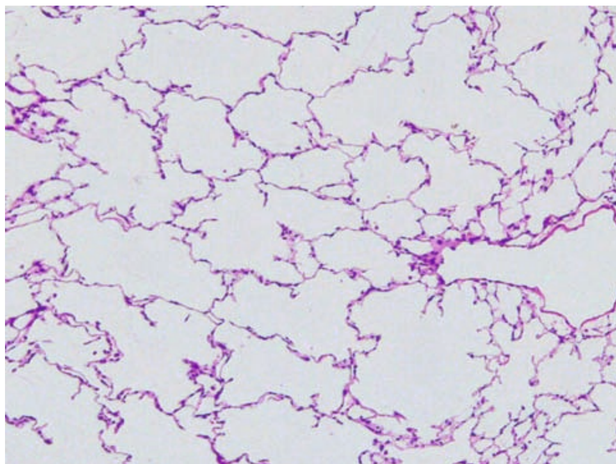
THE ROLE OF THE ANTIOXIDANT PROTEIN SESTRIN 2 IN EMPHYSEMA DEVELOPMENT IN MICE

ATHANASIOS FYSIKOPOULOS



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The role of the antioxidant protein sestrin 2 in emphysema development in mice

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CONTENTS

TABLE OF CONTENTS Page

LIST OF FIGURES V

LIST OF TABLES VI

LIST OF ABBREVIATIONS VII

1. INTRODUCTION 1

1.1 Definitions 1

1.1.1 COPD 1

1.1.2 Chronic bronchitis 2

1.1.3 Small airways disease 2

1.1.4 Emphysema 3

1.1.5 Pulmonary hypertension. 3

1.1.6 Epidemiology – Prevalence – Incidence 3

1.2 COPD clinical manifestations 4

1.2.1 Cough and sputum 4

1.2.2 Dyspnea 4

1.2.3 Systemic manifestations 5

1.3 Risk factors 6

1.3.1 Host factors 6

1.3.1.1 Genetic factors 6

1.3.1.2 Airway hyperresponsiveness 6

1.3.1.3 Gender 7

1.3.2 Environmental Factors 7

1.3.2.1 Cigarette smoke 7

1.3.2.2 Air pollution 8

1.3.2.3 Occupational exposures 9

1.3.2.4 Other factors 9

1.4 Pathophysiology of COPD 9

1.4.1 Lung inflammation 9

1.4.2 Protease/anti-proteinase balance 13

Other proteases. 15

Metalloproteases. 15

1.4.3 Oxidative/nitrosative stress 16

1.4.4 Apoptosis 18

1.5 COPD in other animal species. 20

In horses 20

In dogs and cats 21

CONTENTS

1.6 Sestrin 2 21

1.7 Emphysema models 27

1.7.1 Cigarette smoke-induced emphysema 27

1.7.2 Elastase-induced emphysema 28

2. AIMS OF THE STUDY 31

3. MATERIALS 32

3.1 Solutions and substances 32

3.2 Consumables 33

3.3 Hemodynamics–Lung Function setup 34

3.4 Histology 35

3.4.1 Equipment 35

3.4.2. Consumables 36

3.5 Smoke generation and exposure system 36

3.6 ROS measurements 37

3.7 Cell culture 37

4. METHODS 38

4.1 Animals 38

4.1.1 Approvals for the experiments 38

4.1.2 Sestrin 2 KO mice 38

4.1.3 Housing 38

4.1.4 Sestrin 2 study/smoke exposure mice groups: 39

4.1.5 sestrin 2 study mice groups: elastase-induced emphysema 39

4.2 Experimental designs 39

4.2.1.Experimental design of tobacco smoke exposure of RRJ141 mice 39

4.2.2 Experimental design of elastase-induced emphysema in RRJ141 mice 40

4.2.3 Elastase intratracheal instillation in mice 41

Elastase 42

Anaesthesia and elastase instillation. 43

4.3 *In vivo* investigations 44

4.3.1 Anaesthesia and mouse preparation 44

4.3.2 Lung function tests 44

4.3.3 Hemodynamic measurements 45

4.4 Alveolar morphometry 46

4.5 Immunohistochemistry and immunofluorescence 49

CONTENTS

4.6 Heart preparation and heart ratio calculation 50

4.7 Quantification of lung elastin by image analysis (sestrin 2 mice smoke exposure experiment) 51

4.8 RT-PCR 52

4.9 Western blotting 53

4.10 Cell cultures and preparation of conditioned media 54

4.11 ROS measurements 55

4.12 Statistical analyses 55

5. RESULTS 56

5.1 Regulation of sestrin 2 and PDGFRβ mRNA in lungs of mice by smoke exposure. 56

5.2 Sestrin 2 inactivation protects mice from smoke-induced emphysema. 57

5.2.1 Sestrin 2 inactivation protects from a decline in lung function in smoke-exposed mice. 57

5.2.2 Sestrin 2 inactivation protects from the development of pulmonary emphysema in smoke-exposed mice. 58

5.2.3 Smoke exposure does not result in the development of pulmonary hypertension and right heart hypertrophy in sestrin 2 knockout mice. 61

5.2.4 Inactivation of Sestrin preserves the number of intact elastin fibers per area in the lungs of smoke-exposed mice. 62

5.3 Results of the investigations of the role of PDGFRβ in elastase-induced emphysema in sestrin 2 knockout mice 63

5.3.1 Inhibition of PDGFRβ accentuates the decline in lung function of sestrin 2 knockout mice treated with elastase. 64

5.3.2 Inhibition of PDGFRβ worsens the emphysema of sestrin 2 knockout mice treated with elastase. 64

5.3.3 Effect of the inhibition of PDGFRβ in hemodynamics of elastase-induced emphysema in wild type and sestrin 2 knockout mice. 68

5.4 Sestrin2 inactivation leads to increased proliferation and decreased apoptosis in alveolar wall ATII cells. 69

5.5 PDGFRβ signaling contributes to lung maintenance through the induction of KGF. 72

5.5 Superoxide anions are required for the induction of PDGRβ signaling. 72

6. Discussion 74

6.1 The mouse model of smoke-induced emphysema 74

6.2 Structural and functional changes in wild type mice after smoke exposure 76

6.3 sestrin 2 knockout mice did not exhibit the same functional and structural changes as wild type mice after smoke exposure 80

CONTENTS

6.4 Cigarette smoke exposure caused pulmonary hypertension and right heart hypertrophy in WT but not in the sestrin 2 KO mice. 83

6.5 Sestrin 2 is a repressor of PDGFRβ signaling and alveolar maintenance programs. 84

6.6 Conclusion 85

8. SUMMARY 87

9. ZUSAMMENFASSUNG 89

10. BIBLIOGRAPHY 91

11. DECLARATION 144

12. ACKNOWLEDGEMENTS 145

14. PUBLICATIONS 147

CONTENTS

LIST OF FIGURES

Figure 1. Outline of sestrin 2 functions under stress and normal conditions. ... 25

Figure 2. Macroscopic lesions of emphysema in mice lungs after PPE instillation. ... 30

Figure 3. Experimental plan of sestrin 2 mice smoke exposure. ... 40

Figure 4. The smoke generator used in the experiments. ... 40

Figure 5. Time schedule of elastase-induced emphysema in sestrin 2 KO and WT mice. ... 41

Figure 6. Mice groups in the elastase-induced emphysema in sestrin 2 mice experiment. ... 41

Figure 7. Lung function and hemodynamics body measurement setup. ... 44

Figure 8. Lung fixation setup. ... 47

Figure 9. Image analysis for alveolar morphometry. ... 49

Figure 10. Schematic illustration of the heart. ... 51

Figure 11. Regulation of sestrin 2 and PDGFRβ in cigarette smoke-exposed and control mice. ... 56

Figure 12. Dynamic lung compliance measurement in wild type and sestrin 2 knockout mice after smoke exposure. ... 57

Figure 13. Alveolar morphometrical evaluation of lungs of WT and sestrin 2 KO mice after 8 months of smoke exposure. ... 59

Figure 14. Representative images of lungs of wild type and sestrin 2 knockout mice exposed to room air (control) or cigarette smoke (se). ... 60

Figure 15. Hemodynamic evaluation in wild type and sestrin 2 knockout mice after 8 months of smoke exposure. ... 62

Figure 16. Number of intact elastic fibers per area in lungs of wild type and sestrin 2 knockout mice after smoke exposure and their respective controls. ... 63

Figure 17. Representative images of lungs of WT and sestrin 2 KO lungs, of mice exposed to cigarette smoke (smoke-exposed). ... 63

Figure 18. Lung function in WT and sestrin 2 KO mice after elastase treatment ± imatinib. ... 64

Figure 19. Morphometric evaluation of the effect of PDGFRβ inhibition on the elastase-induced emphysema induction in WT and sestrin 2 KO mice. ... 65

Figure 20. Representative images of lungs from WT and sestrin 2 KO mice after elastase or saline application combined with imatinib or placebo treatment. ... 68

Figure 21. Effect of imatinib in the hemodynamics of wild type and sestrin 2 knockout mice treated with elastase. ... 69

Figure 22. Staining and quantification of ATII cells in lungs of WT and sestrin 2 KO control and smoke-exposed mice. ... 70

Figure 23. Quantification of apoptotic (CC3 positive) cells in the lungs of WT and KO smoke-exposed mice. ... 71

Figure 24. Lung sections stained with anti-cleaved caspase-3 (CC3-Cy3) for immunofluorescence confocal microscopy. ... 71

Figure 25. KGF expression in WT and sestrin 2 KO-MLFs. ... 72

Figure 26. Induction of PDGFRβ by O_2^- in WT- and KO-MLFs. ... 73

Figure 27. Possible participation of sestrin 2 in the pathogenesis of emphysema through the disruption of lung maintenance. 79
Figure 28. Suggested pathways affected by sestrin 2 inactivation, leading to prevention from emphysema and PH. 86

LIST OF TABLES

Table 1. Classification of Matrix Metalloproteinases (MMPs) according to their substrates.............. 12
Table 2. Tissue Inhibitors of MMPs and their effect on MMPs 12
Table 3. The main MMPs involved in the pathogenesis of COPD. 16
Table 4. Experimental animals. 38
Table 5. Hematoxylin-Eosin staining protocol 48
Table 6. Weigert's elastic stain protocol. 52
Table 7. Temperature profile of the PCR reaction. 53
Table 8. Primers used for sestrin 2, PDGFRβ and KGF. 53

ABBREVIATIONS

LIST OF ABBREVIATIONS

˙OH	Hydroxyl radical
µg	Microgram
µl	Microliter
4E-BP-1	Eukaryotic translation initiation factor 4E-binding protein 1
ADAMs	A disintegrin and metalloproteases
ADAMTS	ADAMs with thrombospondin motifs
AIDS	acquired immunodeficiency syndrome
Akt	v-akt murine thymoma viral oncogene homolog 1, Protein Kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Apaf-1	apoptotic peptidase activating factor 1
APMA	4-aminophenylmercuric acetate
ARE	Antioxidant response element
ATI	Alveolar epithelial type I
ATII	Alveolar epithelial type II
ATS	American thoracic society
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Bax	BCL2 (B cell leukemia/lymphoma 2)-associated X protein
BSA	Bovine serum albumin
BTS	British thoracic society
BW	Body weight
CCL	CC-chemokine receptor ligand
CCR	CC chemokine receptor
CD-	Cluster of differentiation of T-lymphocytes,

ABBREVIATIONS

$C_{L,dyn}$	Lung dynamic compliance
CO	Carbon monoxide
CO_2	Carbon dioxide
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
CXCL	CXC-Chemokine Ligand, and respective numbers
Cys-SH	Cysteine thiol group
DAPI	4',6-diamidino-2-phenylindole
DEPTOR	DEP domain-containing mTOR-interacting protein
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Electron paramagnetic resonance
ERK	Extracellular regulated map kinase
ERS	European respiratory society
ESR	Electron spin resonance
FADD	Fas-associated protein with death domain
Fas	Fas cell surface death receptor
FasL	Fas ligand
Fe^{+2}	Ferrous iron
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOLD	Global initiative for chronic obstructive lung disease

ABBREVIATIONS

GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
HE	Haematoxylin and eosin
HIV	Human immunodeficiency virus infection
HME	Human macrophage elastase (MMP12 synonym)
HNE	Human neutrophil elastase
i.p.	Intraperitoneal
ICAM-1	intercellular adhesion molecules
IFN	interferon
IL-	Interleukin
KC	Cxcl1 synonym
kg	Kilogram
KGF	Keratinocyte growth factor
KO	Knockout
KY	Kentucky
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
m^3	Cubic meter
MAPK	Mitogen activated protein kinase
MCP1	monocyte chemoattractant protein 1, synonym for CCL2
mg	Milligram
MIP-1γ	macrophage inflammatory protein-1 gamma, synonym for CCL9
MLI	Mean linear intercept
mLST8	Mammalian lethal with SEC13 (yeast secretory pathway gene) protein 8
mmHg	Millimeter of mercury

ABBREVIATIONS

MMP	Matrix metaloproteinase
MMPSF	Matrix metaloproteinase soluble factor
MMRC	Mutant mouse regional resource centers
mPAP	Mean pulmonary artery pressure
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NO_x	mono-nitrogen oxides
NOXA	synonym phorbol-12-myristate-13-acetate-induced protein 1
Nrf2	nuclear factor erythroid 2-related factor 2
O_2^-	Superoxide anion
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-B	Platelet-derived growth factor b (ligand)
PDGFR	Platelet-derived growth factor receptor
PDGFRβ	Platelet-derived growth factor receptor β
PEEP	Positive end-expiratory pressure
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKK	phosphatidylinositol kinase-related kinase
PMA	Phorbol-12-myristate-13-acetate
Ppa	Mean pulmonary arterial pressure
PPE	Porcine pancreatic elastase

ABBREVIATIONS

PRAS40	Proline-rich Akt substrate of 40 kDa
Protor -1	protein observed with Rictor-1
PTM	Pneumotachometer
PUMA	p53 upregulated modulator of apoptosis
RA	Room air
raptor	Regulatory-associated protein of mTOR
rictor	Rapamycin-insensitive companion of mammalian target of rapamycin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
RV	Right ventricle
RV/LS+S	Ratio of right ventricle of left ventricle plus septum
RVP	Right ventricular pressure
RVSP	Right ventricular systolic pressure
S6K1	S6 kinase 1
SAP	Systemic arterial pressure
SE	Smoke-exposed
SEM	standard error of the mean
SERPINA1	Serpin peptidase inhibitor, clade A
Sesn2	sestrin 2
sin1	mammalian stress-activated protein kinase interacting protein
SO_2	Sulfur dioxide
SOD	Superoxide dismutase
TACE	TNF-α converting enzyme
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinases and respective numbers

ABBREVIATIONS

TNF	Tumor necrosis factors
TNFR	tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TPA	Tissue plasminogen activator
TPM	Total particulate matter
TRAIL	TNF-related apoptosis-inducing ligand
U	Units of specific activity
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World health organization
WT	Wild type
α-2M	alpha-2-macroglobulin

1. INTRODUCTION

1.1 Definitions

1.1.1 COPD

Chronic obstructive pulmonary disease is defined by the American Thoracic Society (ATS) and the European Respiratory Society as “a preventable and treatable disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces significant systemic consequences“ (Siafakas, 2006). According to the Global Initiative for Chronic Obstructive Lung Disease, COPD is “a preventable and treatable disease with some significant extra-pulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (Celli et al., 2004).

This chronic innate and adaptive inflammatory response results in a tissue remodeling process that affects both the large and small airways and may produce lesions similar to four different pathologic conditions: chronic bronchitis (central airways) (Di Stefano et al., 1996; Kemeny et al., 1999; Mullen et al., 1985; O’Shaughnessy et al., 1997; Reid, 1960; Saetta et al., 1993; Saetta et al., 1994; Saetta et al., 1997), small airways disease (Hogg et al., 1994; Hogg et al., 2004; Wright et al., 1984), emphysema (parenchyma) (Leopold and Gough, 1957; McLean, 1957a; McLean, 1957b; McLean, 1958; Retamales et al., 2001) and pulmonary hypertension (Elwing and Panos, 2008; Peinado et al., 2008; Weitzenblum and Chaouat, 2005; Wright et al., 2005). The phenotype of the disease in patients is dependent on the combination of the presence of these conditions and their severity and prominence. The main etiologic factor of COPD in the developed world is cigarette smoking while other factors like environmental or professional exposure to particles and gases, air pollution, biomass smoke, asthma and airway hyperresponsiveness are also important. The classification of COPD into four stages

according to the airflow limitation (in subjects with an $FEV_1/FVC < 0.70$ measured with post-bronchodilator lung function) is:

- Stage 1 (mild, $FEV_1 \geq 80\%$ predicted)
- Stage 2 (moderate, $50\% < FEV_1 < 80\%$ predicted)
- Stage 3 (severe, $30 < FEV_1 < 50\%$ predicted)
- Stage 4 (very severe, $FEV_1 < 30\%$ predicted)

as adopted by the GOLD and the ATS/ERS guidelines (Celli et al., 2004).

1.1.2 Chronic bronchitis

Chronic bronchitis was defined at the CIBA Guest Symposium in 1959 as the presence of a productive cough for more than 3 months in two successive years in subjects after the exclusion of other causes of chronic cough, (e.g. tuberculosis, lung cancer, heart failure). Chronic bronchitis is seen in 14-74% of patients with COPD (Agusti et al., 2010; Burgel et al., 2009; de Oca et al., 2012; Kim et al., 2011b). The chronic cough is dose-related to cigarette smoking (Janson et al., 2001). Cigarette smoke is the stimulus (Fischer and Voynow, 2002; Gensch et al., 2004; Shao et al., 2004; Verra et al., 1995) that produces an inflammatory response in the epithelium of the central (cartilaginous) airways and the mucus producing glands located there (Hogg, 2004). This leads to goblet cell metaplasia and an increase of goblet cell numbers (Jeffery, 2000) which in turn results in an excessive production and secretion of mucus combined with a decreased elimination caused by impaired mucociliary action, occlusion of the distal airways and reduced peak expiratory flow (Danahay and Jackson, 2005; Vestbo, 2002). Chronic bronchitis further worsens the decline in quality of life (Kim et al., 2011a; Kim et al., 2011b), lung function (Vestbo et al., 1996), increases exacerbations (Burgel et al., 2009; Kim et al., 2010; Seemungal et al., 2009) and mortality (Guerra et al., 2009).

1.1.3 Small airways disease

The small airways of the lungs (less than 2 mm in diameter) are considered to be the site where obstruction occurs and where increased resistance of the lungs is present (Hogg et al., 1968; Yanai et al., 1992). The pathological lesions of small airways disease (chronic bronchiolitis, respiratory bronchiolitis) are similar to those of chronic bronchitis (thickening of the airway walls, increased number of goblet cells,

goblet cell metaplasia, smooth muscle hypertrophy) (Cosio et al., 1978; Saetta et al., 2000). Combined with airway wall inflammation, that could lead to the destruction of the alveolar walls directly attached to the outer surface of small airway walls (Saetta et al., 1985), this results in the deformation of the airway walls and the decrease of their lumen's diameter. In addition, the hyperproduction of mucus, also facilitated by the secretion of neutrophil elastase (by the increased number of neutrophils in the site), a known secretagogue (Nadel, 1991), contributes to the obstruction.

1.1.4 Emphysema

Emphysema is the abnormal, permanent enlargement of the airways distal to the terminal bronchiole, accompanied by destruction of their walls, without any obvious fibrosis (Snider et al., 1985). The emphysematous lesions were first described by J. Laennec, the inventor of the stethoscope, in his book De l'Auscultation Médiate (1819). The elastic recoil of the emphysematous lungs is decreased and this leads to a decreased expiratory flow. Lesions can also be found in subjects with normal lung function. The form of emphysema usually associated with cigarette smoking is the centriacinar type in which the destruction is localized in the central part of the acinus and confined to the respiratory bronchioles while the surrounding areas appear normal. Centriacinar emphysema is more severe in the upper lung lobes (Mahadeva and Shapiro, 2005).

1.1.5 Pulmonary hypertension.

Pulmonary hypertension (PH) can be associated with COPD. Recent studies have shown that the pathophysiology of pulmonary hypertension in COPD is more complex than permanent medial hypertrophy of pulmonary vessels (Barbera et al., 2003; Naeije, 2005; Peinado et al., 2008; Shujaat et al., 2007; Wright et al., 2005). It is now suggested that there is extensive vascular remodeling that leads to more severe PH than previously accepted. The increase in mean pulmonary artery pressure (mPAP) is 25 to 35 mmHg with a normal cardiac output, even though pressures higher than 40 mmHg may also be observed. The severe PH in COPD can eventually lead to right sided heart failure by increasing the right ventricular afterload (Naeije, 2005).

1.1.6 Epidemiology – Prevalence – Incidence

COPD is one of the major causes of mortality and morbidity worldwide and is prevalent in developed as well as in developing countries. It was the sixth leading cause of death in 1990, became fourth since 2000 and is predicted to be third by 2030 (Molina París, 2013). COPD accounts for 2.9 million deaths per year, slightly under HIV/AIDS, and for ≈ 2% of disability caused by disease. Studies have reported a prevalence of COPD that ranges from 0.2% in Japan (Tatsumi, 2001) to 37% in the USA (Ohar et al., 2010). Reported prevalence varies according to the method used to diagnose and classify COPD and when spirometry is used with combination of the GOLD criteria the estimates are greater (Hnizdo et al., 2006; Lindberg et al., 2005a; Lindberg et al., 2006).

While the concept of an existence of a susceptible group of smokers is popular, the notion that only 15% of smokers develop COPD (Fletcher Charles, Peto Richard, Tinker Cecily, 1978) might be misleading (Rennard and Vestbo, 2006). Most probably, with enough smoking, 50% of all smokers could develop COPD even if not presenting disabling respiratory problems but still fulfilling the diagnostic criteria (Rennard and Vestbo, 2006). The incidence of COPD was greater in men and in ages over 75 years, but it has started to increase in women and adolescents, most probably due to a change of cultural standards and smoking habits.

1.2 COPD clinical manifestations

1.2.1 Cough and sputum

The main characteristic symptoms of COPD are dyspnea, chronic cough, and sputum production although the last two do not necessarily mean that the disease is already established, as they can precede it for many years without a decrease in lung function. Chronic cough or sputum are present in 15-44% in men and 6-17% in women and increase with age (Berkhof et al., 2012). Changes in these symptoms are closely related to smoking status (Viegi et al., 2007).

1.2.2 Dyspnea

Dyspnoea is the unpleasant sense of breathlessness and the major symptom of COPD while at the same time the main reason for which subjects seek medical consultation. Dyspnea is a symptom of cardiovascular and respiratory pathological conditions that is usually described by patients as short breath, air hunger, difficult

breathing. Dyspnea suggests an imbalance between the demand on one side and the ability on the other for ventilation. It is therefore present when the need for alveolar ventilation is increased (e.g. increase of metabolic rate) or when the ventilation ability and gas exchange is compromised (e.g. cardiovascular and respiratory disease). As lung volume increases greater breathing effort is required to maintain a constant respiratory volume. In the late onset of the disease this may be absent or related to exercise, but in advanced stages it can also be observed at rest (Antoniu, 2010).

1.2.3 Systemic manifestations

Although COPD is considered primarily a lung disease, studies have shown that it also presents extra-pulmonary manifestations. One of the common complaints of patients is exercise limitation that could be attributed to the airflow limitation and the subsequent increased breathing effort and dynamic hyperinflation (Celli et al., 2004; Pauwels et al., 2001; Siafakas et al., 1995). However, studies have also attributed it to skeletal muscle dysfunction (Donaldson et al., 2012; Furness et al., 2012; Hussain and Sandri, 2013; MacNee, 2013; Roca and Mihăescu, 2012; Seymour et al., 2012; van den Borst et al., 2013). Patients with severe emphysema have either a great weight loss or changes in body mass index which is also an important predictor of increased mortality, independent of poor lung function. This weight loss is associated with increased TNF-α levels (circulating, soluble receptors, released from circulating cells) (Agustí et al., 2003; Di Francia et al., 1994).The weight loss is also attributed to increased metabolism and skeletal muscle loss, particularly lower limb muscles. The muscle weakness results from chronic hypoxia, immobility and increased metabolic rate. Due to the breathlessness and the fatigue, patients often remain inactive by choice or adopt a sedentary way of life.

In addition to the lung inflammation, a similar inflammatory response is observed on a systemic level. This response involves a systemic oxidative stress, inflammatory cells and cytokines in the systemic circulation (neutrophils, lymphocytes, TNF-α and its receptors, IL-6, IL-1β, GM-CSF). Finally, other extra-pulmonary effects are cardiovascular, nervous system and osteoskeletal.

1.3 Risk factors

The risk factors for COPD can be attributed either to the patient (host related) or to the environment (exposures) and the combination of both leads to the development of the disease.

1.3.1 Host factors

1.3.1.1 Genetic factors

The best documented hereditary deficiency that increases the risk of developing COPD is that of α1-antitrypsin responsible for the disease in 1-3% of patients (Stoller and Aboussouan, 2005). A1-antitrypsin (α1-AT), encoded by the gene SERPINA1, first described in 1963 by Laurell and Eriksson, is a serine protease inhibitor that inhibits neutrophil elastase, proteinase 3, and cathepsin G and is part of the protection of the lung against elastolytic damage. Furthermore, it blocks the pro-inflammatory effects of human neutrophil peptide and regulates expression of TNF-α, interleukin-6, interleukin-8, interleukin-1β and monocytes chemoattractant protein 1 (MCP-1). The allele associated with the most severe form of disease is Z (population frequency 1%) although not all PI Z smokers have been seen to develop COPD (Silverman, 2002). In the most severe form of α1-AT deficiency (piZZ) the Z α1-AT also polymerizes and acts as a chemoattractant for neutrophils (Mulgrew et al., 2004; Parmar et al., 2002) leading to a further amplification of inflammation. In patients suffering from this form there is an increase of neutrophil numbers and a decrease in anti-elastase concentration which nevertheless remains active. Other genes that have been studied in this context are genes encoding matrix metalloproteinases and in particular, MMP 9 and MMP 12, TGF-β1 (Celedón et al., 2004), TNF-α etc.

1.3.1.2 Airway hyperresponsiveness

Airway hyperresponsiveness and childhood asthma have been characterized as markers of susceptibility to lung function decline. In a lot of cases, COPD in adult smokers has been traced back to episodes having occurred in childhood (Gold et al., 1989; O'Connor et al., 1995). Airway hyperresponsiveness accelerates the decline in FEV1 (Rijcken et al., 1995). The degree of its severity correlate with an increased decline (Tashkin et al., 1996; Tracey et al., 1995).

1.3.1.3 Gender

Historically, men have been considered to be more susceptible to the development of COPD. But it has been demonstrated that during the past decades, while death rates in males have been stabilizing, they have been increasing in women (Mannino et al., 1997). Data suggests that women are more susceptible to COPD (greater susceptibility to the effects of cigarette smoke, more likely to produce antibodies, more severe small airway disease, greater airway hyperresponsiveness, larger airways) (Chen et al., 1991; Leynaert et al., 1997; Lombardi et al., 2011; Prescott et al., 1997; Ucińska et al., 2012).

1.3.2 Environmental Factors

1.3.2.1 Cigarette smoke

Cigarette smoke is the most important risk factor for the development of COPD. Active smoking accounts for most cases of COPD in developed industrialized countries, but passive smoking is also a risk factor. There is a causal relationship between active smoking and COPD morbidity and mortality. An increase in the number of cigarettes smoked increases the respiratory symptoms and lung function decline and smoking cessation decreases the severity of the symptoms ((US) and Surgeon General, 2004; Doll et al., 2004; Eagan et al., 2004) and returns the rate of decline to that of never-smokers.

Cigarette smoke is a complex mixture of >5000 chemicals (Talhout et al., 2011). It is constituted by a particulate phase (either solid or liquid droplets, more stable, $>10^{17}$ free radicals/gram) in a gaseous phase (less stable, $>10^{15}$ free radicals/puff). More than 200 of these chemical substances have been characterized as toxic to humans and animals (DeMarini, 2004; Husgafvel-Pursiainen, 2004). Cigarette smoke can be distinguished in three types: mainstream smoke (smoke that comes from the mouth end of the cigarette and inhaled by the smoker), side-stream smoke (smoke that comes off the burning end of the cigarette when the smoker puffs) and environmental smoke (the mixture of the two previous ones when the smoker exhales, diluted in the surrounding air). There are significant differences between the three. Side-stream smoke contains higher levels of many hazardous

chemicals than mainstream smoke (Brunnemann et al., 1990; Evans and Sefton, 1992; Grimmer et al., 1987; Pakhale and Maru, 1998; Rodgman et al., 2000).

Furthermore, other factors affecting the smoke composition is the smoking pattern, the type of wrapping material (paper or not, type of paper), the additives, the type of tobacco ((US) and Surgeon General, 2004). There are numerous chemical compounds in cigarette smoke like semiquinones, hydroquinones and metals that increase the oxidative burden, counteract the antioxidant firewall and lead to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The tar phase of smoke contains transition metals like iron that can promote the formation of hydroxyl radicals via Fenton/Haber-Weiss reactions in the lung epithelial lining fluid or form complexes with organic molecules that undergo redox cycling (Kirkham and Rahman, 2006; Li et al., 2008). The most important effects of smoke will be discussed in the oxidative stress section of this introduction. Cigarette smoke also leads to the release of numerous inflammatory mediators. The participation of smoke in the pathogenesis of COPD involves:

I. Damage to nucleic acids, proteins and lipids (Birben et al., 2012; Bowler et al., 2004).
II. Pro-inflammatory activity (Witherden et al., 2004)
III. Inactivation of protease inhibition (Janoff and Carp, 1977)
IV. Depletion of antioxidants (Rahman, 2005)
V. Participation in carcinogenesis (Massion and Carbone, 2003; Witschi et al., 1997)
VI. Decreased binding of steroid receptors (Barnes, 2010)

1.3.2.2 Air pollution

Outdoor and indoor pollution has a clear association with COPD (Bernstein et al., 2004; Bernstein et al., 2008). Outdoor pollution is furthermore one of the exposures present throughout a person's life span. Gaseous or particulate pollutants either emitted directly into the atmosphere (SO_2, NO_x species, CO particulate matter) or formed as secondary products of chemical reactions (ozone, particulates, etc.) deriving from a variety of sources (indoor and outdoor) can cause adverse health effects. A correlation between pollutant levels and lung function decline has been reported by a number of studies (Hogervorst et al., 2006; Holguin et al., 2007). A

major source of indoor pollution is the use of biomass fuel as domestic energy source and cooking which accounts for 50% of deaths from COPD affecting mostly women (75%) (Peña et al., 2000). According to the World Health Organization (WHO), about 50% of households and 90% of rural households worldwide use biomass fuel or coal.

1.3.2.3 Occupational exposures

About 15% of COPD cases can be attributed to occupational exposure (Balmes et al., 2003; Blanc and Torén, 2007). People being exposed to toxic gases in the working environment, farmers exposed to grain dust and factory workers exposed to fumes and dust have a greater risk of developing COPD (Becklake, 1989; Bergdahl et al., 2004; Degano et al., 2012; Diaz-Guzman et al., 2012; Jaén et al., 2006; Lindberg et al., 2005b; Montnémery et al., 2001; Schachter et al., 2009; Shaikh et al., 2012; Sunyer et al., 2005; Voisin and Wallaert, 1992; Zock et al., 2001). It has also been reported that in diagnosed patients past occupational exposures increase the likelihood of developing COPD (Trupin et al., 2003).

1.3.2.4 Other factors

While a decrease in lung function is unavoidable with age, COPD prevalence, morbidity and mortality all increase also in elderly individuals. Studies since the early 1970s have shown a correlation between lung function impairment and socioeconomic status (Bakke et al., 1995; Cohen et al., 1977; Hole et al., 1996; Marmot et al., 1984). Poor socioeconomic status is correlated with other factors that can increase the risk for COPD like intrauterine growth retardation, childhood lung infections, poor nutrition (lower antioxidant intake), increased use of biomass fuel, high work exposures and increased smoking rates, poor access to medical care and, poor housing conditions.

1.4 Pathophysiology of COPD

The three main mechanisms that contribute to the pathogenesis of COPD are inflammation (lung and systemic), oxidative stress and protease-antiprotease imbalance. They do not constitute clearly separated entities but are integrally related with each other.

1.4.1 Lung inflammation

Inflammatory cells. Lung inflammation in COPD affects the small and central airways, the lung parenchyma and the pulmonary arteries. Cigarette smoke inhalation triggers an inflammatory reaction affecting the whole lung without necessitating established airflow limitation that includes neutrophils, macrophages, T-lymphocytes, eosinophils and mast cells (O'Donnell et al., 2006).

The airflow limitation that characterizes COPD succeeds cellular and structural changes of the airways and the inflammatory response triggered by cigarette smoke affects the entire tracheobronchial tree (central and peripheral airways, lung parenchyma and pulmonary arteries). In early stages, the walls of the central airways of smokers are infiltrated by macrophages, T-lymphocytes and low numbers of neutrophils while the latter ones are increased in the lumen. In the peripheral airways (respiratory bronchioles) the infiltrate of the walls consists of macrophages and mononuclear cells. As the inflammation progresses and COPD is established, the numbers of macrophages, T-lymphocytes in the walls as well as that of neutrophils in the lumen, are further increased (Keatings et al., 1996; Saetta et al., 1993). Furthermore, the ratio of CD4+/CD8+ T-lymphocytes is shifted towards the CD8+ which infiltrate the airway walls, parenchyma and the adventitial layer of the pulmonary arteries (O'Shaughnessy et al., 1997; Saetta et al., 1999).

Neutrophils. Two leukocyte-endothelial adhesion molecules, E-selectin and ICAM-1 expressed as ligands for leukocytes cell receptors are involved in the recruitment of neutrophils in sites of inflammation in the lung. ICAM-1 serves as a ligand for CD11b/CD18 whose expression is increased in neutrophils of subjects with a rapid FEV1 decline. Their expression is upregulated by cytokines produced by macrophages, such as IL-1β and TNF-α. Other neutrophil chemotactic factors are IL-8, LTB4 and CCL5. The recruited neutrophils adhere to the endothelial cells and then migrate into the lung where they secrete serine proteases (neutrophil elastases, cathepsin G and proteinase-3) and matrix metalloproteinases (MMP-8 and MMP-9). Although these molecules could play a role in the alveolar destruction process, the negative correlation between the numbers of neutrophils and the degree of destruction (Finkelstein et al., 1995) and the fact that in diseases with more pronounced neutrophilia, elastolysis is not a main feature, suggests that neutrophils do not play an integral part in the generation of emphysema (Barnes, 2004). These secreted molecules are secretagogues and serve in the role of neutrophils localized

in the bronchial glands of COPD patients in the development of mucus hypersecretion and airflow obstruction. Neutrophils localized in the smooth muscle of peripheral airways of COPD patients along with CD8+ lymphocytes further worsen the observed airflow limitation (Baraldo, 2004).

Macrophages. Macrophages along with neutrophils are the two main cell types which produce elastolytic enzymes in the airways of smokers. But macrophages are the most abundant cells in airway lavage under normal conditions and chronic inflammation (Kuschner et al., 1996; Linden et al., 1993). During smoke exposure there is a 5- to 10-fold increase in the numbers of cells in BALF out of which macrophages constitute 98% (Merchant et al., 1992; Shapiro, 1994) and after careful quantification a 25-fold increase in macrophage numbers was observed in tissue and alveolar space (Retamales et al., 2001). Macrophages are recruited with the involvement of MCP-1 and its receptor CCR2 in the airway epithelium (de Boer et al., 2000) and they localize in sites of alveolar wall destruction in emphysematous lungs (Finkelstein et al., 1995; Meshi et al., 2002). The severity of COPD correlates to the numbers of macrophages in the airways (Di Stefano et al., 1998).

Macrophages participate in both the inflammation and emphysema by secreting inflammatory proteins and exhibiting elastolytic activity, a behavior enhanced in COPD patients and further increased by cigarette smoke exposure (Lim et al., 2000; Russell et al., 2002a). Macrophages release reactive oxygen species, destructive extracellular matrix proteases, inflammatory cytokines and chemotactic factors, mucus gland activators and smooth muscle constrictors. The transcription factor NF-κB in activated macrophages from COPD patients is the key regulator of most of the macrophage–produced inflammatory proteins (Birrell et al., 2008; Caramori et al., 2003; Di Stefano et al., 2002; Kent et al., 2008; Kersul et al., 2011; Li et al., 2012; Winkler et al., 2012). Included in the extracellular matrix proteases are at least 20 proteolytic enzymes, the matrix metalloproteinase enzymes (MMPs) that hold a key role in tissue remodeling and repair during development and inflammation. They break down collagen, laminin and elastin (McCawley and Matrisian, 2001). The family members share a 40-50% similarity in amino acids and contain similar structural domains. Their classification according to their substrate specificity and amino acid similarity is shown in Table 1 (Visse and Nagase, 2003):

Table 1. Classification of Matrix Metalloproteinases (MMPs) according to their substrates.

Subclass of MMPs	MMPs
collagenases	MMP-1, MMP-8, MMP-13
gelatinases	MMP-2, MMP-9
stromelysins	MMP-3, MMP-10, MMP-11
membrane-type MMP	MMP-14 to MMP-25
matrilysin	MMP-7
macrophage metalloelastase	MMP-12

They are secreted as inactive pro-enzymes and are activated either on the cell membrane surface or the extracellular space by enzymatic cleavage of their N-terminal domain. Their activity depends upon the disruption of the interaction between an active site zinc ion and a cysteine residue in the prodomain (activation) or the formation of a complex on the C-domain of the pro-MMP with a protein of the family of the tissue inhibitors of MMPs (TIMPs, 1 to 4), also produced by macrophages. The inhibition of MMPs by the different TIMPs is shown in Table 2 (Brew and Nagase, 2010).

Table 2. Tissue Inhibitors of MMPs and their effect on MMPs

TIMP	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MMPs or proMMPs inhibited	MMP-14, MMP-16, MMP-19, MMP-24, Pro-MMP-9	All MMPs, Pro-MMP-2	All MMPs, Pro-MMP-9, Pro-MMP-2	Most MMPs, Pro-MMP-2

In the case of COPD, the two most important MMPs are MMP-9 and MMP-12 as they are responsible for the greater part of the elastolytic activity of macrophages. Several cytokines, mediators, surface molecules (HME, LPS, PMA, ConA, IL-6, IL-1β, TNF-α, α5β1integrin, MMPSF, APMA, EDTA, alpha 2-macroglobulin, TPA, IL-1 and epidermal growth factor) (Belaaouaj et al., 1995; Brew and Nagase, 2010; Busiek et al., 1992; Gomez et al., 1997; Leber and Balkwill, 1998; O'Keefe et al., 1998; Visse and Nagase, 2003; Watanabe et al., 1993; Xie et al., 1998) affect the secretion of MMP-9. It has been demonstrated that the equilibrium between TIMPs

and MMPs is a mechanism that determines the progression of COPD itself (Ohnishi et al., 1998; Shapiro, 1994; Vignola et al., 1998). A particular case of interest is the relation between TIMP-1 and MMP-9 and how IL-10 produced by activated macrophages affects it. In healthy subjects IL-10 results in increased production of TIMP-1 and decreased production of MMP-9 while the situation is reversed in smokers with established emphysema where the increase of MMP-9 appears to be an early feature of the disease (Abbal et al., 1998; Finlay et al., 1997; Lim et al., 2000; Martinez et al., 1997; Mautino et al., 1999; Robinson et al., 1996).

In addition to its elastolytic activity, MMP-12 is also involved in the recruitment of neutrophils in the lung through the release of TNF-α and upregulation of VCAM-1 (Churg et al., 2002; Churg et al., 2003; Dhami et al., 2000; Murugan and Peck, 2009) or the fragmentation of elastin that acts as a chemoattractant for monocytes through the interaction with the elastin-binding protein (Kelly et al., 1987). It is also involved in the degradation and inactivation of α1-AT (Gronski et al., 1997). Another MMP with great significance that has been involved in the pathogenesis of emphysema is MMP-1 which has also been shown to have increased expression in lungs of COPD patients compared to healthy controls (D'Armiento et al., 1992; Fujita et al., 2001; Iizuka et al., 2005). Macrophages are also potentially involved in the resistance to corticosteroid treatment due to reduced HDAC activity through increased secretion of TNF-α and IL-8 (Adcock et al., 2005; Barnes, 2006; Barnes, 2010; Mizuno et al., 2011; Sundar et al., 2013). Finally, macrophages as neutrophils could theoretically be involved in mucus hypersecretion through the activity of IL-1 and LTB4.

1.4.2 Protease/anti-proteinase balance

The hypothesis that an imbalance between proteases and anti-proteases may be an integral part of the pathogenesis of emphysema and consequently of COPD has been based on the observations that α1-AT in humans and studies performed with the intratracheal instillation of elastases in animals leads to the development of emphysema (Churg et al., 2003; Hautamaki et al., 1997; Senior et al., 1977; Shapiro et al., 2003). This imbalance leads to the breakdown of several connective tissue components particularly elastin which is an important target for these enzymes. This results in a loss of elasticity of the lung parenchyma. Elastin is secreted by several cell types as tropoelastin, a precursor. Monomers of tropoelastin in the extracellular

space form elastin polymers by cross linking under the action of lysyl oxidase. These cross-links are known as desmosines and have served as evidence of elastin degradation (Harel et al., 1980). Studies have demonstrated that the excretion of desmosine is increased in smokers with a rapid decline in lung function and there is a positive correlation between the annual rate of decline in FEV 1 and desmosine levels in the urine (Gottlieb et al., 1996). In animals challenged with an intratracheal instillation of elastases lung elastin is quickly depleted and the elastin content is restored yet the architecture is abnormal and resembles to the elastic fibers of human patients with emphysema (Goldstein and Starcher, 1978; Kuhn et al., 1976). A study evaluating BALs from COPD patients reported a direct correlation between NE burden and an inverse correlation with antielastase activity in emphysema (Fujita et al., 1990), supporting further the protease/antiprotease imbalance hypothesis.

Although many proteases that break down elastin have been implicated in this process (Stockley and Campbell, 2001), neutrophil elastase has been of great interest due to the observations that patients with α1-AT develop early onset emphysema, the results from animal models of emphysema using neutrophil elastase (Janoff et al., 1977; Senior et al., 1977) and its localization on elastin fibers and emphysematous lungs (Damiano et al., 1986). While α1-AT inhibits neutrophil elastase, the alveolar macrophage elastase remains active and degrades elastin (Chapman and Stone, 1984; Chapman et al., 1984). However, α1-AT is ineffective in inhibiting NE already bound to elastin (Morrison et al., 1990). Smokers exhibit a reduction of about 40% α1-AT function when compared to non-smokers (Gadek et al., 1979). However the decrease of activity in BALF was temporary and non-significant 1 hour after smoking (Abboud et al., 1985). The mechanism of α1-AT functional inactivation is the oxidation of methionine in its active site (Carp et al., 1982; Gadek et al., 1979). The reduction of activity of anti-elastase activity by smoke is also complemented by the recruitment of increased number of neutrophils that in turn secrete NE. The severity of the emphysema correlates to the increase of NE (Kidokoro et al., 1977). Neutrophil elastase is a serine protease with multiple functions and properties:

- Degrades elastin.
- Is stored in azurophilic granules of neutrophils.
- Is expressed on the surface of cells primed by cytokines.

- Induces the expression of MUC5AC through a mechanism dependent on the generation of ROS.
- Is a potent secretagogue and induces hypersecretion of mucus by submucosal and gland cells, a property inhibited by some antibiotics like erythromycin (Goswami et al., 1990) and flurithromycin (Gorrini et al., 2001).
- Renders macrophages incapable of clearing apoptotic cells (including neutrophils) by cleaving the phospatidylserine receptor on their surface.
- Increases the inflammatory response to endotoxin by the inactivation of CD14 (receptor for LPS).
- Its inactivation in mice increases their susceptibility to infections.

Other proteases.

Other serine proteases: Cathepsin G and Proteinase 3 are also stored in neutrophilic granules and have similar properties to those of NE (Sommerhoff et al., 1990; Witko-Sarsat et al., 1999). Cathepsins B, C, D, H, L and S may also contribute to emphysema (Reilly et al., 1989; Reilly et al., 1991; Shi et al., 1992; Turk et al., 2001). They are overexpressed in mouse lungs when interferon gamma is inducibly targeted (Chapman et al., 1997; Wang et al., 2000). Alveolar macrophages from COPD patients secrete more cysteine proteases than healthy smokers or non-smokers do (Russell et al., 2002b).

Metalloproteases.

Metalloproteases include MMPs, A disintegrin and metalloproteases (ADAMs), and ADAMs with thrombospondin motifs (ADAMTS). They serve in the modulation of tissue structure and function under both normal and pathological conditions and are involved in intracellular communication, cell differentiation during embryogenesis and development and tissue remodeling after injury (Flannery, 2006; Malemud, 2006). Pro-inflammatory cytokines like IL-6, TNF-α, IL-4 and IL-13 and inhibitors like TIMPs and a-2M regulate their function. As mentioned before, the fragmented elastin fibers also have a pro-inflammatory property and serve as chemoattractants for monocytes (Houghton et al., 2006; Hunninghake et al., 1981; Senior et al., 1980).

Table 3 summarizes (Barnes, 2004; Hayashi et al., 1996; Lagente et al., 2005; Nénan et al., 2005; Newby, 2006; Risinger et al., 2006; Yu and Stamenkovic, 2000) the main characteristics of the most important MMPs involved in the pathogenesis of COPD.

Table 3. The main MMPs involved in the pathogenesis of COPD.

MMP	Source	Substrate	Additional function
MMP-12	Alveolar macrophages	Elastin	Pro-inflammatory
MMP-9	Alveolar macrophages, bronchial epithelial cells, mast cells, eosinophils and neutrophils	Elastin, collagen IV, V, VII, X, gelatin, pro-MMP-9 and pro-MMP-13	Activation of the latent TGF-β and IL-8
MMP-1	Alveolar macrophages, alveolar epithelial type II and bronchial epithelial cells	Collagen I, II, III, IV, X	Activation of pro-MMP-9 and pro-MMP-2
MMP-2	Bronchial epithelial and airway smooth muscle cells	Collagen I, II, III, IV, VII, X, XI, XIV, elastin, fibronectin, gelatin	Regulation of smooth muscle cell proliferation

Abbreviations: MMP: matrix metalloproteinases, TGF-β: transforming growth factor beta, IL-8: interleukin 8.

It has also been demonstrated that some MMPs interact with their respective inhibitors or those of others and this interaction can further worsen the reaction to cigarette smoke as in the case of MMP-12 degrading α1-AT and NE degrading in turn TIMPs (Shapiro et al., 2003).

Since MMPs attack substrates either exclusively other than elastin or in addition to it, the studies focused on them have speculated that emphysema may be related to the degradation of the lung matrix as a whole and not only elastin (Churg et al., 2012).

1.4.3 Oxidative/nitrosative stress

Oxidative stress is defined as “a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage” (Sies 1985, 1986). Once the inflammatory cells are recruited in the air space they are activated and generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). Activated macrophages, neutrophils and eosinophils generate O_2^- which is then converted into H_2O_2 under the catalytic action of SOD and can then react to ·OH in the presence of Fe^{2+}. ROS are also produced intracellularly mainly through the NADPH oxidase system and mitochondria (Macnee, 2006). Cigarette smoke contains high concentrations of oxidants and free radicals. The O_2^- and NO of the gas phase of cigarette smoke react quickly to a form the highly reactive peroxynitrite. Due to its great diffusion coefficient peroxinitrite can cause greater damage in the tissues around its site of production. Radicals of the tar phase react rapidly with molecular oxygen in a redox-dependent manner to form O_2^-, ·OH and H_2O_2.

COPD patients exhibit higher levels of oxidative and carbonyl stress markers like nitrotyrosine, hydrogen peroxide, carbon monoxide, myeloperoxidase (MPO) (Ichinose et al., 2000; Montuschi et al., 2001; Paredi et al., 2000).

Oxidative stress can stimulate the release of inflammatory chemotactic factors like IL-8 in the lungs of smokers (Gilmour et al., 2003), activate the NF-kB pathway, and directly damages protein (carbonylation), lipids (peroxidation), carbohydrates and DNA (Aoshiba et al., 2012; Wistuba et al., 1997).

This increased release of ROS from peripheral neutrophils and monocytes depletes the antioxidant firewall in patients with COPD in both plasma and lungs. Antioxidants such as GSH uric acid ascorbic acid have been found to be reduced in smokers. In the case of GSH, cigarette smoke inhalation results in the formation of GSH conjugates in the airways that leads to an antioxidant deficiency and an injurious lung response. Oxidative stress also leads to autophagy for the removal and degradation of damaged mitochondria and oxidized proteins as a defense mechanism. However, excessive autophagy finally leads to cell death.

Nevertheless, while ROS were initially from their discovery considered harmful, they have also been shown to act as messaging molecules, participating in signal transduction. Studies have demonstrated that ROS production after ligand binding of growth factors as PDGF and EGF is necessary for the physiological tyrosine kinase signaling by altering the phosphatase-kinase balance (Bae et al., 1997; Sundaresan et al., 1995) or that they play a role in enzymatic activation (Guo

et al., 2010). These types of ROS effects indicate a role in the regulation of intracellular signaling pathways.

1.4.4 Apoptosis

“Apoptosis” is a Greek word that means “to fall/drop of” (απόπτωσις, από=of/from, πτώσις=falling). It is the term used to describe the morphological changes in a complex and tightly regulated process of programmed cell death. These changes include cell shrinkage, nuclear condensation, blebbing of the membrane, fragmentation into membrane bound apoptotic bodies and changes of the membrane that lead to phagocytosis of the cell that undergoes cell death (Kerr et al., 1972; Wyllie et al., 1980).

Apoptosis has the opposite effect of cell division. However, it complements the latter in the homeostasis of cell populations and allows the elimination of unwanted, heavily damaged or infected cells. The various stimuli that trigger apoptosis are direct induction by immune cells, activation of death receptors, growth factor deprivation, loss of contact with the extracellular matrix and various heavy damages caused by stress. There are three main apoptotic pathways:

1) **the extrinsic pathway**. It is triggered by extracellular signals, namely death ligands like the TNF, FasL and TRAIL that bind to their receptors on the cell surface (Strasser et al., 2000). The receptors are then multimerized and form the death inducing signaling complex (DISC) that contains adaptor molecules like the Fas associated death domain (FADD). This results in the cleavage of pro-caspase-8/10 into their active forms, caspase-8/10 (Muzio et al., 1996; Muzio et al., 1998) that exhibit a protease activity and cleave specific substrates and start activating other caspases (3, 6, 7) (Hirata et al., 1998; Scaffidi et al., 1998) that finally cause DNA fragmentation (Tang and Kidd, 1998).

2) **the intrinsic pathway**. It is mainly triggered by processes that cause DNA damage, like oxidative stress (Roos and Kaina, 2006; Roos and Kaina, 2013). This leads to the activation of tumor suppressor p53 routes which induces cell cycle associated (like p21,14-3-3σ) or apoptosis-related (Bax, NOXA, PUMA, Fas) gene transcription. If the DNA damage was severe enough these factors will translocate to the outer membrane of mitochondria where channels that allow pro-apoptotic factors like DNAase, cytochrome-C and anti-apoptotic factors inhibitors to move to the

cytoplasm. Consequently the apoptosome (cytochrome-C, Apaf-1 and caspase-9) is formed (Li et al., 1997; Liu et al., 1996; Zou et al., 1997) and caspase-9 is activated which in turn activates caspase-3 and initiates apoptosis (Slee et al., 1999).

3) **the endoplasmatic reticulum pathway**, is triggered by cell signals such as hypoxia and apoptosis is induced through caspase-7-mediated caspase 12 (Rao et al., 2001; Szegezdi et al., 2003) activation.

In human studies increased apoptosis has been demonstrated in several lung structural cells: endothelial cells (Imai et al., 2005; Kasahara et al., 2001; Segura-Valdez et al., 2000; Yang et al., 1996), alveolar epithelial cells (Imai et al., 2005; Kasahara et al., 2001; Segura-Valdez et al., 2000; Yang et al., 1996; Yokohori et al., 2004), interstitial cells (Segura-Valdez et al., 2000), inflammatory cells (Segura-Valdez et al., 2000), mesenchymal cells (Imai et al., 2005), airway epithelial cells and BAL T-cells (Hodge et al., 2005). The samples in these studies came from lung tissue sections or BALs from emphysema or COPD patients. In addition, the pro-apoptotic factors (Bax, Bad) were detected in emphysema patients but not in controls (Imai et al., 2005), and expression of VEGF and VEGFR2 mRNA and protein were significantly reduced in emphysema (Kasahara et al., 2001). Another study has shown decreased VEGF levels in induced sputum from COPD with severity of the disease (Kanazawa and Yoshikawa, 2005).

In COPD patients the phagocytosis of apoptotic airway epithelial cells by alveolar macrophages is decreased compared to controls (Hodge et al., 2003). The mechanism behind this is the cleavage of the phospatidylserine receptor on macrophages by neutrophil elastase secreted by activated neutrophils (Keatings et al., 1996; Lacoste et al., 1993; Vandivier et al., 2002). Furthermore, the increased numbers of CD8+ cells in the lungs of COPD patients (Saetta et al., 1998; Saetta et al., 1999) are able to induce apoptosis through perforins (Barry and Bleackley, 2002; Liu et al., 1999). Neutrophil elastase has also been shown to induce apoptosis of small airway and alveolar epithelial cells via the intrinsic pathway and decreasing AKT phosphorylation (Suzuki et al., 2005). A1-AT also can inhibit active caspases and consequently apoptosis of lung endothelial cells exposed to smoke (Aldonyte et al., 2008) and staurosporine (Petrache et al., 2006). The MMPs also might be involved in apoptosis since the Fas ligand is their (MMP-7) substrate (Kayagaki et al., 1995; Powell et al.). Apoptotic lung epithelial cells have exhibited elastolytic

activity in an emphysema model in mice (Aoshiba et al., 2003). Apoptosis is also linked to oxidative stress. In a rat model of emphysema induced by VEGF receptor blockade, apoptosis was increased in areas of the lung where oxidative stress was present while the oxidative markers were reduced after the blockade of apoptosis (Tuder et al., 2003). Mice exposed to smoke for a period of 6 months showed enhanced alveolar oxidative stress; and increased number of apoptotic alveolar septal cells when compared to wild type mice (Rangasamy et al., 2004). Oxidative stress is also associated with reduced levels of VEGF in sputum of COPD patients (Kanazawa and Yoshikawa, 2005).

1.5 COPD in other animal species.

Pathological conditions similar to COPD are also seen in other animal species, most prominently in horses and small companion animals (dogs and cats) with respective differences in etiology, pathophysiology, symptomatology and treatment.

In horses, COPD is now referred to as recurrent airway obstruction (RAO) or with its more empirical name “heaves”. It constitutes the most common cause of chronic cough in this animal species. Other clinical manifestations are exercise intolerance and in more severe cases expiratory dyspnea. Susceptible (genetically predisposed) horses exposed to airborne agents most notably organic dust containing molds (*Aspergillus fumigatus*, *Faenia rectivirgula*, *Thermoactinomyces vulgaris*) (Séguin et al., 2010; Séguin et al., 2012), bacterial endotoxins, proteases among others, found in the housing stables and the hay used for feeding, develop the disease. In the stables horses can also be exposed to high levels of gases such as ammonia that also contribute to the induction of the disease. Removal of the animals outdoors or in stable with less dust burden can cause remission but the disease may persist either due to irreversible structural alterations (Kaup et al., 1990) or to mild airway inflammation (Bureau et al., 2000). RAO affects a considerable number of horses in Great Britain (14%) (Hotchkiss et al., 2007). While the immunological mechanism of the disease is not fully understood, and the clinical symptoms show similarities with human asthma, RAO is a much more complex condition than an allergy. The pathophysiology of the disease presents several discrepancies with asthma. For example in the BALF of affected horses it is neutrophils that predominate (Art et al., 2008) and there is absence of increased histamine levels (McGorum et al., 1993), while additionally a delayed bronchospasm

occurs after antigen challenge (Deaton et al., 2007). T-lymphocytes and epithelial cells play a central role in the pathogenesis of the disease by recruiting neutrophils into the airways. These cells along with macrophages most likely produce MMPs (MMP-1, MMP-8, MMP-9, MMP-13) (Nevalainen et al., 2002; Raulo et al., 2001). Finally, the structural changes of the small airway walls associated with bronchiolitis lead to airway obstruction. This obstruction commonly leads to emphysema, predominantly of the distensive type (Marinkovic et al., 2007).

In dogs and cats chronic bronchitis (CB) results in a chronic cough for more than two months that cannot be attributed to other causes like chronic heart failure, bronchopneumonia or pulmonary neoplasia. The disease more commonly affects small or toy, older and overweight dogs, although it can also be reported in larger breeds (Rozanski, 2014). The cough is typically dry, non-productive that mostly occurs after excursion (exercise intolerance) or at night produced by secretion accumulation. The animals are also presented with wheezing, abdominal push, crackles during auscultation, cyanosis and even syncope in severe cases (Johnson, 2008). Irritation of the trachea also leads to coughing. The most common etiological factors are recurrent airway inflammation from infections or irritant inhalation. Glandular hypertrophy and goblet cell hyperplasia lead to increased mucus production. Epithelial edema, thickening and metaplasia lead to airway narrowing. In more advanced cases mild emphysema is also present, making the condition more similar to the human disease. However dogs do not present clinical signs of emphysema (Johnson, 2006).

1.6 Sestrin 2

Sestrin 2 belongs to a family of highly conserved proteins that are ~48-60 kDa in size. Mammalian cells express 3 sestrin isoforms: i) sestrin 1 (SESN1 or PA26), ii) sestrin 2 (SESN2 or Hi95) and iii) sestrin 3 (SESN3). Sestrins were initially discovered via subtractive cDNA cloning experiments to identify p53 target genes (Velasco-Miguel et al., 1999) (sestrin 1) and hypoxia upregulated genes (sestrin 2 and sestrin 3); after attention was drawn to p53 and its mutations and/or inactivation in cancer (Budanov et al., 2010). Sestrin 2 was discovered 2 years (2001) later than sestrin 1 as a close homologue of the latter. The name of the protein family comes from Sestri Levante, a town located in the Italian Riviera where, during a human genetics course, the high amino sequence homology between the 3 family members

(Budanov et al., 2004) was discovered (Peeters et al., 2003). The sestrin 2 gene is located on chromosome 4 in the mouse and chromosome 1p35.3 in humans.

Their role is believed to be the regeneration of peroxiredoxins (Prxs), a family of thiol-containing peroxidases that catalyze the destruction of endogenously produced peroxides in eukaryotes (Seaver and Imlay, 2001) and decompose RNS (Barr and Gedamu, 2003; Bryk et al., 2000; Chen et al., 1998; Wong et al., 2002), even though that is a matter of debate where sestrin 2 is concerned (Woo et al., 2009). Peroxiredoxins are inactivated through an overoxidation of a reactive cysteine (peroxidatic cystein, Cys-SH) in their catalytic center (Rabilloud et al., 2002; Yang et al., 2002). Under conditions of high peroxide concentrations, Prxs are overoxidized and yield sulfinic acid (Cys-SO_2H) and cannot be reduced by typical reductants like glutathione. Normally this peroxidatic cysteine is oxidized to sulfenic acid that forms a disulfide bridge with the resolving cysteine of the other subunit of the Prx dimer, which is consequently reduced by thioredoxin (Chae et al., 1994a; Chae et al., 1994b; Woo et al., 2003). But the slow formation of the disulfide bridge allows further oxidation of the sulfenic acid to sulfinic acid (Cys-SO_2H) that cannot be reduced (Wood et al., 2003). As a consequence Prxs become inactive and accumulate in the oxidative stress-exposed cells (Budanov et al., 2004). This mechanism allows peroxide, which acts as a signaling molecule, to convey its message to redox-sensitive components of signaling pathways by inactivating peroxidase activity (Georgiou and Masip, 2003; Wood et al., 2003), an apparent evolutional advantage of eukaryotes that use peroxide for that purpose (Wood et al., 2003). While both sulfiredoxins (Srx) and sestrins have been reported to be responsible for this regeneration (Budanov et al., 2004; Chang et al., 2004), it was later demonstrated that neither sestrin 2 has the reductase activity of sulfiredoxin nor exists a sequence similarity between the two (Woo et al., 2009). The mechanism by which sestrin 2 participates in the regeneration of Prxs was shown to be via the autophagic degradation of the Kelch-like ECH-associated protein 1 (Keap-1), a repressor of the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) (Itoh et al., 2010), which then activates Srx (Bae et al., 2013). In conclusion, the antioxidant role of sestrin 2 is the promotion of the antioxidant Nrf2 signaling, which activates Srx among other target genes and finally leads to the regeneration of Prxs.

Sestrins are also implicated in the mammalian target of Rapamycin (mTOR) inhibition via a p53-activation mechanism through a redox-independent manner

(Budanov and Karin, 2008). mTOR, a serine/threonine protein kinase that belongs to the PIKK (phosphatidylinositol kinase-related kinase) family and regulates cell growth and proliferation (Corradetti and Guan, 2006; Fingar and Blenis, 2004; Guertin and Sabatini, 2005; Hay and Sonenberg, 2004; Soliman, 2005; Thomas, 2006; Wullschleger et al., 2006), forms two distinct signaling complexes: i) mTORC1 (mTOR along with raptor, PRAS40 and mLST8) that regulates cell growth and protein synthesis (Guertin and Sabatini, 2005; Yang and Guan, 2007), and ii) mTORC2 (mTOR with rictor, sin1, Protor-1 and mLST8) that regulates the actin cytoskeleton and cell spreading (Astrinidis et al., 2002; Sarbassov et al., 2004; Yang and Guan, 2007). mTORC1 phosphorylates the S6K1 and 4E-BP-1 to enhance translation within the ribosomal translation initiation complex and cap-dependent mRNA translation respectively (Choi et al., 2003; Nojima et al., 2003; Schalm and Blenis, 2002; Wullschleger et al., 2006). The mTOR signaling pathway has been shown to have several upstream regulators: growth factors (Ma et al., 2005; Potter et al., 2002; Roux et al., 2004), hypoxia (Arsham et al., 2003; Brugarolas et al., 2004; Hudson et al., 2002), ROS (Bae et al., 1999; Sarbassov and Sabatini, 2005; Smith et al., 2005), etc., including sestrin 2 (Budanov and Karin, 2008). Sestrin 2 is a potent inhibitor of mTOR through the TSC1:TSC2 complex via stimulation of AMPK, a major TSC2 kinase and by phosphorylation of TSC2 which also depends on AMPK (Budanov and Karin, 2008). mTOR acts as a crucial regulator of cell growth and proliferation but also exerts anti-inflammatory (Säemann et al., 2009; Weichhart et al., 2008; Weichhart et al., 2011) and angiogenic effects (Advani, 2010).

As p-53 target genes, sestrins participate in protection of cells from various insults. Under normal physiological conditions, sestrins offer a baseline protection from any damage resulting from processes like respiration and DNA replication. Under genotoxic stress and DNA damage save energy that can be directed towards DNA repair by minimizing new protein and membrane synthesis through attenuating TORC1 activity via AMPK activity enhancing, that leads to inhibition of anabolic pathway also resulting in arrest of lipid and protein synthesis. This also inhibits proliferation and promotes the removal of cells with DNA damage. Under oxidative stress, the induction of sestrin transcription results in ROS scavenging and peroxiredoxins regeneration and furthermore, through the inhibition of TORC1, in damaged mitochondria elimination (Jin, 2006). An additional effect of TORC1 shutdown is the improvement of mitochondrial respiration and the reduction of ROS

production (Bonawitz et al., 2007). Finally in hypoxic conditions, the TORC1 inhibition by sestrin counteracts apoptosis and once more saves energy for the cells to adapt.

These effects of sestrin 2 activation are summarized in Fig. 1.

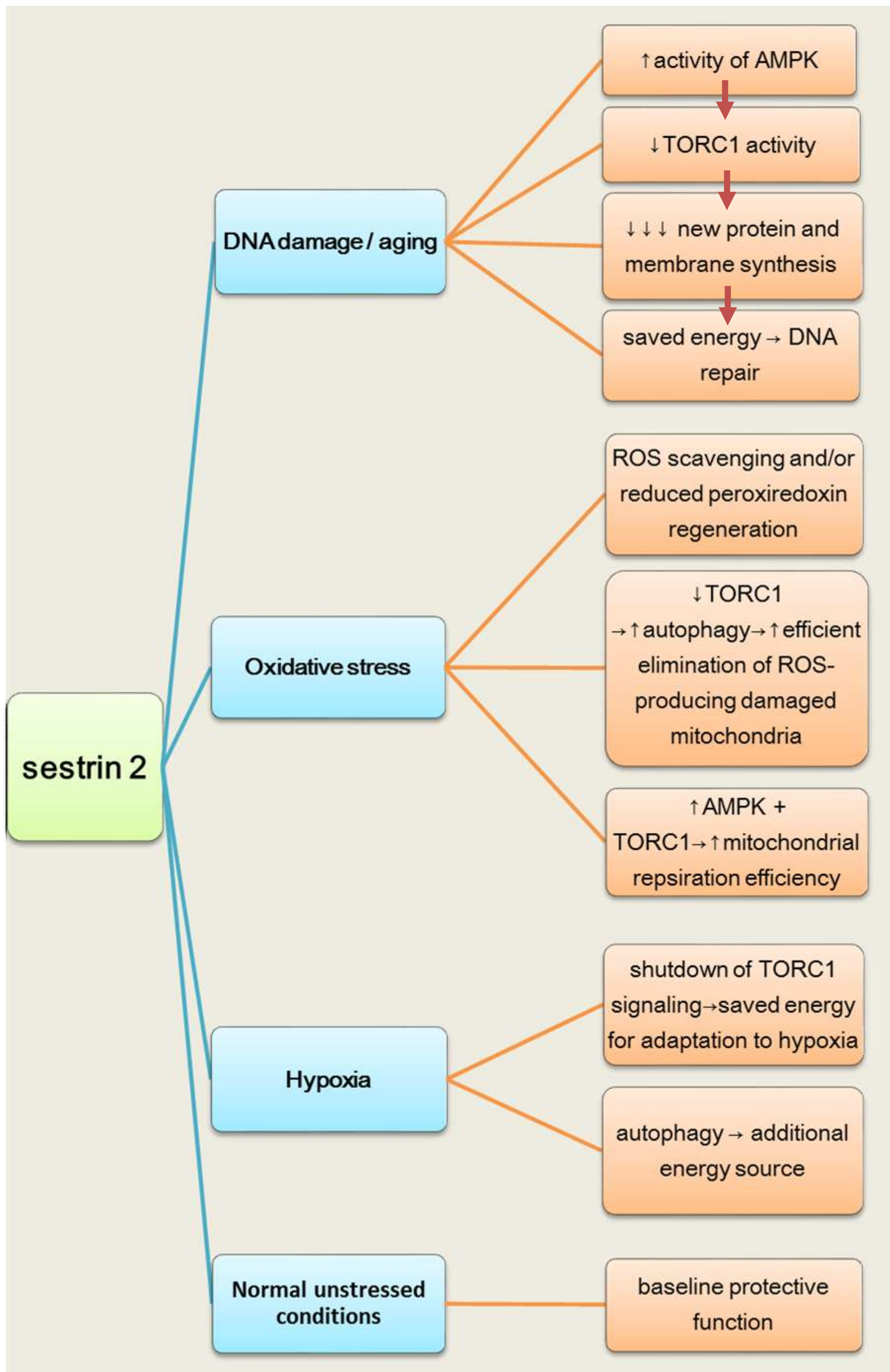


Figure 1. Outline of sestrin 2 functions under stress and normal conditions.

Besides mTOR, sestrin 2 has been implicated in pulmonary emphysema by regulating the TGF-β pathway, also involved in tissue remodeling and regeneration. Sestrin 2 mutational inactivation partially rescued the emphysema phenotype in a mouse model (Ltbp4S$^{-/-}$ mice), an effect correlated with the upregulation of TGF-β and mTOR pathways. In addition the effect of sestrin 2 inactivation on mTOR is TGF-β- and not redox-dependent (Wempe et al., 2010).

Furthermore, sestrin 2 silencing increases PDGFRβ expression in human glioblastoma U87 cells and results in PDGFRβ accumulation due to slow degradation (Liu et al., 2011). A similar upregulation has been demonstrated in mouse embryonic fibroblasts lacking peroxiredoxin II (PrxII) (Choi et al., 2005) which theoretically requires sestrin 2 for its regeneration in some cellular contexts. PDGF is a potent mitogen and chemoattractant involved in the recruitment of mesenchymal cells (Betsholtz, 2004) and wound repair (Gao et al., 2005). PDGFRβ is a tyrosine kinase that has five immunoglobulin domains extracellularly and a split tyrosine kinase domain in the cytoplasm. Binding of the ligand to the receptor leads to receptor dimerization and activation of the tyrosine kinase domains. This leads to autophosphorylation of several tyrosine residues in the cytoplasmic domains of the receptor and the creation of docking sites for several signaling proteins and adaptors (Heldin and Westermark, 1999). After activation the receptor is internalized and either degraded in lysosomes or recycled back to the cell surface for reuse. Most cell types do not express simultaneously the ligand and the receptor, indicating a paracrine mode of action. However, fibroblasts and vascular smooth muscle cells express both, making an autocrine action possible. PDGFRβ participates in embryonic development while in the adult it mainly plays a central role in wound healing. Activation of the PDGFRβ receptor regulates important processes such as ECM production proteins crucial in repair and angiogenesis through controlling of the differentiation of fibroblasts into myofibroblasts and vascular smooth muscle cell and pericyte recruitment and proliferation respectively (Abramsson et al., 2003; Betsholtz, 2004; Gao et al., 2005). PDGFβ and PDGFRβ null mutants die near birth from widespread hemorrhages (Levéen et al., 1994; Soriano, 1994) due to diminished numbers of vascular smooth muscle cells and pericytes. During embryonic development, PDGFβ/PDGFRβ signaling seems to participate in the induction of proliferation of these types of cells during their recruitment to new vessels where they express the receptor and the sprouting epithelium expresses the ligand. In the null

mutants the new vessels are unable to attract pericytes which serve to support the vessel wall and control the formation of normal diameter and function (Betsholtz, 2004). Thus the newly formed capillaries are weak and increased blood flow can lead to rupturing. Finally, PDGFRβ signaling plays an important role in all stages of wound healing where it participates in the recruitment of inflammatory cells immediately after injury (Heldin and Westermark, 1999; Trengove et al., 2000; Vogt et al., 1998), granulation tissue formation (Uutela et al., 2004), blood vessel maturation (Lindahl et al., 1997; Sundberg et al., 1997) and in the production by fibroblasts of ECM proteins necessary for tissue repair and remodeling (Jinnin et al., 2005; Lin et al., 2006; Rhee and Grinnell, 2006).

1.7 Emphysema models

The two emphysema models used in the experiments conducted were the smoke-induced and elastase-induced emphysema in the mouse. While other rodent species (rats, hamsters, guinea pigs) have been used to model the disease, the mouse is the most favorable one for studying emphysema. Many reasons make this species an ideal choice: the mouse genome has been sequenced as well as the human one and only a few differences have been demonstrated (about 300 genes) between them (Waterston et al., 2002), a good knowledge of its anatomy and physiology, the variety of naturally occurring strains, extensive antibody availability, high reproductive turnover, short life span, low cost of maintenance and housing.

1.7.1 Cigarette smoke-induced emphysema

Since cigarette smoke is by far the most common cause of COPD in developed countries, it has been one of the most attractive experimental models to be used on a variety of animal species (guinea pigs, rabbits, dogs, rats, mice). The effectiveness of the model depends on numerous factors such as the mouse strain, type of exposure (whole-body chambers, nose-only exposure systems), type of smoke used (mainstream, side-stream), duration of exposure, smoke concentration, brand and number of cigarettes, sex and age of mice used in the experiments. In general, a smoke exposure of at least 6 to 8 months, 5-6 hours/day, 5 days/week will induce lesions in mice similar to those of a mild form of emphysema in humans (Guerassimov et al., 2004; Martinez et al., 2007; Takubo et al., 2002). Immediately after the beginning of cigarette smoke exposure neutrophils are recruited,

macrophages accumulate in tissue destruction occurs. Physiologically, the residual volume, functional residual capacity, total lung capacity and compliance are increased, flow decreases in the lower lung volumes (Wright et al., 2008). Histologically, goblet cell metaplasia occurs (Bartalesi et al., 2005), small airway walls are thickened (Bracke et al., 2006; Churg et al., 2006) (mostly collagen and fibronectin and not smooth muscle participates in this thickening), lymphocytes aggregate around the bronchioles (Bracke et al., 2006; Bracke et al., 2007). Smoke also produces vascular remodeling in this animal species (Nadziejko et al., 2007; Seimetz et al., 2011). In the end, this model produces a disease severity similar to a GOLD stage 1 or 2 (Wright et al., 2008). Although the underlying mechanisms between this animal model and human COPD may not all be identical, the smoke-induced emphysema model is a useful tool for the investigation of cellular and molecular mechanisms involved in the development and progression of emphysema, while allowing to study the effects of the most common risk factor of COPD, cigarette smoke.

1.7.2 Elastase-induced emphysema

The intratracheal instillation of tissue-degrading enzymes has also been used to produce emphysema-like lesions. After the initial report of the early development of emphysema in α1-antitrypsin deficient patients (Laurell and Eriksson 1963) and the description of emphysema induction in rats by instillation of papain (Gross 1965) a lot of other studies followed that contributed to the formulation of the protease-antiprotease hypothesis. Although numerous enzymes have been employed, the observation that only those that attack intact elastin can produce emphysema, led to the conclusion that the fundamental pathological abnormality was the destruction of the elastin framework. Most studies use porcine pancreatic elastase (PPE) or human neutrophil elastase (HNE). PPE is the most used, since it has produced the most consistent and impressive results (airspace enlargement), is easy to obtain and is much less expensive than HNE (Antunes and Rocco, 2011). Different protocols exist (single to repeated instillations of elastase, dose of elastase administered) but a single administration of elastase is adequate to produce emphysema-like lesions within 21 days in mice. The severity of the disease depends on the dose selected. Thus, it is possible to rapidly produce severe emphysema with this model. These

characteristics make the elastase-induced emphysema model a very useful tool in the investigation of new drug interventions.

While the mechanism and basic principle on which this model works initially appears simple as a digestion of lung tissue by the enzyme, when the half-life of elastase was measured (Stone et al., 1988) it was found to be only about 50 minutes. Since an intervention with an inhibitor did not prevent progression, it is suggested that other processes are involved than just the enzyme activity of elastase bound to the matrix. Several elastase inhibitors and elastin protectors have only partially protected from emphysema (Cantor et al., 2000; Lafuma et al., 1991; Lungarella et al., 1986), suggesting a more complicated mechanism. The elastase instillation also leads to an inflammatory response involving cells and mediators (neutrophils, macrophages, TNF-α, IL-1b, IL-6,IL-8) (Gamze et al., 2007). It is also speculated that it can also mediate apoptosis (extrinsic pathway) through a TNF/TNFR interaction (Lucey et al., 2002). Additional evidence also suggests that the application of elastase acts not only as a mechanistic attack on elastin but involves abnormal repair of collagen (Hoffman et al., 2010; Ito et al., 2005; Kononov et al., 2001; Lucey et al., 1998; Rubio et al., 2004).

Elastase, depending on the instillation techniques, can produce more homogenously distributed lesions in the lung and produces panacinar emphysema, more closely related to genetic emphysema in humans, with lesions that are stable and do not regress like in the smoke model. Moreover, it also produces systemic effects similar to those seen in human COPD patients (Fournier and Lewis, 2000; Lüthje et al., 2009; Mattson et al., 2002; Supinski and Kelsen, 1982). Overall, while this model employs partially different mechanisms than the smoke exposure one and results should be extrapolated to human disease with caution, it is a very valuable screening technique for new interventions.

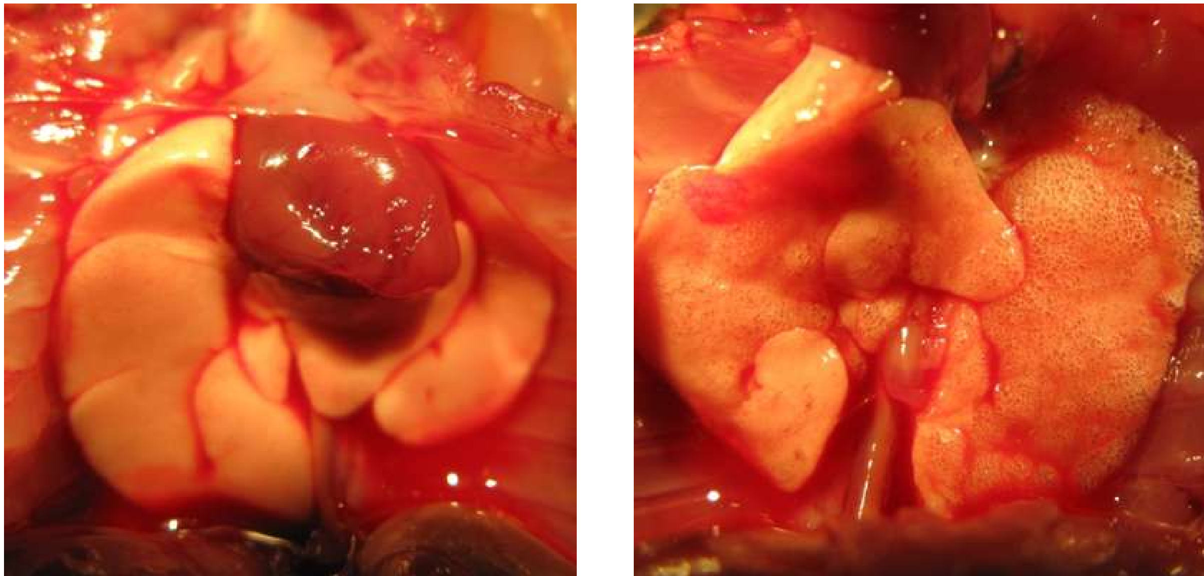


Figure 2. Macroscopic lesions of emphysema in mice lungs after PPE instillation.

Left panel: control lung, right panel: emphysematous lung (0.6U of elastase, 3 weeks after instillation), (photographs taken from own experiments).

2. AIMS OF THE STUDY

The main objective of this thesis was to investigate sestrin 2 as a new possible therapeutic target for the treatment of COPD and its role in the development of emphysema.

In this context, the smoke-induced emphysema model and the elastase-induced emphysema were employed to:

1. Establish if the mutational inactivation of sestrin 2 would protect mice from developing smoke-induced emphysema/COPD.
2. Elucidate the involvement of setrin 2 in disrupting alveolar maintenance programs in regards to PDGFRβ and mTOR signal transduction pathways.
3. Determine whether PDGFRβ constitutes a central player in emphysema pathogenesis by employing the elastase-induced emphysema in combination with a PDGFRβ inhibitor (imatinib).
4. Investigate whether PDGFRβ activation is ROS-dependent, since sestrin 2 appears to contribute to the redox homeostasis.

3. MATERIALS

3.1 Solutions and substances

- Heparin: Heparin-Natrium-25000-ratiopharm®, B. Ratiopharm GmbH, Ulm, Germany
- Ketamin hydrochloride 100 mg/ml, Ketamin 10%®, Belapharm GmbH & Co KG, Vechta, Germany
- Xylazin hydrochloride, Xylazin 2%®, Ceva Tiergesundheit GmbH, Düsseldorf, Germany
- Sterile physiological saline solution (for injections) Braun, Melsungen, Germany
- Isoflurane Isofluran Baxter®, Baxter Germany GmbH, Unterschleißheim, Germany
- Sodium chloride NaCl 0,9% Ecotainer®, Braun, Melsungen, Germany
- Formaldehyde solution 3.5-3.7%, Otto Fischar GmbH & Co, Saarbrücken, Germany
- 50% 2 propanol, 1%povidone-iodine (10% available iodine), Braunoderm ®, B. Braun Melsungen, AG Melsungen, Germany
- Isotonic saline, isotonic sodium chloride solution 0,9% Diaco ®, Serag-Wiessner KG, Naila, Germany
- Xylocain1%, AstraZeneca GmbH, Wedel, Germany

In the elastase-induced emphysema experiment

- Elastase: PPE, ET947, Elastin Products Company, Inc., Owensville, Missouri, USA
- Elastase Substrate:Succinyl-Ala-Ala-Ala-pNitroanilide,NS945, Elastin Products Company, Inc., Owensville, Missouri, USA
- Imatinib, Glivec® 400 mg, Novartis International AG, Basel. Switzerland
- Tris HCL, Carl Roth GmbH & Ko, Karlsruhe, Germany
- Tris, Carl Roth GmbH & Ko, Karlsruhe, Germany
- Sodium acetate, NaOAc Carl Roth GmbH & Ko, Karlsruhe, Germany

3.2 Consumables

- Syringes (single use): Inject® Luer Solo 0,001-1ml, 2ml, 5ml, 10ml, 20ml, Original Perfusor® Spritze 50ml, Braun, Melsungen, Germany
- Needles (single use): BD Microlance™ 3, 24G 1” (0,55x25mm), 26G ½” (0,45x13mm), 30G ½” (0,3x13mm), Becton Dickinson, Heidelberg, Germany
- Single use examination gloves NitraTex, Ansell, Munich, Germany
- Eye cream, Bepanthen® Eye and Nose ointment, Bayer Vital Leverkusen, Germany
- Medical adhesive bands Durapore® 3M St. Paul, MN, USA
- Gauze balls (size 6), Fuhrman Verrbandstoffe GmbH, Much, Germany
- i.v. catheter’s cannula used as a tracheal tube: Vasofix® Safety 22G Braunüle® Braun, Melsungen, Germany
- Surgical sutures: a) Nr. 12 Coats GmbH, Kenzingen, Germany, b) non-absorbable size 5-0 ETHIBOND EXCEL® Ethicon GmbH, Norderstedt, Germany, c) size 5-0, 6-0 and 7-0 ProleneTM, Ethicon GmbH, Norderstedt, Germany, d) Black merciful 100% cotton AMANN, Bönnigheim,Germany
- Polyester Thread 5/0, FSSB, Jestetten, Germany
- 3-way stopcocks Discofix®-Braun, Melsungen, Germany
- Closing cones: Combi-Stopper Intermedica GmbH, Klein-Winternheim, Germany
- Perfusor-tubing 150 cm Perfusor-Line 150,Braun, Melsungen, Germany
- Surgical instruments T.S.E., Berlin, Germany
- Tubes for cryogenic transportation and storage of biological material, Cryo.s™, Greiner Bio-One GmbH, Frickenhausen, Germany
- Eppendorf tubes, SafeLock 1,5ml, Sarstedt AG & Co, Nümbrecht, Germany
- Glass capillary tubes for hematocrit, Hirschmann Laborgeräte GmbH & Ko KG, Eberstadt, Germany
- Hematocrit-sealing kit, Brand GmbH & Ko KG, Wertheim, Germany
- Pipettes: Eppendorf Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
- Pipette tips: 200µl, 1000µl, Sarstedt, Nümbrecht, Germany
- Falcon tubes: 15ml, 50ml Cellstar® Tubes, Greiner Bio-One GmbH, Frickenhausen, Germany
- Parafilm Wrap Bemis Flexible Packaging, Neenah WI, USA

- Scalpels: Feather® Disposal Scalpel No. 10, 11, Pfmmedical AG Köln, Germany
- Paper Napkins: Tork, Mannheim, Germany
- Micro-tubes 1.5ml, Sarstedt, Nümbrecht, Germany

3.3 Hemodynamics–Lung Function setup

- Centrifuge for hematocrit: Hematocrit 210, Hettrich Zentrifugen, Tuttlingen, Germany
- Centrifuge Mikro 200R, Hettrich Zentrifugen, Tuttlingen, Germany
- Mouse ventilator, MiniVent Type 845, Hugo Sachs Elektronik, Hugstetten, Germany
- Tracheal tube, Vasofix® Safety, 20G shortened ca 1,5 cm, B. Braun Melsungen AG, Melsungen, Germany
- Carotis catheter, Micro Cannulation System, FST Fine science tools GmbH, Heidelberg, Germany
- Jugular catheter, Hyman Mouse Pressure Catheter 14 cm, Nu MED Inc. Hopkinton, USA
- Software for lung function measurement, Pulmodyn W, Hugo Sachs Elektronik, Hugstetten, Germany
- Lab bath circulator, Lauda E100 + Lauda MA6, Delran, New Jersey, USA
- PET-Tubes Tygon®, Saint-Gobain Performance Plastics, Charny, France
- Gauze 5 x 4 cm Purzellin® Lohmann und Rauscher, Rengsdorf, Germany
- Gauze balls size 6 Fuhrman Verrbandstoffe GmbH, Much, Germany
- 3 way stopcock Discofix®-Braun, Melsungen, Germany
- Computer with Labtech Notebook PC-Based Data Acquisition Software for Windows, OMEGA Engineering, INC., Stamford, Connecticut, USA
- Transbridge TBM4M, Transducer Amplifier, World Precision Instruments, Inc, Berlin, Germany
- Combitrans disposable transducer for arterial blood pressure measurement Braun, Melsungen, Germany
- DT BNC Box USB 9800 Series, Data Translation®, Hugo Sachs Elektronik Hugstetten, Germany

- Differential Pressure Transduser, MPX Typ 399/2, Hugo Sachs Elektronik Hugstetten, Germany
- Blood analyzer ABL 330 Radiometer, Copenhagen, Denmark

3.4 Histology

3.4.1 Equipment

- Dehydration machine: Fully Enclosed Tissue Processor TP1050, Leica Microsystems, Nussloch, Germany
- Cassette & slide printer, Sakura, IDent^{TM}, Alphen aan den Rijn, The Netherlands
- Embedding center: heated paraffin embedding module Leica EG1140H / Cold plate for modular tissue embedding system Leica EG1150C, Leica Microsystems, Nussloch, Germany
- Rotating Microtome: Fully automatic rotating microtome RM2165, Leica Microsystems, Nussloch, Germany
- Waterbath for paraffin section, HI 1210, Leica Microsystems, Nussloch, Germany
- Analytical balance, PL303 Mettler-Toledo GmbH, Giessen, Germany
- pH meter, Labor-pH-Meter 766, Knick GmbH & Co. KG, Berlin-Zehlendorf, Germany
- Magnetric Stirrer, RH basic 2 IKAMAG®, IKA®-Werke GmbH & CO. KG, Staufen / Germany
- Microscope slides drying table, Flattening table for clinical histopathology HI 1220, Leica Microsystems, Nussloch, Germany
- Incubator for drying histological slides, Memmert Oven 100-800, Memmert GmbH & Co KG, Schwabach, Germany
- Image analysis workstation, Leica Q 550 IW, Leica Microsystems, Nussloch, Germany, comprised of:
- Microscope, Leica DMLA, Leica Microsystems, Nussloch, Germany
- CCD camera, Leica DC300F, Leica Microsystems, Nussloch, Germany
- PC, T3500, Dell

- Image analysis software with appropriate macros for the necessary analyses (SeptaeAutoanalyze, Vascular muscularization, Cell counting), Leica Qwin, Leica Microsystems, Nussloch, Germany

3.4.2. Consumables

- Embedding cassettes: a) macro Rotilabo®, extra high 12mm, Carl Roth GmbH &Ko, Karlsruhe, Germany b) small, Tissue Tek® Uni-Cassette®, Sakura Finetek Europe B.V Zoeterwoude, Netherlands
- Microscope glass slides, Superfrost Plus® - frosted white, R. Langenbrinck, Emmendingen, Germany
- Coverslips, 24x36 mm, R. Langenbrinck, Emmendingen, Germany
- Single use examination gloves Touch ‘n’ Tuff®, Ansell, Munich, Germany
- Di-sodium hydrogen phosphate dehydrate, Merck, Darmstadt, Germany
- Potassium chloride, ROTH, Carl Roth GmbH + Co, Karlsruhe, Germany
- Sodium chloride, Sigma-Aldrich, Steinheim, Germany
- Potassium dihydrogenphosphate, Merck, Darmstadt, Germany
- Hydrogen peroxide 30% pro analysi Merck, Darmstadt, Germany
- Ethanol 96%, 99.6% Otto Fischar, Saarbrücken, Germany
- Ethanol 70%, SAV Liquid Production GmbH, Flintsbach, Germany
- Isopropanol(≥99.8%), Methanol (≥99.8%), Sigma-Aldrich, Steinheim, Germany
- Resorcin Fuchsin Chroma, Münster, Germany
- Xylol Roth, Karlsruhe, Germany
- Nuclear Fast red, Kernechtrot Aluminiumsulfate Chroma, Münster, Germany
- Roti-Histol (Xylolersatz) Roth, Karlsruhe, Germany
- Microtome blades: a) MX35 Premier, ThermoScientific, Dreieich, Germany, b) Leica 819, Leica, Leica Microsystems, Nussloch, Germany
- Mounting medium, Pertex®, Medite GmbH, Burgdorf, Germany

3.5 Smoke generation and exposure system

- Smoke generator, Custom-made, Burghart Tabaktechnik GmbH, Wedel, Germany
- Vacuum pump for smoke generator, Jun-Air, Ahrensburg, Germany

- Pump for smoke transfer to exposure chamber, TSE, Bad Homburg, Germany
- Millipore filter, Millipore, Schwalbach, Germany
- 3R4F Standard cigarettes, University of Kentucky, Lexington,KY, USA
- Computer program for monitoring smoke, TSE, Bad Homburg, Germany

3.6 ROS measurements

- Electron Spin Resonance (ESR) spectrometer, EMX micro Bruker, Karlsruhe, Germany
- Superoxide dismutase–polyethylene glycol from bovine erythrocytes, Sigma-Aldrich, Steinheim, Germany
- 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), Noxygen Denzlingen, Germany
- Tempol, R&D System GmbH, Wiesbaden-Nordenstadt, Germany

3.7 Cell culture

- Dulbecco's modified Eagle's medium (DMEM) - High Glucose, Sigma-Aldrich, Steinheim, Germany
- 10% fetal calf serum (FCS)
- 2 mM glutamine, Gibco, Invitrogen, Karlsruhe, Deutschland
- 1% penicillin/streptomycin, Gibco, Invitrogen, Karlsruhe, Deutschland
- 0.2 µm Millipore filter, Millipore, Schwalbach, Germany
- cut-off filtration membrane, Vivaspin 20, Sartorius, Göttingen, Germany

4. METHODS

4.1 Animals

4.1.1 Approvals for the experiments

All the experiments performed in the context of this study were carried out according to the EU Directive 86/609/EEC and the rules and regulations of the Justus-Liebig University of Giessen, and were approved by the Regierungspräsidium Giessen. The corresponding animal experiment approved proposals can be found under the references: GI 20/10, Nr.37/2011, entitled “Charakterisierung der Rolle von sestrin 2 bei der Entstehung des Zigarettenrauch-induzierten Lungenemphysems mit Hilfe transgener Mausmodelle” for the smoke-induced emphysema experiment and GI 20/10, Nr.28/2012, entitled “Rolle von sestrin 2 für die Entstehung des Lungenemphysems” for the elastase-induced emphysema experiment.

4.1.2 Sestrin 2 KO mice

All experiments in this study employed 8-10 week old male and female sestrin 2 KO mice (RRJ141/Sesn2$^{Gt(RRJ141)Byg}$) and C57BL/6J WT mice. The RRJ141 (C57BL6J/129P2) mice were rederived from frozen embryos distributed by the UC-Davis branch of the Mutant Mouse Regional Resource Centers (MMRC). They were then back-crossed to C57BL6J mice for at least 10 generations. Mouse tail DNA and primers complementary to sequences flanking the gene trap (RRJ141, BayGenomics, gene trap vector pGT0Lxf) insertion site were used for genotyping. The mice used in the experiments are summarized in Table 4.

Table 4. Experimental animals.

Mouse Line	Genotype	Background
sestrin 2 KO	Sesn2$^{-/-}$	C57BL/6J
WT	C57BL/6J	C57BL/6J

4.1.3 Housing

All animals were housed under controlled conditions (20-23ºC and 40-70% humidity) of an equal daylight/dark cycle of 12 hours. Food (Altromin®Standard Diet Food) and water was supplied *ad libitum.* The animals were randomly allocated to the experimental groups of all studies and were kept in polycarbonate type 2 and 3 cages with woodchip bedding and red shelters.

4.1.4 Sestrin 2 study/smoke exposure mice groups:

9-15 (equally distributed male and female) mice to smoke-exposed or unexposed (control) groups with parallel groups for: (i) lung function tests (dynamic compliance) ii) hemodynamics (right ventricular and systemic arterial blood pressure) measurements, iii) alveolar morphometry, (iv) protein and mRNA analysis v) elastin quantification by image analysis.

4.1.5 sestrin 2 study mice groups: elastase-induced emphysema

8 (3 female, 5 male) mice were assigned to elastase treated or saline treated (control) groups with parallel groups for: (i) lung function tests (dynamic compliance and resistance measurements), ii) hemodynamic (right ventricular and systemic arterial blood pressure) measurements, iii) alveolar morphometry, (iv) protein and mRNA analysis.

4.2 Experimental designs

4.2.1.Experimental design of tobacco smoke exposure of RRJ141 mice

Sestrin 2 KO and WT mice were exposed to the primary smoke of the standardized 3R4F research cigarettes; (Kentucky, Lexington, KY, USA). The smoking machine employed ensured a constant total particulate matter (TPM) concentration of 140 mg/m^3 of cigarette smoke in a mixture with fresh air in the chamber and the mice were exposed for 6 h/day, 5 days/week for up to 8 months. Age-matched control mice were kept under identical conditions except for the smoke exposure.

Experimental plan / time table of analysis

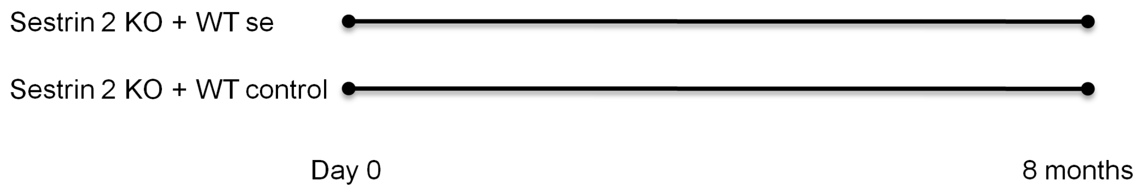


Figure 3. Experimental plan of sestrin 2 mice smoke exposure.

At the end of the experiment (8 months) the mice were subjected to lung function and hemodynamic measurements, heart ratios, alveolar morphometry, mRNA and protein analysis, elastin quantification by image analysis. (se: smoke-exposed)

Figure 4. **The smoke generator used in the experiments.**

4.2.2 Experimental design of elastase-induced emphysema in RRJ141 mice

Sestrin 2 KO and WT mice started being treated with imatinib or placebo on Day 0. Three days later mice received either PPE (24U/kg of BW in 100µl of saline) or 100µl of saline alone. The imatinib/placebo (drinking water) by oral gavage treatment continued for six weeks after the elastase instillations. At that timepoint the

mice were sacrificed and the investigations were performed according to Fig.5 (time plan). The mice were divided into groups according to Fig. 6 (groups).

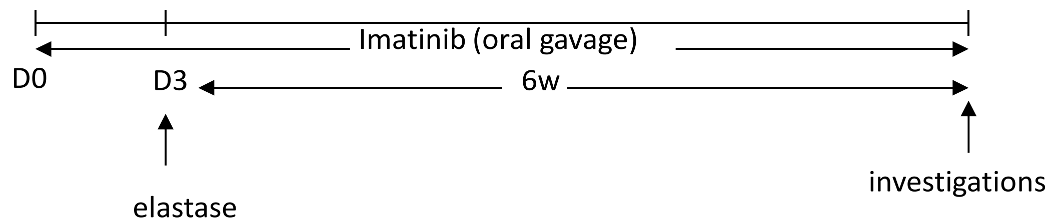


Figure 5. Time schedule of elastase-induced emphysema in sestrin 2 KO and WT mice.

Six weeks after the elastase instillations, the mice were subjected to lung function and hemodynamic measurements, alveolar morphometry, mRNA and protein analysis.

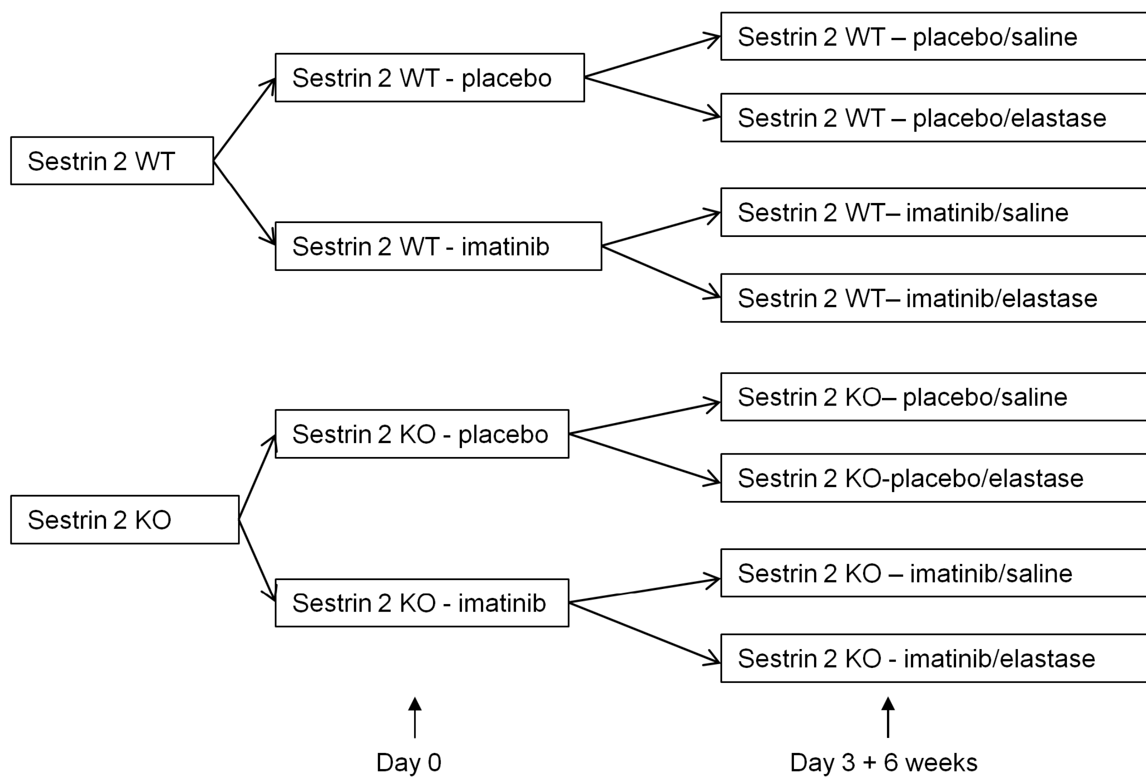


Figure 6. Mice groups in the elastase-induced emphysema in sestrin 2 mice experiment.

4.2.3 Elastase intratracheal instillation in mice

Elastase

The elastase used was PPE (ET947, EPC, USA). The activity of the enzyme was measured using the assay with Suc-Ala-Ala-Ala-pNA as a substrate, according to the following protocol:

Protocol for elastase activity assay

DETERMINATION OF PORCINE PANCREATIC ELASTASE ACTIVITY

Assay with Suc-Ala-Ala-Ala-pNA (EPC No. NS945) as substrate (44).

Materials Required

1. Tris buffer; 0.1 M Tris pH 8.3 at 25ºC. Dissolve 6.75 g Tris-HCl and 8.14 G Tris base in 900 ml H_2O. Determine pH at 25ºC. Titrate if necessary to pH 8.3 with 0.1 M HCl or 0.1 M NaOH. Dilute to 1000 ml with H_2O.
2. NaOAc-NaCl buffer; 0.05 M NaOAc pH 5, containing 0.1 M NaCl. Combine 14.8 ml of 0.2 M HAc and 35.2 ml of 0.2 M NaOAc and 100 ml 0.2 M NaCl. Bring to 200 ml with H_2O. Titrate to pH 5 at 25ºC.
3. Substrate solution; 2.5 mM in 0.1 M Tris pH 8.3. Utilizing a 25 mg vial of N-Suc-Ala-Ala-Ala-pNA (EPC No NS945), dissolve the contents with 22 ml of tris buffer. (Note: Use about 10 ml of the buffer for 5 flushes of the substrate vial.) Dissolve with stirring. Store at 5ºC.
4. Elastase solution; dissolve 1.0 mg per ml in the NaOAc-NaCl buffer. Prepare a secondary solution of 0.10 mg per ml in the same buffer. Keep both solutions cold in an ice bath.

Procedure

1. Adjust the spectrophotometer to 410 nm and cell temperature to 25ºC.
2. Equilibrate 2.5 ml of Tris buffer and 0.5 ml of substrate solution to 25º in the cell.
3. Add 0.005 ml of the 0.10 mg ml elastase solution, mix and determine the rate increase in absorbency at 1 minute intervals. The rate increase should be ca. 0.025 – 0.040 Δ_{410} nm per minute.

Calculation of Specific Activity

Є, 1%, 280 = 19.5 for porcine pancreas elastase

mg/ml = A_{280} x 0.51

Vol = 1.505 ml A=410 nm T=25ºC Light Path=1.0 cm

8.8=mM extinction coefficient of pNA at 410 nm

$$\frac{Units}{mg} = \frac{\Delta A_{410} \times 1.5005ml}{8.8 \times 0.0005mg}$$

From the Manufacturer, modified for volume used:

http://www.elastin.com/methods/PPEassayusingNS945assubstrate.aspx

The prepared stock solution of elastase for the instillation had a concentration of 0.6 U/100 µl.

Anaesthesia and elastase instillation.

Prior to intubation, the animals were anaesthetized with a mixture of 5% isoflurane (Baxter) in O_2 in an anaesthetic chamber. The animals were monitored at all times. The onset of surgical anaesthesia was controlled by using the loss of the pedal reflex as an index. Eye cream was applied to the corneas to prevent damage from dryness. The animals were placed on the mounting support from which they were suspended by their front incisors in a manner so that the neck and trachea were straightened. Anaesthesia was maintained by the use of a self-made face mask (1ml syringe) with a mixture of 2–3% isoflurane in oxygen. To illuminate the larynx and make the vocal chords visible one of the fiber optic arms of a strong external halogen light source (Schott KL200) was positioned very close to the front of the neck of the animal while the other fiber optic arm was used to illuminate the oral cavity. The intubation was performed under a dissecting microscope. After the displacement of the tongue the larynx and vocal chords were clearly visible and the intubation tube was inserted in the trachea. The tube used was an intravenous catheter (Neoflon outer sheath, 20G x $1^{1/4”}$, 33mm length, B. Braun, Germany) that was carefully pushed between the vocal chords. 100 µl of the elastase solution followed by 200 µl of air for even distribution in the lungs was instilled through the endotracheal tube using a 1ml syringe and another intravenous catheter of smaller diameter (Neoflon outer sheath, 24G gauge, 19 mm length; Becton Dickinson, Sweden) attached to it. After the instillation the mice were placed in a cage under an infrared lamp and were allowed to recover.

4.3 *In vivo* investigations

4.3.1 Anaesthesia and mouse preparation

After the end of each experiment the mice were anticoagulated with heparin (1000 U/kg, i.p) about 20 minutes before the beginning of the surgical interventions and anesthetized with ketamine (60 mg/kg body weight) and xylazine (10 mg/kg body weight) intraperitoneally. Their temperature was maintained at a physiological level (≈37°C) throughout the experiment. After the evaluation of the depth of anesthesia the mice were transferred to a Single-Chamber Plethysmograph for mice (Fig.7), placed in the supine position fixed on a rubber pad and subjected to the *in vivo* investigations.

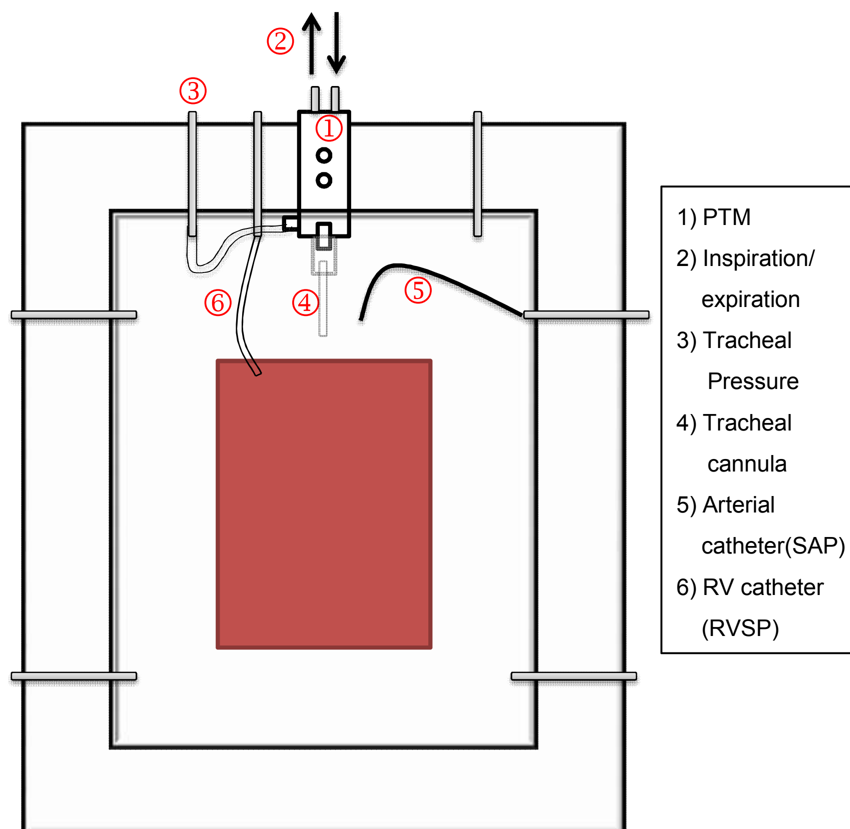


Figure 7. **Lung function and hemodynamics body measurement setup.**

PTM: pneumotachometer, SAP: systemic arterial pressure, RVSP: right ventricular systolic pressure.

4.3.2 Lung function tests

After the disinfection of the skin using an alcohol-based povidone-iodine solution (Braunoderm®), also used to keep the hair away from the surgical field) an incision

on the ventral surface of the neck was performed. The removal of the skin (approximately $1cm^2$) and subcutaneous tissue revealed the (left and right) submandibular salivary glands. Following the preparation and the removal of theses glands on the side, the muscle layer (*M. sternohyoideus* and *M. thyreohyoideus*, left and right) covering the ventral surface of the trachea was visible. The muscles were removed on the side and the trachea was prepared. A surgical thread was guided from under the trachea and a loose double-knot was made over it. Following tracheostomy (small incision between cartilageous rings), the animals were intubated with a tube (Vasofix® Safety Cannula for vein catheter support 20G, cut at the same length for all experiments) which was fixed with the thread. The ventilation of the lungs began immediately using a mouse ventilator for mice, set to a stroke volume of 10µl/g of BW, a respiratory rate of 150 breaths/min and a PEEP of 2 cmH_2O for the prevention of lung derecruitment. The tracheal cannula was connected to a pneumotachometer and the pressure difference produced in it was converted by a differential pressure transducer (VALIDYNE DP 45-14, Hugo Sachs Electronics, March-Hugstetten, Germany) into an electrical signal that is proportional to the respiratory flow. The measurement of the dynamic compliance ($C_{L,dyn}$) was performed in the living mouse by using the HSE PULMODYN software (Hugo Sachs Electronics, March-Hugstetten, Germany). The duration of the measurement was approximately 5 minutes.

4.3.3 Hemodynamic measurements

After the completion of the compliance measurements the head of the mouse was moved and tilted to the right. The left *A. carotis* was prepared by passing two curved forceps under it and carefully pulling the connective tissue and the *N. vagus* aside. Two surgical sutures (cranial and caudal) were passed under the vessel. A square knot was made to the cranial suture and it was then fixed on the upper right corner of the water bed using tape with appropriate tension. A simple loose knot was made in the caudal suture and the carotid artery was occluded with a microsurgery clip the nearest possible to the entrance of the artery in the thoracic cavity. The measuring equipment was at this point fixed to a value of 0 mmHg. A small incision was made on the artery and an arterial catheter was introduced in the vessel and fixed with the caudal suture. The surgical clip was removed and the SAP measurement was initiated.

The right *V. jugularis* was prepared in a way similar to that for the left carotis. The caudal suture was pulled lightly during the incision of the vessel and the insertion of the catheter (PE-10 tube) as a means of occlusion to prevent bleeding through the incision. For the same reason after the introduction of the tip of the catheter into the vessel the simple knot of the caudal suture was tightened with enough tension to stop the bleeding but still permit the unobstructed advance of the catheter through it until it reached the right ventricle.

Both catheters (Fig. 7, points 5 and 6) used for the hemodynamic measurements are fluid filled and the pressures are converted into electrical signals with the use of transducers and interpreted by the computer software LabTech Pro®. The measurement of the RVSP and SAP lasted for approximately 10 minutes. After the end of the lung function test and the hemodynamics measurements, the mice were euthanized by exsanguination through the heart.

4.4 Alveolar morphometry

The abdominal surface of the body was again disinfected with Braunoderm®. An incision was made to open the abdomen and extended medially to reveal the xiphoid process of the sternum. The thoracic cavity of the mice was opened by holding that process up, making a small incision in the diaphragm and cutting on the medial line of the body until the jugular notch was reached. After separation of the diaphragm from the inferior thoracic aperture using a pair of microsurgery scissors, the two sides of the thoracic cage were pulled and fixed aside using needles. Elevating and slightly tilting the heart from its apex the *A. pulmonalis* was displayed and isolated with the use of a curved forceps passing behind it and between the vessel and the base of the heart. A surgical suture was passed through the same point and a square knot was tied loosely over the pulmonary artery. A cannula was inserted in the artery through a small incision in the right ventricle and was fixed by tightening the knot. Isotonic saline solution was administered through that cannula into the lungs to remove the remaining blood that exited through a second small incision in the left ventricle kept open with the help of microsurgery scissors. The right lung was removed and kept in liquid nitrogen for molecular investigations. The tracheal tube was removed and another 20G cannula connected to a 50ml syringe through an extension set was inserted in the trachea.

The left lung was fixed by infusing 4.5% formaldehyde solution at an inflating pressure of 22 cmH_2O (Fig. 8). The lungs were removed from the thoracic cavity after 20 minutes and were immersed in respective fixative solution overnight. The second day they were transferred in 0.1M PBS, the third day in 50% ethanol and the fourth in 70% ethanol. Afterwards the lungs were placed in histological microcassettes and dehydrated using an automated dehydration unit (TP 1050, Leica Microsystems, Nussloch, Germany) with a program specific for alveolar structure maintenance.

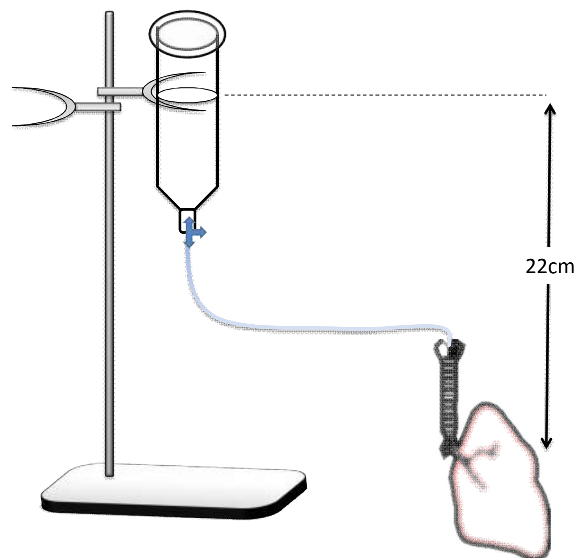


Figure 8. Lung fixation setup.

The lungs were afterwards embedded in paraffin. The blocks were placed in a cooling plate (EG 1150C Leica Microsystems, Nussloch, Germany) and subsequently to a cool chamber until the paraffin became solid. The following step was the preparation of 3μm thick sections on histological slides at the same level for all lungs (entrance of the bronchus in the lung).

The sections were stained with hematoxylin-eosin staining. The steps followed (removal of paraffin with xylene, rehydration of tissue with decreasing strengths of alcohol, staining of the nuclei with hematoxylin, blueing of the nuclei for better contrast, staining of non-nuclear elements with eosin, dehydration with increasing strengths of alcohol, “clearing” with xylene and placement of the coverslip) are given in the protocol described in Table 5.

Table 5. Hematoxylin-Eosin staining protocol

Step	Duration in minutes	Reagent/Solution	Effect
1	60	Incubation at 60ºC	Removing of paraffin
2	10	Xylol	
3	10	Xylol	
4	10	Xylol	
5	5	Absolute ethanol 99,6%	Hydration of the section
6	5	Absolute ethanol 99,6%	
7	5	Ethanol 96%	
8	5	Ethanol 70%	
9	2	Aqua dest.	
10	20	Mayer’s Hematoxylin solution	Staining of nuclei
11	5	Wash under running tap water	Rinsing-“blueing”
12	1	Ethanol 96%	Differentiation
13	4	Alcoholic Eosin-y solution	Staining of non-nuclear elements
14	rinse	Aqua dest.	Rinsing
15	2	Ethanol 96%	Dehydration
16	2	Ethanol 96%	
17	5	Absolute ethanol 99,6%	
18	5	Isopropyl alcohol 99,8%	
19	5	Xylol	„Clearing“ of tissue
20	5	Xylol	
21	5	Xylol	
22		Mount with Pertex / Corbit	Apply the cover slip

The stained sections were evaluated using the Leica Qwin software and the macro for alveolar morphometry. As a first step the white balance of the slides was set so that the program could later automatically identify the parts of the section corresponding to air (white part). The second step was the scanning of the section with the 5x objective to produce a mosaic picture of the section and to exclude the surrounding space of the lung and the big airways and vessels form the following

analysis of the individual fields. A total of 30-100 fields from each lung were analyzed with the 10x objective in the third step. The program identified the airspace (white color) and the lung structures (septal, airway and vessel walls, red color). Since emphysema is defined as “a condition of the lung characterized by abnormal, permanent enlargement of the air spaces distal to the terminal bronchiole,” and it involves the lung parenchyma, the airway structures proximal to the terminal bronchiole and vessels as well as the dead space surrounding the lung were excluded from the analysis. Fig.9 shows how the software analyses the photos taken with the microscope. Large airways proximal to the terminal bronchiole and vessels are excluded (white part), while the parenchymal part taken into account for the measurement (yellow part) is analyzed for the measurements performed by the program: Airspace%, Septal Wall Thickness and Mean Linear Intercept. The average values of all the fields for every lung were calculated and used to calculate the average values for each experimental group.

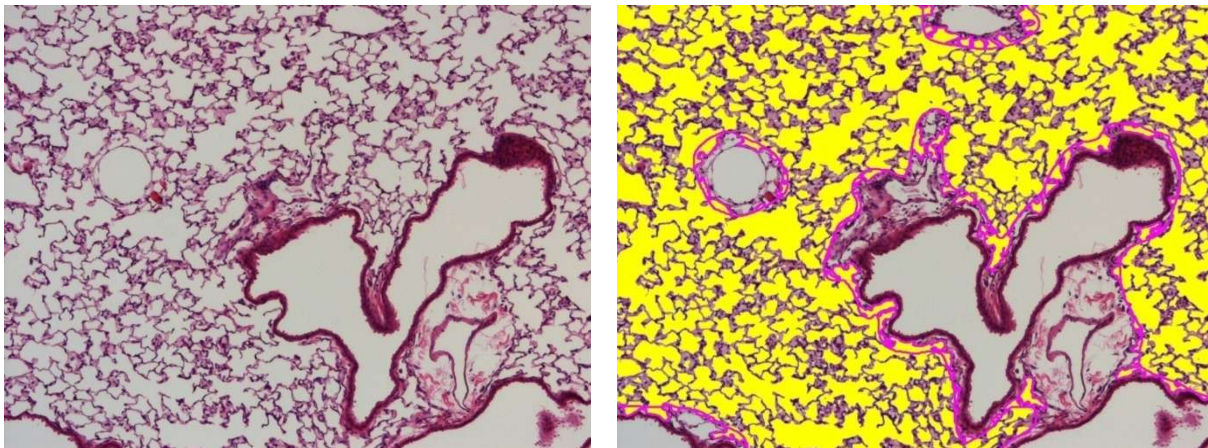


Figure 9. Image analysis for alveolar morphometry.

Yellow color: airspace mask, red: lung structures mask used by the software for image analysis.

4.5 Immunohistochemistry and immunofluorescence

5-μm tissue sections were deparaffinized, rehydrated, and then the antigen was retrieved by boiling in citrate buffer, pH 6.0 (Invitrogen), for 20 minutes in a microwave, and allowed to cool down for 30 minutes. Following rinsing in distilled H_2O and PBS for 5 minutes, the inactivation of the endogenous peroxidases was ensured by treatment of the slides with H_2O_2Block (Thermo Scientific) for 10-15 minutes and with Ultra V Block (Thermo Scientific) for 5 minutes. Steps of rinsing in dH_2O and soaking in PBS for 5-10 minutes followed, and then blocking nonspecific

protein-binding sites was achieved with incubation with 2% BSA in PBS for 1 hour. Exposure to the specific antibodies was performed with 2% BSA in PBS at 4°C overnight. The excess antibody was removed, the secondary antibody (biotin-labelled) (KPL) was applied to the slides (30 minutes, room temperature) followed by treatment with streptavidin peroxidase (KPL) (30 minutes, room temperature). After washing, incubation with AEC Chromogen Single Solution (Thermo Scientific) followed (10-20 minutes, room temperature). Counterstaining was done with Mayer's Haematoxylin Solution (Sigma) and finally the coverslips were mounted with Immuno Histo Mount (Santa Cruz Biotechnology). For the TTF1 staining, the staining procedure followed involved the M.O.M. Kit (Mouse on Mouse Kit, Vector Laboratories) according to the manufacturer's instructions. (Performed by collaboration partner: AG von Melchner, University of Frankfurt Medical School).

4.6 Heart preparation and heart ratio calculation

Just before the explantation of the lungs, the hearts were removed by cutting off the vessels connecting them to the body and removing the adjacent connective tissue. The hearts were then dissected under a stereoscope. The atria and vessel trunks were removed to the level of the mitral and tricuspid valves. The right ventricle was excised and separated from the left ventricle and septum. The two parts of the heart (right ventricle and left ventricle plus septum) were dried for at least 3 days at room temperature and then weighed separately to obtain the right ventricle to left ventricle plus septum ratio (Dumitrascu et al., 2006) according to the following equation:

$$\text{Heart ratio} = \frac{Right\ \ ventricle}{Left\ \ ventricle\ + septum}$$

Fig. 10 illustrates the heart structures used in the calculation.

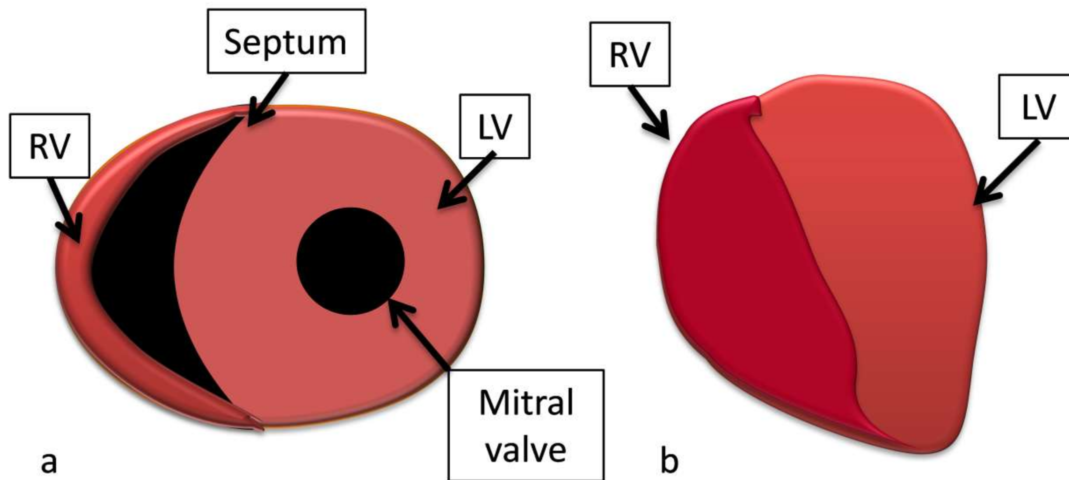


Figure 10. Schematic illustration of the heart.

Structures used in the determination of right heart hypertrophy by calculation of the heart ratio (right ventricle to left ventricle plus septum). a) transverse plane, dorsal view (after removal of atria and main vessels), b) sagittal plane, left lateral view

4.7 Quantification of lung elastin by image analysis (sestrin 2 mice smoke exposure experiment)

Paraffin sections of 3µm thickness, were analysed using the macro for cell counting of the Leica QWin software and the 40x objective. The slides were stained with Weigert's resorcin-fuchsin solution and counterstained with nuclear fast red according to the protocol presented in Table 6. Paraffin wax was removed through an initial step of heating and then consecutive changes in xylol. The tissue was rehydrated with passes in decreasing strengths of alcohol. The tissue was afterwards rinsed and rehydrated with increasing strengths of alcohol. The next step was the "clearing" the tissue in xylol and finally the coverslips were placed. Depending on the size of the lung section, 175-370 images were analysed.

The number of intact elastin fibres for each image that covered an area of 0.07598346mm^2, was counted (Seimetz et al., 2011). The number of intact fibres per area was then calculated according to the following equation:

$$\text{intact fibers} = \frac{\sum \text{intact fibers}}{\sum \text{images} \ \text{x} \ 0{,}07598346 \ \text{mm}^2}$$

Table 6. Weigert's elastic stain protocol.

Step	Duration	Reagent/Solution	Effect
1	60	Incubation at 60ºC	Removing of paraffin
2	10	Xylol	
3	10	Xylol	
4	10	Xylol	
5	5	Absolute ethanol 99,6%	Hydration
6	5	Absolute ethanol 99,6%	
7	5	Ethanol 96%	
8	5	Ethanol 70%	
9	10-24 hrs	Resorcinol fuchsine solution acc. to Weighert.	Staining of elastic fibers
10	2x rinses	Distilled Water	Rinsing
11	10	Nuclear fast red aluminium sulphate solution	Counterstaining
12	2x rinses	Distilled Water	Rinsing
13	1	Ethanol 70%	Dehydration
14	2	Ethanol 96%	
15	3	Ethanol 96%	
16	5	Isopropyl alcohol 99,8%	
17	5	Isopropyl alcohol 99,8%	
18	5	Xylol	„Clearing" of tissue
19	5	Xylol	
20	5	Xylol	
21		Mount with Pertex / Corbit	Mounting the cover slip

4.8 RT-PCR

RNA isolation was performed using the TriReagent (Sigma) protocol according to the manufacturer's instructions. Gene expression analysis through Real-time RT-PCR was performed using SYBR Green (ABgene, Epsom, UK) and/or TaqMan chemistry (Life Technologies) in an Opticon 2 qPCR machine (MJ Research). Random priming and Superscript II reverse transcriptase (Invitrogen) were used to synthesize cDNA from total RNA. cDNA derived from 7.5-15.0 ng of total RNA, 1×

ABsolute SYBR fluorescein mix (ABGene), and 5 pmol of gene-specific primers in a total volume of 25 µl were used to run PCR reactions as triplicates on 96-well plates. For the normalization of the reactions, RT-PCR reactions for RNApolII were carried out simultaneously using the primers 5'-ATGAGCTGGAACGGGAATTTGA-3' and 5'-ACCACTTTGATGGGATGCAGGT-3'. The temperature profile of the reactions is shown in Table 7.

Table 7. Temperature profile of the PCR reaction.

Temperature	Duration	No. of cycles
94°C	10 min	
94°C	15 seconds	40 cycles
61°C	30 seconds	
72°C	30 seconds	

The primers used for expression analysis are given in Table 8.

Table 8. Primers used for sestrin 2, PDGFRβ and KGF.

Gene	Forward primer	Reverse primer
sestrin 2	5'-ATA ACA CCA TCG CCA TGC AC-3'	5'-TTG AGG TTC CGT TCC AGG AG-3'
PDGFRβ	5'-CCA CCA TGA AAG TGG CTG TC-3'	5'-TCC ACC AGA TCA CCG TAT CG-3'
KGF	5'-CGA GCG ACA CAC CAG AAG TT-3'	5'-CAC GGT CCT GAT TTC CAT GA-3'

4.9 Western blotting

The detection of the KGF and PDGFRβ proteins from mouse lung fibroblast lysates and concentrated conditioned media was performed using Western blotting. The cells were lysed with an ice-cold RIPA buffer (cell signaling, # 8906) supplemented with PMSF (1mM, final concentration) for 20 minutes and cleared of debris by centrifugation (20 minutes, 13000 RPM at 4ºC). The conditioned medium was tenfold concentrated by passing through a 3-kDa cut-off filtration membrane. Protein concentration was measured by spectrophotometry and equal

protein concentration was adjusted by addition of RIPA buffer. Equal amounts of protein were separated by polyacrylamide gel electrophoresis (PAGE) (NuPAGE, 4-12% Bis-Tris precast gels, invitrogen), containing sodium dodecyl sulfate (SDS) for protein denaturation. Gels were run with NuPAGE® MOPS SDS Running Buffer (invitrogen). The total amount of protein loaded per sample was 10 µg. Protein samples were supplemented with NuPAGE® LDS Sample Buffer4x, 1x end concentration). 5% of mercaptoethanol was added and the samples were heated to 95°C for 3 minutes. 5 µl of a marker (Seeblue plus2, invitrogen) were applied as a standard. The proteins were then transferred to a nitrocellulose (Biorad) membrane using a semi-wet blotting system (XCell II™Blot Module, invitrogen) by electrophoresis (35 Volts, 1.5 hrs).

The membrane was afterwards blocked in TBST/5% dry fat free milk buffer for 1 hour for non-specific binding saturation. An overnight incubation with the primary antibody (dissolved in 5%milk buffer) followed. The following day the membrane was washed 3 times and incubated with the secondary antibody for 1 hour. After three washing steps the membrane was incubated with a chemiluminescent reagent (ECL: mix ECL I and ECL II 1:1 shortly before use) for fluorescent detection of horseradish peroxidase (HRP) conjugates. ECL I: Luminol (2.5 mM), cumaric acid (0.4 mM), TRIS-HCl pH 8.5 (100 mM), ECL II: H_2O_2 (0.028%), TRIS-HCl pH 8.5 (100 mM). The protein bands were visualized and analyzed using the FluorChem Q Imaging system (Alpha Innotech).

For the reincubation of the membrane with β-actin the membrane was stripped with an one hour incubation in stripping buffer (RestoreTM PLUS Western Blot Stripping Buffer, Meridian, Rockford USA). Afterwards, the already described steps for washing, blocking and incubation with a primary and seconday antibody were followed.

4.10 Cell cultures and preparation of conditioned media

Mouse lung fibroblasts (MLFs) were cultured in Dulbecco's modified Eagle's medium (DMEM, High Glucose, Sigma) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). For the preparation of MLF conditioned media (CM) 90% confluent cultures were used after incubation of the cells in serum-free DMEM for 48 hours. Subsequently, the MLF CM was filtered using a 0.2 µm Millipore filter and was used immediately or at a later time

point after shock-freezing in liquid nitrogen and storage at −70°C. MLF CMs used in western blot analysis were concentrated after passing through a 3-kDa cut-off filtration membrane (Vivaspin 20, Sartorius).

4.11 ROS measurements

The release of superoxide from MLFs measurement by EPR was performed as previously described (Veit et al., 2013). Briefly, EPR measurements were performed at −170°C using an EMXmicro Electron Spin Resonance (ESR) spectrometer (Bruker, Karlsruhe, Germany) and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen) as the spin probe for detection of intra- and extracellular superoxide production. To assess the superoxide signal as part of the total CMH signal, parallel samples that contained either CMH alone or combined with superoxide dismutase (SOD) conjugated to polyethyleneglycol (PEG-SOD) were measured to avoid issues arising from the reaction of CMH with superoxide and peroxynitrite. For this purpose, incubation of duplicate samples of $2×10^5$ cells were incubated for 2 hours at 37°C with 15 U/ml PEG-SOD (Sigma) and afterwards CMH (500 µM) ± PEG-SOD was added. The samples were then incubated for another 20 minutes followed by shock-freezing and storage in liquid nitrogen. The frozen samples were used for spectrometry using specific conditions (g-factor= 2.0063, center field= 3349.95G, microwave power= 200 mW, sweep time= 20 seconds, sweep number= 5).

4.12 Statistical analyses

All data presented are expressed as means ± SEM. Analysis of variance (ANOVA) with the Newman-Keuls post-test for the comparison of multiple groups was performed using the Prism software. For comparison of two groups the Student's t-test was employed. A P value <0.05 was considered statistically significant for all analyses. The significance differences between two groups indicated in the graphs as asterisks represent p-values as follows: * $p<0.05$,** $p<0.01$,*** $p<0.001$.

5. RESULTS

Portions of this study were performed in collaboration with the group of Professor Harald von Melchner, University of Frankfurt Medical School. Data which are important for the concept and interpretation of the present study but were not perfromed by myself but by the Frankfurt group are given in addition to the own results. If such data are given, it is explicitly stated that these data were provided by Prof. Harald v. Melchner.

5.1 Regulation of sestrin 2 and PDGFRβ mRNA in lungs of mice by smoke exposure.

The first step in the elucidation of the role of sestrin 2 in the development of smoke-induced emphysema was the determination of regulation of sestrin 2 and PDGFRβ mRNA by smoke. Gene expression analysis from lung homogenates of WT and sestrin 2 KO mice after 8 months of smoke exposure, demonstrated that sestrin 2 mRNA was upregulated in the lungs of mice when compared to respective controls (Fig. 11a). In contrast, PDGFRβ mRNA was downregulated in the lungs of the WT mice while no such downregulation was observed in the lungs of sestrin 2 KO smoke-exposed mice (Fig. 11b).

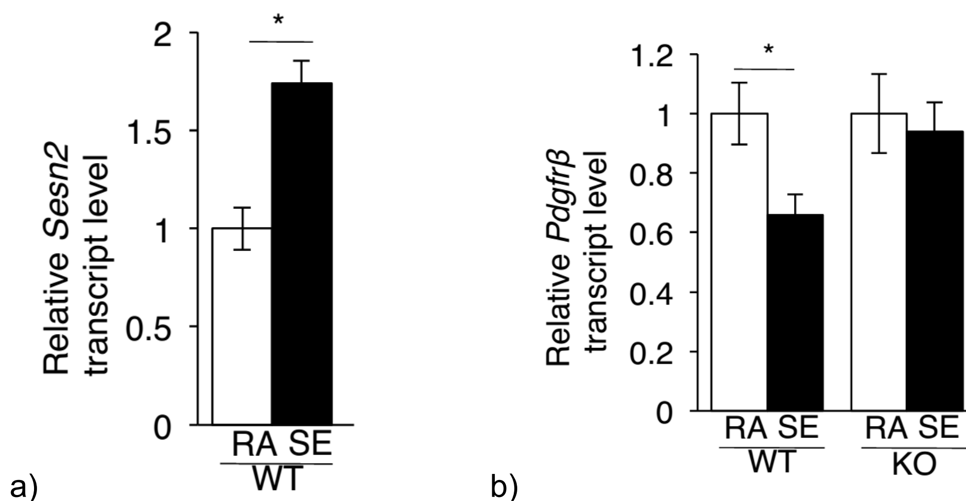


Figure 11. Regulation of sestrin 2 and PDGFRβ in cigarette smoke-exposed and control mice.

qRT-PCR analysis of a) sestrin 2, b) PDGFRβ. Sestrin 2 is upregulated and PDGRβ is downregulated in the lung of mice after smoke exposure. * $p<0.05$. Sesn2: sestrin 2 mRNA, PDGRβ: PDGFRβ

mRNA, RA: room air, SE: smoke-exposed, WT: wild type, sestrin 2 KO: sestrin 2 knockout. Data were provided by Prof. von Melchner, University of Frankfurt Medical School.

5.2 Sestrin 2 inactivation protects mice from smoke-induced emphysema.

5.2.1 Sestrin 2 inactivation protects from a decline in lung function in smoke-exposed mice.

To estimate the extent of the effect of smoke exposure on lung function, dynamic lung compliance measurements were performed. Results from WT and sestrin 2 KO mice revealed that the sestrin 2 inactivation protected the mice from developing emphysema that would impair their lung function. While the wild type mice exhibited a decline in lung function as indicated by the significant increase in dynamic lung compliance, the same effect of smoke was not observed in the lungs of the KO mice (Fig. 12). While there were no significant differences between the smoke-exposed WT and sestrin 2 KO mice groups, the absence of a significant effect of smoke exposure on the sestrin 2 KO mice is clearly demonstrated by the absence of a significant change in lung compliance between the two sestrin 2 KO mice groups (control and smoke-exposed).

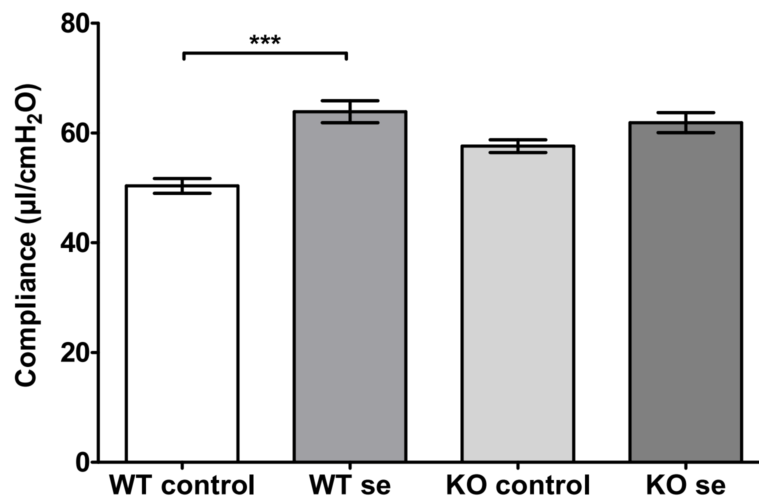
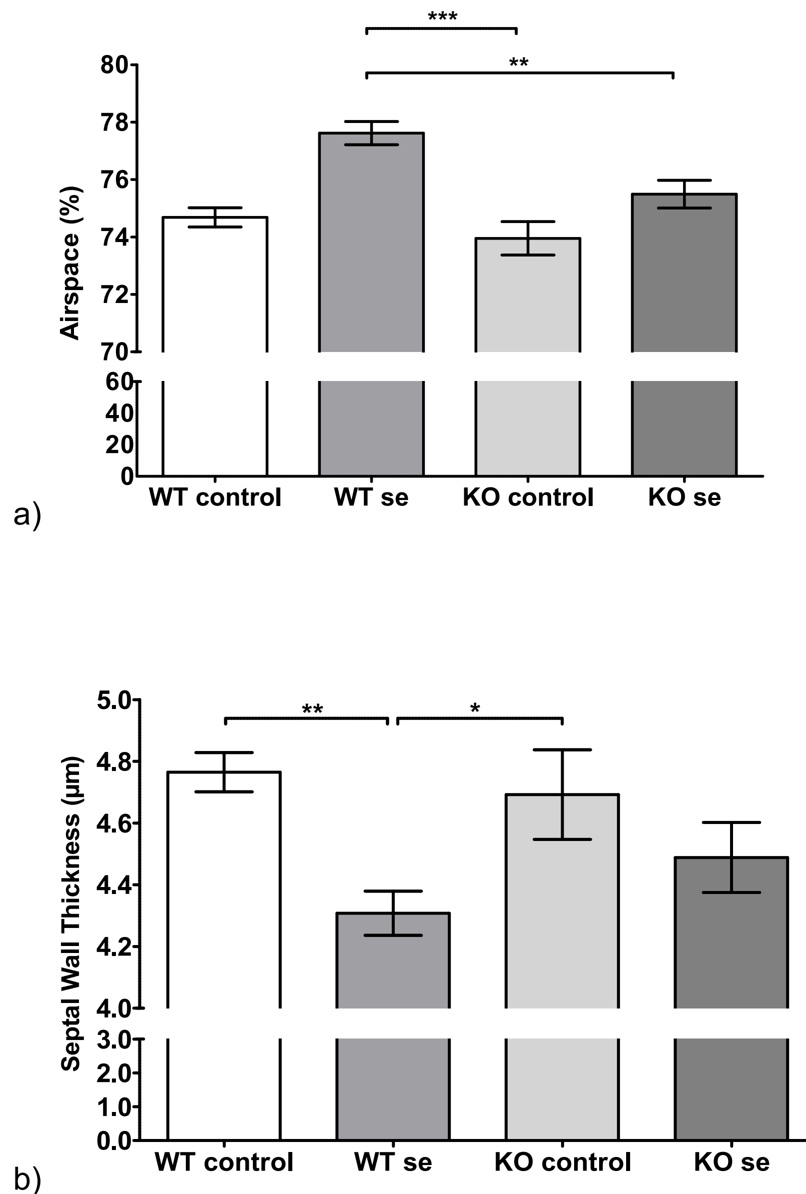


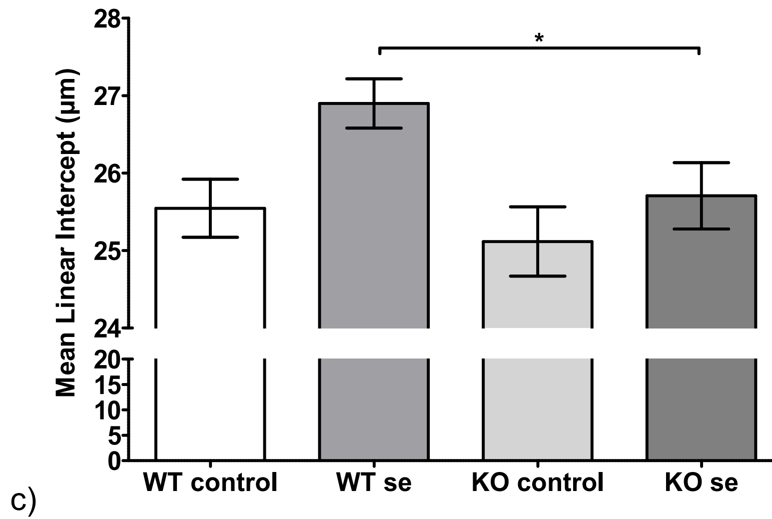
Figure 12. Dynamic lung compliance measurement in wild type and sestrin 2 knockout mice after smoke exposure.

Impaired lung function indicated by an increase of dynamic lung compliance in WT mice after 8 months of smoke exposure. *** $p<0.001$. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed n= 9-15 mice/group.

5.2.2 Sestrin 2 inactivation protects from the development of pulmonary emphysema in smoke-exposed mice.

Lungs of mice from all experimental groups underwent morphometric evaluation (alveolar morphometry). Lung tissue destruction and airspace enlargement were evaluated by analyzing 3 parameters: a) airspace %, b) septal wall thickness (SWT) and c) mean linear intercept (MLI). Smoke exposure resulted in a significant increase of airspace % and MLI and a decrease of SWT in the lungs of WT mice. No significant changes were observed in the sestrin 2 KO mice. The results indicating structural alterations in WT mice but not in KO mice are presented in Fig. 13 and representative images of the lungs are given in Fig. 14.





c)

Figure 13. Alveolar morphometrical evaluation of lungs of WT and sestrin 2 KO mice after 8 months of smoke exposure.

Parameters evaluated: a) airspace %, b) septal wall thickness c) mean linear intercept .* $p<0.05$, ** $p<0.01$,*** $p<0.001$. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed, n= 9-15 mice/group.

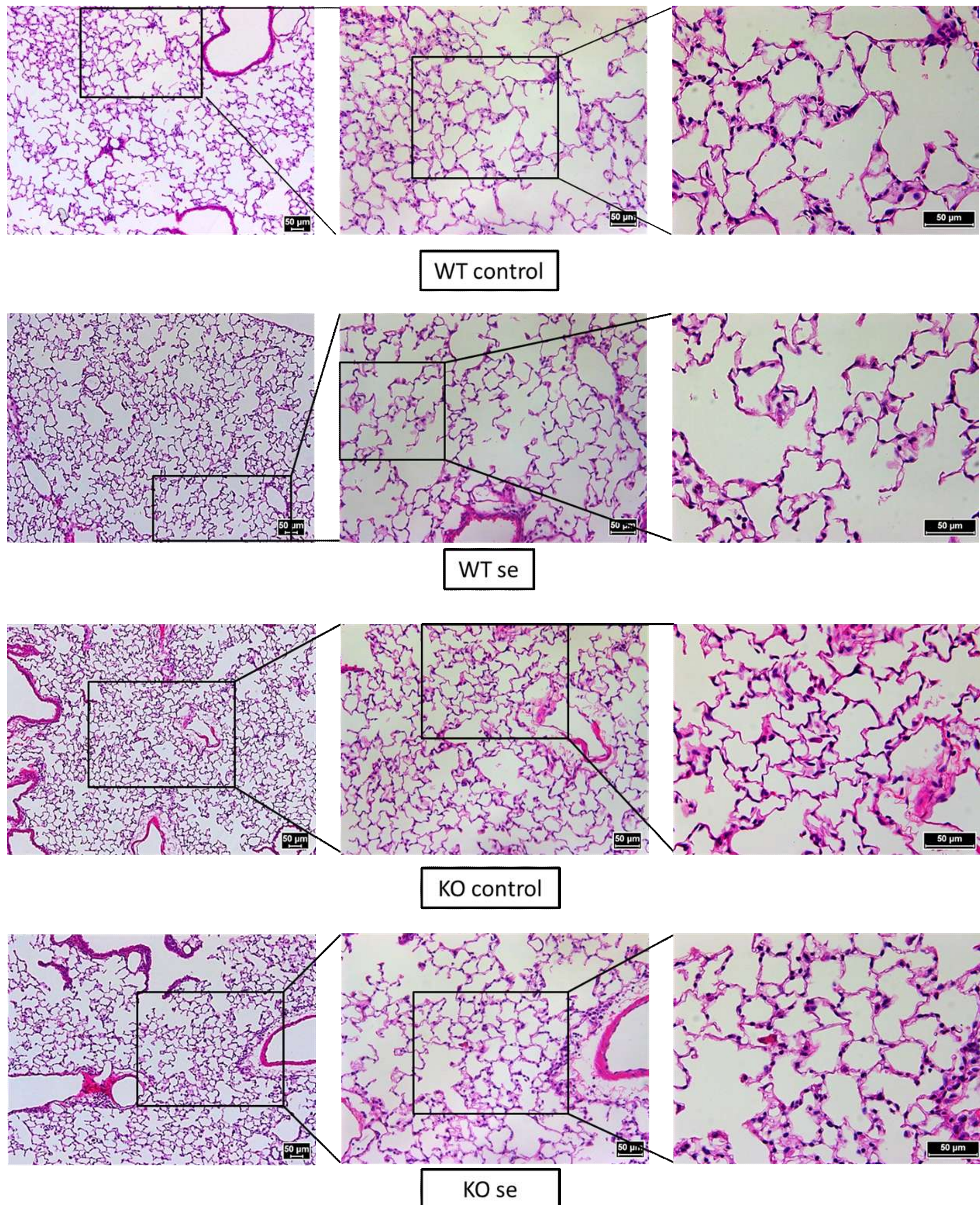


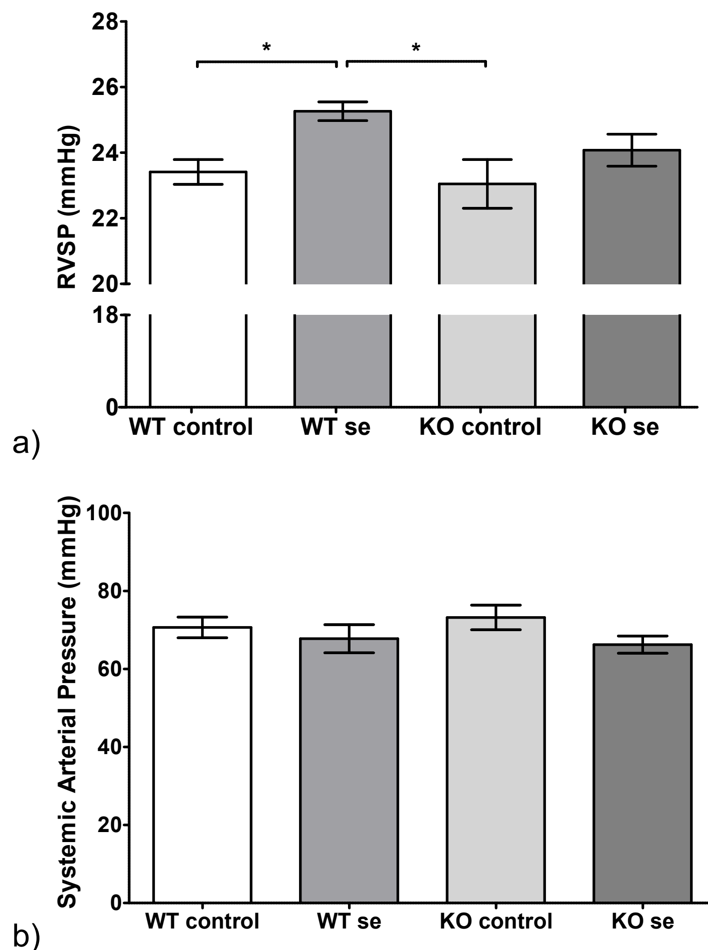
Figure 14. Representative images of lungs of wild type and sestrin 2 knockout mice exposed to room air (control) or cigarette smoke (se).

Alveolar morphometry (H&E staining). Magnifications appearing in the panel from left to right: 10x, 20x, 40x. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed.

5.2.3 Smoke exposure does not result in the development of pulmonary hypertension and right heart hypertrophy in sestrin 2 knockout mice.

After the end of eight months smoke exposure, the wild type mice developed pulmonary hypertension as shown by the significant increase of the right ventricular systolic pressure (RVSP) in the smoke-exposed mice when compared to the control ones. This is in contrast to the sestrin 2 KO mice where no significant increase of the RVSP was observed (Fig. 15a). The knockout mice did not exhibit a significant difference between smoke-exposed and control groups. No significant differences were observed concerning the systemic arterial pressure (SAP) in all groups (Fig. 15b).

The heart ratio calculation demonstrated that while smoke exposure led to the development of right heart hypertrophy in the wild type mice, it didn't have the same effect on the KO mice (Fig. 15c).



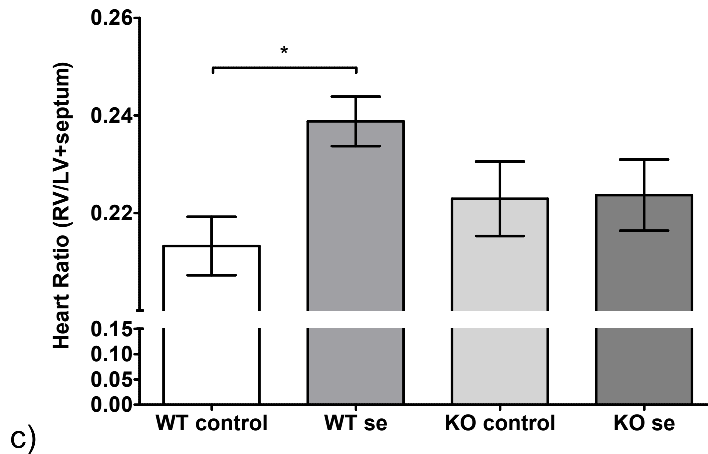


Figure 15. Hemodynamic evaluation in wild type and sestrin 2 knockout mice after 8 months of smoke exposure.

Parameters evaluated: a) right ventricular systolic pressure, b) systemic arterial pressure, c) right heart hypertrophy (heart ratios). * $p<0.05$. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed n= 9-14 mice/group.

5.2.4 Inactivation of Sestrin preserves the number of intact elastin fibers per area in the lungs of smoke-exposed mice.

After eight months of smoke exposure the number of intact elastic fibers per area in the lungs of wild type mice was significantly reduced when compared to their respective controls and both the groups of knockout mice. In the knockout mice, smoke exposure had no effect and the average number of intact elastic fibers per area remained at the same level in the control and smoke-exposed groups (Fig. 16 and 17).

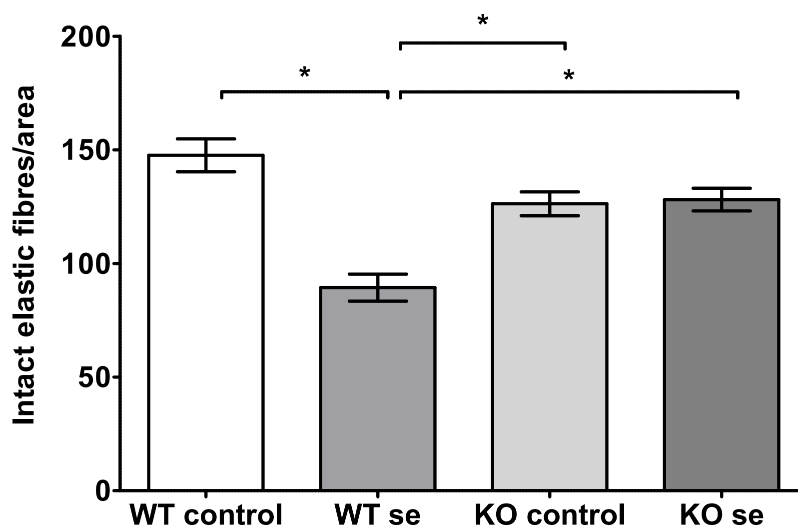
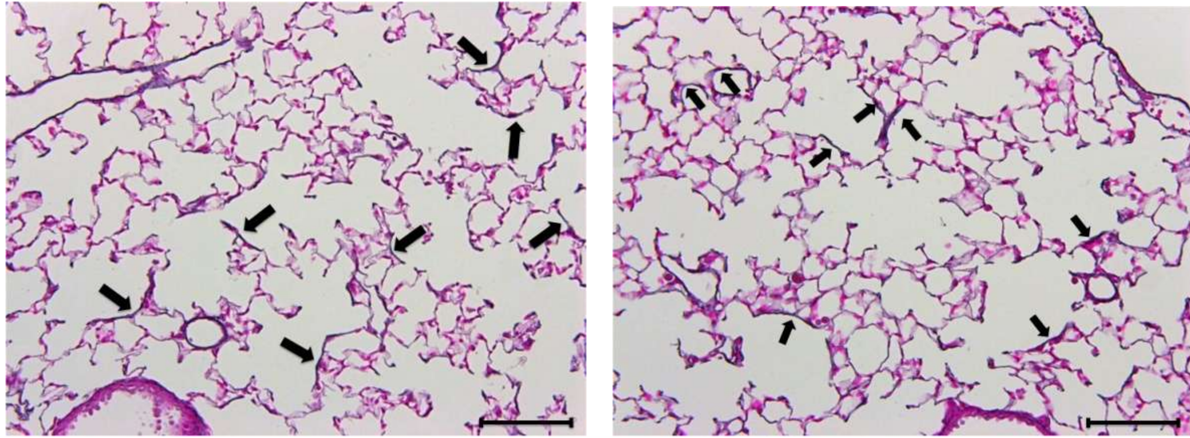
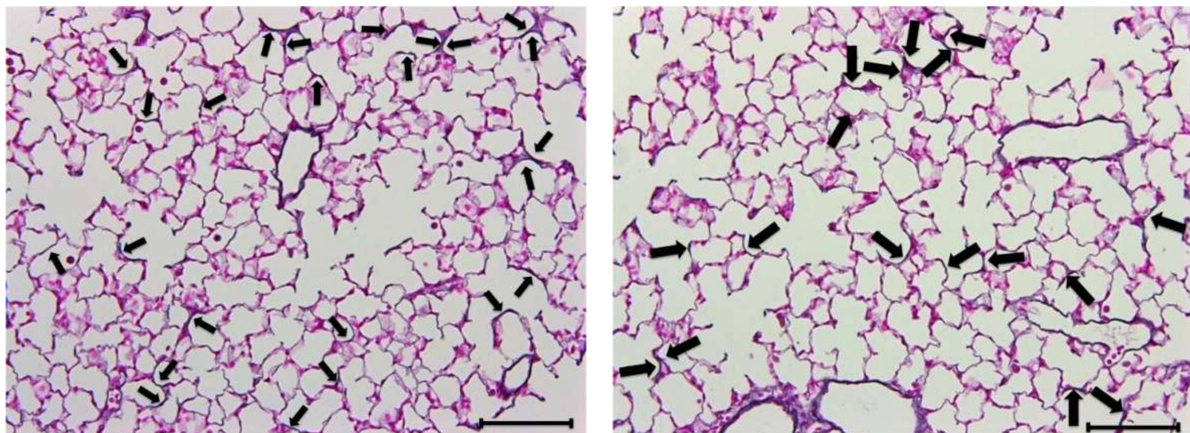


Figure 16. Number of intact elastic fibers per area in lungs of wild type and sestrin 2 knockout mice after smoke exposure and their respective controls.

Results of manual counting of intact elastic fibers after staining. * $p<0.05$. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed n= 5.



Smoke-exposed WT



Smoke-exposed KO

Figure 17. Representative images of lungs of WT and sestrin 2 KO lungs, of mice exposed to cigarette smoke (smoke-exposed).

Arrows mark intact elastic fibers. Scale bars: 100 µm. WT: wild type, KO: sestrin 2 knockout, n= 5.

5.3 Results of the investigations of the role of PDGFRβ in elastase-induced emphysema in sestrin 2 knockout mice.

To elucidate furthermore the importance of PDGFRβ in the regulation of lung maintenance, the elastase-induced emphysema model was employed in combination with imatinib (a PDGFRβ inhibitor), or placebo.

5.3.1 Inhibition of PDGFRβ accentuates the decline in lung function of sestrin 2 knockout mice treated with elastase.

The instillation of elastase caused similar changes in the lung functional measurements in both wild type and sestrin 2 knockout mice. Mice treated with elastase exhibited significantly higher values of dynamic lung compliance compared to their respective controls, 6 weeks after elastase instillation. Sestrin 2 knockout mice treated with elastase and imatinib had a significantly higher compliance compared to the elastase-placebo treated group. Such an effect was not observed in the wild type mice, where treatment with imatinib did not have an effect on lung function (Fig. 18).

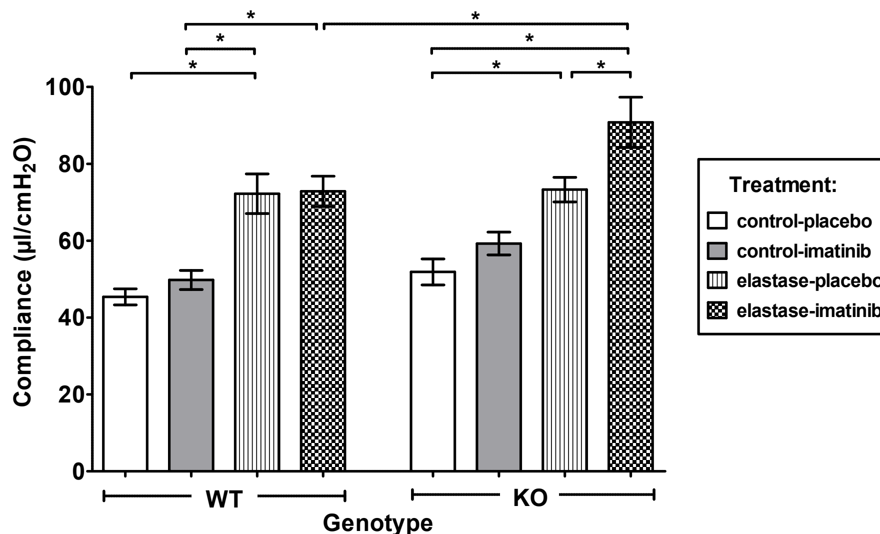


Figure 18. Lung function in WT and sestrin 2 KO mice after elastase treatment ± imatinib.

*** p<0.05,. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed, n= 5-7.**

One way ANOVA was used to prove the hypothesis that imatinib treatment affected lung compliance only in knockout mice. Additional p-values are given to support the expected, negligible effect of the treatment in the WT mice.

5.3.2 Inhibition of PDGFRβ worsens the emphysema of sestrin 2 knockout mice treated with elastase.

Consistent with the results obtained from the lung function measurements, elastase application resulted in an increase of the airspace %, septal wall thickness and mean linear intercept. Inhibition of PDGFRβ further increased the elastase

lesions in the knockout but not in the wild type mice with regards to airspace % and mean linear intercept but not to septal wall thickness.

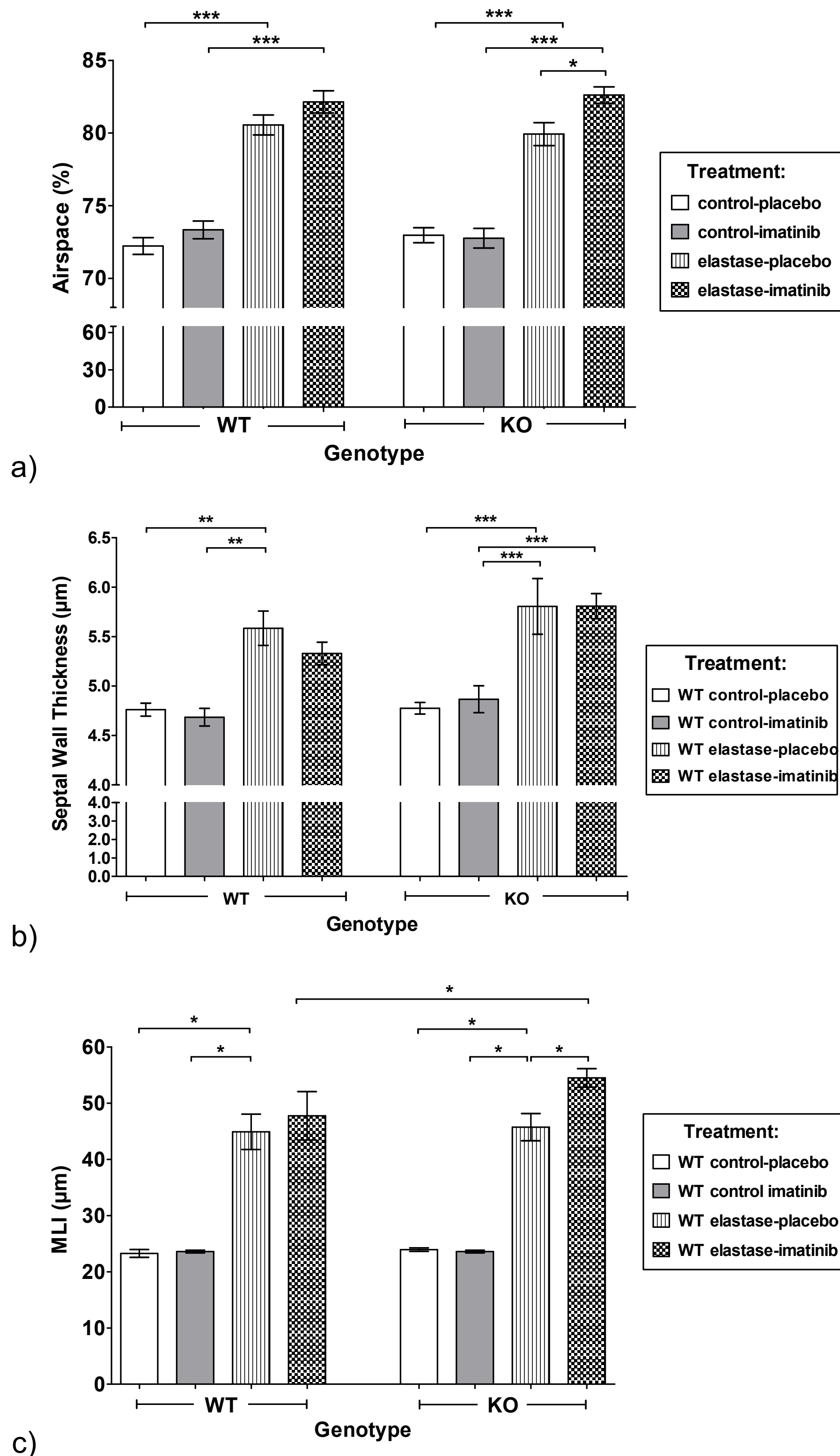
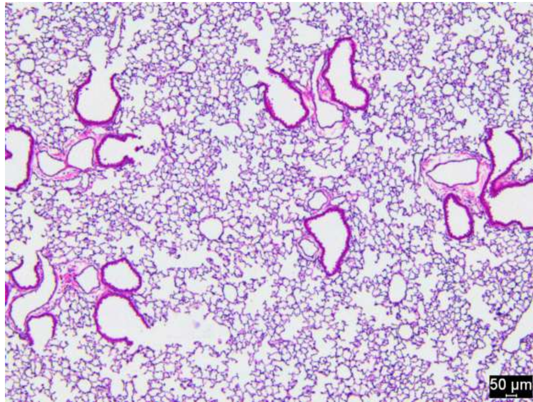


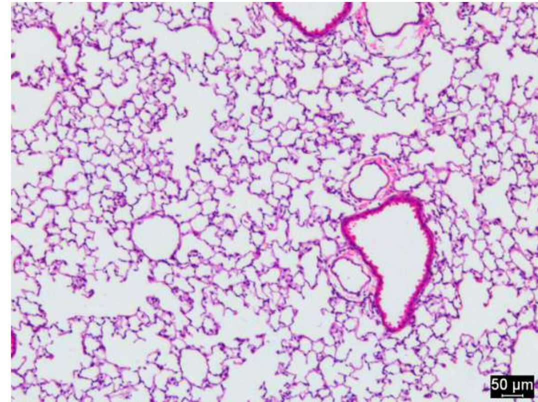
Figure 19. Morphometric evaluation of the effect of PDGFRβ inhibition on the elastase-induced emphysema induction in WT and sestrin 2 KO mice.

PDGFRβ inhibition further increased the differences in the evaluated parameters in the KO lungs while no effect was observed in the WTs. Parameters evaluated: a) Airspace %, b) septal wall thickness c)

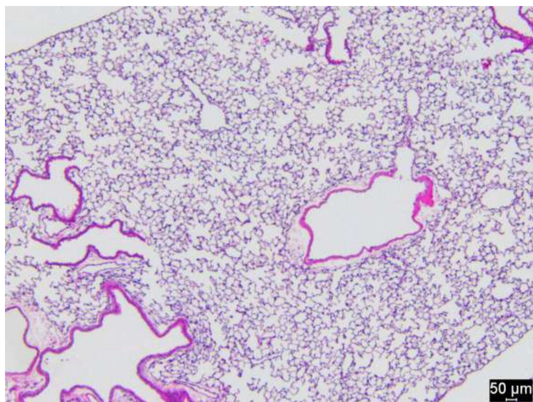
mean linear intercept.* p<0.05, ** p<0.01,*** p<0.001. WT: wild type, KO: sestrin 2 knockout, se: smoke-exposed, n= 5-7. One way ANOVA was used to prove the hypothesis that imatinib treatment affected Airspace%, septal wall thickness and mean linear intercept only in knockout mice. Additional p-values are given to support the expected, negligible effect of the treatment in the WT mice.

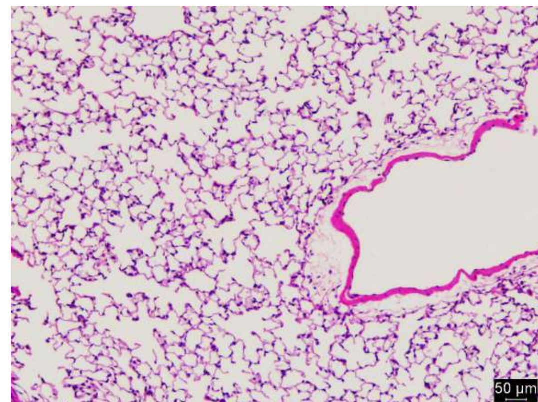
Representative images from lungs of all groups in this study are presented in Fig. 20.



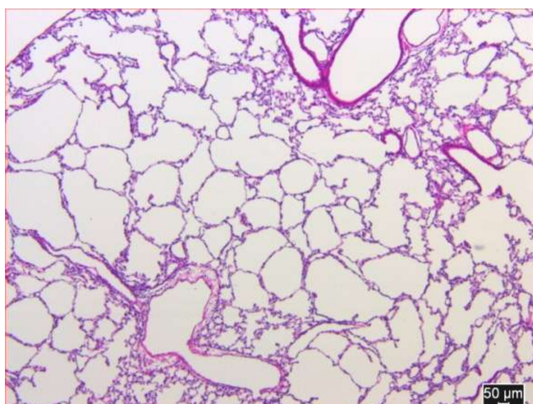


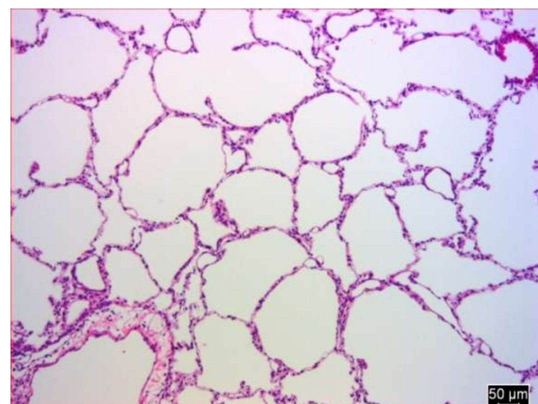
KO control-placebo



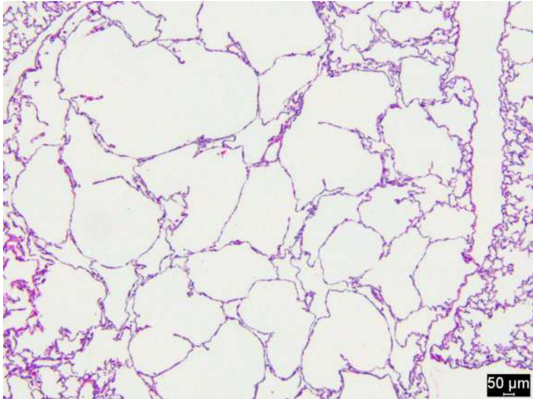


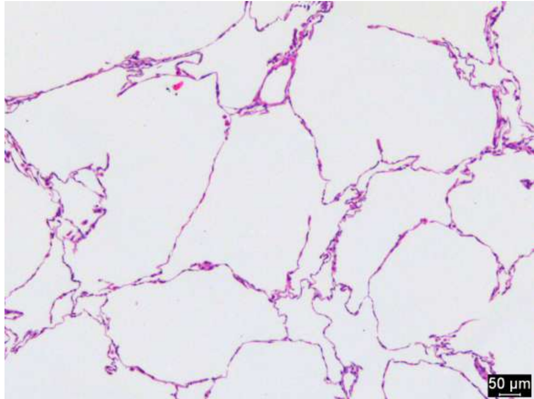
KO control-imatinib



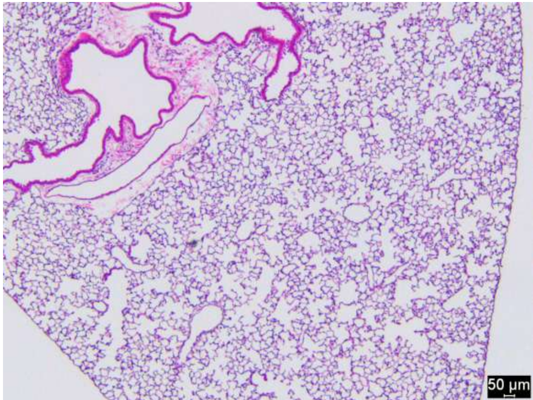


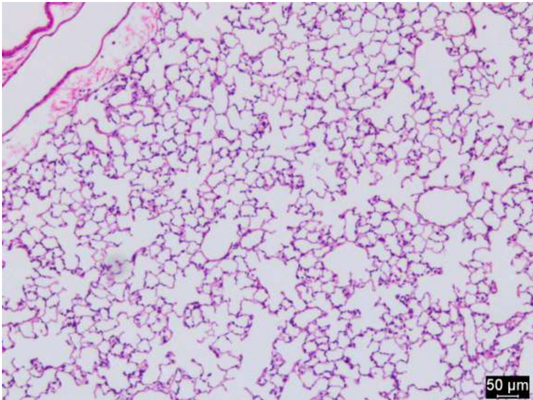
KO elastase-placebo



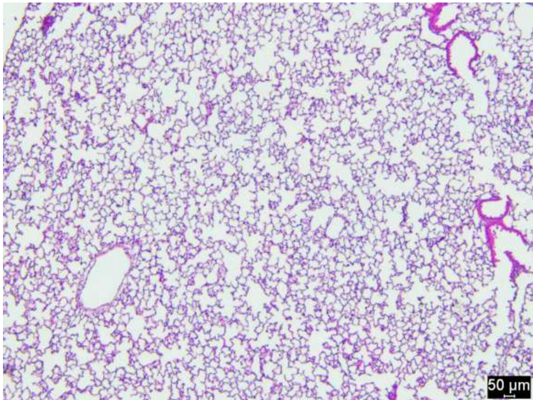


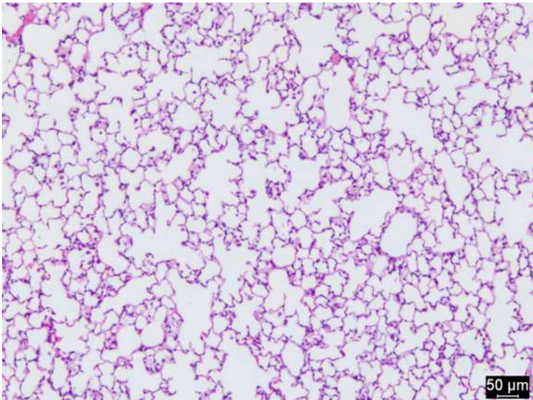
KO elastase-imatinib



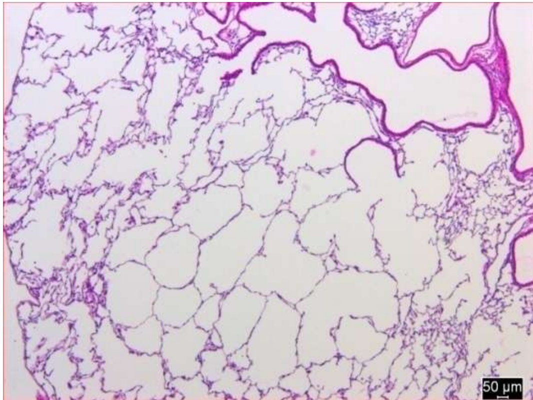


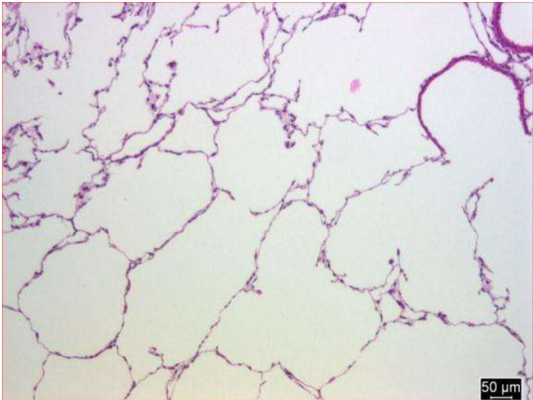
WT control-placebo



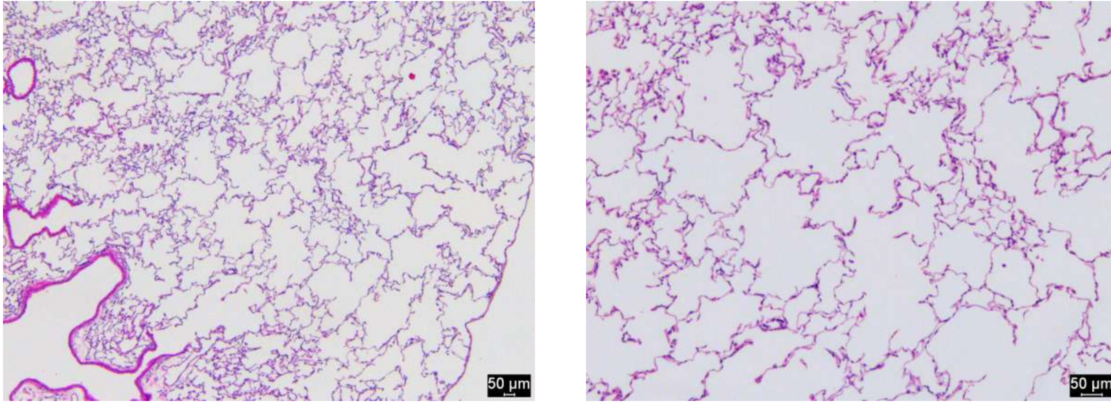


WT control-imatinib





WT elastase-placebo



WT elastase-imatinib

Figure 20. Representative images of lungs from WT and sestrin 2 KO mice after elastase or saline application combined with imatinib or placebo treatment.

Pages 66-67. HE staining. Magnifications presented for each lung (from left to right): 5x, 10x. WT: wild type, KO: sestrin 2 knockout.

5.3.3 Effect of the inhibition of PDGFRβ in hemodynamics of elastase-induced emphysema in wild type and sestrin 2 knockout mice.

The effect of imatinib on RVSP in the sestrin 2 knockout mice treated with elastase produced significantly higher values when compared to their respective controls and the mice treated with elastase alone (Fig. 21a). While in the wild type imatinib tended to lower the blood pressure (the difference however, was not significant) in the WT mice, RVSP was significantly increased compared to the placebo-treated group in the sestrin 2 KO mice. The absence of significant changes in the SAP between all groups is shown in Fig. 21b.

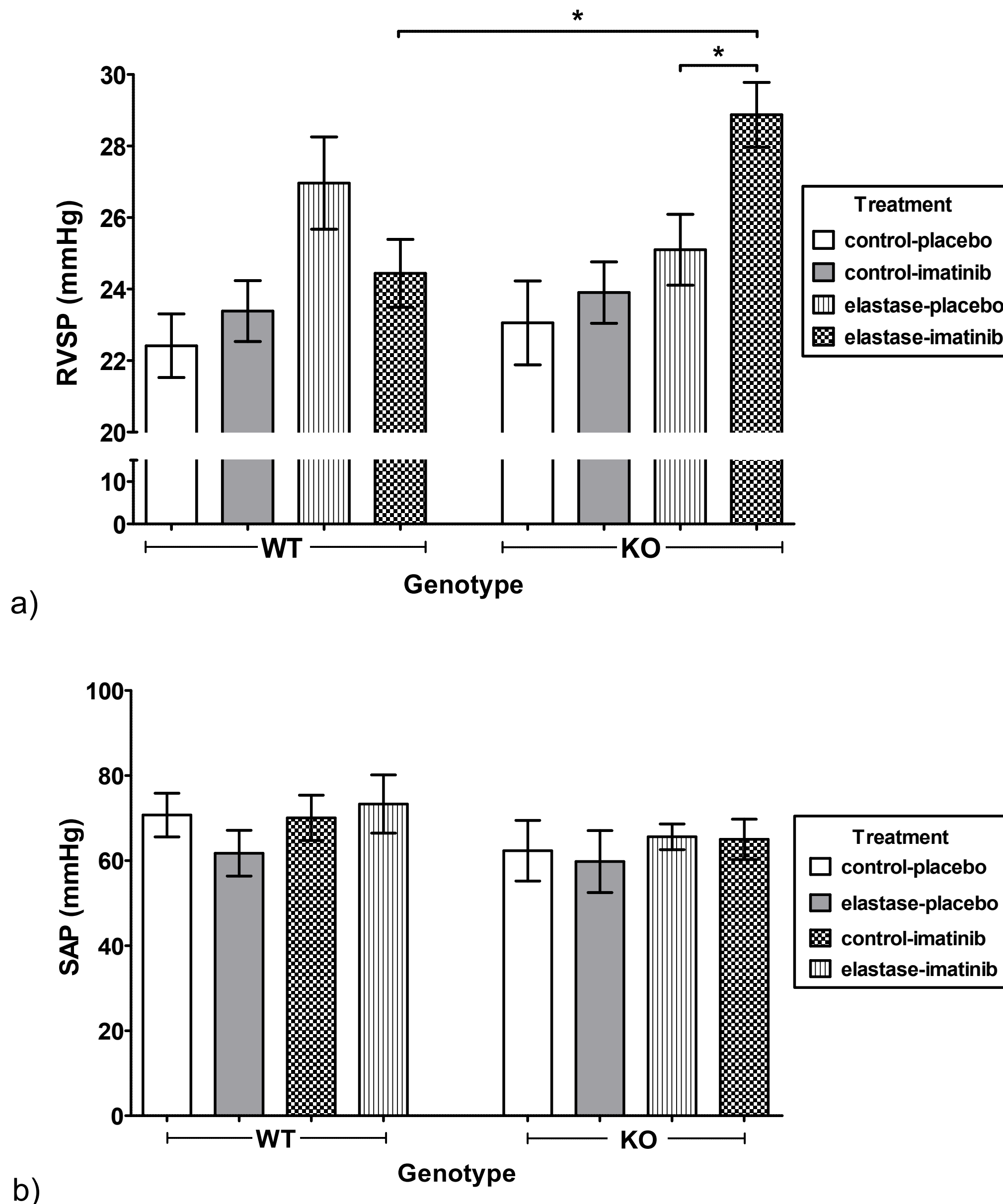


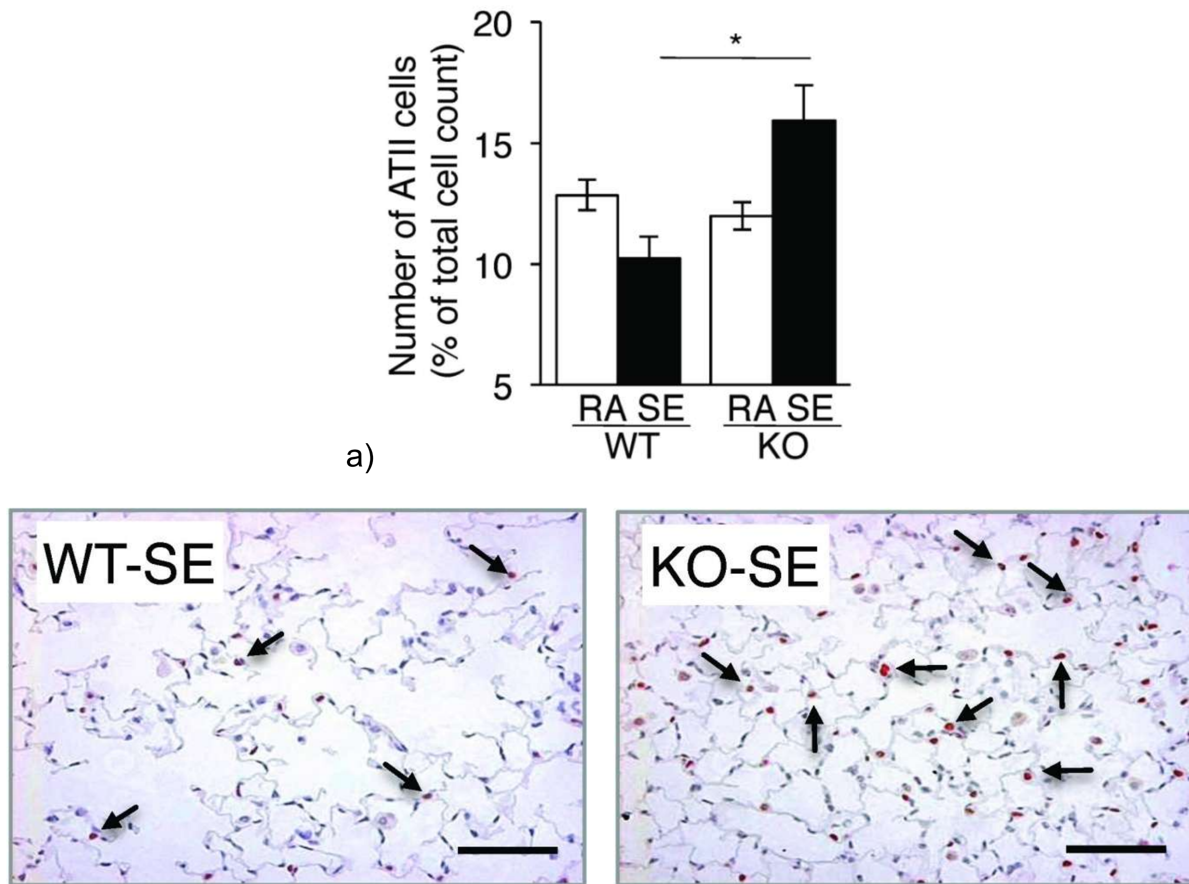
Figure 21. Effect of imatinib in the hemodynamics of wild type and sestrin 2 knockout mice treated with elastase.

Parameters evaluated: a) right ventricular systolic pressure, b) systemic arterial pressure.* $p<0.05$, ** $p<0.01$ WT: wild type, KO: sestrin 2 knockout. n=5-7. One way ANOVA was used to prove the hypothesis that imatinib treatment affected right ventricular systolic pressure and systemic arterial pressure only in knockout mice. Additional p-values are given to support the expected, negligible effect of the treatment in the WT mice.

5.4 Sestrin2 inactivation leads to increased proliferation and decreased apoptosis in alveolar wall ATII cells.

The quantification of ATII cells in the lungs of control and smoke-exposed, WT and sestrin 2 KO mice revealed that cigarette smoke caused a significantly increased

number of ATII cells in the lungs of KO mice after 8 months of smoke exposure, when compared to the lungs of WT smoke-exposed mice (Fig. 22).



b)

Figure 22. Staining and quantification of ATII cells in lungs of WT and sestrin 2 KO control and smoke-exposed mice.

a) Quantification of ATII cells in lungs of WT and sestrin 2 KO control and smoke-exposed mice. b) Representative images from immunostaining used for the quantification. Black arrows indicate TTF1 positive cells. Scale bars: 50µm. RA: Room air (control), SE: Smoke-exposed.* $p<0.05$. Data were provided by Prof. von Melchner, University of Frankfurt Medical School.

In contrast, the number of apoptotic cells in the lungs of smoke-exposed sestrin 2 KO mice was found reduced when compared to the respective WT groups. The frequency of apoptotic cells was obtained by counting 400±50 cells from 6 randomly chosen fields (Fig. 23 and 24).

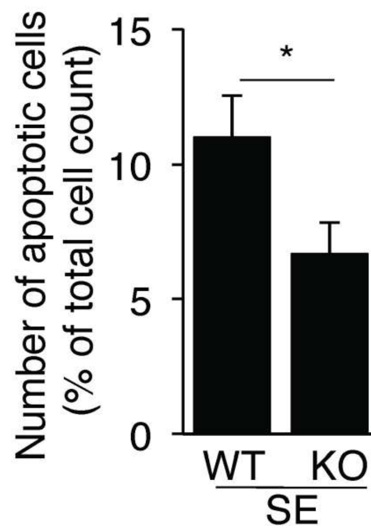


Figure 23. Quantification of apoptotic (CC3 positive) cells in the lungs of WT and KO smoke-exposed mice.

SE: Smoke-Exposed, KO: sestrin 2 knockout. Results are represented as means ± s.e.m. of n=3 mice/group.* p<0.05. Data provided by Prof. von Melchner, University of Frankfurt Medical School.

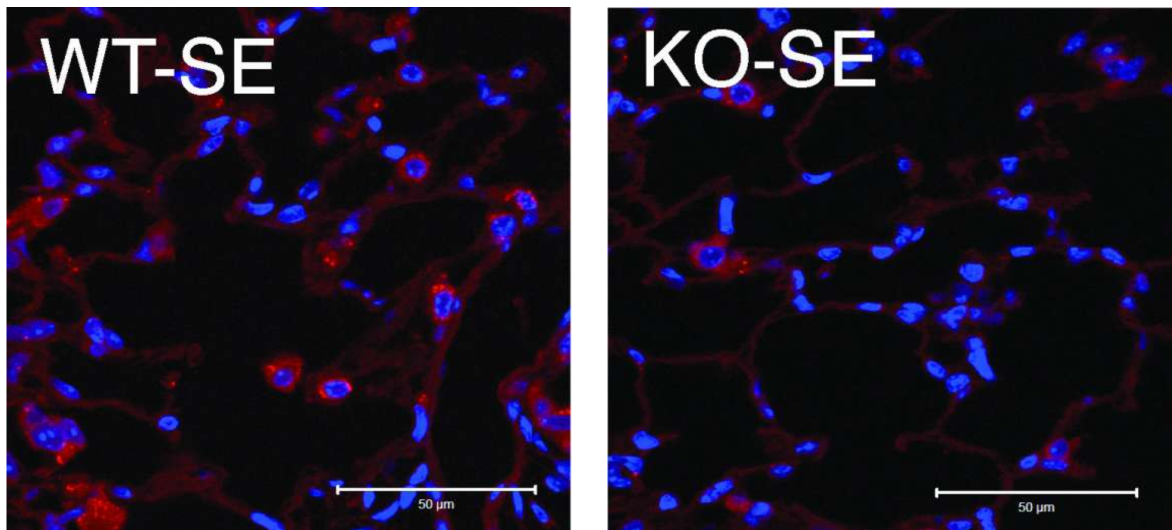


Figure 24. Lung sections stained with anti-cleaved caspase-3 (CC3-Cy3) for immunofluorescence confocal microscopy.

Red stain marks the apoptotic cells and nuclei are stained with blue (DAPI). Scale bars: 50µm. Data provided by Prof. von Melchner, University of Frankfurt Medical School.

5.5 PDGFRβ signaling contributes to lung maintenance through the induction of KGF.

KGF has long been identified as a stimulator of ATII cell proliferation (Ware and Matthay, 2002) and PDGF-BB as its inducer (Chedid et al., 1994). Thus, KGF regulation was investigated in sestrin 2 KO-MLFs. Fig. 25a shows an over 10-fold upregulation of KGF mRNA in the fibroblasts. Since the fibroblasts release KGF into the culture medium, a comparison of the cytokine levels in conditioned medium (CM) from KO-MLFs and WT-MLFs showed significantly higher levels in the KOs. When PDGF-BB was added, KGF expression was even more induced, suggesting that PDGF-BB is an upstream regulator of KGF expression (Fig. 25b).

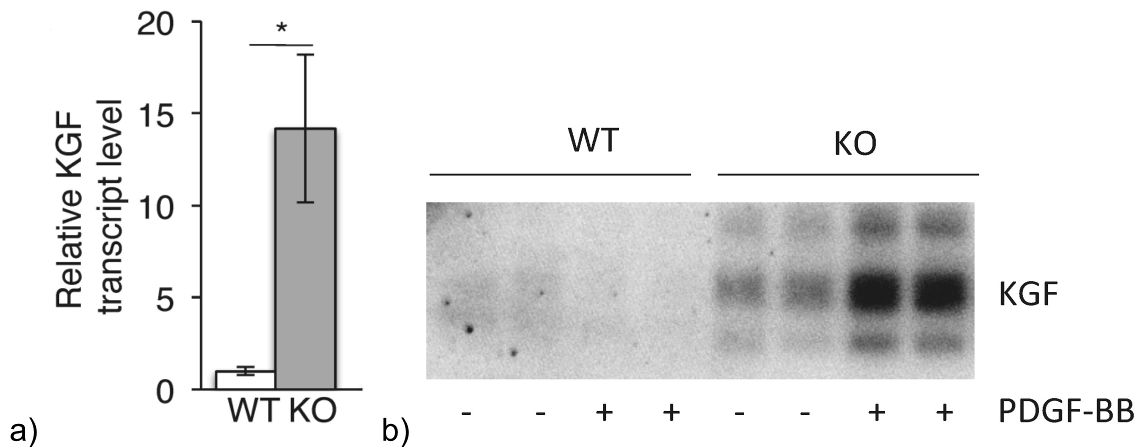


Figure 25. KGF expression in WT and sestrin 2 KO-MLFs.

a) mRNA expression in MLFs, b) western blot of 10-fold concentrated CM. * $p<0.05$ Data provided by Prof. von Melchner, University of Frankfurt Medical School. The multiple bands detected for KGF represent differently glysosylated forms of the factor (Maas-Szabowski et al., 1999). Main band size: 19 kDa.

5.5 Superoxide anions are required for the induction of PDGRβ signaling.

Since ROS accumulation has been demonstrated in sestrin 2 KO-MLFs (Budanov et al., 2002; Wempe et al., 2010) the next step in the investigations was to determine whether there is a ROS dependent mechanism involved in the activation of PDGFRβ. Electron paramagnetic resonance spectroscopy showed that levels of

O_2^- were 4 times higher in KO-MLFs when compared to the WTs (Fig. 26a). When WT-MLFs were exposed to phorbol 12-myristate 13-acetate (PMA), a stimulator of NADPH oxidase O_2^- production, PDGFRβ expression was upregulated. The addition of tempol, a superoxide dismutase mimetic led to downregulation, identifying O_2^- as the responsible ROS subspecies for the induction of PDGFRβ (Fig. 26b and 26c).

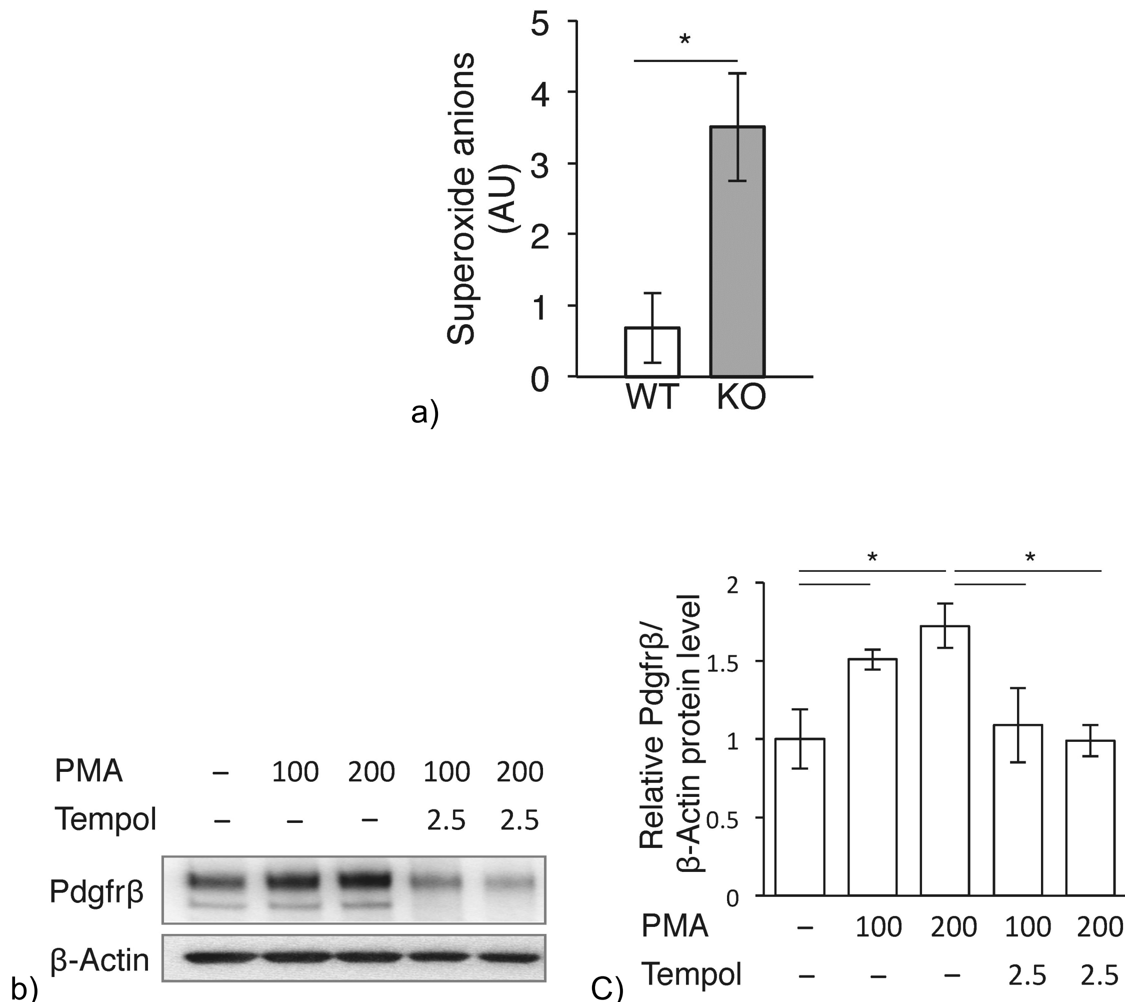


Figure 26. Induction of PDGFRβ by O2– in WT- and KO-MLFs.

a) EPR measurements of O_2^- levels in KO-MLFs of six independent measurements. AU, arbitrary units. b) Representative Western blot of PDGFRβ expression in WT-MLFs after exposure to PMA ± tempol. c) PDGFRβ levels quantified by densitometry of three separate experiments. *p<0.05. The second band detected for the PDGFRβ represents a partially glysosylated, immature form of the receptor which is approximately 170 kDa compared to the fully glycosylated, mature form of 180 kDa in size (upper band) (Hart et al., 1987).

6. Discussion

6.1 The mouse model of smoke-induced emphysema

Cigarette smoke is the main cause of COPD, a pathological condition that is the fourth ranking cause of death worldwide (Miniño, 2010). While 95% of cases can be attributed to smoking only a small percentage of smokers is susceptible to the development of the disease (Fletcher and Peto, 1977). Not only smoke affects smokers who are directly exposed to a cigarette's mainstream smoke but also causes problems to non-smokers by second-hand smoke exposure as was recently pointed out (Leberl et al., 2013), being responsible for 1% of deaths worldwide. Thus, the smoke-induced emphysema model has been favorably used to mimic the disease in several animal species, with rodents being the most commonly used. A number of studies has used mainstream smoke as the irritant at a TPM concentration comparable to that of 140mg/m^3 (Eppert et al., 2013; Leberl et al., 2013; Miller et al., 2010; Motz et al., 2010a; Motz et al., 2010b; Rangasamy et al., 2004; Seimetz et al., 2011; Witschi et al., 1997; Woodruff et al., 2009; Yao et al., 2010) as was the case with the present study.

The present study also used the standardized Kentucky 3R4F cigarettes, allowing a respective comparison of the own results with other published studies using the same reference cigarettes. As the 3R4F has equivalent and comparable smoke chemistry and toxicity to its predecessor, the 2R4F (Roemer and Schramke, 2012), the number of studies that the own results can be compared to, is even higher. It is also noteworthy that these cigarettes were designed to represent typical products of the tobacco market.

Additionally, the majority of studies that used comparable smoke concentrations and exposure duration employed the same mouse strain, the C57BL/6J (Eppert et al., 2013; Miller et al., 2010; Motz et al., 2010b; Seimetz et al., 2011; Woodruff et al., 2009; Yao et al., 2010). Diversity in the level of susceptibility, lung structure, lung function and pathophysiological mechanistic responses to smoke has been observed between species and between strains of the same species. Guinea pigs have proven to be very susceptible but the number of commercially available antibodies to perform molecular investigations is limited; rats have demonstrated a resistance to developing emphysema after smoke exposure

developing minimal disease and at high concentrations of smoke causes particle overload effects (Stevenson et al., 2007), while mice are in the middle with different strains exhibiting different levels of susceptibility. In particular, the C57BL6 strain also used in this study is the strain used in a large number of studies (Eppert et al., 2013; Miller et al., 2010; Motz et al., 2010b; Seimetz et al., 2011; Woodruff et al., 2009; Yao et al., 2010) since its deficiency in antielastase (decreased serum concentration of a_1-protease inhibitor for elastase (Cavarra et al., 2001; Gardi et al., 1994), high contents of elastase and cathepsin G in lysosomes (Gardi et al., 1994) make it mildly susceptible and thus popular in similar experimental designs. The chronic smoke exposure of a duration of eight months in the own study can be compared to the manifestation of pathological changes seen in humans developing COPD after a long history of smoking, notably emphysema and pulmonary hypertension (Churg and Wright, 2009; Leberl et al., 2013; Seimetz et al., 2011; Wright and Churg, 2008).

While it has already been demonstrated by previous studies of our research group (Seimetz et al., 2011; Weissmann et al., 2014) that cigarette smoke exposure induces similar functional and structural alterations in mice there are some limitations and considerations to be taken into account when extrapolating the results to the human disease. In this regard, mice do not exhibit the symptoms of the disease that humans exhibit and upon which the diagnose of COPD is based like chronic cough, phlegm and persistent shortness of breath (Fehrenbach, 2002). Moreover, in our smoke-induced emphysema, only cigarette smoke was used as a contributing factor, while the pathogenesis of the human disease is multifactorial (Fehrenbach, 2002). Another limitation of our model is that it does not result in the airway obstruction seen in humans, but more subtle alterations (March et al., 2006). Nevertheless, the aim of the own study was the investigation of sestrin 2 in alveolar maintenance, so the choice of this model was appropriate. Additionally, the smoke exposure used in this study resulted in a mild form of emphysema in the wild type mice comparable to a GOLD stage I/II COPD, rather than the stage III/IV that is normally diagnosed in human patients (Churg and Wright, 2009; Wright et al., 2008). However, it does offer an insight to pathophysiological mechanisms involved and thus potential early interventions that might prove to be useful and easier to design (Churg et al., 2008; Churg et al., 2011; Nikota and Stämpfli, 2012). In addition, the alterations observed in mice after 8 months of smoke exposure and human patients with developed COPD and a history of smoking are similar (Seimetz et al., 2011). Also, with regard to the

temporal onset of emphysema development, a chronic exposure to cigarette smoke should be used to relate to the human disease, which also manifests after a long-term exposure (Leberl et al., 2013).

6.2 Structural and functional changes in wild type mice after smoke exposure

After eight months of smoke exposure the wild type mice displayed a significant increase in dynamic lung compliance. The impairment of lung function mirrors the structural alterations. A correlation between the degree of histopathological changes and lung function has already been demonstrated (Hogg, 2008; Nakano et al., 2000). Histopathologically the lungs of the WT mice exhibited an increased airspace and thinner alveolar walls (increased airspace & and reduced SWT) from the loss of tissue and enlarged alveoli from the rupture of neighboring walls (increased MLI). The parameters used for the structural evaluation of the lungs used in this study have also been used in other similar investigations (Bracke et al., 2006; Canning and Wright, 2008; Maeno et al., 2007; Seimetz et al., 2011). The damage of elastic fibers in the parenchyma resulted in a loss of elasticity of the airway walls that also manifested functionally as an increase in compliance. This alveolar wall destruction could be attributed to increased apoptosis after smoke exposure and its interaction with inflammation, protease/antiprotease balance and oxidative stress. Apoptosis and proliferation of alveolar cells are increased in emphysematous lung tissue (Imai et al., 2005). These cell types (alveolar epithelial type I and II, endothelial and myofibroblasts) are in close proximity and have significant contacts amongst them (Sirianni et al., 2003). Consequently, severe damage to one of them could cause a disruption of the other and furthermore the matrix and interstitium. A concordant destruction of these cells is required for the disruption of the alveolar wall.

Cigarette smoke causes lung inflammation characterized by an influx of inflammatory cells, notably macrophages, neutrophils and lymphocytes, predominantly CD8+ T-lymphocytes. Neutrophils release elastase, reactive oxygen species and cytokines that can result in the structural changes characterizing emphysema (Chung and Adcock, 2008). It has already been reported by Wang and colleagues in a transgenic mouse model that CD8+ cells via expression of IFN-γ cause epithelial cell DNA damage and apoptosis correlated to activation of death

receptor and mitochondrial apoptosis pathway that finally lead to alveolar enlargement and increased compliance (Wang et al., 2000). In their model, cathepsin S was instrumental in the IFN-γ induced DNA damage, apoptosis, emphysema and protease accumulation (Zheng et al., 2005). Additionally there is a correlation between the number of lymphocytes and tissue destruction, and CD8+ cells produced type and cytokines, that link CD8+ cells and IFN-γ overexpression to emphysema, increased compliance and increased accumulation of inflammatory cells. The resulting tissue, lung matrix and elastin degradation would lead to the modification of the parameters quantifying (functionally and morphometrically) the emphysema seen in the lungs of the WT mice after smoke exposure. Sestrin 2 by repressing PDGRβ (and consequently KGF induction) and mTOR signaling could contribute to this process by inducing apoptotic and pro-inflammatory effects, respectively.

Cigarette-smoke exposure also results in shifting the protease-antiprotease balance towards the side of proteolysis by increasing neutrophil elastase, cathepsins, matrix metalloproteinases and decreasing α1-antitrypsin airway epithelium-derived secretory leukoprotease inhibitor and tissue inhibitors of matrix metalloproteinases. This shift will eventually lead to the breakdown of connective tissue components, for the most part elastin. The same fragments of elastic fibers produced also act as a chemotactic for monocytes leading to macrophage accumulation and further tissue breakdown. Macrophages will produce matrix metalloproteinases (MMP-2, MMP-9, MMP-12), cathepsins (K, L, S) and neutrophil elastase (Petruzzelli et al., 1997; Punturieri et al., 2000). This activity of the macrophages of COPD patients is greater at a baseline level when compared to healthy smokers and is even more amplified by smoke exposure (Lim et al., 2000; Russell et al., 2002a; Russell et al., 2002b). Macrophage numbers are also increased by a 25 fold factor in lung tissues from COPD patients compared to normal smokers (Retamales et al., 2001) where they localize to sites of alveolar wall destruction (Finkelstein et al., 1995; Meshi et al., 2002).The lung matrix destruction ensuing from the interaction between neutrophils and macrophages and the proteolytic enzymes they produce (Maeno et al., 2007), promotes airspace enlargement quantified in histological sections as an increase in airspace percentage and mean linear intercept (mean distance between alveolar walls) and a decrease of septal wall thickness, changes observed in a significant degree in the lungs of the WT mice used in the present study.

Another contributing factor to the thinning the alveolar wall is the cigarette-induced apoptosis of ATII cells, an event demonstrated and quantified in the present study. Apoptosis has been shown to be increased in emphysematous lung tissue. Imai and colleagues showed increased expression of pro-apoptotic proteins like Bax and Bad and activation of caspase-3, while the counteracting Bcl-2 could not be detected in neither healthy nor damaged lungs (Imai et al., 2005). Kasahara and colleagues also showed increased apoptosis of epithelial and endothelial cells in lungs with emphysema when compared to healthy lungs from smokers and nonsmokers, while they couldn't demonstrate a difference in apoptosis between the lungs of healthy smokers and smokers without emphysema (Kasahara et al., 2000). Hodge et al. also reported an increased apoptosis in lungs of smokers even after smoking cessation (Hodge et al., 2005). The fact that apoptosis persists after smoking cessation (Hodge et al., 2005) further suggests that smoke is not necessary for the induction of apoptosis after COPD establishment but that it is the interaction between the rest of the pathophysiological mechanisms that regulates this process. This interaction between the two most prominent mechanisms of alveolar destruction (protease/ anti-protease imbalance and apoptosis) has already been demonstrated by studies showing that apoptosis signals are substrates of proteases as in the case of the FAS ligand and MMP7 in prostate involution (Powell et al.). Neutrophil elastase causes the impaired clearance of apoptotic cells in cystic fibrosis and bronchiectasis by cleaving the phospatidylserine receptor in macrophages. In addition, the production of ceramide stimulated by cigarette smoke, also mediates apoptosis, proteolysis and alveolar destruction. Additionally, the antiprotease α1-antitrypsin has been reported to inhibit staurosporine-induced caspase 3 activation (Petrache et al., 2006) providing further evidence for the interaction between apoptosis and the protease/antiprotease balance. The loss of tissue and the resulting thinning of the alveolar walls render them susceptible to rupturing from the application of mechanical forces during respiration which could also contribute to the impaired lung function of the smoke-exposed mice.

Since the alveolar septal walls include vessels and lung maintenance requires all of its components to be intact, the vascular compartment might also contribute. Liebow demonstrated thin and almost avascular septal walls in emphysematous lungs. He further hypothesized that the disruption of alveolar walls could be induced by the reduced blood flow (Liebow, 1959). G.W. Wright suggested a "vasculonecrotic

nature" of emphysema injury (Wright and Kleinerman, 1963). Such a contribution has also been supported by studies demonstrating the involvement of VEGFR blockade in the development of emphysema (Kasahara et al., 2000; Morissette et al., 2009; Tang et al., 2004). Other studies have demonstrated the connection between VEGF and mTOR and the importance of mTOR in emphysema development mainly through its inactivation by Rtp801 (Seimetz et al., 2011; Yoshida et al., 2010).

The upregulation of sestrin 2 in the lungs of the WT mice by smoke could theoretically contribute to the development of emphysema through the repression of PDFGRβ and mTOR (Wempe et al., 2010) that in turn results in amplification of mechanisms (endothelial and alveolar epithelial cell apoptosis, inflammation and protease/anti-protease imbalance) that lead to the disruption of lung maintenance programs (Fig. 27).

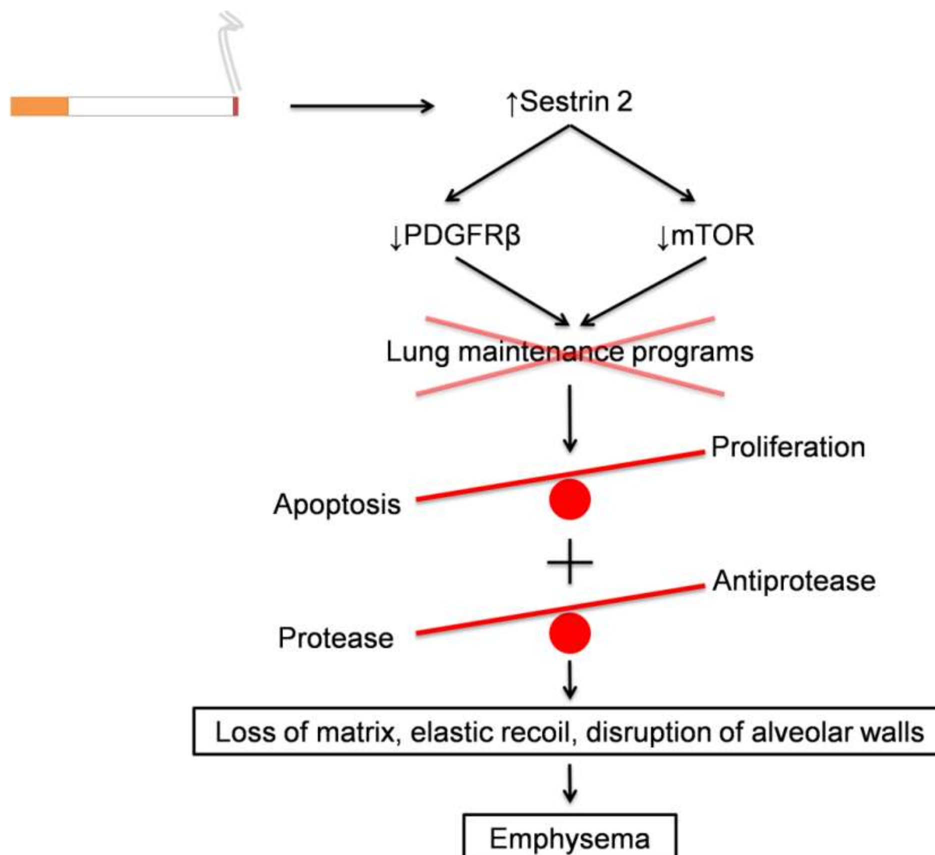


Figure 27. Possible participation of sestrin 2 in the pathogenesis of emphysema through the disruption of lung maintenance.

The repression of PDGFRβ and mTOR signaling leads to the shifting of injury/repair mechanisms towards the latter, leading to the development of pulmonary emphysema.

6.3 sestrin 2 knockout mice did not exhibit the same functional and structural changes as wild type mice after smoke exposure.

After 8 months of smoke exposure, sestrin 2 KO mice exhibited normal lung function and physiological lung structure (alveolar morphometry and intact elastin fiber content) and activation of lung maintenance programs (KGF upregulation, ATII cell proliferation, elastin production). The basic structure of the lung's primary anatomical and functional unit, the alveolus, is the alveolar interstitium. This structure acts as the scaffold on which the cellular components of the alveolar wall proliferate and differentiate. Elastin together with collagen are the two prominent components of the extracellular matrix network and the thin basement membrane (80–90 nm width) to which the airway epithelial cells are attached (Roche et al., 1989). Being primarily composed of elastin, collagen and proteoglycans it is also accountable for the lung's basic mechanistic feature: elasticity. Additionally to the collagen that provides tensile strength and elastin being responsible for the distensibility of the lungs, ATII cells also contribute to the elastic recoil through the production of surfactant and its effects on surface tension. The destruction of elastin, the cleavage of alveolar walls, the disturbances in surface tension can lead to both an impaired lung function and a respective histopathological appearance of the lungs. The preservation of intact elastin fibers, the decreased apoptosis and increased proliferation of ATII cells contributes to the prevention of emphysema development in the lungs of the sestrin 2 KO mice.

The cells primarily in charge of the production and maintenance of the alveolar interstitium are the lung fibroblasts. After any type of injury the repair of lung parenchyma relies with the interactions of epithelial cells, mesenchymal cells as well as endothelial cells and particularly between fibroblasts and ATII cells who are the most active participants in lung maintenance in the adult lung. Our study has provided evidence that the inactivation of sestrin 2 in the sestrin 2 KO lungs resulted, through the upregulation of PDGFRβ signaling, in the initiation of lung maintenance programs. One of the major requirements for alveolar regeneration is the formation of the structural scaffold made up by elastin, which then drives alveogenesis (Wendel et al., 2000). This process is then completed with the repopulation of the walls by all the cellular components that comprise it, an event promoted by pathways induced by the inactivation of sestrin 2: PDGFRβ, KGF, (present study) TGF-β and mTOR (Wempe et al., 2010). The mechanism of protection of the sestrin 2 knockout mice from

developing emphysema can be attributed to the induction of proliferation and counteraction of ATII cell apoptosis resulting from the inactivation of sestrin 2. This inactivation can lead to enhanced PDGFRβ signaling which in turn stimulates the production of KGF by the pulmonary fibroblasts. KGF is a potent mitogen for a number of epithelial cells including ATII cells in the lung that mediates their maturation and proliferation (Deterding et al., 1996; Schmeckebier et al., 2013). While the growth factor is produced by fibroblasts, its receptors are expressed only in epithelial cells revealing a paracrine function and an important role in mesothelial-epithelial interactions (Danilenko, 1999).

After the disruption of the alveolar epithelial lining by cigarette smoke, the restoration process involves the proliferation of ATII cells and their differentiation into ATI cells (Bachofen and Weibel, 1974; Haschek and Witschi, 1979; Rannels and Rannels, 1989). KGF has been demonstrated to be an important endogenous trigger of alveolar repair in different lung injury models. Charafeddine et al. showed that increased expression of KGF in rabbit lungs after hyperoxic injury resulted in an increased ATII cell proliferation (Charafeddine et al., 1999) and Ray et al. achieved protection by inducibly expressing KGF in mouse lungs using the same type of injury. Additionally, the peak of KGF levels in BALF coincided with the peak of ATII cell proliferation after bleomycin injury in rats (Adamson and Bakowska, 1999). In another study, administration of exogenous KGF was correlated to declined levels of p53, Bax, and Bcl-x proteins that trigger cell death as well as plasminogen activator inhibitor-1 after oxygen-induced lung injury in mice (Barazzone et al., 1999).

The effect of KGF is also mediated by TGF-β. Yildirim et al. showed, using a TGF-β1 neutralizing antibody, that recombinant KGF induced elastin expression mediated by TGF-β1 in a mouse model of emphysema (Yildirim et al., 2010). TGF-β has also been shown to activate elastin transcription in human lung fibroblasts that is dependent on phosphatidylinositol 3-kinase/Akt activity (Kuang et al., 2007). The repair of the elastic fibers is complex for the elastin producing cells because the process of damaged fiber replacement requires a sophisticated coordination of all the molecules that constitute the microfibrils as well as the elastin cross-linking enzymes also shown to be inhibited by cigarette smoke *in vitro* (Laurent et al., 1983). This inability of the cells in the adult damaged lungs might also be a failure of gene reactivation in the required ratios and order necessary for the assembly of the complex elastic fiber, a mechanism that appears to work flawlessly in lung

development (Shifren and Mecham, 2006). In the sestrin 2 KO mice, the upregulation of KGF also enhanced by the amplified PDGFRβ signaling, could then have led to the protection from the disease phenotype.

As mentioned before, the fragments produced during the breakdown of elastin seen in emphysema act as chemotactic factors for monocytes and macrophages *in vitro* (Hunninghake et al., 1981; Senior et al., 1980; Senior et al., 1984) and have been shown to promote the progression of smoke-induced and elastase-induced emphysema in mice (Houghton et al., 2006). While the wild type mice in our study exhibited a significantly reduced number of intact elastic fibers and consequently an increase in the numbers of fragments, the lungs of sestrin 2 knockout mice showed practically no difference before and after cigarette smoke exposure, indicating that one of the promoting and perpetuating mechanisms that sustain the pathogenesis of and development of emphysema was disrupted. Additionally, the induction of PDGFRβ expression and the amplification of its signaling in the lungs of the sestrin 2 knockout mice played a key role in the remodeling and injury repair via the induction of KGF and elastin expression.

Chen et al. detected activated autophagic proteins and increased autophagy in the lungs of COPD patients with emphysema. Sestrin 2 has been reported in previous studies to be a negative regulator of mTOR through AMPK phosphorylation and thus suppress cell growth and proliferation in cancer cells (Budanov and Karin, 2008; Sanli et al., 2012). While under low hydrogen peroxide concentrations it can act as an antioxidant through regenerating peroxiredoxins and maintaining cell viability through the Nrf2/ARE pathway, under elevated concentrations of hydrogen peroxide it induces apoptosis through the p53 pathway (Budanov et al., 2002; Budanov et al., 2010; Sablina et al., 2005; Shin et al., 2012). Recently the role of sestrin 2 in induction of apoptosis and the activation of the AMPK/p38/BAX signaling pathway in colorectal cancer was investigated and it was demonstrated that quercetin-induced apoptosis requires the expression of sestrin 2 as does the inhibition of mTOR through AMPK phosphorylation (Kim et al., 2014). In this sense the induction of mTOR in the sestrin 2 KO mice also contributes to the restoration of the alveolar wall.

6.4 Cigarette smoke exposure caused pulmonary hypertension and right heart hypertrophy in WT but not in the sestrin 2 KO mice.

Cigarette smoke resulted in hemodynamic alterations (significant increase of RVSP and significant right heart hypertrophy) in the WT mice after 8 months of exposure. Smoke exposure leads to vascular remodeling and loss of vessels (Seimetz et al., 2011). In the mild form of emphysema produced in the cigarette smoke model hypoxic vasoconstriction is most likely not involved in the development of the hemodynamic alterations since these mice have been shown neither to be exposed to hypoxia nor to suffer from hypoxemia (Seimetz et al., 2011). Such mechanisms and chronic hypoxia can, however, be a driving factor of vascular remodeling in severe, end-stage COPD (Voelkel et al., 2011). The vascular remodeling induced by cigarette smoke in mice is mainly restricted to the wall media with an increased degree of muscularization. However, the vascular remodeling seen in COPD involves all layers of the vessel wall with intimal alterations being the most prominent, and is not exclusively attributed to prolonged hypoxic vasoconstriction (Elwing and Panos, 2008). Inflammatory cells also participate in this process. An increase of predominantly CD8+ activated T lymphocytes infiltration in the adventitia of pulmonary arteries causes an impairment of endothelium-dependent vascular relaxation and intimal thickening (Peinado et al., 2008). The loss of expression of enzymes and proteins that are physiologically expressed in the endothelium such as prostacyclin synthase, NO synthase and VEGF and VEGFR can lead to EC dysfunction, EC apoptosis and intimal fibrosis (Voelkel et al., 2011). In patients with mild-to-moderate COPD endothelial dysfunction resulting from this imbalance between these vasodilating mediators and vasoconstrictors such as ET-1 and angiotensin (Peinado et al., 2008) also triggers hemodynamic alterations. In summary, the significant elevation in RVSP seen in the WT mice after 8 months of smoke exposure is most probably related to vascular remodeling, inflammation and a loss of vessels as previously shown (Seimetz et al., 2011). The increase in RVSP as seen in WT mice was not observed in the sestrin 2 KO mice. This protection may be attributed to the enhanced mTOR signaling in the KO lungs (Wempe et al., 2010). mTOR has been shown to have anti-inflammatory and angiogenic effects. It inhibits the production of inflammatory cytokines as IL-12, IL-23, TNF-α and IL-6 through the transcription factor NF-κB and simultaneously induces the release of the anti-inflammatory IL-10 through STAT3 (Weichhart et al., 2008). It also promotes

angiogenesis through inhibiting IL-12 and inducing the expression of angiogenic factors such as VEGF, PGDFβ and TGF-α (Advani, 2010). Thus, the activation of mTOR signaling in the KO lungs might counteract the effects of smoke in the vasculature by counteracting inflammation and rebuilding the lost vessels. Additionally, PDGFRβ can contribute to the maturation and stability of these new vessels by pericyte recruitment and smooth muscle cell proliferation (Betsholtz, 2004). The effect of sestrin 2 inactivation on the vasculature in this study is currently under investigation.

6.5 Sestrin 2 is a repressor of PDGFRβ signaling and alveolar maintenance programs.

The present study indicated that the inactivation of sestrin 2 induced PDGFRβ signaling and that PDGFRβ can be a key switch in lung injury repair. Additional evidence for this conclusion was provided by the exacerbation of lesions in the sestrin 2 knockout lungs that underwent an elastase application, after the inhibition of PDGFRβ by imatinib. It can be suggested that imatinib abolished PDGFRβ participation in the injury repair process which necessitates ECM proteins, proliferation of fibroblasts and smooth muscle cells and new vessel formation. This could explain why emphysema was exacerbated in these mice, as indicated by the worsening of lung function (increased compliance) and structural lesions in their lungs (alveolar morphometry). The absence of a significant effect on lung structure and function in the WT mice, where PDGFRβ signaling is steady might be attributed to a lower susceptibility to imatinib (Hägerstrand et al., 2006).

The upregulation of sestrin 2 in the lungs of smoke-exposed WT mice led to the suppression of PDGFRβ and KGF expression, an effect not seen in the lung homogenates of the sestrin 2 KO mice, which resulted in increased elastin production. The resulting reduced lung pathology corroborates the results of a previous study using a genetic model of emphysema of impaired TGF-β signaling and elastic fiber structure (Wempe et al., 2010), which identified sestrin 2 as a repressor of both TGF-β and mTOR. The amplification of PDGFRβ signaling after the inactivation of sestrin 2 in the lungs of KO smoke-exposed mice also indicated the key role of this pathway in the protection against emphysema. The effect of the inactivation of an antioxidant which would theoretically lead to ROS accumulation, known to participate in the pathogenesis of COPD, appears contradictory at a first

glance. The crucial role of the participation of ROS in signal transduction has been demonstrated in the context of a plethora of pathways (Alexander et al., 2010; Bae et al., 1999; Choi et al., 2005; Finkel, 2011; Frijhoff et al., 2014; Martindale and Holbrook, 2002; Sundaresan et al., 1995). Along these lines, the present study also reported that PDGFRβ signaling requires O_2^-. The employment of PMA which activates O_2^- generation by NADPH oxidase supports this conclusion. The different, spatial, cellular and subcellular effects of ROS and their time-dependent generation can explain the “first glance” contradiction. For example, sestrin 2 could potentially be involved in an intrisinc regulation of ROS production and accumulation but its upregulation by smoke in COPD lungs leads to an elimination of the amounts of ROS (O_2^-) needed for the activation of signaling pathways that regulate alveolar maintenance. In addition, it has to be taken into account that sestrin 2 also activates Nrf2 through the degradation of Keap1 without requiring its catalytic function (Bae et al., 2013).

6.6 Conclusion

The current study revealed sestrin 2 as a potential therapeutic target for the treatment of COPD in mice. Sestrin 2 is upregulated in mice after smoke exposure and promotes the development of emphysema by repressing alveolar maintenance and repair mechanisms. The mutational inactivation of sestrin 2 in mice prevents from the development of emphysema/COPD through the initiation of protective mechanisms beginning with the upregulation of PDGFRβ and mTOR signaling and concluding with the production of elastin through KGF secretion (Fig. 28).

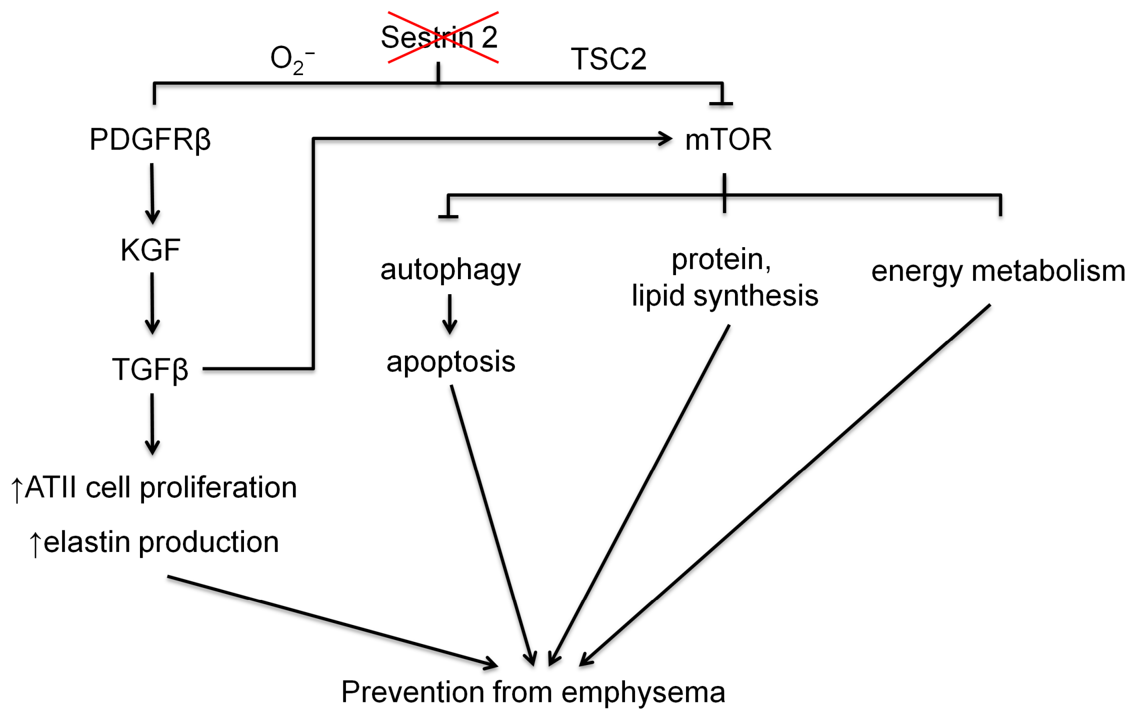


Figure 28. Suggested pathways affected by sestrin 2 inactivation, leading to prevention from emphysema and PH.

The inactivation of sestrin 2 induces alveolar maintenance programs that prevent/ protect from the development of emphysema in mice. PDGFRβ plays a central regulatory role in this mechanism that begins with the activation of PDGFRβ and can act via mTOR and TGF-β in maintaining the lung parenchyma.TSC2: tuberous sclerosis complex 2, PDGFRβ: Platelet-derived growth factor receptor beta, KGF: keratinocyte growth factor, TGF-β: transforming growth factor beta, mTOR: mammalian target of rapamycin, ATII: alveolar epithelial cells type II.

8. SUMMARY

Chronic obstructive pulmonary disease (COPD) is the fourth ranking cause of death worldwide. Despite the extensive efforts that have been made so far, the disease remains still incurable due to a lack of therapeutic targets that can prevent from the development or even reverse already established disease. Only symptoms can be alleviated to some degree, by the current treatments available. Thus, it still causes high morbidity, mortality, disability, health resources depletion and socioeconomic burden. One of the major components of COPD is pulmonary emphysema caused by the destruction and of alveoli after exposure to noxious particles and gases, with cigarette smoke and smoke from heating and cooking being the most prominent. The disruption of signaling pathways responsible for lung integrity maintenance is thought to be a key point in the development of emphysema. Reactive oxygen species generated after the exposure to smoke could disrupt these alveolar maintenance programs. It has already been reported that the inactivation of the sestrin 2 gene, encoding the member of a family of highly conserved antioxidant proteins, can partially rescue the disease phenotype in a genetic mouse model of emphysema. This primarily contradictory finding that deletion of an antioxidant protein can protect from a genetically induced emphysema was attributed to the restoration of impaired TGF-β signaling. Sestrin 2 has also been shown to inhibit mTOR. This led to the hypothesis that sestrin 2 might play a role in emphysema development in a chronic model of tobacco smoke exposure. To this end, wild type and knockout mice were exposed to smoke of a 140mg/m^3 total particulate matter (TPM) concentration, for 6 hours/day, 5 days/week for a period of 8 months. The wild type mice developed pulmonary emphysema as indicated functionally by a significant increase in pulmonary compliance and pulmonary hypertension indicated by a significant increase in right ventricular systolic pressure. Morphometric evaluation also revealed significant structural changes: increase in airspace% and mean linear intercept, and decrease in alveolar septal wall thickness. In contrast the loss of function of sestrin 2 resulted in the prevention of emphysema development in the knockout mice which demonstrated no significant changes in these parameters when compared to respective controls. Sestrin 2 and PDGFRβ mRNA were up- and down-regulated respectively in the lungs of wild type mice after smoke exposure. Furthermore, sestrin 2 knockout mice exhibited increased numbers of alveolar epithelial type II cells and decreased numbers of apoptotic cells after smoke

exposure when compared to wild type mice. This study demonstrated that the mutational inactivation of sestrin 2 induces PDGFRβ signaling which preserves lung integrity by initiating a cascade of events also involving KGF and mTOR signaling that results in increased ATII cell proliferation and elastin production. If these data are transferrable to the human situation, a sestrin 2 antagonist could be beneficial in the treatment of COPD.

9. ZUSAMMENFASSUNG

Derzeit gehört die chronisch obstruktive Lungenerkrankung (COPD) zu der vierthäufigsten Todesursache weltweit. Trotz bisherigen umfangreichen Bemühungen, gibt es bis heute noch keinen Therapieansatz, der die Entwicklung der COPD-Erkrankung verhindert oder gar eine bereits etablierte Erkrankung heilen kann. Bisher können lediglich ein Teil der Symptome behandelt werden. Folglich verursacht COPD eine hohe Morbidität, Mortalität und Invalidität resultierend in einer Erschöpfung von Gesundheitsressourcen und einer sozioökonomische Belastung. Zu den Hauptmerkmalen der COPD gehört das Lungenemphysem, charakterisiert durch eine Zerstörung der Alveolen aufgrund einer Exposition gegenüber schädlicher Partikel und Gase. Zu den bedeutendsten Faktoren scheinen eine Inhalation von Zigarettenrauch und eine Verbrennung von Biomasse zum Kochen und Heizen zu sein. Derzeit wird eine Dysregulation von Signalwegen zur Erhaltung der Lungenintaktheit mit der Entwicklung eines Lungenemphysems in Verbindung gebracht. Reaktive Sauerstoffspezies, die nach einer Rauch-Exposition generiert werden, könnten diese alveolaren Instandhaltungsmechanismen zerstören. Bisherige Studien berichteten, dass eine Inaktivierung des Sestrin-2 Gens, welches ein Mitglied der Familie von hoch konservierten antioxidativen Proteinen kodiert, zu einem partiellen Schutz des Krankheitsphänotypes in einem genetisches Emphysem-Maus-Modell führte. Diese zunächst widersprüchliche Erkenntnis, dass eine Inaktivierung eines antioxidativen Proteins vor einem genetisch induzierten Emphysems schützt, wurde auf die Restoration des beeinträchtigten TGF-β Signalweges zurückgeführt. Zusätzlich wurde gezeigt, dass Sestrin-2 mTOR inhibiert. Im Rahmen dieser Dissertation sollte daher die Hypothese untersucht werden, ob Sestrin-2 eine Rolle bei der Entwicklung eines Lungenemphysems in einem Zigarettenrauch- induzierten Emphysemmodell der Maus spielen könnte. Hierfür wurden Wildtyp- und Knockout-Mäuse 6 Stunden pro Tag, 5 Tage die Woche für 8 Monate bei einer Rauchpartikelkonzentration von 140 mg/m³ beraucht. Rauch-exponierte Wildtyp-Mäuse entwickelten ein Lungenemphysem und eine pulmonale Hypertonie funktionell gekennzeichnet durch eine signifikant erhöhte Lungencompliance und durch einen signifikanten Anstieg des systolischen rechtsventrikulären Druckes. Morphometrische Analysen zeigten zusätzlich signifikante strukturelle Veränderungen: eine Erhöhung des Gesamtluftraums der Alveolen und des mittleren Abstandes zwischen den Alveolarsepten, sowie eine niedrigere alveolare

Septendicke. Eine Inaktivierung von Sestrin-2 in Knockout-Mäusen verhinderte die Entwicklung eines Lungenemphysems, da im Vergleich zu den respektiven Kontrolltieren keine signifikanten Veränderungen in den genannten Parametern zu sehen waren. Sestrin-2 und PDGFRβ mRNA waren im Lungenhomogenat von Wildtyp-Mäusen nach Rauch-Exposition entsprechend hoch- und herunterreguliert. Des Weiteren zeigten Rauch-exponierte Sestrin-2 Knockout-Mäuse eine erhöhte Anzahl an Alveolarepithelzellen Typ II und eine verminderte Anzahl an apoptotischen Zellen im Vergleich zu Wildtyp-Mäusen. Mit dieser Studie konnte gezeigt werden, dass ein Knockout von Sestrin-2 den PDGFRβ- Signalweg induziert und somit die Lungenintaktheit während einer Rauch-Exposition erhalten bleibt. Hierbei wurden Ereigniskaskaden initiiert, die in einer erhöhten ATII Zellproliferation und Elastinproduktion resultierten. Diese Ergebnisse werden unter anderem mit dem KGF und dem mTOR-Signalweg in Verbindung gebracht. Vorausgesetzt, dass man diese Ergebnisse auf den Menschen übertragen kann, könnte ein Sestrin-2 Antagonist eine neue Behandlungsstrategie für COPD-Patienten sein.

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11. DECLARATION

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided to the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

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Athanasios Fysikopoulos

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PUBLICATIONS-ORAL PRESENTATIONS-POSTER PRESENTATIONS

14. PUBLICATIONS

- “Stimulation of soluble guanylate cyclase prevents cigarette smoke-induced pulmonary hypertension and emphysema”.(Weissmann N, Lobo B, Pichl A, Parajuli N, Seimetz M, Puig-Pey R, Ferrer E, Peinado VI, Domínguez-Fandos D, **Fysikopoulos A**, Stasch JP, Ghofrani HA, Coll-Bonfill N, Frey R, Schermuly RT, García-Lucio J, Blanco I, Bednorz M, Tura-Ceide O, Tadele E, Brandes RP, Grimminger J, Klepetko W, Jaksch P, Rodriguez-Roisin R, Seeger W, Grimminger F, Barberà JA.) Am J Respir Crit Care Med. 2014 Jun 1;189(11):1359-73. doi: 10.1164/rccm.201311-2037OC.
- “Sestrin-2, a repressor of PDGFRβ signalling, promotes cigarette smoke-induced pulmonary emphysema in mice and is upregulated in patients with COPD”(Juliana Heidler*, Athanasios Fysikopoulos*, Frank Wempe*, Michael Seimetz, Thorsten Bangsow, Ana Tomasovic, Florian Veit, Susan Scheibe, Alexandra Pichl, Friederike Weisel, K C Kent Lloyd, Peter Jaksch, Walter Klepetko, Norbert Weissmann, and Harald von Melchner) Dis. Model. Mech. 2013 dmm.013482; doi:10.1242/dmm.013482. (Equal contribution for first authorship).
- “Trypacidin, a spore-borne toxin from Aspergillus fumigatus, is cytotoxic to lung cells”. Gauthier T, Wang X, Sifuentes Dos Santos J, **Fysikopoulos A**, Tadrist S, Canlet C, Artigot MP, Loiseau N, Oswald IP, Puel O. PLoS One. 2012;7(2):e29906. doi: 10.1371/journal.pone.0029906. Epub 2012 Feb 3.
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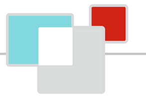
ORAL PRESENTATIONS

- Efficacy of fipronil-(S)-methoprene, metaflumizone combined with amitraz, and pyriprole commercial spot-on products in preventing Culex pipiens pipiens from feeding on dogs 10th International Symposium on Ectoparasites (ISEP) June 3 – 5, 2009 Toulouse, France.
- "Sestrin 2: how an antioxidant can repress alveolar maintenance" UGMLC meeting, June 13, 2014, Giessen.

PUBLICATIONS-ORAL PRESENTATIONS-POSTER PRESENTATIONS

POSTER PRESENTATIONS

- Sestrin-2, a repressor of PDGFRβ signalling, promotes cigarette smoke-induced pulmonary emphysema in mice and is upregulated in patients with COPD" (Juliana Heidler, **Athanasios Fysikopoulos**, Frank Wempe, Michael Seimetz, Thorsten Bangsow, Ana Tomasovic, Florian Veit, Susan Scheibe, Alexandra Pichl, Friederike Weisel, K C Kent Lloyd, Peter Jaksch, Walter Klepetko, Norbert Weissmann, and Harald von Melchner) ECCPS / PVRI Joint Symposium January 29, 2014, Bad Nauheim.
- Inactivation of sestrin 2 protects against emphysema in mice exposed to tobacco smoke (Frank Wempe, **Athanasios Fysikopoulos**, Thorsten Bangsow, Juliana Heidler, Michael Seimetz, Alexandra Pichl, Norbert Weissmann, and Harald von Melchner), 2nd International Symposium of the Excellence Cluster Cardio Pulmonary System, June 17, 2011, Bad Nauheim.



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