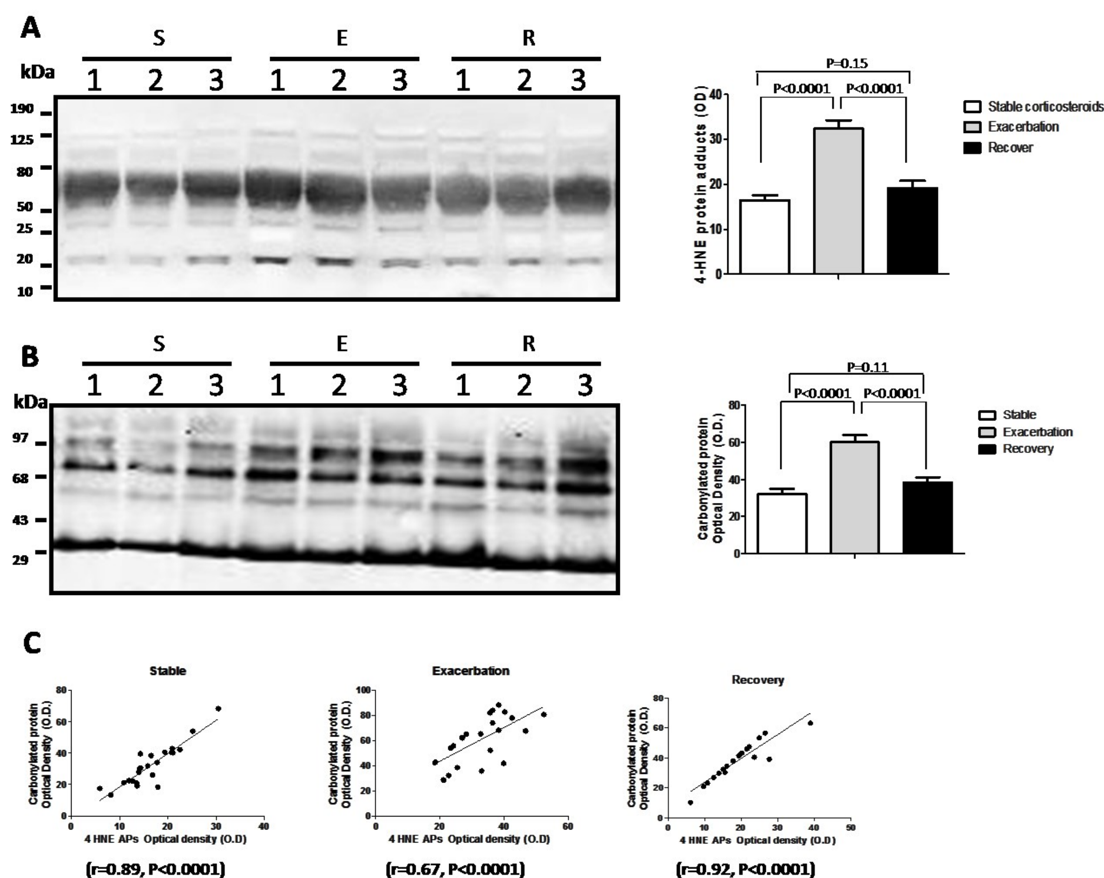


Fig. 7.1 *Oxidative damage on proteins in serum before, during and after a corticosteroids withdrawal induced asthma exacerbation.* **A)** Immunochemical detection of 4HNE-Protein Adducts (4HNE-PAs). The picture illustrates a western blot analysis of 4-HNE PAs in serum samples from three patients. The signals of 4HNE-PAs level were determined by densitometry of the scanned images. All samples were normalized per proteins content (50μg). Blots were developed under the same conditions. Shown is a representative of two replicate experiments. On right side quantitative data for all individuals (n=23). **B)** Immunochemical detection of carbonylated proteins. Panel B illustrates a representative oxyblot for the detention of carbonylated protein in serum from three asthmatics. Each western blot included all the conditions, so that in all experiments, samples were developed under same conditions. Densitometry was performed on scanned gels by using the ImageJ software. Derivatized bands were quantified by measuring the optical density of the bands in comparison to the signal form the negative control. The graph (on the right) shows the Rel. O.D. of carbonylated protein in all the individuals (n=23). **C)** Correlation between 4HNE PAs and carbonylated proteins. The graphs show a positive correlation between level of 4-HNE PAs and carbonylated protein in all the conditions. Legends: S= Stable, E=Exacerbation, R= Recovery.

TABLE 15. SERUM OXIDATIVE DAMAGE ON PROTEINS AND CYTOPROTECTIVE RESPONSE BEFORE, DURING AND AFTER CORTICOSTEROIDS WITHDRAWAL INDUCED EXACERBATION

	Stable	Exacerbation	Recovery
4-HNE PAs	16.43±1.15	32.37±1.90	19.22±1.56
Carbonylated proteins	32.21±2.71	60.11±3.8	38.57±2.71
HO-1	0.83±0.15	3.20±0.48	2.41±0.60
Trx-2	0.22±0.04	0.42±0.08	1.24±0.15
Sirt-1	2.82±0.23	0.94±0.12	2.38±0.19

Cyto-protective proteins in serum before, during and after corticosteroids withdrawal-induced asthma exacerbation. Serum HO-1 was significantly increased during exacerbation. In particular, 3.8 fold higher if compared to stable condition P<0.0001. No significant difference between the HO-1 levels measured during the recovery and stable condition (**Fig. 7.2 A, Table 15**). HO-1 is a recognized marker of oxidative stress its increase in serum demonstrates an increase of oxidative stress during the corticosteroids withdrawal which is in line with that observed for oxidative markers.

We found significantly 3 times lower levels of Sirt-1 during corticosteroids withdrawal induced exacerbation if compared to stable condition P<0.0001. The level of Sirt-1 during the recovery was not significantly different from stable condition P=0.15 (**Fig. 7.2 B, Table 15**).

We found significantly higher levels of Trx-2 during exacerbation and recovery if compared to stable condition. Specifically, 1.9 times and 5.6 times higher P=0.03 and P<0.0001, respectively (**Fig. 7.2 C**, **Table 15**).

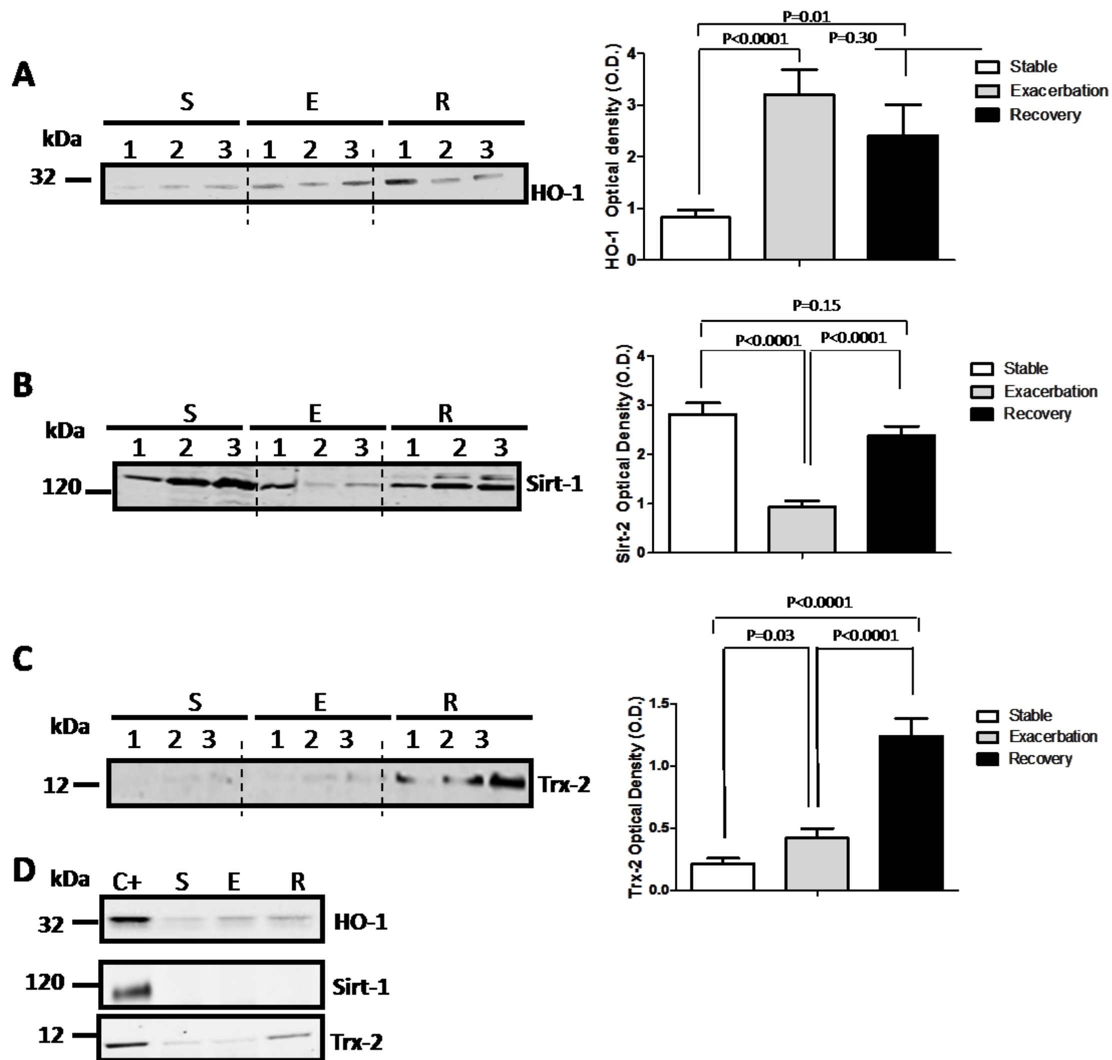


Fig. 7.2 *Cyto-protective proteins in serum before, during and after a corticosteroids withdrawal induced asthma exacerbation.* **A)** Immunochemical detection of HO-1. Panel A illustrates a representative Western blots for HO-1 in serum samples from three subjects. The signals of HO-1 level were determined by densitometry of the scanned images. All samples were normalized per protein (50µg). Red ponceau was used as loading protein control. Blots incubated only with secondary antibody were used as negative control. **B)** Immunochemical detection of Sirt-1. Panel B illustrates a representative Western blots for Sirt-1 in serum samples from three subjects. **C)** Immunochemical detection of Trx-2. Panel C illustrates a representative Western blots for Trx-2 in serum samples from three subjects. **D)** Westerblot for HO-1 and Trx-2 of microvescicle from plasma samples. As positive control (C+) for HO-1 was used whole Hela cells lysates for HO-1, 293T cells for Trx-2 and K-562 for Sirt-1. Shown a rapresentative blot of replicate experiments. Legends: S= Stable, E=Exacerbation, R= Recovery.

HO-1 and Trx-2 Exosomial release in serum. Cyto-protective proteins expressed in response to oxidative stress are normally expressed inside the cells. However, is possible find these in extracellular environment potentially due to a mechanism of release. We confirmed the presence of HO-1, Trx-2, Sirt-1 in low density membrane pellet obtained after ultracentrifugation of serum samples by western blot analysis (**Fig. 7.2 D**).

To our knowledge, this report is the first to describe the involvement of oxidative damage on proteins and antioxidant response in corticosteroid withdrawal-induced asthma exacerbation.

Chapter 8

8. Oxidative stress and asthma: clinical implication, conclusion and future directions

8.1. Clinical implication

In all the 4 studies described it has been shown an increase in oxidative modified proteins parallel a decrease in the anti-oxidant response during asthma exacerbation. In the previous introductive chapters we have seen that there is wide evidence of redox unbalance in asthma. In particular, protein bromination, lipid peroxidation, and NO production have all been related with the pathophysiology of asthma.[516] Stable end-products of distinct oxidation pathways may be used as dependable indices of oxidative stress. Elevated levels of 3-bromotyrosine and F2-IsoPs have been detected in urine and exhaled breath condensates of asthmatics. Increased NO production in the airways seems due to the upregulation of inducible nitric oxide synthase (iNOS) and the release from storage pools of GSNO.[517] Exhaled breath condensate pH assays may be of significance in monitoring the airway redox status.

Anti-oxidant treatment may represent a safe and effective alternative. Several therapeutic strategies have been used to develop small antioxidant molecule inhibitors of redox-regulated transcription factors. PNRI-299 selectively inhibits AP1 transcription but not NF-kB or thioredoxin.[518] PNRI-299 effectively reduces airway eosinophil infiltration, mucus hypersecretion, and IL-4 levels. MOL 294 inhibits both NF-kB and AP1 via inhibition of thioredoxin. Intranasal administration of MOL 294 markedly reduces airway eosinophilia and mucus hypersecretion.[519]

SOD therapy provides a connection between antioxidants and airway hyper-responsiveness. Transgenic mice that overexpress SOD have decreased allergen-induced physiologic alterations in the airway in comparison to controls.[520] SOD mimetics reduce PARP immunofluorescence, providing evidence of a role for SOD in inhibition of apoptosis and inflammation.[521] Also, SOD mimetics lessen the ovalbumin-

induced airway hyper-responsiveness to methacholine.[522] Exogenous EC-SOD given intratracheally to mice treated with asbestos, decreases neutrophil influx and oxidative matrix degradation.[523] Moreover, SOD mimics attenuate allergen-induced asthmatic bronchospasm.[524] However, clinical trials on the effects of SOD mimetics in patients with asthma have not been performed.

Other potential strategies comprise the glutathione peroxidase mimetic. Ebselen is a nontoxic seleno-organic drug and an effective reductant of hydroperoxides. Ebselen inhibits airway inflammation by reducing neutrophil recruitment and chemokine expression. Resveratrol, a phytoalexin that is found in seeds of grapes, has been reported to have antioxidant, anti-inflammatory, and anticarcinogenic properties.[525] Resveratrol effectively inhibits oxidative damage and scavenges free radicals. Resveratrol induces GSH synthesis. In primary lung epithelial cells, resveratrol (10 mM) attenuates cigarette smoke-mediated GSH depletion.

Epidemiological studies suggest associations between low dietary antioxidant intake, reduced lung function, and increased respiratory symptoms in asthmatics. A large cross-sectional study in Third National Health and Nutrition Examination Survey (NHANES III) shows that selenium and serum vitamin C is lower in young asthmatics.[526] Asthmatics have lower levels of coenzyme Q(CoQ).[527] CoQ increases SOD activity and thus therapy with CoQ may benefit in asthma.[528] In a study asthmatics who received corticosteroids, supplementation with CoQ [Q-Gel_ (120 mg), 32 weeks] improved asthma control and enabled reduction of corticosteroid dose.[529] These studies all support the concept that antioxidant supplementation and/or reduction in oxidant production or exposures will be beneficial in the treatment of asthma.

8.2. Conclusion and future directions

Asthma is a chronic inflammatory airway disease, and it is clear from multiple lines of evidence that the airway inflammation is defined by alterations of the airway redox. The studies described in this thesis show that redox mediated post-transcriptional modifications lead to protein structure–function changes that are present even in mild asthmatics and in larger extent during an exacerbation. Here it has been shown that the

abnormalities in redox are magnified in the asthmatic airway in response to exacerbating factors, including microbial infection, exposure to inhaled oxidizing pollutants, or allergen triggers in atopic individuals. During leukocyte activation, such as following allergen exposure, a respiratory burst occurs, generating $O_2^{\cdot -}$ and its dismutation product H_2O_2. Fenton/Haber–Weiss reactions affect endogenous proteins. Oxidative modifications of MnSOD intensify the oxidative milieu in the mitochondria, with potential adverse consequences on cellular respiration. As eosinophils and/or neutrophils enter the inflamed airway, H_2O_2 is used in eosinophil peroxidase and/or myeloperoxidase-mediated reactions that oxidatively modify susceptible proteins. Among those proteins is catalase. This allows more H_2O_2 to accumulate at the site of inflammation and further promotes peroxidase systems to produce high levels of nitrating, halogenating, and oxidizing injurious species. The greater toxic nitrogen oxides and airway acidity is accompanied by loss of beneficial nitrogen oxides, in particular nitrosothiols, which have adverse effects on smooth muscle relaxation and airway reactivity. In addition to injury of macromolecules, RNS and ROS amplify specific cytokine signal transduction by processes that include inhibition of deactivating signals. The loss of downregulatory signal transduction measures further amplifies the inflammatory milieu and contribute to Th2 lymphocyte polarization. Thus, alteration of redox participates in the pathophysiology of asthma. Future therapy targeting redox will require the definition of the clinical pharmacology of antioxidant compounds. Furthermore, identification of noninvasive biomarkers of oxidative stress in patients with asthma will be critical for enabling assessment of treatment outcomes. In line with this, the data described in this thesis provide a compelling rationale to develop therapeutic strategies for asthma that aim to correct the redox abnormalities.

Chapter 9

9. Proteomic investigation of N-Lysin Acetilation and Carbonlylation

9.1.Introduction

This chapter will show preliminary data obtained at the centre of proteomics research in Southampton (UK) while studying the relation within oxidative stress and protein acetylation.

Lysine acetylation is a reversible post-translational modification (PTM) that occurs on proteins involved in the regulation of various cellular processes.[530,531] Acetylation is dynamically controlled by lysine acetyltransferases (KATs, also known as histone acetyltransferases or HATs), and by lysine deacetylases (KDACs, also known as histone deacetylases or HDACs), **Fig. 9.1**.[532]

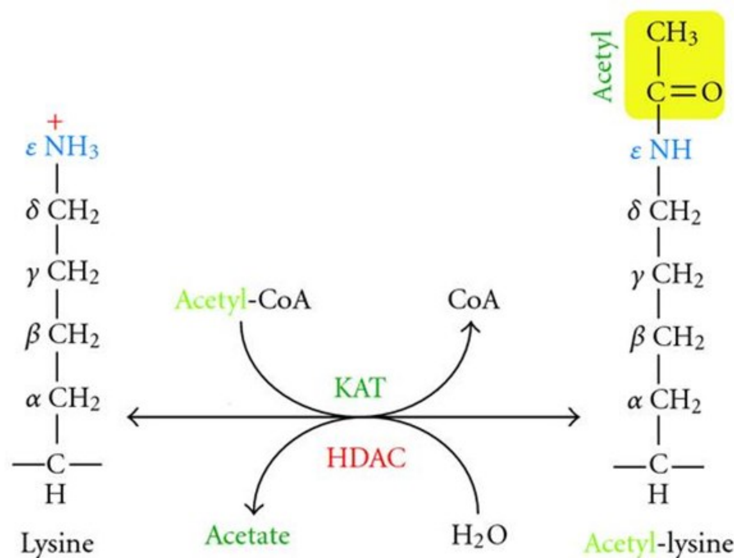


Fig. 9.1 *Acetylation and deacetylation at the ε-amino group of a lysine residue.* A KAT is responsible for transfer of an acetyl moiety (in yellow) from acetyl-CoA to the ε-group of a lysine residue, whereas an HDAC removes the acetyl group from acetyl lysine, releasing acetate.

The human and mouse genomes each encodes 18 different KDACs, of which 11 are classified as zinc-dependent deacetylases.[533] The remaining seven are NAD+-dependent deacetylases, known as Sirtuin 1–7 (Sirt1–7).[534] Sirtuins are localized to specific sub-cellular compartments: Sirt3, 4, and 5 in the mitochondria, Sirt6 and 7 in the nucleus, and Sirt1 and 2 in both the cytoplasm and nucleus. Sirtuins are important regulators of mammalian physiology whose functional roles are believed to be conserved from yeast to mammals (See **Chapter 3**).[535]

Despite important regulatory functions of acetylation, until recently only a limited number of endogenous acetylation sites were known. Owing to the extreme complexity of mammalian proteomes, and possibly low stoichiometry of modified sites, mapping endogenous acetylation sites has been a challenging task. Using antibody-based affinity enrichment, a proteomic survey discovered nearly 300 acetylation sites on mitochondrial proteins.[536]

Oxidative stress and redox status of the cells can regulate nuclear chromatin remodeling (histone acetylation/deacetylation) leading to gene expression. Oxidative stress also altered histone acetylation/deacetylation which increased the activation of NF-κB and AP-1, leading to the release of the pro-inflammatory cytokine IL-8 in human alveolar epithelial cells. ROS generation has been reported to regulate histone acetylation differentially in different cell types. However, the relationship between induced oxidative stress, inflammation and acetylation remains unknown and was therefore investigated.We applied high resolution mass spectrometry (MS) for evaluating endogenous acetylation proteins targets in human fetal lung fibroblasts exposed to oxidative, deacetylating and inflammatory conditions. Our preliminary data show that 47 acetylation proteins are identified as differently regulated in fibroblasts. A majority of these proteins are enzymes that participate in the regulation of metabolic pathways, cellular clcle, and transcription.

9.2.Methods and Results

Experimental Procedure. To identify substrates of acetylation we used Human Fetal Lung Fibroblast Cells (MRC-5 Line). MRC-5 cells were treated with 0.1 mM H_2O_2, 10 ng/ml TGF-β, and 2nM Fk228 for 48h (**Fig. 9.2**). FK228 (Romidepsin, depsipeptide) is a potent and selective inhibitor of class I histone deacetylases (HDACs) with IC50 values of 36, 47, 510 and 14,000 nM for HDAC1, HDAC2, HDAC4 and HDAC6, respectively. Romidepsin was dissolved to 5 mg/mL in 4:1 propylene glycol and ethanol and then diluted to 100 µg/mL in dimethyl sulfoxide (DMSO) and stored at -20°C. After lysis cells undergone western blot. For MS analysis proteins obtained from cell lysates were digested into peptides using trypsin. Acetylated peptides were enriched from the resulting complex peptide mixture with an anti-acetyllysine antibody.

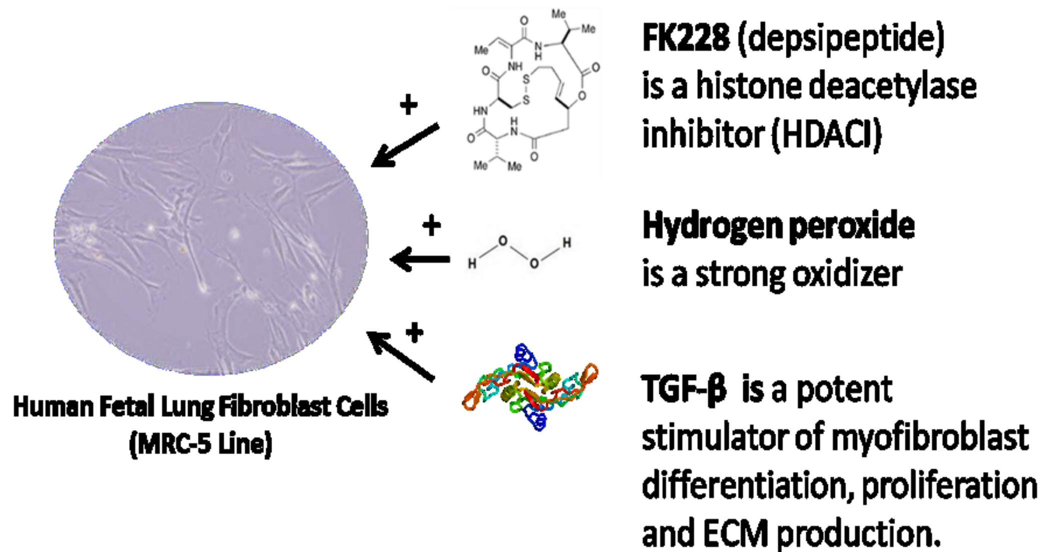
Immunoprecipitation and immunoblotting. Immunoprecipitation of N-Acetylated Proteins was performed according to standard procedure with an anti-acetyllysine antibody conjugated to beaded agarose (Immunechem). Immune complexes were washed five times in ice-cold PBS and eluted with 40 µl SDS sample buffer. Eluates were resolved by 4–12% gradient SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked using 3% BSA in PBS for 1hr and then incubated with After electrophoresis and 2h blotting onto a PVDF membrane, the blot was incubated with: Rabbit Anti Acetyl-Lysine Histon 1:2000 in TPBS, Mouse Anti α-actin smooth muscle 1:500 in TPBS, Rabbit Anti β-actin smooth muscle 1:1000 in TPBS**.** After the membranes were washed three times in TPBS and incubated for 60 min at room temperature with IRDye 680LT conjugates secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) 1:15,000 diluted in TPBS with 0.5% BSA. Blots incubated with only secondary antibody were used to evaluate aspecific secondary antibody binding. After three further washes in TPBS, bound antibodies were visualized using infrared fluorescence detection using the Odyssey Imager and software as recommended by the manufacturer (LI-COR Biosciences, Lincoln, NE, USA). After stripping the blot was re-probed for β-Actin in order to normalize for variable protein loading. Optical density was obtained by using Odyssey LICOR software, in which the values are expressed relative to β-Actin.

MS sample preparation. MRC-5 treated and untreated cells were cultured for 48 hours. MRC-5 lysates were incubated for 15 minutes on ice, and were cleared by sonication followed by centrifugation (17,000 g, 20 minutes at 4 degrees). Protein concentration of the cleared lysates was measured with Direct Detect Spectrometer (Mille Pore). Proteins were acetone precipitated and re-dissolved in 6M urea/2M thiourea/10 mM HEPES (pH 8), reduced with 1 mM dithiothreitol (DTT), alkylated with 5.5 mM chloroacetamide (CAA) and subsequently digested with trypsin. Peptides were purified using reversed-phase Sep-Pak C18 cartridges (Waters). The peptides were re-dissolved in immunoprecipitation (IAP) buffer and incubated with an anti-acetyllysine antibody overnight at 4°C on a rotation wheel. The immunoprecipitates were washed three times with IAP-buffer followed by additional 3 washes with distilled water. Residual water was removed and acetylated peptides bound to antibodies were eluted by 0.1% TFA in water.

Fractionation of peptides and mass spectrometric analysis. Peptides from immunoaffinity purification were fractionated with isoelectric focusing[537] using the Agilent 3100 OFFGEL Fractionator (Agilent). Peptides were purified using reversed phase C18 micro StageTips. The peptides were eluted from stage tips with 40 µl of 40% acetonitrile, 0.5% acetic acid into a 96 well plate. Acetonitrile was removed by speed-vac Concentrator Plus (Eppendorf) and the volume was reduced to ~5 µl. Peptide fractions were analyzed on a Synapt G2-S QuanTof mass spectrometer (Waters) equipped with a nanoflow UPLC system system (Waters) as described. The MS analyss was operated as MSE. MSE is an approach that acquires MS1 and MS2 mass spectra in an unbiased and parallel manner. It increases both the number of peptides detected and the reproducibility of the peptides sampling during an LC-MS experiment. During data acquisition, the energy of the gas-filled travelling-wave collision cell is dynamically switched between a low-energy and an elevated-energy status. The MSE raw data files are then processed by three different algorithms in ProteinLynx Global SERVER (PLGS).

Peptide identification and computational analysis. Peptide identification and computational analysis. Raw data files were processed and analyzed using ProteinLynx Global Server (PLGS) (Waters) version 3.0. Data were searched against the Human Uniprot protein database using an Ion Accounting algorithm. Spectra were searched with strict trypsin specificity, and allowing up to 1 missed cleavage sites. Minimum required peptide length was 6 amino acids. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein, oxidized methionine and acetylation of lysine were searched as variable modifications. The false discovery rate (FDR) for peptides and sites was estimated using a target-decoy approach. Statistical analysis was performed using the R software environment. Annotation enrichment analysis was performed using the String.db database.

Experimental procedure



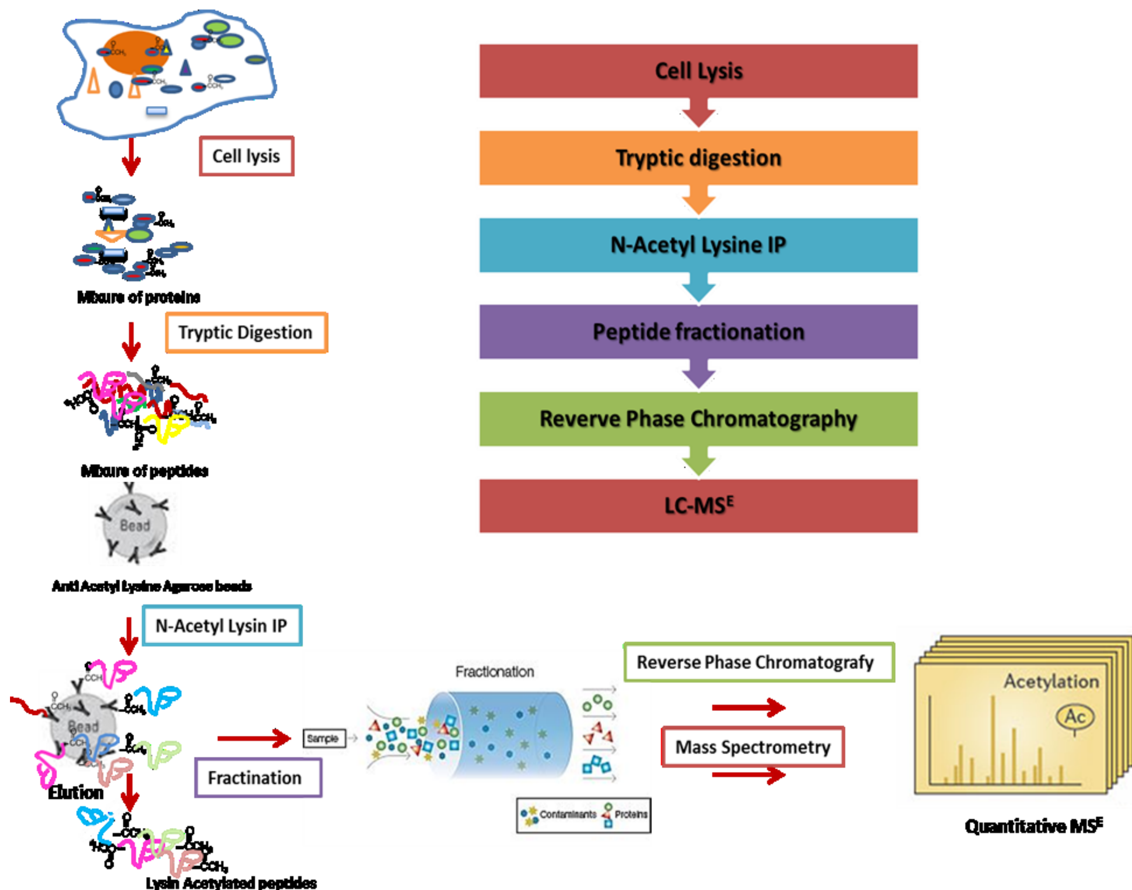


Fig. 9.2. *Experimental procedure synopsys.* C. Folisi.

9.3. Results

Immunoblotting for N- Lysin Acetylated proteins. For this experiment we used lysates from different cell lines and we immuno-blotted against N-Lysin Acetylated Proteins (Acetylated Lysine Rabbit Cell Signaling #9814P). As positive control we used Fibroblasts treated with deacetylases inibitor FK228. We could appreciate only 6 main immuno-reactive bands of which the more intense were those from histones (10-20 kDa), **Fig. 9.3**.

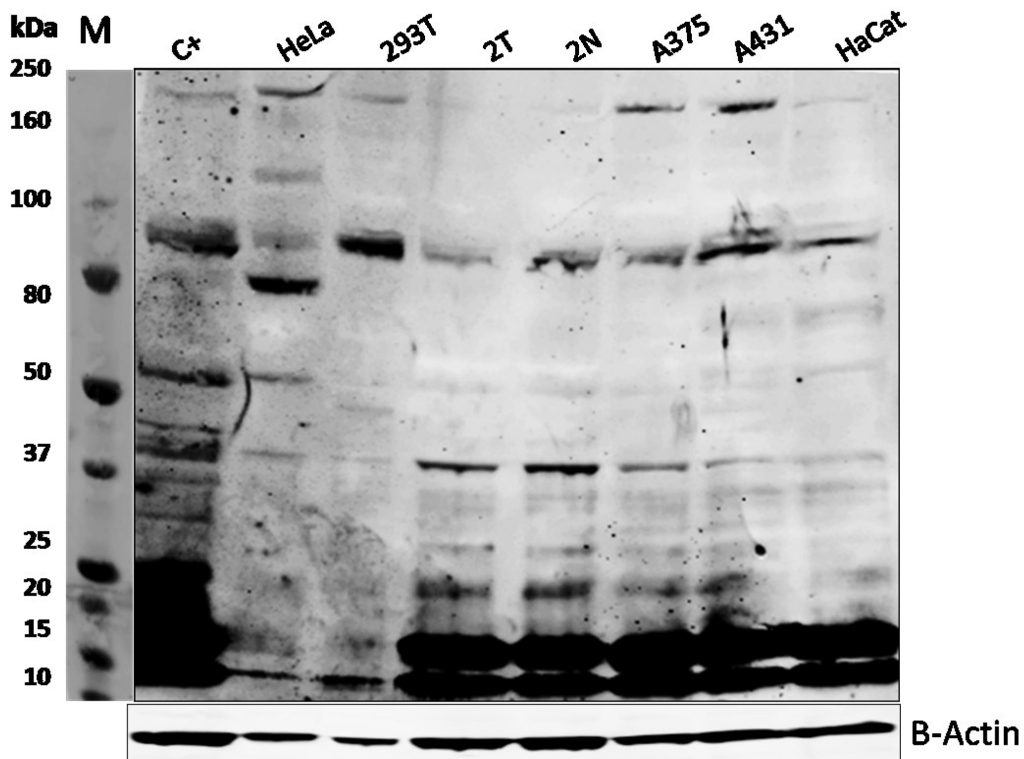


Fig. 9.3 Western Blot for N-Lysin Acetylated Proteins of lysates from MRC-5 treated with FK228 (C+), Hela Cells,He 293 Cells Line, MRC-5 treated and untreated with TGF-β (2T and 2N), A375, A431 and HaCat . Wb conditions: 24μg proteins/Lane. Primary Antibody: Acetylated Lysine Rabbit Cell Signaling #9814P 1:500 TPBS 5% BSA, Overnight 4°C Secondary Antibody: Biotin 1:2000 2hrs/Avidin 1:5000 1hr. Nitrocellulose membrane. M= molecular weight standards marker

Immunoprecipitation and immunoblotting. In order to confirm that the immuno-precipitation of acetylated proteins procedure functioned effectively and in order to obtain a greater number of immune-reactive bands we immunoblotted protein lysates before and after the immuno-precipitation of N-Lysin Acetylated proteins. For these

experiments we used He 293 and MRC-5 cell lines. In the third lines of the blot showed in **Fig. 9.4** we can clearly appreciate an increased immuno-reactivity after IP. This confirmed that the IP procedure used was functioning properly.

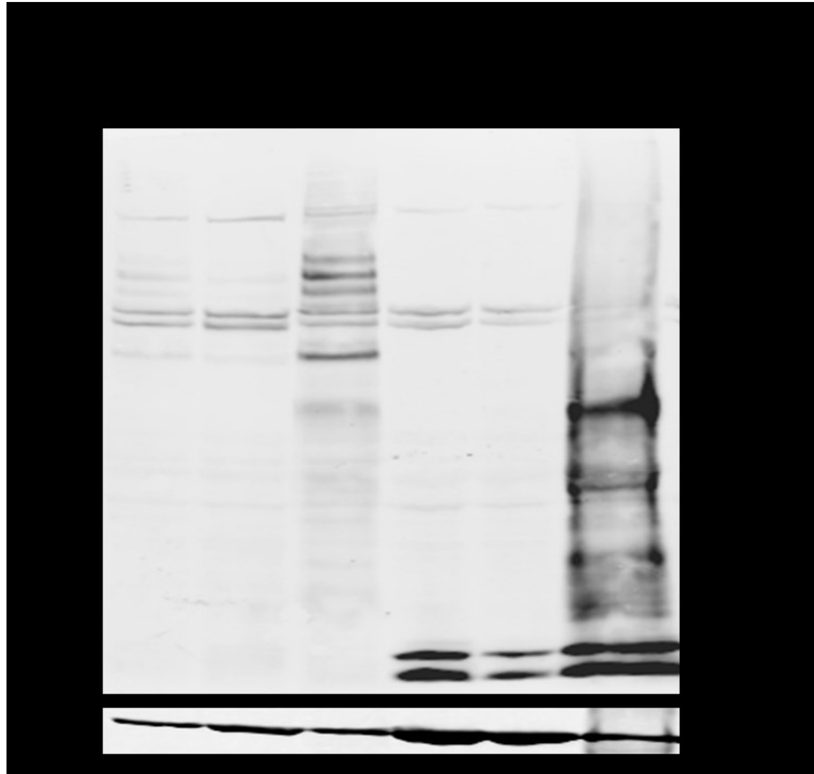


Fig. 9.4 Western Blot for N-Lysin Acetylated Proteins of lysates from He 293 Cells Line and MRC-5 before and after IP of Acetylated Proteins. Conditions: Overnight incubation with Anti-acetyl Lysine Agarose (Immunechem ICP0388 (2 mg proteins/50μL beads). WB Conditions: 15μg proteins/lane, 2hs blotting in CAPS buffer, 1hr blocking with BSA 3% Primary Antibody: Acetylated Lysine Rabbit Cell Signaling #9814P 1:500 TBST 5% BSA, Overnight 4°C. Secondary Antibody: Biotin 1:2000 2hs/Avidin 1:2000 1hr. Legend: 1= whole cell lysate before IP, 2= whole cell lysate after IP, 3= enriched IP eluted fration after N- Lysin Acetylated Proteins IP.

Immunoblotting for N-Lysin Acetylated Histones, α-Actin Smoot Muscle (ASM), and Carbonylated Protein. For this experiment we used MRC-5 treated with 1 mM H_2O_2, 10 ng/ml TGF-β, and 2nM Fk228 for 48h (**Fig. 9.5** and **9.6**).We could appreciate that FK228 was able to increase the acetylation status of the Histones, TGF-β increased the ASM expression and Hydrogen Peroxide increased the Carbonylated proteins detection after their derivatization with DNPH.

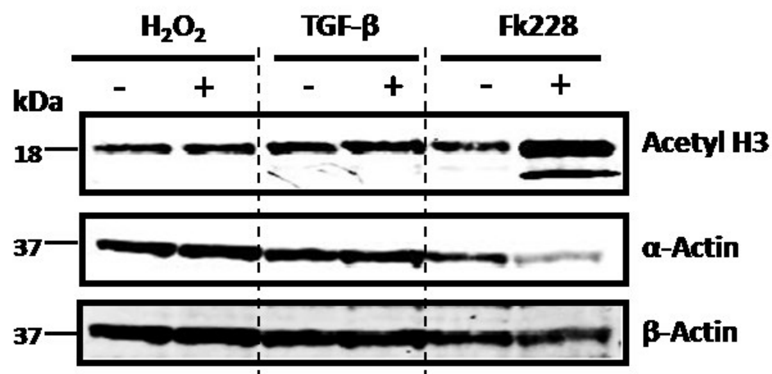


Fig.9.5. *N-Acetyl Lysine Histones and α-Actin in un-treated and treated MRC-5.* Western blot of 50 µg proteins from MRC-5 whole cells lysates in Tetraethylammonium bromide (TEAB) buffer. MRC-5 were treated with 0.1 mM H_2O_2, 10 ng/ml TGF-β, and 2nM Fk228 for 48h. Lysates from untreated MRC5 were used as a control (-). After electrophoresis and 2h blotting onto a PVDF membrane, the blot was incubated with Anti-Acetyl-Lysine Histon, Anti-α-Actin smooth muscle and Anti-β-actin smooth muscle.

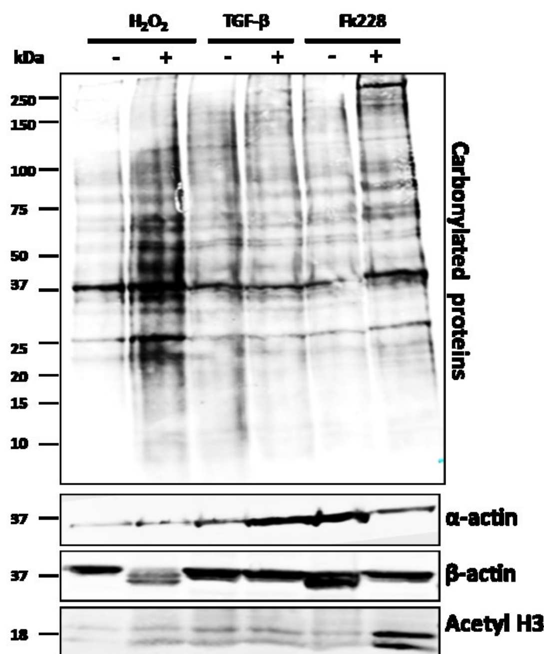


Fig. 9.6 *Protein carbonylation, Acetyl Lysine H3, and α-Actin Smoot Muscle in un-treated and treated MRC-5.* Western blot of 30 µg proteins from MRC5 whole cells lysates in TEAB buffer. MRC-5 were treated with 0.1 mM H_2O_2, 10 ng/ml TGF-β, and 2nM Fk228 for 48h. Untreated MRC-5 were used as a control (-).

MS analysis of acetylated proteins in whole untreated MRC-5 cells lysates.

Raw data files were processed and analyzed using ProteinLynx Global Server (PLGS) (Waters) version 3.0. Data were analysed by using http://string-db.org/

STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context High-throughput Experiments (Conserved) Coexpression Previous KnowledgeSTRING quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable. The database currently covers 5.214.234 proteins from 1133 organisms. In **Fig 9.7** is illustrated the string interface. **Fig. 9.8** and **Table 16** show the result obtained for N-Lysine Acetylated Proteins in untreated MRC-5.

Fig. 9.7. *String Data Base Search for proteins interection interface.*

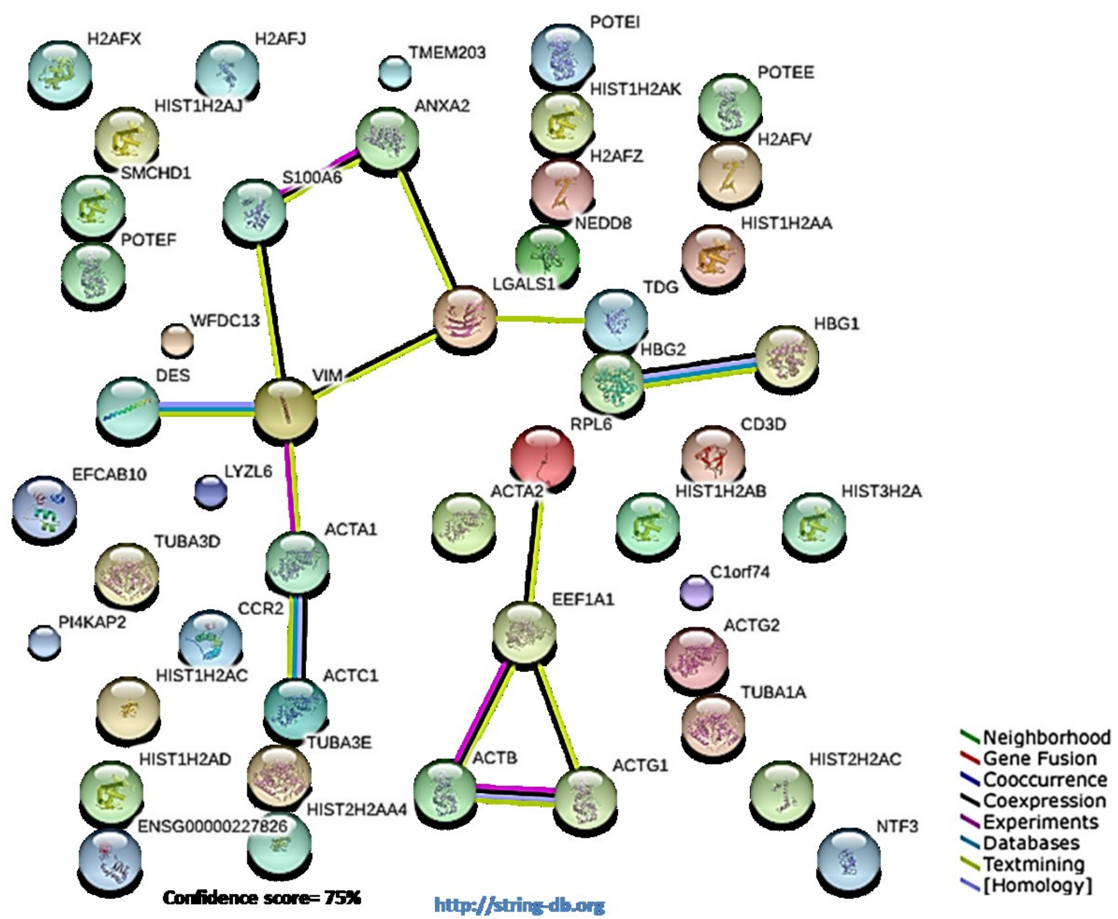


Fig. 9.8 *N-Lysine Acetylated Proteins in MRC-5 whole Lysate and their interections.*

TABLE 16 LIST OF ACETYLATED PROTEINS IN MRC-5

RPL6	60S ribosomal protein L6 (TAX-responsive enhancer element-binding protein 107)(TAXREB107)(Neoplasm-related protein C140); Specifically binds to domain C of the Tax-responsive enhancer element in the long terminal repeat of HTLV-I (288 aa)
LGALS1	Lectin, galactoside-binding, soluble, 1; May regulate apoptosis, cell proliferation and cell differentiation. Binds beta-galactoside and a wide array of complex carbohydrates. Inhibits CD45 protein phosphatase activity and therefore the dephosphorylation of Lyn kinase (135 aa)
VIM	Vimentin; Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells (466 aa)
ACTA2	Actin, alpha 2, smooth muscle, aorta; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (By similarity) (377 aa)
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8; Ubiquitin-like protein which plays an important role in cell cycle control and embryogenesis. Covalent attachment to its substrates requires prior activation by the E1 complex UBE1C- APPBP1 and linkage to the E2 enzyme UBE2M. Attachment of NEDD8 to cullins activates their associated E3 ubiquitin ligase activity, and thus promotes polyubiquitination and proteasomal degradation of cyclins and other regulatory proteins (81 aa)
HIST1H2AB	Histone cluster 1, H2ab; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
ACTC1	Actin, alpha, cardiac muscle 1; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (By similarity) (377 aa)
CCR2	Chemokine (C-C motif) receptor 2; Receptor for the MCP-1, MCP-3 and MCP-4 chemokines. Transduces a signal by increasing the intracellular calcium ions level. Alternative coreceptor with CD4 for HIV-1 infection (374 aa)

LYZL6	Lysozyme-like 6 (148 aa)
C1orf74	UPF0739 protein C1orf74 (269 aa)
ACTG2	Cctin, gamma 2, smooth muscle, enteric; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (By similarity) (376 aa)
H2AFZ	H2A histone family, member Z; Variant histone H2A which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling. May be involved in the formation of constitutive heterochromatin. May be [...] (128 aa)
HIST1H2AA	Histone cluster 1, H2aa; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (131 aa)
CD3D	CD3d molecule, delta (CD3-TCR complex); The CD3 complex mediates signal transduction (171 aa)
TUBA1A	tubulin, alpha 1a; Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the Alpha-chain (By similarity) (451 aa)
WFDC13	WAP four-disulfide core domain 13 (93 aa)
H2AFV	H2A histone family, member V; Variant histone H2A which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling. May be involved in the formation of constitutive heterochromatin. (128 aa)
TUBA3E	tubulin, alpha 3e; Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an

Continued	exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity) (450 aa)
HIST1H2AC	Histone cluster 1, H2ac; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
TUBA3D	Tubulin, alpha 3d; Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity) (450 aa)
HBG1	Hemoglobin, gamma A (147 aa)
HIST1H2AJ	Histone cluster 1, H2aj; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (128 aa)
EEF1A1	Eukaryotic translation elongation factor 1 alpha-like 7; This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis (By similarity) (462 aa)
HIST1H2AK	Histone cluster 1, H2ak (130 aa)
ACTG1	Actin, gamma 1; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (By similarity) (375 aa)
HIST2H2AC	Histone cluster 2, H2ac; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (129 aa)
HBG2	Hemoglobin, gamma G; Gamma chains make up the fetal hemoglobin F, in combination with alpha chains (147 aa)

HIST1H2AD	Histone cluster 1, H2ad; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
ANXA2	Annexin A2 pseudogene 1; Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids (By similarity). It binds two calcium ions with high affinity (By similarity). May be involved in heat-stress response (By similarity) (357 aa)
POTEE	POTE ankyrin domain family, member E (1075 aa)
ACTB	Actin, beta; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in All eukaryotic cells (By similarity) (375 aa)
POTEF	POTE ankyrin domain family, member F (1075 aa)
SMCHD1	Structural maintenance of chromosomes flexible hinge domain containing 1; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
ACTA1	Actin, alpha 1, skeletal muscle; Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (By similarity) (377 aa)
HIST3H2A	Histone cluster 3, H2a; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
S100A6	S100 calcium binding protein A6; May function as calcium sensor and contribute to cellular calcium signaling (Potential). May function by interacting with other proteins and indirectly play a role in the reorganization of

Continued	the actin cytoskeleton and in cell motility. Binds 2 calcium ions. Calcium binding is cooperative (90 aa)
HIST2H2AA4	Histone cluster 2, H2aa4; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (130 aa)
DES	Desmin; Desmin are class-III intermediate filaments found in muscle cells. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures (470 aa)
H2AFX	H2A histone family, member X; Variant histone H2A which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling. Required for checkpoint-mediated arrest of cell cycle progression in resp [...] (143 aa)
H2AFJ	H2A histone family, member J; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling (129 aa)
TMEM203	Transmembrane protein 203 (136 aa)
TDG	Hymine-DNA glycosylase; In the DNA of higher eukaryotes, hydrolytic deamination of 5-methylcytosine to thymine leads to the formation of G/T mismatches. This enzyme corrects G/T mispairs to G/C pairs. It is capable of hydrolyzing the carbon-nitrogen bond between the sugar-phosphate backbone of the DNA and a mispaired thymine. In addition to the G/T, it can remove thymine also from C/T and T/T mispairs in the order G/T >> C/T > T/T. It has no detectable activity on apyrimidinic sites and does not catalyze the removal of thymine from A/T pairs or

	from single-stranded DNA. It can also r [...] (410 aa)

Continued

POTEI	POTE ankyrin domain family, member M (1075 aa)
NTF3	Neurotrophin 3; Seems to promotes the survival of visceral and proprioceptive sensory neurons (270 aa)
ENSG00000227826	HLA class II histocompatibility antigen, DR beta 4 chain Precursor (266 aa)
PI4KAP2	Phosphatidylinositol 4-kinase, catalytic, alpha pseudogene 2 (592 aa)
EFCAB10	EF-hand calcium binding domain 10 (149 aa)

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11.Abbreviations

AA: Arachidonic acid

ACQ: Asthma Control Questionnaire

AHR: Airway hyper-responsiveness

ASM: Airway smooth muscle

ARE: Antioxidant-response elements

AUC: Area under the curve

BAL: Broncho Alveolar Lavage

BALF: Broncho Alveolar Lavage Fluid

BHR: Bronchial responsiveness

BMDC: Bone-Marrow derived dendritic cells

BS: Broncho Alveolar Lavage from stable asthmatics

BU:Biological Units

CAT: Catalase

$C0_2$:Dioxide Carbone

COPD: Chronic obstructive pulmonary disease

COX: Cyclooxigenase

CSC:Cigarettes smoke condensate

Ctrl: Control

DNPH:2,4-dinitrophenylhydrazine

EAR: Early allergic response

ECP: Eosinophil cationic protein

ED: Emergency department

EDN:Eosinophil-derived neurotoxin

EPA: Eicosapentanoic acid

EPO: Eosinophil peroxydase

F2-ip: Isoprostanes

FEV_1: Forced expiratory volume in I second

FEV_1% FEV: as percentage of predicted value

FVC: Forced vital capacity

FVC% FVC: as percentage of predicted value

4-HNE PAs: 4-Hydrossyl-2-nonenal Protein Adducts

g/d: grams per day

GM-CSF:granulocyte-macrophage colony-stimulating factor

GINA: Global Initiative on Asthma

GPx: Glutathione Peroxidase

GSH: Reduced glutathione

GSH-Px: Glutathione peroxidase

H3:Histone 3

HDL: High density lipoprotein

HDM: House Dust Mite

HRP:Horse Radish Peroxidase

HSP: Heat Shock Protein

HO: Heme Oxygenase

15-HETE: 15-hydroxyeicosatetraenoic

H_20_2: Hydrogen peroxide

ICS: Inhaled corticosteroids

Ig: Immunoglobuline

IL: Interleukine

IS: Induced sputum

ISS: Induced sputum stable

ISE: Induced sputum exacerbation

LA: Linoleic acid

LAR: Late Asthmatic Response

LOX: 5-lipo-oxygenase

LPS:lipopolysaccharides

LT: Leukotrien

LT HDM: Low endotoxin HDM

LTRA:Leukotriene modifiers

mg/d: Milligrams per day

MDA: Malondialdehyde

MBP: Major basic protein

NAC: N-Acetyl Cysteine

NLHBI: National Heart, Lung, and Blood Institute

Nrf: Nuclear factor erythroid 2–related factor 2

NO: Nitric oxide

$0_2^{\cdot-}$:Superoxide radical

0_2:Oxygen

OCS:Oral corticosteroids

PAF: Platelet-activating factor

PBMC: peripheral blood mononucleocytes

PBS: Phosphate Buffered Saline

PC_{20}:Concentration required to produce a 20% fall in FEV_1

PD_{20}: Dose required to produce a 20% fall in FEV

PEF: Peak expiratory flow

PG: Prostaglandin

PRR: Pattern recognition receptors

PVDF: polyvinylidene difluoride

ROS:Reactive oxygen species

RANTES: Regulated on activation, T-cell expressed and secreted, eotaxin

Rel O.D: Relative Optical density

RNS: Reactive nitrogen species

RV:Rhinovirus

SABA: Short-acting β2-agonists

SD: Standard deviation

SDS:Sodium Dodecyl Sulphate

SE: Standard error

Sirt: Sirtuin

SOD: Superoxide dismutase

SO_2: sulphur dioxide

Th2: T-helper 2 cells

TBARS: Thiobarbituric acid-reactive species

TGF-β: Transforming growth factor

TNF-α:tumour necrosis factor

Trx: Thioredoxin

Trx-R: Thioredoxin Reductase

WB: Western blot

WURSS: Wisconsin Upper Respiratory Symptom Survey.

X/XO: Xanthine/Xanthine Oxidase

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