UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Scienze Mediche

Ciclo XXIX

Chronic Renal Injury is an Under-recognized COPD Comorbidity That is Linked to Endothelial Injury

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Riassunto

I pazienti con broncopatia cronica ostruttiva (BPCO) hanno spesso albuminuria, indicativa di danno endoteliale, associata con ipossiemia. La severita' dell' albuminuria e' direttamente correlata al grado di ipossiemia nei pazienti con BPCO. Sebbene sia gia' noto che l' insufficienza renale cronica e' piu' frequente nei pazienti con BPCO rispetto ai controlli, non si sa se la BPCO sia associata a danno renale cronico.

In questo studio abbiamo testato le seguenti ipotesi: 1) Il fumo di sigaretta provoca un danno all' endotelio sia polmonare che vascolare. Questo danno spiega l' associazioinbe tra albuminuria e BPCO; 2) L' albuminuria indotta dai danni da fumo di sigaretta e' collegata ad aumento del pathway stress ossidativo-advanced glycation end products (AGEs)-recettore per AGEs (RAGE); 3) l' Enalapril, un ACE inibitore dalle proprieta' antiossidanti, limita la progressione del danno polmonare ed endoteliale tramite la riduzione dell' attivazione del pathway AGEs-RAGE nelle cellule endoteliali in polmone e rene.

Per testare queste ipostesi abbiamo utilizzato due approcci: uno in cui si sono studiate diverse coorti di pazienti con BPCO e controlli, ed uno in cui si e' studiato un modello murino di enfisema indotto da esposizione a fumo di sigaretta.

Studio in umani

Abbiamo selezionato 26 pazienti con BCPO. 24 fumatori senza BPCO, e 32 non fumatori senza BPCO che si erano sottoposti a biopsia renale o intervento di nefrectomia. In questi soggetti abbiamo valutato le lesioni renali (glomerulari e tubulari tramite microscopio ottico, e vascolari tramite microscopio elettronico), insieme alla filtrazione glomerulare.

Abbiamo poi selezionati 15 tra questi soggetti (5 con BCPO, 5 fumatori senza BCPO, e 5 non fumatori senza BPCO) per effettuare colorazione ad immunofluorescneza per RAGE ed AGEs nelle cellule endoteliali nelle sezioni

renali. Su altri 15 soggetti che si erano sottoposti a pneumectomia (5 con BCPO, 5 fumatori senza BCPO, e 5 non fumatori senza BPCO) abbiamo effettuato la stessa colorazione ad immunofluorescenza per AGEs e RAGE nelle cellule endoteliali polmonari.

In una differente coorte di fumatori sani, provenienti dal Lovelace Respiratory Research Institute (LRRI, Albuquerque, New Mexico, USA) abbiamo misurato i livelli di albumina nelle urine e correlati con il declino della funzionalita' respiratoria.

Studio in modello murino di enfisema

Abbiamo esposto topi wild-type a fumo di sigaretta per 1 e 6 mesi. A meta' delle esposizioni (15 giorni e 3 mesi, rispettivamente), abbiamo iniziato a trattare i topi con Enalapril fino al completamento delle esposizioni al fumo e sacrificio degli animali.

Nell' esperimento cronico abbiamo misurato il danno renale e vascolare, la microalbuminuria, lo sviluppo di enfisema polmonare e rimodellamento delle vie aeree, i livelli di AGEs e RAGE, e l' apoptosi delle cellule endoteliali. Nell' esperimento acuto abbiamo misurato l' infiammazione polmonare, lo stress ossidativo, i livelli di AGEs e RAGE, e la microalbuminuria. Inoltre abbiamo testato l' efficacia dell' Enalapril nel revertire/prevenire i suddetti fenomeni polmonari e renali.

Risulltati nello studio in umani

I pazienti con BPCO avevano danno renale particolarmente evidente a livello dei glomeruli e dei capillari. In particolare i glomeruli dei pazienti con BPCO avevano un diametro maggiore di molto inferiore rispetto ai controlli fumatori e non. Inoltre le cellule endoteliali renali dei pazienti con BPCO mostravano strati di danno e rigenerazione sovrapposti.

La colorazione ad immunofluorescenza ha dimostrato che nelle cellule endoteliali di reni e polmoni dei pazienti con BPCO vi e' una elevata attivazione di RAGE and AGEs rispetto ai controlli fumatori e non. Nella coorte di fumatori sani del LRRI, abbiamo riscontrato che non vi era alcuna correlazione tra i livelli di microalbuminuria e declino della funzionalita' respiratoria.

Risulltati nello studio in topi

Topi esposti al fumo di sigaretta per 6 mesi avevano glomeruli renali atrofici e appiattimento e accorciamento dei podociti renali. La microalbuminuria compariva nelle urine dei topi a partire del primo mese di esposizione al fumo e si manteneva elevata per tutti i 6 mesi di esposizione al fumo. Sia dopo 1 che dopo 6 mesi di fumo i livelli di AGEs e RAGE nelle cellule endoteliali di rene e polmone aumentavano rispetto ai topi che non erano stati esposti al fumo. Inoltre, topi esposti a fumo per un mese mostravano livelli elevati di stress ossidativo sia in rene che polmone, e topi esposti al fumo per 6 mesi avevano aumento dell' apoptosi delle cellule endoteliali renali e polmonari. Il trattamento con Enalapril ha migliorato: 1) il danno renale cronico; 2) la microalbuminuria a partire dalle 2 settimane di trattamento; 3) lo stress ossidativo renale e polmonare; 4) l' apoptosi delle cellule endoteliali renali e polmonari; 5) l' enfisema polmonare ed il rimodellamento delle vie aeree. Inoltre, il trattamento con Enalapril ha diminuito i livelli di RAGE ed AGEs nelle cellule endoteliali di rene e polmone sia nell' esperimento acuto che nel cronico.

Per concludere, i pazienti con BPCO manifestano lesioni endoteliali nel rene che sono correlate ad aumento dei livelli di RAGE ed AGE nell' endotelio di entrambi gli organi. Questi dati sono stati confermati con un modello murino, che inoltre ha evidenziato una protezione dal danno renale e polmonare grazie alla terapia con Enalapril.

Dunque, la presenza di albuminuria potrebbe identificare pazienti con BPCO in cui la terapia con ACE inibitori possa migliorare la funzionalita' renale e polmonare tramite la riduzione del danno endoteliale.

Introduction

Chronic Obstructive Pulmonary Disease

Over the last decades, the progressive control of communicable diseases as the most important causes of morbidity and death in the World, has resulted in three important changes. First, an increase in the World's population; second, a simultaneous improvement in longevity and thirdly, an increase in the prevalence of diseases that are non-communicable (NCD). An NCD is a medical condition or disease that is by definition non-infectious and non-transmissible among humans. The four most important NCDs are cardiovascular diseases, cancers, chronic lung diseases, and diabetes, leading to 17 million, 7.6 million, 4.2 million, and 1.3 million deaths, respectively, in 2008 according to the World Health Organization (WHO Global Health Observatory (http://www.who.int/gho/ncd/mortalitymorbidity/ncd_total/en/index.html).

Besides having an important genetic determinant, these diseases have in common the precipitating effect of damaging environmental exposures (as is the case for indoor and outdoor pollution and cigarette smoke) as well as behavioural changes such as high caloric intake leading to obesity. For the foreseeable future, these four NCD's will dominate the health landscape and demand intense efforts to prevent their onset and to slow their effects once developed. For this reason, the WHO's World Health Report identified tobacco smoke, alcohol consumption, overweight, physical inactivity, high blood pressure, and high cholesterol as important risk factors for NCDs (World Health Organization. The world health report 2002: reducing risks, promoting healthy life. Available from: (http://www.who.int/whr/2002/en/index.html).

One of these NCD's are the chronic lung diseases of the airways and other structures of the lung with asthma and chronic obstructive pulmonary

disease (COPD) representing the most important and numerous of them. This review summarizes our current knowledge on COPD and specifically attempts to single out the unmet needs surrounding its unfortunate emergence as one large contributor to poor health around the World. This is a call to action for COPD as an important medical need in the World

Definition and clinical characteristics

COPD is a lung disease that causes limitation to airflow in and out of the lungs. It results from an abnormal airway and parenchymal response to the inflammation caused by the inhalation of toxic particles contained primarily in cigarette smoke, the combustion of biomass for cooking and heating purposes and from environmental pollution. Although an accelerated loss of lung function is seen in close to half of subjects who develop COPD, the other half reach the disease state most likely from alterations in the pre-or post-natal period of lung development, a concept that should lead us to pay more attention to lung heath during this important period of life. Symptoms include breathing difficulty, cough, mucus (sputum) production and wheezing, that may be present for years before the diagnosis is suspected and made. Patients with COPD are at increased risk of developing several co-morbid diseases compared with age and gender matched patients without COPD, a topic that we shall expand later in this review. Once present, the disease tends to progress, causing decrease in functional capacity, disability and death. Although most patients with COPD die from cardiovascular disease or cancer, a substantial proportion do so from respiratory failure, a slow phase of the disease punctuated by exacerbations, the need to supplement oxygenation and in some cases mechanical ventilation.

The diagnosis of limitation to airflow is made during a forced vital capacity manoeuvre measured using a spirometer. This simple test is reliable, affordable and well tolerated and its use should be encouraged at all levels of care 30. In addition, the degree of airflow limitation, measured with the forced expiratory volume in one second (FEV1) is used to determine the severity of the disease, assess the response to medications, and follow disease progression31. Guidelines have been produced regarding the standardization of spirometry, the diagnosis and management of COPD and different spirometric criteria exist. When evaluating a patient for possible COPD, the spirometry is performed pre and post administration of an inhaled bronchodilator (e.g., albuterol 400 mcg) to determine whether airflow limitation is present and whether it is partially or fully reversible. Airflow limitation that is irreversible or only partially reversible with bronchodilator is the characteristic physiologic feature of COPD. Screening spirometry is not currently recommended. In contrast, spirometry should be performed in patients with symptoms suggestive of COPD, as described above32. It has been shown that primary-care spirometry testing increases the number of individuals correctly diagnosed as having COPD. Also, it does not only improve the accuracy of diagnosis but also results in significant improvements in management, without input from secondary care33.

A recommendation statement from the US preventive services task force, published in 2013, focused on the role for spirometry as screening and diagnostic tool in COPD, highlighting that the opportunity costs (time and effort required by both patients and the health care system) associated with screening for COPD using spirometry are large even in populations at higher risk. The statement points out that spirometry can lead to substantial over diagnosis of COPD in "never smokers" older than age 70 years, and

that it produces fewer false-positive results in other healthy adults37. The USPSTF concludes that there is at least moderate certainty that screening for COPD using spirometry has no net benefit. Since 4 out of 5 cases of COPD result from tobacco use, an early intervention strategy of providing evidence-based therapies proven to increase smoking cessation rates and smoking abstinence is likely to be more effective than an early detection strategy of performing spirometry on patients who do not recognize or report respiratory symptoms38-40. However, the document does encourage the use of spirometry in subjects with respiratory symptoms, those with family history of respiratory diseases and environmental exposure. On a personal clinical note, we would like to point out that the spirometry not only help detects the fixed obstruction of COPD, but importantly, the reversibility that characterises asthma and it may suggest the presence of restrictive pulmonary diseases, an increasingly important clinical problem in every day practice.

Epidemiology

In the World, more than 3 million people died of COPD in 2012, which is equal to 6% of all deaths globally that year, with more than 90% of COPD deaths occurring in low- and middle-income countries. The prevalence of COPD hovers around 10% across the World, but varies in different regions as has been determined in the well conducted population studies of PLATINO in Latin-America and BOLD in the rest of the globe. In the United States, COPD is currently the 4th cause of death and accounting for more than 125,000 adult deaths per year 3-5. The age-adjusted mortality rates varied dramatically by state, from a low of 27.1 per 100,000 in Hawaii to a high of 93.6 per 100,000 in Oklahoma. The Third National Health and

Nutrition Examination Survey (NHANES III) data—the most recent United States survey that included spirometry—showed a prevalence of COPD in adults of 6.8 percent[1]. The disease now affects men and women almost equally, due in part to increased tobacco use among women in high-income countries. Unfortunately, Over 50 percent of people with spirometric evidence of COPD, have never been diagnosed. This proportion is even higher among people with mild disease, which is most amenable to intervention[2]. COPD is responsible for about 700,000 hospitalizations annually in the United States. In recent years, the hospitalization rate among women has increased and is now similar to the rate among men. As smoking prevalence has declined over the last two decades in the United States, a stabilization in the number of admissions to hospitals and in the number of deaths has been documented. However, with close to 15% smoking rates in the country, COPD as a health problem is here to stay.

COPD primarily affects individuals over 40 years of age, with the prevalence increasing with age, with a five-fold increased risk for those aged over 65 yrs. compared with patients aged less than 40 yrs. The prevalence of COPD increases with smoking status (by a factor of five), yet never smokers comprise a substantial proportion (20 to25 % in population studies) of individuals with COPD, suggesting the existence of other risk factors, such as passive smoking, or factors of occupational exposure19. In the United States, the age-adjusted prevalence is usually higher among non-Hispanic whites compared with non-Hispanic blacks or Hispanics. The annual age-adjusted prevalence is higher in women than in men3.

A dose response relationship between educational level and COPD is present in both never smokers and smokers, suggesting that educational level might be a risk factor for COPD independent of smoking. Some possible mechanism explaining the adverse effects of low social economic

status on COPD among never smokers might be poor dietary habits (low in antioxidants and fresh fruit) 20,21, poor housing conditions 22, more occupational dust exposure and indoor air pollution from biomass combustion in low socioeconomical status group 23.

Cost of COPD to Society

The growing burden of COPD exacts an economic cost, as people are less productive, less able to work for more years of their lives, and die prematurely. The aavailable data suggest that COPD presents a large and under-estimated mortality-related cost to healthcare systems and to society[6]. The financial burden related to COPD in 1998 included an estimated direct medical cost of $14.7 billion[3-5] and an estimated indirect cost related to morbidity (loss of work time and productivity) and premature mortality of an additional $9.2 billion, for a total of $23.9 billion annually. By 2002, this cost had increased to $32.1 billion annually[3-5]. In 2014, the American College of Chest Physicians reported the total and state-specific medical and absenteeism costs of COPD among adults aged ≥18 years in the United States for 2010 and projections through 2020[7]. The report finds that in 2010, the total national medical costs attributable to COPD were estimated at $32.1 billion dollars annually, absenteeism costs were $3.9 billion for a total burden of $36 billion. In addition, an estimated 16.4 million days of work were lost due to COPD each year. The burden of the medical cost for COPD was shared as follows: 18% paid for by private insurance, 51% by Medicare, and 25% by Medicaid. The study also projects a rise in medical costs from $32.1 billion in 2010 to $49 billion by 2020[7].

Direct health care costs, accounting for nearly two-thirds of total COPD dollars, are those related to the detection, treatment, prevention, and

rehabilitation of a disease, which include: physician office visits, hospitalizations, home care, and medications[8], with a direct relationship between the severity of COPD and the overall cost of care[8]. Hospitalization was identified as the most important cost variable across all severity stages of COPD, accounting for roughly 45%–50% of the total direct cost generated by the patients[9,10]. In a survey of the burden of COPD in the United States, it was shown that almost one-fifth of patients were limited in their ability to work normally, and 6% of patients missed time from work due to COPD. In the same survey, it was reported that the total number of work days lost by patients was approximately 18.7 days per patient of working age. Work loss among the caregivers of patients with COPD was reported by 7% of respondents, with a total of 54 days lost (mean of 1 -7 days per caregiver reporting work loss)14.

There is a strong association between COPD exacerbations and hospitalization. Although the length of stay for a COPD-related hospitalization has decreased over the past 10 years, the number of admissions to the hospital has remained stable since 1999 [11]. Unfortunately, with increasing age and disease progression, exacerbations only become more frequent and severe[11]. In small single center reports and in large observational and interventional studies, anywhere from 20 to 77% of COPD patients experienced an exacerbation during one year[12]. Importantly, the most severe episodes require visits to the emergency department or hospitalizations, the cost of which is estimated to be $7100[13]. In 2006, an estimated 672,000 hospital discharges for COPD were reported[11]. No matter how it is analyzed, the cost of caring for patients with COPD is alarming. So much so, that CMS has singled out 30 days re-admissions as one target to evaluate burden of care and quality of health delivery for hospitals across the country.

Effects on the individual patient

Although the economic cost is important, more important is the effect of COPD itself on the patients. Due to its slow and usually progressive nature, there are important physical and psychological effects that profoundly affect patients with COPD. The cardinal symptom of dyspnea, often worsened by effort, leads to considerable modifications in lifestyle, such as careful planning of activities, limitation or total elimination of outings and holidays to avoid crowds, hills and stairs. The loss of work and subsequent social isolation related to limitations in activities of leisure have a profound effect on their personal experience.

Arne et al interviewed patients with COPD to explore their perspectives at the time of diagnosis. A feeling of "shame" proved to be a main theme and was related to the notion that their disease was self-inflicted. This shame was felt as a personal obstacle to seeking advice[15]. Patients also experience fear, particularly fear of breathlessness, death and dying. Shackell et al found that patients' anxiety and fears of breathlessness and dying, extended into the night and were aggravated by feelings of isolation, vulnerability and frustration[16]. This fear and sense of helplessness can lead to panic and evidence suggests that patients with chronic breathlessness often call for emergency assistance during the night likely related to exacerbation and panic of this fear[17]. The stigma of COPD is stigmatized not only by the patients, but also from members of society because it has resulted primarily from what is now considered an abnormal behaviour (smoking).In addition, the most severely affected patients are marked with oxygen equipment that singles them in the crowds and affects bodily changes, thereby disrupting their social interactions[18]. Feeling stigmatized

by the community affects self-esteem and further limits engagement in social activities, thus having implications for social support, which may lead to isolation. Stigma from health care professionals may affect health care access. Patients with COPD who feel stigmatized may hesitate to seek care for fear of judgment or negative repercussions associated with having the condition. Patients may also decrease their use of specific treatments that are associated with stigma or that show that they have COPD[18].

Not least of all is the effect of COPD on care givers, who may also experience psychological problems. They describe stress in relation to feeling restricted, anxious and profoundly helpless in the face of dyspnea, accompanied by a sense of preoccupation with their relative and a debilitating hypervigilance[17]. Thus, COPD ceases to be a disease affecting a single person, but rather a disease affecting the surrounding family conglomerate.

Under-diagnosis and mismanagement as a health care problem

Currently, COPD is primarily suspected and certainly diagnosed late in the clinical course of the disease itself and as detailed above, late in life. Well-conducted studies worldwide have shown that most spirometrically detected cases of COPD were not diagnosed. This not only is true in less developed regions. A study conducted by Damarla and co-workers showed that whereas 73% of patients admitted to a tertiary referral hospital in Boston, United States with the diagnosis of congestive heart failure, had had an echocardiogram within 7 years of the diagnosis, only 23% of patients with a diagnosis of COPD, had had a confirmatory spirometry over the same time span. Further, close to 20% of the patients with the diagnosis of COPD, who had had a spirometry, actually had a restrictive and not an obstructive pattern on the forced vital capacity maneuver. Many of undiagnosed COPD patients are isolated at home, are in nursing or senior-assisted living

facilities, or are present in oncology and cardiology clinics as patients with lung cancers and coronary artery disease (CAD) [24]. Misdiagnosis potentially results not only in an individual patient being misinformed and incorrectly educated about their condition, but can also lead to incorrect management. Several reasons underlie the failure to diagnose COPD in a timely fashion. Respiratory symptoms are often not felt or are underestimated by the patient, who considers his symptoms as logical effects of age or smoking habits [25,26]. As a consequence, the patient does not consult his doctor until his symptoms are aggravated, mainly due to exacerbations. Also the general practitioner (GP) can underestimate the situation in these occurrences, diagnosing the episode only as an independent acute event (acute bronchitis) rather than an epiphenomenon of an unrecognized chronic problem, thus neglecting to look further into the clinical history. The result is that up to 80% of subjects affected with airways obstruction have never had a diagnosis of COPD, and even among those with severe obstruction fewer than half have already been diagnosed [27]. A missed diagnosis influences the timing of therapeutic intervention, thus contributing to the evolution into more severe stages of the illness. Hill et al reported that, although more than three-quarters of the patients with COPD report at least one respiratory symptom, two-thirds ware unaware of their diagnosis. These findings suggest that adults who attend a primary care practice with known risk factors for COPD are important targets for case detection and early intervention[28].

Given that the majority of reported COPD costs in United States are associated with unscheduled care, specifically inpatient hospitalizations, improving the long-term management of the disease in primary care could reduce the burden of the disease. Improving disease management in the United States may require an increase in the availability and usage of

interventions that can prevent exacerbations, reduce the risk of hospitalizations, improve symptom control, delay disease progression and reduce the risk of comorbidities in patients with COPD[14].

Underdiagnosis of COPD

Compared with the diagnosis and follow up of other non-communicable diseases, such as coronary artery disease (CAD, which requires electrocardiogram, echocardiogram, stress test, cardiac catheterization or angiogram), diabetes mellitus (DM, which requires several blood tests such as glycohemoglobin test, fasting plasma glucose test or an oral glucose tolerance test) and lung cancer, with its very complex diagnostic algorithms, the diagnosis of COPD, which relies on spirometry, is relatively simple. Spirometry is objective, noninvasive, sensitive to early change and reproducible. With the availability of portable meters it can be performed almost anywhere and, with the right training, it can be performed by anybody.

Despite the proven value of a spirometry, its routine use is something health care providers have been slow to accept. While no physician would give insulin to a diabetic without measuring blood sugar or an antihypertensive to a patient without measuring blood pressure, these same physicians often prescribe powerful beta agonists, anticholinergics and even corticosteroids without performing spirometry.

Many smokers manifest respiratory symptoms (cough, sputum production and even dyspnea) without meeting the spirometric definition required to confirm a COPD diagnosis — those whose FEV_1:FVC after bronchodilator use is equal to or greater than the conventionally accepted cut-off of 0.70. The study—SPIROMICS (Subpopulations and Intermediate Outcome

Measures In COPD Study), with the aim of shedding light on symptomatic smokers, collected data on symptoms, pulmonary function and biomarkers, between 2010 and 2015 on 2,736 current smokers and former smokers with a smoking history of more than 20 pack-years, from multiple centers in the United States[34]. This study revealed that about half of the smokers had COPD-like symptoms despite having normal spirometry readings. CT lung scans also revealed that many had thickening of the airways, which occurs in people with chronic bronchitis. Symptomatic smokers had more frequent respiratory illnesses or flare-ups that required the use of respiratory medications or medical attention, including hospitalization, than nonsmokers and asymptomatic smokers[34]. Another study found that smokers and nonsmokers without COPD but with signs of emphysema on CT scans had higher all-cause mortality rate than those without lung damage[35]. The same group also recently showed that respiratory diseases and lung cancer were the most common causes of death in people with lung damage at CT scan but no airflow obstruction, including those who were not heavy smokers[36]. To date, the best way to treat, if at all, symptomatic smokers without airflow limitation is still unknown. The great majority of the clinical trials have only studied COPD as defined by spirometry. Going forward, more studies aimed at studying the natural course of patients with COPD like symptoms but without spirometric evidence of obstruction would be recommendable. Likewise, the effect of therapies aimed at those symptoms deserve attention.

Management of COPD

The goals of effective COPD management are to: 1) prevent disease progression; 2) relieve symptoms; 3) improve exercise tolerance; 4)

improve health status; 5) prevent and treat complications; 6) prevent and treat exacerbations; and 7) reduce mortality. To date, treatment with medications alone is not enough to completely control COPD symptoms, and most importantly to significantly alter the progression of disease in the majority of patients. Thus, a reduction of therapy once symptom control has been achieved is not normally possible in COPD. Further deterioration of lung function usually requires the progressive introduction of more treatments, both pharmacologic and nonpharmacological, to attempt to limit the impact of these changes. Promotion of a healthy lifestyle and non-pharmacological interventions in the treatment of chronic obstructive pulmonary disease (COPD) has received great attention in recent decades.

The first step to take is to reduce the risk factors for COPD. Namely, a reduction of total personal exposure to tobacco smoke, occupational dusts and chemicals, and indoor and outdoor air pollutants are important goals to prevent the onset and progression of COPD. Smoking cessation is the single most effective and cost-effective way to reduce the risk of developing COPD and stop its progression. A brief tobacco dependence treatment is effective and every tobacco user should be offered at least this treatment at every visit to a health care provider.

Whilst smoking cessation is crucial in the treatment and prevention of progression of the disease, physical activity is an important element in the treatment of COPD[41,42]. Physical activity has the potential to prevent or delay onset of other chronic diseases, and indeed in patients with COPD, physical activity is associated with a relatively reduced risk of hospitalization and death[43,44]. The level of daily physical activity is, however, remarkably reduced in individuals with COPD compared to healthy individuals[45,46]. A simple test that can help clinicians evaluate the functional capacity of patients with COPD is the validated 6 minute walk distance test. The

longest of two walks conducted 30 minutes apart, asking the patient to cover as much distance as possible over 6 minutes (6MWD), using a 30 meters corridor has proven to be a very good predictor of risk of death and hospitalization. A distance walked of 350 meters or lower identifies individuals at higher risk of death. The predictive value of risk of death is improved if the 6MWD is integrated with the body mass index, the FEV1 and the dyspnea score measured with the modified Medical Research Council scale, to provide the BODE index single multidimensional score.

Indeed physical inactivity, smoking, alcohol consumption and obesity are cardiovascular risk factors and associated with the onset of other chronic diseases including type 2 diabetes, hypertension and dyslipidaemia[42,44]. Thus, a general health campaign promoting a healthy lifestyle in patients with COPD should focus on physical activity, smoking cessation, a limited alcohol intake and a balanced caloric intake.

COPD and comorbidities

Patients with COPD are frequently afflicted with other co-morbidities. Several well-conducted studies have shown that this co-occurrence is more frequent in patients with COPD that in patients without that diagnosis. The exact reason why this occurs is not known, but it is likely that the exposure to a common injurious agent, such as cigarette smoke, by the nature of its systemic invasive properties, affects other susceptible organs. However, not every smoker gets COPD and therefore the manifestations of multiple diseases affecting certain smokers who do develop COPD, supports the concept of increased susceptibility for that particular group, or the absence of compensatory mechanism capable of controlling the multiple sites

affected by the substances contained in cigarette smoke and other pollutants.

The most important co-morbidites to consider are: coronary artery disease, lung cancer, Gastro-esophageal reflux, anxiety, depression, osteoporosis and muscle disuse atrophy. All of these should be thought of, screened or actively thought for in patients with COPD.

COPD, kidney, and microalbuminuria

Chronic obstructive pulmonary disease (COPD), a major cause of mortality[1], is associated with comorbidities, most importantly cardiovascular disease[2;3]. Albuminuria, a marker of endothelial dysfunction/injury and inflammation[4], is associated with worse cardiovascular outcomes in patients with diabetes mellitus[5], hypertension[6], and the general population[7]. Approximately 24% of COPD patients (versus 4% of controls) have persistent albuminuria[8;9]. Albuminuria severity correlates with degree of hypoxemia in stable COPD patients and during exacerbations[8;9], and is associated with systemic inflammation[10] and increased mortality risk independent of cardiovascular comorbidities[11].

Several studies have suggested a link between COPD and renal dysfunction. In a cohort of smokers screened for lung cancer, there was an association between emphysema severity and the estimated glomerular filtration rate (eGFR)[12]. COPD patients have a higher prevalence of renal failure than age-matched controls without COPD[13]. A study of over 900,000 subjects followed for 10 years reported a 2-fold increase in the risk of dying from renal failure in smokers versus non-smokers after adjusting for potential confounders[14]. These results support the notion that the link between COPD and renal dysfunction is due to the vascular effects of

cigarette smoke (CS). However, no prior studies have assessed whether COPD is associated with structural renal lesions.

Aims

We tested the hypothesis that CS exposure causes simultaneous injury to the lungs and kidneys by increasing tissue oxidative stress levels leading to injury to both the pulmonary and renal endothelium (and other pulmonary and renal cells) in humans and mice. This hypothesis could explain the albuminuria developing in some COPD patients. We also tested the hypothesis that CS-induced pulmonary and renal endothelial cell (EC) injury is associated with increased EC levels of advanced glycation end-products (AGEs) and the receptor for AGEs (RAGE). The rationale for this second hypothesis is that oxidative stress leads to the generation of AGEs which activate the RAGE[15], and both AGEs and RAGE have been linked to COPD pathogenesis in humans and mice[16;17]. Also, in diabetic nephropathy, increased renal EC levels of AGEs and RAGE are linked to both endothelial dysfunction/injury and albuminuria[18;19].

We also assessed whether increased urinary albumin:creatinine ratios (UACRs) occur in a subset of an upstream longitudinal cohort of smokers, and whether UACRs correlate with rate of decline in forced expiratory volume in 1 second (FEV_1) in these subjects. In mice, we investigated whether CS also increases UACRs, and the increased UACRs are associated with increased renal and pulmonary oxidative stress levels leading to increased AGEs and RAGE levels in pulmonary and renal ECs. We also assesses whether these changes in mice are associated with the development of pulmonary and renal endothelial injury and chronic end-organ lesions. Angiotensin-converting enzyme inhibitors ([ACEi] such as

Enalapril) which have anti-oxidant properties[20;21], are used to treat patients with microalbuminuria secondary to diabetes mellitus and hypertension. Thus, we also assessed whether treating CS-exposed mice with Enalapril ameliorates the pulmonary and renal lesions induced by CS by reducing AGEs and RAGE levels in ECs, and generalized EC injury.

Materials and Methods

Human Studies

Inclusion criteria: Patients were diagnosed as having COPD and included in the study if they had ≥ 20 pack-year smoking history and spirometrically-confirmed airflow limitation with $FEV_1/FVC < 0.7$. Among 6736 patients at Brigham and Women's Hospital (BWH) that had a renal biopsy (from 2011 to 2015) or a nephrectomy (from 2008 to 2015), 26 COPD patients met these criteria. Renal samples were also available from 32 non-smokers without COPD [NSC] and 24 ever-smokers [SC]) without COPD. Smoker controls had a cigarette smoking history of ≥ 10 pack years. Current smokers were defined as active smokers at the time of the study or smokers who had stopped smoking less than 1 year before being studied. NSC were all never-smokers in the nephrectomy cohort. NSC in the renal biopsy cohort included never-smokers (76%) and smokers (24%) who had a < 10 pack-year history and had stopped smoking for > 2 years before the renal biopsy. DLCO adjusted by the alveolar volume (DLCO/VA) measurements were only available in the medical records for 58% of COPD patients, 67% of smoker controls without COPD (SC), and 62.5% of non-smokers controls without COPD (NSC). The % predicted DLCO/VA values of the COPD group were significantly lower (median: 77; interquartile range [IQR]: 44-91%) than those of the SC (median: 88; IQR: 78-95%; $P = 0.036$) and NSC (median: 91; IQR: 78.5-98%; $P = 0.01$) groups.

Exclusion criteria: Samples with an incomplete evaluation and allograft renal biopsies were excluded. Samples from subjects younger than 50 years of age, and subjects that did not have pulmonary function tests (PFTs) or had acute kidney injury before the procedure (defined by Kidney Disease: Improving Global Outcomes [KDIGO] criteria as either an increase

in serum creatinine of ≥ 0.3 mg/dl within 48 h, or a 1.5 fold increase over baseline values in the 7 days prior to the procedure) were also excluded. Patients with physician-diagnosed diabetes mellitus (DM), autoimmune diseases, or vasculitis or with PFTs indicating restrictive lung diseases were excluded. As none of the subjects had physician-reported DM, glycated hemoglobin levels were not measured on any of the subjects studied. However, all the subjects studied in both cohorts had plasma glucose levels within the normal reference range in their medical records. In the biopsy cohort, subjects with renal cancer and subjects that had undergone a renal transplant were excluded (**Figure 1**).

Nephrectomy samples: The nephrectomy cohort was used to analyze pathologic changes in renal sections. Non-neoplastic parenchymal changes in tumor nephrectomy specimens are routinely evaluated using light microscopy by the Renal Pathology Service at BWH. These changes are described in detail in the final pathology reports. All the pathology reports were generated by this service and filed in the medical records before this study was initiated. The parameters that were recorded on the tissue sections included the main renal pathology diagnosis, the percentage of each kidney that had global glomerulosclerosis, tubular atrophy, or interstitial fibrosis, and the extent of vascular sclerosis (scored as none [1], mild-moderate [2], or severe [3]). Pertinent information regarding the main cause of renal injury and the extent of overall chronic renal injury was provided to us by a renal pathologist (VB). Also, Masson's Trichrome staining (a histochemical stain that is commonly used to evaluate connective tissue deposition in renal sections[1]) was performed on 5 μm thick renal sections from a subset of the subjects.

Renal biopsy samples: The renal biopsy cohort was used to assess ultra-structural abnormalities in 65-87 nm sections of kidneys from COPD patients and controls. Renal biopsies undergo preparation and examination by electron microscopy (EM) as part of patients' clinical care. For many of the subjects that underwent a renal biopsy, the renal pathologist could identify only non-specific changes rather than pathologic findings of a specific renal pathology (and these subjects are listed in the "others" category). EM digital image files were also reviewed by a renal pathologist (VB) for each case to evaluate the presence of glomerular capillary wall remodeling and double contour formations due to repetitive endothelial cell (EC) injury. Double contours consist of subendothelial widening, cellular interposition, and new basement membrane formation. The frequency of such capillary wall duplications and the severity of double contour was scored (no double contours were scored as 1; rare double contours were scored as 2; and several double contours were scored as 3).

Estimated glomerular filtration rate (eGFR): The eGFR was calculated for all the patients in whom the values of serum creatinine at the time of the nephrectomy or biopsy were available. Three independent eGFR calculators available online (http://egfrcalc.renal.org/; http://nephron.com/cgi-bin/CGSI.cgi; www.davita.com) were used to calculate the eGFR from the following parameters: age, sex, serum creatinine, and race (black or white). The three calculators gave comparable results (coefficient of variation $\pm$ 5%), and the results were averaged for each subject. None of the subjects included in the analysis of eGFR had acute kidney injury before the procedure as defined using KDIGO criteria.

Analysis of lung and kidney paraffin-embedded samples: We used an additional human cohort for these studies as unstained sections from the nephrectomy and renal biopsy cohorts used for pathologic and ultrastructural assessments were not available. Lung samples from 5 COPD patients, 5 SC, and 5 NSC (all of which were never-smokers) were obtained from subjects undergoing resection of tissue for lung nodules or localized carcinoma, at least 10 cm away from the primary lesion (**Table 3**). Renal samples were obtained from 5 COPD patients, 6 SC, and 5 NSC (all of whom were never-smokers) who underwent a unilateral nephrectomy for the presence of a renal tumor (**Table 4**). None of the subjects included in the immunostaining staining studies were taking angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers at the time of the procedure.

Pulmonary and renal sections were processed in the same manner. The sections were deparaffinized, and antigen retrieval was performed by boiling the slides immersed in 0.01 M sodium citrate and 2 mM citrate buffer (pH 6.0) in a microwave for 10 minutes. To identify pulmonary and renal advanced glycation end products (AGEs) and their receptor (RAGE), pulmonary and renal sections were triple immunostained with: 1) rabbit anti-AGEs IgG followed by a goat anti-rabbit IgG conjugated to Alexa-488; 2) goat anti-RAGE IgG followed by a rabbit anti-goat IgG conjugated to Alexa-647; and 3) ovine anti-von Willebrand factor (vWF) IgG (as a marker of endothelial cells) followed by a donkey anti-ovine IgG conjugated to Alexa-546. All of the primary antibodies listed were purchased from Abcam (Cambridge, MA). All of the secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Lung sections were also immuno-stained with three isotype-matched non-immune control primary antibodies. Lung sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Images of the immuno-stained lung sections were captured and analyzed using a confocal microscope (Olympus Corporation, Center Valley, PA)[2].

Analysis of urinary albumin-creatinine ratios (UACRs) in ever-smokers in the Lovelace Smokers Cohort: Urine samples from smokers were obtained from subjects in the Lovelace Smokers Cohort (LSC), an observational study of ever-smokers being conducted in the greater Albuquerque metropolitan area in New Mexico[3;4]. Subjects (n = 2400) were followed for over 5 years and at least three spirometries were used to calculate the annual rate of change in FEV_1 (**Table 5**). A previous report from our group showed that there is an association between the use of ACEi and protection from rate of FEV_1 decline in rapid FEV_1 decliners (> 40 ml FEV_1 decline/year)[5]. We selected 148 subjects having a broad range of rate of FEV_1 declines in whom to measure UACRs on first-void morning urine specimens. None of the subjects selected for this study were taking ACEi or angiotensin receptor blockers or had diabetes mellitus at the time of study and 28% had COPD at baseline as defined by spirometrically-confirmed airflow limitation with $FEV_1/FVC < 0.7$. We measured urinary albumin:creatinine ratios (UACRs) using a commercially-available ELISA kit for albumin (R&D Systems, Minneapolis, MN) or colorimetric assay for creatitine (Bioassays systems, Hayward, CA). The assays were performed following the manufacturer's instructions.

Animal Studies

All experiments conducted on mice were approved by the Harvard University Medical School Institutional Animal Care and Use Committee.

CS exposures and Enalapril treatments: C57BL/6 strain wild type (WT) mice (males and females aged 10-12 weeks) were exposed to either filtered air or cigarette smoke (CS) using a Teague TE-10z device (Teague Enterprises, Woodland, CA) for 2 h per day, 6 days-a-week for up to 24 weeks, as described previously[6]. This model has been shown to induce lesions similar to COPD in humans[7]. A therapeutic dosing strategy was used to assess the efficacy of Enalapril in established CS-induced pulmonary and renal injury. Vehicle (endotoxin-free normal saline) or a solution of Enalapril in endotoxin-free normal saline (25 mg of Enalapril/Kg body weight; Selleck Chemicals, Houston, TX) were delivered to mice 6 days-a-week using intra-peritoneal (i.p.) injections beginning at the mid-point of either 4 wk acute CS exposures or 24 wk chronic CS exposures. Urine was collected weekly during the exposures. Five cohorts of mice were studied in total, 2 cohorts of mice were studied in the acute CS exposures (10-15 mice/treatment group), and 3 cohorts of mice were studied in the chronic treatments (8-10 mice/group).

At the end of the acute CS exposures, mice were euthanized, BAL was performed[7], kidneys were removed and one kidney was frozen at -80^{o}C and the other kidney was fixed in 10% formaldehyde for light microscopy and immunostaining experiments. Leukocytes were counted in bronchoalveolar lavage (BAL) samples[7], and UACRs were measured using commercially-available kits (Genway, San Diego, CA for albumin and Bioassays Systems, Hayward, CA, for creatinine).

At the end of the chronic CS exposures, mice were euthanized, kidneys were removed, and one was frozen at -80^{o}C and the other one was either: 1) fixed in 10% formaldehyde and then embedded in paraffin for further light microscopy or immunostaining and confocal microscopy analyses; or 2) fixed in EM fixative (0.1 M sodium cacodylate buffer [pH 7.4]

containing 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% picric acid, 1% osmium tetroxide and 1.5% potassium ferrocyanide) for 1 h for EM analysis. The samples for EM analyses were washed in water containing 1% uranyl acetate, and then dehydrated and infiltrated with a 1:1 mixture of Epon and propyleneoxid, and incubated in an embedding mold filled with fresh Epon for 24-48 h at 60°C. Lungs were removed and frozen at -80^{o}C or inflated at 25 cm H_2O pressure and fixed in 10% saline buffered formaldehyde to measure chronic COPD-like lung pathologies, or used for immunostaining assays. UACRs were measured on urine samples from mice exposed chronically to air or CS using commercially-available kits (see above).

Chronic Enalapril experiment:

Analysis of glomerular major diameter: In the 24 wk experiment, sections of kidneys from 3 mice exposed to air, 4 mice exposed to CS and treated with saline, and 3 mice exposed to CS and treated with Enalapril were deparaffinized and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. For each sample, at least 20 randomly-selected glomeruli were evaluated using a Leica epi-fluorescence microscope (Leica Microsystems, Buffalo Grove, IL). The major diameter of the glomeruli was measured using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Analysis of podocyte base width: In the 24 wk experiment, sections of kidneys from 3 mice exposed to air, 4 mice exposed to CS and treated with normal saline, and 3 mice exposed to CS and treated with Enalapril were fixed in EM fixative and processed, as described above. Sections were examined using a Tecnai G² Spirit BioTWIN EM (FEI Electron Optics International, Hillsboro, OR). Images of the tissue sections were captured at

10000 X magnification and the width of the podocyte foot processes was measured using MetaMorph software.

Analyses of air-space enlargement and small airway fibrosis (SAF): Airspace enlargement and SAF were measured in sections of inflated lungs from mice exposed to air for 24 weeks, or exposed to CS for 24 weeks and treated 6 days-a-week with either normal saline or a solution of Enalapril in normal saline (25 mg/kg body weight) during the second 12 weeks of the CS exposure (n = 5-11 mice/group). Distal airspace size was measured in Gills-stained lung sections (as mean alveolar chord length in microns), and SAF was measured around small airways having a luminal diameter between 300 and 699 microns in Masson's Trichrome-stained lung sections, exactly as described previously[6]. Images of the lung sections were captured at 200 X magnification (to measure airspace enlargement) or 400 X magnification (to measure SAF) magnification. Images of the lung sections were analyzed using Scion Image software (for airspace enlargement)[6] or MetaMorph software (for SAR).

TUNEL staining in endothelial cells in the lungs and kidneys of mice: In the 24 wk experiment, pulmonary and renal sections from mice exposed to air, or mice exposed to CS and treated with either saline or Enalapril (4-5 mice/group) were deparaffinized. To identify pulmonary and renal Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL)-positive endothelial cells, sections of lungs and kidney were double immunostained with: 1) a commercial TUNEL kit (Apoalert DNA Fragmentation Detection Kit from Clontech Laboratories, Mountain View, CA) using TdT Terminal Transferase-negative TUNEL as a negative control; and 2) an ovine anti-von Willebrand factor (vWF) IgG followed by donkey anti-ovine IgG conjugated to Alexa-546 (to detect endothelial cells; ECs). The primary antibody was purchased from Abcam, and the secondary

antibody was obtained from Invitrogen. Lung sections were also immuno-stained with an isotype-matched non-immune control antibody. Lung sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images of the immuno-stained lung sections were captured and analyzed using a confocal microscope (Olympus Corporation, Chelmsford, MA). Images of the lung sections were captured at 600 X magnification using a Leica epi-fluorescence microscope, and analyzed using MetaMorph software.

Immunostaining for AGE and RAGE in pulmonary and renal endothelial cells in mice: In the 24 week experiment, pulmonary and renal tissue sections of mice exposed to air, or mice exposed to CS and treated with saline or Enalapril (3-5 mice/group) were deparaffinized, and antigen retrieval was performed by boiling the slides immersed in 0.01 M sodium citrate and 2 mM citrate buffer (pH 6.0) in a microwave for 10 minutes. Tissue sections were triple immunostained with: 1) rabbit anti-AGEs IgG followed by goat anti-rabbit IgG conjugated to Alexa-488; 2) goat anti-RAGE IgG followed by rabbit anti-goat IgG conjugated to Alexa-647; and 3) ovine anti-von Willebrand factor (vWF) IgG followed by donkey anti-ovine IgG conjugated to Alexa-546 (vWF was used as a marker of endothelial cells [ECs]). All of the primary antibodies listed were purchased from Abcam, and all of the secondary antibodies were obtained from Invitrogen. Lung sections were also immuno-stained with three appropriate isotype-matched non-immune control antibodies. Lung sections were counterstained with DAPI. Images of the immuno-stained lung sections were captured and analyzed using a confocal microscope (Olympus Corporation)[2]. The expression of RAGE in lung and kidney endothelial cells was quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA) as intensity of RAGE fluorescence per endothelial area (measured in pixel^2). For each

sample, at least 10 randomly-selected high-magnification fields in which endothelial cells were present were evaluated. The results were expressed as fold change over the mean results for air-exposed mice.

AGEs levels in murine lungs and kidneys: In the 24 week experiment, AGEs levels were measured in homogenates of frozen lungs and kidneys from the mice. A commercially-available kit was used to measure AGEs levels (Cell Biolabs, San Diego, CA) in tissue homogenates. AGEs levels were corrected for total protein concentrations measured in the samples using a commercially-available kit (Life Technologies, Carlsbad, CA). Data are presented as fold change over the mean results for air-exposed mice.

Acute enalapril experiment:

Oxidative stress and AGEs levels, and apoptosis rates in murine lungs and kidneys: Mice were exposed to air or CS for 4 weeks. In CS-exposed mice, treatment with either endotoxin-free normal saline or a solution of Enalapril in endotoxin-free normal saline (25 mg Enalapril/Kg body weight) on 6 days-a-week was initiated at the mid-point of the exposures and continued for the second 2 weeks of the CS exposures. Oxidative stress, AGEs, and active caspase-3 levels were then measured in homogenates of frozen lungs and kidneys from the mice as outlined below.

Oxidative stress levels were measured as thiobarbituric acid reactive substances (TBARS) using a commercially-available kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. Commercially-available kits were used to measure AGEs levels (Cell Biolabs, San Diego, CA) in tissue homogenates. The results for TBARS and AGEs levels were corrected for total protein concentrations measured in the samples using a commercially-available kit (Life Technologies, Carlsbad, CA). Data are presented as fold change over the mean results for air-exposed mice.

Apoptosis was measured in pulmonary and renal samples by quantifying intracellular levels of active caspase-3 in cytoplasmic fractions isolated from whole lung and renal samples using a NE-PER™ Nuclear and Cytoplasmic Extraction kit (Thermo Fisher scientific, Cambridge, MA). Active caspase-3 levels in these samples were quantified using a quenched fluorogenic substrate that is specific for active caspase-3, assays standards of recombinant active caspase-3, and fluorimetery, exactly as described previously[8]. The results for active caspase-3 levels were expressed as fold change relative to the mean activity detected in tissue samples from air-exposed mice.

ACE-I and Angiotensin II (Ang-II) levels in serum samples, lungs, and kidneys from mice: ACE-I and Ang-II levels were measured in serum samples and homogenates of lungs and kidneys from mice (n = 6-13 mice/per group) in the acute (4 week) study using commercially-available ELISA kit (R&D Systems, Minneapolis, MN).

Results

Human studies

Human renal pathology analyses: **Tables 1** and **2** show demographic and clinical characteristics of the patients that underwent a nephrectomy or a renal biopsy, respectively. Among COPD patients in the biopsy cohort, 57.1% had GOLD stage I-II and 42.9% had GOLD stage III-IV COPD. Among both cohorts, 61.5% of COPD patients had GOLD stage I-II COPD and 38.5% had GOLD stage III-IV COPD. COPD patients did not differ from SC in age, sex ratios, race, ethnicity, pack-year smoking histories, or current smoking status. A similar proportion of COPD patients and controls in both cohorts had hypertension and cardiovascular disease (defined as physician-diagnosed coronary artery disease, congestive heart failure, myocardial infarction, or cardiomyopathy).

Fig. 2 shows representative images of Masson's Trichome-stained renal sections from COPD patients and controls in the nephrectomy cohort. The COPD patients had a higher percentage of sclerotic glomeruli and more global glomerulosclerosis (**Fig. 3A)** and tubulo-interstitial fibrosis (**Fig. 3B;** median: 15%; IQR: 10-22.5%) than SC (median: 6%; IQR: 1-15%) and NSC (median: 10%; IQR: 5-15%). COPD patients also had greater vascular sclerosis (**Fig. 3C;** median: 3%; IQR: 2.5-3%) than SC (median: 2%; IQR: 1-2.7%) and NSC (median: 2%; IQR: 1.5-2%). Most COPD patients had evidence of repetitive renal EC injury with glomerular capillary wall remodeling visible as double contours in the glomerular basement membranes. **Fig. 4** shows representative images of absent, rare, and frequent double contours occurring in NSC, SC, and COPD kidneys, respectively. NSC had no or few double contours, most SC had a few double contours, but 57% of COPD patients had frequent double contours (**Fig. 5A**). COPD patients had a higher double contour frequency score (median: 2.7; IQR: 2-3) than either SC (median: 2; IQR: 1.2-2) or NSC

(median: 2; IQR: 1-2; **Fig. 5B**). In the nephrectomy cohort, 80% of the COPD patients, 25% of SC, and 8% of NSC had at least moderately-severe chronic renal injury as defined by glomerular, and/or tubular and/or interstitial pathologies. In the biopsy cohort, 50% of COPD patients had severe double contours compared with 17% of SC, and 0% of NSC.

Relationships between eGFR, FEV_1, and double contour frequency: When the biopsy and nephrectomy cohorts were combined, the eGFR was significantly lower in COPD patients than either the SC or NSC (**Fig. 6A)**. None of the subjects studied had evidence of acute kidney injury prior to the procedure as determined by Kidney Diseases Improving Global Outcome criteria. The eGFR had a significant positive correlation with the FEV_1 % predicted (**Fig. 6B**). Subjects with the highest double contour frequency score (which were mostly COPD patients) had significantly lower FEV_1 % predicted values and lower eGFRs than patients with intermediate or low double contour frequency scores (**Figs. 6C** and **6D**).

UACRs in smokers and rate of FEV_1 decline: Albumin was detected in urine samples from most smokers in the LSC. However, UACRs did not correlate with rate of FEV_1 decline in this cohort of smokers (not shown).

Animal Studies:

Effects of CS exposure: CS exposure increased UACRs in WT mice from baseline (median: 110; IQR: 87-128 μg/mg) as early as after 4 weeks of CS exposure (median: 250; IQR: 230- 663 μg/mg) and remained elevated after 24 weeks of CS exposure (median: 368; IQR: 307-534 μg/mg; **Fig. 7**). After 24 weeks of CS exposure, WT mice had smaller glomeruli than air-exposed

mice (**Fig. 8A**) and pronounced widening of podocyte foot processes (**Fig. 8B**), an invariable feature of glomerular diseases associated with proteinuria[27;28]. CS exposure did not induce ultra-structural lesions in the renal tubules in mice (not shown). As expected, CS-exposed mice developed emphysema and small airway fibrosis (**Figs. 9A and 9B**).

Enalapril in the chronic experiment: Mice exposed to CS for 24 weeks had more TUNEL-positive ECs in pulmonary small vessels than air-exposed mice and more TUNEL-positive ECs in renal capillaries (median: 4; IQR: 3.0-8.0 fold change vs. air) than in air-exposed mice; **Fig. 10**). CS-exposed mice had higher AGEs levels (**Fig. 11A**) in homogenates of lungs (median: 5.4; IQR: 3-6.6 fold change vs. air) than air-exposed mice. CS-exposed mice also had higher AGEs levels in homogenates of kidneys (median: 4.2; IQR: 3.4-9 fold change vs. air) than air-exposed mice. CS-exposed mice had higher RAGE staining in pulmonary ECs (median: 7.8; IQR: 5.4- 29.6 fold change vs. air-exposed mice). CS-exposed mice also had higher RAGE staining in renal ECs than air-exposed mice (**Fig. 11B**), and the greatest RAGE staining was present in glomerular ECs.

Enalapril therapy initiated at the mid-point of 24 week CS exposures reduced the progression of endothelial injury (assessed with TUNEL staining) in lungs and kidneys (Enalapril median: 2; IQR: 1.9-2.6 fold change vs. air; **Fig. 10**), glomerular shrinkage (**Fig. 8A**), ultrastructural podocyte injury (**Fig. 8B**), emphysema development (Enalapril median: 25.1; IQR: 2.1-0.8 μm; **Fig. 9A**), and small airway fibrosis (Enalapril median: 15.0; IQR: 13.8-16.6 μm; **Fig. 9B**). Enalapril therapy limited the CS-induced increases in pulmonary AGEs levels (Enalapril median: 2.3; IQR: 1.2-4.3 fold change vs. air-exposed mice; **Fig. 11A**). Enalapril also reduced the CS-induced increases in renal AGEs levels (Enalapril median: 2.3; IQR:

1.4-2.4 fold change vs. air-exposed mice; **Fig. 11A**), and RAGE staining in pulmonary ECs (Enalapril median: 0.5; IQR: 0.2-0.8 fold change vs. air-exposed mice) and renal ECs (**Fig. 11B**).

Enalapril in the acute experiment: Enalapril therapy that was initiated at the mid-point of 4 week (acute) CS exposures reduced pulmonary inflammation **(Fig. 12A**) and UACRs (Enalapril median: 0.2, IQR: 0.1-0.3 μg/mg) when compared with saline-treated mice (**Fig. 12B**). Enalapril therapy also reduced tissue oxidative stress levels in lungs (Enalapril median: 0.95; IQR: 0.4-1.3 fold change vs. air-exposed mice; **Fig. 12C**), and kidneys (Enalapril median: 0.7; IQR: 0-1.7 fold change vs. air-exposed mice; **Fig. 12C**), AGEs levels in homogenates of lungs (Enalapril median: 3.2; IQR: 1-5.4 fold change vs. air-exposed mice; **Fig. 13A**) and kidneys (Enalapril median: 0.8; IQR: 0.7-3.5 fold change vs. air-exposed mice; **Fig. 13A**), and active caspase-3 levels in lungs and kidneys (not shown). Enalapril therapy also reduced RAGE staining in ECs in lungs and kidneys (not shown).

In CS–exposed mice, Enalapril therapy increased ACE-I levels in lungs and increased (rather than decreased) serum angiotensin-II (Ang-II) levels when compared with saline-treated mice (not shown). Ang-II was not detectable in renal samples in any experimental condition.

AGEs and RAGE in human pulmonary and renal ECs: We evaluated whether AGEs and RAGE levels are elevated in ECs in lungs and kidneys from COPD patients as well as in ECs in these organs from CS-exposed mice. Pulmonary and renal sections from COPD patients had greater AGEs and RAGE immuno-staining in ECs (identified by co-staining sections for

vWF) than SC and NSC (**Figs. 14 and 15,** respectively). None of the subjects studied were taking ACEi.

Discussion

We show that chronic renal lesions (with injury to glomeruli, and the renal tubules and interstitium) are more frequent in COPD patients than controls. Studies of samples from humans and CS-exposed mice provide evidence that pulmonary and renal/endothelial injury in small vessels (likely mediated by increases in the oxidative stress-AGES-RAGE pathway) may explain the coincident pulmonary and renal lesions detected in COPD patients. To our knowledge, this is the first time that chronic pulmonary and renal lesions have been described in synchrony and linked to CS exposure. Therapeutic intervention with an ACEi limited the progression of both renal and pulmonary disease in CS-exposed mice, suggesting that ACEi might limit disease progression in human COPD patients with endothelial dysfunction/injury as evidenced by microalbuminuria.

COPD, smoking, and renal and pulmonary injury

The association between human COPD and renal dysfunction has been suspected but no prior study has assessed whether COPD patients have chronic renal lesions to explain the links between albuminuria, renal function, smoking, and COPD. An increased odds ratio for renal failure was reported in COPD patients versus age-matched controls[13], and COPD prevalence was inversely related to renal function in vascular surgery patients[29]. An indirect association between high resolution computed tomorgaphy-determined emphysema (but not airway dimension) and decreased eGFR was reported in smokers screened for lung cancer[12]. Persistent microalbuminuria was reported in 24% of COPD patients versus 4% of age-matched controls, correlated with the degree of hypoxemia[9], and increased during COPD exacerbations[8]. The presence of microalbuminuria was associated with increased risk of death in COPD patients[11], and current smoking is linked to death from renal failure in subjects without COPD[14].

However, none of these prior studies investigated whether COPD patients have renal pathologies to explain these associations or the mechanism involved.

The presence of renal endothelial injury in COPD patients is supported by the pathologic and ultrastructural abnormalities to glomeruli, and the presence of more frequent double contours (a marker of repetitive renal endothelial injury[30]) in kidneys from COPD patients versus SC. Chronic glomerular injury in humans can induce secondary injury to the tubules and interstitial fibrosis[31], and we also detected tubular and interstitial lesions in kidneys from COPD patients that were greater than those detected in SC. The renal lesions detected in COPD patients could not be explained by differences in age, pack-year smoking history, current smoker status, or co-morbidities between the COPD patients and controls. African American race is associated with an increased prevalence of chronic kidney disease[32]. However, it is noteworthy all of the subjects studied herein were non-hispanic whites except for two hispanic whites in the biopsy cohort. Thus, it is unlikely that our findings were related to race or ethicity differences between the study groups.

The eGFR was lower in COPD patients than controls, independent of their smoking history, and eGFR correlated directly with FEV_1. Patients with the highest double contour frequency score had lower eGFRs and lower FEV_1 percent predicted values, thereby linking renal EC injury to pulmonary and renal injury. However, we cannot determine whether there is a causal link between the pulmonary and renal lesions. Likely, CS exposure leads to generalized endothelial injury affecting both the lungs and kidneys leading to coincident, chronic injury to both organs. It is possible that in COPD patients, changes caused by or linked to COPD per se (such as hypoxemia, chronic systemic inflammation, and increased oxidative stress levels)

contribute to the progression of renal injury by increasing the severity of renal endothelial injury[33]. To investigate this possibility, additional studies are needed in larger COPD cohorts with a range of COPD severities.

In CS-exposed mice, endothelial injury was detected in the lungs and kidneys and associated with increased UACRs, podocyte and glomerular injury, and COPD-like lung lesions. Podocytes are specialized epithelial cells attached to glomerular capillaries by numerous interdigitating and elongated foot processes. The width of the podocyte foot processes is small in health and widening is an early, uniform response of podocytes to injury and is associated with albuminuria[28;34].

Few prior studies have linked pulmonary endothelial injury to COPD[35;36], and none have linked renal endothelial injury to COPD. Ever-smokers were reported to have more severe myointimal hyperplasia of small arteries, arteriolar hyalinosis, and glomerular sclerosis than never–smokers[37], but COPD patients were not included in this prior study. Herein, there were no significant differences in double contour frequency or other measures of renal injury between SC and NSC, but our sample sizes were small and additional studies of larger cohorts are needed to determine whether SC also have significant chronic renal lesions. Although the reason for the differences in renal lesions in SC in the two studies are not clear, Lhotta et al. included smokers with diabetes mellitus[37] whereas we excluded subjects with diabetes mellitus from our study.

In a subset of an upstream cohort of smokers (78% of whom did not have without COPD at baseline), UACRs reached microalbuminuria thresholds (30 versus 20 mg/g in women versus men, respectively) only in 2 male smokers studied and UACRs were not related to rate of decline in FEV_1. Likely, endothelial dysfunction/injury of greater severity or duration is needed for emphysema and chronic renal injury to develop in smokers.

Rate of FEV_1 decline in the LSC could have reflected increases in small airway disease rather than emphysema development as various COPD cohort studies have shown that loss of FEV_1 in early disease relates primarily to small airway disease[38;39]. Additional longitudinal studies of smokers are needed to further explore associations between albuminuria and the development of pulmonary and renal lesions.

Potential mechanisms linking renal and pulmonary endothelial injury

Prior studies of renal injury in diabetic mice[40] led us to investigate whether CS increases tissue oxidative stress levels to promote the generation of AGEs[41], thereby causing RAGE activation which, in turn, increases RAGE expression[42]. Oxidative stress, AGEs, and RAGE levels were increased in pulmonary and renal tissue homogenates in CS-exposed mice. Immunostaining studies showed that the increases in AGEs and RAGE levels occurred mostly in glomerular and pulmonary ECs in COPD patients and CS-exposed mice. Increases in AGEs and RAGE levels have been linked to COPD previously[16;17] but not to endothelial dysfunction/injury and microalbuminuria. Interestingly, soluble RAGE (sRAGE, generated by proteolytic shedding of RAGE from cell surfaces) functions as a decoy receptor blocking the binding of RAGE ligands to transmembrane RAGE[43]. Reduced plasma sRAGE levels are linked to the presence of emphysema in humans[44]. Single nucleotide polymorphisms in the RAGE (*AGER*) locus are linked to COPD development[45;46]. CS-exposed *AGER*$^{-/-}$ mice are protected from emphysema development[17]. Thus, reducing RAGE expression or signaling could be an important therapeutic approach for COPD patients.

Therapeutic implications

The presence of microalbuminuria is an indication for ACEi therapy in patients with hypertension and diabetes mellitus[6]. However, the efficacy of ACEi in COPD patients with albuminuria has not been tested even though its potential use is supported by observational studies associating ACEi use with lower rates of FEV_1 decline in smokers[47] and a trend to lower mortality in COPD patients on oxygen therapy[48]. ACEi inhibit ACE-I (a metalloproteinase, expressed by pulmonary ECs[49]), which cleaves the C-terminal dipeptide from inactive Ang-I generating biologically active Ang-II[50]. However, in animal models of diabetes mellitus, ACEi attenuate tissue injury by reducing endothelial injury via their anti-oxidant properties[20;51]. ACEi reduce the production of reactive carbonyl precursors for AGEs, chelate transition metals, and inhibit various oxidative steps thus reducing AGEs generation[52]. Herein, Enalapril therapy in CS-exposed mice reduced the progression of chronic COPD-like lung and renal lesions and these changes were associated with reductions in tissue oxidative stress levels, and AGEs and RAGE staining in ECs in both organs. Serum and pulmonary levels of Ang-II (the product of ACE-I) were reduced in CS-exposed mice and (surprisingly) not reduced further by Enalapril. Enalapril increased serum ACE-I levels likely by reducing pulmonary endothelial injury as ACE-I is expressed highly by pulmonary ECs[49]. These results suggest that Enalapril mediates its disease-modifying effects in mice by inhibiting activation of the oxidative stress-AGEs-RAGE-endothelial injury pathway rather than by inhibiting ACE-I. In humans, while the ACEi, Fosinopril, did not improve quadriceps function or exercise performance in COPD patients selected for having weak quadriceps[53], Enalapril augmented the beneficial effects of pulmonary rehabilitation in improving exercise capacity in patients with moderately-severe COPD[54]. However, microalbuminuria and AT-II levels were not measured in these clinical trials.

We hypothesize that patients having microalbuminuria are the subset that responds best to ACEi therapy.

Study limitations

This study has several limitations. First, UACRs were not measured in the patients whose kidney samples were included in the EM studies and a large number of kidney samples from subjects that may have had COPD could not be analyzed because spirometry had not been performed to confirm the diagnosis of COPD. Thus, a selection bias is likely, and we may not have identified all possible confounders due to the fact that only a small percentage of patients undergoing major surgery in our hospital had physician-diagnosed COPD or had pre-operative pulmonary function testing performed. This issue reflects the common problem of under-diagnosis of COPD in smokers both in hospital and primary care settings[55]. Although all patients selected met strict spirometric criteria for COPD, our results need to be confirmed in additional larger COPD cohorts in the future. Second, we acknowledge that our human studies are correlative, but similar perturbations in the oxidative stress-AGEs-RAGE-endothelial injury pathway were induced by exposing mice to CS. Third, the sample size in the human nephrectomy cohort in which the pathologic assessment for renal glomerular, tubular, and interstitial abnormalities was performed was too small to correlate renal injury with COPD severity which would have linked the oxidative stress-AGEs-RAGE-endothelial injury pathway to COPD progression. Finally, only one ACEi was studied, and its choice was one of convenience as Enalapril is delivered once daily to mice, and widely used in humans. Future studies will determine whether other ACEi have similar effects.

Conclusions

COPD patients and CS-exposed mice have EC injury associated with increases in tissue oxidative stress-AGEs-RAGE-endothelial injury pathway in lungs and kidneys. Enalapril reduces the progression of CS-induced pulmonary and renal injury in mice, and these changes are associated with reduced activation of the tissue oxidative stress levels-AGEs-RAGE-endothelial injury pathway in both organs. Our results provide a rationale for clinical trials testing the efficacy of ACEi in limiting the progression of COPD in patients with albuminuria.

Figures

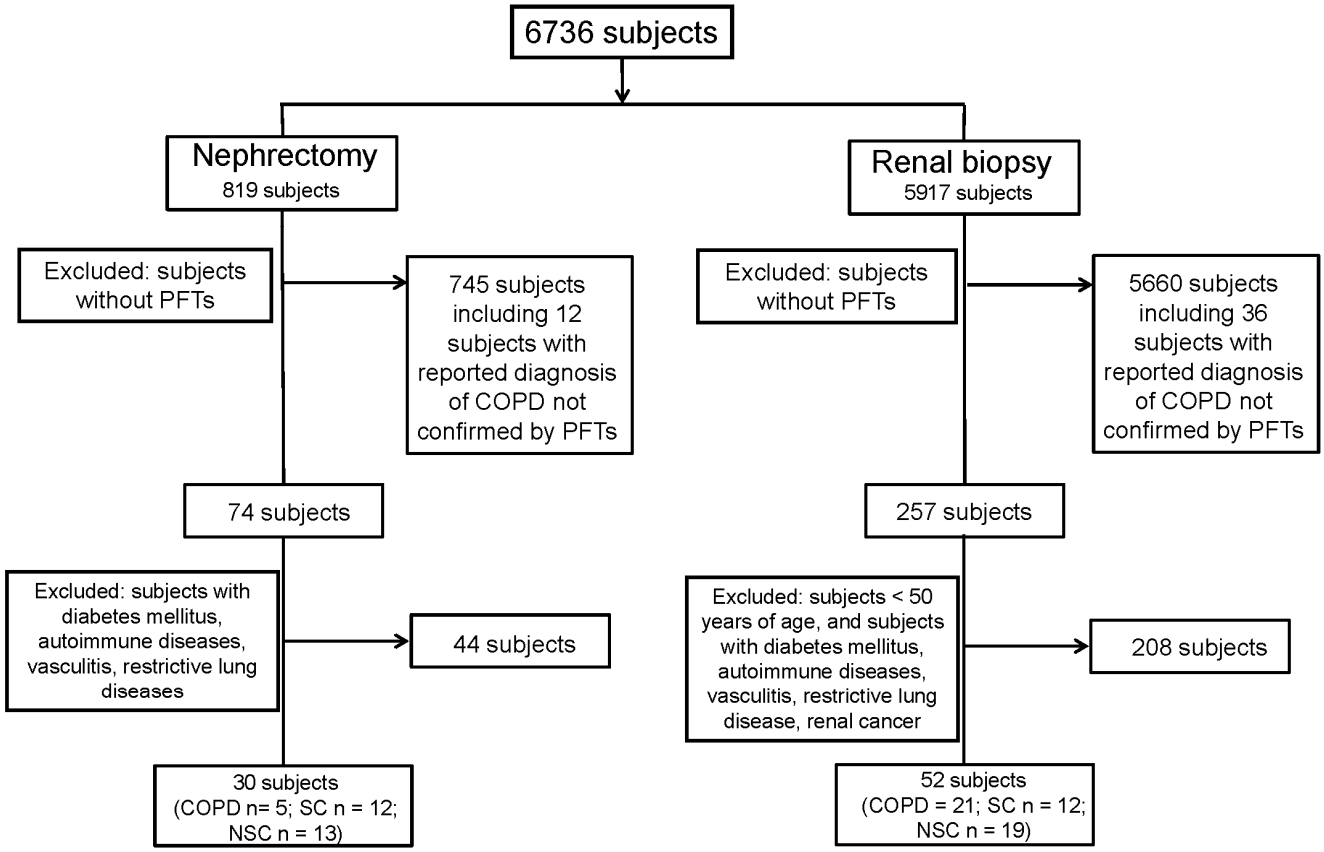

Figure 1: Consort figure showing the process used to select the subjects that underwent a renal biopsy or a nephrectomy for inclusion in the study. From 819 subjects that underwent nephrectomy and 5917 subjects that underwent kidney biopsy, 745 and 5660 were excluded, respectively, because pulmonary function tests (PFTs) were not available. After excluding subjects under 50 years of age and subjects with AKI, autoimmune diseases, vasculitis, diabetes mellitus, restrictive lung diseases, (and renal cancer in the biopsy cohort) 30 subjects (nephrectomy cohort) and 52 subjects (renal biopsy cohort) remained in the study.

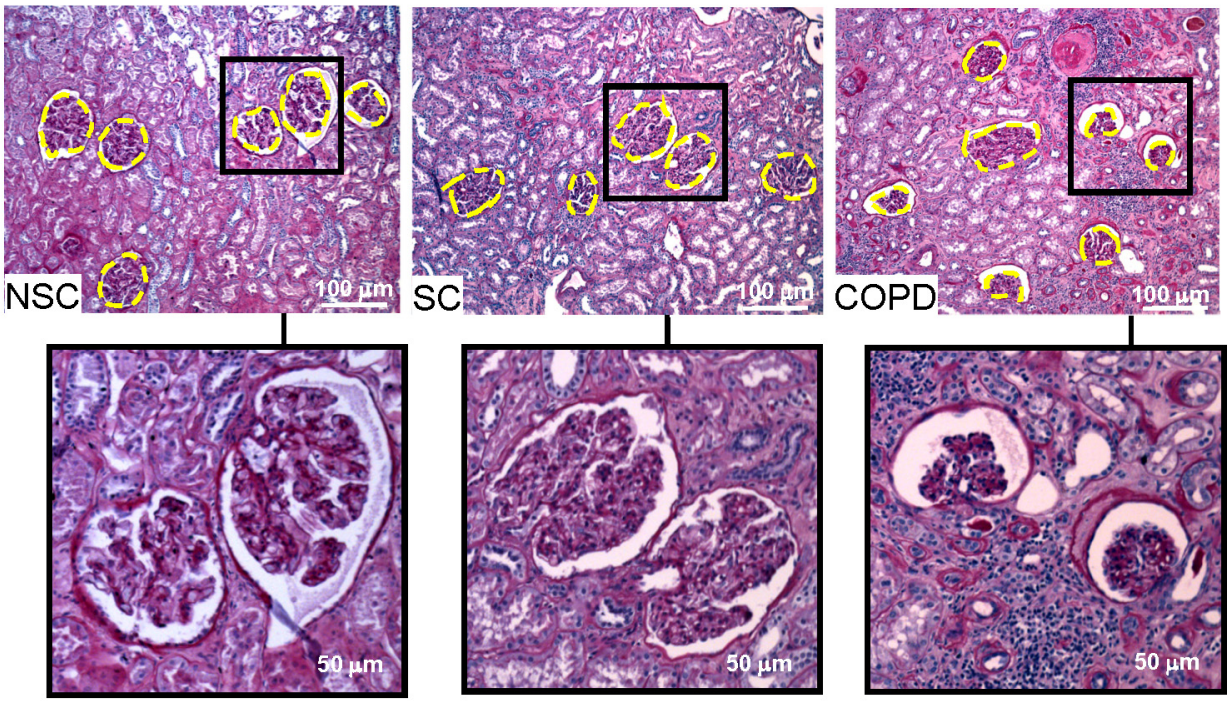

Figure 2: COPD patients have renal injury: Representative images of Trichrome-stained renal sections from COPD patients, SC and NSC in the nephrectomy cohort. The COPD patient has hypoperfused glomeruli with a very widened Bowman space and a small shriveled tuft. The dotted yellow lines trace the glomerular perimeters. The insets show glomeruli at a higher magnification.

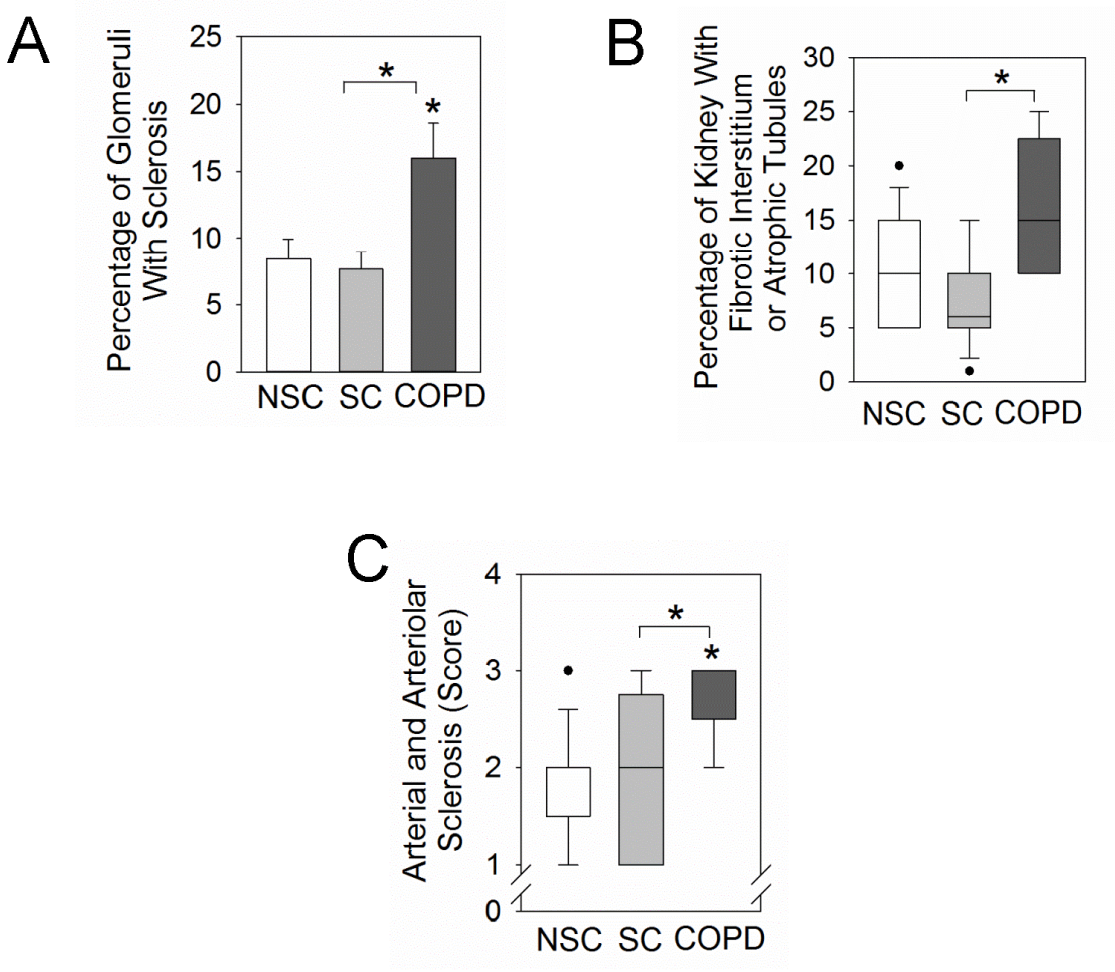

Figure 3: ***A*****:** The percentages of glomeruli having glomerular sclerosis in each group. **B:** The percentages of the renal interstitium having fibrosis, and renal tubules that were atrophied. **C:** The arterial and arteriolar sclerosis score in each group. In **C**, scores of 1, 2, and 3 represent none or mild, moderate, and severe sclerosis, respectively. In this and subsequent figures, bar graphs show mean + SEM, and boxes in box plots show the median values and 25th and 75th percentiles, and error bars show the 10th and 90th percentiles. A Student's t-test (**A**) or a Mann-Whitney U test (**B** and **C**) were used to perform the statistical analyses; asterisks indicate $P < 0.05$ vs. NSC or the group indicated. In **A**-**C**, 5-13 subjects/group were studied.

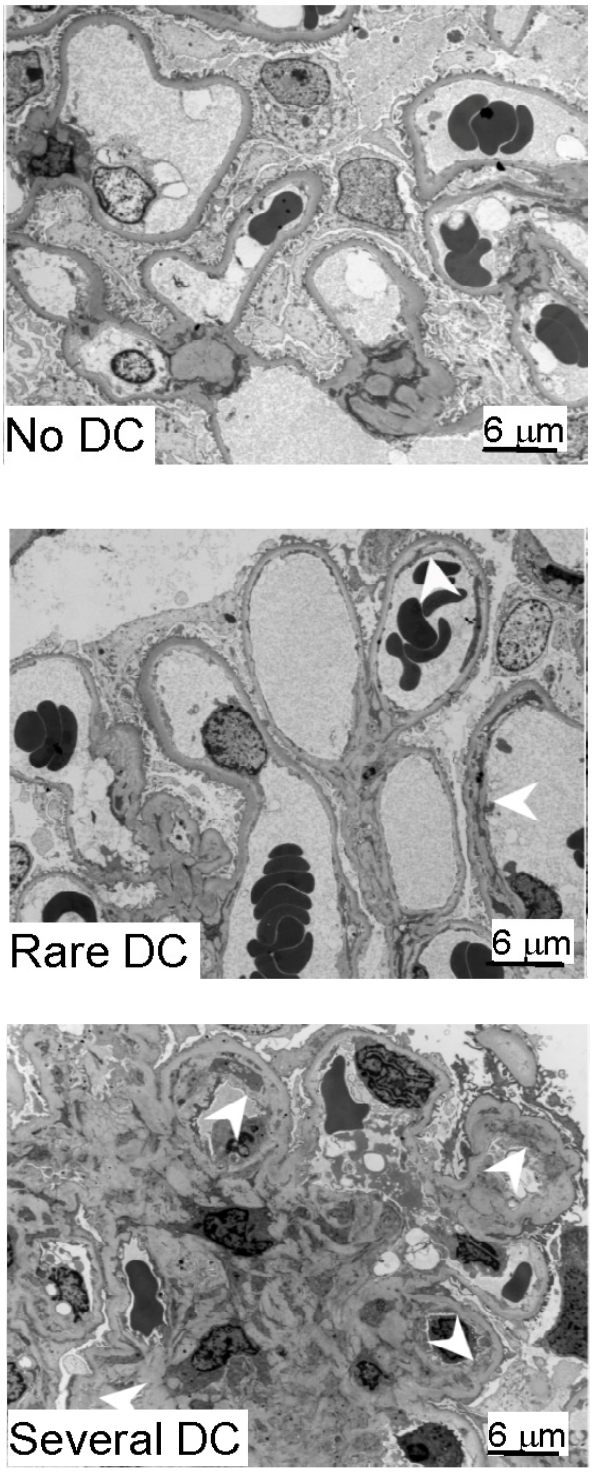

Figure 4: COPD patients have repetitive renal endothelial cell injury: Representative electron photomicrographs of glomerular capillary tufts with no, rare, or several double contours indicated by white arrowheads. Double contours are capillary wall remodeling pathologies indicative of repetitive cycles of endothelial cell injury followed by remodeling.

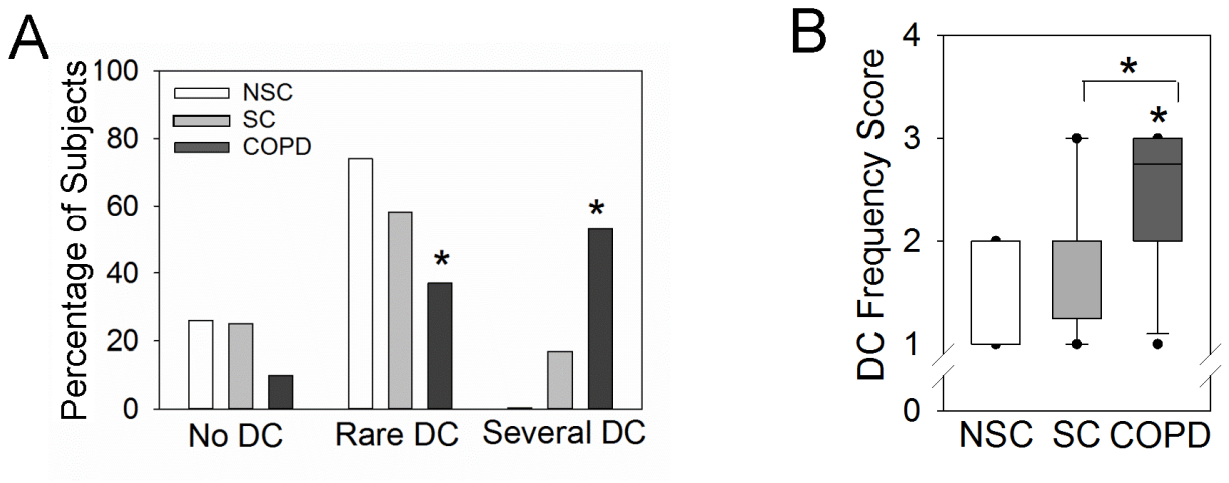

***Figure 5*: A:** The mean (+ SEM) percentage of subjects having no, rare, or several double contours around renal capillaries as assessed using EM. **B:** Double contour frequency in COPD, SC, and NSC. Scores of 1, 2, and 3 represent no, rare, and several double contours, respectively. A Student's t-test (**A**) or a Mann-Whitney U test (**C**) were used to analyze the results; asterisks indicate $P < 0.05$ vs. NSC or the group indicated. In **A** and **B**, 12-21 subjects were studied per group.

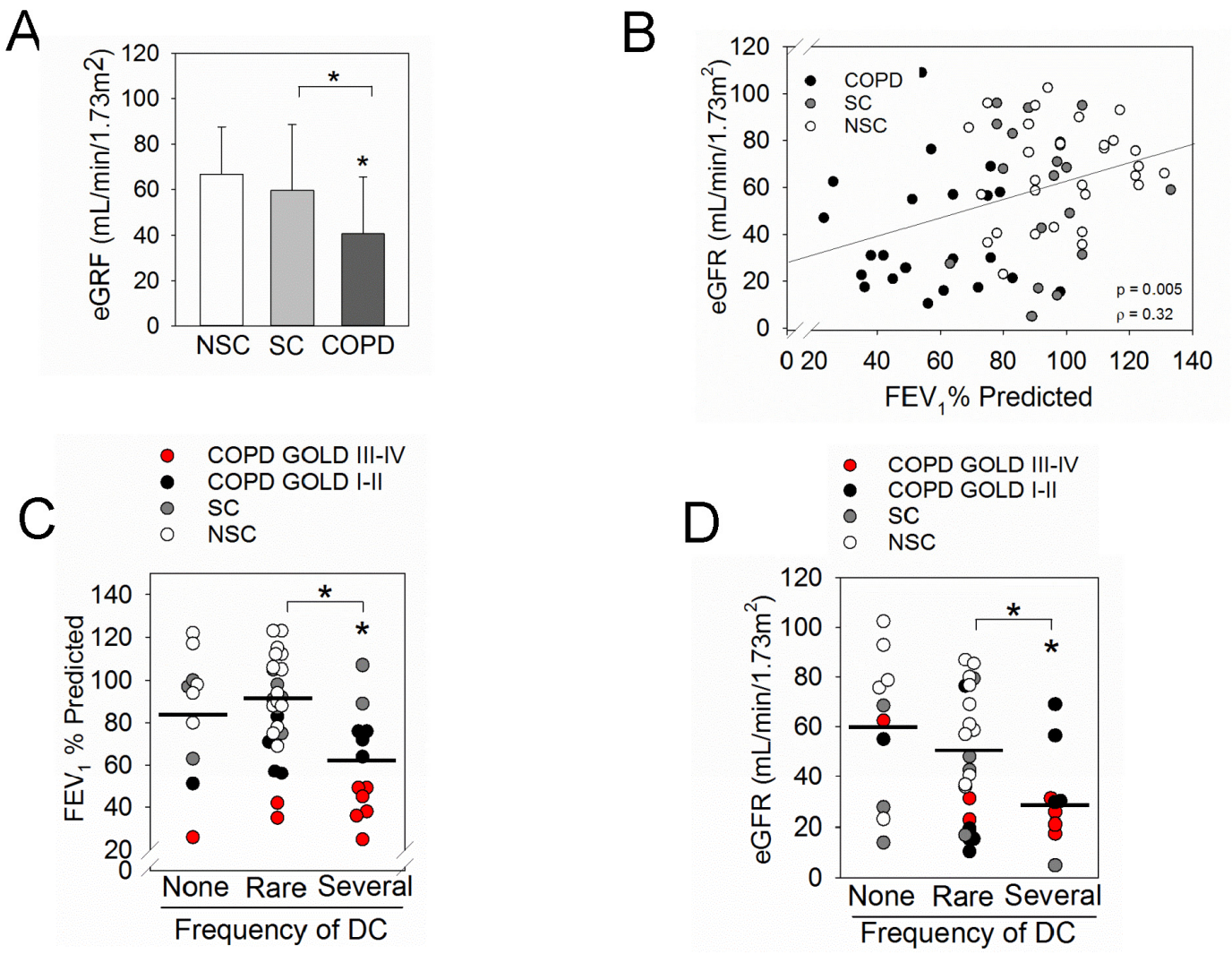

Figure 6: COPD patients have lower estimated glomerular filtration rates (eGFRs) compared with controls and eGFR values correlate with FEV_1 and with renal endothelial injury. **A:** The eGFR values for COPD patients, SC, and NSC in the combined nephrectomy and renal biopsy cohorts are shown. The data shown are mean values and the error bars are SEM. A Student's t-test test was used to analyze the results; asterisks indicate $P < 0.05$ vs. NSC or the group indicated. **B:** eGFR was directly correlated with FEV_1 % predicted in the renal biopsy cohort. White circles represent NSC, gray circles represent smokers without COPD, and black circles represent COPD patients. The Pearson statistical correlation test was used to analyze the data. **C**: The FEV_1 % predicted in subjects having no, rare, or several double contouring of the glomerular endothelial cells. **D**: The eGFRs of subjects having no, rare, or several double contours around the glomerular endothelial cells. In **C** and **D**, data are parametric and the horizontal bars in the scatter plots show the mean values. A Student's t-test test was used to perform the pair-wise comparisons; asterisks indicate $P < 0.05$ vs. no double contour group or the group indicated. In **C** and **D**, the red dots indicate patients with COPD GOLD III-IV, the black dots indicate patients with COPD GOLD I-II, the gray dots indicate SC, and the white dots indicate NSC.

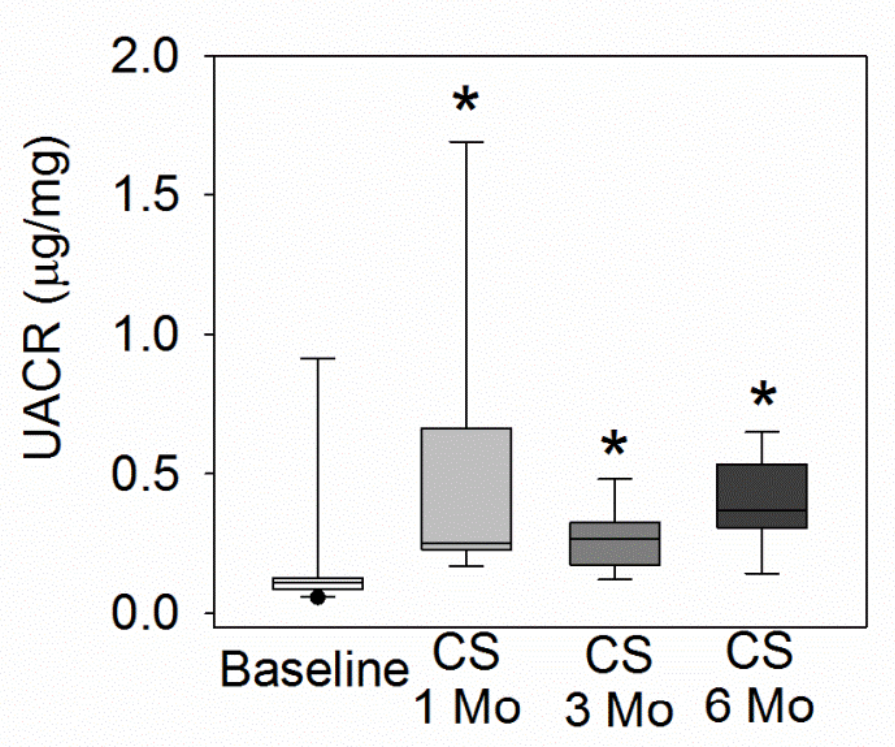

Figure 7: Chronic CS exposure increases urinary albumin-creatinine rations in WT mice: Urinary albumin-creatinine rations were measured in urine samples from C57BL/6 WT mice exposed to CS for 6 months. Data were analyzed using a Mann-Whitney U test, and 3-4 mice/group were studied. Asterisks indicate $P < 0.05$ vs. the air-exposed group.

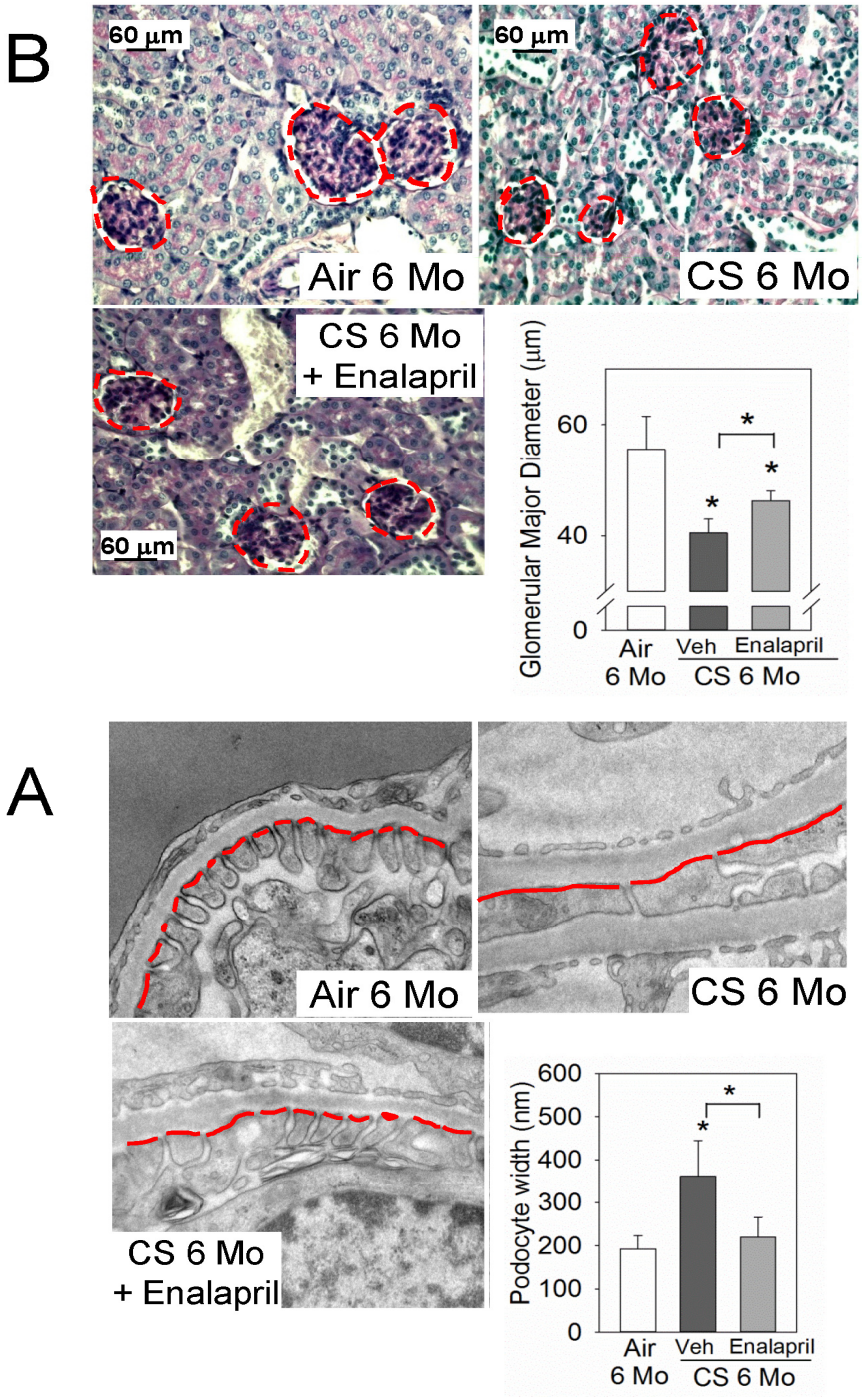

Figure 8: Chronic CS induces chronic renal lesions in WT mice. A: Images of glomeruli in hematoxylin and eosin-stained renal sections from mice exposed to air for 6 months or mice exposed to CS for 6 months and treated with saline or Enalapril 6 days-a-week beginning at the 12 week time point and continued for the second 12 weeks of the CS exposures. The dotted red lines trace the glomerular perimeters. The images are representative of 3-4 mice/group. The bar graph shows the mean (+ SEM) glomerular major diameters. **B:** EM images of podocytes from air-exposed mice or CS-exposed mice treated with saline or Enalapril as outlined above. The red lines trace the widths of the base of the podocytes. The bar graph shows mean (+SEM) podocyte widths. Data were analyzed using a Student's t-test, and 3-4 mice/group were studied. Asterisks indicate $P < 0.05$ vs. the air-exposed group or vs. the group indicated.

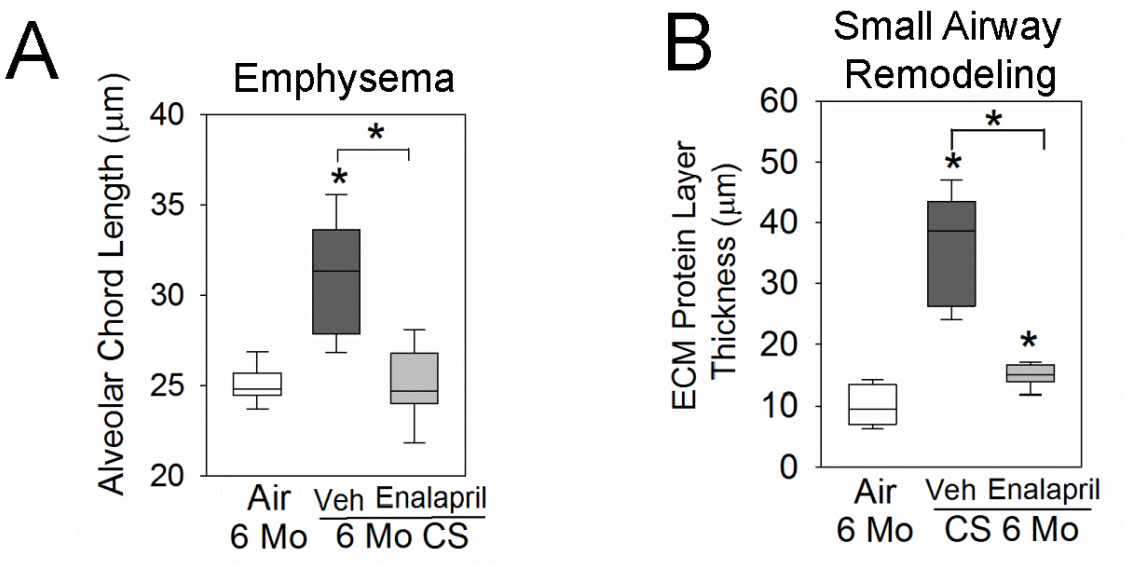

Figure 9: Chronic CS induces chronic pulmonary lesions in WT mice: Airspace enlargement (in **A**) and small airway fibrosis (in **B**) were quantified in the 3 experimental groups. Data were analyzed using a Mann-Whitney U test, and 7-16 mice/group were studied per group. Asterisks indicate $P < 0.05$ vs. the air-exposed group or vs. the group indicated.

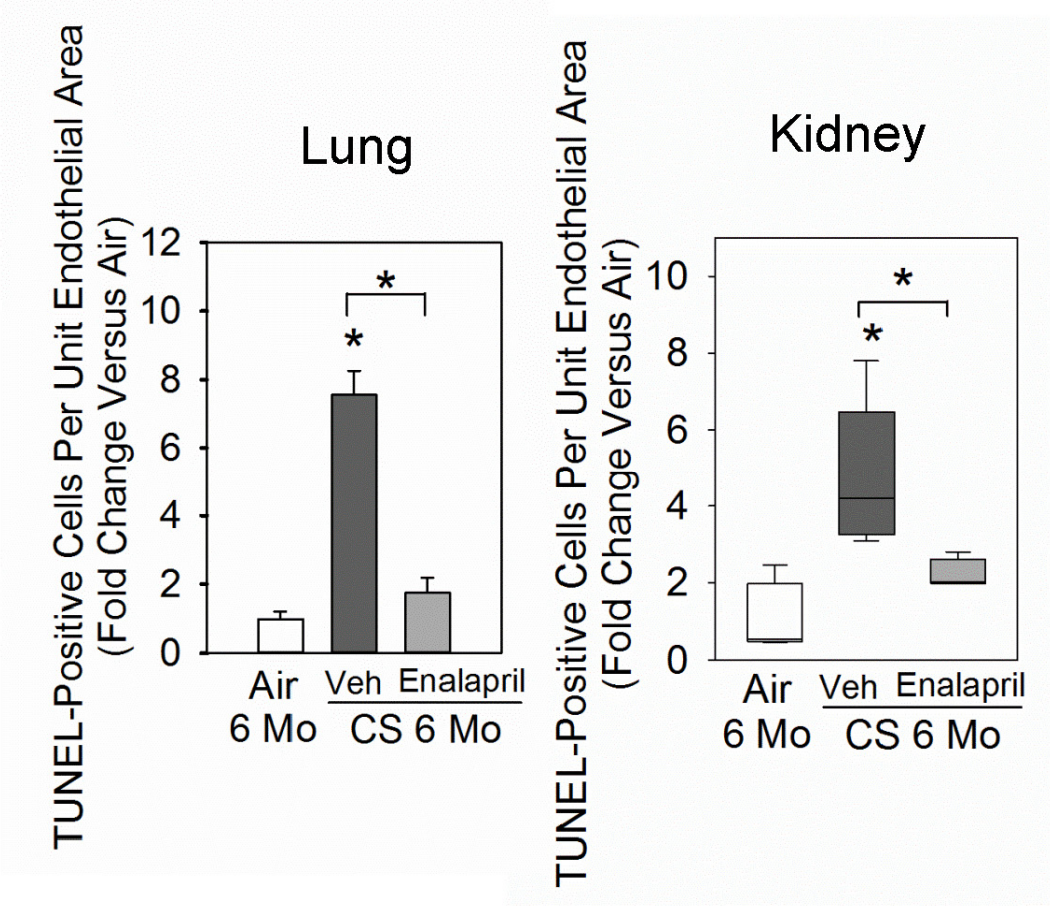

Figure 10: Chronic CS exposure induces injury to pulmonary and renal ECs in mice which is abrogated by Enalapril therapy: C57BL/6 WT mice were exposed to air or CS for 24 weeks. In CS-exposed mice, Enalapril or vehicle therapy was initiated at the 12 week time point as described in the legend to Fig. 3. TUNEL-positive ECs (identified by staining lung and kidney sections with a green color to detect TUNEL-positive cells and a red color to detect von Willebrand Factor [vWF]-positive ECs) were counted in 3-5 mice/group. Data were analyzed using a Mann-Whitney U test (in the right panel) or Student's t-test (left panel) . Asterisks indicate $P \leq 0.05$ vs. air-exposed group or the group indicated.

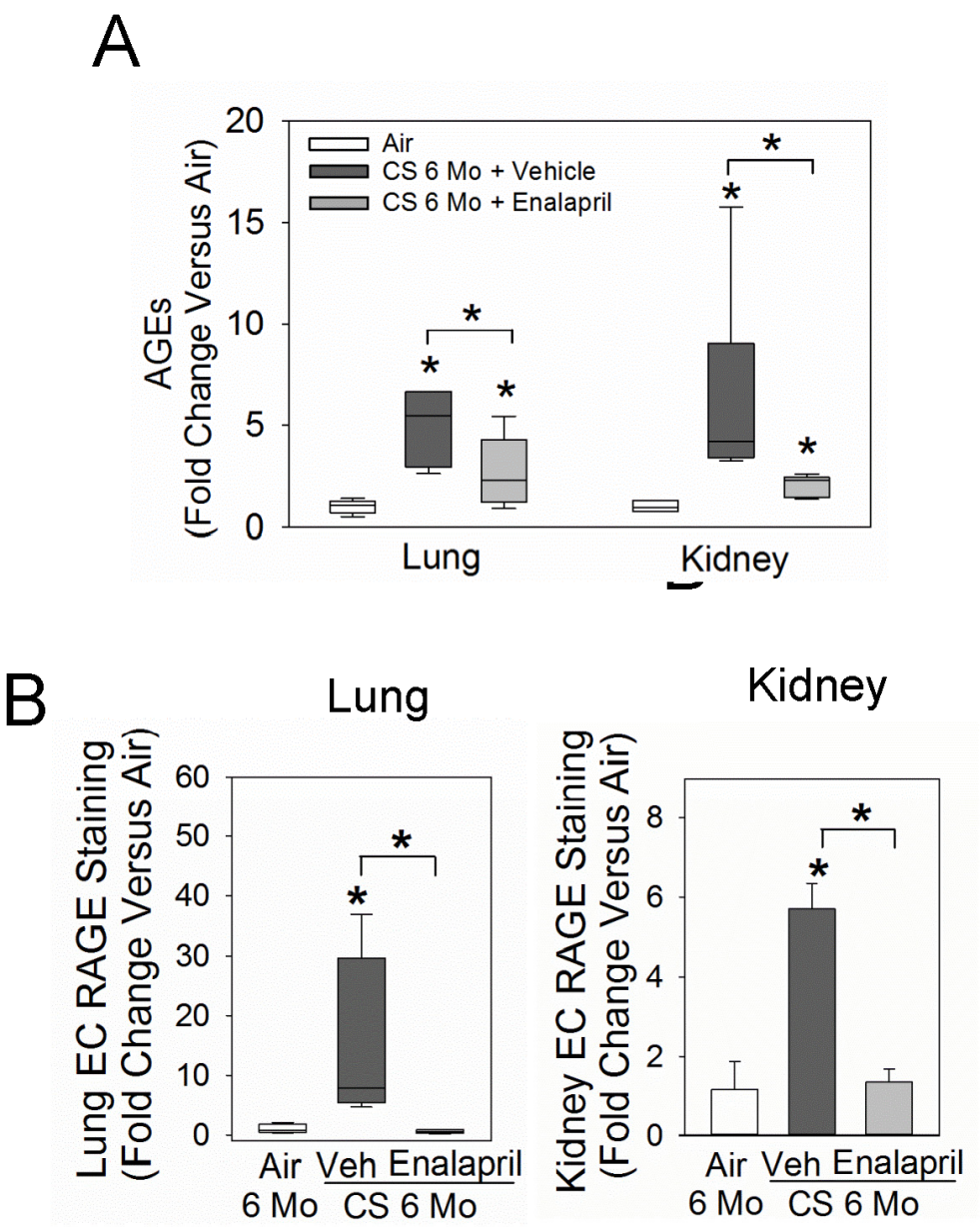

Figure 11: Chronic CS exposure increased renal EC AGEs and RAGE staining which are abrogated by Enalapril therapy: C57BL/6 WT mice were exposed to air or CS for 24 weeks. In CS-exposed mice, Enalapril or vehicle therapy was initiated at the 12 week time point as described in the legend to Fig. 3. **A:** Tissue AGEs levels measured in homogenates of lungs and kidneys from 5-8 mice/group. **B:** RAGE immunostaining levels in vWF-positive ECs in sections of lungs or kidneys from 3-4 mice/group. Data were analyzed using a Mann-Whitney U test (**A**; and the left panel of **B**) or Student's t-test (right panel of **B**) . Asterisks indicate $P \leq 0.05$ vs. air-exposed group or the group indicated.

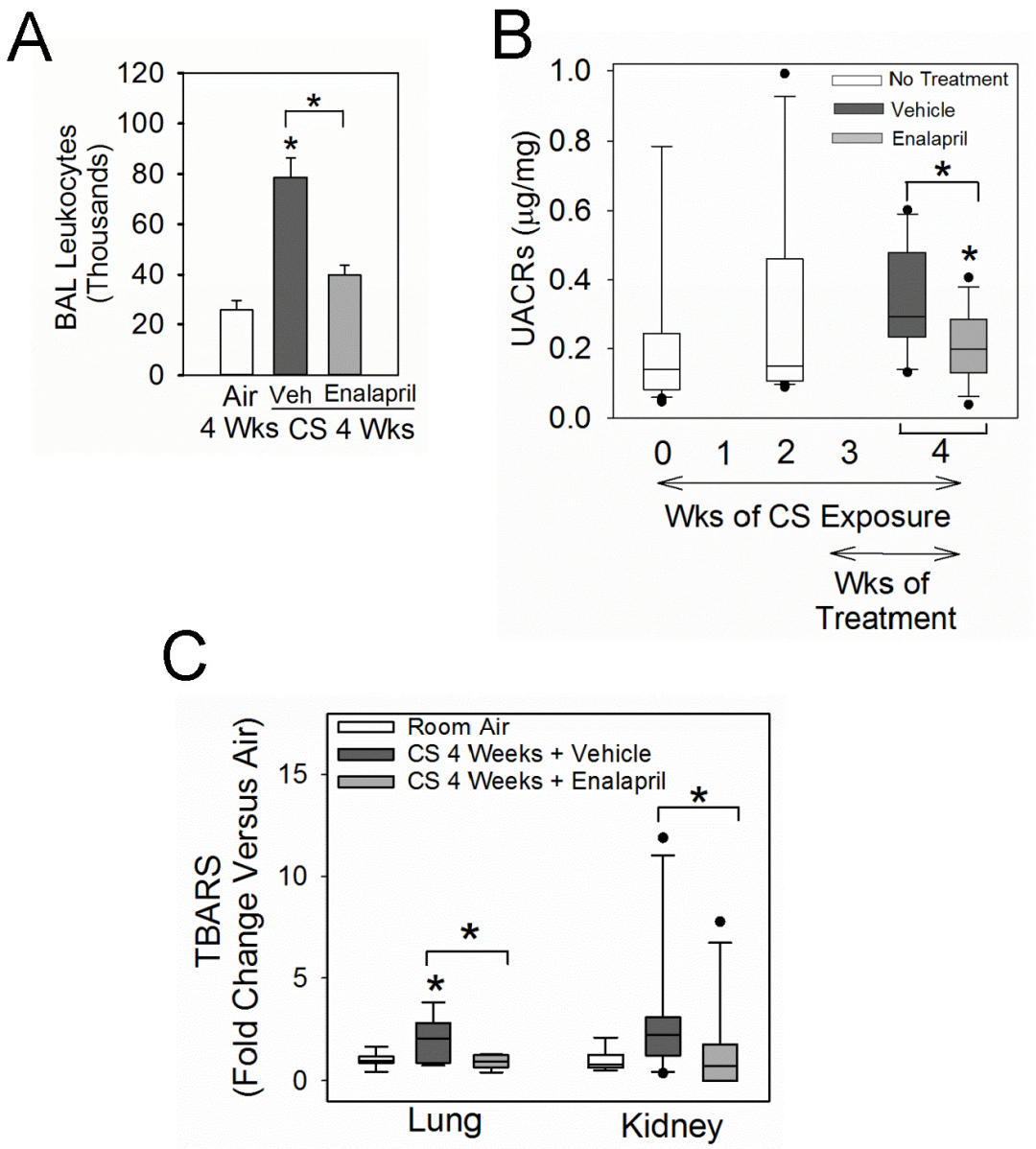

Fig. 12: Enalapril therapy reduces pulmonary inflammation, UACRs, and tissue oxidative stress in the kidneys and lungs of mice exposed acutely to CS: C57BL/6 WT mice were exposed to air or CS for 4 weeks. In CS-exposed mice, Enalapril therapy versus vehicle (6 days-a-week) was initiated beginning at the mid-point of the 4 week CS exposures. **A:** Total leukocyte counts in BAL samples from 5-7/mice group. **B:** UACRs in 10-13 mice/group. **C:** oxidative stress levels measured as TBARs in homogenates of lungs and kidneys from 5-11/mice/group. Data were analyzed using a Student's t-test (**A**) or a Mann Whitney U test (**B** and **C**). * indicates $P \leq 0.05$ vs. air-exposed group or the group indicated.

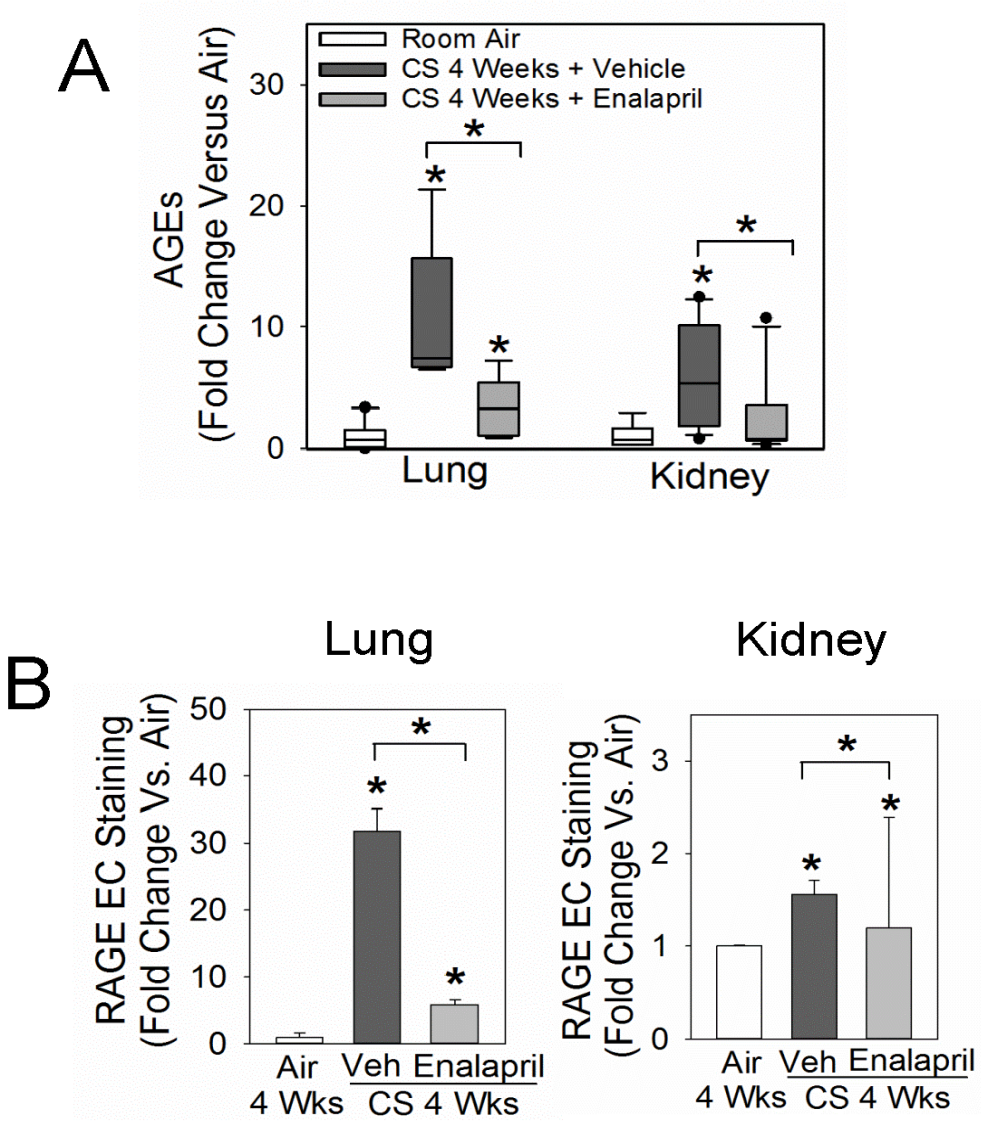

Figure 13: Enalapril therapy reduces AGEs levels, and EC RAGE staining in the kidneys and lungs of mice exposed acutely to CS: C57BL/6 WT mice were exposed to air or CS for 4 weeks. In CS-exposed mice, Enalapril therapy versus vehicle (6 days-a-week) was initiated beginning at the mid-point of the 4 week CS exposures. **A:** Tissue AGEs levels in homogenates of lungs and kidneys from 5-17 mice/group. **B:** RAGE imunostaining levels in vWF-positive ECs in sections of lungs (left panel) or kidneys (right panel) from 3-4 mice/group. Data were analyzed using a Student's t-test (**B**) or a Mann Whitney U test (**A).** * indicates $P \leq 0.05$ vs. air-exposed group or the group indicated. Two-tailed tests were used for all analyses except for the vehicle versus Enalapril comparison in the right panel of **B (*** indicates $P < 0.05$ using a one-tailed test).

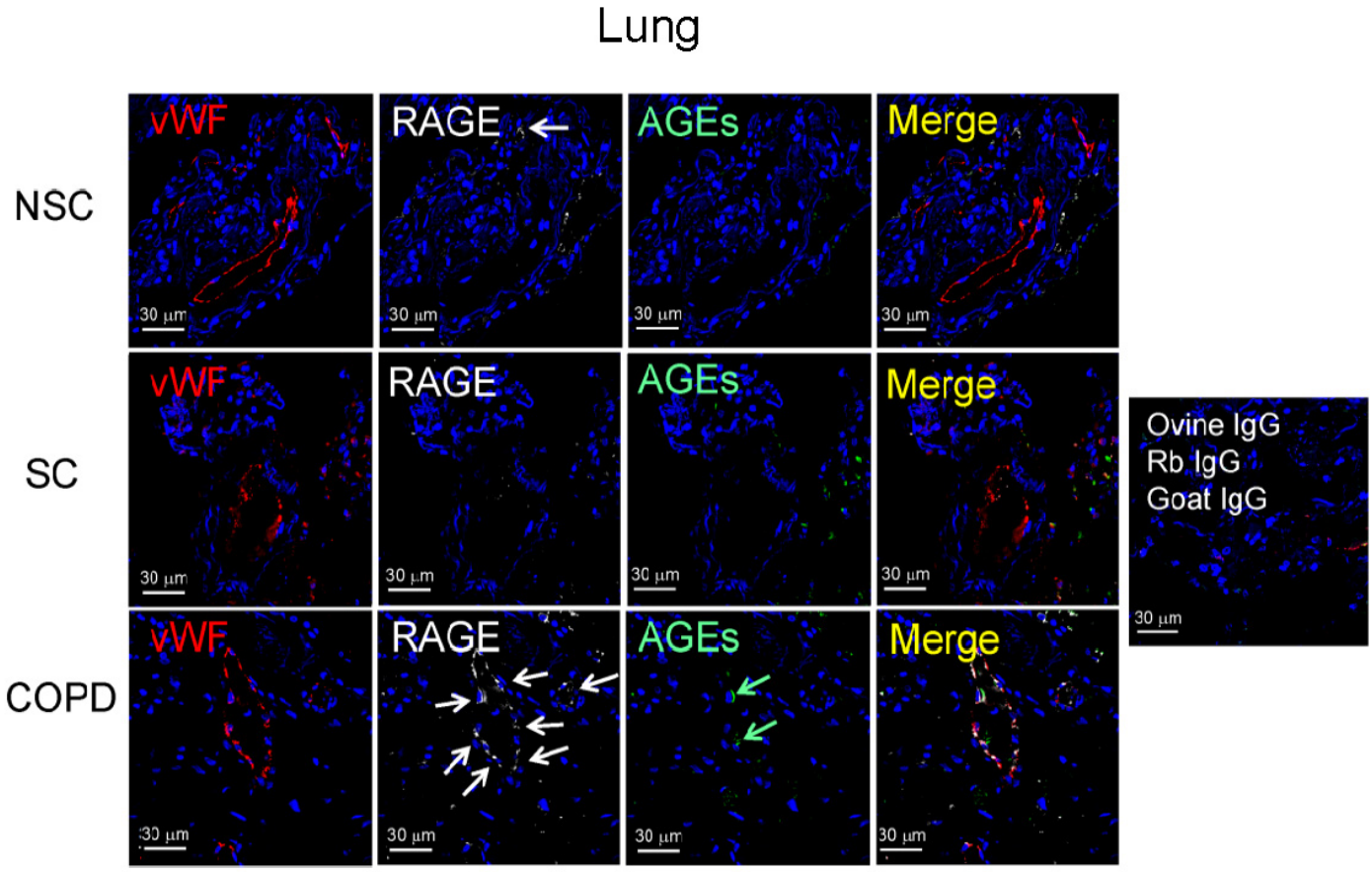

Figure 14: AGEs and RAGE levels are increased in pulmonary ECs in COPD patients. Confocal images of triple-color immunofluorescence staining of sections of lungs from COPD patients, SC, and NSC. ECs in the sections were labeled with a red fluorophore for vWF. Sections were also stained with a green fluorophore for AGEs and gray color for RAGE, and nuclei were counterstained with DAPI (blue). The merged images showing co-localized staining in yellow (fourth panels). White arrows indicate RAGE staining and green arrows indicate AGEs staining in ECs. The fifth panels show a section of a sample from a COPD patient stained with isotype-matched non-immune control antibodies. The images shown are representative of 4-5 subjects/group.

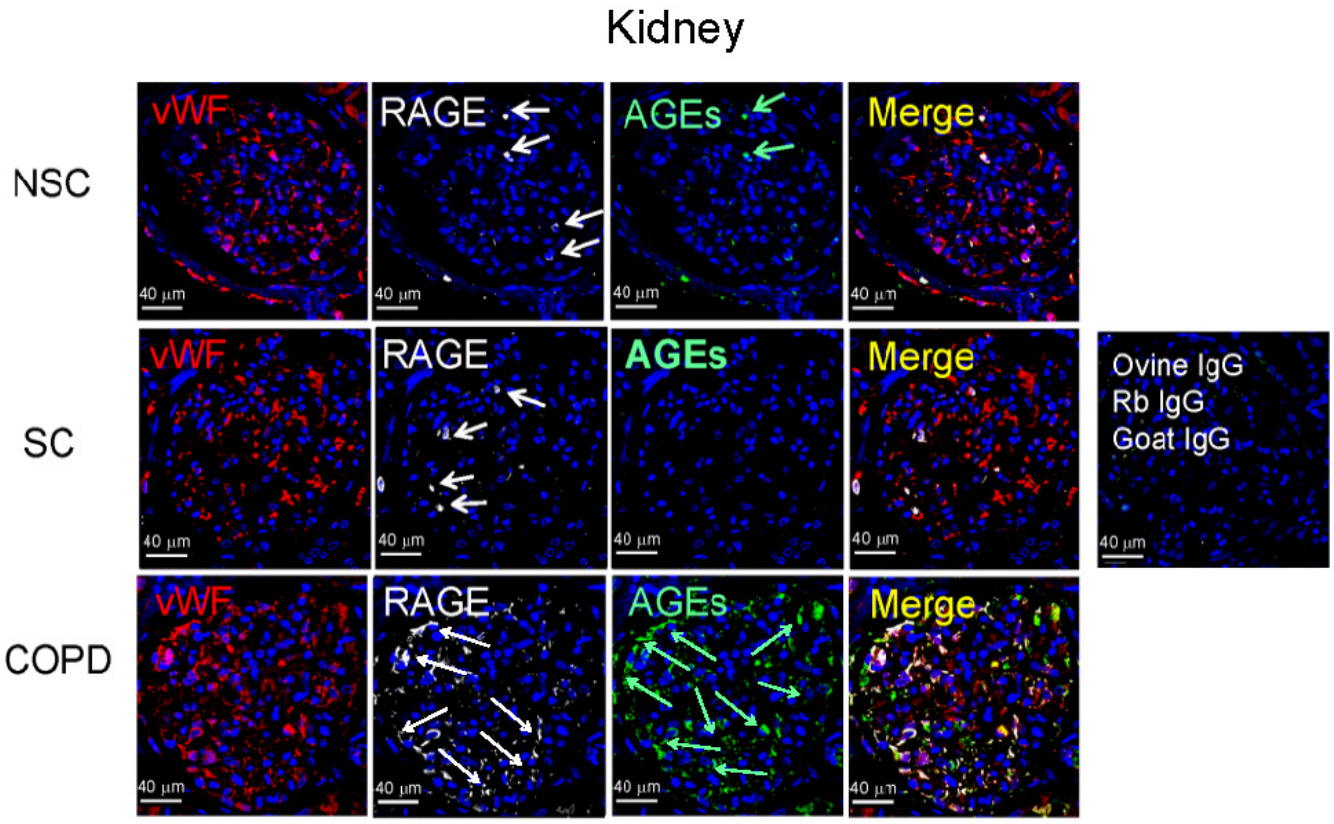

Fig. 14: AGEs and RAGE levels are increased in renal ECs in COPD patients. Confocal images of triple-color immunofluorescence staining of sections of kidneys (glomeruli) from COPD patients, SC, and NSC. ECs in the sections were labeled with a red fluorophore for vWF. Sections were also stained with a green fluorophore for AGEs and gray color for RAGE, and nuclei were counterstained with DAPI (blue). The merged images showing co-localized staining in yellow (fourth panels). White arrows indicate RAGE staining and green arrows indicate AGEs staining in ECs. The fifth panels show a section of a kidney sample from a COPD patient stained with isotype-matched non-immune control antibodies. The images shown are representative of 4-5 subjects/group.

Footnotes

***Abbreviations used:** ACE: angiotensin converting enzyme; angiotensin converting enzyme inhibitor (ACEi); AGEs: advanced glycation end products; BAL: bronchoalveolar lavage; COPD: Chronic Obstructive Pulmonary Disease; CS: cigarette smoke; EC: endothelial cells; eGFR: estimated glomerular filtration rate; EM: electron microscopy; FEV: forced expiratory volume; FITC: fluorescein; FVC: forced vital capacity; GOLD: Global initiative for Obstructive Lung Disease; HRCT: high-resolution computerized tomography; KDIGO: Kidney Disease: Improving Global Outcomes; LSC: Lovelace smokers cohort; NSC: non-smoker controls; RAGE: receptor of advanced glycation end-products; SC: smoker control; TBARS: thiobarbituric acid reactive substances; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; UACR: urinary albumin-creatinine ratio; vWF: von Willebrand factor; WT: wild-type

Tables

Table 1: Demographic and clinical characteristics of the nephrectomy cohort.

B: Characteristics of the nephrectomy cohort	NSC* (N = 13)	SC* (N = 12)	COPD† (N = 5)	P value ††
Males (%)	46	75	60	**N.S§**
Age (yrs)	66 ± 3	65 ± 3	70 ± 6	**N.S.**
Pack-yrs of smoking‖	0 (0)	24 ±18	51± 21	***P* < 0.001‡**
Current smokers (%)	0	8	40	**N.S.**
FEV_1 (% of predicted)	97 ±17	92 ±16	56 ± 21	***P* ≤ 0.001****
FEV1/FVC (%)	78 ± 5	80 ± 6	57± 15	***P* = 0.002****
Hypertension (%)	61	50	40	**N.S.**
Cardiovascular disease (%)	23	17	20	**N.S.**
Obesity (%)	0	8	0	**N.S.**

Table 1 legend: *The demographic and clinical characteristics of non-smoker controls (NSC), smoker controls (SC), and COPD patients that underwent nephrectomy are shown.
†All COPD patients had forced expiratory volume in 1 second/ forced vital capacity ratio (FEV1/FVC) < 0.7 whereas smokers without COPD and non-smoker controls had FEV1/FCV > 0.7. NSC were all never-smokers. SC were defined as subjects that had > 10 pack-year smoking history. Current smokers were defined as active smokers at the time of the nephrectomy, or had stopped smoking < 1 year before the nephrectomy.
‡The results for age, FEV1 % predicted, and pack-year smoking history are expressed as mean + SEM.
‖The pack-year smoking histories of the COPD patients and SC groups were significantly different from those of NSC by design as assessed using a one-way ANOVA followed by pair-wise Student's t-tests; P < 0.001 for both comparisons. The pack-year smoking histories of the COPD patients and SC were not significantly different (P = 0.07).
§Pairwise comparisons showed no significant differences (NS) in the percentage of males, the ages, the proportion of current smokers, or the presence of hypertension, cardiovascular disease, or obesity between the COPD vs. the SC, the COPD vs. the NSC, or the SC vs. the NSC groups.
**The FEV1 % predicted and the FEV1/FVC in the COPD group were significantly different from those of the SC and the NSC groups by design (P < 0.001 for both comparisons). The FEV1 % predicted and the FEV1/FVC in the SC group were not significantly different from that of the NSC group (P > 0.3 for this comparisons).
††Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV1 % predicted, and pack/years) followed by pair-wise comparisons using Student's t-tests or Mann-Whitney U tests. The chi-square test was used to analyze categorical variables.

Table 2: Demographic and clinical characteristics of the biopsy cohort.

	NSC* (N = 19)	SC* (N = 12)	COPD (N = 21)	P value§
Males (%)	63	50	62	**N.S.‡**
Age (yrs)	63 ± 9	69± 12	68 ± 8	**N.S.**
Pack-yrs of smoking†	1.5 ± 3	27±18	40 ± 27	***P* < 0.001**
Current smokers (%)	0	17	19	**N.S.**
FEV_1 (% of predicted)l	99±17	91±12	56 ± 20	***P* < 0.001**
FEV1/FVC (% of predicted)l	82 ± 4	78 ± 5	60 ± 9	***P* < 0.001**
Hypertension (%)	53	67	71	**N.S.**
Cardiovascular disease (%)	10.5	25	14.3	**N.S.**
Obesity (%)	5	8	5	**N.S.**

Table 2 legend: The demographic and clinical characteristics of the non-smoker controls (NSC), smoker controls (SC), and COPD patients that underwent a renal biopsy are shown along with the percentages of subjects with comorbid diseases. All subjects were non-hispanic whites except for one subject in the COPD group and one subject in the NSC group who were both hispanic whites. Information about race was not available for two subjects.

*All COPD patients had forced expiratory volume in 1 second/ forced vital capacity ratio (FEV1/FVC) < 0.7 whereas SC and NSC had FEV1/FCV > 0.7.

NSC included never-smokers (76%) and smokers (24%) that had a < 10 pack-year history and had stopped smoking for > 2 years before the renal biopsy. The mean + SD pack years of the smokers in the NSC group was 5.6 + 2.7.

SC were defined as subjects that had > 10 pack-year smoking history. Current smokers were defined as active smokers at the time of the biopsy, or had stopped smoking < 1 year before the biopsy.

†The pack-year smoking histories of the COPD and SC groups were significantly different from those of the NSC by design as assessed using a one-way ANOVA followed by pair-wise Student's t-test (P < 0.001 for both comparisons). The pack-year smoking histories of the COPD and SC groups were not significantly different (P = 0.2).

‡Pairwise comparisons showed no significant differences (N.S.) in percentage of males, the ages, the percentages of current smokers, or the presence of hypertension, cardiovascular diseases, or obesity between COPD vs. SC, COPD vs. NSC, and SC vs. NSC.

The percentage of current smokers in the COPD vs. SC and NSC groups was not significantly different (P = 0.7 and P = 0.1, respectively). The percentage of current smokers in the SC vs. NSC was not significantly different (P = 0.3).

lThe FEV1 % predicted and the FEV1/FVC in the COPD group were significantly different from that of the SC and NSC by design (P < 0.001 for both of these comparisons).

§Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV1 % predicted, and pack/years) followed by pair-wise comparisons using Student's t-tests or Mann-Whitney U tests. The chi-square test was used to analyze categorical variables.

Table 3: Demographic data of the subjects included in the lung tissue cohort for immunofluorescence staining studies

Table E3. Lung tissue cohort for immunostaining studies				
Characteristics	**NSC* (N = 5)**	**SC* (N = 5)**	**COPD† (N = 5)**	**P value††**
Males (%)	20	60	40	N.S.§
Age (yrs) ‡	69 ± 20	62 ± 10	63 ± 10	N.S.
Pack-yrs of smoking	0	34 ±19	60 ± 38	$P \leq 0.005$ l
Current smokers (n)	0	4	2	N.S.
FEV_1 (% of predicted value)	N/A‡‡	83 ± 15	55 ± 29	$P = 0.01$**
FEV_1/FVC (%)	N/A‡‡	79 ± 2	49 ± 11§	$P = 0.004$**

Legend to Table 3: Table of the demographic and clinical characteristics of non-smoking controls (NSC), smoker controls without COPD (SC), and COPD patients that underwent resection of tissue for lung nodules or localized carcinoma.

*NSC were all never-smokers. SC had a cigarette smoking history of ≥ 10 pack years. Current smokers were defined as active smokers at the time of the study or smokers who had stopped smoking less than 1 year before being studied.

†All COPD patients had forced expiratory volume in 1 second/forced vital capacity (FEV_1/FCV) < 0.7 whereas SC and NSC had FEV_1/FCV ≥ 0.7.

‡The results for age, FEV_1 % predicted, and pack-year smoking history are expressed as mean ± SEM.

lThe pack-year smoking histories of the COPD patients and SC groups were significantly greater than those of the NSC by design ($P \leq 0.005$ for both comparisons using Student's t-tests). The pack-year smoking histories of the COPD patients and SC groups were not significantly (N.S.) different ($P = 0.2$)

§Pairwise comparisons showed that there were no significant differences in the percentages of males, age, or the proportion of current smokers in the COPD vs. SC, COPD vs. NSC, and SC vs. NSC groups.

The percentages of current smokers in the COPD group compared with SC and NSC groups were similar. There was a strong trend towards a higher percentage of current smokers in the SC than that of the NSC group ($P = 0.053$ using the Chi-square test).

**The FEV_1 % of predicted value and FEV_1/FVC ratios were significantly lower in the COPD group than the SC group.

††Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV_1 % predicted, and pack-year smoking histories) followed by pair-wise comparisons using Student's t-tests or Mann-Whitney U tests. The Chi-square test was used to analyze categorical variables.

‡‡The FEV_1% predicted and FEV_1/FVC were not available (N.A.) for the NSC. However, all of the NSC were never-smokers and none had any respiratory symptoms at the time of the surgery.

Table 4: Demographic data of the subjects included in renal tissue cohort for immunofluorescence staining studies

Table E4. Renal tissue cohort for immunostaining studies				
Characteristics	**NSC* (N = 5)**	**SC* (N = 6)**	**COPD† (N = 5)**	**P value‡‡**
Percentage of males	80	83	40	N.S.**
Age (yrs) ‡	68±8	65 ± 11	69 ± 11	N.S.
Pack-yrs of smoking[I]	0	27 ± 21	48 ± 20	$P \leq 0.004$
FEV_1 (% of predicted value)	N/A[II]	90 ± 21	54 ± 21	$P \leq 0.04$§
FEV_1/FVC (%)	N/A[II]	77 ± 5	56 ± 15§	$P < 0.001$§

Legend to Table 4: The table shows the demographic and clinical characteristics of NSC, SC, and COPD patients that underwent nephrectomy.

* NSC were all never-smokers. SC had a cigarette smoking history of ≥ 10 pack years.

†All COPD patients had forced expiratory volume in 1 second/forced vital capacity (FEV_1/FCV) < 0.7 whereas SC and NSC had FEV_1/FCV ≥ 0.7. All the COPD and SC in this cohort were former smokers.

‡The results for age, FEV_1 % predicted, and pack-year smoking history are expressed as mean ± SEM.

[I]The pack-year smoking histories of the COPD patients and SC groups were significantly greater than those of the NSC by design ($P \leq 0.004$ for both comparisons using Student's t-tests). The pack-year smoking histories of the COPD patients was not significantly different from that of the SC ($P = 0.1$).

§The FEV_1 % predicted and the FEV_1/FVC values of the COPD group were lower than those of the SC and NSC groups by design ($P \leq 0.04$ for both comparisons using one-way ANOVA followed by the Student's t-test for the pair-wise comparisons).

** Pairwise comparisons showed no significant (N.S.) differences in the percentage of males and age between the COPD vs. SC, COPD vs. NSC, and SC vs. NSC groups.

‡‡Statistical analyses included one-way ANOVA tests for continuous variables (age, FEV_1 % predicted, and pack-year smoking histories) followed by pair-wise comparisons using Student's t-tests or Mann-Whitney U tests. The Chi-square test was used to analyze categorical variables.

[II]The FEV_1% predicted and FEV_1/FVC were not available (N.A.) for the NSC. However, all of the NSC were never-smokers and none had any respiratory symptoms at the time of the surgery.

Table 5: **Demographic characteristics for the UACR cohort**

Characteristics	Lovelace Smokers Cohort (N = 148)
Males (%)	42
Age (yrs) *	55 ± 0.7
Pack-yrs of smoking*	38 ± 1.3
Current smokers (%)	59
Baseline FEV_1 (% of predicted) *	91.5 ± 1.4
FEV_1/FVC (%)*	73 ± 0.8

Legend to Table 5: *The results for age, FEV_1 % predicted, FEV_1/FVC (%) and pack-year cigarette smoking history are expressed as mean ± SEM.
None of the subjects studied were taking ACE inhibitors or angiotensin receptor blockers. Patients with physician-diagnosed diabetes mellitus were also excluded from the analysis.

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