Senescence, DNA damage and repair in chronic obstructive pulmonary disease (COPD)

INAUGURAL DISSERTATION

Submitted to the

Faculty of Medicine

In partial fulfillment of the

requirements for the PhD-Degree

Of the Faculties of Veterinary Medicine and

Medicine of the Justus Liebig University

Giessen

By

Manish Kumar

Of Patna, India

Giessen 2014

From the Max Planck Institute for Heart and Lung Research,
Department of Lung Development and Remodeling
Bad Nauheim, Germany
&
The Faculty of Medicine of the
Justus-Liebig-University Giessen
University Hospital Giessen and Marburg
Department of Internal Medicine
Giessen, Germany

First Supervisor and Committee Member: Prof. Dr. Robert Voswinckel
Committee Member: Prof. Dr. Heinz-Jürgen Thiel

Date of Doctoral Defense: 6.08.14

Contents

List of Figures

List of tables

List of abbreviation

ACT	*Aqueous Cigarette Tar*
ATM	*Ataxia Telangiectasia Mutated*
ATR	*Ataxia Telangiectasia Mutated Related*
53BP1	*p53 Binding Protein 1*
CF	*Circulating Fibrocytes*
CHK 1/2	*Cell Cycle Checkpoint Kinases 1/2*
COPD	*Chronic Obstructive Pulmonary Disease*
CSE	*Cigarette Smoke Extract*
DAPI	*4',6-diamidino-2-phenylindole*
DDR	*DNA Damage Response*
DSB	*Double Strand Breaks*
eNOS	*endothelial Nitric Oxide Synthase*
ETS	*Environmental Tobacco Smoke*
FDG	*Fluorescein di-b-D-Galactopyranoside*
FEV_1	*Forced Expiratory Volume*
FVC	*Forced Vital Capacity*
GOLD	*Global Initiative for Chronic Obstructive Lung Disease*
HLF-1	*Human Lung Fibroblasts 1*
HMGA	*High Mobility Group A protein*
HO	*Hydroxyl radical*
H_2O_2	*Hydrogen peroxide*
HP1	*Heterochromatin Protein 1*
iNOS	*inducible Nitric Oxide Synthase*
MDC1	*Mediator of DNA damage checkpoint protein 1*
MEFs	*Mouse Embryonic Fibroblasts*
MS	*Mainstream Smoke*
NBS1	*Nijmegen Breakage Syndrome 1*
O_2^-	*Superoxide Anions*
PBMC	*Peripheral Blood Mononuclear Cell*
S A- β- Gal	Senescence Associated Beta-D Galactosidase
SAHF	*Senescence Associated Heterochromatin Foci*
SASP	*Senescence Associated Secretory Phenotype*
WHO	*World Health Organization*

1 Introduction

1.1 COPD

Chronic obstructive pulmonary disease (COPD) is a slowly progressing syndrome of airflow limitation caused by chronic inflammation of the airways and lung parenchyma [1]. Chronic obstructive bronchitis, obstruction of small airways, and emphysema, with enlargement of air spaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways constitutes the syndrome of patients with COPD (**Figure. 1**). It is to be noted that the extent of emphysema and obstructive bronchitis within individual patients can vary.

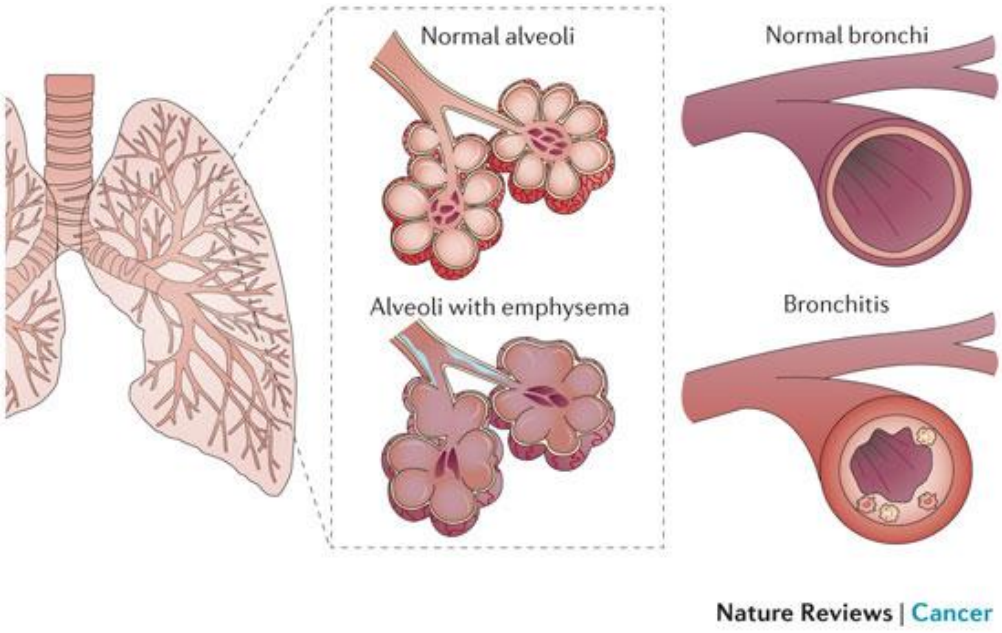


Figure 1. COPD is characterized by bronchitis and emphysema **[2]**

COPD is characterized by emphysema and chronic bronchitis. Emphysema comprises of the enlargement and destruction of the alveoli limiting the surface area for gas exchange. Chronic bronchitis is narrowing down of the small airways and deposition of mucus within them limiting the air flow inside.

1.1.1 Chronic bronchitis

The inflammation of the bronchial epithelium with hypertrophy of the mucus glands and increased goblet cells characterizes chronic bronchitis. Chronic cough and sputum formation for at least three months is indicative of chronic bronchitis. In chronic bronchitis, there is a destruction of the airway cilia leading to the impaired efficiency of the mucociliary escalator. Mucus viscosity and mucus production are increased. There is also an increased susceptibility to infection. Repeated infections and inflammation cause irreversible damage of the airways structure due to narrowing and distortion of the peripheral airways.

1.1.2 Emphysema

The small air sacs, which constitute the lung, and where the exchange of oxygen and carbon dioxide takes place, are called alveoli. Any extrinsic or intrinsic damage to the alveoli, which results in air becoming trapped, may cause them to expand and rupture. Emphysema is characterized by the destruction of alveolar walls and loss of immanent lung elasticity. Emphysema leads to a progressive reduction of alveolar surface area, where exchange of oxygen and carbon dioxide between gas and blood takes place. Hyperinflation of the lung flattens the diaphragm. This leads to less effective contraction and impaired breathing mechanics. Over time, this results in severe airflow limitation and severe decrease of the forced expiratory volume.

1.1.3 Diagnosis of COPD

The clinical diagnose of COPD comprises patient history taking (for cigarette smoke or other toxin exposure as well as chronicity of the symptoms) and spirometery and is characterized by airway obstruction where the ratio of Forced Expiratory Volume (FEV_1) and Forced Vital Capacity (FEV_1/ FVC) is less than 70% [3]. A short comparison of the spirometric definition of COPD is given in **Table 1.**

Table 1. The spirometric definition and grading of COPD [4]

GOLD spirometric criteria for COPD severity		
I. Mild COPD	* FEV1/FVC < 0.7 * FEV1 > or = 80% predicted	At this stage, the patient is probably unaware that lung function is starting to decline
II. Moderate COPD	* FEV1/FVC < 0.7 * FEV1 50% to 79% predicted	Symptoms during this stage progress, with shortness of breath developing upon exertion.
III. Severe COPD	* FEV1/FVC < 0.7 * FEV1 30% to 49% predicted	Shortness of breath becomes worse at this stage and COPD exacerbations are common.
IV. Very Severe COPD	* FEV1/FVC < 0.7 * FEV1 < 30% predicted or FEV1 < 50% predicted with chronic respiratory failure	Quality of life at this stage is gravely impaired. COPD exacerbations can be life threatening.

1.1.4 Epidemiology

Chronic obstructive pulmonary disease (COPD) represents a major health and economic burden with increasingly aging populations as shown in **Figure 2**.
65 million people have moderate to severe chronic obstructive pulmonary disease according to the WHO. In 2005, more than 3 million people died of COPD which corresponds to approximately 5% of deaths globally. Most of the information available on COPD prevalence, morbidity and mortality comes from high-income countries. Even in those countries, accurate epidemiologic data on COPD are difficult and expensive to collect. It is known that almost 90% of COPD deaths occur in low- and middle-income countries.

COPD had been previously reported to be more prevalent in men than women. Due to the increased tobacco use among women in high income countries and the higher risk of exposure to indoor air pollution (such as biomass fuel used for cooking and heating) in low income countries; the disease now affects men and women almost equally.

In 2002 COPD was the fifth leading cause of death. Total deaths from COPD are projected to increase by more than 30% in the next 10 years unless urgent action is taken to reduce the underlying risk factors, especially tobacco use. Estimates show that COPD becomes in 2020 the third leading cause of death worldwide [5].

Cause of Death	Rank in 2002	Rank in 2030
Ischaemic heart disease	1	1
Cerebro vascular disease	2	2
Lower Respiratory Infections	3	5
HIV/AIDS	4	3
COPD	5	4

Figure 2. COPD mortality worldwide in comparison to major diseases [6]

1.1.5 Cigarette smoke

Cigarette smoking is the primary cause of COPD. The tobacco smoke is a mixture of up to 4,700 chemicals with about 10^{10} particles/ml aerosolic components. The cigarette smoke components include about 60 known carcinogens and with each puff of cigarette the smoker takes in 10^{17} oxidant molecules [7]. Tobacco smoke is broadly divided into the mainstream and the side stream smoke. The mainstream is divided into a particulate solid phase (tar) and the gas phase (toxic gases, volatile organic compounds, free radicals, etc.).

The solid phase contains very high concentrations of free radicals (approx. 10^{17} spins·g^{-1}) with long lifetimes. The particulate phase is comprised of at least 3,500 chemical compounds and a high proportion of them are toxic, carcinogens or mutagens, (e.g. benzene, 2-napthylamine, ^{210}Po, ^{226}Ra, ^{228}Ra, nickel, cadmium, benzo[a]pyrene, etc) [8]. The side stream smoke comprises of the solid and gas phases, containing higher concentrations of toxic and carcinogenic compounds and other volatile and semi volatile compounds [9]. The existence of the free radicals and oxidants in the gas phase remains in a steady state in which they are continuously formed or destroyed and their concentration increases as the smoke ages [10]. A few water components of the cigarette tar (ACT) can produce superoxide anions ($O_2^{\bullet -}$), which subsequently result in the formation of H_2O_2 and the reactive hydroxyl radical ($HO^{\bullet}$). These free radicals further cause oxidative stress. This leads to damage of the cellular membrane lipids, proteins, enzymes and most importantly the DNA. The side stream smoke consists of similar chemical components in the solid and gas phases and is also rich in highly reactive and short-lived free radicals. Passive smoking (or environmental tobacco smoke, ETS) has been proven to be a health hazard for non-smokers and is burden of major lung diseases [11].

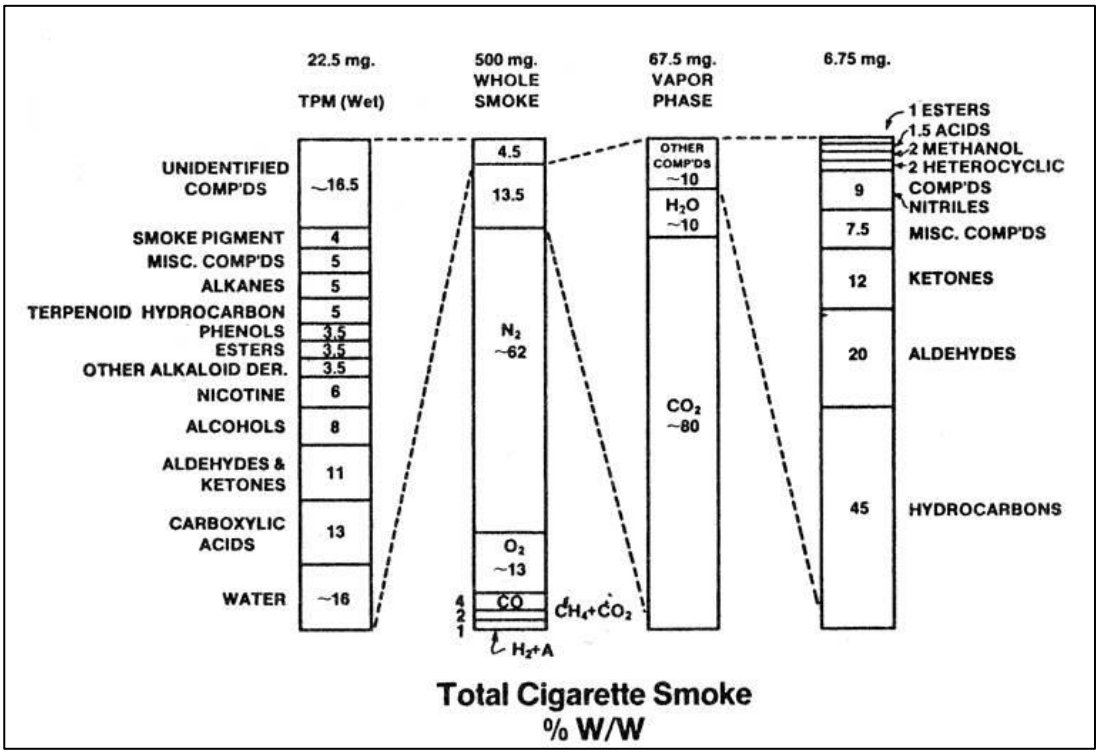


Figure 3. Major components of mainstream cigarette smoke [12]

Detailed figure showing the composition of cigarette mainstream smoke (MS). The figure shows four vertical bars, the second vertical bar representing the main chemical constituents of MS smoke, labelled WHOLE SMOKE, dominated by N2 (nitrogen) ~62% by weight, and O2 (oxygen) ~13% by weight. The 4.5% at the top of this symbolic cigarette is in the "TPM (Wet)" category, the main components of which are shown in the first vertical bar. The main constituent in the "VAPOR PHASE," which constitutes 13.5% of the total, is shown in the third vertical bar.

1.1.6 Risk factors and current therapies

The main precipitating factors are cigarette smoke, environmental pollution by inorganic and organic dust (e.g. due to open fireplaces), genetic predisposition, recurrent pulmonary infections, socioeconomic status and aging. Even if smoking or pollutant exposure is stopped, the condition often progresses at an accelerated rate compared with the normal age-related decline in FEV_1. Hallmarks of COPD are chronic, self-perpetuating inflammation of the airways and gas exchange regions, loss of gas exchange tissue leading to emphysema, and collapse of small airways leading to increasing shortness of breath. Today, there is no causal treatment which could hold the progression of the disease. The currently available therapeutic armamentarium comprises bronchodilators such as muscarinic antagonists and beta adrenergic receptor agonists [13], anti-inflammatory drugs including inhaled and oral steroids and phosphodiesterase-4 inhibition [14, 15], and interventional or surgical procedures to relieve air trapping [16, 17]. These treatments improve symptoms like breathlessness and exercise intolerance and they may reduce the frequency of exacerbations of the disease, but their effects are often very limited.

The fact of ongoing inflammation and tissue destruction despite of smoking cessation in COPD is an intriguing finding, which may be explained at least in part by the effects of premature cellular senescence and its associated secretory phenotype also known as Senescence Associated Secretory Phenotype or SASP.

1.2 Cellular senescence

Leonard Hayflick noticed in 1961 for the first time, that human tissue derived primary fibroblasts, which were maintained for multiple passages in culture, ceased to divide indefinitely. He discovered, that after a limited number of divisions, the cell proliferation gradually grinded to a complete halt [18]. The proliferation of the fibroblasts in culture showed three distinct phases: 1. Lag phase of slow proliferation during culture establishment, 2. Log phase where

the cells show rapid proliferation and 3. Stationary phase in which the cells gradually accomplish permanent arrest [18]. The possible causes of the transition to phase 3 were described by Hayflick [19] as “the finite lifetime of diploid cell strains *in-vitro* may be an expression of aging or senescence at the cellular level.” The term cellular senescence thus represented an irreversible, stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity. The primary cells kept in culture cease to divide further after a replicative senescence. The replicative senescence, or Hayflick’s limit, occurs due to the fact, that each time when the cell divides, the telomeres at the chromosome ends become shorter. Telomeres are subject to attrition due to the fact that the DNA polymerase fails to completely replicate the lagging strands. In the early 1970s, Olovnikov [20] and Watson [21] independently described this so-called “end replication problem”, which contributes to telomere shortening. Thus, telomeres reflect the replicative history of a primary cell as a molecular clock [22].

The telomere capping provides a protective and structural integrity at the end of the chromosomes. If the telomere shortening reaches a crucial minimal length, their protective structure is compromised. The cell recognizes this crucial loss in the chromosome as DNA damage and thus triggers a DNA damage response (DDR). DDR is associated with the appearance of DNA damage foci, that recruit important proteins of the DNA- repair machinery, such as γ-H_2AX (a phosphorylated form of the histone variant H_2AX) and the DDR proteins 53BP1(p53 Binding Protein 1), NBS1 (protein responsible for Nijmegen Breakage Syndrome 1) and MDC1 (Mediator of DNA damage Checkpoint protein 1). It has also been reported that the DNA damage kinases ATM (Ataxia Telangiectasia Mutated) and ATR are activated in senescent cells [23]. The amplification of the DDR signal activates the cell cycle checkpoint kinases CHK1 and CHK2. DDR-associated factors communicate with the cell cycle machinery via phosphorylation and activation of several cell cycle proteins, including CDC25 (a family of phosphatases) and the key regulator of cell cycle arrest p53. In addition, differential expression of p53 isoforms has been linked to replicative senescence [24]. Together, these factors can either induce a transient proliferation arrest, allowing cells to repair their damage, or in case where the DNA damage seems to be irreparable,

cells are destined to undergo either senescence or apoptosis. The molecular decision making that determines the fate of these cells with irreparable DNA damage to senescence or apoptosis still remains elusive. The cell type, the intensity and duration of the stress signal, as well as the nature of the damage, are likely to be important determinants [25].

1.2.1 Markers of cellular senescence

Senescent cells may be confused with quiescent or terminally differentiated cells as the distinction is not always straightforward. No marker or hallmark of senescence identified thus far is entirely specific to the senescent state. Further, not all senescent cells express all possible senescence markers. Nonetheless, senescent cells display several phenotypes, which, in aggregate, define the senescent state (**Figure 4**).

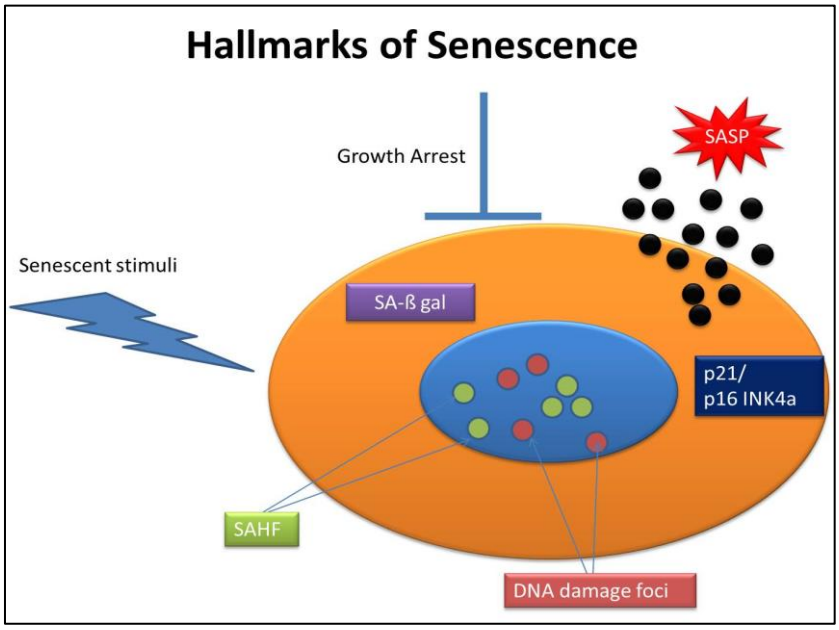


Figure 4. Hallmarks of senescence

Markers of senescence include senescence-associated beta-galactosidase activity at pH 6, formation of senescence associated heterochromatin foci, DNA damage foci, expression of cyclin dependent kinase inhibitors such as p16 INK4a or p21. If senescence induction includes DNA damage, the senescent cell releases several cytokines termed senescence associated secretory phenotype or SASP.

Salient features of senescent cells are:

a) The senescence growth arrest is permanent and irreversible without genetic interventions [19].

(b) Senescent cells increase in size, sometimes enlarging more than twofold relative to the size of nonsenescent counterparts [19]. Senescent cells lose their original morphology. They look larger than their controlled counterparts and have a much larger flattened cytoplasm that contain many vacuoles and cytoplasmic filaments [26, 27], a bigger nucleus and nucleoli and are sometimes multinucleated [28, 29]. In some cases, senescent cells display an increase in the number of lysosomes and golgi [30].

(c) Senescent cells express a senescence-associated β-galactosidase [31] SA- β-galactosidase activity is expressed from GLB1, the gene encoding lysosomal beta-D-galactosidase. The levels of lysosomal-β-galactosidase protein increase during senescence [32]. The SA- β-galactosidase activity in senescent cells is believed to be present due to higher lysosomal mass in senescent cells [33].

(d) Most senescent cells express p16INK4a and p21 [34] . p16 INK4a and p21 are key inhibitors of cyclin-dependent kinases (CDKs), the expression of which leads to cell cycle arrest.

(e) Cells that senesce with persistent DDR signaling harbor persistent nuclear foci, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) and are distinguishable from transient damage foci [35]. DNA-SCARS foci contain activated DDR proteins.

(f) Senescent cells with persistent DDR signaling secrete growth factors, proteases, cytokines and other factors that have potent autocrine and paracrine activities [36].

(g) Senescence-associated heterochromatic foci (SAHF). Senescent cells exhibit increased heterochromatinization that reflects a compact chromatin structure, and are enriched for repressive histone modifications, the histone variant macro-H2A, HP1 proteins and HMGA proteins. SAHF can be visualized in cells as dense bright nuclear spots through DAPI staining.

1.2.2 Premature cellular senescence

Senescence can also be induced in the absence of any detectable telomere attrition or dysfunction by a variety of conditions, which will be discussed in the following section. The term premature explains the fact, that the senescence achieved in these cells is not caused by the replicative limit. Evidence for the existence of premature senescence in vivo has been accumulating rapidly and altogether points to the fact, that senescence plays an important and critical role in tumor suppression. Different ways of premature senescence is diagrammatically represented in **Figure 5.**

Premature senescence is primarily due to environmental factors that exert cellular stress. Various factors like nutrients, growth factors, oxygen levels, absence of other cell types and extracellular matrix components, belonging to the original environment of the cells, can be detrimental for the acclimatization of the explanted culture in the new artificial environment. Changes in one or more of these factors can induce a culture shock, resulting in stress-induced senescence [37]. This type of cell cycle arrest is independent of telomere length.

1.2.3 Stress-Induced Premature Senescence (SIPS) *in-vitro*

Stress induced premature senescence or SIPS is primarily due to the cell culture medium that exerts cellular stress. Various factors like nutrients, growth factors, oxygen levels, absence of other cell types and extracellular matrix components belonging to the original environment of the cells can be detrimental for the acclimatization of the explanted culture in the new artificial environment. Changes in one or more of these factors can induce a culture

shock, resulting in stress-induced senescence [37]. This type of cell cycle arrest is independent of telomere length. Mouse embryonic fibroblasts (MEFs) undergo senescence after a limited number of passages in culture, despite their retaining of long telomeres. Murine cells, in contrast to most human cells, express telomerase [38] and have long telomeres [39]. Oxidative stress induces cessation of replication in cultured human cells [40-42] while the replicative potential of human melanocytes and epithelial cells depends largely on the composition of the culture medium used, as well as on the use of feeder layers [43-45]. Senescence of MEFs can be bypassed also by inactivation of p53 or simultaneous ablation of RB family genes [46-48]. Thus, the long term culture of mammalian cells requires not only telomere maintenance, but also optimal culture conditions [49].

1.2.4 Oncogene-Induced Premature Senescence (OIPS) *in-vitro*

Transfection of the GTPase HRas, also known as transforming protein p21 or HRAS, can induce cell cycle arrest in primary cells [50]. Cells arrested via HRas showed striking phenotypic resemblance to those cells which underwent replicative senescence. This phenomenon of oncogene mediated senescence has eventually come to be known as OIPS [51]. hTERT expression can rescue replicative senescence but not OIPS, confirming its independence from telomere attrition [52]. OIPS occurs in the early stages of tumor development both in mouse models and in humans [53-56]. These observations strongly indicate that OIPS checks the proliferation of oncogenically stressed cells and maintains the tumor in premalignant state; by contrast, the absence of OIPS, which is caused by the mutation of the senescence-inducing pathways, leaves the road to oncogene-driven malignant progression unimpeded [53, 54]. Detection of senescence markers could be of prognostic value for those premalignant lesions, which are characterized by normal cell morphology and lack of invasive growth and are often associated with senescence. Senescence associated with the premalignant tumors is not paradoxical in context to the growth of tumor as only a fraction of the cells within a tumor are able to propagate successfully, while many undergo apoptosis or senescence triggered by the stress due to the aberrant intracellular and extracellular

conditions that are characteristically present in tumors [57]. Hence, it is the balance between cellular proliferation and apoptosis or senescence that determines the growth rate of a particular tumor [36].

1.2.5 Tumor suppressor loss-Induced Premature Senescence (TIPS)

Premature senescence can also be triggered by the loss of tumor suppressor molecules in mouse and human cells. PTEN (Phosphatase and tensin homolog) gene deficient MEFs undergo senescence, which is accompanied by induction of p53. Concomitant loss of p53 allows these cells to override the cytostatic effects of PTEN deletions [41]. Similarly, loss of NF1 causes senescence *in-vitro*, which is eventually accompanied by decreases in ERK and AKT activities [58]. Another example is VHL, loss of which triggers senescence in an RB- and p400-dependent manner [59].

1.2.6 Senescence Associated Secretory Phenotype (SASP)

The fact that the culture medium of senescent cells is enriched with secreted proteins has been shown in the past [60, 61]. When cells become senescent, they often display a senescence-associated secretory phenotype consisting of cytokines, growth factors and proteases, which collectively has been termed SASP by the Campisi group [62]. Daniel Peeper termed the same phenomenon SMS (Senescence Messaging Secretome) [63]. Contribution of senescence might seem to be passive, but the recent discovery of the SASP strongly suggests that senescence might have a more active and pathologically diverse role to play [63, 64]. The physiological role of SASP has been proposed to be a wound healing mechanism [65]. The initial observation of SASP implied, that senescence might not just be a tumor suppressor mechanism, but rather a double-edged sword within the tumor microenvironment [36]. SASP factors might contribute to signal immune cells for the removal of senescent cells. If this removal process is impaired, or if the number of senescent cells in a tissue is too high, the senescent cells might persist and maintain the secretory phenotype, exposing the local tissue persistently to the SASP. The secretion of these senescence-associated factors has the potential to detrimentally alter the local microenvironment,

leading to tissue dysfunction associated with ageing and disease. However, It is also to be noted that SASP occurs in case senescence involves DNA damage. If senescence is mediated by factors not involving any DNA damage, there is no SASP response (**Figure 5**).

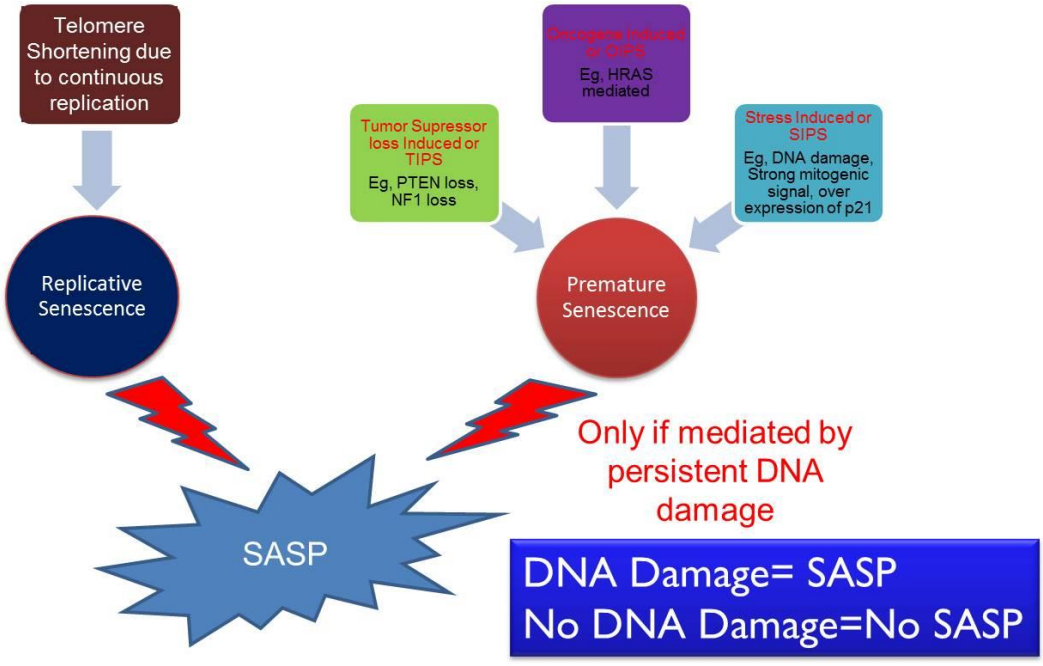


Figure 5. Several factors could lead to senescence, but SASP response occurs only when senescence involves DNA damage.

1.2.7 Molecular induction of SASP

Cellular senescence is most often the result of nuclear DNA damage fuelling a chronic DNA damage response (DDR). The DDR pathway is triggered usually by ionizing radiation or other genotoxic events, resulting in DNA double-strand breaks. The DDR pathway initiates with the phosphorylation of histone H_2AX by ATM (Ataxia Telangiectasia Mutated) that occurs at or near the DNA double-stranded break site and is required for phosphorylation of 53 Binding Protein-1(53-BP1) by ATM and localization of 53BP1 to nuclear repair foci [66]. 53BP1 function is important for coupling ATM to several of its downstream targets, including p53 and SMC1 (Structural Maintenance of Chromosomes protein 1). In the case of the checkpoint homolog 2 (Chk2)

kinase, the coupling mechanism to ATM seems to be largely independent of 53BP1 and may involve another undefined member of the BRCT repeat family of proteins [66]. Upstream elements of the DDR signalling pathway like ATM, NBS1 (Nibrin) and CHK2 are necessary for full blown SASP, and additional crosstalk occurs between the DDR and cytokine secretion in an autocrine loop, meaning that the secreted cytokines both control and are controlled by the DDR [67].

p53-knock out cells embark SASP response in the absence of senescence upon persistent DNA damage and in contrast, cells induced to senesce by $p16^{INK4a}$ over expression, but in the absence of DNA damage, do not initiate a SASP response [67]. This emphasizes the fact, that persistent DNA damage response is the major cause for SASP (**Figure 6**). It is also to be noted, that DDR signaling drives only a subset of SASP factors, but those include the potent inflammatory cytokines IL-6 and IL-8. Development of SASP is a slow process. SASP initiates only upon persistent DNA damage of sufficient magnitude. Delayed SASP might allow cells to attempt DNA repair before initializing the immune clearance signal through SASP.

As a summary, senescence can be of replicative and premature type. Telomere attrition due to repeated replication leads to replicative senescence whilst the premature senescence occurs due to genotoxic stress, oncogene insertion or loss of a tumor suppressor. Stress induced senescence via the chronic or intense DNA damage leads to a DDR that engages ATM, NBS1 and CHK2, leading to cellular senescence via the cell cycle effectors p53 and pRB [67]. Persistent DDR in turn is responsible for the SASP response.

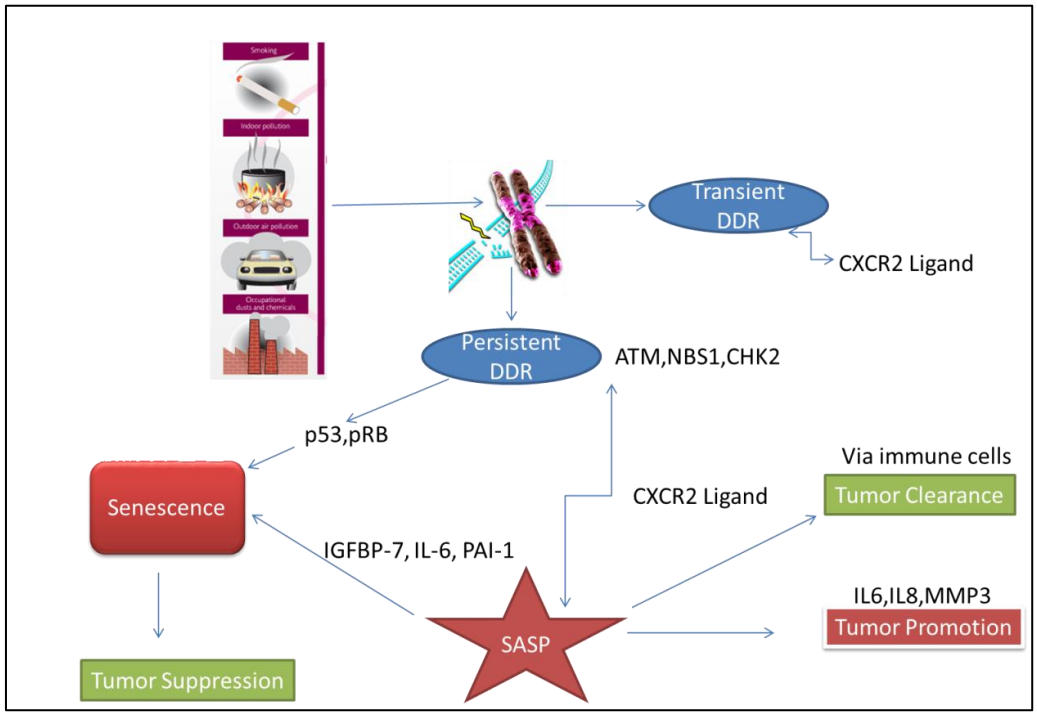


Figure 6. Genotoxic stress, including cigarette smoke, may lead to persistent DNA double breaks which if unrepaired may lead to senescence and inflammation.

DNA double strand breaks lead to a transient DNA damage response. However, if the DNA damage is persistent, this leads to activation of chemokine pathway. Persistent DNA damage may further drive the cell to senescence. Senescent cells activate a self-amplifying secretory network (SASP) in which CXCR2-activation reinforces growth arrest.

1.2.8 Paradoxical role of senescence:

Although senescence represents a halt in cell division and thought to possess tumor suppressive capabilities, it has been shown that senescent cells could promote tumor formation and may have a role in tissue repair as well. Cell cycle arrest is the major mechanism by which cellular senescence suppresses malignant tumorigenesis [64, 68, 69]. However, some of the factors secreted by senescent cells help to reinforce the senescence growth arrest in an autocrine manner as well, for example the pro-inflammatory cytokines IL (interleukin)-6 and IL-8, but also factors such as the pro-apoptotic protein IGFBP (insulin-like growth factor binding protein)-7 and PAI (plasminogen activator inhibitor)-1. Many evidences confirm the tumor suppressor nature of senescence response in both mice and humans [70]. It seems paradoxical

that senescent cells secrete factors that may also promote cancer progression [36, 62]. Examples of such SASP factors include amphiregulin and GRO (growth-related oncogene)-α, which stimulate cell proliferation; VEGF (vascular endothelial growth factor), which stimulates angiogenesis; and the pro-inflammatory cytokines IL-6 and IL-8, which can induce an epithelial-to-mesenchyme transition and epithelial cell migration and invasion [71]. There are a few evidences where senescence has been linked to tissue repair or regeneration [72, 73].

These evidences suggest that senescent cells feature a paradoxical phenomenon and the effect might be context dependent. Senescence has thus been viewed as a form of antagonistic pleiotropy, in which it is beneficial early in life, but detrimental at a later old stages of life [64].

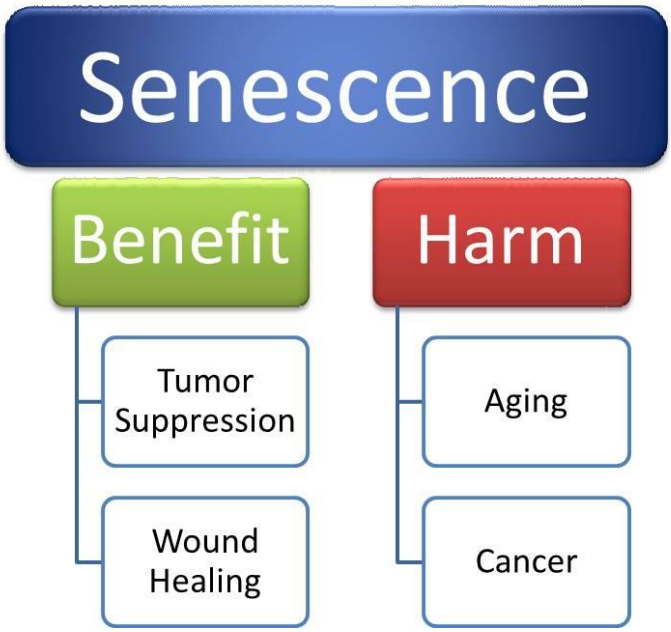


Figure 7. Senescence as an example of antagonistic pleiotropy

Benefits of senescence include tumor suppression and wound healing early in life and the harms include aging and cancer which could be detrimental later in life.

1.2.9 COPD, aging and cellular senescence

Aging has an influence on development of COPD and at the same time COPD has been reported to be a disease of premature lung tissue aging [74, 75]. The progressive decline of tissue homeostasis after a certain span (reproductive age) of life is termed as aging. Aging leads to an increasing susceptibility of disease and causes the failure of organs due to oxidative stress induced premature senescence and the replicative exhaust due to telomere shortening (replicative senescence). Environmental stress, such as cigarette smoke or other pollutants accelerate the aging of lung cells through oxidative stress, thereby inducing accelerated progression of COPD in some patients. The striking fact that only 25% of the cigarette smokers develop COPD points towards additional “hits” by infections as well as to genetic variability and predisposition for the disease. It has also been suggested that human beings possessing different length of telomeric DNA might elicit variable susceptibility for the disease [76].

It has been reported that cigarette smoking causes premature cellular senescence in lungs. *In-vitro* exposure of human lung epithelial cells to cigarette smoke extract results in an increased expression of SA-β-gal (senescence-associated β-galactosidase), a marker of cellular senescence [77]. Cultured lung fibroblasts from patients with emphysema show increased expression of SA-β-gal and decreased proliferative capacity *in-vitro*, when compared with those from healthy smokers [78, 79].

In several health disorders related to age, cigarette smoking is considered to be an important risk factor. Cigarette smoking is associated with increased systemic inflammation and oxidative stress [80]. This also supports the fact that extra pulmonary manifestations of COPD might include muscle wasting, cardiovascular disease or osteoporosis [81]. It is not mere coincidence but an established fact that these manifestations are also common characteristics of aging [82]. Elderly individuals (more than 60 years) possess a higher COPD disease rate than younger groups, independent of their history of exposure to tobacco smoke. The aging lung normally shows progressive distal air space

enlargement, with loss of gas-exchanging surface area and the support of the alveolar attachments for peripheral airways [83]. Lung function declines in elderly healthy individuals normally but is accelerated in patients with COPD [84]. One of the prominent possible causes for the lung function decline might be the elastin fiber fragmentation which also is associated with age [84, 85]. Although the structural changes in the lung are thought to be non-destructive, in contrast with smoking-induced emphysema [85] , they do have functional consequences, resulting in a loss of elastic recoil of the lungs, an increase in residual volume and functional residual capacity or over-inflation of the lungs. This loss of elastin fibers is similar to that which occurs with aging in the skin, resulting in loss of elasticity and skin wrinkling which is enhanced by smoking [86]. Interestingly, the degree of skin wrinkling correlates with quantitative measurements of emphysema by CT (computed tomography) scanning [87]. Thus cigarette smoking seems to cause elastolysis both in the lungs and systemically in the skin [88], suggesting that cigarette smoke may accelerate the aging process [89].

1.2.10 Similarities between the secretory profile of senescescent cells and inflammation in COPD

Patients with COPD show severe increase in inflammatory molecules along with various others which was collectively termed COPD associated secretory phenotype or CASP. The link between senescence and COPD arises from the fact that each of them primarily is the result of oxidative abuse. Oxidative stress via Cigarette smoke/noxious gas causes persistent DNA damage in alveolar cells further leading to premature pulmonary senescence. Senescence, mediated via persistent DNA damage, leads to a secretory phenotype as discussed above (**Figure 6**). It is interesting to note that both senescence and COPD display a prominent secretory phenotype associated with it. The factors that have been reported to be upregulated in COPD show clear resemblance to that of SASP, suggesting a significant link between the two.

Below, a review of the striking similarities between the secretory phenotypes of senescence (SASP) and COPD is displayed. A summarized version is tabularized for ease in **Table 2**.

1.2.11 Interleukins and chemokines

IL-1, IL-6, IL-8 (CXCL-8), GROα, GROβ, GROγ, have been previously shown to be upregulated in senescescent cells [62]. These are upregulated in COPD as well [90, 91]. Monocyte Chemoattractant Protein 2 or MCP 2, Macrophage Inflammatory Protein (MIP)-1α and 3α levels are increased in senescent cells [62, 92]. Similarly in COPD, MCP-1, and IL-8 were increased in sputum, with further increases during exacerbations, and the bronchiolar epithelium overexpressed MCP-1, its receptor CCR2, MIP1α, and IL-8. MCP-1 and CCR2 were involved in the recruitment of macrophages and mast cells into the airway epithelium in COPD [93, 94]. Inflammatory cytokines such as the colony-stimulating factors (CSFs, including GM-CSF and G-CSF) are secreted at high levels by senescent fibroblasts [62]. Strikingly the concentrations of GM-CSF in BAL fluid are also increased in stable COPD and significantly elevated during exacerbations [95]. Macrophage migration inhibitory factor (MIF) is upregulated in senescence and has recently been forwarded as a critical regulator of inflammatory conditions and it has been hypothesized that MIF may have a role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD).

1.2.12 Growth factors

Growth factors like epithelial growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and angiogenin have been shown to be upregulated significantly in senescent cell culture media. EGF, bFGF, VEGF and angiogenin have also been reported to be upregulated in COPD [96, 97]. The insulin-like growth factor (IGF) and its IGF receptor have a major role in SASP response. Senescent endothelial, epithelial, and fibroblast cells express high levels of almost all the IGF-binding proteins

(IGFBPs), including IGFBP-2, 3, 4, 5, and 6 [62, 92, 98, 99]. Recently, activation of the BRAF oncogene in primary fibroblasts was shown to lead to the secretion of IGFBP-7, which acts through autocrine/paracrine pathways to induce senescence and apoptosis in neighboring cells [100]. Strikingly, lung fibroblasts from emphysema patients also showed upregulation of insulin-like growth factor-binding protein-3 (IGFBP-3) and IGFBP-related protein-1 (IGFBP-rP-1) [78].

1.2.13 Proteases and their regulators

Senescent cells secrete a myriad of proteases in addition to soluble signaling cytokines and growth factors. The main proteases are the matrix metalloproteinases (MMPs). MMPs are a large family of zinc-dependent proteinases that regulate the destruction of extracellular matrix components [101]. The MMP family members that are consistently upregulated in human and mouse fibroblasts undergoing replicative or stress-induced senescence are stromelysin-1 and -2 (MMP-3 and -10, respectively) and collagenase-1 (MMP-1) [102-106]. Similarly in COPD there is an increase in bronchoalveolar lavage concentrations and macrophage expression of MMP-1 (collagenase) and MMP-9 (gelatinase B) in patients with emphysema [107-109]. Alveolar macrophages from normal smokers express more MMP-9 than those from normal subjects [110], and there is an even further increase in cells from patients with COPD [111], which has greatly enhanced elastolytic activity [112]. MMP-9 and the ratio of MMP-9 to TIMP-1 are increased in induced sputum of patients with COPD [113, 114]. MMP-8 and MMP-9 do not only act as secreted enzymes, but they are also bound to cells where they exert elastolytic activity.

Another family of proteases present in the SASP comprises serine proteases and regulators of the plasminogen activation pathway. Members of this family include urokinase or tissue-type plasminogen activators (uPA or tPA, respectively), the uPA receptor (uPAR), and inhibitors of these serine proteases (PAI 1 and 2) [92, 115]. Indeed, a >50-fold increase in plasminogen activator activity has been reported in senescent endothelial cells and lung and skin fibroblasts [92, 116, 117]. PAI-1 is also upregulated in fibroblasts and endothelial cells from aged donors [92, 118-120]. Induced sputum of COPD

patients also has been shown to contain significantly increased u-PAR, PAI-1, and IL-8 compared to the control subjects [121].

1.2.14 Shed receptors or ligands

Shed receptors include ICAM-1 (Intercellular Adhesion Molecule 1), -3, osteoprotegerin, TRAIL-R3, sTNFR1, Fas, STNFR2, uPAR and EGF-R, which are present at high levels in the extracellular milieu of senescent fibroblasts and are also found to be upregulated in COPD [122-124]. In fact, osteoprotegerin in sputum might be a potential biomarker in COPD [125].

1.2.15 Non protein factors, extra cellular matrix and reactive oxygen species

Non protein factors upregulated upon senescence include prostaglandin E2 (PGE2) [126] and Cox-2, the enzyme responsible for the production of PGE2 and other prostaglandins. These act in an autocrine or paracrine way. Similarly, it has been reported that the concentration of prostaglandin PGE2 in exhaled breath of COPD patients increases significantly [127]. This is likely to be derived from cyclooxygenase-2 (COX-2), which is expressed in alveolar macrophages [128]. There is also an increased COX-2 expression in alveolar macrophages from patients with COPD compared with normal control subjects [129].

Fibronectin is a large multidomain glycoprotein found in connective tissue, on cell surfaces, and in plasma and other body fluids. It interacts with a variety of macromolecules, including cell-surface receptors, components of the cytoskeleton, and other ECM molecules. Through its interactions with cell-surface receptors, primarily integrins, fibronectin can affect cell adhesion, survival, growth, and migration. Fibronectin production is upregulated in prematurely aging Werner syndrome fibroblasts [130]. Moreover, cells undergoing premature cellular senescence in culture and *in-vivo* show increased fibronectin expression [131]. Data from previous studies suggest a similar profile of ECM molecules including fibronectin in COPD [132].

Senescent cells have been shown to release nitric oxide and reactive oxygen species due to alterations in inducible nitric oxide synthase (iNOS), endothelial

nitric oxide synthase (eNOS), and superoxide-dismutase activities [92, 133-137]. These reactive molecules are known modulators of cellular phenotype, such as the differentiation of monocytes. In addition, these molecules can enhance cancer cell aggressiveness and can promote aging and age-related degeneration [138, 139]. Similarly inflammatory and structural cells that are activated in the airways of patients with COPD also produce ROS, including neutrophils, eosinophils, macrophages, and epithelial cells [140]. Superoxide anions ($O_2 \cdot^-$) are generated by NADPH oxidase, and this is converted to hydrogen peroxide (H_2O_2) by superoxide dismutases. H_2O_2 is then dismuted to water by catalase. $O_2 \cdot^-$ and H_2O_2 may interact in the presence of free iron to form the highly reactive hydroxyl radical (·OH). $O_2 \cdot^-$ may also combine with NO to form peroxynitrite, which also generates ·OH [141]. Nitrosylation and oxidation of lung proteins is a prominent finding in COPD and emphysema. The genetic ablation as well as pharmacological inhibition of inducible NOS prevented and reversed cigarette smoke induced emphysema in mice [142].

Table 2. Similarities between the secretory profile of senescent cells and cytokine profiles of the lungs in COPD

SASP factors	**Secretory profile for Senescent Cells [62, 92]**	**Secretory Profile of COPD lung**
Interleukins		[90, 91, 143]
IL-6	↑	↑
IL-1a, -1b	↑	↑
IL-13	↑	↑
Chemokines (CXCL, CCL)		[93, 94]
IL-8	↑	↑
GRO-α,-β,-γ (Growth-Related Oncogene)	↑	↑
MCP-2 (monocyte chemoattractant protein)	↑	↑
MIP-1a, 3a (macrophage inflammatory protein)	↑	↑
Other inflammatory factors		[95, 144]
GM-CSF (granulocyte macrophage colony stimulating factor)	↑	↑
MIF (macrophage migration inhibitory factor)	↑	↑
Growth factors and regulators		

EGF (endothelial growth factor)	↑	↑[145]
bFGF (basic fibroblast growth factor)	↑	↑[132, 146]
VEGF (vascular endothelial growth factor)	↑	↑[96]
Angiogenin	↑	↑[96]
IGFBP-3, 1, 2, 5 (Insulin like growth factor binding protein)	↑	↑[78]
Proteases and regulators		
MMP-1, -3, -10, -12, -13, -14 (matrix metalloproteinase)	↑[102-106]	↑[107-110]
PAI-1, -2, uPAR (plasminogen activator inhibitor) (urokinase-type plasminogen activator)	↑[98, 117-119, 147]	↑[148, 149]
Cathepsin B	↑	↑[150]
Soluble or shed receptors or ligands		
ICAM-1, -3 (intercellular adhesion molecule)	↑	↑[122, 151]
OPG (osteoprotegerin)	↑	↑[152]
TRAIL-R3, sTNFRI, Fas sTNFRII (tumor necrosis factor–related apoptosis-inducing ligand) (soluble tumor necrosis factor receptor)	↑	↑[153]
uPAR (urokinase-type plasminogen activator receptor)	↑[115]	↑[115, 149]
EGF-R (endothelial growth factor receptor)	↑	↑[97]
Nonprotein factors		
PGE2 (prostaglandin E2)	↑	↑[127, 129]
Nitric oxide	↑	↑[135, 141, 142]
Reactive oxygen species	↑	↑[154]
Extracellular Matrix proteins		[132, 155]
Fibronectin	↑	↑
Collagens	↑	↑
Laminin	↑	↑

1.3 DNA damage and DNA repair response

Oxidative stress leads to damage of various molecules within the cell, DNA being most crucial. DNA damage has been shown to be accumulated in various tissues such as brain, liver, muscle, kidney, liver etc. Evolution facilitated the eukaryotic cells to possess advanced multiple mechanisms to counter the DNA damage and control stress or age-associated damage to their genomes.

Damaged DNA has been shown to accumulate during aging in many tissues, including brain, muscle, kidney and liver. Cellular Senescence involves DNA damage as well. However, it remains elusive if DNA damage is entirely a product of the aging process or the cause. Furthermore, several studies suggest that dietary (calorie) restriction reduces the amount of age-associated oxidative DNA damage. Although, senescence could exist independent of any DNA damage, many of the aforementioned triggers of senescence usually elicit the DDR. DNA damage engages the p53 and pRB pathways. Thus, it is not surprising that the DNA damage response (DDR) is an important effector pathway through which senescence is established. In fact, telomere attrition, oncogene activation, or ionizing radiation all induce senescence through activation of the DDR [23]. A simplified DDR response is represented in **Figure 8**.

End replication problem of the DNA leads to the progressive telomere shortening and is a well-documented trigger of the DNA damage response. It has been reported that replicative senescent cells could accumulate several markers of DNA damage including phosphorylated γ-H2AX, SMC1, RAD17, CHK1 and CHK2 [23]. The study further revealed that γ-H2AX was enriched at subset of sub telomeric regions of chromosomes that have been shown to have particularly short telomeres. In contrast, inactivation of the DNA response through expression of dominant negative forms of ATM, ATR, CHK1 and CHK2 enabled replicative senescent cells to resume DNA replication. Further, it has been shown that DNA damage occurring at telomeres cannot be effectively repaired, which results in

the presence of chronic DNA damage foci, a persistent DDR and establishment of senescence [156].

Oncogene mediated senescence have also been reported to involve DDR. Furthermore, it has been demonstrated that oncogenic activation indeed initiates a DDR by causing hyper-replication that results in DNA double-strand breaks and improperly terminated replication forks [157, 158]. Inactivation of CHK2 not only abolishes H-RAS-induced senescence but also results in cell transformation, highlighting the importance of an intact DDR for establishment of OIS [158].

Finally, it is to be emphasized that the DDR is not only important for the induction of a permanent proliferation arrest, but is also critically important to the generation of the SASP. It has been reported that inactivation of critical DDR mediators including NBS1, ATM and CHK2 prevents the SASP in response to radiation-induced senescence [67]. In addition, SASP factors including the chemokine IL8 and ligands of CXCR2 provide a feedback loop that enhances the DDR [159]. Therefore, the DDR is not an independent process, but rather a fully integrated branch of the other effector pathways that establish senescence.

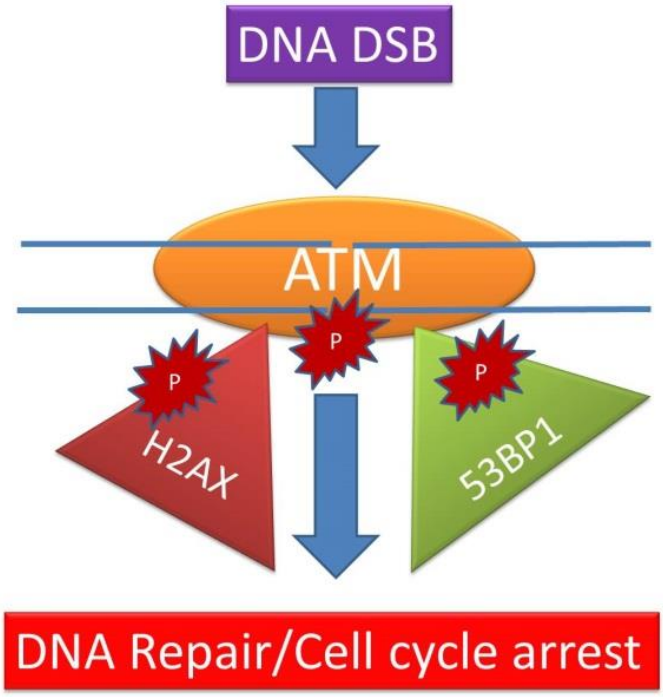


Figure 8. DNA damage response (DDR)

DNA double strand breaks trigger the activation of ATM which phosphorylates H_2AX and further recruits 53BP1 at the site of DNA double strand breaks. The DDR drives the cell either to repair the DNA damage or halt the cell cycle, depending upon the extent and nature of DNA damage.

1.4 Hypothesis

The close comparison between the factors that are up regulated during SASP response and in COPD draws attention to the striking similarity between the phenotypic state of cellular senescence and that of the pulmonary cells during COPD. Thus, the understanding of the phenomenon of senescence in COPD and the possible molecular mechanism that leads to SASP during senescence could also be very relevant in understanding the pathogenesis and inflammatory phenotype of COPD. Having this information in the background, two questions were asked.

1. Is there an involvement of pulmonary **cellular senescence** in the pathogenesis of COPD?

   And if yes,

2. Does this senescence involve persistent **DNA damage**?

1.5 Aims of study

The overall goal of this thesis was to investigate markers of senescence, DNA damage and repair in an *in-vitro* model of cigarette smoke induced senescence and in a cigarette smoke-induced mouse emphysema model.

In particular, the aims of this thesis are:

1. Establishment of an *in-vitro* model to study the effects of cigarette smoke induced premature senescence.

2. To evaluate the markers of senescence, DNA damage and repair in an *in-vitro* model of cigarette smoke extract (CSE) induced premature cellular senescence.

3. To evaluate the markers of senescence, DNA damage and repair in the murine model of cigarette smoke induced emphysema.

4. To compare the extent of DNA damage with the development of emphysema in tobacco-smoke exposed mice.

2 Materials

2.1 Reagents and chemicals

Reagent	Company
Acetic acid, glacial 99%	Sigma, USA
Acrylamide solution	Sigma, USA
Agarose	Carl Roth, Germany
Ammonium Persulfate	Sigma, USA
β mercaptoethanol	Sigma, USA
Bromophenol Blue	Roche, Germany
Bovine serum albumin	Carl Roth, Germany
BSA solution (2mg/ml)	Bio-Rad, USA
Chloroform	Carl Roth, Germany
DAPI	Invitrogen, USA
Dimethyl sulfoxide	Sigma, USA
Digest All 2 (Trypsin)	Invitrogen, USA
DNA ladder (100bp,1Kb)	Fermentas, Germany
Ethanol absolute	Carl Roth, Germany
Ethidium Bromide	Carl Roth, Germany
Ethylenediamine- Tetracetic acid (EDTA)	Carl Roth, Germany
Fluorescence mounting medium	Dako, Germany

Formaldehyde	Carl Roth, Germany
Glycerol	Sigma, USA
Glycine	Sigma, USA
Hydrochloric acid	Carl Roth, Germany
IQ SYBR® Green Supermix	BioRad, USA
Isopropanol	Carl Roth, Germany
JM109 competent cells	Promega, USA
Luria Broth agar	Sigma, USA
Luria Broth	Sigma, USA
Lipofectamine 2000	Invitrogen, USA
Methanol	Carl Roth, Germany
Milk powder	Carl Roth, Germany
N,N,N´,N´-Tetramethyl-1,2-diaminomethane (TEMED)	Sigma, USA
Ponceau S solution	Sigma, USA
Potassium dihydrogen phosphate	Carl Roth, Germany
Potassium phosphate monobasic	Carl Roth, Germany
Rainbow protein molecular weight marker	Amersham Biosciences, USA
RIPA lysis buffer	Thermo Scientific, Germany
Rnase Away	Invitrogen , USA
Sodium chloride	Carl Roth, Germany

Sodium citrate tribasic trihydrate	Carl Roth, Germany
Sodium dodecyl sulfate (20% w/v)	Carl Roth, Germany
Sodim hydroxide	Carl Roth, Germany
Stripping Buffer	Thermo Scientific, Germany
TOPRO-3	Invitrogen, USA
Tris base	Sigma, USA
Tris 1.5M (pH 8.9)	Amresco, Germany
Tris 0.5 M (pH 6.8)	Amresco, Germany
Trizol reagent	Invitrogen, USA
Tween 20	Sigma, USA
Xtreme gene siRNA transfection reagent	Roche, Germany
Xylol	Carl Roth, Germany

2.2 Kits

Names	Company
Dc Protein assay kit	BioRad, USA
Plasmid maxi prep kit	NucleoBond, Germany
InProm-II reverse transcriptase kit	Promega, USA
In situ apoptosis	Roche, Germany

NovaRed substrate kit	Vector, USA
Rneasy minikit	Qiagen, Germany
Supersignal west femto	Thermo Scientific, Germany
Senescence β-galactosidase staining kit	Cell signalling technology, Germany

Agarose electrophoresis chambers	Biometra, USA
BioDoc analyzer	Biometra, USA
Cell culture incubator	Heraeus, Germany
Centrifuge	Heraeus, Germany
CFX96 tm real-time PCR detection system	BioRad, USA
Fluorescence microscope	Leica, Germany
Fujifim image	Fujifilm, Japan
Light microscope	Hund, Germany
Precellys Homogenizer	PeQLab, Germany
Microplate reader Infinite 200	TECAN , Germany
PCR thermocycler	Eppendorf, USA
Power supply	BioRad, USA
Vacuum Pump	SBG, Germany
Water bath (cell culture)	BioRad, USA
Western blot chambers	BioRad, USA

2.3 Cell culture medium and reagents

Names	Company
Dulbecco's phosphate buffered saline (DPBS)	PAA, Germany
Endothelial growth medium (EGM-2)	Lonza, USA
Fetal calf serum	PAA, Germany
OptiMEM-I+ GlutaMax-I	Gibco, Germany
Pencillin/Streptomycin	Sigma, USA
Smooth muscle cell medium (SmGM)	Lonza, USA
Trypsin/EDTA	Sigma, USA

2.4 Other materials

Names	Company
Cell scrapers	BD Falcon, USA
Cell culture dishes (10cm, 3cm, 6well, 48well,96well)	Greiner bio-one, Germany
Cell culture flasks (75cm^2, 25cm^2)	Greiner bio-one, Germany
Centrifugal protein concentrators	Millipore, Germany
Filter tips (10, 100, 1000 µl)	Greiner bio-one, Germany
Gel blotting paper	Whatman, USA
Microcentrifuge tubes	Eppendorf, USA

Nitroceullulose membrane	Pall Corporation, USA
Polypropylene tubes (15ml, 50ml)	Greiner bio-one, Germany
Precellys Tubes with beads	Precellys, Germany
Real time PCR plates	BioRad, USA
Tips (10, 100, 1000 µl)	Greiner bio-one, Germany
Tissue culture chamber slides	BD Falcon, USA

2.5 Microscopes

Names	Company
Confocal microscope	Axio Imager Z.1, Germany
Fluorescence	Leica DMI 3000 B, Germany
Fluorescence	Leica DM 6000 B, Germany
Light Microscope cell culture	Axiovert 25, Germany
Stereo microscope	Leica MZ 16FC, Germany
Binocular	Leica S6, Germany

2.6 Smoke generating system

Name	Company
Vacuum pump for smoke generator	TSE, Germany
Pump for removing smoke	TSE, Germany

Smoke chamber	TSE, Germany
Millipore filter	Millipore, Germany
Cigarettes	University of Kentucky, USA
Computer program for monitoring smoke	TSE, Germany
Smoke Generator	TSE, Germany

2.7 Antibodies

The primary and secondary antibodies used in this study are listed below:

2.7.1 Primary antibodies

Antigen	Purpose/Dilution	Isotype	Supplier
p21	WB 1:1000 IF 1: 200	Mouse monoclonal	BD (556430) Germany
53BP1	IF 1:200	Rabbit polyclonal	Bethyl Biotech, USA (A300-272A-2)
Caspase - 3/CPP32 Mab	IF 1:200	Mouse monoclonal	BD (611048) Germany
Anti phospho-Histone H2A.X (ser139)clone JBW301	IF 1:200	Mouse IgG1	Millipore(05-636) Germany

HP1	IF 1:200	Rabbit	Cell signaling (2619) Germany
NF kappa B	WB 1:1000	Rabbit	Abcam (ab7971) Germany
CD11b/CD18	IF 1:200	Mouse	Millipore (MAB1387Z) Germany
CD 34	IF 1:200	Mouse	SerotecMCA (1825GA) Germany
Von Willebrand factor	IF 1:200	Human	Dako (A0082) Germany
CD45 PE	IF 1:200	Mouse	eBioscience (12-0451) Germany
Monoclonal anti-beta actin	WB-1: 5000	Mice	Sigma (A5441) USA
ADFP	IF 1:200	Rabbit	Abcam (ab52355) Germany
pro SP-C polyclonal	IF 1:200	Rabbit	Millipore (AB3786) Germany

2.7.2 **Secondary Antibodies**

Antigen	Purpose/Dilution	Isotype	Supplier
Alexa Fluor 488 Anti-Rabbit	ICC 1:1000	Goat IgG	Invitrogen, USA
Alexa Fluor 594 Anti-Rabbit	ICC 1: 1000	Goat IgG	Invitrogen, USA
Alexa Fluor 488 Anti-Mouse	ICC 1: 1000	Goat IgG	Invitrogen, USA
Alexa Fluor 594 Anti-Mouse	ICC 1: 1000	Goat IgG	Invitrogen, USA
Alexa Fluor 646 Anti-Mouse	ICC 1: 1000	Goat IgG	Invitrogen, USA
Anti-Rabbit IgG HRP-linked	WB 1:10000	Donkey IgG	GE Health care, UK
Anti-Mouse IgG HRP-linked	WB 1:10000	Sheep IgG	GE Health care, UK

2.8 Buffers and solutions

The buffers and solutions used in this work are listed in Table 2.11. Unless specified otherwise the solutions were prepared in distilled and autoclaved water. Freshly prepared solutions for an application were not autoclaved.

2.8.1 Compositions of buffers and solutions

Buffer/Medium/Solution	Compositions
Agarose gel loading buffer	0.25% bromophenol blue [w/v] 0.25% xylene cyanol FF [w/v]
Alkaline phosphatase (NTMT) buffer	1 ml NaCl (5 M) 510 mg $MgCl_2$, $6H_2O$ 50 µl Tween 20 5 ml Tris (1 M; pH 9.5)
Antigen retrieval buffer	0.1 M Tris/HCl buffer (pH 9.0)
AEC stock	1 tablet AEC (20 mg) dissolve in 7.5 ml N,N dimethyl formamide store at -20℃

Buffer/Medium/Solution	Compositions
AEC working solution	50 µl AEC stock 900 µl dH_2O 100 µl 0.5 M acetate buffer (pH 4.9)
Acetate buffer (pH 4.9)	85 g CH_3COONa, $3H_2O$ 900 ml dH_2O adjust pH with glacial acetic acid dH_2O q.s. to 1 l
Blocking buffer (for in situ)	5% goat serum in PBS [v/v]
B-Block	2% BSA [w/v] 10% goat serum in PBST [v/v] 0.1% Tween 20 [v/v]
DEPC-Water	0.01% DEPC [v/v] in dH_2O incubate overnight at RT and then autoclave for 60 min.
DNase I solution	100 mg DNase I dissolve in 10 ml 10 µM $MgCl_2$ solution, filter and store at -20°C

Buffer/Medium/Solution	Compositions
PBS (1x)	8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH_2PO_4 dissolve in 800 ml of dH_2O, adjust pH to 7.4 and add H_2O q.s. to 1 l
PCR buffer (10x) without $MgCl_2$	20ml KCl (1 M) 4ml TrisHCl (1 M), pH 9 0.4ml Triton X100 sterile distilled water q.s. to 40 ml
PFA in PBS (4%)	4 g PFA dissolve in 100 ml PBS (add few drops of NaOH). Heat at 55℃ until PFA is dissolved. Cool and adjust the pH to 6-7
PBT	0.1% Tween in PBS [v/v]

Buffer/Medium/Solution	Compositions
PBT/glycine	0.2% glycine in PBT [w/v]
PT	0.3% Triton X100 in PBS [v/v]
PBA	5% BSA [w/v] 0.02% NaN_3 [w/v]
PBAT	0.3% Triton X100 in PBA [v/v]
PBDT	0.1% DMSO and 0.1% Triton X100 in PBS
PEM	0.1 M PIPES 1 mM $MgSO_4$, $7H_20$ 2 mM EGTA
RIPA buffer	2.5 ml 10% SDS in water 15 ml NaCl (5 M) 5 ml NP40 25 ml 10% deoxycholate in water [w/v] 1 ml EDTA (0.5 M) 25 ml Tris (1M, pH 8.0) dissolve in DEPC-treated water q.s. to 500 ml. (Not autoclaved).

Buffer/Medium/Solution	Compositions
SADO mix	50 ml HEPES Na (200 mM; pH 7.6) 50 ml NaCl (1.3 M) 5 ml KCl (300 mM) 5 ml NaH_2PO_4 (100 mM) 1 ml glucose (2 M)
SSC (20x)	17.53 g NaCl 8.82 g Na citrate Dissolved in 80 ml DEPC-treated water and adjusted the pH to 7. DEPC-treated water q.s. to 100 ml
SSC/Formamide/Tween	5 ml SSC (20x) 25 ml formamide 50 µl Tween 20
TBST (10x)	8 g NaCl 0.2 g KCl 25 ml Tris (1 M; pH 7.5) 1 ml Tween 20 dH_2O q.s. to 100 ml
TAE running buffer (1x)	0.04 M Tris base 0.002 M glacial acetic acid 0.002 M EDTA, $2H_2O$
TBS	40 g NaCl 1.8 g tris base dissolved in 4.5 l dH_2O adjusted the pH 7.6 dH_2O q.s. to 5 l
TBST	0.1% Tween 20 in TBS [v/v]

Buffer/Medium/Solution	Compositions
Transformation buffer (KCM buffer)	500 mM KCl 150 mM $CaCl_2$
Transfer buffer (20x)	163.2 g bicine 209.3 g bis Tris 2 g EDTA
Transfer buffer (1x)	250 ml 20x transfer buffer 1 l methanol dH_2O q.s. to 5 l

2.9 Antibiotics

Antibiotics that were used in this study are listed below.

Antibiotics	Working concentration liquid culture	Working concentration agar plates
Ampicillin	100µg/ml	100µg/ml
Streptomycin	15µg/ml	30µg/ml
Penicillin	20µg/ml	20µg/ml

3 METHODS

3.1 Culture conditions

For cell culture experiments cells were kept at 37 °C in humidified chambers containing 5 % CO_2 (carbon dioxide); media were exchanged according to individual needs (about every 24 to 72 h). In case of HLF-1 cells, DMEM culture medium containing 10 % FCS (fetal calf serum), 1 % L-glutamine and 1 % penicillin / streptomycin was used.

3.2 Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared following some modification from the method described by Carp and Janoff [160] Briefly, three 100-mm cigarette (Research Grade Cigarette, University of Kentucky) was combusted with a Variable Speed Pump or Water Jet Filter pump. The smoke was bubbled through 30 ml serum containing media at a speed of 1 cigarette / 2min. The prepared suspension was then filtered through a 0.22-µm pore filter. This solution was considered to be 100% CSE within 30 min of preparation to obtain the desired concentration.

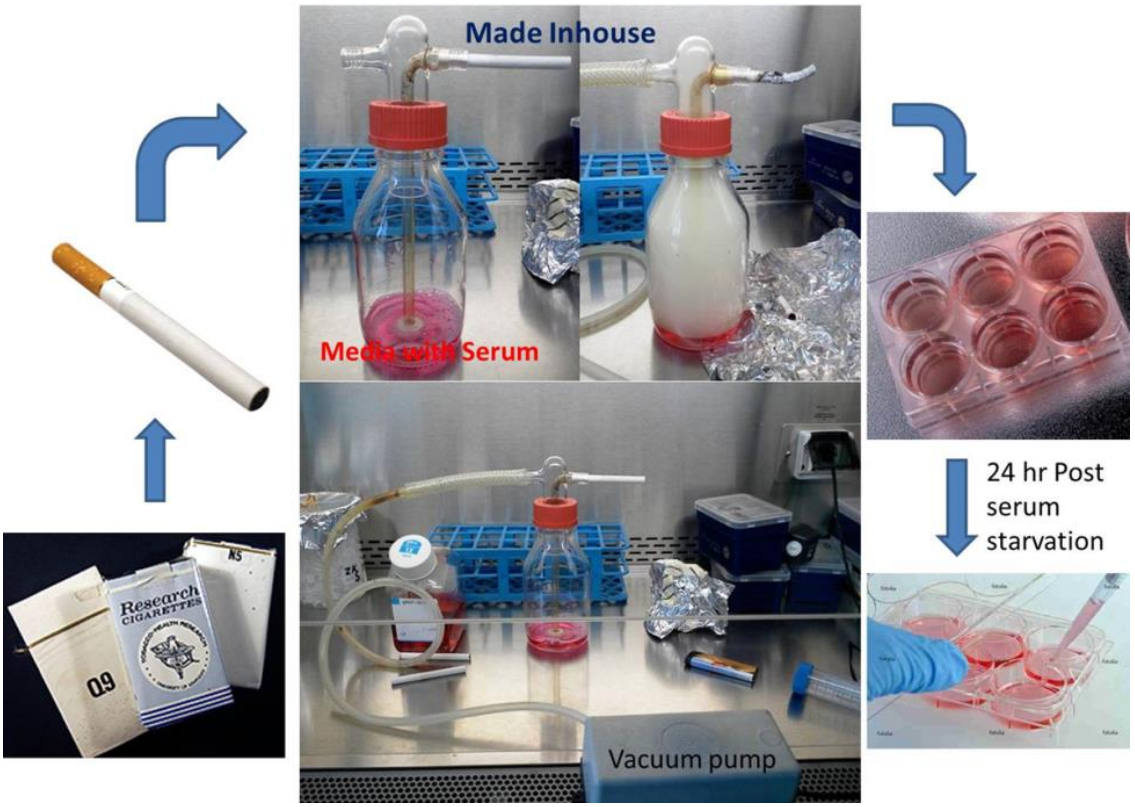
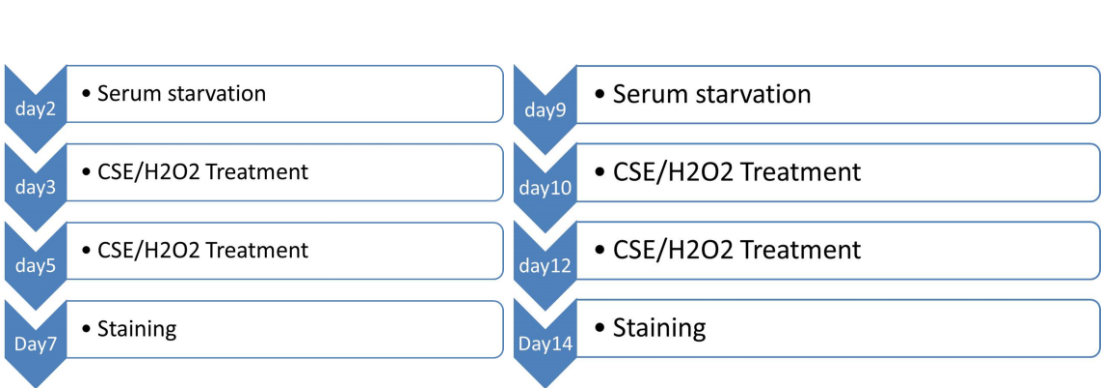


Figure 9. Setup for the preparation of the cigarette smoke extract

An in-vitro set was devised to determine if cigarette smoke indeed induced senescence on various cell types of interest. The cell culture media was bubbled with cigarette smoke. The cigarette smoke extract was freshly used for treating cells after 24 hours of serum starvation.

The cells were treated post 24 hours of serum starvation with several concentrations of the prepared cigarette smoke extract as per **scheme 1.**



***Scheme 1*. Cigarette smoke exposure to different cell types.**

Human lung fibroblasts and rat pulmonary arterial smooth muscle cells were treated by cigarette smoke extract on day 3 and day 5. Staining was performed on day 7. Fibrocytes were differentiated by day 7 from the PBMCs isolated from the human blood and later on treated with CSE/H_20_2 on day 10 post 24 Hours serum starvation. Second exposure was given on day 12 and staining was carried out on day 14.

3.3 Cigarette smoke exposure *in-vivo*

Wild type mice C57BL/6J were exposed to the mainstream smoke of 3R4F cigarettes at a concentration of 140 mg particulate matter/m^3 for 6 h/day, 5 days/week for up to 8 months. To assure age-matched controls, respective control groups were kept under identical conditions as smoke-exposed mice but without smoke exposure the time course of COPD development was correlated with the markers of senescence.

For alveolar morphometry and staining, lungs were fixed in chest by infusion of 4.5% formaldehyde solution at 22 cm H_2O of inflating pressure via the trachea. During fixation, tracheal pressure of 12 cm

H_2O was maintained. For both alveolar and vascular morphometry, lungs were isolated from the chest cavity after 20 minutes and allowed to immerse overnight in respective fixative solution. Thus fixed lungs were transferred to 0.1 M phosphate buffered saline the following day.

After this, the lung lobes were individually placed in histological cassettes and dehydrated in an automated dehydration station and then embedded in paraffin blocks. Staining was done on 3µm lung sections for alveolar staining.

3.4 Removal of (residual) lungs

Mice were anesthetized by intraperitoneal injection with ketamine/xylazine (20µl ketamine/20µl xylazine/40µl NaCl) and sacrificed for investigation. Mice were killed by an overdose of isoflurane gas and exsanguination via the vena cava inferior. After sacrifice, mice were given first incision in longitudinal ventral area from trachea to abdomen, diaphragm was opened and tracheal area was cleaned.

For molecular biology the trachea was incised and cannulated. The thoracic cavity was opened by sternotomy, and a second cannula was placed into the pulmonary artery, allowing the removal of residual intrapulmonary blood. Then, the apex of the left ventricle was incised, and the lung was flushed via the pulmonary artery using 20 ml of 0.9 % NaCl solution at a constant pressure of 25 cm H_2O while being inflated with N_2 (nitrogen) gas via the trachea at a pressure of 15 cm. Afterwards, the organ was removed in total and immediately frozen in liquid nitrogen.

For alveolar morphometry, lungs were fixed in chest by infusion of 4.5% formaldehyde solution at 20 cm H_2O of inflating pressure via the trachea for 20 minutes. Lungs were isolated from the chest cavity and allowed to immerse overnight in respective fixative solution. Thus

fixed lungs were transferred to 0.1 M phosphate buffered saline the following day.

After this, the lung lobes were individually placed in histological cassettes and dehydrated in an automated dehydration station and then embedded in paraffin blocks. Staining was done on 3µm lung sections for alveolar staining.

3.5 Protein isolation from tissues

100 mg of tissue was weighed and put into 500µl of RIPA buffer (Thermo Scientific) containing protease and phosphatase inhibitor cocktail. Tissues were homogenized by precellys lysing kit, followed by centrifugation for 30min at 4°C (13,000xg). Supernatants were transferred to 1,5ml tubes and directly quantified or stored at -80°C.

3.6 Protein isolation from cells

Protein isolation from cells was carried out using RIPA buffer (Thermo Scientific) containing protease and phosphatase inhibitor cocktail (Thermo Scientific). Media was removed from the wells, followed by a PBS wash. 75µl of RIPA buffer was directly added to each well of 6 well plates and after 10min of incubation at 4°C; plates were scratched with cell scrapers and supernatants were put into 1,5ml tubes. The tubes were centrifuged for 30min at 4°C (13,000xg). Supernatants were transferred to 1,5ml tubes and directly quantified or stored at -80°C.

3.7 Protein estimation

Protein quantification was carried out using Bio-Rad *DC* Protein Assay kit. It is a colorimetric assay based on Lowry's method involving reaction of protein with an alkaline copper tartrate solution and Folin reagent giving rise to a characteristic blue color showing absorbance at 750nm. Different concentrations of BSA in range of 0.125-2mg/ml were used as standard. Protein samples were prediluted in range of the standard and the absorbance was measured at 750nm using a micro plate reader (Tecan). Exact concentrations were calculated in accordance to the standards.

3.8 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE is carried out to separate various proteins in a sample according to their molecular weights for further immunoblot analysis. Protein samples from cells and tissues were equalized to same concentrations and mixed with 5X gel loading buffer at a ratio of 4:1 and denatured at 95°C for 10min. Protein samples were loaded along with molecular weight marker into the wells of 7% or 10% (depending on protein sizes to be separated) polyacrylamide gels. Gels were run in vertical electrophoretic assembly using 1X running buffer at 100-120V for 2-3 hrs. Buffers used are as follows:

5X gel loading buffer components	**Final concentration**
Tris-HCl (2M, pH-6.8)	375mM
SDS	10% (w/v)
Glycerol	50%(v/v)
β-Mercaptoethanol	12.5%(v/v)
Bromophenol Blue	0.02%(w/v)

1X SDS Running buffer components	Final concentration
Tris	25mM
Glycine	250mM
SDS 10% (w/v)	0.1%(w/v)

Stacking gel (5%)

Gel Component	Final concentration
Tris-HCl (0.5M, pH-6.8)	125mM
Acrylamide/Bis-acrylamide 40% (w/v)	6%
SDS 10% (w/v)	0.10%
APS 10% (w/v)	0.05%
TEMED	0.10%
Water up to the final volume	

Resolving gel

Gel component	Percentage of gel		
	8%	**10%**	**12%**
Tris-HCl (1.5M, pH-8.8)	375mM	375mM	375mM
Acrylamide/Bis-acrylamide 40% (w/v)	10%	10%	12%
SDS 10% (w/v)	0.10%	0.10%	0.10%
APS 10% (w/v)	0.05%	0.05%	0.05%
TEMED	0.10%	0.10%	0.10%
Water up to the final volume			

3.9 Immunoblotting

Proteins separated on gel were transferred to a nitrocellulose membrane by electrophoretic transfer. The transfer was carried out for 1hr at 100V in transfer buffer.

Blotting buffer components	Final concentration
Tris	25mM
Glycine	192mM
Methanol	20%(v/v)

After the transfer, membranes were removed and blocked in blocking buffer for 1hr on shaker at RT, followed by overnight incubation in primary antibodies diluted in blocking buffer (for dilutions, Appendix Table 2) at 4°C. Following day, membranes were washed 3 times for 10min with 1xTBST buffer and subsequently incubated in secondary HRP-conjugated antibodies diluted in blocking buffer for 1hr at RT. After 1hr of incubation, membranes were washed thrice for 10min in 1x TBST and incubated with ECL substrate (Thermo Scientific) in the Image reader (Fujifilm) to detect the signal. The time of exposure was determined on the basis of signal intensity.

1xTBST (1xTBS, 0.1% (v/v) Tween 20)

Tris buffer saline (TBS) components	Final Concentration
Tris 20mM	**20mM**
Sodium	137mM
Tween	0.1%
HCl	To set the pH
Water	up to the final volume

Blocking buffer (5% non-fat dry milk powder in 1xTBST)

In order to reprobe the membranes for housekeeping genes, membranes were stripped in a stripping buffer (Thermo Scientific) for 30min at 37°C, washed and incubated with the primary antibodies.

Densitometry analysis of the immuno-blots

Western blots were quantified using the multi gauge software (Fujifilm). Expression was quantified using bands intensity values (in arbitrary units) which were normalized to the housekeeping gene (β-actin).

3.10 Transfection with over expression plasmid

Transfection refers to the process of introducing foreign DNA or RNA into mammalian cells. In this study, lipofection was used as the method of transfection, lipofection refers to use of synthetic cationic lipid to facilitate the delivery of DNA into the cells. These cationic lipids tend to form liposomes in aqueous solution. Liposomes, thus formed, interact with negatively charged DNA to form lipid-DNA complexes. The complex fuses with the plasma membrane of cells, resulting in the uptake of DNA and further expression of the gene carried by the plasmid DNA.

HEK 293 cells were cultured to 70-80% confluence in 6well plates (for protein isolation) or chamber slides (for immunofluorescence). Plasmid and lipofectamine were diluted in antibiotic free opti-MEM medium and mixed within 5 min at ratio of 1:2 (DNA in µg to transfection reagent in µl). The mixture was incubated at RT for 30min and added to the cells which were cultured in antibiotic free transfection medium (opti-MEM medium + basal growth medium with 0.1% FCS). After 5hrs, medium was changed to the normal growth medium up to 48hrs, followed by protein isolation or immunofluorescence staining.

3.11 Immunohistochemistry

4µm thick sections were cut from paraffin embedded lung tissues. Sections were incubated at 65°C for 20min, followed by deparaffinization in xylene and rehydration in series of grade-decreasing ethanol solutions. Sections were washed with PBS and incubated in boiling 10mM citrate buffer for 8min at 630watt in microwave for antigen retrieval. When needed, antigen retrieval was carried out by using 0.25% trypsin for 10min at 37°C. After blocking endogenous peroxidases activity by treatment with 15% hydrogen peroxide for 20min, Nova RED horseradish peroxidase (HRP)-substrate kit was used for immunohistochemical staining according to the manufacturer's instructions. Sections were kept in serum blocking for 1hr, followed by overnight incubation with primary antibody at 4°C. After washing with PBS, sections were incubated with biotinylated secondary antibody for 10min, followed by again a PBS wash and incubation with streptavidin conjugated HRP for 5min. Sections were washed and color development was carried out using a substrate/chromogenic mixture, followed by counterstaining with hematoxylin. Stained sections were examined under Leica DM 2500 microscope using Leica Q Win imaging software

3.12 Senescence associated β-galactosidase cell staining

The growth media from the cells was removed. The plate was then rinsed once with 1X PBS (2 ml for a 35 mm well). 1 ml of 1X fixative solution to each 35 mm well was added. The cells were then allowed to fix for 10-15 minutes at room temperature. The plate was then rinsed two times with 1X PBS (2 ml for a 35 mm well). 1 ml of the b-galactosidase staining solution to each 35 mm well was added and the plate was incubated at 37°C overnight in a dry incubator (no CO2).

While the b-galactosidase staining solution was still on the plate, the cells were checked under a microscope (200X total magnification) for the development of blue color. For long-term storage of the plates, the b-galactosidase staining was removed and the cells were overlaid with 70% glycerol and stored at 4°C.

3.13 Senescence detection by fluorescence

The ImaGene Green C12 FDG lac-Z Gene Expression Kit (I-2904) was used for fluorescence staining of senescent cells as per the manufacturer's protocol.

Cells were grown on coverslips inside a culture dish with appropriate cell culture medium to the desired confluency. The medium was removed from the dish and the cells were covered with pre-warmed 33 µM substrate containing culture medium. The cells are then incubated for 20-60 minutes under desired conditions. The coverslips then can be removed and examined in the fluorescence microscope.

3.14 Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Statistical comparisons of samples were performed by Student's t test for comparing two groups or by one-way ANOVA followed by the Newman-Keuls post-hoc test for multiple comparisons. Difference with $p<0.05$ between groups was considered significant.

4 RESULTS

Classically, COPD primarily has been considered a lung disease that is mainly caused by cigarette smoking. However, COPD has important manifestations beyond the lungs, the so-called systemic effects. These include unintentional weight loss, skeletal muscle dysfunction, and an increased risk of cardiovascular disease, osteoporosis, and depression, among others. Low-grade, chronic systemic inflammation is one of the key mechanisms underlying these systemic effects. Because these extra-pulmonary manifestations of COPD are common and/or may have significant implications for the patient wellbeing and prognosis both lung resident cell types as well as the systemic cells are believed to be involved in the pathogenesis of or response to COPD. It was hence an aim to investigate lung resident cells and systemic cells. It had been hypothesized that the primary cause of increased inflammation and pathogenesis is the onset of DNA damage and premature senescence upon exposure to cigarette smoke.

4.1 Characterization of lung resident and systemic cells.

Isolation and characterization of different lung resident and systemic cells were carried out with the aim to decipher the markers of cellular senescence and DNA damage upon treatment with cigarette smoke exposure over them. Cell types that were investigated further for our studies were namely human lung fibroblasts and circulating fibrocytes from human blood and are shown in **Figure 10.**

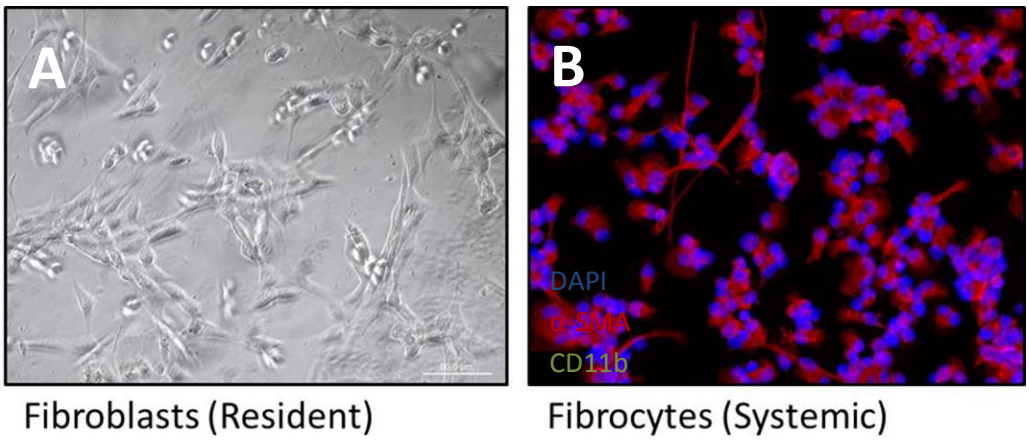


Figure 10. Characterization of lung resident fibroblasts and systemic human circulating fibrocytes.

***(A)**. Representative images of primary lung fibroblasts characterized by spindle shape morphology in bright field **(B).** Circulating fibrocytes were isolated from the PBMCs and characterized by the presence of CD 11b and alpha smooth muscle actin (α-SMA).*

.

4.2 Cigarette smoke extract exposure induced cellular senescence in-vitro

Morphologic changes are characteristic features of the senescent phenotype that occur at both the cellular and organism level. Senescent cells showed morphologically flattened and enlarged cell shapes. Human lung fibroblasts were exposed to cigarette smoke exposure *in-vitro* as described in **Scheme 1** in the methods section**.** Cigarette smoke exposure *in-vitro* could induce characteristic morphological senescent phenotype as shown in **Figure 11.**

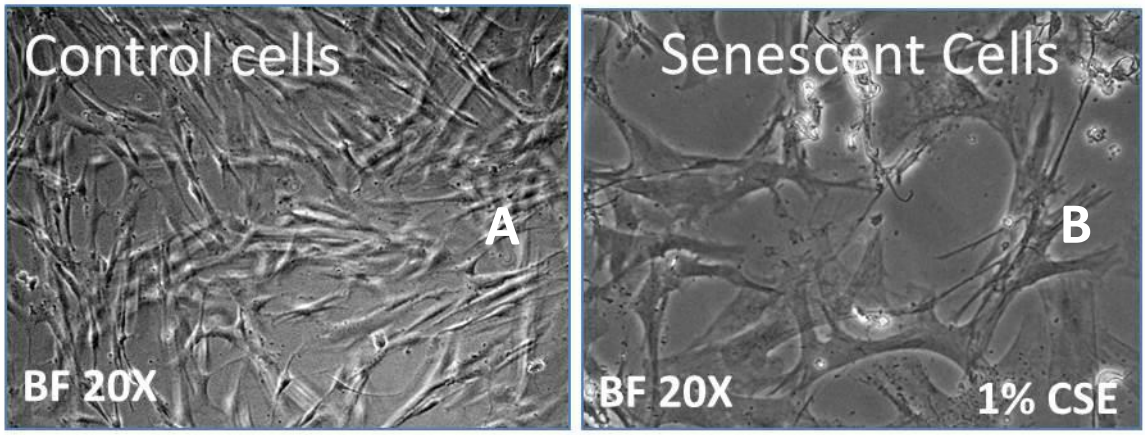


Figure 11. Morphological changes in Human Lung Fibroblasts 1 (HLF-1) cells after chronic exposure to cigarette smoke in-vitro.

HLF-1 cells were exposed to 1 % cigarette smoke and their morphology was examined through bright field microscopy. The morphological changes upon CSE treatment resembled closely to senescent phenotype. ***(A).*** *Normal HLF-1 cells as control. (**B**). HLF-1 cells treated with 1% CSE showed flattened and enlarged phenotype resembling senescent phenotype.*

Senescence-associated (beta)-galactosidase is widely used as a biomarker of senescence. The pH 6 β-galactosidase activity detected in senescent cells can be attributed to a rise in the level of the classic lysosomal enzyme. Furthermore, there are evidences that this is a consequence of an increase in lysosomal mass in senescent cells [161]. β-D-galactosidase is an eukaryotic lysosomal hydrolase. It acts by cleaving β-linked terminal galactosyl residues from many substrates like the glycoproteins, gangliosides, glycosaminoglycan and many other artificial substrates. The β-D-galactosidase has an optimum pH 4 for its activity which is closer to the natural pH of the lysosome. Lysosomal β-galactosidase activity can be detected in situ in most mammalian cells by means of a cytochemical assay, normally carried out at pH 4, using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Dimri et al. (1995) described a pH 6 β-galactosidase activity, which was found specifically in senescent human fibroblast cultures, but not in quiescent or terminally differentiated cells. Furthermore, this pH 6.0 activity enabled identification of senescent fibroblasts and keratinocytes in biopsies of aged human skin, and subsequently became known as senescence associated β-galactosidase (SA-β-galactosidase).

Different cell types were exposed to CSE or H_2O_2 (as described in **scheme 1**). A sub lethal dose of CSE could indeed induce senescence, as evident by senescence associated β-galactosidase activity. Hydrogen peroxide was used as a positive control. Senescence was detected by the presence of beta galactosidase activity at pH-6 (**Figure 12**).

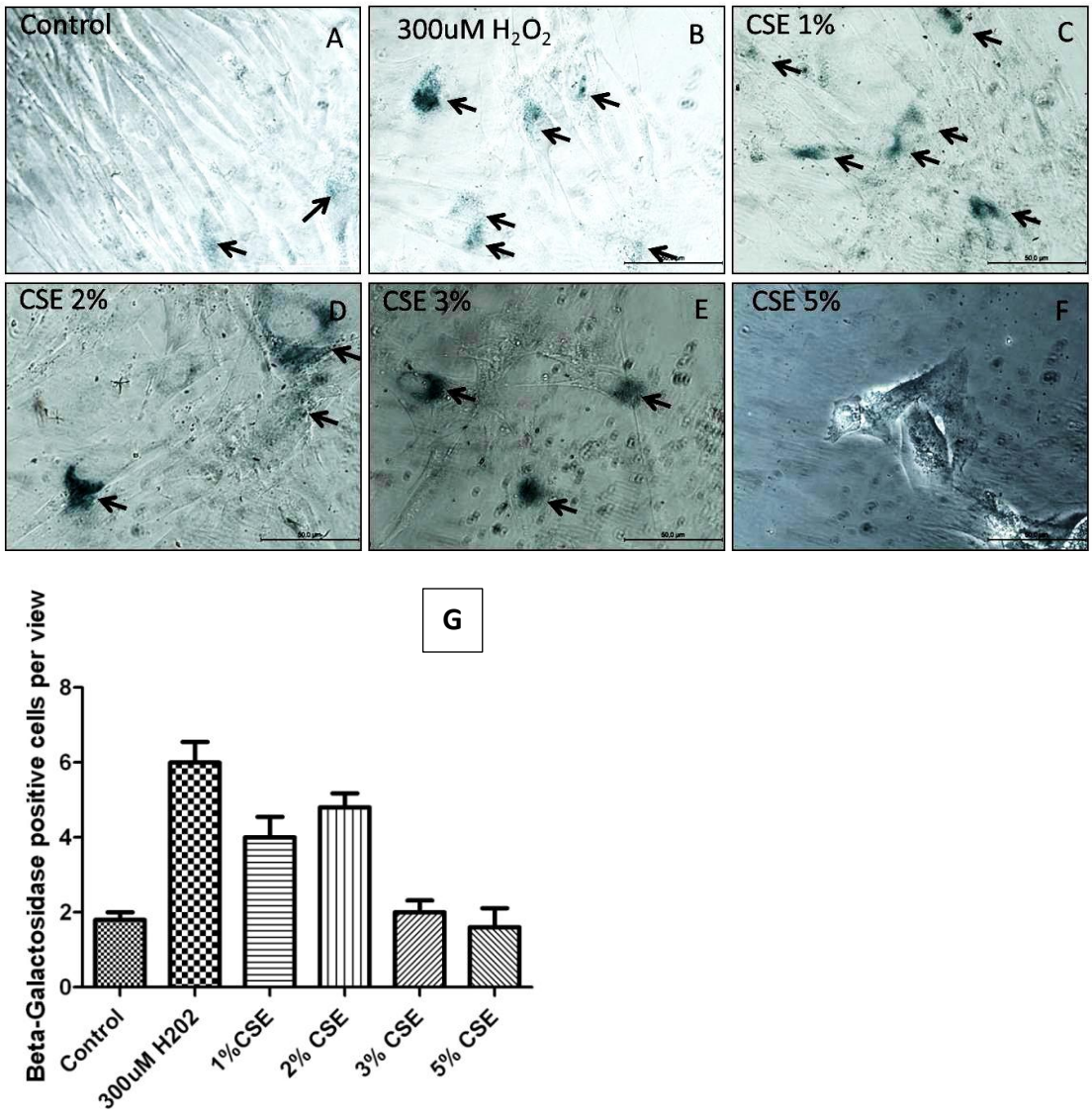


Figure 12. Increased senescence upon cigarette smoke treatment Human Lung Fibroblasts 1 cells (HLF-1).

HLF-1 cells were treated with different doses of cigarette smoke extract (CSE) leading to senescence. H_2O_2 treatment was done as a positive control. Senescence was detected by β-galactosidase activity using x-gal. Higher β-galactosidase activity indicates higher senescence. Representative bright field images of ***(A).*** *Control HLF-1 cells* ***(B).*** *HLF-1 cells treated with 300uM H_2O_2* ***(C)****. HLF-1 cells treated with 1% CSE* ***(D).*** *HLF-1 cells treated with 2% CSE* ***(E).*** *HLF-1 cells treated with 3%CSE* ***(F).*** *HLF-1 cells treated with 5% CSE* ***(G).*** *Quantitative analysis of the senescescent cells were performed by counting blue cells in 10 random fields.*

In addition, the modified version of a cell permeable fluorescent β-galactosidase substrate, fluorescein di-b-D-galactopyranoside (FDG), supplied by Molecular Probes as *ImaGene Green*™ C12FDG *lacZ* Gene Expression Kit (I-2904) was used to assess senescence (**Figure 13**). Once fluorescein di-b-D-galactopyranoside (FDG) reacts with the β-galactosidase, it turns into a fluroscencent compound which is not further permeable to the cells. Hence the substrate could be used as a marker of β-galactosidase activity at pH 6 indicative of cellular senescence.

β-D- galactosidase activity at pH 6 was found to be increased with C12FDG indicative of increased cellular senescence after chronic exposure to cigarette smoke extract *in-vitro*.

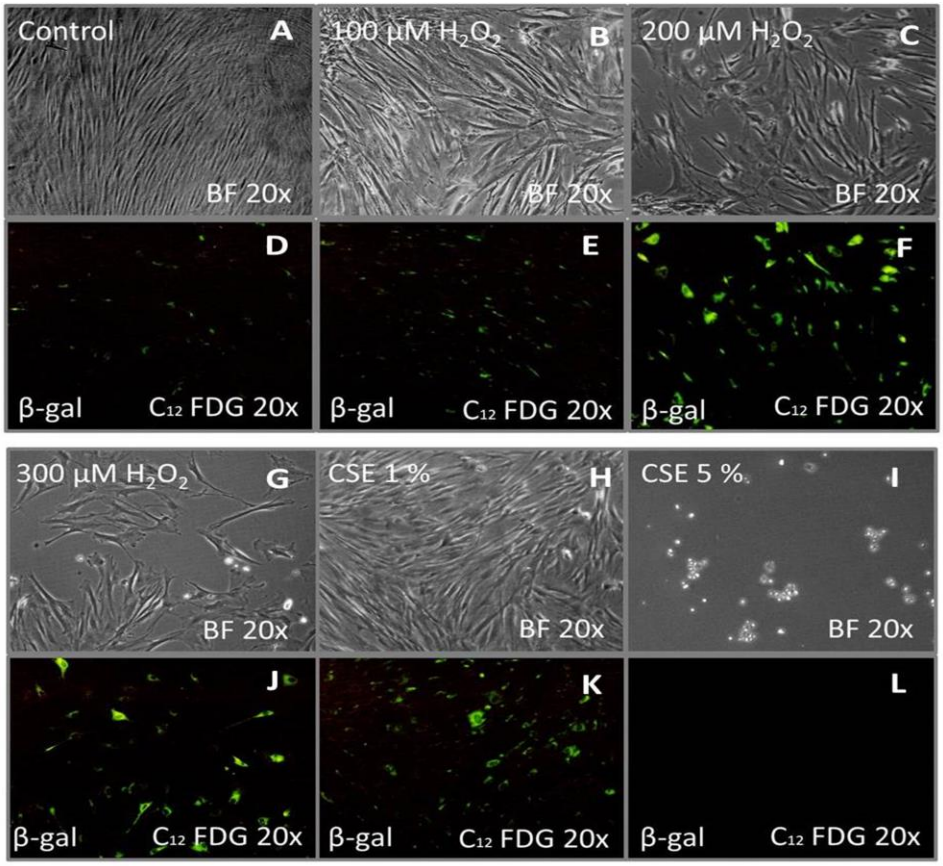


Figure 13. Increased senescence upon cigarette smoke treatment in Human Lung Fibroblasts (HLF-1).

HLF-1 cells were treated with different doses of H_2O_2 and cigarette smoke extracts (CSE). Senescence induced by treating the cells by H_2O_2 was used as a positive control. Senescence was detected by β-galactosidase activity using a fluorescent dye C_{12} FDG. Higher β-galactosidase activity indicates higher fluorescence. Induction of senescence was found to be concentration dependent.

***A & D.** HLF-1 cells untreated as control. **B & E.** HLF-1 cells treated with 100µM H_2O_2. **C & F.** HLF-1 cells treated with 200µM H_2O_2. **G & J.** HLF-1 cells treated with 300µM H_2O_2. **H & K.** HLF-1 cells treated with 1% CSE. **I & L.** HLF-1 cells treated with 5%CSE*

While the human lung fibroblasts underwent senescence upon CSE treatment, the human circulating fibrocytes rather proliferated and were resistant to CSE challenge as shown in the **Figure 14** below.

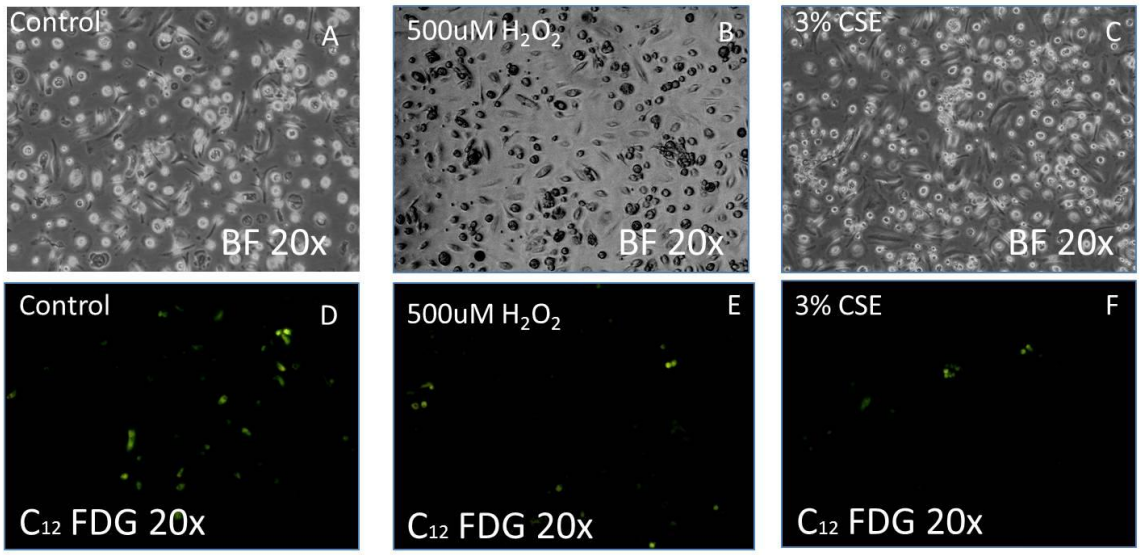


Figure 14. Circulating fibrocytes did not undergo increased senescence upon CSE treatment

Circulating fibrocytes were isolated from human blood and treated with hydrogen peroxide and different doses of cigarette smoke extract. Senescence was detected by β-galactosidase activity using C_{12}FDG (in green). Higher β-galactosidase activity indicated higher senescence. There was no increase in senescence upon CSE treatment with reference to the controls.

A & D. *Representative images of control circulating fibrocytes untreated.*

B & E. *Representative images of circulating fibrocytes treated with 500uM of hydrogen peroxide.*

C & F. *Representative images of circulating fibrocytes treated with 3% Cigarette smoke extract.*

4.3 Cigarette smoke extract exposure lead to increased hetero-chromatinization

Senescence leads to hetero-chromatinization within the nucleus and this also may serve as a marker of senescence. Cells treated with cigarette smoke extract at sub lethal doses showed an increased amount of nuclear DNA domains stained densely by DAPI. This represents increased heterochromatinization inside their nucleus, so called senescence associated heterochromatin foci (SAHF) (**Figure 15**).

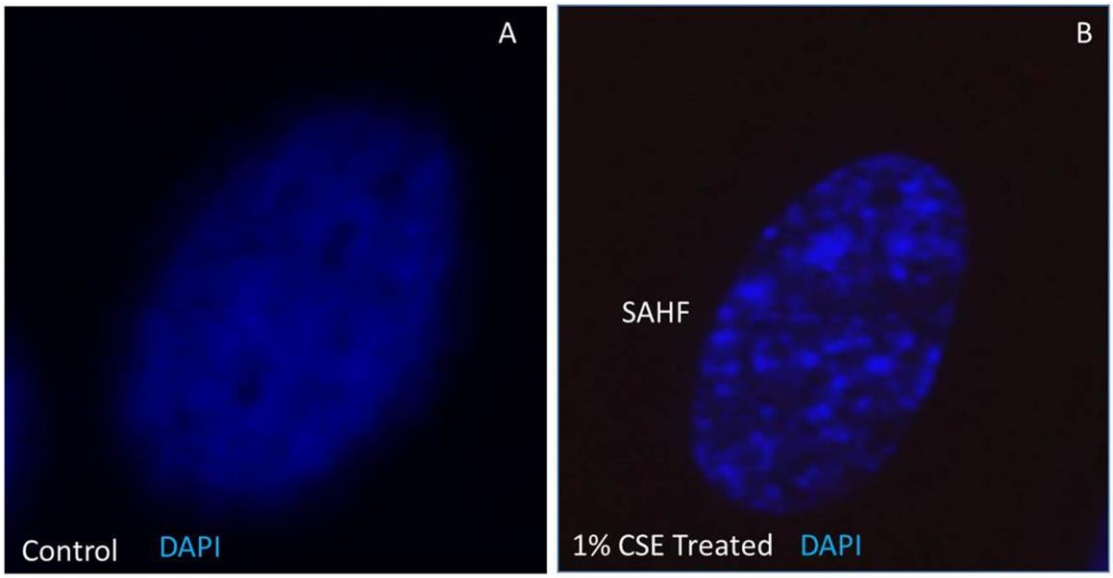


Figure 15**. **CSE lead to the formation of senescence associated heterochromatin foci

Cells were treated with CSE and observed under light microscope for nucleus with condensed chromatin foci indicative of increased heterochromatinization (SAHF) with DAPI staining (blue).

***A.** Represents a normal nucleus with diffuse DAPI (blue) staining.*

***B.** Represents nucleus with DAPI (blue) punctate staining indicative of the formation of SAHF upon 1% CSE treatment.*

4.4 Senescence induced by cigarette smoke extract exposure *in-vitro* involves accumulation of DNA double strand breaks

Keeping in mind the chronic inflammation in COPD, it was obvious to think that pulmonary cellular senescence, if any in COPD, must involve factors that lead to a SASP response. SASP occurs only when senescence involves factors that lead to DNA damage. Cigarette smoke exposure is the primary cause of COPD. Hence, investigations were carried out to check if cigarette smoke extract *in-vitro* caused DNA damage.

Nuclear Specific marker of DNA double strand break (dsb), 53 BP1 foci were checked upon by immunofluorescence microscopy. 53BP1 forms foci on the site of DNA double strand breaks and is a specific marker for DNA dsb. 53 BP1 was found to form DNA damage foci in the cells that were treated with cigarette smoke extract. p21, which is a maker of cell cycle arrest, was localized in parallel. Both 53BP1 and p21 were found to be upregulated (**Figure 16**).

What was interesting to note, was that not all the cells that were positive for 53 BP1 were positive for p21, but all the cells positive for p21 were positive for 53BP1. This indicated that not all the cells that undergo DNA damage necessarily undergo cell cycle arrest, but only those cells, in which the DNA damage has been persistent and accumulated beyond repair, decide to halt.

It was also noted that a higher dose of CSE not necessarily lead to formation of higher DNA damage induced 53 BP1 foci. A higher dose of CSE rather induced apoptosis **(Figure 17).**

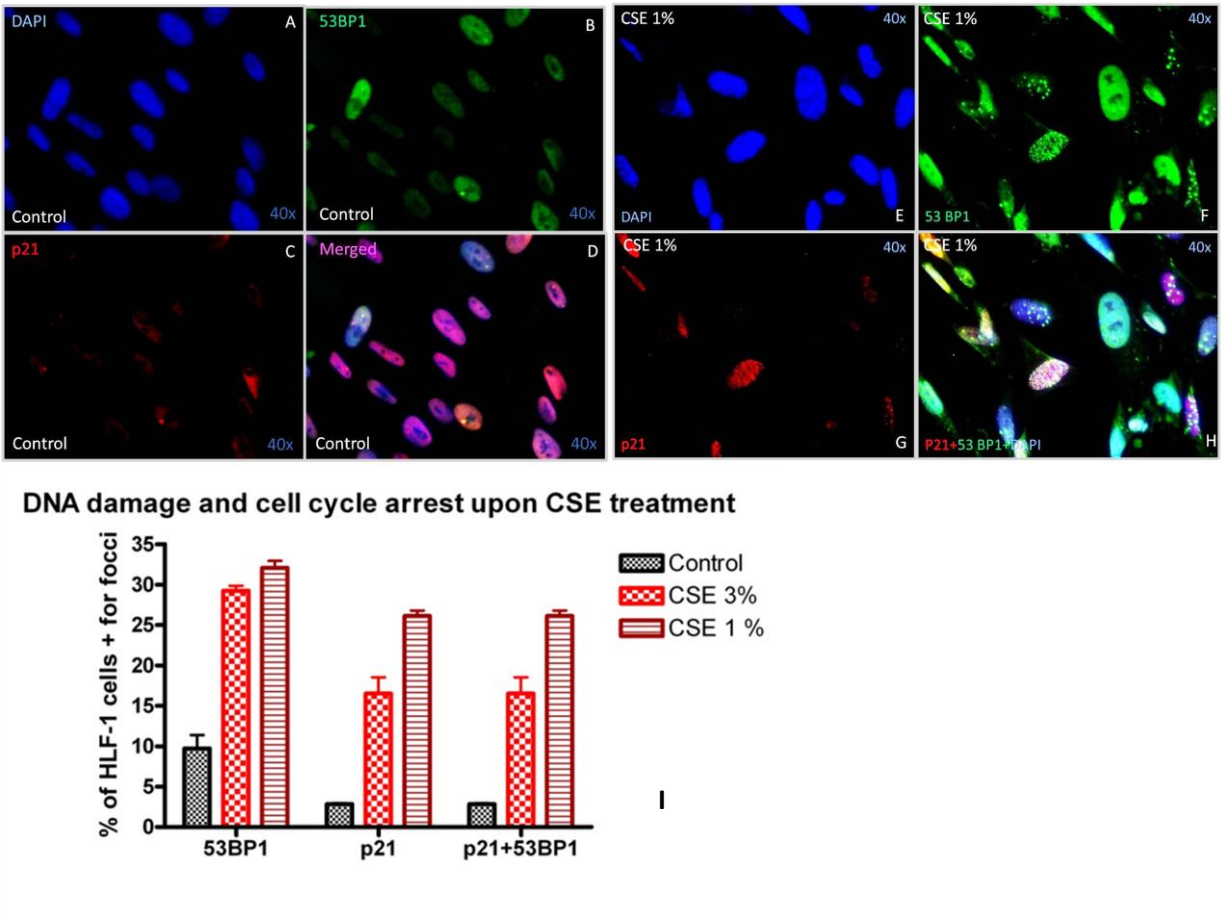


Figure 16. Human Lung Fibroblast-1 (HLF-1) cells treated with CSE display nuclei with 53 BP1 positive DNA damage foci indicative of DNA double strand breaks and higher expression of p21 indicative of cell cycle arrest.

A. *Control HLF-1 cells stained with DAPI (blue)* ***B.*** *Control cells stained with 53BP1 (green).* ***C.*** *Control HLF-1 cells stained with p21 (red)* ***D.*** *Represents a merged image of A, B and C.* ***E.*** *s HLF-1 cells treated with 1% CSE stained with DAPI (blue)* ***F.*** *HLF-1 cells treated with 1% CSE stained with 53BP1 (green)* ***G.*** *HLF-1 cells treated with 1% CSE stained with p21 (red)* ***H.*** *Merged image of E, F and G.* ***I.*** *Quantification of 53 BP1 and p21 positive cells in control and CSE treated cells.*

4.5 Treatment with increased doses of CSE lead to apoptosis

It was also noted that while a lower dose of CSE lead to the formation of 53 BP1 foci, the higher dose lead to activation of Caspase 3 (**Figure 17**).

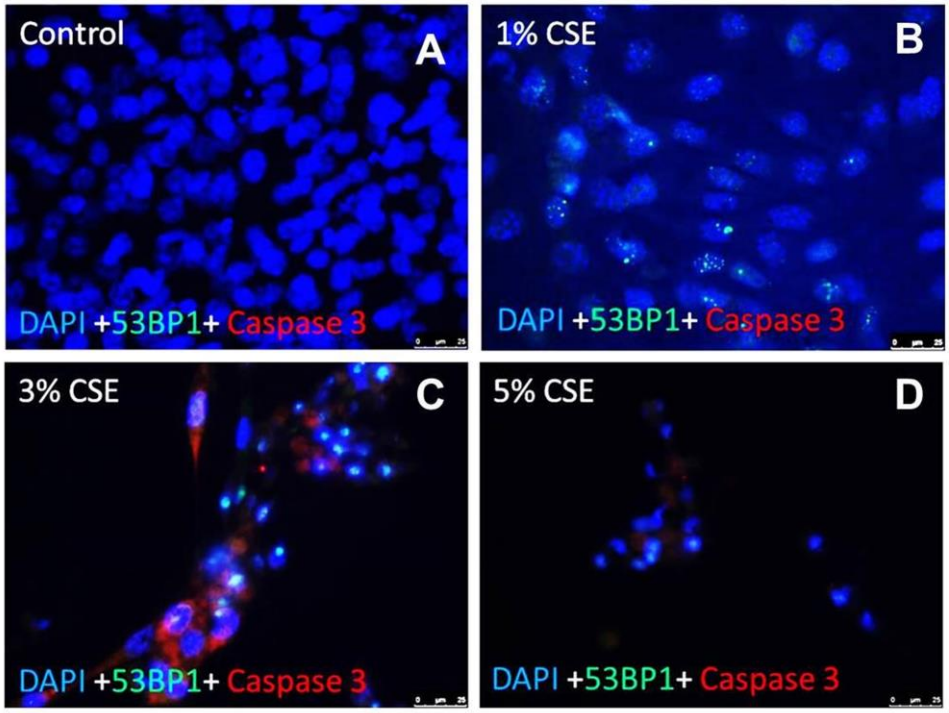


Figure 17 Chronic exposure to lower concentration of cigarette smoke extract lead to accumulation of the DNA damage while higher concentration of CSE lead to apoptosis in Human Lung Fibroblast -1 (HLF-1) cells.

*HLF-1 cells were cultured and treated with different dose of CSE.**A**. HLF-1 cells stained with DAPI as control **B.** HLF-1 cells with exposure to lower dose of CSE (1%) having DNA double strand break inside the nucleus with recruitment of 53 BP1 positive foci (green dots) **C.** HLF-1 cells exposed to higher dose of CSE (3%) and **D**. 5% lead to apoptosis as evident from activated Caspase 3 (CPP32) staining in red.*

4.6 Activation of γH2A.X and DNA damage foci in-vitro

Activation of a DNA damage response leads to the formation of DNA damage foci comprising of the activated H2A.X (gamma-H2A.X). Double-strand breaks (DSBs) are the most deleterious DNA lesions, which, if left unrepaired, may have severe consequences for cell survival. Persistent DNA strand breaks are the major trigger of cellular senescence. This is because DNA DSBs may lead to chromosome aberrations, genomic instability, or cell death.

DSB induction could occur due to various physical, chemical, and biological factors. Cells respond to DNA damage by activating the so-called DNA damage response (DDR), a complex molecular mechanism developed to detect and repair DNA damage. The formation of DSBs triggers activation of many factors, including phosphorylation of the histone variant H2AX. This leads to the formation of gamma H2AX foci within the nucleus of the cell. Phosphorylation of H2AX plays a key role in DDR and is required for the assembly of DNA repair proteins at the sites of DNA damage as well as for activation of checkpoints proteins which arrest the cell cycle progression. Formation of gamma H2AX foci can be used as a specific marker of DNA double strand breaks.

Hence we stained for gamma-H2A.X. Significant gamma H2AX foci formation were observed in the CSE treated cells (**Figure 18**).

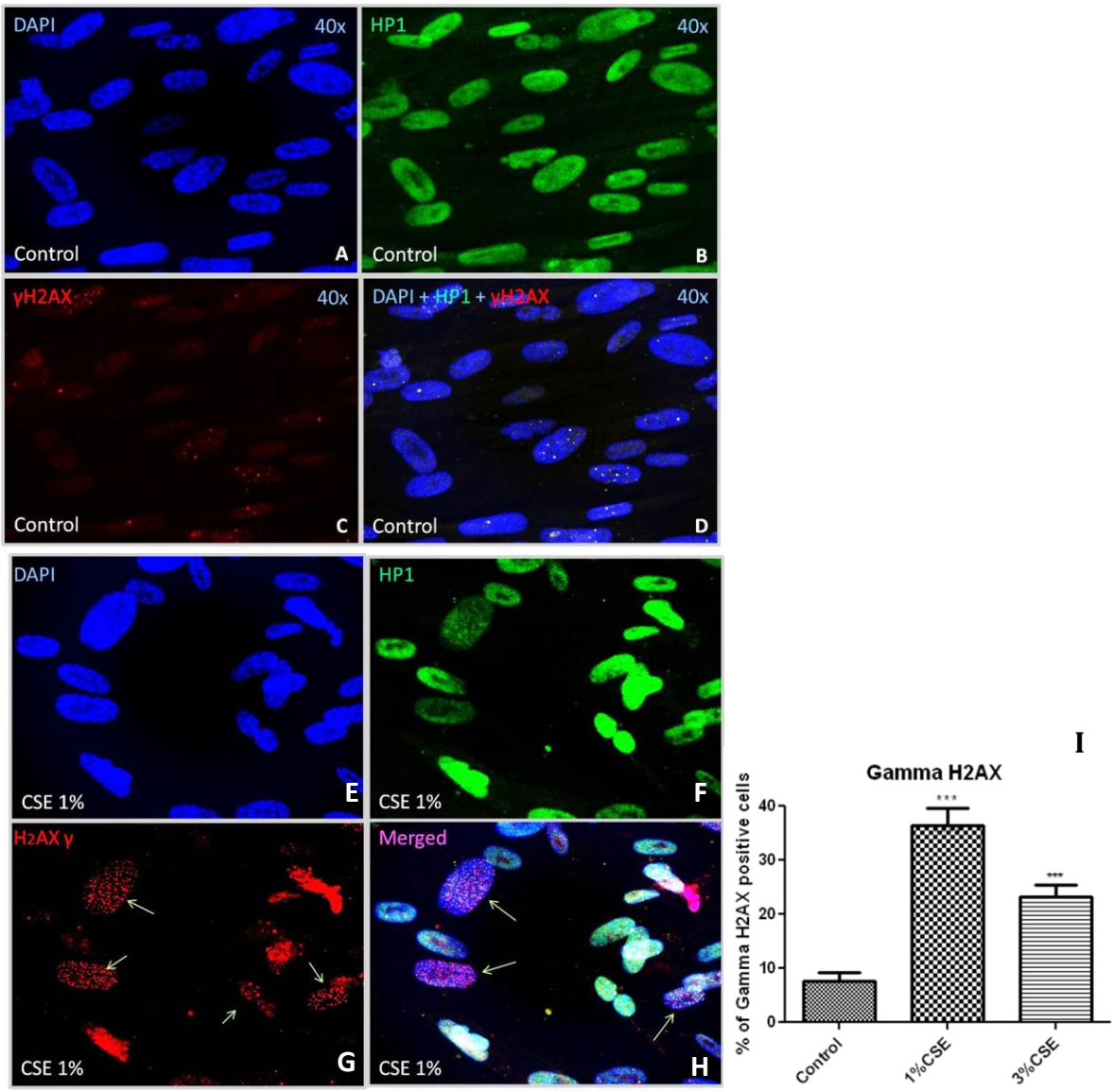


Figure 18. Human Lung Fibroblast-1 (HLF-1) cells treated with cigarette smoke extract shows formation of gamma H2AX positive DNA damage foci indicative of DNA double strand breaks and higher expression of HP1 indicative of heterochromatin formation.

HLF-1 cells were cultured and treated with different dose of CSE ***A.*** *Control HLF-1 cells stained with DAPI in blue* ***B.*** *Control cells stained with H_2AX in red.* ***C.*** *Control HLF-1 cells stained with HP1 in green* ***D.*** *Merged image of A, B and C.* ***E.*** *HLF-1 cells treated with 1% CSE stained with DAPI in blue* ***F.*** *HLF-1 cells treated with 1% CSE stained with H_2AX in red* ***G.*** *HLF-1 cells treated with 1% CSE stained with HP1 in green* ***H.*** *Merged image of E, F and G.* ***I.*** *Quantification of H_2AX and HP1 positive cells in control and CSE treated cells.*

4.7 Mouse model of pulmonary emphysema

There are no animal models available which exactly mimic chronic obstructive pulmonary disease. Mice, exposed to cigarette smoke exposure chronically over a long period of time, resemble a valid animal model to study emphysema pathophysiology [142]. However, the model does not exhibit much chronic bronchitis; hence calling it a model of COPD may not be precise. We utilized this animal model in which mice were exposed to cigarette smoke for different times (2, 6 or 8 months) and lung sections were examined for formation of emphysema (**Figure 19**).

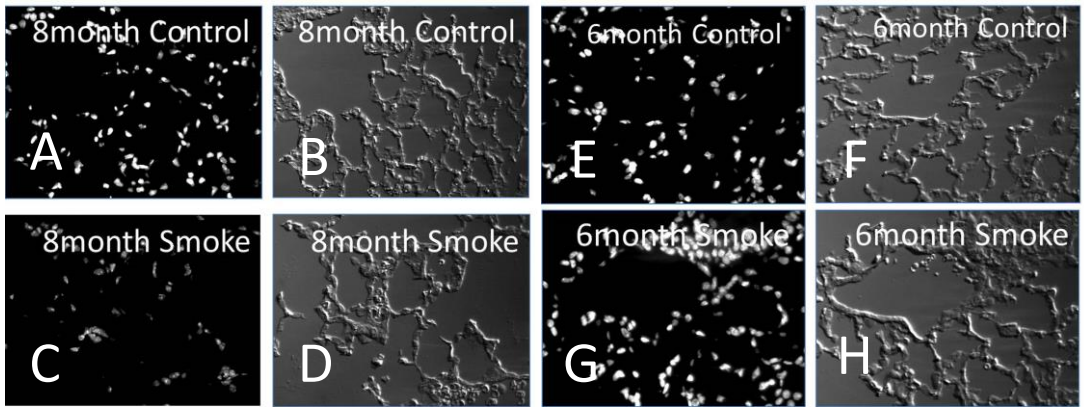


Figure 19. Mice were exposed to cigarette smoke upto 8 months and the lung paraffin sections (5µM) were viewed to characterize emphysema

A. *Lung sections from 8 month old control mice stained with DAPI* ***B.*** *Differential Interference Contrast (DIC) image of lung sections from 8 month old control mice.* ***C.*** *Lung sections from 8 month smoke exposed mice, stained with DAPI* ***D.*** *DIC image of Lung sections from 8 month smoke exposed mice* ***E.*** *Lung sections from 6 month old control mice stained with DAPI* ***F.*** *DIC image of lung sections from 6 month old control mice* ***G.*** *Lung sections from 6 month smoke exposed mice stained with DAPI* ***H.*** *DIC image of lung sections from 8 month smoke exposed mice.*

4.8 Gamma H2AX foci was found to be upregulated in cigarette smoke exposed mice model of emphysema

To correlate the in-vitro findings to that in the in-vivo model, lung sections from mice with chronic cigarette smoke exposure were also examined for gamma H2AX as shown in **Figure 20.** Gamma H2AX was significantly upregulated in lung sections from mice exposed to cigarette smoke.

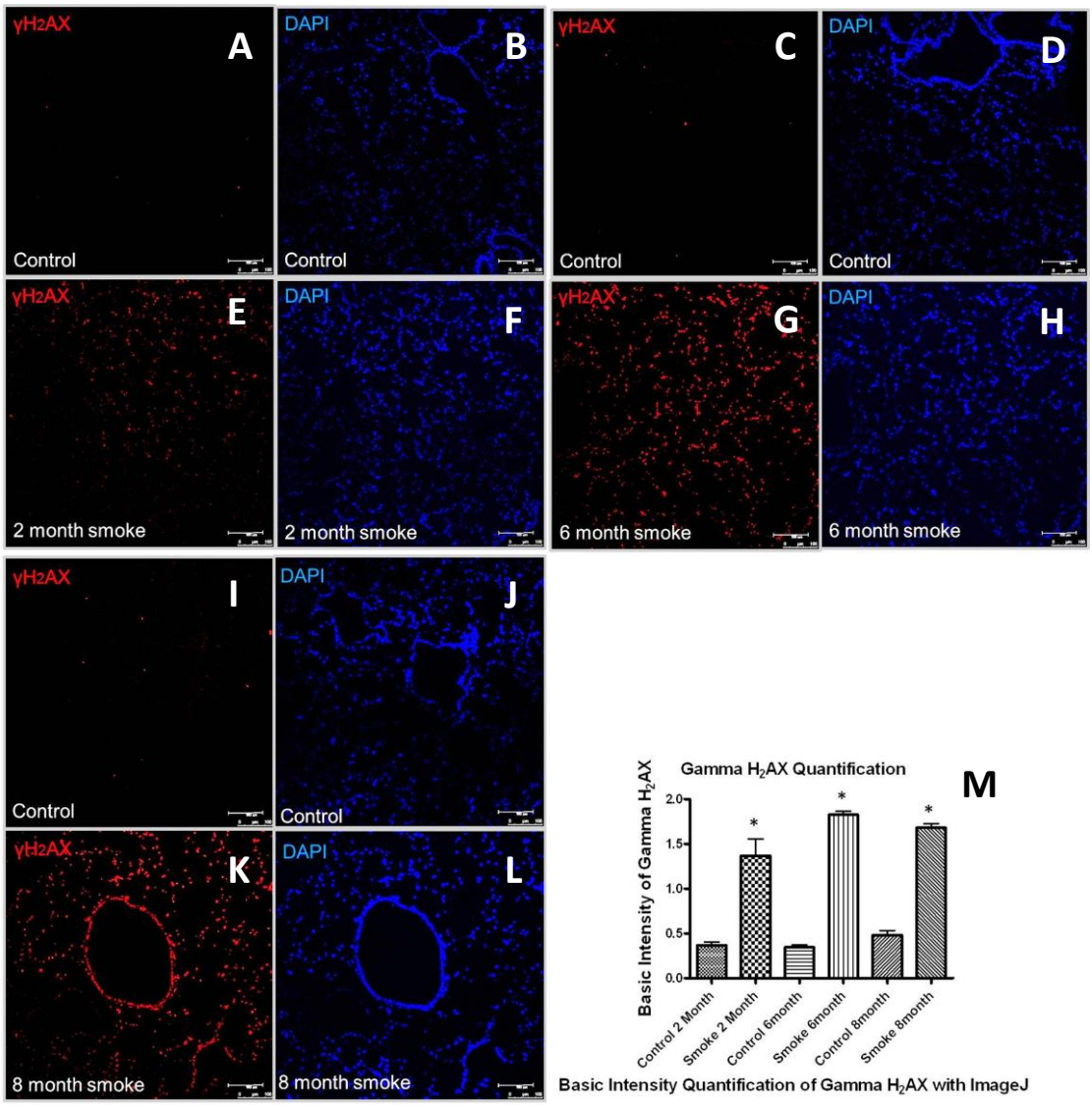


Figure 20. Increased γH$_2$AX foci indicating DNA damage-repair foci in mice lungs upon cigarette smoke exposure.

Mice were exposed to cigarette smoke for 2, 6 and 8 months respectively and the lung paraffin sections (5μM) were stained with γ H_2AX. The sections were examined by confocal microcopy for DNA damage. ***A.*** *Lung paraffin sections from 2 month control mouse, stained with γ H_2AX (red)* ***B.*** *Lung paraffin sections from 2 month control mouse, stained with DAPI (blue).* ***C.*** *Lung paraffin sections from mouse exposed to cigarette smoke for 2 month, stained with γ H_2AX (red).* ***D****. Lung paraffin sections from 2 month control mice, stained with DAPI (blue).* ***E.*** *Lung paraffin sections from 6 month control mice, stained with γ H_2AX.* ***F.*** *Lung paraffin sections from 6 month control mice, stained with DAPI (blue).* ***G.*** *Lung paraffin sections from mice exposed to CS for 6 month, stained with γ H_2AX (red).*

H. *Lung paraffin sections from mice exposed to cigarette smoke for 6 month smoked lung paraffin sections, stained with DAPI (blue).* ***I.*** *Lung paraffin sections from 6 month control stained with γ H_2AX (red).* ***J.*** *Lung paraffin sections from 6 month control stained with DAPI (blue).* ***K.*** *Lung paraffin sections from mice exposed to cigarette smoke for 8 month stained with γ H_2AX (red).* ***L.*** *Lung paraffin sections from mice exposed to cigarette smoke for 8 month stained with DAPI (blue).* ***M.*** *Basic fluorescence Intensity quantification of γ H_2AX with ImageJ (n = 4, mean ± SEM).*

4.9 53BP1 foci and p21 was found to be upregulated in cigarette smoke exposed mice model of emphysema

Antibodies for 53 BP1 and p21 did not work well enough on the lung paraffin sections. Hence lung cryo sections from the aged matched control and smoke exposed mice were prepared. The lungs sections revealed that 53BP1 foci were upregulated in the cigarette smoke exposed mice. Expression of p21 revealed that p21 was significantly upregulated upon exposure to cigarette smoke in our *in-vivo* model.

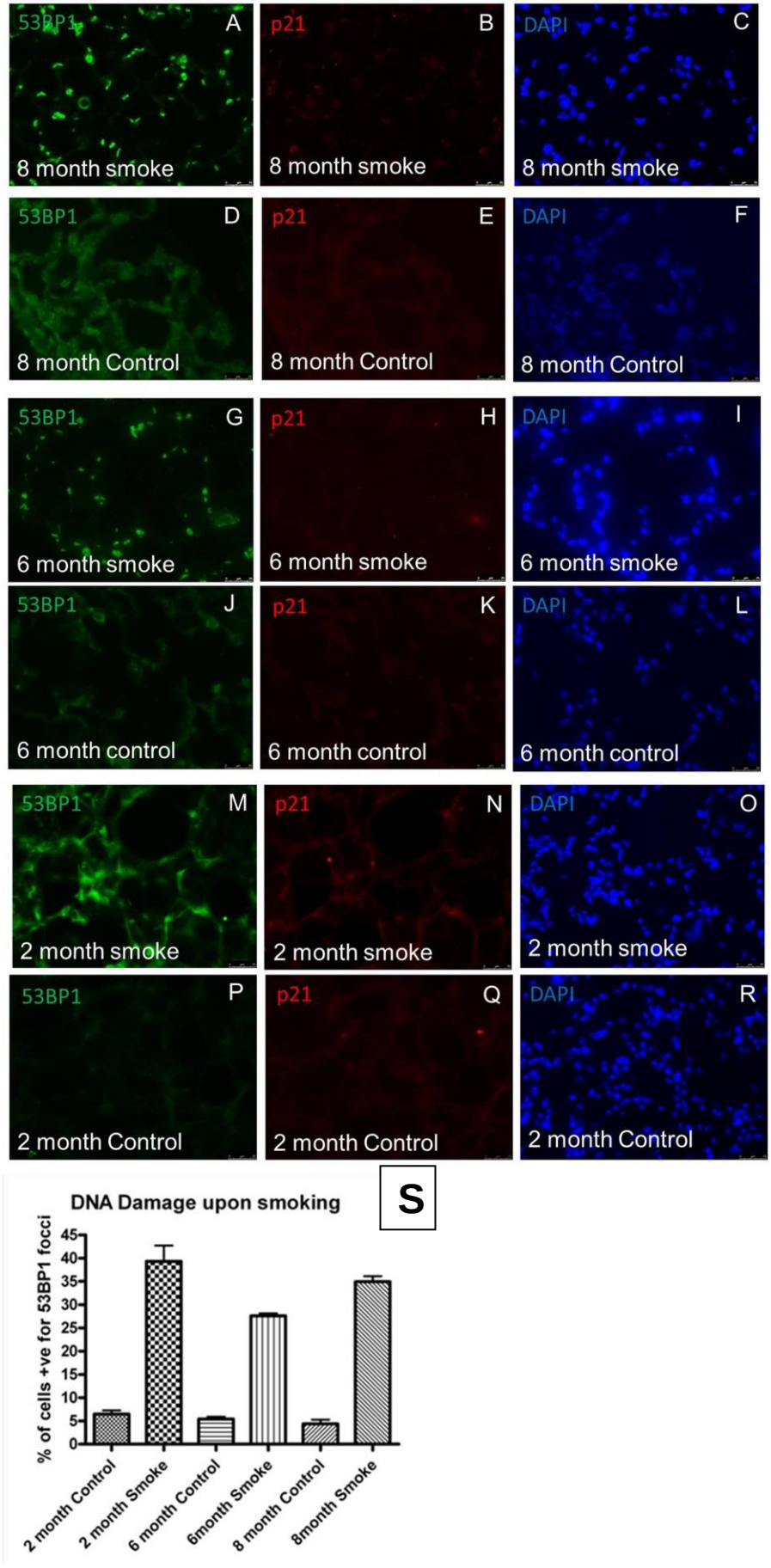


Figure 21. Increased 53BP1 indicating DNA double strand breaks in mice lungs upon cigarette smoke exposure.

Mice were exposed to cigarette smoke for 2, 6 and 8 months respectively and the lung cryo sections (5µM) were examined for DNA double strand breaks by 53BP1 and cell cycle arrest with p21.

A. *Lung cryo sections from mice exposed to cigarette smoke for 8 months stained with 53BP1 (green).* ***B .*** *Lung cryo sections from mice exposed to cigarette smoke for 8 months with p21 (red).* ***C.*** *Lung cryo sections from mice exposed to cigarette smoke for 8 months with DAPI* ***D.*** *Lung cryo sections from 8 month old mice stained with 53BP1.* ***E.*** *Lung cryo sections from 8 month old mice stained with p21.* ***F.*** *Lung cryo sections from 8 month old mice stained with DAPI.* ***G.*** *Lung cryo sections from mice exposed to cigarette smoke for 6 months stained with 53BP1.* ***H.*** *Lung cryo sections from mice exposed to cigarette smoke for 6 months stained with p21.* ***I.*** *Lung cryo sections from mice exposed to cigarette smoke for 6 month stained with DAPI.* ***J.*** *Lung cryo sections from 6 month old mice stained with 53BP1.* ***K.*** *Lung cryo sections from 6 month old mice stained with p21.* ***L.*** *Lung cryo sections from 6 month old mice stained with DAPI blue.* ***M.*** *Lung cryo sections from mice exposed to cigarette smoke for 2 months stained with 53BP1.* ***N.*** *Lung cryo sections from mice exposed to cigarette smoke for 2 months stained with p21.* ***O.*** *Lung cryo sections from mice exposed to cigarette smoke for 2 months stained with DAPI.* ***P.*** *Lung cryo sections from 2 month old mice stained with 53BP1 (green).* ***Q.*** *Lung cryo sections from 2 month old mice stained with p21 (red)* ***R.*** *Lung cryo sections from 2 month old mice stained with DAPI.* ***S.*** *Represents quantification of 53 BP1 foci (n =5, mean ± SEM).*

4.10 Increased inflammation in the lungs upon smoke exposure

Western blot analysis of the protein isolated from the lung homogenate of mice exposed to cigarette smoke and their aged matched controls that were not exposed to cigarette smoke revealed that cigarette smoke exposure induces inflammation as evident from increased NF-Kappa B expression (**Figure 22**).

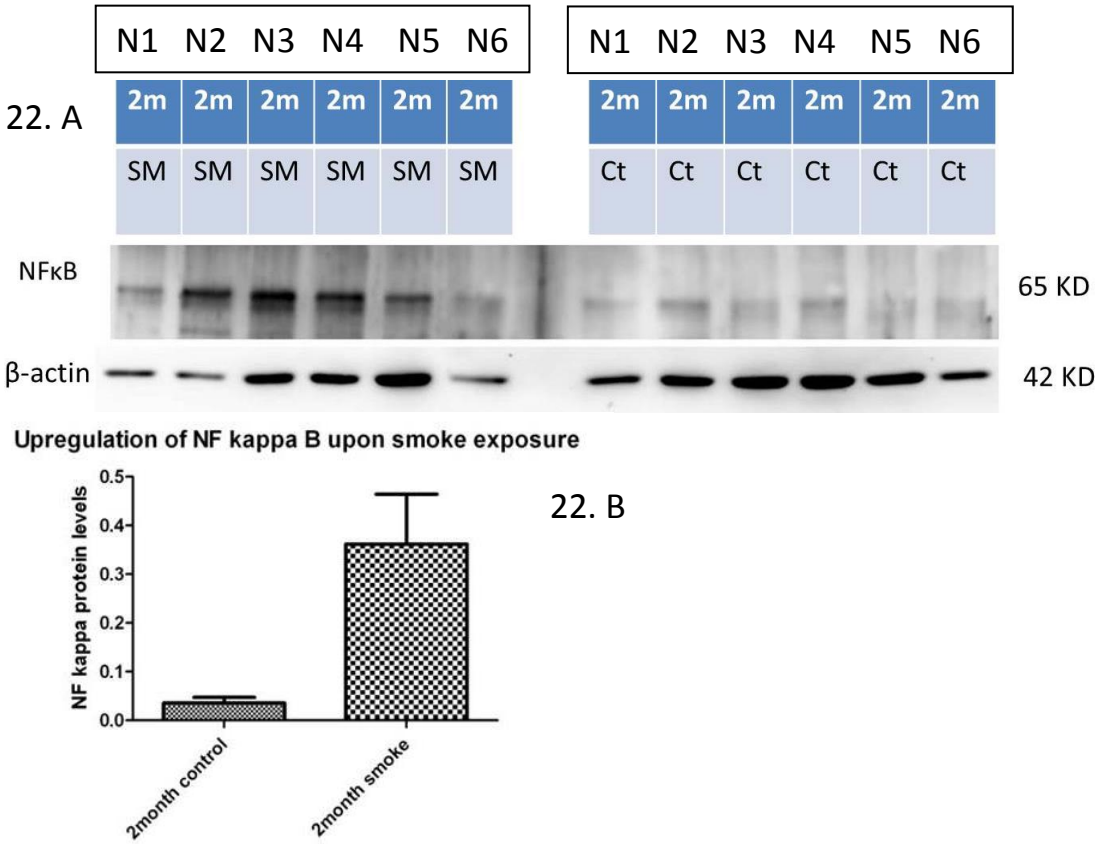


Figure 22. Increased NF kappa B expression in mouse lungs upon cigarette smoke exposure.

Mice (n=6 in each group) were exposed to cigarette smoke for 2 months and the lung homogenates were examined for the expression of NF kappa B through western blotting. ***22. A.*** *Western blots indicating the upregulation of NF kappa B in the lung homogenate of cigarette smoke exposed mice (SM) compared to the aged match control (Ct) mice* ***22.B.*** *Quantification of the western blots by densitometry analysis*

4.11 Increased cell cycle arrest (p21) in mouse lungs upon smoke exposure

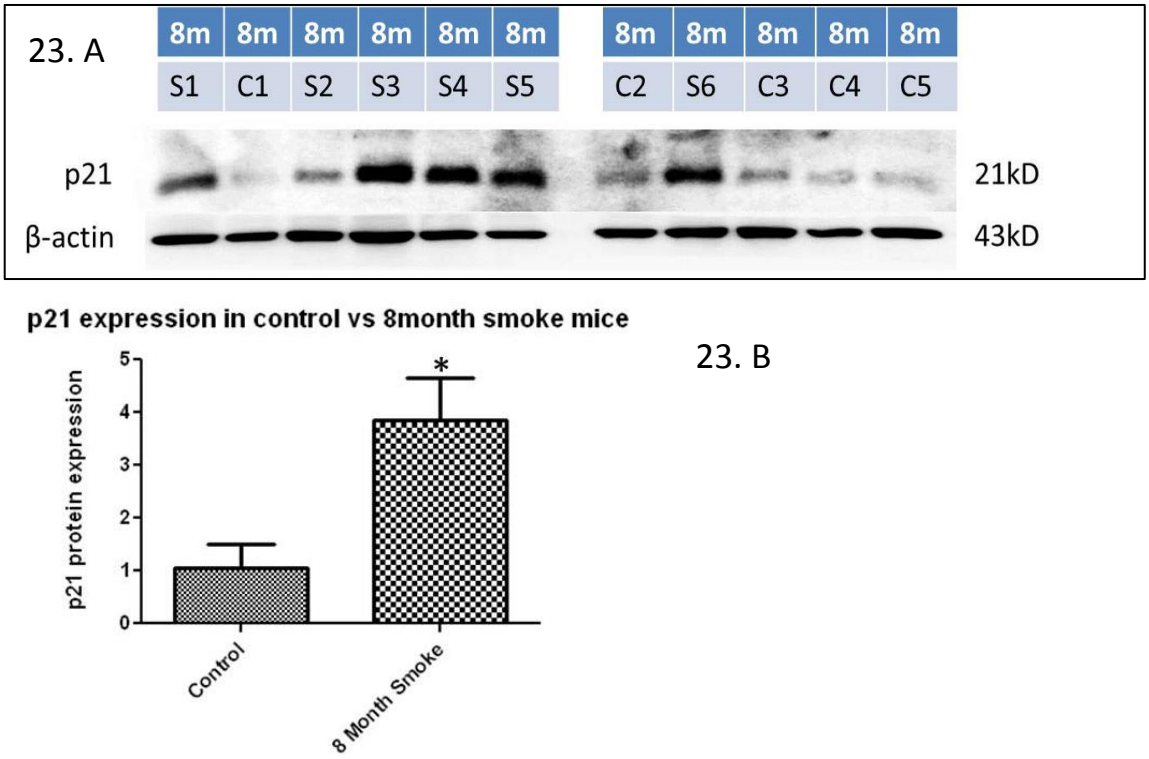


Figure 23. Increased p21 expression in mice lungs upon cigarette smoke exposure.

Mice (n=6 each group) were exposed to cigarette smoke for 8 months and the lung homogenate were examined for the expression of p21 through western blotting.

23. A. *Western blots indicating the upregulation of p21 in the lung homogenate of cigarette smoke exposed mice(SM) compared to their aged match control mice (C).*

23. B. *Quantification of the western blots by densitometry analysis*

4.12 Short hairpin RNA (ShRNA) for ATM blocks IL6 release in CSE induced senescent cells

RNA interference is a powerful technology that allows to suppress gene expression [162]. However, in most mammalian cells this provokes a strong cytotoxic response [163]. This non-specific side effect could be bypassed by using synthetic short (21- to 22-nucleotide) interfering RNAs, or short hairpin RNA, which can mediate strong and specific suppression of gene expression.

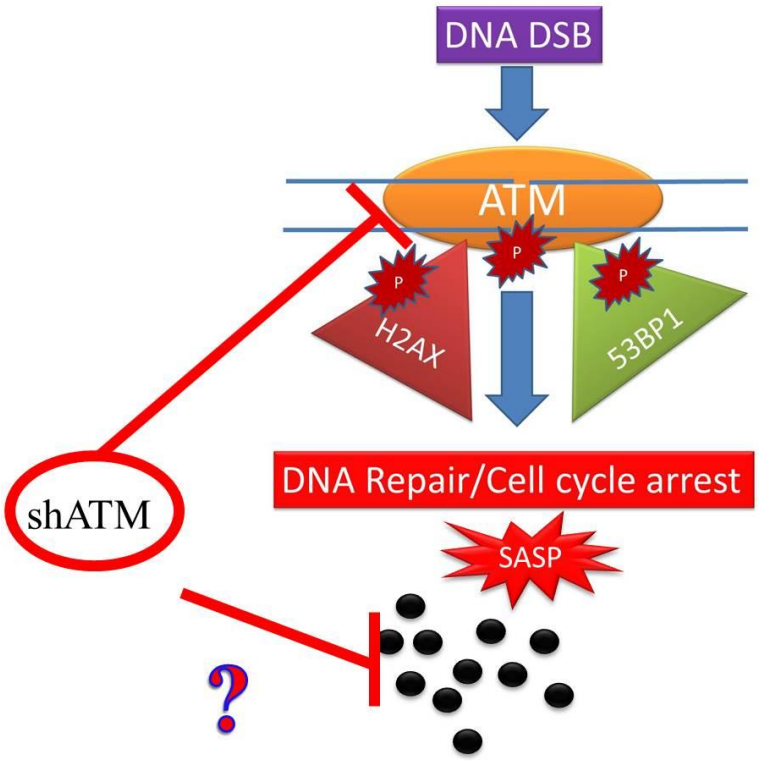


Figure 24. *Strategy to block ATM with shRNA to block SASP response*

The figure explains a strategy to knock down ATM to observe any changes in SASP response. ATM is central to DNA damage response. If the SASP response in senescence indeed is due to the DDR response, knocking down the central player of the DDR response would inhibit SASP response.

Since the first application of RNA interference (RNAi) in mammalian cells, the expression of short hairpin RNAs (shRNAs) for targeted gene silencing has become a benchmark technology. We employed it to suppress the central key player of the DDR response pathway, ATM with the aim to investigate if inflammation induced by cigarette smoke *in-vitro* is indeed caused by the DDR signalling and not senescence per se.

Short hairpin RNA or shRNA against ATM were used to knock down ATM in HEK 293 cells and IL-6 release was checked upon subsequently. IL-6 release was diminished upon CSE treatment by blocking ATM (**Figure. 25**).

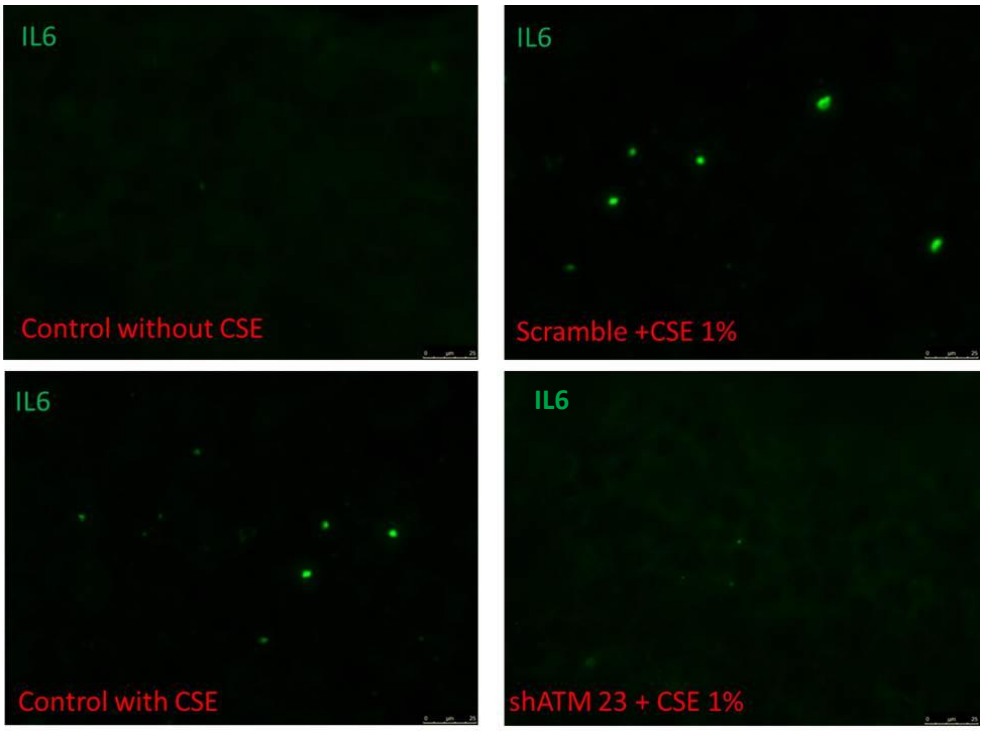


Figure 25. shATM suppresses IL6 release in CSE induced senescent cells in-vitro

*HEK 293 cells were transfected with shRNA for ATM and scrambled control. These cells were treated with 1 % CSE and later stained for IL-6. While the control cells show no IL6 release **(A)**, the CSE treated HEK293 cells and CSE treated HEK 293 cells transfected with the scramble showed IL-6 release **(B &C)**. The shRNA for ATM suppressed the release of IL-6 upon CSE treatment.*

5 Discussion

Researchers in the past have described several theories for the pathogenesis of COPD [1, 164-167]. Laurell and Eriksson described the association between α-1 protease inhibitor (PI) deficiency and pulmonary emphysema [168] Further experimental studies conducted by Gross and others demonstrated that infusion of elastolytic enzymes into the lungs induced emphysema[169-171]. In contrast, a proteolytic enzyme that does not degrade elastin could not induce emphysema. These observations led to the protease-antiprotease theory of emphysema. The initial attention largely focused on α-1 protease inhibitor and neutrophil elastase. Later, other enzymes and inhibitors were believed to play an important role. Numerous serine proteases in addition to leukocyte elastase, including chymotrypsin and proteinase 3, have elastolytic activity and may contribute to the development of emphysema [172]. Similarly it was observed that metalloproteases and cysteine proteases may also have elastolytic activity as they were found in the lung of COPD patients and believed to contribute in the development of emphysema[173]. Further, inhibitors of these enzymes are also present in the normal lung, thus expanding the concept of protease-antiprotease balance.

Variations in antioxidants may also predispose one to COPD. There is considerable evidence for increased oxidative stress in COPD [174-176]. Oxidative stress is derived from cigarette smoke and from inflammation caused by the activation of macrophages and neutrophils. Epidemiological evidence suggests that reduced dietary intake of antioxidants may also be a factor causing COPD. The population surveys have linked a low dietary intake of the antioxidant ascorbic acid with declining lung function [177, 178].

The increased oxidative stress in the airways of COPD patients may play an important pathophysiological role in the disease by amplifying the inflammatory response in COPD. This may reflect the activation of NF-κB and activator protein-1, which then induce a neutrophilic inflammation via increased expression of IL-8 and CXC chemokines, TNF-α and MMP-9. Oxidative stress may therefore serve to amplify the ongoing chronic

inflammatory response in COPD and may be an important mechanism leading to increased inflammation during acute exacerbations.

5.1 COPD a disease of elderly

Inspite of the numerous scientific findings supportive of the various hypotheses for pathogenesis of COPD, there is still a fundamental lack of knowledge about the molecular mechanisms underlying the causes of COPD.

With the exception of those who have homozygous alpha-1-antitrypsin deficiency, in the vast majority of COPD patients there is no in-vivo evidence of significant protease–antiprotease imbalance at the cellular or organ level, indicating that there must be some other processes additionally in play. It is important to note that the prevalence of COPD increases exponentially with aging. Before 40 years of age COPD is extremely rare, but by 70 years of age the prevalence is as high as 30%[179, 180], even among ex-smokers or lifetime nonsmokers, indicating a strong link between aging and COPD. Patients diagnosed with chronic obstructive pulmonary disease have limited therapeutic options.

The existing therapies are inadequate because no causative treatments exists that could hold disease progression. Airflow limitation, measured by reduced FEV_1, progresses very slowly over several decades. So most patients with symptomatic COPD are in middle age or are elderly. Thus, the prevalence of COPD is at least partly also an age dependent phenomenon. The prevalence of the disease in elderly suggests an intimate relationship between the pathogenesis of COPD and aging.

5.2 Aging hypothesis of COPD

Aging is characterized as a condition that basically satisfy four principles: it is intrinsic (gene dependent), universal, progressive, and usually detrimental to the host[181]. Aging lung results in both structural and functional lung

changes that comply with these four principles in healthy subjects [182]. Cellular senescence is a vital biological state that implements a halt on the replicative capacity of any impaired or damaged cell via a network of programmed processes. As a result, senescence has evolved as a potent barrier to tumorigenesis and contributes to other physiological processes such as aging and wound healing.

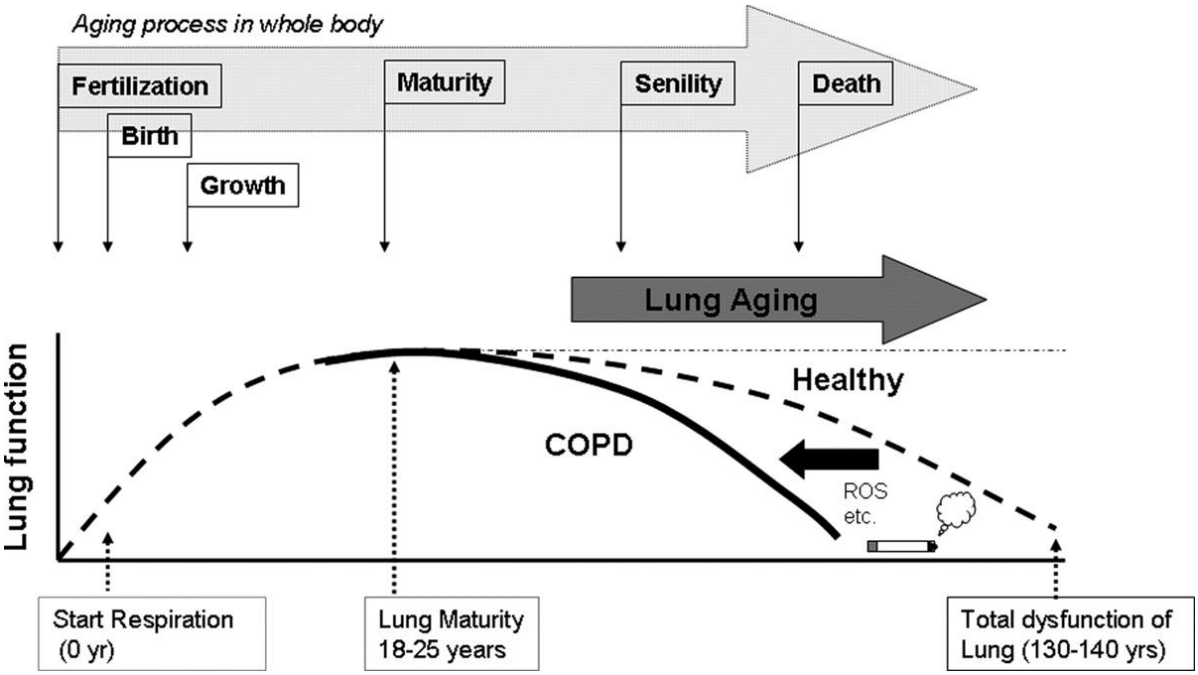


Figure 26. Hypothesis of development of COPD by an accelerating lung aging [183].

Aging is defined as the progressive decline of homeostasis, and this is the result of failure of organ maintenance from DNA damage, oxidative stress, and telomere shortening. During aging, pulmonary function deteriorates progressively and pulmonary inflammation is increased with structural changes in the lung parenchyma and small airways. Environmental exposure, for example cigarette smoke, may accelerate aging-dependent defective lung function [183].

Several clinical observations support the hypothesis, that accelerated aging may play a role in the pathogenesis of COPD. First, lung function starts to decline in healthy individuals over 30 years of age. This is associated with several structural changes, such as progressive distal air space enlargement, with loss in the gas exchange surface area and the supporting alveolar attachments in the peripheral airways[182]. It is postulated that smoking induces elastin fiber degradation and matrix remodeling in the lungs similar to that which occurs with aging in the skin, resulting in skin wrinkling[184-186]. It has also been shown in the past, that an in-vitro exposure of human epithelial cells to cigarette smoke extract results in an increased expression of senescence-associated [beta]-galactosidase (SA-[beta]-gal), a marker of cellular senescence[77]. Further, it has been shown that senescence marker protein-30 (SMP30) is a multifunctional protein which provides protection against deleterious changes related to aging. Transgenic mice with deficiency in SMP30 (i.e. SMP30 knockout mice) contain lungs that demonstrate significantly larger mean linear intercept than wild-type mice when exposed to cigarette smoke, suggesting more emphysematous changes in the lungs[187] Mice with deficiencies in the klotho gene have short life span and age related disorders. Klotho gene knockout mice develop emphysematous changes in the lungs following normal development [188].

Several striking similarities in the profile of aging lung to that of COPD lung suggest that accelerated aging could be one of the root causes of COPD. It has been believed, that premature cellular senescence contributes to accelerate organismal aging. Furthermore, reviewing the similarities between the secretory profiles of DNA damage induced senescent cells and that of COPD, a premature cellular senescence hypothesis may hold value for the pathogenesis of COPD.

Evidence of premature cellular senescence in COPD patients had been reported by a few groups [189, 190], however a systematic study of an animal model of emphysema in a time dependent manner for assessing the senescence hypothesis had been missing. Moreover, it remained elusive whether the pulmonary senescence that occurs in COPD could be linked to

DNA damage, possibly caused by the oxidative stress due to the cigarette smoke.

5.3 COPD involves more than just lungs

Many patients with chronic obstructive pulmonary disease (COPD) suffer from exercise intolerance. In about 40% of the patients exercise capacity is limited by alterations in skeletal muscle rather than pulmonary problems [191]. Increasing evidence suggests that COPD is a complex systemic disease involving more than just the airways and lungs [192]. COPD patients have high rates of comorbidities. These include cardiovascular disease and metabolic disorders which have been linked to the systemic component of COPD inflammation [190]. COPD does not just affect the lung resident cells but systemic cells as well. Data from the past shows evidence for increased systemic markers of oxidative stress in patients with COPD as measured by biochemical markers of lipid peroxidation [192].

While the *in-vitro* investigation of resident fibroblasts with cigarette smoke extract resulted in an increased state of senescence, circulating fibrocytes seemed unaffected by the cigarette smoke even at much higher concentrations. They rather showed enhanced proliferation, which was surprising. However, this is in correlation with the later findings of Wang et al, where they show increased activation of fibrocytes in patients with chronic obstructive asthma through an epidermal growth factor receptor-dependent pathway [193]**.** As this work aimed to focus on cellular senescence, circulating fibrocytes were not further pursued.

5.4 An *in-vitro* model of cellular senescence

In order to investigate the relationship between cigarette smoke, DNA damage and senescence, an in-vitro model of cellular senescence was established and validated. To this end, senescent cells were established by subjecting the cells to hydrogen peroxide. Using common molecular markers of senescence like its morphology, SA ß-gal assay and SAHF formation[29, 69, 106, 119, 194, 195] it was further confirmed that the cigarette smoke induced cells in our experimental model generated senescent cells.

5.5 β-D Galactosidase activity as a marker of senescence

The most widely used biomarker for senescent and aging cells is senescence-associated beta-galactosidase (SA-beta-gal), which is defined as beta-galactosidase activity detectable at pH 6.0 in senescent cells[31]. SA-β-gal activity is expressed from GLB1, the gene encoding lysosomal β-D-galactosidase, the activity of which is typically measured at acidic pH 4.5. Lysosomal β-galactosidase displays maximal activity between pH 4.0 and 4.5 but markedly lower activity at pH 6.0[196]. In fact, β-galactosidase activity is not detectable in proliferating cells by in situ staining with X-gal at pH 6.0, the conditions used to detect SA-β-gal activity, even though lysosomal β-galactosidase activity is readily detectible in these cells at acidic pH. Nevertheless, based on indirect physiological experiments, it has been proposed that increased lysosomal-β-galactosidase activity in senescent cells accounts for SA-β-gal activity [33]. Specifically, the levels of total cellular β-galactosidase activity is higher in late-passage compared to early-passage cells at pH 4.5 as well as at pH 6.0, and maximal β-galactosidase activity is measurable at low pH in both early- and late-passage cells[33, 197-200]. Furthermore, the number and size of lysosomes increase in cells at late passage[201, 202]. These results suggest that lysosomal β-galactosidase activity increases in senescent cells due to increased lysosome content, surpassing a threshold level, so that it is detectable at the suboptimal pH 6.0 [33, 199].

Cells treated with cigarette smoke extract or hydrogen peroxide both contained higher lysosomal content as evident from lysosomal β-galactosidase activity that were detected by the formation of a perinuclear blue precipitate. Bright field microscopy of stained cells also revealed significant differences. While the control cells displayed normal spindly fibroblasts morphology, the senescent cells possessed enlarged, flattened shape with prominent vacuoles.

5.6 Senescent Associated Heterochromatin Foci (SAHF)

In addition to a variety of well characterized morphological and biochemical changes, senescent cells displays profound changes in its chromatin structure. It has been established that senescent cells exhibit excessive heterochromatization [203, 204]. The changed chromatin architecture during cellular senescence plays a very important role in the senescence program. This change in chromatin architecture in senescent cells could be visible at the global level when senescent cells are stained with 4′-6-Diamidino-2-phenylindole (DAPI). Proliferating cells exhibit a diffuse distribution of DNA throughout the cell nucleus. However in DAPI-stained senescent cells, punctate DNA foci become visible (**Figure 14**). These foci have been described as heterochromatic (so-called senescent associated heterochromatin foci (SAHF) [205]. Each SAHF focus in a senescent cell is thought to represent an individual chromosome [206, 207]. It has also been reported that SAHF do not contain pericentromeres and telomeres, pointing to massive heterochromatization of euchromatin in senescent cell [203, 206-208]. Formation of SAHF has been reported by some to be wholly or partly dependent on major effectors of senescence, such as pRB and p53 [203, 208]. There is large-scale reorganization and heterochromatization during formation of SAHF in senescent cells. It has been proposed specifically, that the formation of facultative heterochromatin plays an important role in mediating several aspects of senescence, including the repression of proliferation genes and limiting the DNA damage response [207, 208]. Therefore, the heterochromatic modifications induced by cigarette smoke extract were investigated in this study. Formation of SAHF was clearly

noticeable upon DAPI staining in the CSE treated cells. This suggested that the cigarette smoke extract also led to a change in the chromatin landscape of the cell.

5.7 Cigarette smoke induce DNA double strand breaks

Subsequently, DNA damage profiles for the respective senescence model were investigated. It was found that the senescence induced in our model involved DNA double strand breaks. 53 BP1 is a DNA repair protein that is known to be recruited at the site of DNA double strand breaks at punctate nuclear foci [66, 209]. Our results clearly showed such formation of DNA double strand break foci upon cigarette smoke exposure. We also investigated for p21 and found that it was expressed more in those cells that had more DNA double strand break (DSB) foci [210]. While we noted DNA DSB foci in more number of cells, p21 expression was limited to those cells which had more punctuate foci of 53 BP1. In other words, not all the cells that had 53 BP1 foci, showed increased levels of p21 but all the cells that had increased p21 were 53BP1 positive. These results pointed out, that while a low level of DNA damage could be self-repaired [211] and the cell does not express cell cycle inhibitors, higher expression of p21 for the required cell cycle halt occurs only above a threshold level of DNA damage, which was induced through the cigarette smoke. Investigating γH2AX levels and the presence of heterochromatin protein 1 in the cells upon CSE treatment showed, that the γH2AX foci were co-localized with heterochromatin protein 1 [212], thereby demonstrating the validity of γH2AX as a biomarker.

5.8 Importance of the *in-vitro* study

An *in-vitro* model to study the various effects induced by cigarette smoke provides an easy, efficient and reproducible method of assessing biological changes, which may occur inside the cells. These results could then be extrapolated to animal models of cigarette smoke induced disease to

investigate any similarities and address number of questions. Detection of premature senescence by β-gal or immunofluorescence based (C12FDZ) assay could be used for measuring the number of senescent cells. Since premature cellular senescence in general could be mediated by several factors, it was interesting to understand if persistent DNA damage, that could lead to SASP response and hence inflammation, indeed accumulated upon chronic exposure to cigarette smoke over a long period of time in mice model of emphysema. This could let us understand, if DNA damage indeed may be linked to premature cellular senescence of alveolar cells in case of COPD. Detection of DNA damage and its quantification could be in addition also useful as a prognostic marker of COPD progression.

To sum up the in-vitro data, significantly increased numbers of senescent cells upon cigarette smoke extract treatment of human lung fibroblasts were found. Cellular senescence could be successfully assessed by staining for β-galactosidase and evaluation of senescence associated heterochromatin foci (SAHF), reflecting condensed chromatin, in the nuclei. DNA double strand breaks could be demonstrated and quantified by up regulated γH2AX, 53-BP1and it's quantification. Cell cycle arrest could be demonstrated by upregulation of p21 at protein level. *In-vitro*, senescence of lung fibroblasts could be induced by 100uM H_2O_2 as well as by 1% cigarette smoke extract. PBMC derived circulating fibrocytes were rather stimulated in growth by H_2O_2 than driven towards senescence. The presence of γH2AX foci in fibroblast cells was associated with, apoptosis and pro-inflammatory phenotypic changes. The results of this study also showed that the γH2AX foci were co-localized with DNA damage thereby demonstrating the validity of γH2AX number as a biomarker of DNA damage.

5.9 Studies in the cigarette smoke induced mouse emphysema model

As chronic tobacco smoke exposure is regarded as the major cause of the disease, chronic cigarette smoke exposure of mice [142][213] has been

investigated for a correlation of induction of emphysema and appearance of DNA damage over time. Investigation of DNA damage and cell cycle inhibition was done in mice exposed for 2, 6 or 8 month to cigarette smoke.

Remarkably, the in-vivo results were in close correlation to the in-vitro results. The in-vivo model displayed accumulating DNA damage over time shown by γH2AX and 53BP1 levels in the lung sections. Immunofluorescence-based assays of γH2AX provide a sensitive, efficient and reproducible method of measuring the number of DSBs. Since persistence of γH2AX foci after the initial induction of DNA DSBs indicates, that some of the damage remains unrepaired, the γH2AX foci is widely used as a biomarker of DNA damage in various tissues[214-217]. In the present study, significantly increased numbers of γH2AX foci were detected in the alveolar cells. The presence of γH2AX foci in these cells was associated with cellular senescence. Western blotting is commonly accepted as the gold- standard method for the accurate determination of protein levels. We verified the p21 levels with western blotting and found that p21 indeed was upregulated upon cigarette smoke exposure in the lungs.

5.10 Knocking down of ATM diminished IL6 release upon cigarette smoke exposure

The DNA damage response facilitates recognition of DNA damage sites and initiates the desired cellular programs to maintain the genomic integrity [218]. The cellular programs initiated by DDR ranges from cell cycle checkpoints regulation, transcription, translation, DNA repair, metabolism to cell fate decisions like cellular senescence or apoptosis. Ataxia telangiectasia mutated ATM (Kinase), a master controller of the signal transduction, is central to the DNA damage response [219, 220]. Evidence suggests, that the ATM also is the key regulator of oxidative stress induced vascular endothelial cell senescence [221].

Though only a subset of SASP factors is driven by DDR signalling [67] it becomes very important to understand the role of DDR signalling in SASP

response as the subset of SASP includes IL-6 and IL-8. The cytokine IL-6 becomes particularly interesting for the ability of senescent cells to promote cancel cell invasion [62].

Our results suggest that cigarette smoke exposure induces IL-6 release representing inflammation during senescence. This release of IL-6 could be suppressed by knocking down ATM.

5.11 Conclusions

The results of the present study suggest that DNA damage, in particular DNA-doublestrand breaks DSBs caused by cigarette smoke, contributes to the molecular pathogenesis of emphysema by inducing cellular senescence, apoptosis and pro-inflammatory responses.

The findings may imply, that the DNA damage in the lungs of cigarette smoke exposed mice are amplified and/or remain unrepaired, which results in gradual accumulation of DNA damage in their lungs. The current theory of the pathogenesis of COPD suggests that alveolar destruction is caused by interactions between several pathobiological processes, including inflammation, apoptosis, cell senescence and oxidative stress. By carefully analyzing correlations at both the tissue and cellular levels, we found that DNA damage is correlated with apoptosis, cellular senescence and pro-inflammatory phenotypic changes, thereby underscoring DNA double strand breaks as a molecular link between the pathobiological processes thought to be involved in the alveolar destruction in COPD.

As a limitation of the study it may be that some of the DNA damage observed in may also have been the result of apoptosis, cell senescence and inflammation rather than their cause. However, it is well established that DNA

damage, in particular DSBs, is a strong inducer of apoptosis, cell senescence and pro-inflammatory responses in various types of cells and tissues. Furthermore, It has been suggested that activation of an ATM/γH2AX-mediated signaling pathway in response to DSBs, which are of irreparable nature, may lead to cellular senescence or apoptosis, thereby eliminating the DNA-damaged cells from the tissue and preventing their oncogenic transformation [214]. However, the detrimental factor that decides the fate to apoptosis or senescence isn't known. In the present study, we showed that DSBs caused by cigarette smoke exposure in mice go along with pro-inflammatory responses, such as NF kappa B activation, suggesting that DSBs may be linked to pro-inflammatory responses. These lines of evidence, although not wholly conclusive, support the hypothesis that DNA damage plays a causative role in the apoptosis, cell senescence and pro-inflammatory responses observed in the lungs of COPD patients. However, future studies on animal models of COPD will be needed to show whether this view is correct.

Based on the results of the present study, we hypothesize that the cell senescence and inflammation, which are thought to represent the pathobiological processes of COPD is at least partly linked to DNA damage, particularly double strand breaks. A model is proposed which starts with cigarette smoke inducing DNA double strand breaks and activation of DNA damage response. The DNA damage response acts to repair the damage or drive the cell to arrest depending upon the extent of DNA damage. Chronic or persistent DNA damage lead the cell to senescence or apoptosis where as a transient DNA damage is repaired by the cell [67]. The DNA damage also triggers an inflammatory response. Cellular senescence on the other hand also secretes myriads of cytokines further reinforcing inflammation. Hence there is an establishment of a vicious loop where senescescent cells reinforce inflammation through autocrine and paracrine pathways. Traditional theory of the pathogenesis of COPD suggests that activation of inflammation by inhaled cigarette smoke and other pollutants plays a central role in airway wall thickening, alveolar destruction, airspace enlargement and vascular remodeling[222]. Our hypothesis that DNA damage underlies the molecular

mechanism of COPD seems to suggest answers to several important questions that the traditional theory does not address. The first question is, why does COPD take decades to develop? The answer based on our DNA damage hypothesis would be that the DNA damage caused by long-term smoking needs to accumulate over several decades before COPD develops, by analogy to the development of lung cancer. The second question is, why does inflammation persist after ceasing to smoke? The answer is that it probably persists because smoking-induced DNA damage persists long after smoking cessation, as is reported previously [3, 4]. The third question is why do corticosteroids have little impact on the inflammation in COPD? The answer may be that corticosteroids do not restore the DNA damage. Finally, why is it that some smokers develop COPD while others do not and, why are COPD smokers more prone to develop lung cancer than non-COPD smokers? The answer to the last two questions would be that the greater susceptibility to DNA damage due to smoking may be genetically determined just as greater susceptibility to smoking-induced lung cancer, so that smokers who are more susceptible to DNA damage may be predisposed to both COPD and lung cancer. However, the results of the current study do not answer all these question; longitudinal studies will be needed that include a larger number of COPD patients with different stages of disease severity.

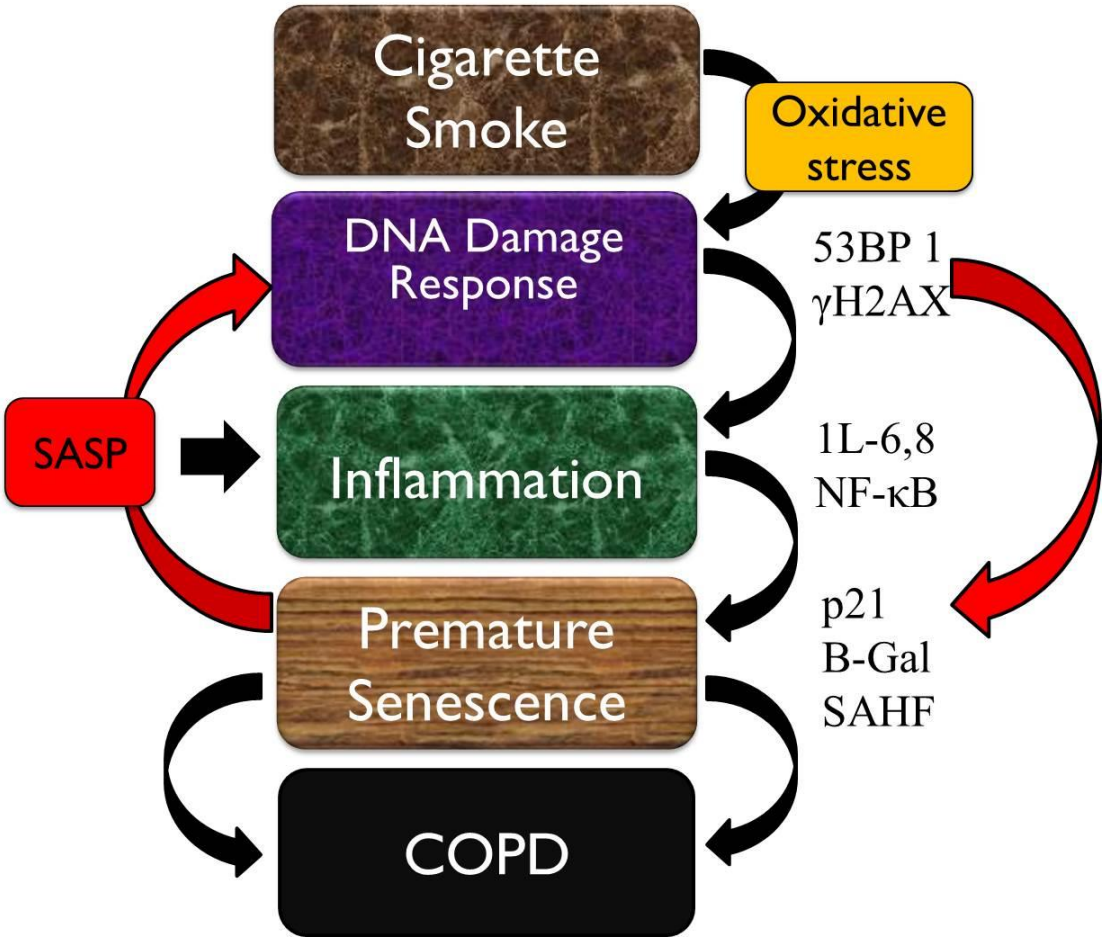


Figure 27 A hypothesis suggesting cigarette smoke induced COPD involving DNA damage mediated premature cellular senescence.

Cigarette smoke causes DNA damage (in particular DNA double strand breaks). If the damage is persistent, DNA damage induces premature senescence and a pro-inflammatory response of the lung resident cells. The senescencent cells further resleases SASP which reinforces inflammation all of which contribute to the development of COPD.

In conclusion, the results of the present study strongly suggest that DNA damage at least partly underlies the molecular pathogenesis of COPD. The DNA damage hypothesis may help to better understand the pathogenetic mechanism of COPD and to target new drugs, such as drugs to prevent DNA damage and to modulate responses to the DNA damage that leads to the pathobiological processes of COPD and emphysema.

6 Summary

Chronic obstructive pulmonary disease (COPD) is an age-associated disease caused mainly by cigarette smoking. Deregulated repair, tissue destruction, inflammation and lung regression are the hallmarks of COPD. Cellular senescence is a signal transduction program leading to irreversible cell cycle arrest. The growth arrest can be triggered by several different mechanisms, including recognition of DNA double-strand breaks by cellular sensors, leading to the activation of cell cycle checkpoint responses and recruitment of DNA repair machinery to damaged foci. The execution of regenerative programs in lung and remote organs is closely linked to viability or senescence of resident cells as well as progenitor cells derived from the circulation. It was proposed here, that COPD might be a disease of premature lung senescence and therefore this work aimed to decipher markers of DNA damage, repair and senescence in lung cell culture and a murine model of cigarette smoke induced emphysema.

In-vitro, cellular senescence could be successfully assessed by staining for β-galactosidase and evaluation of senescence associated heterochromatin foci (SAHF), reflecting condensed chromatin, in the nuclei. DNA double strand breaks and cell cycle arrest could be demonstrated by up regulated 53-BP1, γH2AX and p21. Paraffin lung sections from mice exposed to cigarette smoke were investigated for markers of cellular senescence, DNA damage and repair. Markers of DNA double strand breaks and senescence were up regulated in a time dependent manner, showing a correlation of DNA damage and emphysema progression. These results indicated that the pulmonary senescence in COPD is linked with persistent DNA double-strand breaks due to prolonged cigarette smoking.

7 Zusammenfassung

Die chronisch obstruktive Lungenerkrankung (COPD) ist eine altersbedingte Krankheit, die vor allem durch Inhalation von Zigarettenrauch oder anderen Rauchgasen verursacht wird. Eingeschränkte Geweberegeneration, verstärkte Inflammation und Lungenregression sind zentral an der Pathogenese der COPD beteiligt. Zelluläre Seneszenz ist ein Signaltransduktionsprogramm, welches zu irreversiblem Zellzyklusarrest führt. Die Wachstumshemmung kann durch viele verschiedene Mechanismen ausgelöst werden. Dazu gehören die Erkennung von DNA-Doppelstrangbrüchen durch zelluläre Sensoren, welche zur Aktivierung von Zellzykluskontrollproteinen führen und die Rekrutierung von DNA-Reparaturmaschinerie zu den schadhaften DNA-Loci initiieren. Die Ausführung des regenerativen Programms der Lunge ist eng an Viabilität bzw. Seneszenz der residenten Zellen sowie Vorläuferzellen gebunden. Zur Untersuchung der zellulären Seneszenz als Mechanismus der Entstehung der COPD adressierte diese Arbeit die Induktion von DNA Doppelstrangbrüchen durch Zigarettenrauchextrakt *in-vitro*, sowie die Korrelation von Seneszenz, DNA Doppelstrangbrüchen und Emphysemprogression in einem Mausmodell des Zigarettenrauch- induzierten Lungenemphysems.

Zelluläre Seneszenz konnte erfolgreich anhand der β –Galaktosidase Aktivität und der Analyse der Seneszenz-assoziierten Heterochromatin-Foci (SAHF) in den Zellkernen beurteilt werden. DNA-Doppelstrangbrüche und Zellzyklusarrest wurden durch die spezifische Rekrutierung von 53-BP1 und γH2AX an die DNA-Reparaturpunkte sowie durch Expression des Zellzyklusinhibitors p21 nachgewiesen. Paraffinschnitte von Lungen Zigarettenrauch-exponierter Mäuse wurden in Hinsicht auf Seneszenzmarker, DNA-Doppelstrangreparatur und Zellzyklusinhibition untersucht. Marker der DNA-Doppelstrangbrüche und Seneszenz waren nach längerer Rauchexposition in signifikant mehr Zellen exprimiert, als in den gleichaltrigen Kontrolltieren. Diese Ergebnisse zeigen, dass DNA Doppelstrangbrüche durch Zigarettenrauch-Inhaltsstoffe ausgelöst werden und mit zunehmendem experimentellem Emphysem verstärkt auftreten.

8 References

1. Barnes PJ. Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine* 2000: 343(4): 269-280.
2. Houghton AM. Mechanistic links between COPD and lung cancer. *Nature reviews Cancer* 2013: 13(4): 233-245.
3. Hausen T. [New GOLD guidelines for the therapy of COPD]. *Pneumologie* 2012: 66(12): 768.
4. Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. http://www.goldcopd.org 2007.

5. Raherison C, Girodet PO. Epidemiology of COPD. *European respiratory review : an official journal of the European Respiratory Society* 2009: 18(114): 213-221.
6. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS medicine* 2006: 3(11): e442.
7. MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* 2005: 2(1): 50-60.
8. Hecht SS. Tobacco smoke carcinogens and lung cancer. *Journal of the National Cancer Institute* 1999: 91(14): 1194-1210.
9. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Annals of the New York Academy of Sciences* 1993: 686: 12-27; discussion 27-18.
10. Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environmental health perspectives* 1985: 64: 111-126.
11. Hagstad S, Bjerg A, Ekerljung L, Backman H, Lindberg A, Rönmark E, Lundbäck B. PAssive smoking exposure is associated with increased risk of copd in never smokers. *Chest* 2014: 145(6): 1298-1304.
12. Dube MFG, C.R. . "Methods of Collection of Smoke for Analytical Purposes.". 1982.: Bates: 503556519-.
13. Ray NC, Alcaraz L. Muscarinic antagonist-β-adrenergic agonist dual pharmacology molecules as bronchodilators: a patent review. *Expert Opinion on Therapeutic Patents* 2009: 19(1): 1-12.
14. Lipworth BJ. Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. *Lancet* 2005: 365(9454): 167-175.
15. Kerstjens HA, Timens W. Phosphodiesterase 4 inhibitors: antiinflammatory therapy for chronic obstructive pulmonary disease at last? *Am J Resp Crit Care* 2003: 168(8): 914-915.
16. Sugi K, Kaneda Y, Nawada K, Fujita N, Ueda K, Nawada S, Esato K. [Efficacy of thoracoscopic pulmonary reconstruction in diffuse pulmonary emphysema--selection of surgical procedures based on Xe SPECT findings]. *[Zasshi] [Journal] Nihon Kyobu Geka Gakkai* 1997: 45(3): 372-373.
17. Cooper JD. The history of surgical procedures for emphysema. *The Annals of thoracic surgery* 1997: 63(2): 312-319.
18. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Experimental cell research* 1961: 25: 585-621.

19. Hayflick L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Experimental cell research* 1965: 37: 614-636.
20. Olovnikov AM. [Principle of marginotomy in template synthesis of polynucleotides]. *Doklady Akademii nauk SSSR* 1971: 201(6): 1496-1499.
21. Watson JD. Origin of concatemeric T7 DNA. *Nature: New biology* 1972: 239(94): 197-201.
22. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990: 345(6274): 458-460.
23. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003: 426(6963): 194-198.
24. Fujita K, Mondal AM, Horikawa I, Nguyen GH, Kumamoto K, Sohn JJ, Bowman ED, Mathe EA, Schetter AJ, Pine SR, Ji H, Vojtesek B, Bourdon JC, Lane DP, Harris CC. p53 isoforms Delta133p53 and p53beta are endogenous regulators of replicative cellular senescence. *Nature cell biology* 2009: 11(9): 1135-1142.
25. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. *Nature reviews Cancer* 2008: 8(7): 512-522.
26. Comings DE, Okada TA. Electron microscopy of human fibroblasts in tissue culture during logarithmic and confluent stages of growth. *Experimental cell research* 1970: 61(2): 295-301.
27. Lipetz J, Cristofalo VJ. Ultrastructural changes accompanying the aging of human diploid cells in culture. *Journal of ultrastructure research* 1972: 39(1): 43-56.
28. Cristofalo VJ, Kritchevsky D. Cell size and nucleic acid content in the diploid human cell line WI-38 during aging. *Medicina experimentalis International journal of experimental medicine* 1969: 19(6): 313-320.
29. Mitsui Y, Schneider EL. Increased nuclear sizes in senescent human diploid fibroblast cultures. *Experimental cell research* 1976: 100(1): 147-152.
30. Brunk U, Ericsson JL, Ponten J, Westerma.B. Residual Bodies and Aging in Cultured Human Glia Cells - Effect of Entrance into Phase Iii and Prolonged Periods of Confluence. *Experimental cell research* 1973: 79(1): 1-14.
31. Dimri GP, Lee XH, Basile G, Acosta M, Scott C, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereirasmith O, Peacocke M, Campisi J. A Biomarker That Identifies Senescent Human-Cells in Culture and in Aging Skin in-Vivo. *P Natl Acad Sci USA* 1995: 92(20): 9363-9367.
32. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 2006: 5(2): 187-195.
33. Kurz DJ, Decary S, Hong Y, Erusalimsky JD. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 2000: 113 (Pt 20): 3613-3622.
34. Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. The Parkinson Study Group. *The New England journal of medicine* 1993: 328(3): 176-183.
35. Rodier F, Munoz DP, Teachenor R, Chu V, Le O, Bhaumik D, Coppe JP, Campeau E, Beausejour CM, Kim SH, Davalos AR, Campisi J. DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* 2011: 124(1): 68-81.
36. Young AR, Narita M. SASP reflects senescence. *EMBO reports* 2009: 10(3): 228-230.

37. Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? *Cell* 2000: 102(4): 407-410.
38. Prowse KR, Greider CW. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *P Natl Acad Sci USA* 1995: 92(11): 4818-4822.
39. Kipling D, Cooke HJ. Hypervariable ultra-long telomeres in mice. *Nature* 1990: 347(6291): 400-402.
40. Packer L, Fuehr K. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature* 1977: 267(5610): 423-425.
41. Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. *P Natl Acad Sci USA* 1995: 92(10): 4337-4341.
42. Yuan H, Kaneko T, Matsuo M. Relevance of oxidative stress to the limited replicative capacity of cultured human diploid cells: the limit of cumulative population doublings increases under low concentrations of oxygen and decreases in response to aminotriazole. *Mechanisms of ageing and development* 1995: 81(2-3): 159-168.
43. Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, Wright WE. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes & development* 2001: 15(4): 398-403.
44. Bennett DC, Medrano EE. Molecular regulation of melanocyte senescence. *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society* 2002: 15(4): 242-250.
45. Bennett DC. Human melanocyte senescence and melanoma susceptibility genes. *Oncogene* 2003: 22(20): 3063-3069.
46. Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW, Taniguchi T. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 1994: 77(6): 829-839.
47. Dannenberg JH, van Rossum A, Schuijff L, te Riele H. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes & development* 2000: 14(23): 3051-3064.
48. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes & development* 2000: 14(23): 3037-3050.
49. Mathon NF, Malcolm DS, Harrisingh MC, Cheng L, Lloyd AC. Lack of replicative senescence in normal rodent glia. *Science* 2001: 291(5505): 872-875.
50. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 1983: 304(5927): 596-602.
51. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997: 88(5): 593-602.
52. Wei S, Wei S, Sedivy JM. Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts. *Cancer research* 1999: 59(7): 1539-1543.
53. Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 2005: 436(7051): 660-665.
54. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 2005: 436(7051): 725-730.

55. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M. Tumour biology: senescence in premalignant tumours. *Nature* 2005: 436(7051): 642.
56. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005: 436(7051): 720-724.
57. Collado M, Serrano M. The power and the promise of oncogene-induced senescence markers. *Nature reviews Cancer* 2006: 6(6): 472-476.
58. Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, Hollstein PE, MacCollin M, Cichowski K. A negative feedback signaling network underlies oncogene-induced senescence. *Cancer cell* 2006: 10(6): 459-472.
59. Young AP, Schlisio S, Minamishima YA, Zhang Q, Li L, Grisanzio C, Signoretti S, Kaelin WG, Jr. VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. *Nature cell biology* 2008: 10(3): 361-369.
60. Krtolica A, Campisi J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *The international journal of biochemistry & cell biology* 2002: 34(11): 1401-1414.
61. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. *Current biology : CB* 1999: 9(17): 939-945.
62. Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS biology* 2008: 6(12): 2853-2868.
63. Kuilman T, Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. *Nature reviews Cancer* 2009: 9(2): 81-94.
64. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007: 8(9): 729-740.
65. Rodier F, Campisi J. Four faces of cellular senescence. *The Journal of cell biology* 2011: 192(4): 547-556.
66. Abraham RT. Checkpoint signalling: focusing on 53BP1. *Nature cell biology* 2002: 4(12): E277-279.
67. Rodier F, Coppe JP, Patil CK, Hoeijmakers WA, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR, Campisi J. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nature cell biology* 2009: 11(8): 973-979.
68. Ohtani N, Yamakoshi K, Takahashi A, Hara E. The p16^{INK4a}-RB pathway : molecular link between cellular senescence and tumor suppression. *The Journal of Medical Investigation* 2004: 51(3,4): 146-153.
69. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 2005: 120(4): 513-522.
70. Collado M, Serrano M. Senescence in tumours: evidence from mice and humans. *Nature reviews Cancer* 2010: 10(1): 51-57.
71. Campisi J. Cellular senescence: putting the paradoxes in perspective. *Current opinion in genetics & development* 2011: 21(1): 107-112.
72. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008: 134(4): 657-667.
73. Jun JI, Lau LF. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nature cell biology* 2010: 12(7): 676-685.
74. Karrasch S, Holz O, Jorres RA. Aging and induced senescence as factors in the pathogenesis of lung emphysema. *Resp Med* 2008: 102(9): 1215-1230.

75. Vogelmeier C, Bals R. Chronic obstructive pulmonary disease and premature aging. *Am J Resp Crit Care* 2007: 175(12): 1217-1218.
76. Alder JK, Guo NN, Kembou F, Parry EM, Anderson CJ, Gorgy AI, Walsh MF, Sussan T, Biswal S, Mitzner W, Tuder RM, Armanios M. Telomere Length Is a Determinant of Emphysema Susceptibility. *Am J Resp Crit Care* 2011: 184(8): 904-912.
77. Tsuji T, Aoshiba K, Nagai A. Cigarette smoke induces senescence in alveolar epithelial cells. *American journal of respiratory cell and molecular biology* 2004: 31(6): 643-649.
78. Muller KC, Welker L, Paasch K, Feindt B, Erpenbeck VJ, Hohlfeld JM, Krug N, Nakashima M, Branscheid D, Magnussen H, Jorres RA, Holz O. Lung fibroblasts from patients with emphysema show markers of senescence in vitro. *Respiratory research* 2006: 7: 32.
79. Holz O, Zuhlke I, Jaksztat E, Muller KC, Welker L, Nakashima M, Diemel KD, Branscheid D, Magnussen H, Jorres RA. Lung fibroblasts from patients with emphysema show a reduced proliferation rate in culture. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2004: 24(4): 575-579.
80. Fabbri LM, Rabe KF. From COPD to chronic systemic inflammatory syndrome? *Lancet* 2007: 370(9589): 797-799.
81. Agusti A. Systemic effects of chronic obstructive pulmonary disease: what we know and what we don't know (but should). *Proceedings of the American Thoracic Society* 2007: 4(7): 522-525.
82. Janssens JP, Pache JC, Nicod LP. Physiological changes in respiratory function associated with ageing. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 1999: 13(1): 197-205.
83. Fletcher C, Peto R. The natural history of chronic airflow obstruction. *British medical journal* 1977: 1(6077): 1645-1648.
84. Verbeken EK, Cauberghs M, Mertens I, Clement J, Lauweryns JM, Van de Woestijne KP. The senile lung. Comparison with normal and emphysematous lungs. 2. Functional aspects. *Chest* 1992: 101(3): 800-809.
85. Teramoto S, Ishii M. Aging, the aging lung, and senile emphysema are different. *Am J Resp Crit Care* 2007: 175(2): 197-198; author reply 198.
86. Aizen E, Gilhar A. Smoking effect on skin wrinkling in the aged population. *International journal of dermatology* 2001: 40(7): 431-433.
87. Patel BD, Loo WJ, Tasker AD, Screaton NJ, Burrows NP, Silverman EK, Lomas DA. Smoking related COPD and facial wrinkling: is there a common susceptibility? *Thorax* 2006: 61(7): 568-571.
88. Just M, Ribera M, Monso E, Lorenzo JC, Ferrandiz C. Effect of smoking on skin elastic fibres: morphometric and immunohistochemical analysis. *The British journal of dermatology* 2007: 156(1): 85-91.
89. Bernhard D, Moser C, Backovic A, Wick G. Cigarette smoke--an aging accelerator? *Experimental gerontology* 2007: 42(3): 160-165.
90. Gessner C, Scheibe R, Wotzel M, Hammerschmidt S, Kuhn H, Engelmann L, Hoheisel G, Gillissen A, Sack U, Wirtz H. Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. *Resp Med* 2005: 99(10): 1229-1240.
91. Obot C, Lee K, Fuciarelli A, Renne R, McKinney W. Characterization of mainstream cigarette smoke-induced biomarker responses in ICR and C57Bl/6 mice. *Inhalation toxicology* 2004: 16(10): 701-719.
92. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annual review of pathology* 2010: 5: 99-118.

93. de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *The Journal of pathology* 2000: 190(5): 619-626.
94. Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 2002: 57(7): 590-595.
95. Balbi B, Bason C, Balleari E, Fiasella F, Pesci A, Ghio R, Fabiano F. Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 1997: 10(4): 846-850.
96. Kristan SS, Marc MM, Kern I, Flezar M, Suskovic S, Kosnik M, Korosec P. Airway angiogenesis in stable and exacerbated chronic obstructive pulmonary disease. *Scandinavian journal of immunology* 2012: 75(1): 109-114.
97. de Boer WI, Hau CM, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS. Expression of epidermal growth factors and their receptors in the bronchial epithelium of subjects with chronic obstructive pulmonary disease. *American journal of clinical pathology* 2006: 125(2): 184-192.
98. Wang S, Moerman EJ, Jones RA, Thweatt R, Goldstein S. Characterization of IGFBP-3, PAI-1 and SPARC mRNA expression in senescent fibroblasts. *Mechanisms of ageing and development* 1996: 92(2-3): 121-132.
99. Grillari J, Hohenwarter O, Grabherr RM, Katinger H. Subtractive hybridization of mRNA from early passage and senescent endothelial cells. *Experimental gerontology* 2000: 35(2): 187-197.
100. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. Oncogenic BRAF Induces Senescence and Apoptosis through Pathways Mediated by the Secreted Protein IGFBP7. *Cell* 2008: 132(3): 363-374.
101. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *The Journal of pathology* 2003: 200(4): 448-464.
102. West MD, Pereira-Smith OM, Smith JR. Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity. *Experimental cell research* 1989: 184(1): 138-147.
103. Millis AJ, Hoyle M, McCue HM, Martini H. Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts. *Experimental cell research* 1992: 201(2): 373-379.
104. Zeng G, Millis AJ. Differential regulation of collagenase and stromelysin mRNA in late passage cultures of human fibroblasts. *Experimental cell research* 1996: 222(1): 150-156.
105. Parrinello S, Coppe JP, Krtolica A, Campisi J. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J Cell Sci* 2005: 118(Pt 3): 485-496.
106. Liu D, Hornsby PJ. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer research* 2007: 67(7): 3117-3126.
107. Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX, O'Connor CM. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Resp Crit Care* 1997: 156(1): 240-247.
108. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, Kawakami Y. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Resp Crit Care* 1999: 159(6): 1985-1991.

109. Culpitt SV, Maziak W, Loukidis S, Nightingale JA, Matthews JL, Barnes PJ. Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Resp Crit Care* 1999: 160(5 Pt 1): 1635-1639.
110. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Resp Crit Care* 2000: 162(4 Pt 1): 1355-1360.
111. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, Barnes PJ. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *American journal of respiratory cell and molecular biology* 2002: 26(5): 602-609.
112. Russell RE, Thorley A, Culpitt SV, Dodd S, Donnelly LE, Demattos C, Fitzgerald M, Barnes PJ. Alveolar macrophage-mediated elastolysis: roles of matrix metalloproteinases, cysteine, and serine proteases. *American journal of physiology Lung cellular and molecular physiology* 2002: 283(4): L867-873.
113. Cataldo D, Munaut C, Noel A, Frankenne F, Bartsch P, Foidart JM, Louis R. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *International archives of allergy and immunology* 2000: 123(3): 259-267.
114. Beeh KM, Beier J, Kornmann O, Buhl R. Sputum matrix metalloproteinase-9, tissue inhibitor of metalloprotinease-1, and their molar ratio in patients with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and healthy subjects. *Resp Med* 2003: 97(6): 634-639.
115. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol* 2002: 3(12): 932-943.
116. Comi P, Chiaramonte R, Maier JA. Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. *Experimental cell research* 1995: 219(1): 304-308.
117. West MD, Shay JW, Wright WE, Linskens MH. Altered expression of plasminogen activator and plasminogen activator inhibitor during cellular senescence. *Experimental gerontology* 1996: 31(1-2): 175-193.
118. Mu XC, Higgins PJ. Differential growth state-dependent regulation of plasminogen activator inhibitor type-1 expression in senescent IMR-90 human diploid fibroblasts. *Journal of cellular physiology* 1995: 165(3): 647-657.
119. Mu XC, Staiano-Coico L, Higgins PJ. Increased transcription and modified growth state-dependent expression of the plasminogen activator inhibitor type-1 gene characterize the senescent phenotype in human diploid fibroblasts. *Journal of cellular physiology* 1998: 174(1): 90-98.
120. Martens JW, Sieuwerts AM, Bolt-deVries J, Bosma PT, Swiggers SJ, Klijn JG, Foekens JA. Aging of stromal-derived human breast fibroblasts might contribute to breast cancer progression. *Thrombosis and haemostasis* 2003: 89(2): 393-404.
121. Xiao W, Tong WL, Ma DD. [Higher levels of urokinase plasminogen activator system components in the airways of chronic obstructive pulmonary disease patients]. *Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases* 2006: 29(11): 723-726.
122. Di Stefano A, Maestrelli P, Roggeri A, Turato G, Calabro S, Potena A, Mapp CE, Ciaccia A, Covacev L, Fabbri LM, Saetta M. Upregulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis. *Am J Resp Crit Care* 1994: 149(3 Pt 1): 803-810.

123. Ishii Y. [Role of adhesion molecules in the pathogenesis of COPD]. *Nihon rinsho Japanese journal of clinical medicine* 1999: 57(9): 1965-1971.
124. Popper HH, Pailer S, Wurzinger G, Feldner H, Hesse C, Eber E. Expression of adhesion molecules in allergic lung diseases. *Virchows Archiv : an international journal of pathology* 2002: 440(2): 172-180.
125. To M, Ito K, Ausin PM, Kharitonov SA, Barnes PJ. Osteoprotegerin in sputum is a potential biomarker in COPD. *Chest* 2011: 140(1): 76-83.
126. Lu SY, Chang KW, Liu CJ, Tseng YH, Lu HH, Lee SY, Lin SC. Ripe areca nut extract induces G1 phase arrests and senescence-associated phenotypes in normal human oral keratinocyte. *Carcinogenesis* 2006: 27(6): 1273-1284.
127. Montuschi P, Kharitonov SA, Ciabattoni G, Barnes PJ. Exhaled leukotrienes and prostaglandins in COPD. *Thorax* 2003: 58(7): 585-588.
128. Jiang YJ, Lu B, Choy PC, Hatch GM. Regulation of cytosolic phospholipase A2, cyclooxygenase-1 and -2 expression by PMA, TNFalpha, LPS and M-CSF in human monocytes and macrophages. *Molecular and cellular biochemistry* 2003: 246(1-2): 31-38.
129. Taha R, Olivenstein R, Utsumi T, Ernst P, Barnes PJ, Rodger IW, Giaid A. Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* 2000: 161(2 Pt 1): 636-640.
130. Rasoamanantena P, Thweatt R, Labat-Robert J, Goldstein S. Altered regulation of fibronectin gene expression in Werner syndrome fibroblasts. *Experimental cell research* 1994: 213(1): 121-127.
131. Kumazaki T, Kobayashi M, Mitsui Y. Enhanced expression of fibronectin during in vivo cellular aging of human vascular endothelial cells and skin fibroblasts. *Experimental cell research* 1993: 205(2): 396-402.
132. Kranenburg AR, Willems-Widyastuti A, Moori WJ, Sterk PJ, Alagappan VK, de Boer WI, Sharma HS. Enhanced bronchial expression of extracellular matrix proteins in chronic obstructive pulmonary disease. *American journal of clinical pathology* 2006: 126(5): 725-735.
133. Sato I, Morita I, Kaji K, Ikeda M, Nagao M, Murota S. Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell. *Biochemical and biophysical research communications* 1993: 195(2): 1070-1076.
134. Lee AC, Fenster BE, Ito H, Takeda K, Bae NS, Hirai T, Yu ZX, Ferrans VJ, Howard BH, Finkel T. Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *The Journal of biological chemistry* 1999: 274(12): 7936-7940.
135. van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V, Luscher TF. Enhanced peroxynitrite formation is associated with vascular aging. *The Journal of experimental medicine* 2000: 192(12): 1731-1744.
136. Macip S, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW, Aaronson SA. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *The EMBO journal* 2002: 21(9): 2180-2188.
137. Xin MG, Zhang J, Block ER, Patel JM. Senescence-enhanced oxidative stress is associated with deficiency of mitochondrial cytochrome c oxidase in vascular endothelial cells. *Mechanisms of ageing and development* 2003: 124(8-9): 911-919.
138. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000: 408(6809): 239-247.
139. Finkel T, Serrano M, Blasco MA. The common biology of cancer and ageing. *Nature* 2007: 448(7155): 767-774.

140. MacNee W. Oxidative stress and lung inflammation in airways disease. *European journal of pharmacology* 2001: 429(1-3): 195-207.
141. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *The American journal of physiology* 1996: 271(5 Pt 1): C1424-1437.
142. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel FC, Milger K, Egemnazarov B, Turowska A, Fuchs B, Nikam S, Roth M, Sydykov A, Medebach T, Klepetko W, Jaksch P, Dumitrascu R, Garn H, Voswinckel R, Kostin S, Seeger W, Schermuly RT, Grimminger F, Ghofrani HA, Weissmann N. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell* 2011: 147(2): 293-305.
143. Chung KF. Cytokines as targets in chronic obstructive pulmonary disease. *Current drug targets* 2006: 7(6): 675-681.
144. Korsgren M, Kallstrom L, Uller L, Bjerke T, Sundler F, Persson CG, Korsgren O. Role of macrophage migration inhibitory factor (MIF) in allergic and endotoxin-induced airway inflammation in mice. *Mediators of inflammation* 2000: 9(1): 15-23.
145. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA. Epidermal growth factor system regulates mucin production in airways. *P Natl Acad Sci USA* 1999: 96(6): 3081-3086.
146. Kranenburg AR, De Boer WI, Van Krieken JH, Mooi WJ, Walters JE, Saxena PR, Sterk PJ, Sharma HS. Enhanced expression of fibroblast growth factors and receptor FGFR-1 during vascular remodeling in chronic obstructive pulmonary disease. *American journal of respiratory cell and molecular biology* 2002: 27(5): 517-525.
147. Kortlever RM, Higgins PJ, Bernards R. Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. *Nature cell biology* 2006: 8(8): 877-884.
148. Xiao W, Hsu YP, Ishizaka A, Kirikae T, Moss RB. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. *Chest* 2005: 128(4): 2316-2326.
149. Stewart CE, Sayers I. Characterisation of urokinase plasminogen activator receptor variants in human airway and peripheral cells. *BMC molecular biology* 2009: 10: 75.
150. Burnett D, Crocker J, Stockley RA. Cathepsin B-like cysteine proteinase activity in sputum and immunohistologic identification of cathepsin B in alveolar macrophages. *The American review of respiratory disease* 1983: 128(5): 915-919.
151. Zandvoort A, van der Geld YM, Jonker MR, Noordhoek JA, Vos JT, Wesseling J, Kauffman HF, Timens W, Postma DS. High ICAM-1 gene expression in pulmonary fibroblasts of COPD patients: a reflection of an enhanced immunological function. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2006: 28(1): 113-122.
152. To M, Ito K, Ausin PM, Kharitonov SA, Barnes PJ. Osteoprotegerin in Sputum Is a Potential Biomarker in COPDOsteoprotegerin in COPD. *CHEST Journal* 2011: 140(1): 76-83.
153. Takabatake N, Nakamura H, Inoue S, Terashita K, Yuki H, Kato S, Yasumura S, Tomoike H. Circulating levels of soluble Fas ligand and soluble Fas in patients with chronic obstructive pulmonary disease. *Resp Med* 2000: 94(12): 1215-1220.
154. Langen RC, Korn SH, Wouters EF. ROS in the local and systemic pathogenesis of COPD. *Free radical biology & medicine* 2003: 35(3): 226-235.
155. D'Armiento J, Dalal SS, Okada Y, Berg RA, Chada K. Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. *Cell* 1992: 71(6): 955-961.
156. Hewitt G, Jurk D, Marques FD, Correia-Melo C, Hardy T, Gackowska A, Anderson R, Taschuk M, Mann J, Passos JF. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nature communications* 2012: 3: 708.

157. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LVF, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Orntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 2006: 444(7119): 633-637.
158. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garre M, Nuciforo PG, Bensimon A, Maestro R, Pelicci PG, di Fagagna FD. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 2006: 444(7119): 638-642.
159. Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, Fumagalli M, Da Costa M, Brown C, Popov N, Takatsu Y, Melamed J, di Fagagna FD, Bernard D, Hernando E, Gil J. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* 2008: 133(6): 1006-1018.
160. Carp H, Janoff A. In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. *J Clin Invest* 1979: 63(4): 793-797.
161. Kurz DJ, Decary S, Hong Y, Erusalimsky JD. Senescence-associated beta-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *Journal of cell science* 2000: 113(20): 3613-3622.
162. Sharp PA. RNAi and double-strand RNA. *Genes & development* 1999: 13(2): 139-141.
163. Hunter T, Hunt T, Jackson RJ, Robertson HD. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *The Journal of biological chemistry* 1975: 250(2): 409-417.
164. Sethi S, Murphy TF. Chronic obstructive pulmonary disease. *The New England journal of medicine* 2000: 343(26): 1969-1970; author reply 1970-1961.
165. Hogg JC. Chronic obstructive pulmonary disease: an overview of pathology and pathogenesis. *Novartis Foundation symposium* 2001: 234: 4-19; discussion 19-26.
166. Koczulla AR, Vogelmeier C. [Chronic obstructive pulmonary disease: from pathogenesis to treatment]. *Deutsche medizinische Wochenschrift* 2008: 133(10): 471-475.
167. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* 2005: 2(4): 258-266; discussion 290-251.
168. Laurell CB, Eriksson S. The Electrophoretic alpha(1)-Globulin Pattern of Serum in alpha(1)-Antitrypsin Deficiency. *Copd-Journal of Chronic Obstructive Pulmonary Disease* 2013: 10: 3-8.
169. Gross P, Babyak MA, Tolker E, Kaschak M. Enzymatically Produced Pulmonary Emphysema; a Preliminary Report. *Journal of occupational medicine : official publication of the Industrial Medical Association* 1964: 6: 481-484.
170. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *The American review of respiratory disease* 1977: 115(3): 461-478.
171. Senior RM, Tegner H, Kuhn C, Ohlsson K, Starcher BC, Pierce JA. The induction of pulmonary emphysema with human leukocyte elastase. *The American review of respiratory disease* 1977: 116(3): 469-475.
172. Travis J. Structure, function, and control of neutrophil proteinases. *The American journal of medicine* 1988: 84(6A): 37-42.
173. Takeyabu K, Betsuyaku T, Nishimura M, Yoshioka A, Tanino M, Miyamoto K, Kawakami Y. Cysteine proteinases and cystatin C in bronchoalveolar lavage fluid from

subjects with subclinical emphysema. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 1998: 12(5): 1033-1039.
174. Repine JE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Resp Crit Care* 1997: 156(2 Pt 1): 341-357.
175. Bowler RP, Barnes PJ, Crapo JD. The role of oxidative stress in chronic obstructive pulmonary disease. *Copd* 2004: 1(2): 255-277.
176. Koyama H, Geddes DM. Genes, oxidative stress, and the risk of chronic obstructive pulmonary disease. *Thorax* 1998: 53 Suppl 2: S10-14.
177. Britton JR, Pavord ID, Richards KA, Knox AJ, Wisniewski AF, Lewis SA, Tattersfield AE, Weiss ST. Dietary antioxidant vitamin intake and lung function in the general population. *Am J Resp Crit Care* 1995: 151(5): 1383-1387.
178. Schunemann HJ, Freudenheim JL, Grant BJ. Epidemiologic evidence linking antioxidant vitamins to pulmonary function and airway obstruction. *Epidemiologic reviews* 2001: 23(2): 248-267.
179. Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM. Global burden of COPD: systematic review and meta-analysis. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2006: 28(3): 523-532.
180. Buist AS, McBurnie MA, Vollmer WM, Gillespie S, Burney P, Mannino DM, Menezes AMB, Sullivan SD, Lee TA, Weiss KB, Jensen RL, Marks GB, Gulsvik A, Nizankowska-Mogilnicka E, Grp BCR. International variation in the prevalence of COPD (The BOLD Study): a population-based prevalence study. *Lancet* 2007: 370(9589): 741-750.
181. Strehler BL, Mark DD, Mildvan AS, Gee MV. Rate and Magnitude of Age Pigment Accumulation in the Human Myocardium. *J Gerontol* 1959: 14(4): 430-439.
182. Janssens JP, Pache JC, Nicod LP. Physiological changes in respiratory function associated with ageing. *European Respiratory Journal* 1999: 13(1): 197-205.
183. Ito K, Barnes PJ. COPD as a Disease of Accelerated Lung Aging. *Chest* 2009: 135(1): 173-180.
184. Maclay JD, McAllister DA, Rabinovich R, Haq I, Maxwell S, Hartland S, Connell M, Murchison JT, van Beek EJ, Gray RD, Mills NL, Macnee W. Systemic elastin degradation in chronic obstructive pulmonary disease. *Thorax* 2012: 67(7): 606-612.
185. He J, Turino GM, Lin YY. Characterization of peptide fragments from lung elastin degradation in chronic obstructive pulmonary disease. *Experimental lung research* 2010: 36(9): 548-557.
186. Stone PJ, Gottlieb DJ, O'Connor GT, Ciccolella DE, Breuer R, Bryan-Rhadfi J, Shaw HA, Franzblau C, Snider GL. Elastin and collagen degradation products in urine of smokers with and without chronic obstructive pulmonary disease. *Am J Resp Crit Care* 1995: 151(4): 952-959.
187. Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, Kodama Y, Mori T, Goto S, Takahashi K, Fukuchi Y, Maruyama N, Ishigami A. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. *Am J Resp Crit Care* 2006: 174(5): 530-537.
188. Suga T, Kurabayashi M, Sando Y, Ohyama Y, Maeno T, Maeno Y, Aizawa H, Matsumura Y, Kuwaki T, Kuro OM, Nabeshima Y, Nagai R. Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *American journal of respiratory cell and molecular biology* 2000: 22(1): 26-33.
189. Muller KC, Welker L, Paasch K, Feindt B, Erpenbeck V, Hohlfeld J, Krug N, Nakashima M, Branscheid D, Magnussen H, Jorres RA, Holz O. Lung fibroblasts from patients with emphysema show markers of senescence in vitro. *Respiratory research* 2006: 7.

190. Holz O, Zuhlke I, Jaksztat E, Muller KC, Welker L, Nakashima M, Diemel KD, Branscheid D, Magnussen H, Jorres RA. Lung fibroblasts from patients with emphysema show a reduced proliferation rate in culture. *European Respiratory Journal* 2004: 24(4): 575-579.
191. Wust RC, Degens H. Factors contributing to muscle wasting and dysfunction in COPD patients. *International journal of chronic obstructive pulmonary disease* 2007: 2(3): 289-300.
192. Tkacova R, Kluchova Z, Joppa P, Petrasova D, Molcanyiova A. Systemic inflammation and systemic oxidative stress in patients with acute exacerbations of COPD. *Respiratory medicine* 2007: 101(8): 1670-1676.
193. Wang C-H, Huang C-D, Lin H-C, Huang T-T, Lee K-Y, Lo Y-L, Lin S-M, Chung KF, Kuo H-P. Increased activation of fibrocytes in patients with chronic obstructive asthma through an epidermal growth factor receptor–dependent pathway. *Journal of Allergy and Clinical Immunology* 2012: 129(5): 1367-1376.
194. Adams PD. Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene* 2007: 397(1-2): 84-93.
195. Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *P Natl Acad Sci USA* 2001: 98(21): 12072-12077.
196. Zhang S, McCarter JD, Okamura-Oho Y, Yaghi F, Hinek A, Withers SG, Callahan JW. Kinetic mechanism and characterization of human beta-galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells. *The Biochemical journal* 1994: 304 (Pt 1): 281-288.
197. Ferland G, Perea A, Audet M, Tuchweber B. Characterization of liver lysosomal enzyme activity in hepatocytes, Kupffer and endothelial cells during aging: effect of dietary restriction. *Mechanisms of ageing and development* 1990: 56(2): 143-154.
198. Gerland LM, Peyrol S, Lallemand C, Branche R, Magaud JP, Ffrench M. Association of increased autophagic inclusions labeled for beta-galactosidase with fibroblastic aging. *Experimental gerontology* 2003: 38(8): 887-895.
199. Gary RK, Kindell SM. Quantitative assay of senescence-associated beta-galactosidase activity in mammalian cell extracts. *Analytical biochemistry* 2005: 343(2): 329-334.
200. Yang NC, Hu ML. The limitations and validities of senescence associated-beta-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Experimental gerontology* 2005: 40(10): 813-819.
201. Robbins E, Levine EM, Eagle H. Morphologic Changes Accompanying Senescence of Cultured Human Diploid Cells. *Journal of Experimental Medicine* 1970: 131(6): 1211-&.
202. Brunk U, Ericsson JL, Ponten J, Westermark B. Residual bodies and "aging" in cultured human glia cells. Effect of entrance into phase 3 and prolonged periods of confluence. *Experimental cell research* 1973: 79(1): 1-14.
203. Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 2003: 113(6): 703-716.
204. Howard BH. Replicative senescence: considerations relating to the stability of heterochromatin domains. *Experimental gerontology* 1996: 31(1-2): 281-293.
205. Narita M, Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, Myers MP, Lowe SW. A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. *Cell* 2006: 126(3): 503-514.
206. Funayama R, Saito M, Tanobe H, Ishikawa F. Loss of linker histone H1 in cellular senescence. *The Journal of cell biology* 2006: 175(6): 869-880.

207. Zhang RG, Chen W, Adams PD. Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* 2007: 27(6): 2343-2358.
208. Ye X, Zerlanko B, Zhang R, Somaiah N, Lipinski M, Salomoni P, Adams PD. Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. *Mol Cell Biol* 2007: 27(7): 2452-2465.
209. Morales JC, Franco S, Murphy MM, Bassing CH, Mills KD, Adams MM, Walsh NC, Manis JP, Rassidakis GZ, Alt FW, Carpenter PB. 53BP1 and p53 synergize to suppress genomic instability and lymphomagenesis. *P Natl Acad Sci USA* 2006: 103(9): 3310-3315.
210. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nature Reviews Cancer* 2009: 9(6): 400-414.
211. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA, Jr., Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005: 434(7035): 907-913.
212. Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR. HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. *Nature* 2008: 453(7195): 682-U614.
213. Wright JL, Cosio M, Churg A. Animal models of chronic obstructive pulmonary disease. *American journal of physiology Lung cellular and molecular physiology* 2008: 295(1): L1-15.
214. Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y. GammaH2AX and cancer. *Nature reviews Cancer* 2008: 8(12): 957-967.
215. Qvarnstrom OF, Simonsson M, Eriksson V, Turesson I, Carlsson J. gammaH2AX and cleaved PARP-1 as apoptotic markers in irradiated breast cancer BT474 cellular spheroids. *International journal of oncology* 2009: 35(1): 41-47.
216. Sedelnikova OA, Bonner WM. GammaH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. *Cell cycle* 2006: 5(24): 2909-2913.
217. Jeon JH, Kim SK, Kim HJ, Chang J, Ahn CM, Chang YS. Insulin-like growth factor-1 attenuates cisplatin-induced gammaH2AX formation and DNA double-strand breaks repair pathway in non-small cell lung cancer. *Cancer letters* 2008: 272(2): 232-241.
218. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nature reviews Cancer* 2003: 3(3): 155-168.
219. Rotman G, Shiloh Y. ATM: from gene to function. *Human molecular genetics* 1998: 7(10): 1555-1563.
220. Jung M, Timofeeva O, Cheema AK, Varghese R, Ressom H, Dritschilo A. Human fibroblasts for large-scale "omics" investigations of ATM gene function. *Advances in experimental medicine and biology* 2011: 720: 181-190.
221. Zhan H, Suzuki T, Aizawa K, Miyagawa K, Nagai R. Ataxia telangiectasia mutated (ATM)-mediated DNA damage response in oxidative stress-induced vascular endothelial cell senescence. *The Journal of biological chemistry* 2010: 285(38): 29662-29670.
222. Cosio MG, Saetta M, Agusti A. Immunologic aspects of chronic obstructive pulmonary disease. *The New England journal of medicine* 2009: 360(23): 2445-2454.

9 Acknowledgements

First, I would like to thank my parents for providing me with unconditional love, support and encouragement as I endeavored to complete these studies. Their constant and genuine belief in my abilities provided me with the confidence and drive that I needed to make it through the challenging days and nights in the laboratory. I would also like to thank my brothers Ashutosh Kumar and Anand Hrishikesh for their everlasting support and for encouraging me to explore a new locale.

I would like to express my sincerest gratitude to Bhavna for her relentless encouragement and cheerful optimism. Her continual patience and willingness to listen enabled me to stay focused and keep things in perspective. I am truly honored to have such a remarkable person in my life!

I would like to thank my supervisor, Prof. Robert Voswinckel, for providing me with the opportunity to pursue a PhD in his laboratory. I am extremely grateful for the years of mentorship and thoughtful discussion that he has provided. I would also like to thank Robert for providing exceptional training when I was a young impressionable scientist. I am also grateful to Prof. Werner Seeger for his timely suggestions and support. I would also like to take this opportunity to thank my collaborators and all my lab members for their insightful discussions and cooperation throughout my research work.

Finally, I would like to acknowledge the generosity of Max Planck Institute for heart and Lung Research, Germany for providing me with the financial resources necessary for completion of this work.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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 by 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."

Giessen, March 2014 Manish Kumar