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Effects Of Waterpipe Smoking On The Human Lung

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Spécialité de doctorat : Sciences de la vie et de la santé

Par

Mme Yael Strulovici Barel

Effets de la Fumée de Nargile sur la Santé du Poumon

Thèse présentée et soutenue à Paris, le 6 juillet 2016 :

Composition du Jury :

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Am Israel Chai

Titre : Effets de la Fumée de Nargile sur la Santé du Poumon

Mots clés : nargile, fumée, poumon

Résumé : La Chicha qui sert à fumer du tabac parfumé est utilisée par des millions de personnes. Il y a peu de données sur les effets du chicha sur la santé, peu de réglementation et les utilisateurs pensent que la chicha n'est ni addictif ni nocif. Pour évaluer les effets précoces de la chicha sur les poumons nous avons comparé des fumeurs de chicha occasionnels et des non fumeurs pour les paramètres cliniques et biologiques. L'utilisation de la chicha augmentait la toux et les expectorations ainsi que le niveau sanguin de carboxyhémoglobine. Ces modifications étaient associées à des modifications du métabolome des sécrétions pulmonaires, ainsi que de la modification de l'épithélium pulmonaire dans sa composition et son transcriptome. Les fumeurs présentaient une diminution de la capacité de diffusion qui est un marqueur prédictif du développement de la BPCO. Ils avaient également une augmentation du niveau plasmatique des microparticules endothéliales qui sont un marqueur de la destruction alvéolaire. Notre étude démontre que l'utilisation occasionnelle chez les jeunes de la chicha peut avoir des conséquences sur les maladies pulmonaires.

Title : Effects of Waterpipe Smoking on the Human Lung

Keywords : waterpipe, smoking, lung

Abstract : Waterpipe, an instrument for smoking fruit-flavored tobacco, is used by millions worldwide. There is limited data on its health effects, no regulations to its use, and users believe smoking it is not as harmful or addictive as cigarette smoking. To assess the early effects of waterpipe smoking on lung health, light-use waterpipe smokers with normal spirometry were assessed for lung clinical and biologic abnormalities compared to nonsmokers. Waterpipe smokers had increased cough and sputum, increased blood carboxyhémoglobin levels, abnormal lung epithelial lining fluid metabolome profile, abnormal small airway epithelium (SAE) cell composition, and markedly abnormal SAE and alveolar macrophage transcriptomes. They also had reduced diffusion capacity, a lung function marker of high risk for developing COPD in cigarette smokers, and high plasma levels of total and apoptotic endothelial microparticles, biomarkers of alveolar capillary destruction in COPD cigarette smokers that persists despite smoking cessation. These studies suggest that even young, light-use waterpipe smokers are likely at risk for developing lung disease.

Longue Résumé

La Chicha qui sert à fumer du tabac parfumé est utilisée par des millions de personnes. Il y a peu de données sur les effets du chicha sur la santé, peu de régulation et les utilisateurs pensent que la chicha n'est ni addictif ni nocif. Nous avons émis l'hypothèse que la consommation même occasionnelle de la chicha chez le sujet jeune a des conséquences sur la biologie pulmonaire. Nous avons ainsi comparé 21 sujets jeunes fumeur occasionnel de Chicha à un groupe de 19 non fumeur apparié pour le sexe et l'ethnicité. Les premières anomalies chez le fumeur de cigarette étant présent au niveau des cellules pulmonaires nous avons évalué plusieurs paramètres : (1) taux plasmatique de carboxyhemoglobine (CO), (2) Score de toux et d'expectoration; (3) fonction pulmonaire; (4) Métabolites présent dans les fluides des voies respiratoires basses (ELF); (5) différences cellulaires et de transcriptome des petites voies aériennes (6) composition cellulaire des lavages broncho-alvéolaires (7) le transcriptome et (9) niveau des microparticules endothéliales circulantes. Le groupe d'étude montrait des anomalies dans tous les paramètres étudiés. Comparé au groupe contrôle les fumeurs avaient plus de toux et d'expectoration, un niveau de CO plus élevé, une diminution de la capacité de diffusion du CO, des anomalies du profil métabolique des fluides alvéolaires, une augmentation des cellules sécrétoires et intermédiaires et une diminution des cellules ciliées et basales, des anomalies du transcriptome des cellules pulmonaires et de macrophages alvéolaires et une augmentation des microparticules endothéliales.

LA capacité de diffusion du monoxyde de carbone qui est un paramètre lié à l'emphysème et aux pathologies des petites voies pulmonaires était affectée par l'utilisation de la chicha. Nos précédentes études avaient montré que chez les sujets fumeurs de cigarette la

réduction de la capacité de diffusion malgré une spirométrie normale était associée à un risque de développer un BPCO. Nous avons ainsi évalué le risque de développer une BPCO chez le sujet fumeur avec spirométrie normale par des scanners haute résolution comparant des groupes avec capacité de diffusion diminuée (46) et normale (59). La réduction de la capacité de diffusion était associée à un risque élevé de développer une BPCO dans les 4 ans.

Par ailleurs les niveaux plasmatiques des microparticules endothéliales totales et apoptotiques était élevé dans le groupe d'étude. Dans une étude chez les sujets fumeurs de cigarette, nous avons comparé des non fumeurs (28) à des fumeurs sains (61) et des fumeurs BPCO (49) sur un an. Nous avons montré que le niveau de microparticules endothéliales apoptotiques étaient élevé en continu chez les sujets fumeurs sains et avec BPCO. Un sous groupe des fumeurs sains (17) et BPCO (18) a accepté d'arrêter de fumer. 12 mois après l'arrêt de la cigarette le niveau des microparticules endothéliales totale et apoptotique était retourné à la normale pour les fumeurs sains mais restait élevé chez les fumeurs BPCO. Ainsi le niveau élevé de ces microparticules indiquait des lésions persistantes et irréversibles des capillaires pulmonaires et pourrait servir à évaluer les fumeurs de chicha au long cours.

Au total, l'utilisation occasionnelle de chicha chez le sujet jeune a des conséquence clinique et biologique pulmonaire en relation avec une diminution de la capacité de diffusion. Certaines anomalies mises en évidence dans notre étude (diminution de la capacité de diffusion, Microparticules endothéliales) pourraient prédire la survenue de maladies pulmonaires chroniques obstructives.

Long abstract

Waterpipe, an instrument for smoking fruit-flavored tobacco, is used by millions of people worldwide. There is limited data on the health effects of waterpipe smoking, and no regulations to its use. We hypothesized that even young, light-use waterpipe smokers have abnormalities relevant to lung health. Based on the knowledge that the first abnormalities associated with cigarette smoking are in lung cells long before there are clinical abnormalities, we compared young, light-use waterpipe smokers to nonsmokers, using a variety of lung-related parameters, including: blood carboxyhemoglobin (CO) levels; cough and sputum scores; lung function; metabolites present in lower respiratory tract epithelial lining fluid (ELF); cell differentials and transcriptome of small airway epithelium (SAE); cellular composition of ELF; transcriptome of alveolar macrophages (AM); and levels of total and apoptotic endothelial microparticles (EMPs). Light-use waterpipe smokers displayed abnormalities in all parameters assessed. Compared to nonsmokers, waterpipe smokers had more cough and sputum, higher CO levels, reduced diffusing capacity for carbon monoxide (DLCO), abnormal ELF metabolome profile, increased proportions of SAE secretory and intermediate cells, reduced proportions of SAE ciliated and basal cells, markedly abnormal SAE and AM transcriptomes, and elevated levels of total and apoptotic EMPs.

DLCO, a lung function parameter linked to emphysema and small airway disease, was affected by light-use waterpipe smoking. The relevance of this comes from our studies that demonstrated, in a separate cohort of cigarette smokers with normal spirometry, that reduced DLCO predicted a high risk for developing chronic obstructive pulmonary disease (COPD), a leading cause of death worldwide. We assessed the risk for developing COPD, a clinical disorder characterized by a mixture of small airway disease and parenchymal destruction (emphysema), with a serial lung function in cigarette smokers with normal spirometry and no emphysema as

assessed by HRCT, by comparing smokers with reduced DLCO vs normal DLCO. Despite having normal spirometry, cigarette smokers with reduced DLCO were at significantly higher risk for developing COPD within <4 years compared to those with normal DLCO i.e., the DLCO can be used to identify smokers at high risk for developing COPD, and could be a unique parameter in future studies to assess waterpipe smokers over time.

Plasma levels of total and apoptotic EMPs, indicative of pulmonary capillary endothelial apoptosis, were elevated in light-use waterpipe smokers. The possible importance of this observation was highlighted by a parallel study, where we assessed the stability and reversibility of EMP levels in nonsmokers, healthy cigarette smokers and COPD cigarette smokers at 4 time points over a period of 1 year. The levels of total and apoptotic EMPs remained high with continuous smoking in healthy and COPD cigarette smokers. A subset of the healthy cigarette smokers and COPD cigarette smokers agreed to quit smoking. Following smoking cessation for 1 year, total and apoptotic EMP levels returned to normal nonsmoker levels in healthy cigarette smokers but remained abnormally high in COPD cigarette smokers. High levels of circulating and apoptotic EMPs are indicative of persistent and irreversible destruction of pulmonary capillaries and may be another unique parameter to assess waterpipe smokers over time.

In summary, young, light-use waterpipe smokers have a number of lung clinical and biologic abnormalities compared to nonsmokers, including reduced DLCO, found to predict high risk for developing COPD in cigarette smokers, and elevated plasma levels of total and apoptotic EMPs, a marker of alveolar destruction, shown to be persistent and irreversible in COPD cigarette smokers despite smoking cessation. Together, these studies suggest that even light-use waterpipe smokers may be at risk for developing lung disease.

List of Abbreviations:

AM – alveolar macrophages

COPD – chronic obstructive pulmonary disease

DLCO – diffusion capacity of the lung for carbon monoxide

EMPs – endothelial microparticles

GOLD – global initiative for chronic obstructive pulmonary disease

HRCT – high resolution chest tomography

FEF – forced expiratory flow

FEV1 - forced expiratory volume in 1 sec

FRC – functional residual capacity

FVC - forced vital capacity

SAE – small airway epithelium

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1. Introduction

1.1 Waterpipe Smoking

1.1.1 The waterpipe device

Waterpipe, also known as narghile, hookah, shisha and hubble-bubble, is a device used to smoke tobacco. It dates back over 400 years to the Middle East¹⁻³. There is regional variation in the size and appearance of the device, the type of tobacco smoked, the heating device, and the frequency the water in the device is changed^{2,4-5}. The waterpipe device that is mostly used is the one originating from the Middle East, consisting of a small bowl where a pre-made tobacco mixture with flavors or spices is placed and burned, by a lamp, coal or wood (Figure 1)^{2,5}. The generated smoke is drawn through a pipe connecting the bowl to a base filled with water. The smoke then passes through a hose and mouth piece into the lungs. In the Indian waterpipe (Jajeer), the coal is in direct contact with the tobacco in the waterpipe device head, without the tin or silver foil separator that is used in the Middle Eastern waterpipe device⁶. In the Chinese waterpipe, the tobacco is directly lit without charcoal⁷⁻⁸.

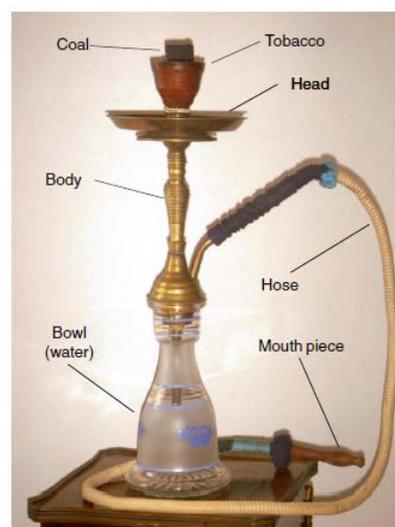


Figure 1. Middle Eastern waterpipe device. Adapted from Akl et al.²

1.1.2 Global prevalence of waterpipe smoking

Waterpipe smoking was originally associated with Middle Eastern culture, but since the 1990s, migration, tourism, curiosity and the social media has facilitated its spread among populations in Europe, the United States and South America. The use of waterpipe is now considered a global epidemic, with millions of daily users worldwide, making it the second most popular form of tobacco use, after cigarettes^{2,4,9}. The raising prevalence of waterpipe smoking is due to its low cost, the lack of waterpipe-specific smoking regulations, and its social acceptance. Many users believe that waterpipe smoke is far less harmful and addictive than cigarette smoke because the smoke passes through water, which they presume acts as a filter^{4-5,8-10}. The setting of waterpipe smoking in public places, such as bars or cafes, with one device shared by several smokers during long smoking sessions (45-60 min), makes it a form of socialization and entertainment that attracts youth and teens^{2,4-5}. Added flavors are used to attract smokers to the pleasant aroma and reduce irritation from the tobacco products accompanying the inhaled nicotine; therefore, the tobacco mixture used for waterpipe smoking a flavored tobacco product, a category that is not regulated⁵. Tobacco-related laws that do not explicitly reference waterpipe smoking, do not regulate waterpipe smoking in public, making it more accessible to the general public and specifically to adolescents^{5,8}.

The common profile of waterpipe smokers includes adults and adolescents in Middle Eastern countries, as well as school and university students who are descendants of Middle Eastern countries living in the West². The Global Youth Tobacco Survey summarizing the smoking habits of half a million young teenagers (13 to 15 years old) from 95 countries found that, while cigarette smoking prevalence is stable or in decline, waterpipe smoking shows a

rising trend. In Middle Eastern countries, as high as 60% of adolescents have tried waterpipe smoking in their lifetime, with the male gender being dominant, and with more women regularly smoking waterpipe than cigarettes as it is considered more acceptable¹¹. In the US it is estimated that 10 to 20% of college student are current waterpipe smokers and that 41 to 48% have tried it. Eleven to 17% of high-school students and 2 to 10% of middle school students are current waterpipe smokers⁵.

1.1.3 Waterpipe smoke components

There are many similar compounds in waterpipe tobacco and cigarette tobacco. Both contain high amounts of nicotine, tar and heavy metals, such as arsenic, chromium and lead, known carcinogens such as polycyclic aromatic hydrocarbons and naphthylamines, primary aromatic amines and carbon monoxide^{1,4,12-14}.

Waterpipe smokers are exposed to higher concentrations of toxins than cigarette smokers due to the higher toxin concentration in the smoke itself and/or the mode of smoking, including the frequency of puffing, the depth on inhalation and the length of smoking session^{1,4}. Studies assessing waterpipe smoking habits found that during one waterpipe smoking session, the smoker inhales 10 times more puffs and up to 200 times more smoke compared to one cigarette smoked. The waterpipe smoker, and those in the local milieu, are also exposed to high levels of second-hand smoke^{3-5,9}. Despite the belief that most hazardous components are filtered by the water in the device, analyses of the water following a waterpipe smoking session showed that only 3% of the total metals and 5% of the nicotine are filtered out by the water while the rest passes through the water, as well as other volatile carcinogens and particles. These results did not vary by the flavor or the type of tobacco used^{4-5,15}.

The combination of charcoal and tobacco is unique to waterpipe smoking. The charcoal burning temperature is twice as high as needed for cigarettes, exposing the waterpipe smoker to large quantities of combustion-generated toxicants in addition to the inhaled toxicants transferred from the tobacco itself^{1,4-5}. Approximately 90% of the carbon monoxide, >95% of the benzo(a)pyrene, and 75 to 92% of the polycyclic aromatic hydrocarbons compounds measured in the waterpipe smoke originate in the charcoal used to heat the tobacco¹⁶. Further, the added flavors expose the waterpipe smoker to various allergens and phenol and its derivatives, which are known to promote DNA mutations and cardiovascular diseases, in quantities up to 1,000 times greater than in smoke from a single cigarette. Finally, the use of a shared mouthpiece during smoking sessions can spread infectious diseases^{5,17-18}. Table I summarizes the toxicants of machine-generated waterpipe smoke compared to machine-generated cigarette smoke. Together, these studies indicate that, compared to cigarette smokers, the waterpipe smokers are exposed to high levels of toxins that penetrate the lungs much deeper^{1,4,19}. However, a caveat to these is that the levels of toxicants were evaluated in the released smoke or water left in the waterpipe bowl, rather than measuring the levels reaching the smoker's lungs. In addition, the assessments were done on a machine-generated smoke programed to imitate waterpipe and cigarette smoking behavior rather than during real waterpipe and cigarette smoking^{15-16,20-22}.

Table I. Comparison of Toxicant Yield in Waterpipe Smoke and Cigarette Smoke^{1,*}

Component	Waterpipe	Cigarette	Waterpipe/ cigarette
Tar (mg)	802	15-29	8-53
Nicotine (mg)	3.09	1-3	6-15
Carbon monoxide (mg)	145	10-23	6-15
Polycyclic aromatic hydrocarbons (ng)			
Benzo(a)pyrene	307	20-40	8-15
Diben(a,h)anthracene	147	4	37
Indeno(1,2,3-cd) pyrene	183	4-20	9-45
Aldehydes (mg)			
Formaldehyde	630	70-100	6-9
Acetaldehyde	2520	500-1400	2-5
Acrolein	892	60-140	6-14
Heavy metals (ng)			
Arsenic	165	40-120	1-4
Chromium	1340	4-70	19-335
Lead	6870	34-85	80-200
Phenols (µg)			
phenol	58.0	22.3	2.6
o-cresol	4.41	5.79	0.8
m-cresol	4.66	4.33	1.1
p-cresol	5.38	10.1	0.5
catechol	316	40.7	7.8
resorcinol	1.69	0.79	2.1
hydroquinone	20	23	23

¹ Comparison of machine-generated waterpipe smoke and machine-generated cigarette smoke using standard protocols for an average 1 hr for a waterpipe session and 5 min for a cigarette consumption.

* Adapted from Maziak et al.¹⁹

1.1.4 Long-term and acute effects of waterpipe smoking on the respiratory system

A number of studies have assessed the long-term effects of waterpipe smoking on pulmonary function, cancer prevalence and other clinical symptoms in older (40 to 60 years old), heavy-use waterpipe smokers (30 to 60 waterpipe-year history), and mostly in waterpipe smokers who already have manifested disease^{3,8,23-26}. Long-term effects of heavy-use waterpipe smoking include increased symptoms of chronic bronchitis (frequent, productive cough), emphysema, a significant reduction in forced expiratory volume in 1 sec (FEV₁) and FEV₁/forced vital capacity (FVC) ratio, and a shorter 6-minute walk distance compared to nonsmokers. A few studies of older waterpipe smokers show an association between chronic obstructive pulmonary disease (COPD) and smoking the traditional Middle-Eastern or the Chinese waterpipe. An association with asthma and waterpipe smoking remains inconclusive^{8,23}. Animal models have demonstrated airway resistance, lung inflammation, and oxidative stress as possible mechanisms leading to the impaired lung function with prolonged exposure to waterpipe smoke^{8,24}.

Meta-analysis reviews of lung function studies in older, heavy-use waterpipe smokers compared to nonsmokers and cigarette smokers showed reduced FEV₁ and a trend to lower FVC and FEV₁/FVC in heavy-use waterpipe smokers compared to nonsmokers^{8,23,25}. There was no difference in lung function in heavy-use waterpipe smokers compared to cigarette smokers. Both cigarette smokers and heavy-use waterpipe smokers had similar prevalence of cough and sputum symptoms, though the symptoms appeared at an earlier age in the waterpipe smokers than in the cigarette smokers (Table II). However, the reviews concluded the quality of evidence in the studies was low with no standardized measure of frequency and length of waterpipe smoking sessions, the type of tobacco smoked, or the exposure to other toxins. Also, there was no distinction between nonsmokers and passive smokers^{23,25}.

Studies conducted in long-term smokers of the Indian waterpipe showed high prevalence of severe respiratory symptoms compared to nonsmokers. Some of the lung function parameters correlated with the duration and amount of waterpipe smoking. Indian waterpipe smoking had a similar effect on the respiratory system as deep inspiration of cigarettes^{26,28}.

A study conducted in China assessed the effects of active Chinese waterpipe smoking in men (>40 years old) and of passive Chinese waterpipe smoking in women compared to active and passive cigarette smokers and nonsmokers. The active waterpipe smokers demonstrated a higher prevalence of cough and sputum and a lower FEV₁/FVC ratio compared to nonsmokers and cigarette smokers⁷. The passive waterpipe smokers had significantly worse lung function compared to nonsmokers and passive cigarettes smokers. Eighteen % of the active waterpipe smokers and 8% of the passive waterpipe smokers had emphysema on CT, compared to 5% of the cigarette smokers, 1% of the passive cigarette smokers and 0% of the nonsmokers⁷.

In a study of n=110 waterpipe-only smoking men (20 to 60 years old), none of the subjects had normal spirometry and many waterpipe smokers had an estimated lung age significantly higher than the chronological lung age²⁴. Restrictive ventilator defects, usually associated with obesity and rare in cigarette smokers, were found in 14% of the waterpipe smokers (Table III)²⁴.

Table II. Long-term effects of waterpipe smoking on pulmonary function^{1,*}

Reference	Population	WP quantity ²	Tobacco type	All healthy?	Comparison	% predicted difference ³				FEV ₁ /FVC difference ³
						FEV ₁	FVC	FEF 25-75		
28	371 men, 301 women average, ages in 30s and 40s	1.17 ± 0.53 WP smoked/week	N/A	Yes	WP vs nonsmokers	-14.6	-21.9	N/A	N/A	-13.8
29	142 men age 35-60 years	36 ± 22 WP years	Tabamel	Yes	WP vs cigarette smokers (normal inhalation)	-3.8 (NS)	-7.03	N/A	N/A	-13.0
24	110 men, age 20-60 years	Median 14 WP years	N/A	Yes	WP vs reference values	Decreased	Decreased (NS)	Decreased	Decreased	Decreased (NS)
25	139 men, 13 women, age 24-65 years	N/A	Moassal	Yes	WP vs cigarette smokers	-1.1 (NS)	N/A	+0.5 ⁴ (NS)	N/A	N/A
30	25 persons average age 49 ± 12.2 years	23.7 ± 8.3 years smoking 1-2 times/day	N/A	Yes	WP vs nonsmokers	-12.2 (NS)	N/A	-2.5 ⁴ (NS)	N/A	N/A
31	397 men, age 18-85 years	37 ± 42 Jurak-years	Jurak	No	WP vs passive cigarette smokers	-2.5 (NS)	+0.9 (NS)	-5.6 ⁴	-7.2 (NS)	
32	788 women, age 44+ years	N/A	N/A	No	WP vs nonsmokers	-6.5	-5.86 (NS)	-3.02 ⁴	-8.63 (NS)	
					WP vs cigarette smokers	+3.01	-0.5 (NS)	+4.49 ⁴	+5.08	
7	1238, mostly men, age 40+ years	28 ± 11.2 years of 17.9 ± 8.9 g tobacco/day	Chinese WP tobacco	Yes	WP vs nonsmokers and cigarette smokers	+5.3 (NS)	N/A	+0.1 (NS)	N/A	N/A
					WP vs nonsmokers and cigarette smokers	-9.4	+6.1	-12.1	N/A	
					WP vs cigarette smokers	-4.0	+7.1	-8.0	N/A	
					WP passive vs never smokers	-9.0	-6.6	-4.5	N/A	
					WP passive vs cigarette-passive	-6.9	-5.5	-3.0	N/A	
33	441 men (20-59 years old), 154 women (17-59 years old)	N/A	Jurak	Yes	WP smokers vs nonsmokers	-0.54 L	-0.43 L	-4.6	N/A	N/A
					Males	-0.41 L	-0.19 L	-11.42	N/A	N/A
					Females	-4.4 (NS)	-9.1	+5.56	N/A	N/A
34	87 men, 45 women, average 33.4 ± 13.29 years	11.12 ± 17.27 WP/week	Moassal	No	WP vs nonsmokers	+1.63 (NS)	-2.28 (NS)	+4.28	N/A	N/A
					WP vs cigarette smokers					

¹ WP= waterpipe; NS = not significant. ² Data is average ± standard deviation, unless otherwise specified. ³ All pulmonary function values are differences (WP value compared to group value). Units are % predicted, except FEV₁/FVC which is % ratio, or otherwise specified; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; FEF = forced expiratory flow between 25% and 75% of the FVC. ⁴ Percent predicted value. * Adapted from El-Zaatari et al. ⁸

Table III. Spirometric Profile of Waterpipe Smokers^{1,2,*}

Parameter	n (%)
	≤ N
FEV ₁ (L)	14 (13%)
FVC (L)	7 (6%)
SVC (L)	40 (36%)
FRC (L)	14 (13%)
TLC (L)	15 (14%)
RV (L)	9 (8%)
PEFR (Ls ⁻¹)	31 (28%)
PEF _{25%} (Ls ⁻¹)	8 (7%)
PEF _{50%} (Ls ⁻¹)	9 (8%)
PEF _{75%} (Ls ⁻¹)	9 (8%)
MMEF (Ls ⁻¹)	18 (16%)
FEV ₁ /FVC	6 (6%)
FEV ₁ /SVC	2 (2%)
	≥ N
FRC (L)	36 (33%)
TLC (L)	23 (21%)
RV (L)	40 (36%)
RV/TLC	33 (30%)
FRC/TLC	16 (14%)
Normal FVC and PEF _{25%} or PEF _{50%} or PEF _{75%} or MMEF <N	15 (14%)
PEF _{25%} <N	8 (7%)
PEF _{50%} <N	9 (8%)
PEF _{75%} <N	9 (8%)
MMEF <N	15 (14%)
Normal FEV ₁ /FVC and TLC and FVC <N or FEV ₁ <N	5 (5%)
FVC <N	3 (3%)
FEV ₁ <N	5 (5%)

¹ Data is presented as number (%) of subjects with ventilator variables outside the normal range or with clinical obstructive ventilatory defect.

² N – normal corresponding to the confidence interval of 95%; FEV₁ – forced expiratory volume in 1 sec; PEFR – peak expiratory flow rate; PEF – peak expiratory flow; MMEF – maximum mid expiratory flow; FVC – forced vital capacity; SVC – slow vital capacity; RV – residual volume; FRC – functional residual capacity; TLC – total lung capacity.

* Adapted from Ben Saad H et al.²⁴

A recent study comparing young, waterpipe-only male smokers to nonsmokers found a significant reduction in FEV₁, FEV₁/FVC and forced expiratory flow (Table IV)³⁵. There was also a significant reduction in fractional exhaled nitric oxide. While of interest, this study did not include a detailed history of waterpipe smoking (i.e., number of years smoked or number of sessions per week) or past or current exposure to other tobacco products.

Table IV. Comparison of Lung Function and Fractional Exhaled Nitric Oxide (FeNO) Parameters in Waterpipe Smokers and a Matched Control Group^{1,*}

Parameter	Waterpipe (n=73)	Control (n=73)	p value
Age (years)	21.54 ± 0.41	21.36 ± 0.19	>0.6
Height (cm)	172.68 ± 0.76	173.71 ± 1.03	>0.4
Weight (kg)	76.26 ± 2.39	72.84 ± 1.48	>0.2
FVC (L)	5.76 ± 0.21	5.54 ± 0.11	>0.3
FEV ₁ (L)	3.80 ± 0.12	4.49 ± 0.07	<0.0002
FEV ₁ /FVC (%)	69.34 ± 1.87	82.83 ± 1.29	<0.0002
FEF _{25%} (L/sec)	6.04 ± 0.25	6.93 ± 0.18	<0.006
FEF _{50%} (L/sec)	3.13 ± 0.19	5.25 ± 0.17	<0.0002
FEF _{75%} (L/sec)	1.13 ± 0.13	2.56 ± 0.13	<0.0002
FEF _{25-75%} (L/sec)	6.86 ± 2.23	4.53 ± 0.19	>0.2
FEF _{75-85%} (L/sec)	1.21 ± 0.24	1.91 ± 0.11	<0.02
PEF (L/sec)	7.29 ± 0.22	7.32 ± 0.18	>0.08
FeNO (ppb)	23.97 ± 2.12	31.38 ± 2.38	<0.03

¹ Data is presented as mean ± standard deviation. FVC – forced vital capacity; FEV₁ – forced expiratory flow in 1 sec; FEF – forced expiratory Flow; PEF – peak expiratory flow.

*Adapted from Meo et al.³⁵

Acute effects of waterpipe smoking measured in heavy-use waterpipe smokers immediately after a waterpipe session include increased respiratory rate, changes in the FVC, FEV₁, FEV₁/FVC and diffusion capacity of the lung for carbon monoxide (DLCO), and acute increase in carboxyhemoglobin levels (Table V)^{8,27}.

Table V Acute Respiratory Effects of Waterpipe Smoking*

Reference	Population ¹	Smoking session duration	Tobacco type and amount	All healthy?	RR bpm ²	% predicted difference ³				FEV ₁ /FVC difference ³
						FEV ₁	FVC	PEFR	FEF 25-75	
36	202 men, >17 years old	45 min	N/A	No	+2	N/A	N/A	N/A	N/A	N/A
37	30 men, 15 women, 18+ years old, ave age 23.4 ± 23.4 years old, range 18-65 years old	30 min	10g Moassal	Yes	+2.3	-1 (NS)	0	-8	-5	-1 (NS)
38	24 men, 18-26 years old	45 min	N/A	Yes	+2	-0.08 L/sec	-0.05 L/sec (NS)	N/A	-0.22 L/sec (NS)	N/A
39	39 men, 23 women, 47 active smokers, 18+ years old, ave age 24.9 ± 6.2	30 min	10g Moassal	Yes	+3.5	+0.1 (NS)	-0.7 (NS)	-3.6	-0.1 (NS)	+1.0 (NS)
34	87 men, 45 women, ave age 33.4 ± 13.29 years, WP exclusive smokers	45 min	20g Moassal	No	N/A	-1.21 ⁴	+1.69 ⁴	N/A	N/A	-2.28 ⁴

¹ WP = waterpipe, ave=average.

² All pulmonary function values are changes (WP value after-WP value before). The units are % predicted, except FEV₁/FVC, which is % ratio; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; PEFR = peak expiratory flow rate; FEF25-75 = forced expiratory flow between 25% and 75% of the FVC; N/A = not evaluated; NS = not significant.

³ RR bpm= resting heart rate (beats per min).

⁴ Statistical significance unspecified.

* Adapted from El-Zaatari et al.⁸

1.1.5 Additional health effects of waterpipe smoking

Studies assessing the health effects associated with heavy-use waterpipe smoking show many acute and long-term effects (Table VI). Several studies have found a significant correlation of waterpipe smoking to lung cancer, and a non-significant increased risk to various types of other carcinomas, including bladder, nasopharyngeal and oesophageal cancers (Table VII)^{3-5,8,40}. Heavy-use waterpipe smoking was significantly associated with various respiratory diseases, heart rate variations, hyperglycemia and hypertriglyceridemia, low birth rate, periodontal disease and chromosomal aberrations.

Table VI. Adverse Health Effects Associated with Waterpipe Smoking^{1,*}

Acute effects

- Increased heart rate
- Increased blood pressure
- Carbon monoxide intoxication
- Impaired pulmonary function (FEF_{25-75%}, PEFR)
- Decreased exercise capacity
- Larynx and voice changes

Long-term effects

- Ischemic heart disease
- Impaired pulmonary function (FEV₁, FVC, FEV₁/FVC, FEF_{25-75%}, PEF, FRC, RV)
- Chronic obstructive lung disease
- Chronic bronchitis
- Emphysema
- Lung cancer
- Oesophageal cancer
- Gastric cancer
- Low birth rate
- Pulmonary problems at birth
- Periodontal disease
- Larynx and voice changes
- Lower bone density and increased fracture risk

¹ FEF – forced expiratory flow; PEFR – peak expiratory flow rate; FEV₁ – forced expiratory rate in 1 sec; FVC – forced vital capacity; PEF – peak expiratory rate; FRC – functional residual capacity; RV – residual volume.

* Adapted from El-Zaatari et al.⁸

Table VII. Studies on Association of Waterpipe Smoking and Cancer*

Reference	Cancer type	Population	Study type	Controlled for cigarette smoking?	Adjusted for other cofounders?	OR (95% CI)	
41	All cancer death	20033 Bangladeshi individuals	prospective community-based	no	yes	adjusted=2.5 (1.08 to 5.82)	
40	Lung	150 Lebanese individuals	case-control	yes	yes	6.0 (1.78 to 20.26)	not significant after adjustment for
6	Lung	751 Indian individuals	case-control	no	no	5.8 (3.9 to 8.6)	
42	Lung	265 Indian individuals	case-control	yes	yes	adjusted=4.44 (1.2 to 16.44)	OR for male, >45 year old heavy smokers
43	Lung	148 Chinese men	case-control	no	no	¹	increased risk with cumulative exposure
44	Lung	1438 Chinese men	case-control	yes	yes	adjusted=1.8 (0.8 to 4.2)	did not control for Chinese long-stem pipe smoking
45	Lung	12011 Chinese men	case-control	yes	yes	¹	did not control for Chinese long-stem pipe smoking
46	Oesophageal	2365 Indian individuals	case-control	yes	yes	adjusted=1.85 (1.41 to 2.44)	higher risk with greater intensity, duration and cumulative waterpipe smoking
47	Oesophageal	330 Indian individuals	case-control	no	yes	adjusted=21.4 (11.6 to 39.5)	
48	Oesophageal	871 Iranian individuals	case-control	yes	yes	adjusted=1.66 (0.65 to 4.22)	OR for >32 waterpipe-years smoking
49	Gastric	928 Iranian individuals	prospective cohort	yes	yes	adjusted=3.4 (1.7 to 7.1)	
50	Gastric	92 Iranian individuals	case-control	N/A	N/A	N/A	statistically significant association was observed
51	Gastric	922 Iranian individuals	case-control	yes	yes	adjusted=1.1 (0.3 to 3.3)	also non-significant for cumulative waterpipe use; also included a small % of n of gastric cancer cases was too small for significance: most
52	Gastric and oesophageal	3064 Yemeni individuals	cross-sectional	N/A	no	not calculated (x ² =2.646, p<0.05)	OR for smoking >153 waterpipe-years; OR also insignificant for lesser
53	Bladder	1134 Egyptian men	case-control	yes	yes	adjusted=1.1 (0.7 to 1.9) for urothelial cancer; 0.5 (0.2 to 1.0) for squamous cancer	
54	Bladder	308 Egyptian men	case-control	yes	yes	adjusted=0.8 (0.2 to 4.0)	
55	Prostate	274 Iranian men	case-control	yes	yes	OR=7.0 (0.9 to 56.9)	waterpipe smoking was also non-significant (but
56	Pancreatic	388 Egyptian individuals	case-control	no	yes	adjusted=1.6 (0.9 to 2.8)	waterpipe smoking was also not exclusive of other
57	Nasopharyngeal	1251 North African individuals	case-control	no	yes	adjusted=0.49 (0.2 to 1.43)	had small number of waterpipe smokers

¹ A single OR was not reported, but there was an increased risk based on mathematical modelling, which was beyond the scope of the paper. * Adapted from El-Zaatari et al.⁸

1.1.6 Limitations of current studies

There is limited data on the long term health effects of waterpipe smoking. While cigarette smoking is a well-established risk factor for lung cancer, COPD and other disorders, there is need for more robust, longitudinal, well-designed studies of the potential health risks associated with waterpipe smoking³. Despite the growing prevalence of young adults smoking waterpipe, most studies assess the effects of heavy-use, long-term waterpipe smoking in older individuals, disregarding the health effects and clinical and biologic abnormalities associated with light-use, short-term waterpipe smoking.

Many of the studies of waterpipe smoking have limited details of the quantity and type of the tobacco used, exposure to other risk factors such as the use of other tobacco products, second-hand exposures to tobacco or other toxins, the frequency and length of the waterpipe smoking session or the number of years of smoking^{5,8-9}. Most of the spirometry studies of waterpipe smoking have low sample size and the subjects had other diseases affecting lung function⁸. In several studies evaluating the association of waterpipe smoking with cancer, almost all of the waterpipe smokers also smoked cigarettes or other tobacco products, making it difficult to isolate the effect of waterpipe smoking⁵⁸.

Another complication in making conclusions about the health effects of waterpipe smoking is the lack of uniformity in the type of waterpipe smoked. Several studies of waterpipe smoking take place in China and India where the waterpipe devices are unique as the tobacco burns directly on the charcoal (India) or is directly lit (China). In contrast, in most other countries, the Middle Eastern device is used, where the tobacco is indirectly heated by the charcoal. Since the carcinogenic potential of the smoke may be related to the temperature achieved during the smoking session, the Chinese and Indian waterpipe likely have a higher carcinogen potential than the Middle Eastern waterpipe⁶. Another difference is the frequency the

water in the bowl is changed: whereas in the Middle Eastern waterpipe the water is changed after each use, in the Indian and Chinese waterpipe, the water is only changed after several days of use, exposing the smoker to higher levels of toxins accumulating from smoking multiple sessions⁶⁻⁷.

Despite these limitations, there is enough evidence to suggest that waterpipe smoking has harmful health effects. In this regard, it has been suggested that the current knowledge should be used to educate the public and design intervention and research to help guide regulations to stop the epidemic of waterpipe smoking from spreading, to ban misleading information, and to limit access of youth and minors to waterpipe smoking^{4,8-9}. As recommended by the World Health Organization, there is need to identify and quantify with confidence the health effects associated with short-term and long-term waterpipe smoking and to explore the interaction of waterpipe smoking with other forms of smoking with standardized exposure methods^{9,25}. In Turkey, one of the leading countries rectifying smoking-related laws to include waterpipe smoking, the public, and specifically school students, is being educated about the health risks associated with waterpipe smoking, leading to a significant reduction (65%) in waterpipe smoking prevalence within the past few years¹⁰.

In our study of the health effects associated with light-use, short-term waterpipe smoking in young individuals (Article 1), we assessed various clinical and biologic lung-related parameters of lung health, specifically in cells directly exposed to waterpipe smoke, in waterpipe smokers compared to nonsmokers. The results of the study add to the growing evidence of harmful effects of waterpipe smoking and specifically demonstrate the abnormalities associated with even light-use waterpipe smoking.

1.2 Chronic Obstructive Pulmonary Disease (COPD)

1.2.1 Definition

COPD is defined as a progressive airflow limitation that is not fully reversible with bronchodilators⁵⁹⁻⁶⁰. The airflow limitation or obstruction is caused by a mixture of small airway disease, caused by lesions that obstruct the small conductive airways, parenchymal destruction (emphysema), reducing the elastic recoil of the lung available to force air out of the lung, or both. The relative contribution of each characteristic varies among affected individuals⁵⁹⁻⁶². The major cause of COPD is chronic inhalation of tobacco smoke. Prolonged exposure to cigarette smoke leads to a chronic inflammation, parenchymal tissue destruction, and disruption of normal repair and defense mechanisms (Figure 2)⁶⁰⁻⁶². These changes lead to defective mucociliary clearance and disruption of the epithelial barrier provided by the innate host defense system, causing air trapping and progressive airflow limitation, breathlessness, and excessive cough and sputum production among other symptoms⁶². The airway obstruction affects the time constant for lung emptying, measured by the air that can be expired in one second (FEV_1), the forced vital capacity (FVC) and their ratio (FEV_1/FVC). The levels of these parameters, measured by pulmonary function test are used to diagnose airway disease^{60,62-63}. COPD is defined by post-bronchodilator FEV_1/FVC ratio <0.7 , while FEV_1 % predicted is used to define severity and assess survival rate (Figure 3)⁶²⁻⁶³. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines mild COPD as GOLD I ($FEV_1 \geq 80\%$ predicted), moderate COPD as GOLD II ($80\% > FEV_1 \geq 50\%$ predicted), severe COPD as GOLD III ($50\% > FEV_1 \geq 30\%$ predicted) and very severe COPD as GOLD IV ($FEV_1 < 30\%$ predicted) (Table VIII).

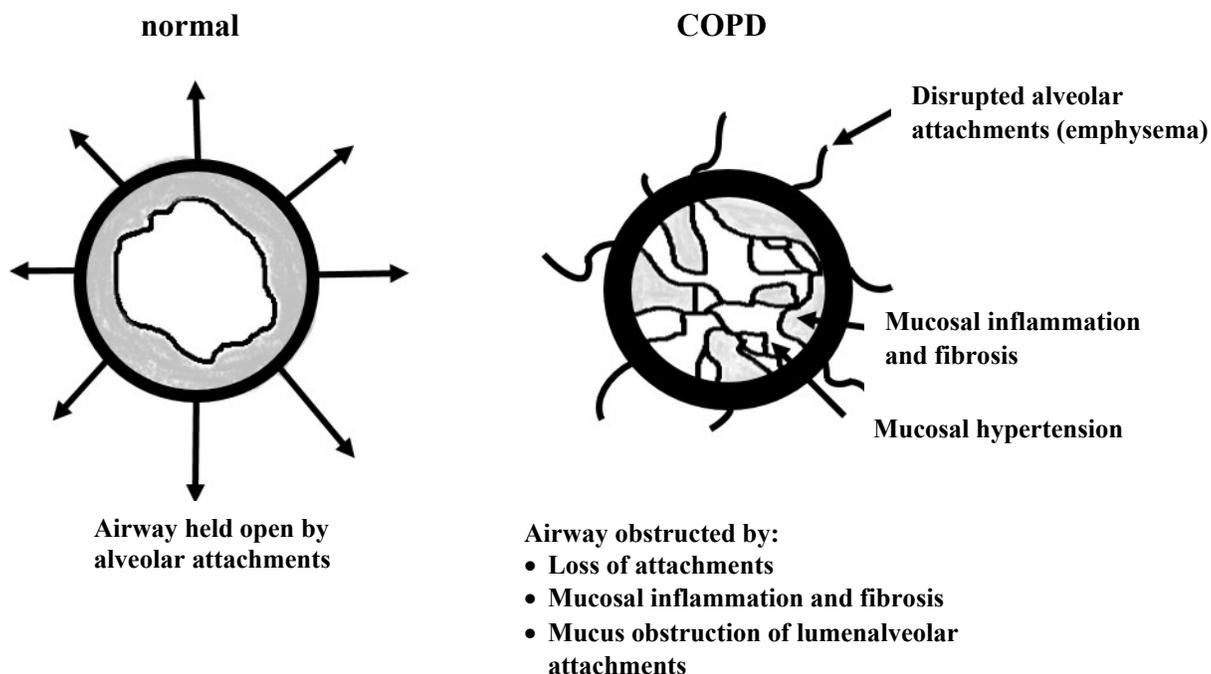


Figure 2. Mechanisms of airflow limitation in COPD. The airway in normal individuals is distended by alveolar attachments during expiration, allowing alveolar emptying and lung deflating. In COPD patients, these attachments are disrupted because of emphysema, trapping gas in the alveoli, contributing to airway closure during expiration, and resulting in hyperinflation. Peripheral airways are also obstructed and distorted by airway inflammation and fibrosis (chronic obstructive bronchiolitis) and by occlusion of the airway lumen by mucus secretions, which may be trapped in the airways because of poor mucociliary clearance. Adapted from Barnes et al.⁶¹

Table VIII. Classification of COPD Severity^{1,*}

	Classification based on post bronchodilator lung function
GOLD I (mild)	FEV ₁ /FVC <0.7 and FEV ₁ ≥ 80% predicted
GOLD II (moderate)	FEV ₁ /FVC <0.7 and 80% > FEV ₁ ≥ 50% predicted
GOLD III (severe)	FEV ₁ /FVC <0.7 and 50% > FEV ₁ ≥ 30% predicted
GOLD IV (very severe)	FEV ₁ /FVC <0.7 and FEV ₁ < 30% predicted or FEV ₁ <50% predicted plus chronic respiratory failure

¹ COPD – chronic obstructive pulmonary disease; GOLD – global initiative for chronic obstructive pulmonary disease; FEV₁ – forced expiratory volume in 1 sec, FVC – forced vital capacity.

* Adapted from GOLD 2015⁶³.

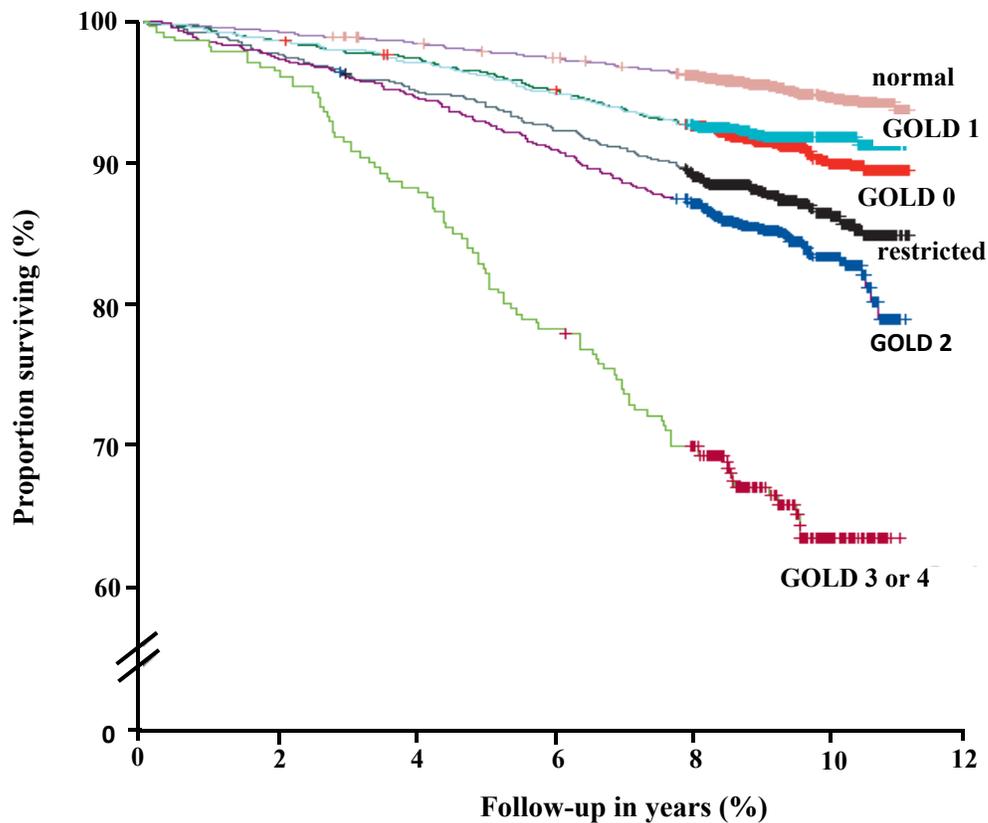


Figure 3. Survival curves, stratified by lung function at baseline. Adapted from Maninno et al.⁶²

1.2.2 Prevalence of COPD

COPD is a global health issue, the 4th leading cause of death worldwide and 3rd in the United States⁶³. The risk for COPD is related to an interaction between genetic factors and environmental exposure, but is also affected by comorbid diseases⁶². Tobacco smoke is the main risk factor for COPD and, in general, the longer people smoke, the higher the risk for developing COPD is. However, for unknown reasons, likely associated with genetics, only a minority of smokers has an excessive decline in FEV₁ leading to COPD and individuals with similar smoking and exposure histories vary in the severity of their disease and response to intervention^{62,64-69}. Other risk factors include occupational hazards such as exposure to various dusts, chemicals, vapors and fumes, indoor or outdoor air pollution and infections⁶².

In the previous decades, the risk for developing COPD among cigarette smokers was estimated to be 15 to 20%, but recent estimates suggest a much higher proportion of smokers, develop COPD, in part due to the worldwide aging population^{60-63,70}. A longitudinal study following lung function in 8045 individuals from the general population for 25 years, found that at least 25% of the smokers developed COPD GOLD II and above while 30 to 40% of smokers developed COPD GOLD I and above⁷¹.

1.2.3 Treatment for COPD

The development of COPD usually takes decades^{65,69,71}. The small airways, the first site with abnormalities associated with cigarette smoke, account for only 10-15% of total airway resistance, therefore small airway impairment might accumulate for many years with very little effect on lung function^{60,71}. Once COPD manifests, there is no therapy that modifies the long-term decline in lung function nor reduces mortality, and pharmacologic therapy can only reduce the symptoms, and improve the health status and exercise tolerance^{61,63,69}. Smoking cessation has a beneficial effect on lung function as measured by a reduction in the excessive decline in FEV₁, mainly if done at an early age^{60,69,72}. Smoking cessation at an older age usually occurs after COPD has manifested, and only has a minor effect on FEV₁ level, with little or no impact on the long term incidence of COPD⁷¹. Regardless of the age of smoking cessation, the rate of decline in FEV₁ is not fully reversible to that of never smokers (Figure 4)⁷².

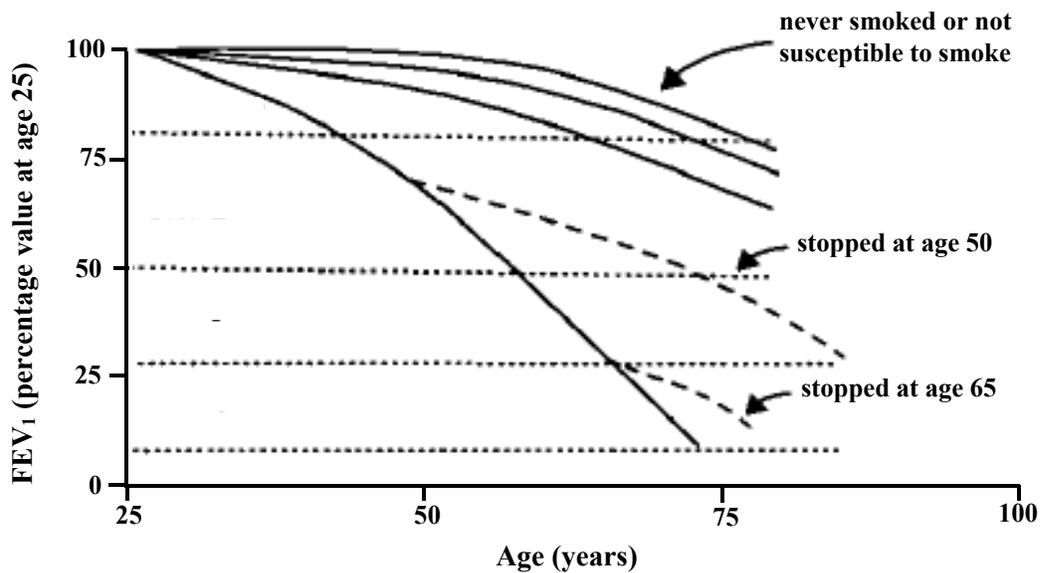


Figure 4. Rate of decline in forced expiratory volume in 1 sec (FEV₁) with age. Adapted from Fletcher et al.⁷²

Since even mild COPD is associated with increased mortality, early detection of COPD is important as it can lead to early therapeutic intervention, including smoking cessation, adequate treatment of the asthmatic component in some patients, and modification of risk factor such as exposure or prevention of complications in patients with established disease^{59,62,66,73}. Various studies assess clinical and genetic markers for identification of those smokers at high risk for developing COPD.

Our study assessing the risk for developing COPD among smokers with normal spirometry but reduced DLCO compared to smokers with normal spirometry and normal DLCO (Article 2) demonstrates the utility of this parameter as a tool for early detection of smokers at risk for developing COPD that can help contribute to early intervention.

1.3 Carbon Monoxide Diffusion Capacity of the Lung (DLCO)

1.3.1 Definition

DLCO is a measurement of the ability of oxygen transfer from the alveoli to the blood. Carbon monoxide acts as a surrogate for oxygen, and is usually used in a test assessing this ability⁷⁴⁻⁷⁵. The DLCO depends on the surface area of the pulmonary microvascular bed available for gas diffusion, the membrane conductivity, the diffusion properties of the alveolar capillary membrane, the binding of carbon monoxide to hemoglobin, the volume of hemoglobin in alveolar capillary blood, and other processes affecting these factors⁷⁶. The DLCO is calculated as the accessible alveolar volume (VA) into which carbon monoxide (CO) is distributed and transferred across the capillary membrane X a rate constant for carbon monoxide removal from alveolar gas (kCO): $DLCO = VA \times kCO^{74,77}$.

1.3.2 Measuring DLCO

Single-breath determination of DLCO measures the uptake of carbon monoxide from the lung over a breath-holding period evaluating the transfer of gas from the alveoli to the red blood cells⁷⁸. The DLCO is a standard noninvasive test to assess the integrity of the alveolar capillary surface area^{77,79}. However, it is not routinely used in lung function assessment as it is expensive, and without expertise and experience, is difficult to reproduce^{66,75}. There are big inter-lab differences in reported DLCO levels caused by differences in DLCO interpretation. Different labs use different methods for calculating DLCO based on variable references for predicted values, and some lack the adjustment for factors affecting the DLCO, such as hemoglobin and carboxyhemoglobin levels^{75,80}. In addition to various factors affecting the actual level of DLCO, the measured level can be biased due to sub-optimal testing caused by submaximal inspired volume, breath hold time and inspiration time.

Therefore, the DLCO test should be conducted under standardized conditions and by experienced technicians^{75,78,81}. To minimize test variability, it is recommended that the maneuver is demonstrated to the subject ahead of time, and that other conditions, such as the subject's position, level of exercise, and room temperature are standardized⁷⁵. DLCO predicted values should be derived from reference individuals recruited from a similar population to that of the tested subjects, measured in a similar setting to that of the tested subjects⁷⁸. Also, to prevent inaccurate measurements, at least two DLCO tests should be performed. The average of two acceptable measures that meet the repeatability requirement as detailed in the ATS/ERS guidelines should be reported as the result⁷⁵.

1.3.3 Adjustment of DLCO levels for carbon monoxide and carboxyhemoglobin and levels

Most regression values for DLCO are derived from studies of groups of lifetime healthy nonsmokers, estimating the inspired carbon monoxide to be 1 to 2% and the hemoglobin level to be 14.6 gL⁻¹ in adult and adolescent males and 13.4 gL⁻¹ in adult females and male and female children <15 years old⁷⁵. In smokers, the carboxyhemoglobin level can be as high as 10 to 15%^{78,82}. Hemoglobin binds to carbon monoxide in a higher affinity than to oxygen, creating carboxyhemoglobin. In smokers, the hemoglobin will be tightly bounded by the excessive carbon monoxide from the cigarette smoke, reducing the overall amount of hemoglobin available for further binding by the fresh carbon monoxide transferred during the DLCO maneuver, leading to less uptake of carbon monoxide and therefore, reduced DLCO level measured^{75,82}. The effect is similar to that of having a reduced blood hemoglobin level, with less hemoglobin available for binding, and is often termed the “anemia” effect^{75,78,82}. In addition, high levels of carboxyhemoglobin increase the backpressure assumed to be 0 during the DLCO maneuver, resulting in overestimation of the driving pressure for carbon monoxide across the air-blood

barrier, leading to underestimation of the DLCO level⁷⁵. It is estimated that in individuals with carboxyhemoglobin >2%, for each 1% increase in carboxyhemoglobin, the DLCO measured is reduced by 0.8 to 1%⁷⁵. The backpressure is responsible for 58.5% and the anemia effect for 41.5% of this reduction in measured DLCO^{75,83}.

To reduce carbon monoxide levels from cigarette smoke affecting DLCO levels measured in smokers, smokers are asked to refrain from smoking or from exposure to other carbon monoxide sources on the day of the test. However, since the time of the last cigarette or exposure may still vary, the measured DLCO level should be adjusted for carbon monoxide level resulting from recent and heavy smoking to compensate for both the back pressure and anemia effect⁸¹. In addition, an adjustment of the measured DLCO level should be made in individuals with low hemoglobin levels^{74,75}.

1.3.4 Factors affecting DLCO levels

The diffusing capacity measures the effectiveness of the alveolar volume and the rate of diffusion per unit volume (kCO); therefore, factors affecting either of these parameters would affect DLCO^{74,78}. A decreased alveolar volume that is associated with age and smoking status will be associated with a decline in FVC as well as a reduced DLCO level⁷⁴. In a study of 1635 never smokers, 775 former smokers and 1392 current smokers (healthy 25 to 74 years old men and women, of European and African origin), FVC was found to have the highest correlation with DLCO ($r=0.53$), followed by height ($r=0.45$)⁸⁴. After adjusting for gender, race and height, DLCO was found to decrease by 0.5% each year in never smokers. DLCO was found to be lower in women but this effect disappeared when adjusting for height, therefore it might only demonstrate a physiologic difference⁸⁴. In young adults, the kCO is the same in both genders but

declines with age at a slower rate in young women than in young men, until the age of 47, when the rate of decline is similar in both genders⁸⁴⁻⁸⁵.

In both males and females, former smokers had lower DLCO levels than never smokers, correlating with pack year history (Figure 5). Current smoking status had a smaller effect on DLCO levels in women, correlating with pack year history and packs per day, with DLCO levels reducing more rapidly than FVC levels (Figure 6). However, the decline in DLCO among smokers might be due to carbon monoxide exposure from the latest cigarette smoked and not a physiological change, as no adjustment for carbon monoxide levels was done to the DLCO measured level⁸⁴. In comparison to European, Africans had lower DLCO levels but the changes in DLCO levels associated with age and height were similar in both races.

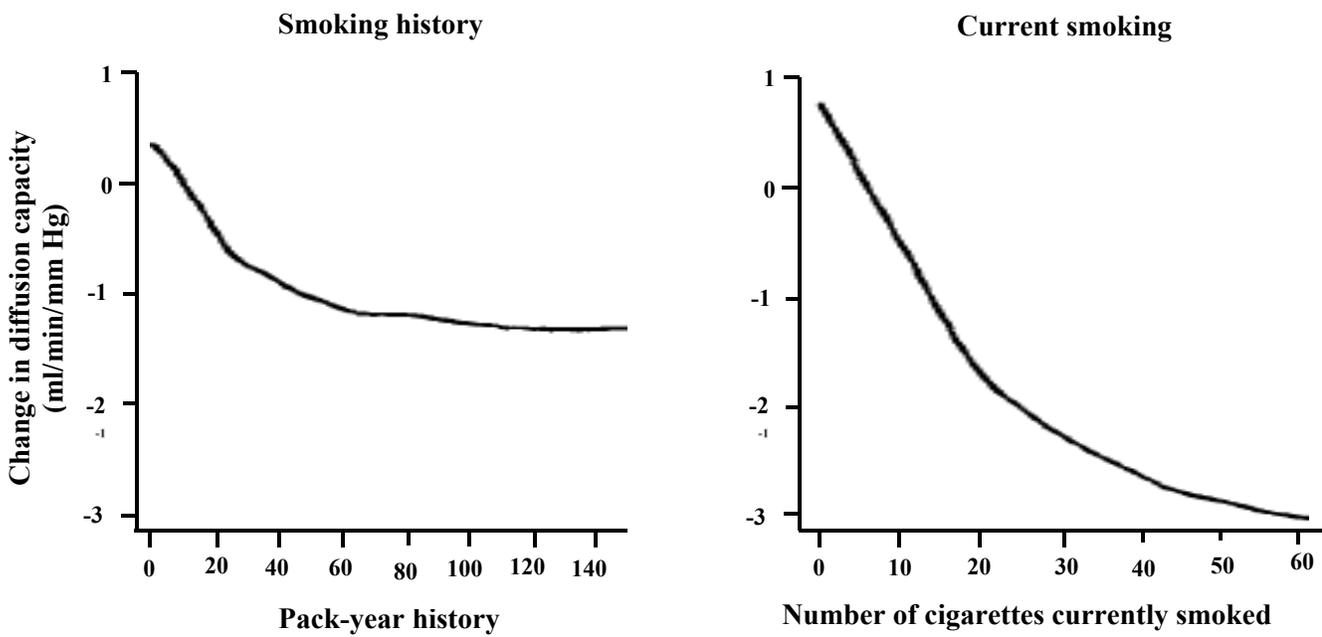


Figure 5. Expected change in diffusion capacity for carbon monoxide (DLCO) based on smoking history (pack year history) and current smoking status (number of cigarettes per day). Data adjusted for gender, race, height, age, weight, and hemoglobin level. Adapted from Neas et al.⁸⁴

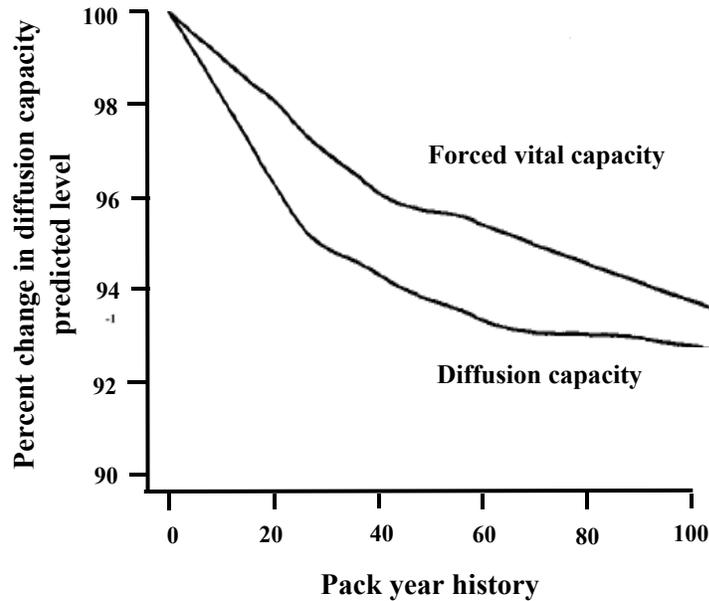


Figure 6. Expected percent change in diffusion capacity for carbon monoxide (DLCO) predicted level based on pack year history. Data adjusted for gender, race, height, age, weight, hemoglobin level and number of cigarettes currently smoked. Adapted from Neas et al.⁸⁴

1.3.5 The use of DLCO level as a diagnostic tool

Reduced DLCO leads to inadequate oxygen levels when there is need for more oxygen (for instance during exercise) as the lung will not have sufficient gas exchanging surface to meet the demand^{74-75,78}. A DLCO % predicted is calculated to assess the function of the lower respiratory tract by comparing the measured DLCO level to a predicted DLCO level derived from reference individual levels and is used in the differential diagnosis of airway obstruction. DLCO % predicted between the lower limit of normal and 60% is considered a mild reduction, DLCO % predicted between 40 and 60% a moderate reduction and DLCO % predicted <40% a severe reduction⁷⁴. A reduction in DLCO levels is observed in a variety of pulmonary disorders affecting the lower respiratory tract, while an elevated DLCO level is associated with asthma, obesity and intrapulmonary hemorrhage (Table IX)⁸⁰. In patients with long-term smoking history and evidence of airway obstruction, normal DLCO suggests chronic bronchitis and reduced DLCO suggests emphysema⁷⁷. Importantly, the DLCO is more sensitive than spirometry to

impairment in gas transfer due to parenchymal destruction and is therefore used for the diagnosis of emphysema^{74,76}.

Reduction in DLCO can be produced by several combinations of reduction in the rate of carbon monoxide removal (kCO) and the accessible alveolar volume. An assessment of both components is essential in the interpretation of DLCO % predicted and can suggest a specific pathophysiological mechanism responsible for the reduction⁷⁵. The single breath estimate alveolar volume should approach the total lung capacity minus the anatomic dead space (~200 ml). When the alveolar volume is low but the alveolar volume/total lung capacity ratio is normal (0.94 ± 0.07), the reduction in DLCO is caused by restrictive lung disease, but when the total lung capacity is normal or increased, and the alveolar volume/total lung capacity ratio is low, the reduction is typically secondary to obstructive lung disease (Figure 7)^{74-75,80}. A low kCO can be caused by emphysema, diffused alveolar-capillary damage associated with connective tissue/autoimmune disease or reduced hemoglobin level⁷⁴.

Table IX. Physiological and Pathological Changes that Affect the DLCO^{1,*}

Extrapulmonary reduction in lung inflation (reduced VA) producing changes in DM or

Reduced effort or respiratory muscle weakness

Thoracic deformity preventing full inflation

Diseases that reduce θV_c and thus reduce DLCO

Anemia

Pulmonary emboli

Other conditions that reduce θV_c and thus reduce DLCO

Hemoglobin binding changes

Valsalva maneuver

Diseases that reduce (in varying degrees) DM and θV_c and thus reduce DLCO

Lung resection

Emphysema

Interstitial lung disease

Pulmonary oedema

Pulmonary vasculitis

Pulmonary hypertension

Diseases that increase θV_c and thus increase DLCO

Polycythaemia

Left to right shunt

Pulmonary haemorrhage

Asthma

Other conditions that increase θV_c and thus increase DLCO

Hemoglobin binding changes

Muller maneuver

Exercise

Supine position (in addition, possibly a slight increase in DM)

Obesity (in addition, a possible DM component)

¹ DLCO – diffusion capacity for carbon monoxide; VA – alveolar volume; DM – membrane conductivity; θ - carbon monoxide-hemoglobin chemical reaction rate; V_c – volume of pulmonary capillary blood.

* Adapted from MacIntyre et al.⁷⁵

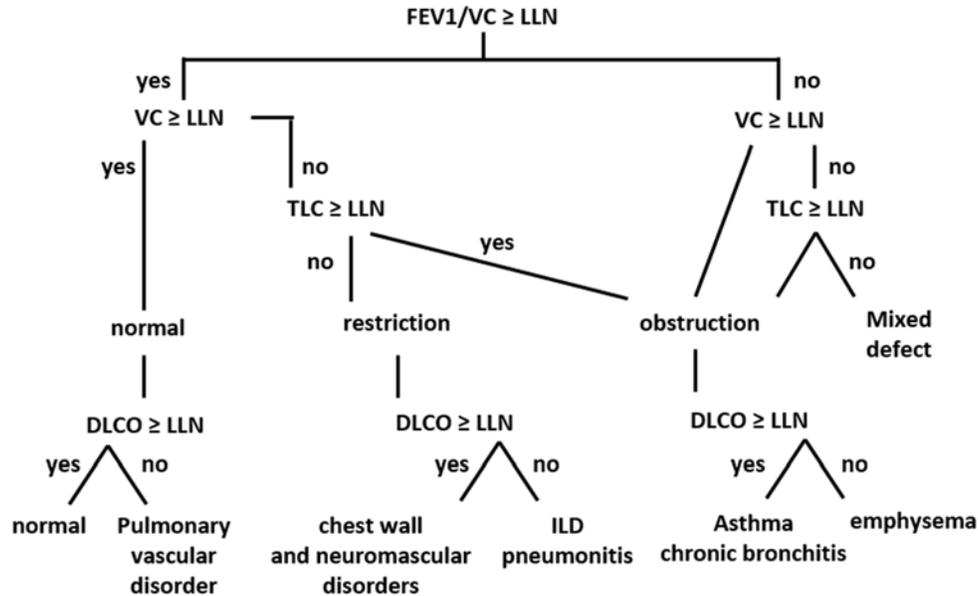


Figure 7. Assessment of lung function in clinical practice. The algorithm presents classic patterns for various pulmonary disorders. Patients may or may not present the classic patterns, depending on their illness, severity or lung function prior to the disease onset. The algorithm includes DLCO measurements with the predicted value adjusted for hemoglobin level. In the mixed defect group, the DLCO patterns are the same as those for restriction and obstruction. The algorithm is not suitable for assessing upper airway obstruction. FEV₁ – forced expiratory volume in 1 sec; VC – vital capacity; LLN – lower limit of normal; TLC – total lung capacity; DLCO – diffusion capacity for carbon monoxide; ILD – interstitial lung disease. Adapted from Pellegrino et al.⁸⁰

1.3.6 Isolated reduced DLCO

Normal spirometry with a reduced DLCO level in a cigarette smoker is usually associated with early small airway disease and/or emphysema, although the reduction can result from anemia, pulmonary vascular disorder, or early interstitial lung disease⁷⁷. High resolution chest tomography (HRCT) in patients with isolated reduced DLCO helps discriminate various patterns of complex mixed obstructive and restrictive abnormalities^{77,86-88}. In a study of 27 individuals with reduced DLCO (<70% predicted), 48% had emphysema on HRCT. In 85% of those, the reduction was associated with a restrictive lung process. The other 52% had interstitial lung disease, pulmonary vascular disease or other isolated findings⁷⁷. The importance of a reduced

DLCO level in individuals with otherwise normal lung function have not been systematically explored, and there are no large studies of subjects with isolated DLCO⁷⁷.

Our study assessed the risk for developing COPD among smokers with normal spirometry, no emphysema on HRCT, but with isolated reduced DLCO compared to smokers with normal spirometry, no emphysema on HRCT and normal DLCO (Article 2). The data demonstrated the importance of DLCO as a marker for early detection of COPD. While the measurement of DLCO is not routine for technical and financial reasons, these results advocate the need to develop an easier to use technique that will allow to follow DLCO levels in cigarette smokers and waterpipe smokers with normal spirometry who are falsely presumed to be normal and help reduce the prevalence of COPD.

1.4. Endothelial Microparticles

1.4.1 Microparticles

Microparticles are small (<1.5 µm) vesicular fragments released from the membrane of various cell types in response to injury, activation or apoptosis⁸⁹⁻⁹². The release, or vesiculation, of these microparticles is caused when a cell membrane loses its normal phospholipid asymmetry, leading to an increase of phosphorylation on the outer layer and blebbing of the membrane causing microparticle formation and shedding by exocytic budding^{89,93}. Though there are several mechanisms suggested (Figure 8), the formation of microparticles is not yet completely understood^{89,94,95}. Typically, there is low grade cell activation caused by normal cell turnover leading to low levels of circulating microparticles found in the blood. However, cellular response to a variety of injury stimuli leads to the generation of microparticles, in some circumstances as a form of defense against sub-lethal complement attack, allowing the cells to shed complement components from the surface^{90,96}. This response to stimuli leads to high levels of microparticles circulating in peripheral blood originating from the plasma membrane of diverse activated or apoptotic cells of platelet, leukocyte or endothelial origin. The cell type from which these microparticles are released and the type of stimuli that released them (cell activation or apoptosis) can be traced by the specific phospholipids and oxidized lipids and the diverse proteins expressed on their membrane^{89,95-96}.

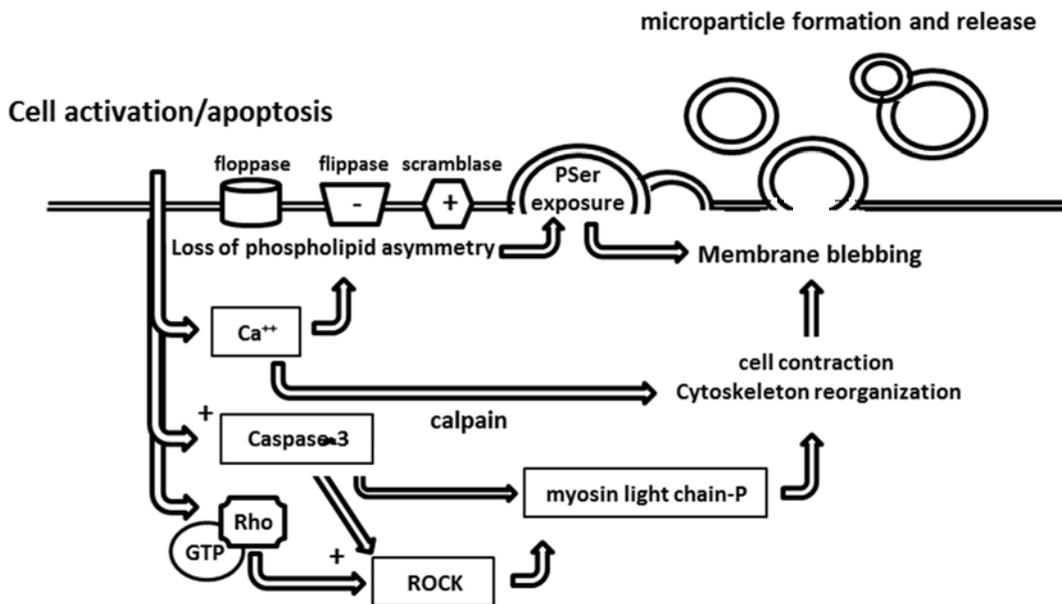


Figure 8. Possible pathways leading to endothelial microparticle release. A resting cell membrane is characterized by its phospholipid distribution, with phosphatidylcholine and sphingomyelin located on its external layer and phosphatidylethanolamine and phosphatidylserine (PSer) on its inner layer. This phospholipid asymmetry is maintained by a transmembrane enzymatic balance of flippase, floppase and scramblase. Cell activation or apoptosis is associated with a release of intracellular calcium by the endoplasmic reticulum that changes the transmembrane steady state. This release leads to PSer externalization and activation of cytosolic enzymes including calpain, leading to the cleavage of cytoskeleton filaments. This pathway results in blebbing and shedding of membrane-derived microparticles into the extracellular fluid. Adapted from Chironi et al.⁸⁹

1.4.2 Endothelial Microparticles

The alveolar tissue is composed of 3 major cell types: type I alveolar epithelial cells, responsible for maintenance of the alveolar structure; type II alveolar cells, the major source of surfactant and the progenitors of type I alveolar epithelial cells; and endothelial cells, lining the capillaries. Together, the type I cells and endothelial cells modulate gas diffusion between the alveoli and blood⁹⁷⁻⁹⁸. Maintenance of an intact monolayer endothelial cell barrier is crucial for normal vascular structure^{89,99}. An intact endothelium monolayer ensures homeostasis by anti-inflammatory, anti-thrombotic and anti-atherogenic properties^{89,97}. The functional integrity of the

vascular endothelium is maintained by continuous regeneration of the endothelial cell layer and the incorporation of endothelial progenitor cells^{96,97}. Under normal conditions, the basal replication rate is 0.1% per day and the release of endothelial microparticles (EMPs), vesicular fragments shed from the endothelial cell membrane, is low grade, local and reversible^{94,96,98}. However, in response to stimuli, the endothelial cells transform to an over pro-coagulant and pro-inflammatory state, releasing higher levels of EMPs^{89,90}. Heterogeneous EMPs may be released from endothelium disturbed by different types of injury and thus, high levels of circulating EMPs are a biologic marker of dysfunctional endothelium and quantification of EMPs in plasma can provide useful information on endothelial cell status^{94,98,100}.

Many factors can injure the endothelium, leading to EMP release, including: inflammation, modification of blood flow, drug toxicity, HIV infection, release of proliferative cytokines and autoimmunity¹⁰¹⁻¹⁰⁴. Elevated levels of circulating EMPs have been reported in vascular diseases, acute coronary syndromes, severe hypertension, metabolic syndrome, type 2 diabetes, end-stage renal disease, pulmonary arterial hypertension, atherosclerosis, heart failure, thrombotic, thrombocytopenic purpura, lupus, multiple sclerosis, sickle cell disease and Other diseases^{92-93,104-107}. Elevated plasma levels of EMPs have also been reported in multiple pro-inflammatory and pro-thrombotic states in asymptomatic individuals, and the quantification of their levels has been used to predict subclinical atherosclerosis burden in individuals with cardiovascular risk factor, and as a predictor of the recurrence of myocardial infraction or death in patients with acute coronary syndromes^{94-95,102}.

Increasing evidence suggest that microparticles not only represent passively released cellular debris, but may also contribute to intercellular signaling mechanisms^{89,108}. EMPs have diverse effects on coagulation, as well as on leukocytes, platelets and endothelium that could

contribute to the pathogenesis of an acute vascular injury¹⁰³. EMPs can impair vascular function and initiate atherosclerosis by promoting endothelial dysfunction and arterial wall inflammation and contribute to plaque progression and rupture⁸⁹; modulate inflammation via leukocyte activation and transendothelial migration¹⁰⁹; decrease release of nitric-oxide by endothelial cells; and increase arterial stiffness¹¹⁰, suggesting that EMPs may also be mediators of disease, not just a marker of vascular injury^{89,108}.

1.4.3 Endothelial microparticles and smoking

Active smoking is established as a cause of endothelial dysfunction and alteration of the biology of endothelial cells^{97,111-115}. Second-hand smoke has also been shown to provoke dysfunctional endothelial cells and an increase in EMP levels¹¹⁶. This endothelium injury is one of the earliest pathological effects of cigarette smoking^{89,114,117}. Cigarette smoke may affect the endothelium by inducing vascular endothelial growth factor (VEGF), a specific growth factor for endothelial cells which induces cell migration and tube formation¹¹². In addition, cigarette smoke reduces endothelial nitric oxide release and surface integrin expression, probably due to excessive generation of reactive oxygen species, affecting normal tube formation and endothelial cell survival. Together, these mechanisms lead to loss of endothelium, resulting in the emphysema observed in COPD^{111,114,118-119}.

In healthy cigarette smokers and in COPD cigarette smokers there is an oxidant/antioxidant imbalance in favor of oxidants^{97,120}. In the respiratory system, the pulmonary vascular endothelium detoxifies xenobiotics arriving through the airways such as those released in cigarette smoke¹²⁰. Excessive xenobiotics exceeding the detoxifying capacity of pulmonary vascular endothelial cells will result in cell impairment, compromising the role of endothelial cells in detoxification which will lead to progression to COPD. Inflammation has an important

role in the pulmonary vascular abnormalities detected in early stage of COPD^{115,120}. In addition to the inflammatory and structural changes in peripheral airways and lung parenchyma, prominent changes also occur in the pulmonary circulation affecting lung microvessels and precapillary arterioles and might be an initiating event that promotes vessel remodeling and pulmonary hypertension in COPD patients^{111,112}. Endothelial impairment promotes the progression of COPD and the progression of COPD may exacerbate the damage of endothelium¹²⁰.

In a previous study we assessed plasma levels of total and apoptotic EMPs in nonsmokers, healthy cigarette smokers with normal spirometry and DLCO and healthy cigarette smokers with early lung destruction as assessed by normal spirometry and low DLCO, the physiologic correlate of emphysema¹²¹. While smokers with normal spirometry and normal DLCO had mild elevated levels of circulating and apoptotic EMPs, smokers with normal spirometry but low DLCO had marked increase of the levels. i.e., there is apoptosis-mediated loss of endothelium before any spirometric evidence of lung disease. Based on the knowledge that smoking is a major cause of COPD and that destruction of alveoli may be initiated, in part, by apoptosis of pulmonary capillaries, we assessed the levels of total and apoptotic EMP levels in a separate cohort of nonsmokers, healthy cigarette smokers with normal spirometry and normal DLCO and COPD cigarette smokers. The consistency of the EMP levels was assessed by measuring the levels in 4 time points over a period of 1 year. Lung function may improve and the rate of decline decrease after a COPD smoker quits smoking, however, airway inflammation persists despite the removal of stimulus^{60,72,115}. Therefore, we hypothesized that COPD smokers maintain high levels of apoptosis even after smoking cessation and followed the plasma levels of

total and apoptotic EMPs in healthy cigarette smokers and COPD smokers who quit smoking for 12 months.

1.5 Study Design and Aims

We hypothesized that even light-use waterpipe smoking is associated with abnormalities in various clinical and biologic lung-related parameters. Based on the knowledge that the first abnormalities associated with cigarette smoking are in lung cells long before there are abnormalities in clinical parameters, we compared young (25 ± 4 years), light-use (3.5 ± 2.5 sessions/week, for an average of 4.1 ± 2.5 years) waterpipe-only smokers ($n=21$) to nonsmokers ($n=19$) matched for gender and ethnicity, using a variety of lung-related parameters, including: (1) blood carboxyhemoglobin levels; (2) cough and sputum scores, assessed using the St. George's respiratory questionnaire (); (3) lung function, including spirometry and DLCO; (4) metabolites present in epithelial lining fluid recovered by bronchoalveolar lavage; (5) cell composition of the small airway epithelium (10^{th} - 12^{th} order bronchi) collected by fiberoptic bronchoscopy; (7) transcriptomes of the small airway epithelium and alveolar macrophages assessed on HG-U133 Plus 2.0 microarrays; and (8) plasma levels of total and apoptotic EMPs.

A reduction in DLCO, a lung function test associated with emphysema and small airway disease, was observed in the young, light-use waterpipe smokers compared to nonsmokers. To assess this parameter as a marker of early disease, we followed a separate cohort of cigarette smokers with normal spirometry, no emphysema, as assessed on HRCT, and normal DLCO ($n=59$) and a group of cigarette smokers with normal spirometry, no emphysema and low DLCO ($n=46$) with a serial lung function for an average of 3.5 years for the risk of developing COPD.

Levels of total and apoptotic plasma endothelial microparticles were found to be elevated in the young, light-use waterpipe smokers compared to nonsmokers. Elevated levels of EMPs, a marker of alveolar capillary destruction, have been previously shown to be associated with a reduction in DLCO in cigarette smokers, probably measuring early alveolar disease. To evaluate

this parameter as an early disease biomarker, we followed plasma levels of total and apoptotic EMPs in nonsmokers (n=29), healthy cigarette smokers (n=61) and cigarette smokers with COPD (n=49) for 1 year at 4 time points (baseline, 3, 6 and 12 months). The effect of cigarette smoking and COPD on EMP levels was assessed by comparing plasma total and apoptotic EMP levels between the groups at each time point. To assess the consistency of the levels, total and apoptotic EMPs were compared at different time points within a group. The reversibility of the elevated total and apoptotic EMP levels measured in the healthy and COPD cigarette smokers compared to nonsmokers was assessed in a subset of the healthy cigarette smokers (n=17) and cigarette smokers with COPD (n=18) who quit smoking for 12 months by comparing the levels in those who quit smoking and those who continued smoking at each time point.

2. Materials and Methods

2.1 Enrollment

Individuals were recruited from the general population of the New York City metropolitan area by posting advertisements in local newspapers and websites. All individuals were evaluated at the Weill Cornell NIH Clinical and Translational Science Center and the Department of Genetic Medicine Clinical Research Facility using Institutional Review Board-approved clinical protocols after giving informed consent. All individuals had their medical history taken and had a physical exam, complete blood count, biochemical profile, serum α 1-antitrypsin levels, HIV test, urine analysis, chest X-ray, EKG, and pulmonary function tests, including FVC, FEV₁, FEV₁/FVC, TLC and DLCO, all carried out under ATS guidelines^{75,122}. Cough and sputum scores were evaluated based on self-reported history using the St. George's respiratory questionnaire¹²³. Smoking assessment included self-reported smoking history including exposure to second-hand smoking or environmental exposure and current smoking status was confirmed using urine nicotine metabolite evaluation (ARUP laboratories, Salt Lake City, UT)¹²⁴.

2.2 Pulmonary Function Test

Pulmonary function tests were done as previously described (DLCO paper). Briefly, individuals were instructed to refrain from smoking as of the night before the testing. Pulmonary function test included spirometry (FEV₁, FVC, FEV₁/FVC) before and after the administration of salbutamol (100 μ g, 4 doses)⁶⁶, lung volumes and DLCO (Viasys Healthcare, Yorba Linda, CA). The DLCO maneuver was carried out 2 to 4 times; the average of the best 2 trials was used. The spirometry and DLCO curves of all pulmonary function tests for all individuals were validated based on ATS/ERS guidelines⁶³. As an additional quality control measure, pulmonary function tests were performed serially in several volunteers during the course of the study. The

DLCO % predicted values were calculated using the Gaensler et al equation¹²⁴, and corrected for hemoglobin and carboxyhemoglobin levels using ATS/ERS guidelines⁶³.

2.3. Sample Collection and Processing

2.3.1 Small airway epithelium, epithelial lining fluid and alveolar macrophages

Small airway epithelium was collected by brushing 10th to 12th order bronchi¹²⁶. The cells were removed from the brush by flicking it into 5 ml of ice-cold LHC8 medium (GIBCO, Grand Island, NY). A 0.5 ml aliquot was used to determine the number and types of cells recovered and 4.5 ml were immediately processed for RNA extraction. The origin of the recovered cells was confirmed as the small airway epithelium based on expression of genes encoding surfactant and club (Clara) cell secretory proteins¹²⁷. Alveolar macrophages and epithelial lining fluid were collected by bronchoalveolar lavage and processed as previously described¹²⁷.

2.3.1.1 RNA processing and quality control

An aliquot of the total RNA extracted from the small airway epithelium and alveolar macrophages was used to determine RNA integrity (Agilent Bioanalyzer, Agilent Technologies, Palo Alto, CA) and concentration (NanoDrop ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE). RNA was hybridized on HG-U133 Plus 2.0 microarrays with probes for >54,000 genome-wide transcripts, using Affymetrix protocols, hardware and software¹²⁸. Microarray quality was verified by signal intensity ratio of GAPDH 3' to 5' probe sets ≤ 3.0 and multi-chip normalization scaling factor ≤ 10.0 ¹²⁹.

2.3.1.2 Transcriptome analysis

For the microarray data, the MAS5 algorithm (GeneSpring version 7.3, Affymetrix Microarray Suite Version 5) was used to normalize the data per array to the median expression value of each sample. Genome-wide analysis was used to compare the expression of the small

airway epithelium and alveolar macrophages in waterpipe smokers to nonsmokers and define a small airway epithelium and alveolar macrophage waterpipe-responsive genes lists using the following criteria: all probe sets expressed in at least 20% of the samples, an expression level fold change ≥ 1.5 and Benjamini-Hochberg corrected for multiple tests¹³⁰ $p < 0.05$. The probe sets found to be differentially expressed between the groups were converted into unique and annotated genes using the Affymetrix site (www.affymetrix.com) and GeneCards (www.genecards.org) and functionally annotated using Gene Ontology and the Human Protein Reference Data Base (www.hprd.org).

2.3.2 Endothelial microparticles

Endothelial microparticles were collected, processed and quantified according to a standard operating procedure to eliminate variability in sample processing as previously described¹²¹. Briefly, blood was collected, processed within 1 hr and stained for the endothelial markers PECAM (CD31) and E-selectin (CD62E) and the constitutive platelet-specific glycoprotein Ib (CD42b) to differentiate endothelium-originated microparticles from platelet-derived microparticles, which also express CD31. EMPs were defined as microparticles $< 1.5 \mu\text{m}$ in size, expressing CD31 or CD62E but not CD42b microparticles. Circulating EMPs are present in low levels in plasma of healthy subjects, reflecting normal endothelial turnover¹³¹. Total EMP levels above the nonsmoker total EMP mean level plus 2 standard deviations were considered abnormally elevated. To assess the presence of relative contribution of pulmonary capillary endothelium to the elevated EMP levels, $\text{CD42b}^- \text{CD31}^+$ EMPs were co-stained with anti-human angiotensin converting enzyme (ACE) that is abundantly expressed on pulmonary capillary endothelium¹¹⁷. EMPs induced by apoptosis express the constitutive CD31 marker, whereas activation-induced EMPs express CD62E. Using this criteria, we assessed the ratio of

CD42b⁻CD62E⁺/CD42b⁻CD31⁺ and EMPs with a low CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ ratio were defined as “apoptotic EMPs”. The percentage of individuals with apoptotic EMPs with CD42b⁻CD62E⁺/CD42b⁻CD31⁺ ratio below the lowest ratio in healthy nonsmokers was quantified. EMP measurements were performed twice to ensure that the measurements were reproducible.

2.4 Metabolite Profiling

Bronchoalveolar lavage fluid was processed as previously described (Article 1). Suspended metabolite extracts from lung epithelial lining fluid were analyzed by LC-MS in two different detection modes (positive and negative ion-monitoring). A Mass Profiler Professional analysis and the molecular formula generator algorithm were used to generate molecular features and score the molecular formulas.

2.5 Chest High Resolution Computed Tomography

HRCT scans were used to determine the percentage of lung affected by emphysema at attenuation -950 Hounsfield Units using the EmphyxJ software application (EmphyxJ, Vancouver, BC, Canada)⁸⁷. Emphysema was defined as >5% lung volume, a value derived from analyses of HRCT in normal nonsmoking individuals with normal lung function.

2.6 Smoking Cessation

All healthy cigarette smokers and COPD cigarette smokers were invited to stop smoking using a combination of varenicline and counseling for 3 months. Smoking status was assessed at baseline and after 3, 6 and 12 months and verified by urine nicotine metabolite levels.

2.7 Statistical Analysis

For comparison of numerical data (e.g., age, urine nicotine levels, relative gene expression and lung function) a 2-tailed Student’s t-test was used. For comparison of categorical

data (e.g., gender and ethnicity) a chi-square test was used, with Yates' correction for low number of subjects when applicable. A pairwise ANOVA was used to compare total and apoptotic plasma EMP levels between groups and at different time points within a group with no correction for multiple test as the number of tests was low (<21). A within-between ANOVA test was used to compare lung function at baseline and at the last visit within the normal spirometry/normal DLCO group and within the normal spirometry/low DLCO group. Gene expression levels were corrected for false discovery rate (Benjamini-Hochberg correction¹³⁰). An unpaired Student's t-test (targeted analysis) and a 1-way ANOVA (untargeted analysis, corrected for false discovery rate (Benjamini-Hochberg correction¹³⁰) were used to compare metabolite profile in waterpipe smokers and nonsmokers (Article 1). To assess if DLCO level can predict the development of COPD, a binomial logistic regression model was implemented in which the response was COPD status ("1"=developing COPD, "0" = not developing COPD). In addition, leave-one-out cross-validation was performed in order to assess the predictive accuracy. Evaluation and fit of the logistic regression model was performed using the "nnet" and "ROCR" packages in the freely available R software¹³²⁻¹³³.

3. Results

3.1 Article 1: Pulmonary Abnormalities in Young, Light-use Waterpipe (Hookah)

Smokers

American Journal of Respiratory and Critical Care Medicine; In Press

Abstract: Waterpipe, an instrument for smoking fruit-flavored tobacco, commonly associated with the Middle East, is becoming a global phenomenon currently used by millions of people worldwide. Many waterpipe smokers believe waterpipe smoking is a safer alternative to cigarette smoking because the smoke is bubbled through water before inhalation. Despite the widespread use, there is only limited data available on the health risks associated with waterpipe smoking, particularly in young, light-use waterpipe smokers. There are no governmental regulations regarding waterpipe use.

Based on the knowledge that the first abnormalities associated with cigarette smoking are in lung cells long before there are abnormalities in clinical parameters such as lung function and lung imaging, we hypothesized that even light-use waterpipe smoking likely mediates abnormalities relevant to lung health. To assess this hypothesis we compared young, light-use waterpipe smokers to nonsmokers, matched for gender and ethnicity, using clinical parameters, including cough and sputum scores, lung function, and chest HRCT, and biologic parameters, including metabolites present in the lung epithelial lining fluid, small airway epithelial cell differentials and transcriptome, alveolar macrophage cellular composition and transcriptome, and plasma microparticle levels derived from pulmonary capillaries undergoing apoptosis. There were abnormalities in all parameters assessed in waterpipe smokers compared to nonsmokers, demonstrating that even light-use waterpipe smoking affects the biology of the human lung with evidence of early disease.

QA1 Pulmonary Abnormalities in Young, Light-Use Waterpipe (Hookah) Smokers

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Abstract

4 **Rationale:** Waterpipes, also called *hookahs*, are currently used by millions of people worldwide. Despite the increasing use of waterpipe smoking, there is limited data on the health effects of waterpipe smoking and there are no federal regulations regarding its use.

Objectives: To assess the effects of waterpipe smoking on the human lung using clinical and biological parameters in young, light-use waterpipe smokers.

Methods: We assessed young, light-use, waterpipe-only smokers in comparison with lifelong nonsmokers using clinical parameters of cough and sputum scores, lung function, and chest high-resolution computed tomography as well as biological parameters of lung epithelial lining fluid metabolome, small airway epithelial (SAE) cell

differential and transcriptome, alveolar macrophage transcriptome, and plasma apoptotic endothelial cell microparticles.

Measurements and Main Results: Compared with nonsmokers, waterpipe smokers had more cough and sputum as well as a lower lung diffusing capacity, abnormal epithelial lining fluid metabolome profile, increased proportions of SAE secretory and intermediate cells, reduced proportions of SAE ciliated and basal cells, markedly abnormal SAE and alveolar macrophage transcriptomes, and elevated levels of apoptotic endothelial cell microparticles.

Conclusions: Young, light-use, waterpipe-only smokers have a variety of abnormalities in multiple lung-related biological and clinical parameters, suggesting that even limited waterpipe use has broad consequences on human lung biology and health. We suggest that large epidemiological studies should be initiated to investigate the harmful effects of waterpipe smoking. 5

The waterpipe, also called *hookah*, *shisha*, or *narghile*, an instrument for smoking fruit-flavored tobacco, is used by millions of people worldwide (1–4). The tobacco is placed in a bowl surrounded by burning charcoal; when the smoker inhales, air is

pulled through the charcoal and into the bowl holding the tobacco (3, 5). The resulting smoke is bubbled through water, carried through a hose, and inhaled. It includes volatilized and pyrolyzed tobacco products, equivalent in a single bowl

waterpipe session over 45–60 minutes to one pack of cigarettes, together with carbon monoxide and charcoal components. In addition to nicotine and its metabolites, urinalyses of waterpipe smokers have identified a variety of compounds that

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2 Author Contributions: Y.S.-B.: was the lead researcher; Y.S.-B., R.S., and R.G.C.: wrote the manuscript; Y.S.-B., R.S., G.W., R.S.D., S.S.G., M.S.M., and M.R.S.: interpreted the data; Y.S.-B., J.S., R.S.D., A.K., T.L.V., F.A.-P., V.S., and J.G.M.: performed data analysis; R.S.D. and A.K.: optimized and performed assays; R.J.K.: was the lead physician; C.H.: oversaw subject recruitment; A.M.A., H.S., and M.M.: contributed to design and analysis of the study; and R.G.C.: was the chief investigator. All authors had input into the manuscript and approved the manuscript version for publication.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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At a Glance Commentary

Scientific Knowledge on the

Subject: Waterpipe smoking is increasing worldwide, mainly among young adults. It is second only to cigarette smoking. Researchers in most studies have assessed older, heavy-use waterpipe smokers with disease manifestation and not young, light-use waterpipe smokers.

What This Study Adds to the

Field: We evaluated multiple lung components, including clinical and biologic abnormalities, in several anatomic components in the lungs of young, light-use waterpipe smokers with no clinical manifestation of disease.

overlap with, but also differ from, those of cigarette smokers (3, 6).

While waterpipe smoking is commonly associated with the Middle East, the use of waterpipes is becoming more prevalent in the United States and worldwide (4, 5, 7). In the United States, 9–20% of young adults report that they have used waterpipes (5, 8), and waterpipe “bars” have become common in many U.S. cities, with increasing waterpipe use among young adults (4, 5). Many waterpipe smokers believe that the water filters out “toxins” from the smoke and that therefore the waterpipe is a safer smoking alternative to cigarettes (9, 10). Despite the increasing prevalence of waterpipe smoking, there is a paucity of data on the health effects of waterpipe smoking and there are no federal regulations regarding its use (5, 7, 11).

On the basis of knowledge that the first abnormalities associated with cigarette smoking are found in lung cells long before there are abnormalities in clinical parameters such as lung function and lung imaging (12–17), we hypothesized that even light-use waterpipe smoking for only a few years, exposing the smoker not only to tobacco smoke but also to the flavorings added to the tobacco and the volatile components of the heated charcoal surrounding the tobacco, likely mediates abnormalities relevant to lung health.

To assess this hypothesis, we compared young, light-use waterpipe smokers with

nonsmokers matched for sex and ethnicity, using a variety of lung-related parameters, including (1) blood carboxyhemoglobin, cough and sputum scores, lung function, and chest high-resolution computed tomography (HRCT); (2) metabolites present in the lower respiratory tract epithelial lining fluid (ELF); (3) cell differentials and transcriptome of the small airway epithelium (SAE); (4) cellular composition of the ELF of the lower respiratory tract recovered by bronchoalveolar lavage (BAL) and transcriptome of alveolar macrophages (AMs); and (5) plasma levels of circulating endothelial microparticles (EMPs) derived from pulmonary capillaries undergoing apoptosis.

Some of the results presented in this article have been reported previously in the form of abstracts (18, 19).

Methods

Self-reported never smokers (“nonsmokers”; $n = 19$) and self-reported waterpipe-only smokers (“waterpipe smokers”; $n = 21$) were recruited from the general population in New York City by posting advertisements in local newspapers, electronic bulletin boards, and waterpipe bars. All subjects were evaluated at the Weill Cornell National Institutes of Health Clinical and Translational Science Center and Department of Genetic Medicine Clinical Research Facility using institutional review board–approved clinical protocols. All subjects were determined to be healthy on the basis of their medical history, physical examination, and detailed laboratory assessments (Table 1; for full inclusion and exclusion criteria, see METHODS section in the online supplement). Urine nicotine and cotinine levels were determined using liquid chromatography–tandem mass spectrometry (ARUP Laboratories, Salt Lake City, UT) (20). All subjects were recruited from the New York metropolitan area. The two study groups had similar environmental exposures; no subject had any industrial exposures; and only one nonsmoker and one waterpipe smoker had a history of exposure to secondhand cigarette smoke. Even though recruitment was open for all waterpipe smokers at least 18 years of age, the waterpipe smokers who volunteered were young and light-use

waterpipe smokers, representative of the rise in waterpipe smoking prevalence in the young adult population in the United States.

All subjects underwent pulmonary function tests performed according to American Thoracic Society guidelines (21, 22), and their cough and sputum scores were based on the St. George’s Respiratory Questionnaire (23). Chest HRCT was used to quantify emphysema (24). Pulmonary function and HRCT quantification are detailed in the METHODS section in the online supplement. The SAE, AM, and ELF samples were collected using fiberoptic bronchoscopy as previously described (25, 26). The metabolites in the lower respiratory tract ELF of waterpipe smokers and nonsmokers were compared in BAL fluid collected from a random subset of the nonsmokers ($n = 5$) and waterpipe smokers ($n = 8$). Total RNA was extracted from the SAE of all subjects, and AM samples were obtained from all nonsmokers and from 19 of the 21 waterpipe smokers (two missing samples due to technical issues during the collection procedure) using TRIzol reagent (Life Technologies, Carlsbad, CA) and RNeasy (RNeasy MinElute RNA Purification Kit; QIAGEN, Valencia, CA) and stored in Ambion RNAsure reagent (Life Technologies) at -80°C . Total RNA processing on Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA), quality control, and analyses were performed as previously described (16). Processing of plasma EMPs as well as quantification and analysis of total EMPs ($\text{CD42b}^{-}\text{CD31}^{+}$), pulmonary-derived EMPs ($\text{CD42b}^{-}\text{CD31}^{+}\text{ACE}^{+}$), and apoptotic EMPs (ratio of $\text{CD42b}^{-}\text{CD62}^{+}$ to $\text{CD42b}^{-}\text{CD31}^{+} < 2$ SD below the average level in nonsmokers) were performed as previously described (27) and as detailed in the METHODS section of the online supplement.

Transcriptome Analyses

Transcriptome analyses were performed as detailed in the METHODS section in the online supplement. SAE and AM waterpipe-responsive gene lists were created with all genes differentially expressed in waterpipe smokers versus nonsmokers using the following criteria: genes expressed in at least 20% of subjects in each group with a fold change greater than or equal to 1.5 ($P < 0.05$ with

Table 1. Demographics and Biologic Samples

Parameter	Nonsmokers	Waterpipe Smokers	P Value
Number of patients	19	21	
Sex, male/female, n	9/10	13/8	>0.3
Age, yr	33 ± 9	25 ± 4	<10 ⁻³
Race, black/white/other, n	6/5/8	8/2/11	>0.3
BMI, kg/m ²	25 ± 5	25 ± 4	>0.7
Alpha-1 antitrypsin, mg/dl	152 ± 27	137 ± 39	>0.1
HIV status	Negative	Negative	NA
IgE, IU/ml	228 ± 526	119 ± 104	>0.3
Blood pressure, systolic/diastolic, mm Hg	115 ± 8/71 ± 12	115 ± 9/65 ± 8	>0.8/>0.1
Heart rate, beats/min	70 ± 11	70 ± 10	>0.9
Smoking history			
Age of initiation, yr	NA	21 ± 5	NA
Duration of smoking, yr	NA	4.1 ± 2.5	NA
Sessions/wk	NA	3.5 ± 2.5	NA
Urinary nicotine,* ng/ml	0	67 ± 193	NA
Urinary cotinine,* ng/ml	0	99 ± 205	NA
Carboxyhemoglobin, %	0 ± 0.7	2.1 ± 1.7	<0.02
Cough score [†]	0.5 ± 0.6	1.3 ± 1.1	<0.008
Sputum score [‡]	0.4 ± 0.5	1.2 ± 1.1	<0.007
Pulmonary function parameters [‡]			
FVC, % predicted	106 ± 12	98 ± 15	>0.06
FEV ₁ , % predicted	105 ± 11	98 ± 13	>0.1
FEV ₁ /FVC, % observed	84 ± 3	86 ± 5	>0.06
FEF _{25-75%} , % predicted	93 ± 16	97 ± 15	>0.4
PEF, % predicted	101 ± 15	103 ± 15	>0.6
TLC, % predicted	95 ± 15	94 ± 14	>0.8
DL _{CO} , % predicted	90 ± 10	82 ± 14	<0.04
Percentage of emphysema, -950 HU	1.5 ± 1.8	0.6 ± 0.6	>0.07
Small airway epithelium			
Number of cells recovered, ×10 ⁶	4.3 ± 2.2	4.8 ± 4.3	>0.6
Percentage of inflammatory cells	1.0 ± 0.7	1.0 ± 1.0	>0.6
Percentage of epithelial cells [§]	98.9 ± 0.8	99.1 ± 0.8	>0.9
Percentage of ciliated cells	70.8 ± 4.6	62.6 ± 8.9	<0.005
Percentage of secretory cells	9.6 ± 4.6	14.5 ± 5.6	<0.005
Percentage of basal cells	11.2 ± 7.5	4.5 ± 4.1	<0.002
Percentage of intermediate cells	8.6 ± 4.4	17.9 ± 6.3	<10 ⁻⁵
BAL cells			
Number of cells recovered, ×10 ⁶	12.6 ± 7.5	8.6 ± 5.2	>0.08
Percentage of macrophages	85.9 ± 10.4	91.8 ± 10.0	>0.05
Percentage of neutrophils	2.7 ± 2.4	1.4 ± 2.3	>0.08
Percentage of lymphocytes	8.9 ± 8.1	5.7 ± 8.1	>0.2
Percentage of eosinophils	0.5 ± 0.7	0.8 ± 1.7	>0.6

Definition of abbreviations: BAL = cells removed by bronchoalveolar lavage; BMI = body mass index; DL_{CO} = diffusing capacity of the lung for carbon monoxide; FEF = forced expiratory flow; FEF_{25-75%} = forced expiratory flow, midexpiratory phase; HU = Hounsfield units; NA = not applicable; PEF = peak expiratory flow; TLC = total lung capacity.

Data are presented as average ± SD. *P* values of numeric parameters were calculated using a two-tailed Student's *t* test. *P* values of categorical parameters were calculated using a χ^2 test. Values represent prebronchodilator measurements.

*Undetectable urine nicotine was defined as less than 2 ng/ml, undetectable cotinine as less than 5 ng/ml.

[†]Cough and sputum scores were each evaluated on a scale of 0–4, where 0 = not at all; 1 = only with chest infections; 2 = a few days per month; 3 = several days per week; and 4 = most days of the week (23).

[‡]Pulmonary function testing parameters are given as percentage of predicted value, with the exception of FEV₁/FVC, which is reported as percentage observed.

[§]As a percentage small airway epithelium recovered.

^{||}Alveolar macrophages were purified by adherence before transcriptome analysis (see METHODS section in the online supplement).

Benjamini-Hochberg correction [28]). The number of differentially expressed waterpipe-responsive genes expressed outside the nonsmoker mean expression level (± 2 SD) divided by the total number of waterpipe-responsive genes was summarized as a percentage and calculated for each subject as a waterpipe transcriptome response score. For both the SAE and AM transcriptomes, the data were depicted (1) using principal component analysis (PCA), collapsing the expression levels of all probe sets present in at least 20% of the subjects' data into a set of linear variables (principal components [PCs]) that summarized the variability between the subjects, with the three components collapsing the largest variability between the groups displayed in a three-dimensional plot; (2) as an SAE and AM waterpipe response score of each subject; and (3) as a fold change of the average expression level in waterpipe smokers compared with nonsmokers of all SAE and AM waterpipe-responsive genes displayed in Gene Ontology functional categories.

Global Index Analysis

To summarize the differences observed in waterpipe smokers compared with nonsmokers, a global index was created that included cough and sputum scores, diffusing capacity of the lung for carbon monoxide (DL_{CO}), SAE PCs, SAE transcriptome response score, AM PCs, AM transcriptome response score, plasma apoptotic EMP levels, and SAE cell differentials. See the METHODS section of the online supplement for index calculations.

Statistical Analysis

For comparison of numerical data (e.g., age, urine nicotine levels, total and apoptotic EMP levels, relative gene expression, lung function, and percentage of emphysema in waterpipe smokers vs. nonsmokers), a two-tailed Student's *t* test was used. Gene expression levels were corrected for false discovery rate (Benjamini-Hochberg correction [28]). For comparison of categorical data (e.g., sex, ethnicity, and number of subjects with abnormal cough and sputum scores, low DL_{CO}, or apoptotic EMP levels), a χ^2 test was used with the Yates correction for low number of subjects when applicable. The differential metabolite profile of the lung ELF samples was assessed using MassHunter Profinder software (Agilent Technologies, Santa

Clara, CA) and compared using an unpaired Student's *t* test (targeted analysis) and Agilent MPP software and one-way analysis of variance (untargeted analysis) corrected for false discovery rate (Benjamini-Hochberg correction [28]), as detailed in the METHODS section of the online supplement.

Results

The study population of nonsmokers and waterpipe smokers was comparable in terms of sex, ethnicity, body mass index, and alpha-1 antitrypsin levels ($P > 0.3$, all comparisons) (Table 1). The waterpipe smokers were younger than the nonsmokers (mean difference, 8 yr) (Table 1). In prior studies, we observed that there were no age-related modifications to cough and sputum scores, SAE cell differentials, DL_{CO} levels, SAE and AM gene expression, or plasma EMP levels in nonsmokers ($r^2 \leq 0.1$, correlation of all parameters with age) (see METHODS section and Figure E1 in the online supplement) (16, 27). Waterpipe smokers smoked an average of 3.5 ± 2.5 sessions per week for an average of 4.1 ± 2.5 years. Carboxyhemoglobin levels were significantly higher in waterpipe smokers than in nonsmokers ($P < 0.02$).

Lung-related Clinical Parameters

Cough and sputum scores were significantly higher in waterpipe smokers than in nonsmokers ($P < 0.008$, both comparisons). Thirty-three percent of waterpipe smokers had an abnormal cough score (≥ 2) compared with 5% of nonsmokers ($P < 0.03$), and 19% of waterpipe smokers had abnormal sputum production (≥ 2) compared with 0% of nonsmokers ($P < 0.04$) (Table 1, Figure 1A). DL_{CO} percentage of predicted value, corrected for hemoglobin and carboxyhemoglobin levels, was lower in waterpipe smokers than in nonsmokers ($P < 0.04$). None of the nonsmokers had a low DL_{CO} level ($< 80\%$ predicted and below the 95% range of normal DL_{CO} calculated per subject based on sex, age, and height using a dataset comprising 405 healthy nonsmokers [29]). In contrast, 38% of the waterpipe smokers had a low DL_{CO} level ($P < 0.009$) (Figure 1B). The HRCT percentage of emphysema was not significantly different between the groups ($P > 0.07$).

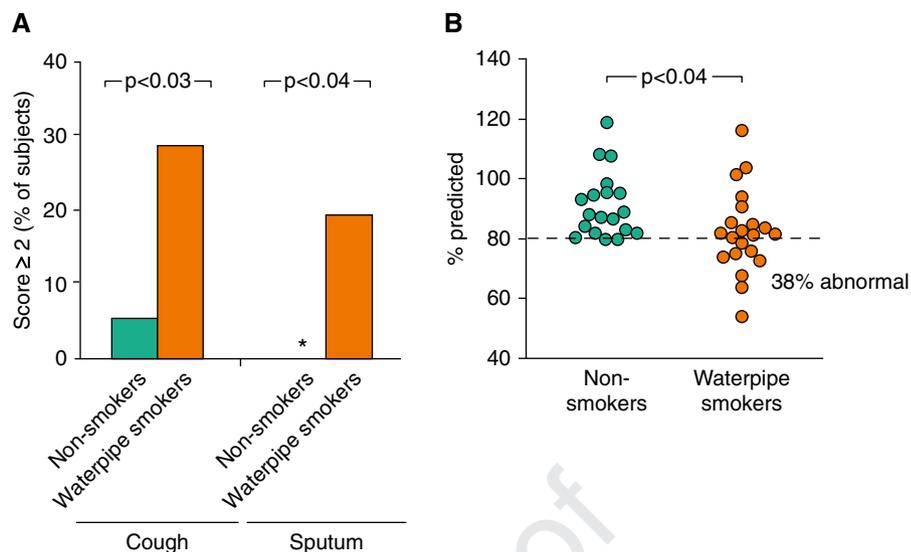


Figure 1. Clinical abnormalities of light-use, young waterpipe smokers compared with healthy nonsmokers. (A) Cough and sputum scores. Shown are the percentages of subjects with abnormal cough and sputum scores (≥ 2 on 0–4 scale). *P* values were calculated using a χ^2 test. *None. (B) Diffusing capacity of the lung for carbon monoxide. *P* value was calculated using a two-tailed Student's *t* test. Dashed line indicates the lower limit of normal.

Metabolite Analysis

Metabolic profiling provided quantification for 1,675 features in the lower respiratory tract ELF; of these, 31 features with significantly different abundance in waterpipe smokers versus nonsmokers were structurally identified ($P < 0.05$) (Table E1; see Figures E2A–E2F for examples).

Small Airway Epithelium

The number of SAE cells recovered and the percentage of total epithelial and inflammatory cells were comparable in waterpipe smokers and nonsmokers ($P > 0.6$, both comparisons) (Table 1). However, the SAE of waterpipe smokers had an altered cellular composition, with a higher percentage of secretory cells and intermediate cells and a lower percentage of ciliated cells and basal cells ($P < 0.005$, all comparisons).

The SAE transcriptome of waterpipe smokers was significantly modified compared with that of nonsmokers, with a marked segregation of the groups based on the genome-wide PCA (Figure 2A). There were 212 probe sets representing 159 unique, annotated genes significantly different between waterpipe smokers and nonsmokers (Figure E3A). Of those, 35% were downregulated and 65% were upregulated (“SAE waterpipe-responsive genes”) (Table E2).

The SAE waterpipe transcriptome response score, a measure of the number of SAE waterpipe-responsive genes differentially expressed in a subject, was significantly higher in waterpipe smokers than in nonsmokers ($P < 10^{-12}$) (Figure 2B). Gene Ontology analysis of the categories of the SAE waterpipe-responsive genes showed a broad distribution dominated by genes related to metabolism, signal transduction, transcription, and transport (Figure 2C). Interestingly, while the SAE transcriptome of cigarette smokers is characterized by upregulation of many oxidative stress-related genes (13–17), very few genes in this category were upregulated in the SAE of waterpipe smokers (categorized as functional category “other” due to the low number of oxidant-related genes) (see DISCUSSION in the online supplement and Table E2).

Alveolar Macrophages

The cell differentials of the lower respiratory tract ELF (AMs, lymphocytes, neutrophils, eosinophils) recovered from the lower respiratory tract by BAL were not statistically different between the groups ($P > 0.05$, all comparisons), and the number of recovered AM cells was also comparable ($P > 0.08$) (Table 1). Genome-wide PCA of the AM transcriptome demonstrated a segregation

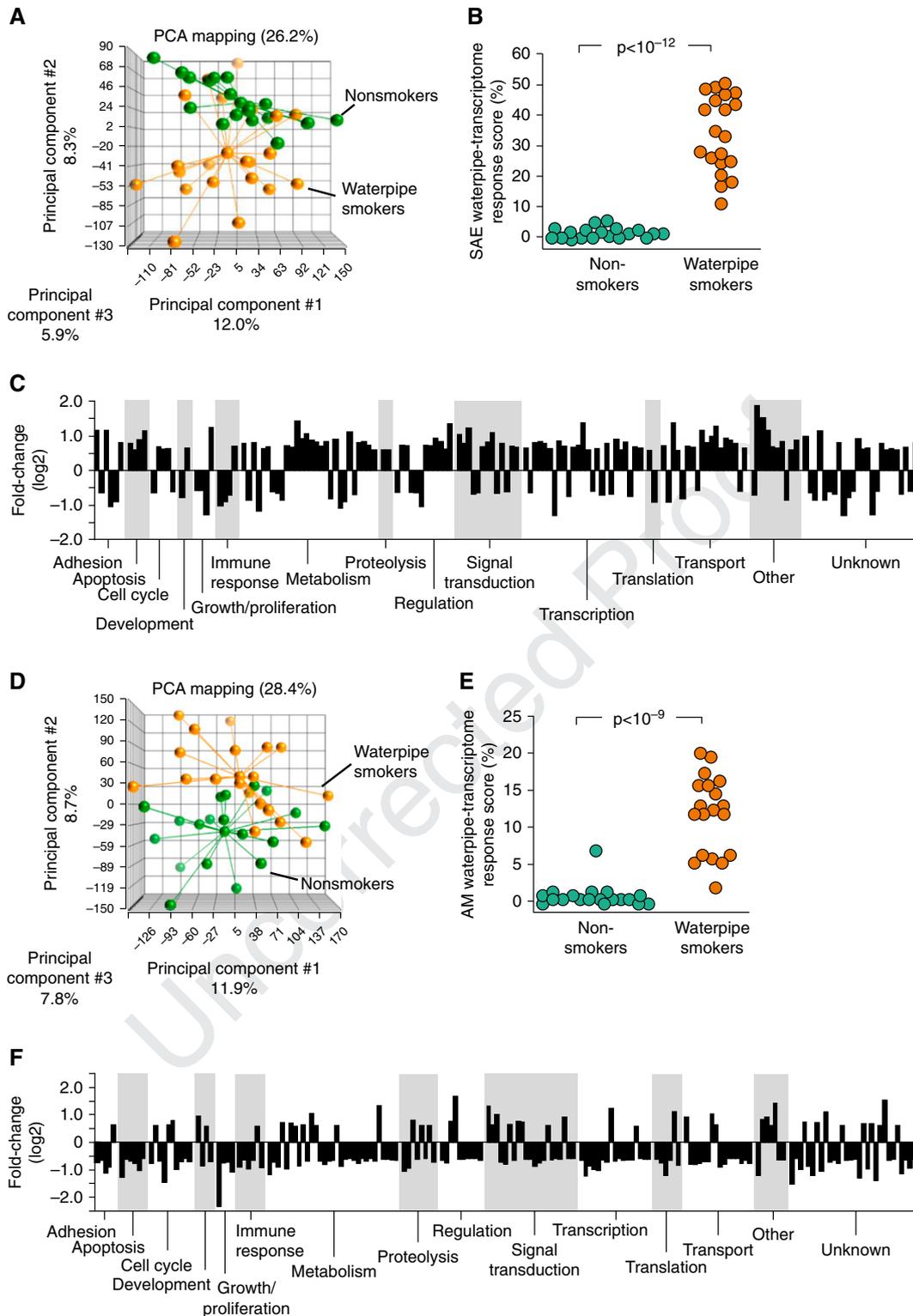


Figure 2. Differential gene expression in the small airway epithelium (SAE) and alveolar macrophages (AMs) of waterpipe smokers compared with nonsmokers. For all panels, the data, normalized by array, were compared in nonsmokers ($n = 19$) and waterpipe smokers ($n = 21$ SAE and $n = 19$ AM samples) for all probe sets “present” in at least 20% of the samples in each group. (A–C) SAE gene expression. Differentially expressed probe sets ($n = 212$, representing 159 unique, annotated genes) identified using criteria of a fold change greater than or equal to 1.5 and $P < 0.05$ with the Benjamini-Hochberg correction (28) (see Table E2 for the complete SAE waterpipe-responsive gene list). (A) Principal component analysis (PCA). Shown are the first three principal components, representing the greatest variability among the groups. Each circle represents a subject, and all subjects in a

of the two groups based on waterpipe smoking status (Figure 2D). Of the probe sets present in at least 20% of samples in each group, 239 probe sets representing 181 unique, annotated genes had significant differential expression between waterpipe smokers and nonsmokers (Figure E3B); 74% were downregulated and 26% were upregulated (“AM waterpipe-responsive genes”) (Table E3), an opposite trend to that observed in the SAE.

As with the SAE transcriptome response score, the AM transcriptome response score was significantly higher in waterpipe smokers than in nonsmokers ($P < 10^{-9}$) (Figure 2E). Gene Ontology analysis of the categories of the AM waterpipe-responsive genes showed a broad distribution; that is, as with the SAE, they were dominated by genes related to metabolism, signal transduction, transcription, and transport (Figure 2F). Among these downregulated genes were many linked to lung inflammation and host defense (see Discussion section in the online supplement and Table E3).

Endothelial Microparticles

Waterpipe smokers showed an increase in plasma total EMP levels compared with the nonsmokers ($P < 0.04$) (Figure 3A). On average, $77 \pm 8\%$ of the plasma EMPs in the waterpipe smokers were of pulmonary origin ($CD42b^- CD31^+ ACE^+$), a percentage comparable to that of nonsmokers ($P > 0.1$) (Figure 3B). The level of EMPs derived from apoptotic cells was increased in the waterpipe smokers compared with nonsmokers ($P < 0.05$), with 45% of waterpipe smokers having apoptotic EMPs (< 2 SD) below the average level in nonsmokers compared with 0% of nonsmokers ($P < 0.008$) (Figure 3C). For global assessment of all parameters compared in waterpipe smokers and nonsmokers, see the RESULTS section in the online supplement and Figure E4.

Discussion

Despite the assumption among waterpipe users that smoking waterpipe is “safer” than smoking cigarettes (9, 10), evaluation of multiple lung components demonstrated a significant number of lung clinical and biological abnormalities in light-use, waterpipe-only smokers compared with healthy lifelong nonsmokers. The waterpipe smokers had increased cough and sputum scores and lower diffusing capacity, as well as biological abnormalities in several anatomic components in the lung, including (1) in the lower respiratory tract ELF, differentially present metabolites; (2) in the SAE, the cell population where chronic obstructive pulmonary disease (COPD) and most lung cancers are initiated (30–33), disarray of the proportions of cell types, with increased numbers of secretory and intermediate cells and decreased numbers of ciliated and basal cells and an abnormal transcriptome; (3) in AMs, the pulmonary representative of the mononuclear phagocyte system, functioning as the scavenger cell in the lower respiratory tract (34, 35), abnormal transcriptome; and (4) in the pulmonary capillary endothelium, an increased proportion of circulating apoptosis-derived EMPs (27, 36).

Clinical Consequences

The use of waterpipes to smoke tobacco is increasing worldwide, mainly among young adults and teens, reaching a global epidemic second only to cigarette smoking. Epidemiological studies suggest that 10–48% of adolescents and young persons in middle school, high school, or universities in the United States, Europe, and other countries admit to ever smoking waterpipes and that 10–35% admit to being current waterpipe smokers (2–5, 7). However, most studies of the long-term effects of waterpipe smoking on pulmonary function, cancer prevalence, and other clinical symptoms have studied older

(ages 40–60 yr), heavy-use waterpipe smokers (30–60 waterpipe-year history), mostly in waterpipe smokers who already have disease manifestation (2, 4, 11, 37–39).

Researchers in a number of studies have assessed lung function in older, heavy-use waterpipe users and found evidence of reduced lung function parameters, including reduced FVC, FEV₁, maximal midexpiratory flow, peak expiratory flow, forced expiratory flow, and midexpiratory phase levels, as well as FEV₁/FVC, compared with nonsmokers, with a correlation between the duration and quantity of waterpipe smoking and the abnormalities of pulmonary function (3, 11, 37, 38). These older, heavy-use waterpipe smokers have a high frequency of cough and sputum compared with nonsmokers, and these symptoms appear at an earlier age than in cigarette smokers (37, 40, 41). An important observation in the present study is that a significant proportion of young waterpipe smokers with a history of fewer than four waterpipe sessions per week for less than 5 years have clinical abnormalities, including an increase in cough frequency and sputum production, and, strikingly, 38% have reduced diffusing capacity. The subgroup of waterpipe smokers with normal HRCT and normal spirometry but low DL_{CO} are of interest, as we have recently demonstrated that cigarette smokers with the same clinical phenotype (normal HRCT and normal spirometry but low DL_{CO}) are at a sevenfold greater risk of developing COPD within 4 years than are those with the same phenotype but with normal DL_{CO} (29).

Biological Changes

There have been a number of analyses identifying compounds that are inhaled in waterpipe smoke, likely placing a significant stress on lung biology (3, 6). Compared with one cigarette, one waterpipe session exposes the smoker to 2–4 times the amount of nicotine, 7–11 times the amount of carbon monoxide, 100 times more tar,

Figure 2. (Continued). group are linked by a vector to a circle representing the average of the principal components in each group (green = nonsmokers, orange = waterpipe smokers). (B) Waterpipe transcriptome response score calculated on the basis of the percentage of the waterpipe-responsive genes each subject expressed outside the normal expression range, defined as mean (± 2 SD) expression in nonsmokers. P values were calculated using a two-tailed Student's t test. (C) Gene categories of all waterpipe-responsive genes. Fold change of mean expression of the waterpipe-responsive genes is compared with nonsmokers, presented on a log₂ scale. (D–F) AM gene expression. The AM data for (D–F) were created as described for the SAE. (D) PCA. (E) AM waterpipe transcriptome response score. (F) Gene categories. Differentially expressed probe sets ($n = 239$, representing 181 unique, annotated genes) were determined using criteria of a fold change greater than or equal to 1.5 and $P < 0.05$ with the Benjamini-Hochberg correction (28) (see Table E3 for the complete AM waterpipe-responsive gene list).

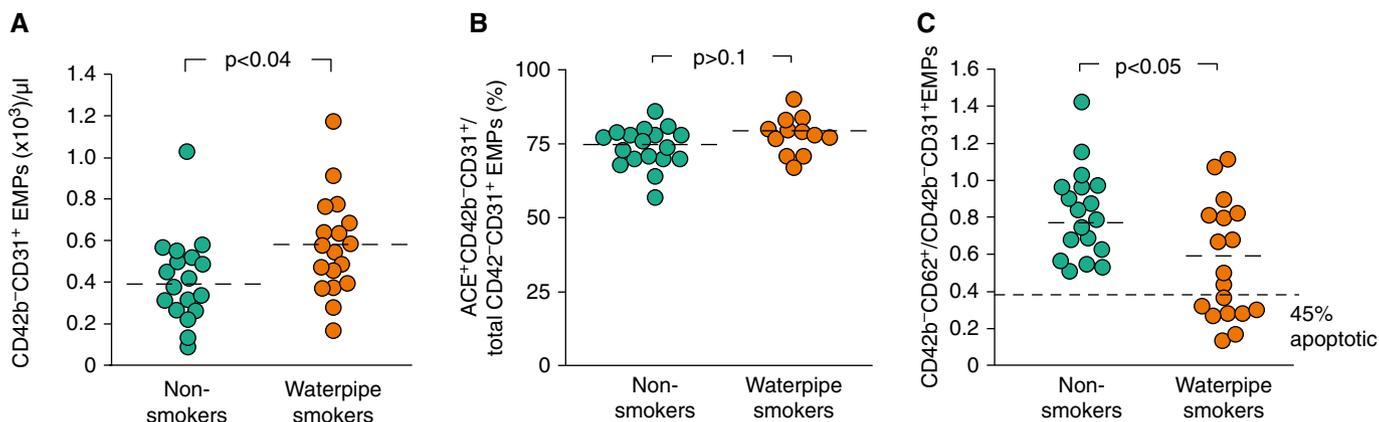


Figure 3. Levels of plasma total endothelial microparticles (EMPs), pulmonary-derived EMPs, and the proportion of apoptotic EMPs. Shown are data for nonsmokers ($n = 19$; green circles) and waterpipe smokers ($n = 20$; orange circles). Each data point represents one subject. Dashed line in each group indicates the group mean. (A) Total $CD42b^-CD31^+$ EMPs. (B) Proportion of $CD42^-CD31^+$ EMPs that express angiotensin-converting enzyme (ACE⁺), a gene highly expressed in the pulmonary capillary endothelium (52). (C) Ratio of circulating activated $CD42b^-CD62^+$ EMPs to $CD42b^-CD31^+$ apoptotic EMPs. The dashed line indicates the level of 2 SD below the mean of $CD42b^-CD31^+/CD42b^-CD62^+$ EMPs in nonsmokers. Values below this line represent elevated levels of apoptotic EMPs. P values were calculated using a two-tailed Student's t test.

17 times the amount formaldehyde, 2–5 times the amount of high molecular weight carcinogenic polyaromatic hydrocarbons, and 3 times the amount of phenol (3). In addition, high levels of benzene, volatile aldehydes, and other toxins originating from flavoring have been detected in waterpipe smoke (3, 6). Consistent with the concept that at least some components of waterpipe smoke reach the lower respiratory tract, metabolomic profiling of lung epithelial fluid demonstrated a variety of metabolites in the lower respiratory tract ELF of waterpipe smokers, with a differential abundance compared with nonsmokers.

The SAE and AM transcriptomes of waterpipe smokers could easily be differentiated from those of nonsmokers, with hundreds of genes up- and downregulated, indicating potential dysregulation of these lung cell populations in response to waterpipe smoking. Waterpipe transcriptome response scores summarizing the waterpipe-modified gene effect on the SAE and AM transcriptomes distinguished not only light-use waterpipe smokers from nonsmokers but also waterpipe smokers with normal spirometry and normal DL_{CO} from those with normal spirometry but reduced DL_{CO} . For both the SAE and AMs, most of these dysregulated genes were metabolism, transcription, and signal transduction related, some of which were previously associated with the pathogenesis of COPD and/or cancer.

Interestingly, there was little overlap among the SAE genes dysregulated in waterpipe smokers compared with the overlap described for cigarette smokers, suggesting that the SAE pathologic phenotypes may be different from those induced in classic cigarette smoking-induced disorders. In this regard, the SAE of waterpipe smokers had an altered cellular composition with a pattern that combined features both similar to and distinct from those commonly observed in cigarette smokers. Similar to SAE changes in cigarette smokers, there was a decrease in the proportion of ciliated cells, the mediator of mucociliary clearance (42), as well as increased numbers of secretory cells resembling mucous cell hyperplasia, in smokers (43). These morphological alterations may be responsible for higher levels of cough and sputum scores observed in waterpipe smokers. However, in contrast to basal cell hyperplasia commonly observed in the airways of healthy smokers, in the SAE of waterpipe smokers there was a significant decrease in the proportion of basal cells, the stem/progenitor cell population of the airway epithelium (44). This was accompanied by an increased proportion of intermediate undifferentiated cells, which are basal cell-derived precursors of the differentiated cell populations (44). The decreased proportion of basal stem/progenitor cells in the airway epithelium is a rather unique phenotype, previously described only in bronchiolitis obliterans (45) and airway epithelial aging

(46). This suggests that waterpipe smoking-induced changes in the SAE transcriptome may have important consequences with regard to the structural organization and maintenance of this anatomic compartment.

While the SAE transcriptome of cigarette smokers is characterized by upregulation of oxidative stress-related genes (13–17), very few genes related to this category were upregulated in the waterpipe smokers, suggesting that passage through water filters out many of the oxidants in waterpipe smoke. Interestingly, while the majority of differentially expressed genes in the SAE were upregulated, the majority of differentially expressed genes in the AMs were downregulated. However, similarly to its effect on the SAE transcriptome, waterpipe smoking induced a unique gene expression pattern in the AMs not previously reported to be evoked by cigarette smoking or other known modulators of the macrophage phenotype (47, 48). Among the downregulated genes were a variety of genes critical for inflammation and host defense functions (see DISCUSSION section in the online supplement for details regarding the specific SAE and AM dysregulated genes). In contrast to cigarette smokers, in whom there is a higher percentage of macrophages recovered compared with nonsmokers (48), there was no significant difference in the proportions of macrophages or other cell types recovered from the BAL of waterpipe smokers compared with nonsmokers, an

observation that may be explained in part by the marked difference in the inhaled smoke composition of waterpipe versus cigarette smoke.

Endothelial cells respond to cell activation, injury, and/or apoptosis by shedding submicron membrane vesicles from their plasma membranes, known as EMPs (27, 49). Apoptotic loss of pulmonary capillaries occurs in association with cigarette smoking (50), and analysis of lung sections of individuals with COPD demonstrates increased DNA fragmentation and endothelial apoptosis in the pulmonary capillaries, representing early lung destruction (50, 51). We have previously shown that cigarette smokers undergo pulmonary endothelial apoptosis as measured by high levels of total EMPs

and an increased proportion of apoptotic EMPs in their plasma (27). The observation that the total level of circulating EMPs and the proportion of apoptotic EMPs were significantly higher in waterpipe smokers than in nonsmokers suggests the possibility of ongoing lung capillary endothelial apoptosis associated with light-use waterpipe smoking.

Implications

The data we present regarding abnormalities in all clinical and lung-related biological parameters used to compare waterpipe smokers with nonsmokers suggests that even light-use waterpipe smoking in young individuals significantly affects lung biology and health. On the basis of this evidence in the context of the increasing use of waterpipe

smoking, together with the accumulating evidence in the literature that older, heavy-use waterpipe smokers have loss of lung function compared with nonsmokers, our findings support efforts to regulate and reduce waterpipe smoking, especially among the young population, and to initiate large epidemiological studies on the harmful effects of waterpipe smoking. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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3.2 Article 2: Risk for COPD with Obstruction of Active Smokers with Normal Spirometry and Reduced Diffusion Capacity

European Respiratory Journal 2015;46:1535

Abstract: Chronic obstructive pulmonary disease (COPD) caused preliminary by cigarette smoking, is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) using spirometry, with normal post-bronchodilator considered “healthy”. In assessing a cohort of 1570 active cigarette smokers, all with normal spirometry, we recognized that a subset had an abnormal diffusion capacity (DLCO), a parameter linked with emphysema and small airway disease. To determine if there is a difference in the risk for developing COPD as defined by the GOLD criteria between the “normal spirometry/low DLCO” and “normal spirometry/normal DLCO” phenotypes, we followed a randomly chosen group with normal spirometry/low DLCO and normal spirometry/normal DLCO with serial lung function over time.

The data revealed that despite appearing normal by GOLD post-bronchodilator spirometry criteria, cigarette smokers with normal spirometry but reduced DLCO are at significantly higher risk for the development of COPD. These results suggest that DLCO measurement could be an additional tool for early detection of smokers at risk for COPD, contributing to early intervention, and that large epidemiologic studies analyzing parameters relevant to “at risk” for COPD should use both spirometry and DLCO to allow for a correct interpretation of their studies.



Risk of COPD with obstruction in active smokers with normal spirometry and reduced diffusion capacity

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ABSTRACT Smokers are assessed for chronic obstructive pulmonary disease (COPD) using spirometry, with COPD defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as airflow limitation that is not fully reversible with bronchodilators. There is a subset of smokers with normal spirometry (by GOLD criteria), who have a low diffusing capacity of the lung for carbon monoxide (DLCO), a parameter linked to emphysema and small airway disease. The natural history of these “normal spirometry/low DLCO” smokers is unknown.

From a cohort of 1570 smokers in the New York City metropolitan area, all of whom had normal spirometry, two groups were randomly selected for lung function follow-up: smokers with normal spirometry/normal DLCO (n=59) and smokers with normal spirometry/low DLCO (n=46). All had normal history, physical examination, complete blood count, urinalysis, HIV status, α_1 -antitrypsin level, chest radiography, forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC), FEV₁/FVC ratio and total lung capacity. Throughout the study, all continued to be active smokers.

In the normal spirometry/normal DLCO group assessed over 45±20 months, 3% developed GOLD-defined COPD. In contrast, in the normal spirometry/low DLCO group, followed over 41±31 months, 22% developed GOLD-defined COPD.

Despite appearing “normal” according to GOLD, smokers with normal spirometry but low DLCO are at significant risk of developing COPD with obstruction to airflow.



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Smokers with normal spirometry but low DLCO have a higher risk of COPD than smokers with normal spirometry and DLCO <http://ow.ly/RWzxB>

For editorial comments see *Eur Respir J* 2015; 46: 1535–1537 [DOI: 10.1183/09031936.01436-2015]

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Introduction

Chronic obstructive pulmonary disease (COPD), the third leading cause of mortality in the USA and Europe, is caused primarily by cigarette smoking [1–3]. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as a chronic disease state characterised by airflow limitation that is not fully reversible with bronchodilators [1, 2]. The GOLD criteria classify COPD into four stages based on post-bronchodilator forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) [2]. With these criteria, if smokers have normal post-bronchodilator spirometry, they are considered to have normal lung function. While the evaluating physician will counsel the patient to stop smoking, the normal post-bronchodilator spirometry reassures both the patient and the physician that the patient does not have COPD and is at no greater risk of COPD than other smokers with normal post-bronchodilator spirometry.

Although the GOLD criteria are widely used [1, 4–6], it has been recognised that some smokers with normal spirometry have low diffusing capacity of the lung for carbon monoxide (*DLCO*), a parameter associated with alveolar destruction and possibly small airway disease, both of which are components of COPD [7–10]. *DLCO* measurement is not part of the GOLD criteria and is not used as a routine screening tool because of the lack of portability, the cost of the equipment, the expertise needed to carry out the measurement and the time involved [1, 11].

In the context that COPD is associated with both airway and alveolar disease [8], we asked: are smokers with normal post-bronchodilator spirometry but low *DLCO* at greater risk of developing COPD than smokers with normal post-bronchodilator spirometry and normal *DLCO*? To answer this question, we evaluated a group of cigarette smokers who answered advertisements in the New York metropolitan region for assessment of lung health. After clinical assessment, we characterised two groups: “normal spirometry/low *DLCO*”, smokers with normal post-bronchodilator spirometry and total lung capacity (TLC) but low *DLCO*; and control “normal spirometry/normal *DLCO*”, smokers with normal post-bronchodilator spirometry, normal TLC and normal *DLCO*. A randomly chosen subset of these groups were asked to return for repeated lung function over time. Strikingly, with an average follow-up of <4 years, compared to smokers with normal spirometry/normal *DLCO*, a significant number of smokers in the normal spirometry/low *DLCO* group developed GOLD criteria-defined COPD, *i.e.* smokers who have normal post-bronchodilator spirometry but low *DLCO* are at a higher risk of developing COPD with obstruction to airflow compared to smokers with normal post-bronchodilator and normal *DLCO*.

Methods

Recruitment, screening and pulmonary function tests

Smokers were recruited from the New York metropolitan area *via* advertisements in newspapers and on websites under a protocol approved by the Weill Cornell Medical College and New York/Presbyterian Hospital Institutional Review Board. Healthy nonsmokers were also recruited to calculate the 95% normal range for pulmonary function tests (PFTs) [12]. All individuals gave their informed written consent prior to any clinical evaluations or procedures. The study population was randomly chosen, using screening assessment and inclusion and exclusion criteria as detailed in the online supplementary material. PFTs were performed according to American Thoracic Society (ATS)/European Respiratory Society (ERS) standards [11, 13], and PFT machine calibrations were performed at the recommended intervals as described in the ATS/ERS guidelines [11] (online supplementary material).

Study groups and assessment

A total of 2302 active smokers were assessed. Based on the inclusion/exclusion criteria, a subset of 1570 active smokers were determined to be eligible. Of these, 1173 were phenotyped as normal spirometry/normal *DLCO* and 397 as normal spirometry/low *DLCO* based on their *DLCO* prediction values (online supplementary material). A subset of these individuals were randomly contacted and asked to return for additional PFT assessments. The groups assessed over time included 59 smokers with normal spirometry/normal *DLCO* and 46 smokers with normal spirometry/low *DLCO* (online supplementary table 1).

Statistical analysis

Statistical analysis was performed as detailed in the online supplementary material.

Role of the funding source

The funding sources of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report or the decision to submit this report for publication.

Results

Study population

Both the normal spirometry/normal DLCO and the normal spirometry/low DLCO groups had a preponderance of males and individuals of African-American descent, but had a similar distribution of sex, age and ethnicity (table 1). The two groups were assessed over a similar time period (online supplementary figure 1) and the age at the last assessment was similar (49 ± 8 versus 50 ± 9 years, respectively; $p>0.9$); there were no differences in the smoking history, cough or sputum scores, Modified Medical Research Council

TABLE 1 Demographics of study groups at baseline

Parameter	Smokers with normal spirometry		p-value
	Normal DLco	Low DLco	
Individuals	59	46	
Males/females	43/16	31/15	>0.6
Age years	45±8	46±8	>0.5
Ethnicity AA/E/H	41/10/8	37/5/4	>0.6
BMI kg·m⁻²	28±5	25±5	<0.002
Smoking history[#]			
Pack-years	24±13	30±15	>0.05
Packs per day	1.0±0.5	1.1±0.6	>0.5
Age of smoking initiation years	17±5	17±4	>0.9
Urine nicotine ng·mL ⁻¹	1102±1290	951±1285	>0.6
Urine cotinine ng·mL ⁻¹	1276±927	1298±894	>0.9
Cough score[¶]	1.2±1.3	1.7±1.5	>0.06
Sputum score[¶]	1.1±1.3	1.3±1.3	>0.3
MMRC score	0.4±0.6	0.5±0.6	>0.2
Emphysema⁺ %	2.0±0.02	2.2±0.04	>0.8
Serology[§]			
α ₁ -antitrypsin mg·dL ⁻¹	152±24	145±21	>0.1
ESR mm·h ⁻¹	13±11	12±10	>0.7
IgE IU·mL ⁻¹	129±208	169±259	>0.4
CRP mg·dL ⁻¹	0.2±0.2	0.3±0.2	<0.005
Hepatitis C negative/positive ^f	46/9	39/6	>0.8
Lung function^{##}			
VC % predicted	114±14	108±14	<0.05
FVC % predicted	111±14	104±14	>0.1
FEV ₁ % predicted	111±15	104±14	<0.03
FEV ₁ /FVC % observed	81±4	79±5	<0.03
TLC % predicted	99±13	94±14	<0.03
RV % predicted	90±25	89±37	>0.8
RV/TLC % predicted	28±7	31±11	>0.1
DLco % predicted	93±10	68±9	<10 ⁻⁴
DLco/VA mL·mHg ⁻¹ ·min ⁻¹ ·L ⁻¹	4.4±0.6	3.6±0.7	<10 ⁻⁶
Assessment over time mean±SD (range)			
Duration of follow-up months	46±21 [5–113]	41±31 [5–146]	>0.4
Number of PFTs	2±1 [2–6]	3±2 [2–8]	<10 ⁻³
Interval between PFTs months	33±18 [5–73]	18±20 [1–127]	<10 ⁻⁶

Data are presented as n or mean±SD, unless otherwise stated. A total of 105 active smokers was enrolled in the study, including 46 individuals with normal history, and physical and general laboratory tests, normal posterior–anterior and lateral chest radiography, and normal spirometry and lung volumes, but low diffusing capacity of the lung for carbon monoxide (DLco), and 59 with normal spirometry, lung volumes and DLco. All were followed over time with full lung function studies. AA: African-American; E: European; H: Hispanic; BMI: body mass index; MMRC: Modified Medical Research Council dyspnoea scale [14]; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; VC: vital capacity; FVC: forced vital capacity; FEV₁: forced expiratory volume in 1 s; TLC: total lung capacity; RV: residual volume; VA: alveolar volume; PFT: pulmonary function test. [#]: current smoking was verified at baseline by urine nicotine and its derivative cotinine; at subsequent visits for lung function testing, active smoking status was verified by questionnaire. [¶]: cough and sputum scores were each evaluated on a scale of 0–4, where 0 represented “not at all”, 1 “only with chest infections”, 2 “a few days a month”, 3 “several days a week” and 4 “most days a week” [15]. ⁺: chest high-resolution computed tomography % emphysema at –950 Hounsfield units. [§]: all individuals tested negative for HIV and had normal levels of α₁-antitrypsin. ^f: data available for 55 out of 59 smokers with normal spirometry and DLco, and 45 out of 46 smokers with normal spirometry but low DLco. ^{##}: DLco corrected for haemoglobin and carboxyhaemoglobin [11].

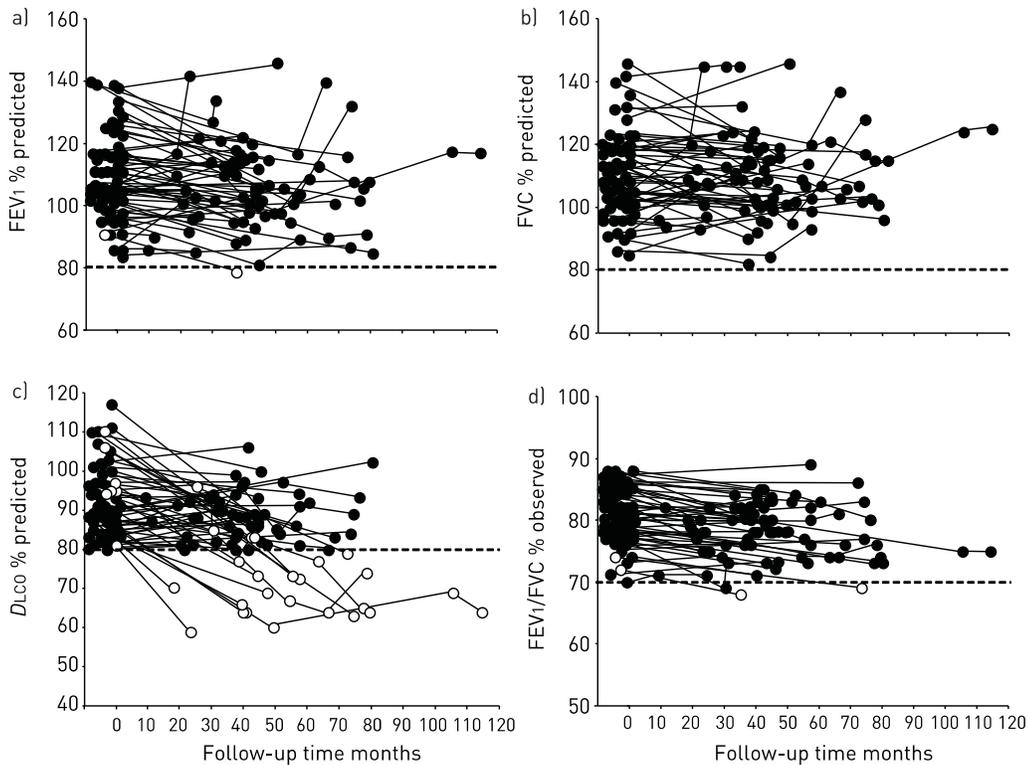


FIGURE 1 Lung function assessment over time of 59 active smokers with baseline normal history, physical examination and laboratory tests, and with normal spirometry, lung volumes, and normal diffusing capacity of the lung for carbon monoxide ($DLCO$). The abscissa shows time in months. Each symbol represents an individual, with lines connecting the follow-up data over time for the same individual. The dashed lines represent the lower limit of normal. Open circles indicate individuals that initially had normal values at baseline but became abnormal over time. Filled circles indicate individuals that had normal values at baseline and remained normal over time. a) Forced expiratory volume in 1 s (FEV_1); b) forced vital capacity (FVC); c) $DLCO$; d) FEV_1/FVC % observed.

dyspnoea (MMRC) scale, or urine nicotine and cotinine levels between the two groups ($p > 0.05$ for all comparisons). Percentage emphysema as assessed by quantitative high-resolution computed tomography (HRCT) was not significantly different between the groups ($p > 0.8$) (online supplementary figure 2). Except for slightly higher C-reactive protein levels in the normal spirometry/low $DLCO$ group, other serology (erythrocyte sedimentation rate, IgE level and hepatitis C positivity/negativity) were not significantly different between the groups ($p > 0.1$ for all comparisons). Body mass index was lower in the normal spirometry/low $DLCO$ group ($p < 0.002$). Comparison of the lung function assessment between the two groups revealed, by definition, a difference in $DLCO$ and $DLCO$ /alveolar volume ($p < 10^{-4}$ for both comparisons). Of the other PFT parameters evaluated, all were within normal range, with the normal spirometry/low $DLCO$ group having a normal but lower vital capacity, FEV_1 , FEV_1/FVC and TLC ($p < 0.03$ for all comparisons). When the groups were divided into African-American, European and Hispanic descendants, there was no significant difference attributed to ethnicity in any of the above parameters within the groups or between the groups ($p > 0.05$ and all comparisons).

Lung function over time

In the normal spirometry/normal $DLCO$ group, the FEV_1 % predicted remained normal in 58 out of 59 individuals and the FVC % predicted remained normal in all 59 individuals throughout the follow-up period (figure 1a and b). The $DLCO$ in this group remained normal in 44 (75%) out of 59 individuals but, interestingly, decreased to the normal spirometry/low $DLCO$ category ($DLCO < 80\%$ predicted) in 15 (25%) out of 59 individuals, suggesting that a significant number of active smokers with normal spirometry/normal $DLCO$ will progress to have low $DLCO$ over an average of < 4 years (figure 1c). Only two (3%) out of the 59 active smokers in the normal spirometry/normal $DLCO$ group developed COPD stage I as defined by the GOLD criteria [3] ($FEV_1/FVC < 0.7$ and $FEV_1 \geq 80\%$ predicted, post-bronchodilators), one individuals at month 34 and the second at month 72 from baseline (figure 1d).

In the normal spirometry/low $DLCO$ group, the FEV_1 % predicted remained normal in 44 out of 46 individuals and the FVC % predicted remained normal in all 46 individuals (figure 2a and b). The $DLCO$

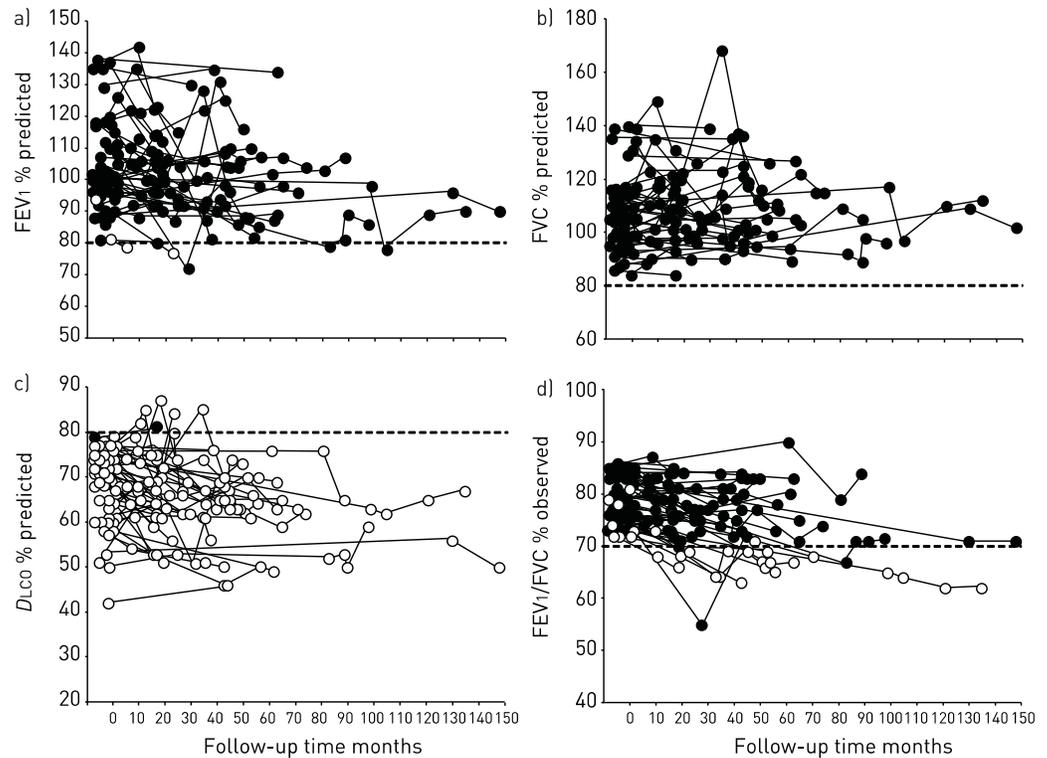


FIGURE 2 Lung function assessment over time in 46 active smokers with normal history, physical examination and laboratory tests, and with normal spirometry, lung volumes, but low diffusing capacity of the lung for carbon monoxide (DL_{CO}). The abscissa shows time in months. Each symbol represents an individual, with lines connecting the follow-up data over time for the same individual. The dashed lines represent the lower limit of normal. Open circles indicate individuals that initially had normal values but became abnormal over time. Filled circles indicate individuals that had normal values at baseline and remained normal over time. a) Forced expiratory volume in 1 s (FEV₁); b) forced vital capacity (FVC); c) DL_{CO} ; d) FEV₁/FVC % observed.

in this group remained low (<80% predicted) in 45 out of 46 individuals (figure 2c). In contrast to the normal spirometry/normal DL_{CO} , 10 (22%) out of 46 active smokers in the normal spirometry/low DL_{CO} group developed airflow limitation consistent with the GOLD criteria for COPD [3] (FEV₁/FVC <0.7), nine with GOLD I (FEV₁ ≥80% predicted post-bronchodilators) and one with GOLD II (FEV₁ ≥50–79% predicted) ($p < 0.009$) (figure 2d and table 2).

Comparison of the last lung function assessment to the baseline lung function within the normal spirometry/normal DL_{CO} group showed no significant difference in the FEV₁ or FVC % predicted ($p > 0.3$ for both comparisons) but a significant decrease in the DL_{CO} % predicted and FEV₁/FVC % observed ($p < 10^{-4}$ for both comparisons) (figure 3a, c, e and g). We did not assess whether this was or was not

TABLE 2 Progression to chronic obstructive pulmonary disease (COPD) in active smokers with normal spirometry/low diffusing capacity of the lung for carbon monoxide (DL_{CO}) versus active smokers with normal spirometry/normal DL_{CO}

Group [#]	At end of evaluation period	
	Normal	With COPD
Normal spirometry, normal DL_{CO}	97 (57/59)	3 (2/59)
Normal spirometry, low DL_{CO}	78 (36/46)	22 (10/46)
p-value [¶]	0.009	

Data are presented as % (n/N) unless otherwise stated. 59 active smokers with normal spirometry/normal DL_{CO} and 46 active smokers with normal spirometry/low DL_{CO} were followed over time with full lung function studies to determine the rate of progression to COPD. [#]: individuals with normal spirometry and lung volumes, and normal DL_{CO} were followed for mean ± SD 45 ± 20 months; individuals with normal spirometry and lung volumes but low DL_{CO} were followed for 41 ± 31 months ($p > 0.4$). [¶]: Chi-squared test.

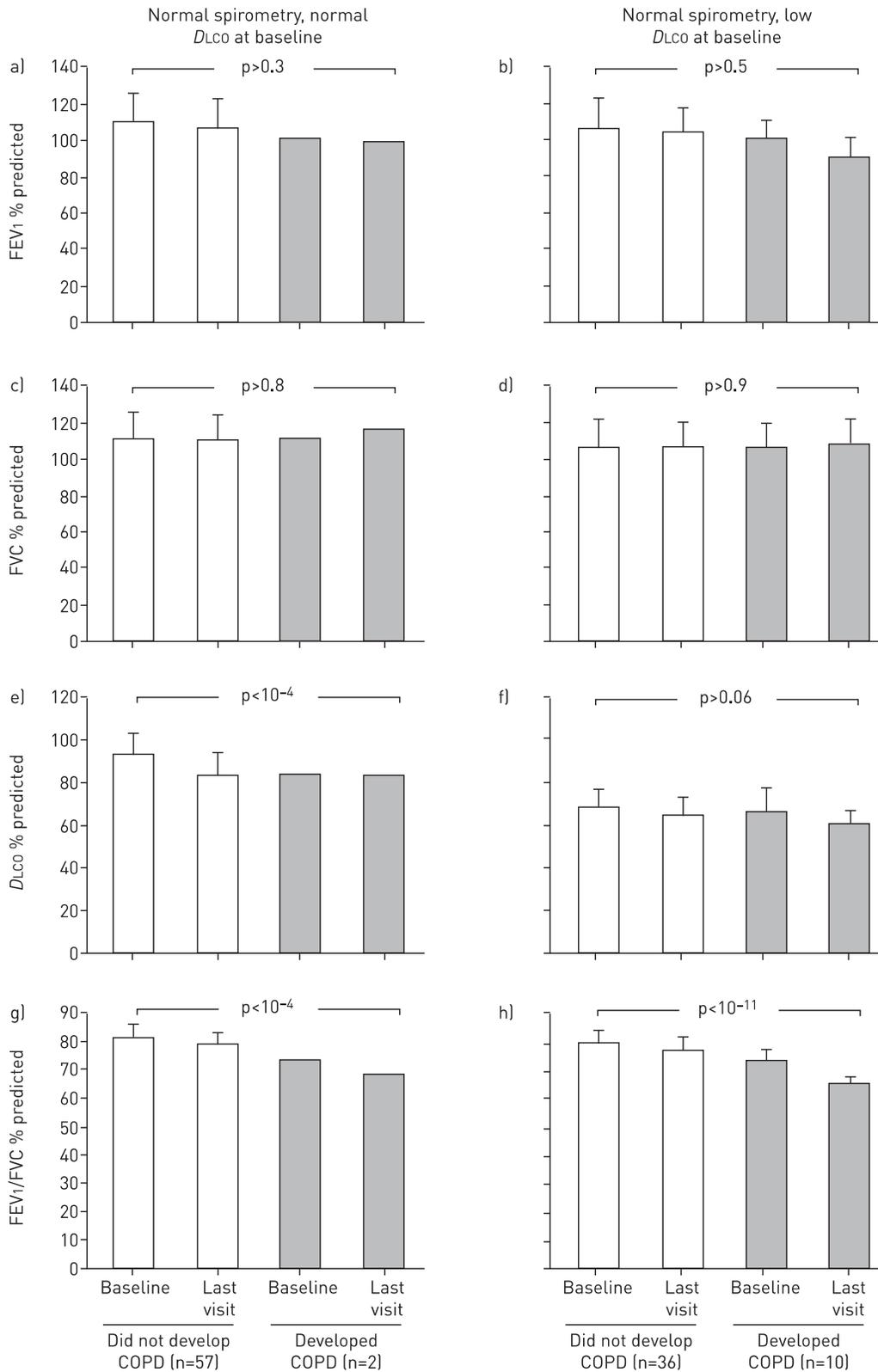


FIGURE 3 Lung function changes from baseline to the last pulmonary function test in the normal spirometry/normal diffusing capacity of the lung for carbon monoxide (*DLCO*) group and normal spirometry/low *DLCO* group comparing individuals who did not develop chronic obstructive pulmonary disease (COPD) to those who did. a and b) Forced expiratory volume in 1 s (FEV₁); c and d) forced vital capacity (FVC); e and f) *DLCO*; g and h) FEV₁/FVC. Data are presented as mean±SD.

associated with symptoms such as cough, sputum or dyspnoea at the last time-point. Comparison of the last lung function to the baseline lung function within the normal spirometry/low *DLCO* group showed no change in FEV₁, FVC or *DLCO* % predicted ($p > 0.06$ for all comparisons) but a significant reduction in FEV₁/FVC % observed ($p < 10^{-11}$) (figure 3b, d, f and h). Comparison of the rate of change of the FEV₁/FVC over time from baseline to last assessment of the normal spirometry/normal *DLCO* group to the normal spirometry/low *DLCO* group showed a significantly greater decrease over time for the normal spirometry/low *DLCO* group (normal spirometry/low *DLCO* $-0.14 \pm 0.18\%$ change in FEV₁/FVC per month, normal spirometry/normal *DLCO* $-0.07 \pm 0.11\%$ change per month; $p < 0.02$).

Assessment of the 46 smokers with normal spirometry/low *DLCO* who were followed over time showed that the distribution of males to females and African-Americans to Europeans or Hispanics was similar in the 10 individuals who developed COPD *versus* the 36 who did not (supplementary table I). The smoking history, cough and sputum scores, and MMRC scale and serology were also similar in both groups and the age at the last assessment was similar (54 ± 7 *versus* 48 ± 9 years, respectively; $p > 0.09$). Percentage emphysema assessed by HRCT was not significantly different between the groups ($p > 0.05$). The 10 individuals who developed COPD had lower, but within the normal range, FEV₁/FVC % observed at baseline compared to the 36 individuals who did not develop COPD ($p < 0.003$). All other lung function parameters were similar between the two groups ($p > 0.05$, all comparisons). On the average, there were no differences in the time of follow-up, number of lung function tests or intervals between lung function tests ($p > 0.1$ for all comparisons). There were no significant differences in any of the parameters or in the prevalence of COPD development between African-Americans, Europeans or Hispanics within and between the low-*DLCO* smokers who developed COPD and those who did not ($p > 0.09$ for all comparisons). The assessment of using *DLCO* levels at baseline as a predictor for development of COPD yielded an area under the curve score of 0.75; *i.e.*, *DLCO* levels can be used to predict COPD development within 41 months with accuracy of 75%.

In addition to using a cut-off of FEV₁/FVC < 0.7 to define developing COPD and *DLCO* $< 80\%$ predicted to define low *DLCO*, a 95% range of normal *DLCO* % predicted and FEV₁/FVC [12] was calculated based on the lung function of a 405 healthy nonsmoker dataset (online supplementary material) and used to compare the study population prevalence of developing COPD. Using the normal range for FEV₁/FVC and *DLCO* % predicted calculated for each sex and ethnicity based on this dataset yielded the same results, with significantly higher prevalence of developing COPD (defined as FEV₁/FVC $< 95\%$ normal) in the normal spirometry/low *DLCO* group *versus* the normal spirometry/normal *DLCO* group (low *DLCO* defined as below the 95% range).

Discussion

Cigarette smoking represents the major risk factor for the development of COPD, although only a fraction of smokers develop the disease [1, 2, 5, 6, 16]. Identification of those smokers at higher risk represents an important step in that the early detection of COPD leads to early therapeutic intervention [1, 2, 17]. Spirometry with bronchodilators is the gold standard tool to screen smokers for COPD [1]. In this study, we focussed on evaluating the addition of the *DLCO* parameter to identify smokers at risk of the development of COPD. We observed that in a population of 2302 active smokers randomly recruited in the New York metropolitan area responding to advertisements to assess lung health in active cigarette smokers, 17% had the phenotype of normal spirometry/low *DLCO*, *i.e.*, the phenotype of low *DLCO* is quite common among active smokers with normal spirometry. Strikingly, of 105 active smokers randomly chosen for follow-up lung function studies over an average of < 4 years, 22% with the normal spirometry/low *DLCO* phenotype developed COPD by the GOLD criteria, compared to only 3% of the normal spirometry/normal *DLCO* phenotype. These observations suggest that the normal spirometry/low *DLCO* phenotype is at higher risk for developing COPD than normal spirometry/normal *DLCO*.

Low *DLCO* in otherwise healthy smokers

DLCO assesses the potential of the lung for gas exchange [18]. A pathologic correlate of decreased *DLCO* in smokers is the destruction of the pulmonary capillary bed and a low *DLCO* in the context of a normal TLC suggests alveolar destruction, *i.e.* emphysema [8, 18]. A good correlation between low *DLCO* and emphysema on chest computed tomography has been reported [19, 20]. Consistent with these observations, active smokers with normal spirometry but low *DLCO* have high circulating levels of endothelial microparticles derived from apoptotic pulmonary capillary endothelium [21]. Decreased *DLCO* has also been correlated with small airway disease in the presence of severe expiratory airflow limitation and hyperinflation [22].

Our observation that 17% of active smokers responding to advertisements to assess lung health had a normal spirometry/low *DLCO* phenotype suggests that, despite a normal spirometry, a significant number of active smokers have a low *DLCO*, an observation consistent with a number of other studies. Interestingly,

while the phenotype of smokers with normal spirometry but low *DLCO* is recognised, there are no data regarding what happens to lung function over time in these individuals.

Risk markers for COPD in smokers

Identification of markers that trigger early intervention in smokers is important in that even mild COPD is associated with increased mortality [23]. Parameters that help identify the “most vulnerable” smokers, include age, sex, cough, sputum production, dyspnoea, continuation of smoking and pack-years of exposure [1, 2, 5, 6, 14, 24–30].

In smokers, the prevalence of COPD increases with age [6]. A 25-year follow-up study found that the incidence of COPD in active smokers was 35.5%, with age being a significant predictor for the development of COPD [5]. Advanced age was found to be significantly related to the incidence of COPD in 7- and 10-year follow-up studies [28, 29]. In the present study, there was no difference in age between the normal spirometry/normal *DLCO* and normal spirometry/low *DLCO* groups or within the normal spirometry/low *DLCO* group, when comparing the individuals who developed COPD and those who did not.

In addition to age, cough and sputum production have been found by prospective studies to identify individuals with higher risk of developing COPD [26, 28]. A study of Japanese male smokers and nonsmokers demonstrated that productive cough was an independent risk factor for the development of COPD [30]. These data contrast with the studies by FLETCHER *et al.* [27] and VESTBO *et al.* [16], which found that mucus hypersecretion in smokers is a benign condition. In our study, there were no differences in cough and sputum scores between the active smokers with normal spirometry/low *DLCO* and normal spirometry/normal *DLCO*. Furthermore, the individuals followed over time with normal spirometry/low *DLCO* who developed COPD did not differ in terms of symptoms compared to those who did not develop COPD.

The data pertaining to sex in the development of COPD are conflicting. Studies of smokers, ex-smokers and nonsmokers over 7 and 10 years did not identify sex as a risk factor [28, 29]. However, a study using the GOLD criteria found that despite similar smoking history, men are more susceptible to development of COPD [25] and male smokers have more emphysema than female smokers [24]. In the present study, the development of COPD was sex-independent.

All individuals in our study continued to be active smokers. Continuation of smoking has been found to be an important risk factor for the development of COPD. In the Lung Health Study, smoking cessation significantly slowed the progression to COPD [1, 2, 5, 17].

Implications

The central observation in this study is that, among active smokers with normal spirometry and normal lung volumes, a decreased *DLCO* is a risk factor for progression to COPD. These observations need to be verified by larger, randomised trials. Furthermore, the identification of the low- *DLCO* phenotype is complicated by ethnic variations in “normal” *DLCO* and significant attention must be paid to quality control. However, with these caveats, the concept that active smokers with normal spirometry/low *DLCO* are at significantly higher risk for the development of COPD over an average period of <4 years than a comparable group of active smokers with normal spirometry/normal *DLCO* has important implications.

First, the data suggest that *DLCO* measurement could be an additional tool for early detection of the smoker at risk for COPD, and thus help contribute to early intervention.

Second, while the measurement of *DLCO* is not presently suitable for routine screening, engineering technology could be developed to make *DLCO* an early, inexpensive, reproducible measurement, suitable for routine office visits and field use for epidemiological studies.

Third, in the past, *DLCO* has not been measured in large epidemiological studies such as SPIROMICS and COPDGene [31, 32]. While there are many reasons for this (mostly cost), the observation that a significant percentage of active smokers have a low *DLCO* and, of these, a significant percentage will develop COPD in an average of <4 years has significant implications for the “risk for COPD” parameters assessed in these studies.

Finally, the findings suggest that in smokers, a normal spirometry post-bronchodilator test may give a false sense of “normal”, in that a significant subgroup may have a low *DLCO* and that subgroup is at a significant risk for developing COPD with obstruction.

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3.3 Article 3: Persistence of Circulating Endothelial Microparticles in COPD Despite Smoking Cessation

Thorax; In press

Abstract: The levels of plasma circulating endothelial microparticles (EMPs) derived from pulmonary capillaries undergoing apoptosis were previously shown to be elevated in smokers with normal spirometry but reduced diffusion capacity (i.e., can be used as a measure of active alveolar destruction). The levels of total and apoptotic EMPs were assessed in healthy cigarette smokers and cigarette smokers with COPD and the persistence of the EMP levels were followed in nonsmokers, healthy cigarette smokers and cigarette smokers with COPD for one year at 4 time points (0, 3, 6 and 12 months). To ask whether these biologic markers of pulmonary capillary endothelial apoptosis associated with smoking are reversible, we studied a subset of the healthy smokers and COPD cigarette smokers who quit smoking, with sampling before smoking cessation and then again after 3, 6 and 12 months following cessation.

The data replicated our previous observation in healthy cigarette smokers, extends it to COPD cigarette smokers and demonstrated that the elevated levels of total and apoptotic EMPs are stable over a period of 12 months in healthy cigarette smokers and COPD cigarette smokers who continued to smoke. In contrast, in healthy cigarette smokers who quit smoking, the plasma level of total and apoptotic EMPs return to the levels of nonsmokers and remain normal for 12 months following smoking cessation. Interestingly, however, and consistent with epidemiologic data of progression of COPD despite smoking cessation, in COPD cigarette smokers who quit smoking, the plasma levels of total and apoptotic EMPs do not return to the levels of nonsmokers, and remain abnormal even following 12 months of smoking cessation, likely reflecting continued lung endothelial injury.

ORIGINAL ARTICLE

Persistence of circulating endothelial microparticles in COPD despite smoking cessation

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ABSTRACT

Introduction Increasing evidence links COPD pathogenesis with pulmonary capillary apoptosis. We previously demonstrated that plasma levels of circulating microparticles released from endothelial cells (EMPs) due to apoptosis are elevated in smokers with normal spirometry but low diffusion capacity, that is, with early evidence of lung destruction. We hypothesised that pulmonary capillary apoptosis persists with the development of COPD and assessed its reversibility in healthy smokers and COPD smokers following smoking cessation.

Methods Pulmonary function and high-resolution CT (HRCT) were assessed in 28 non-smokers, 61 healthy smokers and 49 COPD smokers; 17 healthy smokers and 18 COPD smokers quit smoking for 12 months following the baseline visit. Total EMP (CD42b⁻CD31⁺), pulmonary capillary EMP (CD42b⁻CD31⁺ACE⁺) and apoptotic EMP (CD42b⁻CD62E⁺/CD42b⁻CD31⁺) levels were quantified by flow cytometry.

Results Compared with non-smokers, healthy smokers and COPD smokers had elevated levels of circulating EMPs due to active pulmonary capillary endothelial apoptosis. Levels remained elevated over 12 months in healthy smokers and COPD smokers who continued smoking, but returned to non-smoker levels in healthy smokers who quit. In contrast, levels remained significantly abnormal in COPD smokers who quit.

Conclusions Pulmonary capillary apoptosis is reversible in healthy smokers who quit, but continues to play a role in COPD pathogenesis in smokers who progressed to airflow obstruction despite smoking cessation.

Trial registration number NCT00974064; NCT01776398.

INTRODUCTION

COPD, the third leading cause of mortality in the USA, is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as a chronic lung disorder with airflow limitation that is not fully reversible.¹ There is overwhelming evidence that most cases of COPD are caused by cigarette smoking, with approximately 20% of smokers at risk for COPD if they continue to smoke.² The airflow obstruction that characterises COPD is caused by a variable mixture of small airway disease (bronchitis) and parenchymal destruction (emphysema).^{1,3} Although the airway and alveolar diseases were classically considered as separate

Key messages

What is the key question?

- Is pulmonary capillary apoptosis, as measured by plasma levels of microparticles released from apoptotic endothelial cells (EMPs), reversible in healthy smokers and COPD smokers following smoking cessation?

What is the bottom line?

- Pulmonary capillary apoptosis is reversible in healthy smokers who quit, but not in COPD smokers despite 12 months of smoking cessation, suggesting that the apoptosis continues to play a role in COPD pathogenesis in smokers who progressed to airflow obstruction despite smoking cessation.

Why read on?

- Our longitudinal study demonstrates persistent endothelial stress in subjects with COPD despite smoking cessation and provides a biological correlate to the epidemiological data showing that smoking cessation only has a moderate effect on the continuous decline of lung function in COPD smokers, suggesting EMP levels might serve as a useful biomarker to follow smoking-associated endothelial apoptosis.

entities, it is now recognised that they usually coexist to variable degrees and are closely linked, with the parenchymal destruction evolving around areas of small airway disease.^{3–7}

There is increasing evidence that the pathogenesis of COPD is linked, in part, to apoptosis of pulmonary capillaries.^{8–11} Consistent with this concept, we recently demonstrated that smokers and, to a greater extent, smokers with early evidence of lung destruction (normal spirometry, but low diffusing capacity (DLCO)) have elevated levels of circulating endothelial microparticles (EMPs).¹² Importantly, a significant proportion of these EMPs are derived from pulmonary capillaries and have characteristics of apoptotic EMPs, that is, they are derived from lung endothelial cells that have been induced to undergo apoptosis.^{12–14}



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If elevated levels of circulating apoptotic EMPs are a reflection of active smoking-related injury, to lung endothelium, based on the knowledge that even those COPD smokers who stop smoking continue to have a decline in lung function that is more rapid than that of healthy non-smokers or healthy smokers who quit smoking,¹⁵ we hypothesised that elevated levels of circulating apoptotic EMPs may persist in COPD smokers following smoking cessation, reflecting continuous lung endothelial injury that persists even after the stress of smoking is removed. To assess this hypothesis, we quantified the levels of circulating EMPs, and the fraction represented by apoptotic EMPs, in non-smokers, healthy smokers and smokers with COPD at baseline and at three more intervals over 1 year and then compared those levels with those obtained from a subgroup of healthy smokers and COPD smokers who successfully stopped smoking after baseline assessment. The data demonstrate that circulating EMP levels derived from apoptotic pulmonary capillary endothelial cells remain elevated over 1 year in healthy smokers and COPD smokers who continue smoking. However, while levels of total and apoptotic EMPs return to non-smoker levels in healthy smokers who successfully quit smoking, total and apoptotic EMP levels remain elevated in COPD smokers who quit smoking persisting 12 months of smoking cessation.

METHODS

Human subjects and clinical phenotypes

All subjects were evaluated at the Weill Cornell NIH Clinical and Translational Science Center and Department of Genetic Medicine Clinical Research Facility, under the clinical protocols approved by the Institutional Review Board. Recruitment was from the general population in New York City by posting advertisements in local newspapers and on electronic bulletin boards. All subjects provided written consent prior to enrolment and then underwent thorough medical history, screening and pulmonary function tests. Smoking status was determined based on self-reported history and quantified levels of urine nicotine metabolites. For details and full inclusion/exclusion criteria, see online supplementary methods. A total of 138 subjects were assessed for circulating total and apoptotic EMP levels at baseline, 3, 6 and 12 months (28 non-smokers, 61 healthy smokers and 49 COPD GOLD I/II smokers). See online supplementary figure S1 for study design.

Characterisation of plasma EMPs

EMPs were quantified according to a standard operating procedure as previously described¹² to eliminate variability in sample processing. Briefly, blood was collected, processed within 1 hour and stained for the endothelial markers PECAM (CD31) and E-selectin (CD62E) and the constitutive platelet-specific glycoprotein Ib (CD42b) to differentiate endothelium-originated microparticles from platelet-derived microparticles, which also express CD31. EMPs were defined as microparticles <1.5 µm in size, expressing CD31⁺ or CD62E⁺ but not CD42b. We have previously shown that staining with annexin V is comparable with CD42b⁻CD31⁺ staining,¹² but annexin V was not used because it is not specific for EMPs.¹⁶ Circulating EMPs are present in low levels in plasma of healthy subjects, reflecting normal endothelial turnover,¹⁷ but their levels increase in a variety of vascular-related disorders. As in our previous study,¹² total EMP levels above the non-smoker total EMP mean level plus 2 SDs were considered abnormally elevated. To assess the presence of relative contribution of pulmonary capillary endothelium to the elevated total EMP levels,^{1 12} EMPs were

co-stained with antihuman ACE inhibitors, which is abundantly expressed on pulmonary capillary endothelium (CD42b⁻CD31⁺ACE⁺).¹³ To quantify the proportion of EMPs that originated from apoptotic endothelium, we assessed the ratio of CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMPs in all groups. EMPs induced by apoptosis express the constitutive CD31 marker, whereas activation-induced EMPs express CD62E. Using these criteria, EMPs with a low CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ ratio were defined as 'apoptotic EMPs', and the percentage of subjects with apoptotic EMPs with CD42b⁻CD62E⁺/CD42b⁻CD31⁺ ratio below the lowest ratio in healthy non-smokers was quantified. See online supplementary methods for further details on the EMP analysis.

Assessment after smoking cessation

After the baseline levels of total and apoptotic EMPs were determined, all healthy smokers and COPD smokers were invited to stop smoking using a combination of varenicline and counselling for 3 months (see details in online supplementary methods). A total of 17 healthy smokers and 18 COPD smokers successfully quit smoking as confirmed by urine tobacco metabolite level quantification at 3, 6 and 12 months after the baseline; subjects were considered true quitters only if there were no detectable levels of nicotine metabolites in the urine at months 3, 6 and 12. Healthy smokers and smokers with COPD were treated with exact same prescription of varenicline to prevent any effect it might have on EMP levels. All other healthy smokers and COPD smokers were considered current smokers if urine cotinine level was ≥104 ng/mL at each time point, a level based on our previous study of low-level smoke exposure,¹⁸ where 104 ng/mL was calculated as the induction half-maximal level (ID₅₀) at which the small airway epithelium, the initial site of smoking-related pathology, showed an abnormal response. See online supplementary figure S1 for study design.

Statistical analysis

χ² test, with a Yates' correction for small sample size, was used for comparing demographic parameters and the number of subjects with high total EMP and apoptotic EMP levels, and pairwise analysis of variance was used to compare total and apoptotic EMP levels between groups and within a group, at different time points with no correction for multiple test, as the number of tests was low (<21). In order to eliminate the effect of diseases known to be associated with elevated EMPs, including diabetes^{19 20} and systemic hypertension,²¹ or drugs for COPD, including corticosteroids and bronchodilators, subjects with known disease state or drug treatment were removed from statistical analysis. Removal of those subjects did not alter the results.

RESULTS

Study population

Except for minor differences, the study population of non-smokers was comparable with the healthy smokers and COPD smokers in all demographic parameters (see table 1 for details). At each time point, the non-smokers had undetectable urine nicotine (not shown) and cotinine levels (figure 1). Both the healthy smokers and COPD smokers who continued smoking had urine cotinine levels consistent with tobacco smoking and comparable in both groups at each time point (p>0.07, all comparisons). In healthy smokers and COPD smokers who quit smoking, urine nicotine and cotinine levels were consistent with smoking at baseline and were undetectable at all time points

Table 1 Study Population*

Parameter	Healthy smokers†				COPD smokers‡		
	Non-smokers	All	Who continued smoking	Who quit	All	Who continued smoking	Who quit
n	28	61	44	17	49	31	18
Gender (M/F)	15/13	47/14	37/7	10/7	46/3	30/1	16/2
Ethnicity (B/W/O)§	10/7/11	33/9/19	23/7/14	10/2/5	27/12/10	18/6/7	9/6/3
Age	37±11	44±9	44±9	45±10	53±8	53±7	53±9
BMI	27±5	28±5	27±4	30±4	27±4	25±3	29±5
Smoking history							
Pack-year	–	23±12	24±12	20±8	32±14	32±15	34±12
Pack per day	–	0.8±0.6	1.0±0.6	0.6±0.2	0.8±0.4	0.8±0.5	0.8±0.3
Age of initiation	–	16±3	16±3	16±3	16±3	16±3	16±3
Urine cotinine (ng/mL)	–	1693±961	1828±930	1323±979	1747±980	1953±959	1393±938
Subjects with emphysema (n, %)¶	1 (4%)	0 (0%)	0 (0%)	0 (0%)	13 (27%)	9 (29%)	4 (22%)
Pulmonary function**							
FEV1	106±11	109±11	109±10	107±13	85±16	87±16	82±17
FVC	107±11	111±10	110±10	111±11	108±16	109±17	105±15
FEV1/FVC	83±5	80±5	81±4	79±6	63±6	64±6	63±7
TLC	99±16	96±12	95±12	96±10	99±12	100±13	99±10
DLCO	91±11	89±8	89±9	90±6	71±14	68±13	77±15
GOLD stage (I/II)	–	–	–	–	31/18	20/11	11/7

*Data are presented as mean±SD; all parameters recorded at baseline; health/disease state based on screening and medical history and smoking status based on self-reported history and urine nicotine metabolite levels (detailed in online supplementary methods); non-smokers were comparable with all healthy smokers and all COPD smokers in ethnicity, BMI and all pulmonary function ($p>0.1$, all comparisons), except for FEV1 and DLCO that were lower in all COPD smokers ($p<10^{-7}$, both comparisons), and FEV1/FVC, that was lower in all healthy smokers and, by definition, in all COPD smokers ($p<0.02$, both comparisons). Non-smokers were younger than all healthy smokers and all COPD smokers ($p<0.002$, both comparisons), and there were less female COPD smokers than female non-smokers ($p<0.0002$). There were more COPD smokers with emphysema compared with non-smokers ($p<10^{-4}$); All healthy smokers were comparable with all COPD smokers in ethnicity, age, BMI, all smoking history parameters ($p>0.3$, all comparisons), except for pack-year that was lower in all healthy smokers ($p<10^{-3}$). FVC and TLC were comparable ($p>0.07$, both comparisons), but FEV1, DLCO and, by definition, FEV1/FVC were lower in all COPD smokers ($p<10^{-12}$, all comparisons). There were fewer females among all COPD smokers than among all healthy smokers ($p<0.04$). There were more COPD smokers with emphysema compared with healthy smokers ($p<10^{-4}$).

†Healthy smokers who continued smoking had urine cotinine ≥ 104 ng/mL (see online supplementary methods for details) at baseline, 3, 6 and 12 months. Healthy smokers who quit had undetectable urine nicotine and cotinine levels at 3, 6 and 12 months. The healthy smokers who continued smoking were comparable with those who quit in age, ethnicity, all smoking history ($p>0.3$, all comparisons), except for pack per day that was lower in those who quit ($p<0.03$), and comparable in all pulmonary function ($p>0.1$). There were more females, and the BMI was higher in the healthy smokers who quit group ($p<0.04$, both comparisons).

‡Gold stage defined by GOLD criteria¹; see online supplementary methods for details of subjects on medications; several of those treated were on multiple classes of medications; COPD smokers who continued smoking had urine cotinine ≥ 104 ng/mL at baseline, 3, 6 and 12 months; COPD smokers who quit had undetectable urine nicotine and cotinine levels at 3, 6 and 12 months; The COPD smokers who continued smoking were comparable with those who quit in age, gender, ethnicity, all smoking history and all pulmonary functions ($p>0.3$, all comparisons), except for DLCO that was lower in the COPD who continued smoking compared with those who quit ($p<0.03$). The BMI was lower in the COPD who continued smoking versus those who quit ($p<0.002$). There was no difference in the number of subjects with emphysema between the COPD smokers who quit smoking and those who continued smoking ($p>0.6$).

§B, black, W, white, O, other.

¶Chest high-resolution CT (HRCT); % emphysema at -950 Hounsfield Units (HU); emphysema defined as $>5\%$ lung volume; see online supplementary methods for details.

**Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed. BMI, body mass index; DLCO, diffusing capacity; GOLD; Global Initiative for Chronic Obstructive Lung Disease; TLC, total lung capacity.

after baseline (see online supplementary methods for details of urine nicotine metabolite level criteria for smoking/abstinence).

Total EMP levels

Consistent with our prior study with a different cohort,¹² total EMP levels were higher in healthy smokers compared with non-smokers (figure 2A, $p<0.005$). In addition, COPD smokers had elevated levels of total EMPs compared with non-smokers ($p<0.007$), but lower than those of healthy smokers ($p<0.02$). Twenty-two (36%) healthy smokers and eight (16%) COPD smokers had high levels of total EMPs ($p<0.03$). There was no correlation between the level of total EMPs and any pulmonary function or demographic parameters ($r^2<0.08$, all correlations, see online supplementary figure S2). For the COPD group, total circulating EMP levels were independent of drugs used for treatment, including inhaled and systemic corticosteroids and bronchodilators.

Origin of the circulating EMPs

In our prior study of circulating EMPs,¹² we demonstrated that most of the circulating CD42b⁻CD31⁺ EMPs were positive for

ACE inhibitors, a surface protein more highly expressed on pulmonary capillary endothelium than in other endothelial beds.¹³

In the present study, an average of 75% of the circulating EMPs in all subjects were CD42b⁻CD31⁺ACE⁺. There were similar levels of ACE⁺ EMPs in the healthy smoker group compared with the non-smokers or COPD smokers (figure 2B, $p>0.1$, both comparisons), and higher levels of ACE⁺ EMPs in the COPD smoker group compared with the non-smokers ($p<0.0001$).

EMPs derived from apoptotic endothelium

To quantify the proportion of EMPs originating from apoptotic endothelium, we assessed the ratio of CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMPs in all groups. The CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMP ratio in non-smokers was distributed around a mean of 0.9, significantly higher than that in healthy smokers (mean 0.6; a lower ratio indicates greater number of apoptotic EMPs) and COPD smokers (mean 0.55, $p<0.0001$, both groups compared with non-smokers; $p>0.6$, COPD smokers compared with healthy smokers). CD42b⁻CD62E⁺/

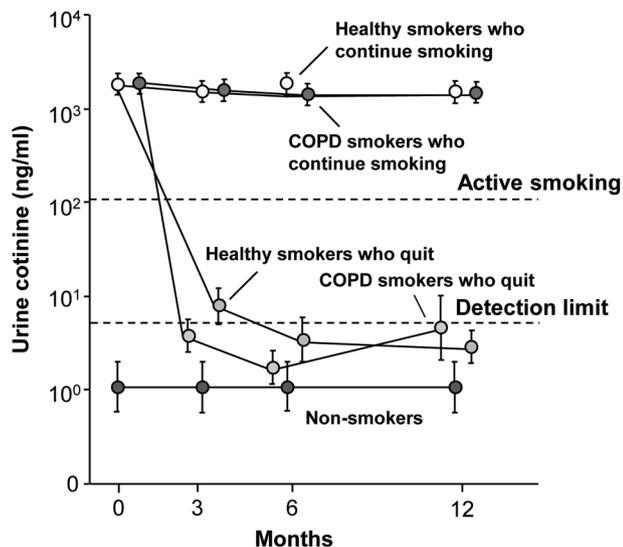


Figure 1 Urine cotinine levels (ng/mL) as a measure of smoking status at baseline and at 3, 6 and 12 months in non-smokers, healthy smokers and smokers with COPD (COPD smokers). Shown are data for non-smokers ($n=28$, green circles), healthy smokers who continue to smoke ($n=44$, yellow circles), healthy smokers who quit smoking following baseline ($n=17$, light blue circles), COPD smokers who continue to smoke ($n=31$, red circles) and COPD smokers who quit smoking following baseline ($n=18$, tan circles). Data represent mean \pm SE. Dashed lines indicate urine cotinine detection level of ≤ 5 ng/mL and urine cotinine level of ≥ 104 ng/mL for active smoking (see online supplementary methods). EMP, endothelial microparticles.

CD42b⁻CD31⁺ EMPs below the lowest level in non-smokers were defined as apoptotic EMPs with 48% of healthy smokers and 45% of COPD smokers having increased levels of apoptotic EMPs ($p>0.7$), that is, even though there are less subjects with total circulating EMPs in COPD smokers compared with healthy smokers (figure 2A), the relative proportion of subjects with apoptotic EMPs was similar (figure 2C), implying that there is active pulmonary capillary apoptosis ongoing in both the healthy smokers and COPD smokers. There was no correlation of CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMP ratio with any lung function or demographic parameter ($r^2=0.09$, all correlations, see online supplementary figure S3). Within the COPD smoker group, there was no correlation of total CD42b⁻CD31⁺ EMP levels or CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMP ratio to the DLCO (% predicted) or % emphysema on high-resolution CT (HRCT) ($r^2=0.04$, all comparisons; not shown), suggesting these parameters are likely measuring different aspects of the destruction process.

Effect of smoking cessation on total EMP levels

The levels of total EMPs were followed for a period of 1 year at baseline, 3, 6 and 12 months in non-smokers, healthy smokers and COPD smokers (figure 3A, B). In non-smokers, total EMP levels were stably low throughout the duration of the study ($p>0.6$, each time point compared with baseline). In healthy smokers who continued smoking, the levels were stably high at each time point ($p>0.1$, each time point compared with baseline; $p<0.05$, each time point compared with the non-smokers at the same time point). In contrast, in the healthy smokers who quit smoking, total EMP levels significantly decreased following smoking cessation to the levels of non-smokers ($p<0.002$, each time point compared with baseline; $p>0.4$, compared with non-smokers at the same time point at 3, 6 and 12 months,

figure 3A). At 12 months, the total EMP levels in healthy smokers who continued smoking were significantly higher compared with healthy smokers who quit ($p<10^{-3}$). Total EMP levels in COPD smokers who continued smoking were stably elevated compared with non-smokers at each time point ($p<0.05$, all comparisons). In contrast to the healthy smokers, in COPD smokers who quit smoking, total EMP levels initially decreased following smoking cessation at month 3, but became elevated again at 6 and 12 months (figure 3B). The levels were not significantly different compared with non-smokers at 3 and 6 months ($p>0.1$, both comparisons), but were significantly elevated at 12 months ($p<0.05$) and similar to those of COPD smokers who continued smoking ($p>0.08$).

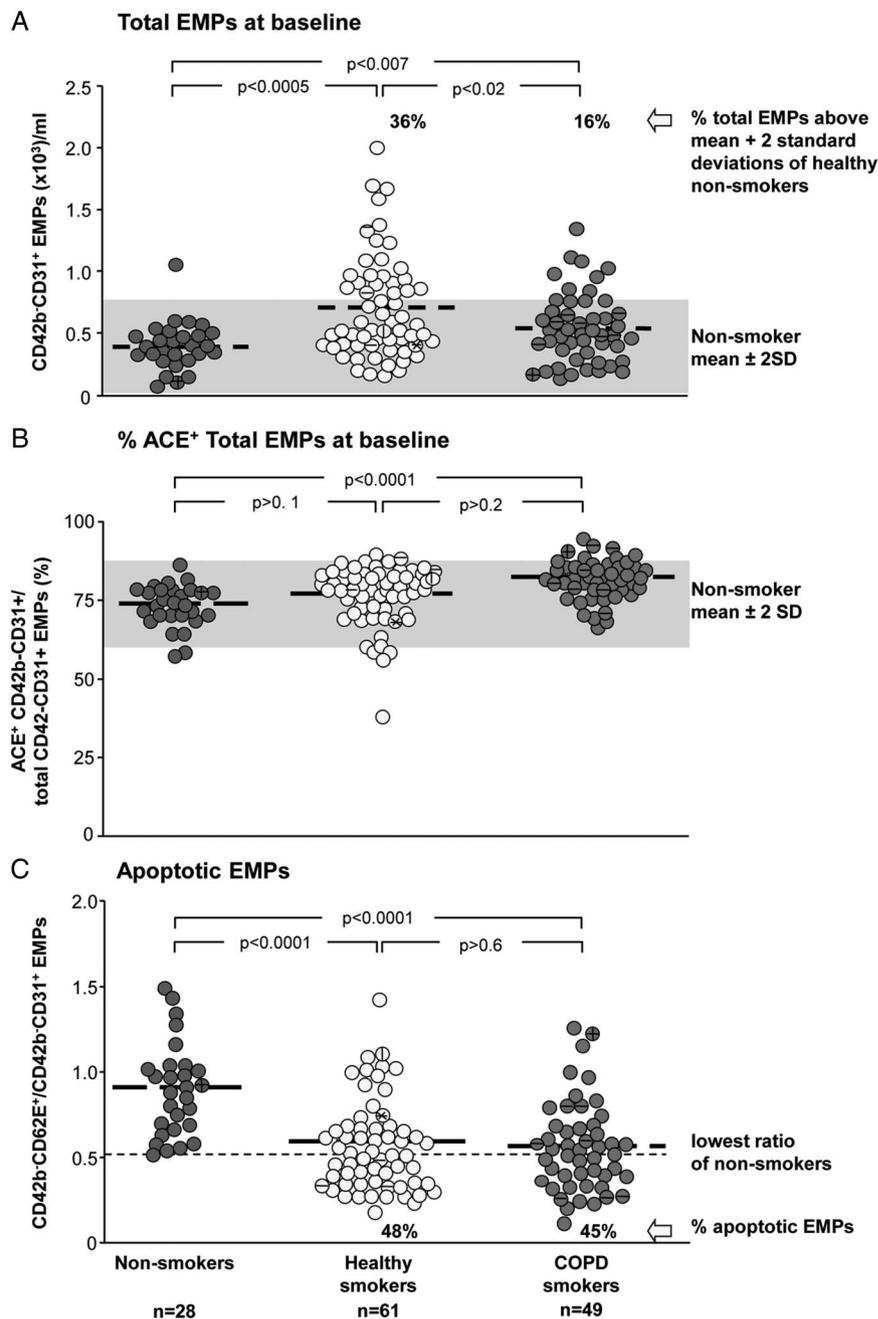
Effect of smoking cessation on apoptotic EMP levels

The ratio of CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMPs was stably high in non-smokers and stably low (ie, EMPs were apoptotic-derived) in healthy smokers who continued smoking ($p>0.1$, each time point compared with baseline within the non-smoker group and within the healthy smoker group; $p<0.01$, healthy smokers who continued smoking compared with non-smokers, at baseline, months 6 and 12; figure 3C). Interestingly, in the healthy smokers who quit smoking group, the ratio increased following smoking cessation ($p<10^{-3}$, within the healthy smokers who quit group, at months 3 and 6 compared with baseline, a p value significant even with correction for multiple tests) to the level of non-smokers at months 3 and 6 ($p>0.1$, both comparisons compared with non-smokers at the same time point) and superseded that of non-smokers at month 12 ($p<0.05$). The ratio was significantly higher (ie, less apoptotic-derived EMPs) in healthy smokers who quit smoking compared with smokers who continued smoking at month 12 ($p<10^{-3}$, a p value significant even with correction for multiple tests). There were no significant changes in the CD42b⁻CD62E⁺/CD42b⁻CD31⁺ ratio in COPD smokers who continued smoking ($p>0.1$, within the COPD smoker group, each time point compared with baseline), and it remained significantly low compared with non-smokers at each time point ($p<0.01$, all comparisons, figure 3D). In contrast to the healthy smoker group, in COPD smokers who quit smoking, there was no change in the ratio ($p>0.1$, within the COPD who quit group, each time point compared with baseline), and the ratio remained significantly low compared with non-smokers at baseline and month 12 ($p<0.05$, both comparisons). The ratio at month 12 was similarly low (ie, more apoptotic-derived EMPs) in COPD smokers who continued smoking and in COPD smokers who quit ($p>0.4$).

DISCUSSION

COPD is a chronic, debilitating disease that is caused primarily by cigarette smoking.¹⁻⁵ Cigarette smoke is very complex, with 10^{14} oxidants and >4000 compounds stressing the lung with each puff.²² The apoptotic loss of pulmonary capillaries in association with smoking is well recognised,⁸⁻¹⁰ although it is not known whether this represents the primary mechanism of lung destruction associated with smoking, a subtype of lung destruction, or is secondary to other mechanisms, such as inflammatory cell-mediated processes.^{5 6 9 10 23} Using circulating, total and apoptotic-endothelial cell microparticles as biomarkers for pulmonary capillary apoptosis, the data in the present study document that pulmonary capillary endothelial apoptosis is a persistent process in smokers with and without COPD. In healthy smokers who quit smoking, the levels of total and apoptotic EMPs return to the levels of non-smokers over time. In contrast, in COPD smokers who quit smoking, the levels of total and

Figure 2 Levels of total circulating endothelium microparticles (EMPs), ACE⁺ EMPs and apoptotic EMPs in plasma at baseline. Shown are data for non-smokers (n=28, green circles), healthy smokers (n=61, yellow circles) and smokers with COPD (n=49, red circles). (A) Total CD42b⁻CD31⁺ EMPs. The grey shaded area indicates the non-smoker mean \pm 2 SDs. The % value above the smoker populations represents the proportion of smokers with EMP levels above that mean. (B) CD42b⁻CD31⁺ACE⁺ EMPs. Proportion of total CD42b⁻CD31⁺ EMPs in plasma that express ACE⁺. The grey shaded area represents the non-smoker mean \pm 2 SDs. (C) Ratio of CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ EMPs. The dashed line indicates the lowest ratio of CD42b⁻CD62E⁺/CD42b⁻CD31⁺ EMPs in non-smokers. The % value below the smoker populations represents the proportion of smokers with a ratio below that level. (A–C) Bold dashed lines represent the mean for each group. Symbols inside the dots: A horizontal line indicates subjects with systemic hypertension; a vertical line indicates subjects with type 2 diabetes mellitus; a star indicates subjects with type 1 diabetes.



apoptotic EMPs remain abnormal over 1 year and were still significantly different compared with non-smoker levels at 12 months. The majority of the COPD subjects assessed for EMPs in this study were GOLD I and GOLD II, providing evidence for ongoing pulmonary endothelial apoptosis even in the earliest stages of COPD. Importantly, the FEV1/FVC ratio of the COPD smokers was 0.63 ± 0.06 , on average, well below the 0.7 ratio threshold definition of COPD GOLD I.

These observations were not altered by the removal of subjects with diseases known to be associated with elevated EMPs, suggesting that smoking has a much stronger effect on EMP levels than hypertension or diabetes.

Endothelial microparticles

Different cell types respond to cell activation, injury and/or apoptosis by shedding submicron membrane vesicles, called microparticles, from their plasma membranes.^{16 24}

Microparticles detected in plasma are of various cellular origins, predominantly derived from platelets, leucocytes and endothelial cells.²⁴ Endothelial microparticles, defined as CD42b⁻CD31⁺ or CD42b⁻CD62E⁺ microparticles, can be generally distinguished from microparticles of other cell types by their size (0.1–1.5 μ m), constitutive expression of the platelet–endothelial cell adhesion marker CD31 (PECAM) and the absence of the platelet-specific glycoprotein Ib marker CD42b.^{24 25} Apoptosis-induced EMPs express CD31, whereas activation-induced EMPs express CD62E. In this regard, a low ratio of CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ EMPs can be used as an index of apoptosis.^{24 25} EMPs variably co-express phosphatidylserine (annexin V).^{25 26}

EMPs can be found in the plasma of healthy subjects;²⁴ however, increased levels are associated with vascular disease and endothelial dysfunction in atherosclerosis and acute coronary syndrome,^{24 27} acute ischaemic stroke,^{24 28} end-stage renal

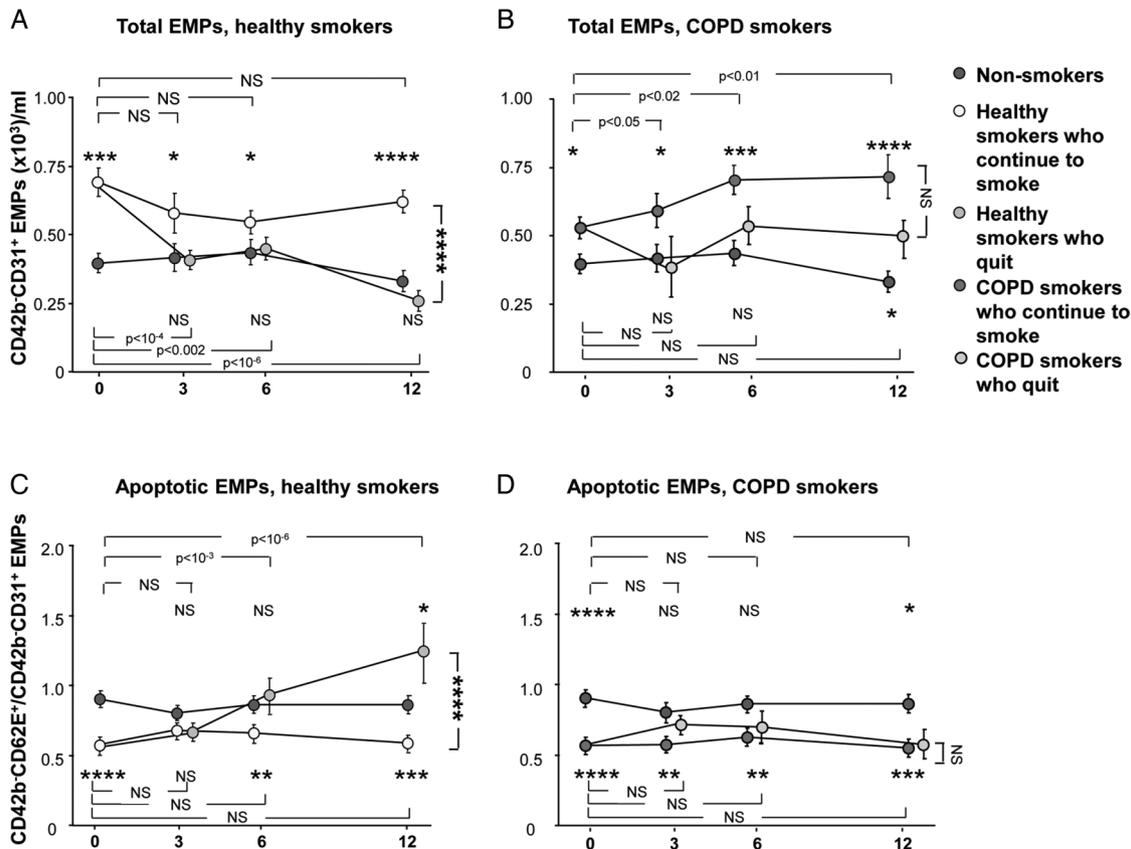


Figure 3 Total circulating CD42b⁻CD31⁺ endothelial microparticles (EMPs) and ratio of CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ EMPs over time in non-smokers (n=28, green circles), healthy smokers who continue to smoke (n=44, yellow circles), healthy smokers who quit smoking following baseline (n=17, light blue circles), smokers with COPD (COPD smokers) who continue smoking (n=31, red circles) and COPD smokers who quit smoking following baseline (n=18, tan circles). (A and B) Total CD42b⁻CD31⁺ EMPs. (C and D) Ratio of CD42b⁻CD62E⁺ to CD42b⁻CD31⁺ EMPs. (A and C) Healthy smokers who continue to smoke and healthy smokers who quit smoking versus non-smokers. (B and D) COPD smokers who continue to smoke and COPD smokers who quit smoking versus non-smokers. (A–D) Data represent mean±SE. p Values comparing each time point to baseline within the same group are shown at the top of the panel (for the group, ie, above the non-smokers at month 12) and at the bottom of the panel (for the group, ie, below the non-smokers at month 12). p Values comparing each time point in a smoker group to the same time point in the non-smoker group are shown above the group, if the group is above the non-smokers at month 12 and below the group if the group is below the non-smokers at month 12. p Values comparing the subjects who continue to smoke with those who quit smoking at month 12 are to the right of the panel. NS, not significant; *, **, ***, **** indicate p<0.05, p<0.01, p<0.001 and p<0.0001, respectively.

failure,²⁹ pre-eclampsia and gestational hypertension,³⁰ hypertension,^{21 24 29} pulmonary hypertension,³¹ metabolic syndrome,³² venous thromboembolism³³ and obstructive sleep apnoea.³⁴ Consistent with our prior study¹² and the data in the present study, Heiss and colleagues³⁵ have shown healthy non-smokers exposed for 30 min to low levels of cigarette smoke had increased circulating EMP levels.

Clinical measures of alveolar capillary destruction

The observation of endothelial apoptosis in the lungs of humans with emphysema is well documented. There is increased DNA fragmentation in the pulmonary capillaries and arteriolar endothelium of subjects with COPD, and increased alveolar endothelial and epithelial cell death in human emphysematous lungs compared with lungs of non-smokers or smokers without emphysema.^{9 10} Lung levels of alveolar epithelial-derived vascular endothelial growth factor are decreased in emphysema, contributing to the complex mechanisms of pulmonary capillary endothelial destruction.¹⁰

The data in the present study add total and apoptotic EMP levels to a growing list of biomarkers that may be useful in assessing active destruction and defining subclinical molecular

phenotypes of lung disease.^{17 36 37} As described in the editorial by Chandra and colleagues,¹⁴ accompanying our study of apoptotic EMPs in healthy smokers and smokers with normal spirometry but low DLCO, the observation of elevated levels of apoptotic EMPs may represent early lung destruction and a subphenotype of subjects with lung destruction. The observation in the present study that, on average, a fraction of smokers with COPD also have elevated levels of total and apoptotic EMPs is consistent with the discussion of the concept of vascular subphenotypes of COPD by Chandra *et al.*¹⁴ In this context, the data on circulating and apoptotic EMPs support the idea that, while the global concept of COPD as an FEV1-defined disorder is useful for epidemiologic studies and as a paradigm for routine clinical care, it is likely masking the concept that there are several subphenotypes of COPD.^{38–42} Consistent with this concept, correlation of the total EMP levels and apoptotic EMP levels with the conventional measures of lung destruction (DLCO and HRCT) was, at best, very weak. This may imply that, while overlapping, each parameter is measuring a somewhat different aspect of the same process and/or that each parameter is assessing a different subpopulation of what is globally referred to as ‘lung destruction’.

Our longitudinal study demonstrates that total and apoptotic EMP levels remain stable over a period of 1 year in subjects with no change to their smoking habits. Intervention with smoking cessation can normalise the levels of total and apoptotic EMPs in healthy smokers, but in contrast, cessation did not lead to significant changes in total and apoptotic EMP levels in COPD smokers in our study. This observation provides a biological correlate for epidemiological data showing that smoking cessation only has a moderate effect to slow the decline of lung function in COPD smokers, that is, we believe that these data show that EMP levels might serve as a useful biomarker to follow smoking-associated endothelial apoptosis. The longitudinal aspect of this study demonstrates persistent endothelial stress in subjects with COPD, despite smoking cessation, and may help to explain the irreversible lung destruction associated with most cases of COPD, as evidenced by lung function of COPD smokers following smoking cessation that does not return to normal,¹⁵ and may serve as a basis for additional studies of the mechanisms of the continuous pulmonary damage leading to this persistent EMP release.

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⁶Eli Lilly and Company

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4. Discussion

Waterpipe is currently the second most popular tobacco product after cigarettes worldwide^{3,5,9,25}. Only limited data is available on the health effects of waterpipe smoking, particularly in light-use, young waterpipe smokers^{3-5,9}. Many users believe it is safer and less addictive than cigarette smoking¹³⁴⁻¹³⁵. Our observation that even young (25±4 years old), light-use (<4 sessions/week for <5 years) waterpipe-only smokers have abnormalities in several clinical and biologic lung-related parameters demonstrates that even light-use waterpipe smoking likely has significant effects on lung health. In comparison to gender and ethnicity matched nonsmokers, the light-use waterpipe smokers had (1) increased cough and sputum; (2) increased carboxyhemoglobin; (3) lower diffusing capacity; (4) an abnormal epithelial lining fluid metabolite profile; (6) an abnormal cell composition and transcriptome of the small airway epithelium, the cell population where COPD and most lung cancers are initiated^{60,136-138}; (7) an abnormal transcriptome of the alveolar macrophage, the pulmonary representative of the mononuclear phagocyte system, functioning as the scavenger cell in the lower respiratory tract¹³⁹⁻¹⁴⁰; and (8) increased plasma levels of total and apoptotic capillary endothelial microparticles, indicative of pulmonary capillary endothelial apoptosis^{117,121}.

4. 1. Lung Function

Millions of people worldwide use waterpipe to smoke tobacco on a daily basis. Most studies assessing the health effects of waterpipe smoking have studied older, heavy-use waterpipe smokers, usually in waterpipe smokers who already have manifested lung disease^{2-3,8,23-24,26}. Several studies assessing the effect of heavy-use waterpipe smoking on the lungs found reduced lung function parameters, including FVC, FEV₁, maximum mid expiratory flow, peak expiratory flow, FEF and FEV₁/FVC and increased cough and sputum symptoms in

waterpipe smokers compared to nonsmokers^{1,8,23-24,26}. In addition, the manifestation of the cough and sputum symptoms was at an earlier age in waterpipe smokers than typically observed in cigarette smokers^{25-26,141}. Our study (Article 1) assessed the effect of waterpipe smoking on lung health in young, light-use waterpipe-only smokers compared to nonsmokers. All individuals had normal spirometry and no emphysema observed on HRCT. Other than waterpipe smoking, the groups did not differ in industrial exposure or second-hand exposure to cigarette smoke.

Compared to the nonsmokers, the light-use waterpipe smokers had increased levels of carbon monoxide and an increase in cough frequency and sputum production. Despite having normal spirometry, the DLCO level was reduced in the light-use waterpipe smokers in comparison to the nonsmokers and 38% of the waterpipe smokers had an isolated reduction of DLCO. This observation of a reduction in DLCO level in, otherwise healthy, young, light-use waterpipe smokers is of interest as in a separate study (Article 2) assessing the risk for developing COPD in cigarette smokers, we observed that cigarette smokers with normal spirometry, but low DLCO were at 7-fold greater risk for developing COPD within <4 years than cigarette smokers with normal spirometry and normal DLCO.

4.2. Reduction in DLCO level

COPD is a global health issue and a leading cause of death worldwide⁶³. A major risk factor for developing COPD is cigarette smoking, but only a subset of cigarette smokers will develop COPD, a tendency likely associated with genetics^{62,66,70-72}. The prevalence of COPD among cigarette smokers is estimated to be at least 20%, but is projected to increase as the population ages^{63,66}. The development of COPD usually takes decades as the impairment caused by the chronic exposure to toxins, such as those existing in cigarette smoke, accumulate for many years with very little effect on lung function^{60,69,71}. However, once COPD manifests, there

is no therapy for the long-term decline in lung function and increased mortality associated with COPD^{61,63}. Smoking cessation taking place at an early age slows down the decline in FEV₁, but has little or no impact on the long term progression of COPD^{60,65,71}. Since even mild COPD is associated with increased mortality, detection of cigarette smokers at risk for developing COPD, can help with prevention by modification of risk factors and early therapeutic intervention^{62,66,81,142}.

The DLCO assesses the potential of the lung for gas exchange⁶⁰ and a reduction in DLCO suggests alveolar destruction, i.e., emphysema^{60,78,80}. Decreased DLCO has also been correlated with small airway disease and high levels of circulating EMPs derived from apoptotic pulmonary capillary endothelium^{79,82}. However, the gold standard to screen cigarette smokers for COPD is using only spirometry levels with bronchodilators, defining smokers with FEV₁/FVC ratio ≥ 0.7 as healthy⁶³. Even though the phenotype of smokers with normal spirometry but low DLCO is recognized, the DLCO parameter is not routinely measured for technical and financial reasons and there are no data regarding what happens to lung function over time in these individuals^{63,66}. In our study (Article 2) we focused on evaluating the addition of the DLCO parameter to the growing list of biomarkers used to identify smokers at risk for the development of COPD. Our observation that cigarette smokers with normal spirometry and no emphysema on HRCT, but with low DLCO are at significantly higher risk for developing COPD than a comparable group of cigarette smokers with normal DLCO suggests that a normal spirometry post-bronchodilators may give a false sense of "normal".

Different risk factors, including advanced age, gender and cough and sputum, have been related to the development of COPD^{70-72,125,142-146}. However, in our study there were no differences in age, gender or cough and sputum scores between the cigarette smokers with

normal spirometry and normal DLCO and those with normal spirometry, but low DLCO. Further, the individuals followed over time with normal spirometry and low DLCO who developed COPD did not differ in these parameters from those with low DLCO who did not develop COPD.

Several studies have raised concerns about the use of a set cutoff for the definition of COPD ($FEV_1/FVC < 0.7$), and for the definition of low DLCO ($< 80\%$ predicted) rather than using cutoff values based on a lower limit of normal calculated for each individual based on their age, gender and height¹⁴⁷⁻¹⁵⁰. In addition to using set values for the definitions of COPD and low DLCO we used several definitions of normal DLCO and FEV_1/FVC to evaluate the risk for developing COPD. These included: (1) a gender and ethnicity-based lower limit of normal for DLCO % predicted and FEV_1/FVC ratio calculated using an internal database of 405 healthy nonsmokers recruited from the general population, comprised of similar gender and ethnicity composition as in our study groups (Article 2); and (2) a lower limit of normal for the FEV_1/FVC ratio based on gender, ethnicity, height and age calculated based on 74187 individuals¹⁴⁷. The results of all analyses, using either cutoff of the FEV_1/FVC ratio to define COPD and/or either cutoff of DLCO % predicted to define normal/low DLCO, were similar. Independent of the method used to determine the normal levels of DLCO, the data demonstrated that cigarette smokers with normal spirometry but low DLCO are at significantly higher risk for developing COPD than a comparable group of cigarette smokers with normal spirometry and normal DLCO (Table X, *appendix V*). These results advocate the need to assess DLCO levels in cigarette smokers and waterpipe smokers with normal spirometry who are falsely presumed to be normal.

4.3. Metabolite Profile

A number of studies have analyzed compounds in waterpipe smoke, finding high levels of nicotine, carbon monoxide, tar, polycyclic aromatic hydrocarbons, heavy metals and other toxins originating from the tobacco, the flavoring and the charcoal^{1,12-14}. Consistent with the concept that at least some components of waterpipe smoke reach the lower respiratory tract, the light-use waterpipe smokers demonstrated an abnormal lower respiratory tract epithelial lining fluid metabolite profile, with a variety of metabolites in the waterpipe smokers with a differential abundance compared to nonsmokers.

4.4. Small Airway Epithelium and Alveolar Macrophage Transcriptome and Cellular Composition

Compared to nonsmokers, the light-use waterpipe smokers displayed an altered small airway epithelial cellular composition. These individuals had less ciliated cells, the mediator of mucociliary clearance¹⁵¹, and basal cells, the stem/progenitor cell population of the airway epithelium¹⁵². In addition, they had more secretory cells¹⁵³ and intermediate, undifferentiated cells, the basal cell-derived precursors of the differentiated cell populations¹⁵². The light-use waterpipe smokers also demonstrated an abnormal small airway epithelium transcriptome compared to the nonsmokers, with hundreds of genes up- and down-regulated. Among the up-regulated small airway epithelium genes in the light-use waterpipe smokers compared to nonsmokers were genes previously associated with different types of cancer, including: CGG triplet repeat binding protein 1 (CGGBP1), a cell cycle regulatory protein associated with the growth of lung and cervical cancer¹⁵⁴⁻¹⁵⁵; pre-mRNA processing factor 4B (PRPF4B), a CDK-like kinase, with homology to mitogen-activated protein kinases, involved in pre-mRNA splicing, signal transduction, cell cycle progression and hepatocarcinogenesis¹⁵⁶⁻¹⁵⁷; and integrin,

beta 1 (ITGB1), a membrane receptor involved in cancer progression that mediates interactions of cells with extracellular matrix¹⁵⁸⁻¹⁵⁹. Among the down-regulated genes in the small airway epithelium of the waterpipe smokers compared to nonsmokers was ankyrin repeat domain 12 (ANKRD12), a gene encoding a member of the ankyrin repeats-containing cofactor family, with low expression linked to poor survival of colorectal cancer¹⁶⁰. Together, these abnormalities suggest that waterpipe smoking-induced changes in the small airway epithelium cell composition and transcriptome may have important consequences with regard to the health of this anatomic compartment.

In contrast to cigarette smokers, where there is a higher % of macrophages recovered compared to nonsmokers¹⁶¹, there was no significant difference in the proportions of macrophage or other cell types recovered from the epithelial lining fluid of waterpipe smokers compared to nonsmokers. The waterpipe smokers also displayed a dysregulated transcriptome of the alveolar macrophages, with hundreds of genes up- and down-regulated in comparison to the nonsmokers. Among the dysregulated genes were genes previously associated with pathogenesis of COPD and/or cancer, including: echinoderm microtubule associated protein like 4 (EML4), a protein found to be involved in lung adenocarcinoma, sarcomas, non-small cell lung cancer and congenital pulmonary airway malformation¹⁶²⁻¹⁶³; ubiquitin protein ligase E3B (UBE3B), with overexpression shown to effect skin carcinogenesis, lung adenocarcinoma, neuroblastoma and risk for coronary artery disease¹⁶⁴; epoxide hydrolase 1, microsomal (EPHX1), a gene encoding a protein that activates and detoxifies toxins in response to environmental carcinogens and is associated with risk for hepatocellular carcinoma and esophageal cancer¹⁶⁵⁻¹⁶⁶; and cell cycle progression 1 (CCPG1), that regulates Rho-mediated signaling events involved in lung cancer¹⁶⁷⁻¹⁶⁸. These gene expression patterns and cell compositions are unique, not previously

reported to be evoked by cigarette smoking^{161,169}.

4.5. Endothelial Microparticles

Increased levels of endothelial microparticles have been associated with vascular disease and endothelial dysfunction in various diseases^{111,113}. Endothelial microparticles are present in the low levels in plasma of healthy individuals⁹¹. In response to cell activation, injury and/or apoptosis the endothelial cells shed EMPs, submicron membrane vesicles from their plasma membranes^{89,117}, therefore high levels of total and apoptotic EMPs in plasma represent early lung destruction^{89,91,103,111,113,130-131,170-171}. The observation that the total level of circulating EMPs and the proportion of apoptotic EMPs are significantly higher in waterpipe smokers compared to nonsmokers suggests an ongoing lung capillary endothelial apoptosis associated with light-use waterpipe smoking.

We have previously shown that cigarette smokers are undergoing pulmonary endothelial apoptosis as measured by high plasma levels of total EMPs and an increased proportion of apoptotic EMPs¹²¹. Cigarette smokers with normal spirometry and low DLCO demonstrated significantly higher levels of total and apoptotic EMPs compared to nonsmokers and cigarette smokers with normal spirometry and DLCO. Using circulating, total and apoptotic endothelial cell microparticles as biomarkers for pulmonary capillary apoptosis, the data in the present study (Article 3) demonstrated that pulmonary capillary endothelial apoptosis is a persistent process in cigarette smokers with and without COPD. Our longitudinal study demonstrates that total and apoptotic EMP levels remain stable over a period of 1 year in individuals with no change to their smoking habits. Intervention with smoking cessation can normalize the levels of total and apoptotic EMPs in healthy cigarette smokers, but in contrast, cessation did not lead to significant changes in total and apoptotic EMP levels in COPD cigarette smokers. The majority of the

COPD individuals assessed for EMP levels in this study were GOLD I and GOLD II, providing evidence for ongoing pulmonary endothelial apoptosis even in the earliest stages of COPD (Article 3).

This observation provides a biologic correlate for epidemiologic data showing that smoking cessation only has a moderate effect on the rate of decline of lung function in COPD smokers, but also adds total and apoptotic EMP levels to a growing list of biomarkers that may be useful in helping to assess active destruction in light-use waterpipe smokers.

4. 6. Implications

Light-use waterpipe smokers demonstrate abnormalities in various clinical- and biologic-lung related parameters. Together, the data suggest that even light-use waterpipe smoking in young individuals significantly affects lung biology and health even before any clinical abnormalities are detected. It is likely that these changes are the earliest biologic correlates of epidemiologic studies linking waterpipe smoking to lung health risk. Some of these abnormalities are similar to those seen in cigarette smokers, but most are unique to waterpipe smokers, suggesting that waterpipe smoking is associated with lung pathology and that it may be different from that associated with cigarette smoking. Based on these evidence, in the context of the increasing use of waterpipe smoking, our findings support the efforts to regulate and reduce waterpipe smoking, especially among the young population, and to use DLCO level and total and apoptotic EMP levels as biomarkers for early detection of disease in waterpipe smokers.

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Appendices

Appendix I – Inclusion and Exclusion Criteria

Appendix II – Article 1 Supplemental Methods and Supplemental References

Appendix III – Article 2 Supplemental Methods and Supplemental References

Appendix IV – Article 3 Supplemental Methods and Supplemental References

Appendix V – Table X

Appendix I - Inclusion and Exclusion Criteria

Nonsmokers

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages
- Willingness to participate in the study
- Self-reported never-smokers, with smoking status validated by the absence of nicotine and cotinine in urine (nicotine <2 ng/ml and cotinine < 5ng/ml)

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

Waterpipe smokers

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)

- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages
- Willingness to participate in the study
- Self-reported waterpipe-only smokers

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

Healthy cigarette smokers with normal spirometry and normal diffusion capacity (DLCO)

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages
- Willingness to participate in the study
- Normal serum α 1-antitrypsin level
- Self-reported current daily smokers with >5 pack-yr, validated by urine nicotine >30 ng/ml and/or cotinine >50 ng/ml
- Normal FEV₁ (\geq 80% predicted), FVC (\geq 80% predicted), FEV₁/FVC (\geq 0.7) based on post-bronchodilator spirometry, TLC (\geq 80% predicted)
- DLCO \geq 80% predicted

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

Healthy cigarette smokers with normal spirometry, but low diffusion capacity (DLCO)

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages
- Willingness to participate in the study
- Normal serum α 1-antitrypsin level
- Self-reported current daily smokers with >5 pack-yr, validated by urine nicotine >30 ng/ml and/or cotinine >50 ng/ml
- Normal FEV₁ (\geq 80% predicted), FVC (\geq 80% predicted), FEV₁/FVC (\geq 0.7) based on post-bronchodilator spirometry, TLC (\geq 80% predicted)
- DLCO <80% predicted and below the 95% range of normal DLCO calculated separately for each individual based on gender and ethnicity

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

COPD cigarette smokers

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Presence of COPD as defined by the GOLD criteria based on post-bronchodilator FEV1/FVC <0.7 (observed); stage I-IV but without evidence of respiratory failure
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery ≤ 30 mm in chest CT scans
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Taking any or no pulmonary-related medication, including beta-agonists, anticholinergics, or inhaled corticosteroids
- Willingness to participate in the study
- Normal serum $\alpha 1$ -antitrypsin level
- Current daily smokers with pack-year ≥ 5 , validated by urine cotinine ≥ 104 ng/ml

Exclusion criteria

- Unable to meet the inclusion criteria
- Individuals in whom participation in the study would compromise the normal care and expected progression of their disease
- Current active infection or acute illness of any kind
- Current alcohol or drug abuse
- Evidence of malignancy within the past 5 years
- Any evidence of interstitial lung disease, pulmonary hypertension, diastolic dysfunction or other disorders associated with a low DLCO
- Individuals with asthma and with recurrent or recent (within three months) acute pulmonary infection
- Individuals with allergies to lidocaine

Appendix II – Article 1 Supplemental Methods and Supplemental References

Supplemental Methods

Inclusion and Exclusion Criteria

Nonsmokers

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages
- Willingness to participate in the study
- Self-reported never smokers, with smoking status validated by the absence of nicotine and cotinine in urine (nicotine <2 ng/ml and cotinine <5 ng/ml)

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

Waterpipe smokers

Inclusion criteria

- Males and females, at least 18 years old
- Provide informed consent
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- HIV negative
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not pregnant (females)
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium or alveolar macrophages

- Willingness to participate in the study
- Self-reported waterpipe-only smokers

Exclusion criteria

- Unable to meet the inclusion criteria
- Current active infection or acute illness of any kind
- Evidence of malignancy within the past 5 years
- Alcohol or drug abuse within the past 6 months

Pulmonary Function, Cough and Sputum Scores and Chest HRCT

All subjects had pulmonary function tests (PFTs), including forced vital capacity (FVC), and forced expiratory volume in 1 sec (FEV1), FEV1/FVC before and after administering salbutamol (100 µg, 4 doses) (1), lung volumes and diffusion capacity of the lung for carbon monoxide (DLCO, Viasys Helathcare, Yorba Linda, CA), all performed according to ATS/ERS guidelines (1, 2). Waterpipe smokers were asked to refrain from smoking as of the night before the testing.

DLCO was performed with the subject in a sitting position. After tidal breathing, a non-forced expiratory maneuver to residual volume was performed, followed by rapid inhalation to TLC. After breath holding for 10 sec, the subjects were asked to exhale (not-forced), not exceeding 4 seconds. For quality control, the spirometry and DLCO curves of all PFTs were evaluated and were similar to those reported by (2, 3): (1) breath hold was for 10 ± 2 ; (2) no evidence of leaks or Valsalva or Muller maneuvers in the curves; (3) inspiration and expiration completed in < 4 sec and sample collection time < 3 sec, with appropriate clearance of dead space volume and proper sampling/analysis of alveolar gas as assessed graphically; and (4) inspiratory vital capacity $> 85\%$ of the largest expiratory forced vital capacity (for spirometry) in 73% of the subjects and $> 80\%$ of the largest expiratory forced vital capacity in 90% of the subjects. Measurement of the DLCO, was carried out 2 to 4 times in all subjects and the average of the best 2 trials was used. The DLCO % predicted value was calculated using Gaensler et al equation (4), and corrected for hemoglobin and carboxyhemoglobin levels using ATS/ERS guidelines (2).

Cough and sputum scores were evaluated based on self-reported history using the St. George's Respiratory Questionnaire as follows: score of "0" = no cough or no sputum; "1" = cough or sputum once a month; "2" = cough or sputum once a week; "3" = cough or sputum 2-3 times a week; "4" = cough or sputum every day of the week (5). Cough or sputum score of 0 or 1 were considered normal, cough or sputum score ≥ 2 were considered abnormal.

All subjects were evaluated for the percentage of lung affected by emphysema using chest high resolution computed tomography (HRCT) with the EmphylxJ software application (EmphylxJ, Vancouver, BC, Canada) allowing automated quantitative analysis of transverse chest CT scans (www.icapture.ubc.ca). HRCT were compared for % emphysema at attenuation - 950 Hounsfield Units (HU). Emphysema was defined as >5% lung volume, value derived from analyses of HRCT in normal nonsmoking individuals with normal lung function.

Sampling of Small Airway Epithelium, Epithelial Lining Fluid and Alveolar Macrophages

Small airway epithelium (SAE) was collected by brushing 10th to 12th order bronchi (6). The cells were removed from the brush by flicking it into 5 ml of ice-cold LHC8 medium (GIBCO, Grand Island, NY). A 0.5 ml aliquot was used to determine the number and types of cells recovered and 4.5 ml immediately processed for RNA extraction. The origin of the recovered cells was confirmed as the SAE based on expression of genes encoding surfactant and Clara cell secretory proteins (6). Alveolar macrophages (AM) and epithelial lining fluid (ELF) were collected by bronchoalveolar lavage (7).

Lung Epithelial Fluid Processing

ELF was filtered through 2 layers of gauze and centrifuged at 1250 rpm for 5 min, 4°C. The supernatant was removed for metabolic analysis of lower respiratory tract ELF (7). Cells were suspended in 5 ml Ack Lysing Buffer (Invitrogen, Grand Island, NY), and then washed twice in RPMI 1640 containing 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml strepto-

mycin and 2 mM glutamine (Invitrogen, Grand Island, NY). Cells were suspended in media (10^6 cells/ml) and an aliquot of 250 μ l fetal bovine serum (InvitrogenTM) was used for total cell count, cell viability assessed by trypan blue exclusion and differential cell count by cytocentrifugation (Cytospin 11; Shandon Instruments, Pittsburgh, PA), stained with DiffQuik (Baxter Healthcare, Miami, FL). The remainder of the cells were seeded in 12-well plastic culture dishes (10^6 per 1 ml/well) and alveolar macrophages were purified by adherence for 12 hr at 37°C in a 5% CO₂ humidified incubator in RPMI 1640 medium supplemented with 10% fetal bovine serum 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mM glutamine (Invitrogen). Non-adherent cells were removed by thorough washing with RPMI 1640 before RNA extraction.

Metabolite profiling

Bronchoalveolar lavage fluid (BAL) collected from a random subset of the nonsmokers (n=5) and waterpipe smokers (n=8) was processed using -70°C 80:20 methanol: deionized water (MeOH, LC-MS grade). The BAL-MeOH mixture was incubated on ice for 10 min, then centrifuged at 5°C and 7000 rpm for 5 min. Supernatants were pooled, vacuum centrifuged until dry (Savant SPD121P, Thermo Scientific, Asheville, NC) and stored at -80°C for analysis. For normalization, post-extraction pellets were solubilized in a minimum volume of 0.2 M NaOH at 95°C for 20 min and protein was quantified (Bio-Rad DC assay, Hercules, CA). At time of analysis, dried lavage extracts were reconstituted in 70% acetonitrile with 0.2% ammonium hydroxide to give an equivalent protein concentration of 8 μ g/ μ L. For LC-MS analysis, 3 μ L of reconstituted extract was assessed.

Suspended metabolite extracts from lung ELF were analyzed by LC-MS using an Agilent Model 1200 liquid chromatography system coupled to an Agilent Model 6230 accurate mass time-of-flight mass spectrometer equipped with dual spray electrospray ionization source analyzer, see Chen et al (8). Metabolite separation was achieved using aqueous neutral phase chroma-

tography on silica hybrid resin-silica (Microsolv Technology, Eatontown, NJ) and analyzed in two different detection modes (positive and negative ion-monitoring). Raw data files were processed using Agilent MassHunter Qualitative Analysis software (Qual, version B06), Mass Profiler Professional software (MPP, version B12.01) and Agilent MassHunter Profinder software (version B06.00). MPP analysis was used to generate molecular features extraction based on elution profiles of identical mass and retention times within a defined mass accuracy (5 ppm). The molecular formula generator (MFG) algorithm in MPP was used to generate and score molecular formulae for accuracy. Of 1675 features in the lower respiratory tract ELF, 537 were present in $\geq 60\%$ samples in any of the groups and searched against the METLIN database (Scripps Research Institute, La Jolla, CA) for structural identification. Of those, 31 features had significantly altered abundance in waterpipe smokers *vs* nonsmokers (present in $\geq 60\%$ samples in any of the group, Benjamini-Hochberg (9) corrected $p < 0.05$) and were structurally identified based on their accurate masses and retention time matched to a database of 610 metabolite reference standards (Supplemental Table I; see Supplemental Figure 2A-F for examples).

Small Airway Epithelium and Alveolar Macrophage RNA processing and Quality Control

An aliquot of the total RNA extracted from the SAE and AM was used to determine RNA integrity (Agilent Bioanalyzer, Agilent Technologies, Palo Alto, CA) and concentration (NanoDrop ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE). For processing on HG-U133 Plus 2.0 microarrays (Affymetrix), total RNA (1 to 2 μg) was used to synthesize double stranded cDNA, and Affymetrix kits (Santa Clara, CA) were used to quantify the biotin-labeled cDNA yield (10, 11). RNA was hybridized on the arrays with probes for $>54,000$ genome-wide transcripts, using Affymetrix protocols, hardware and software (10). Microarray quality was verified by signal intensity ratio of GAPDH 3' to 5' probe sets ≤ 3.0 and multi-chip normalization scaling factor ≤ 10.0 (11).

Small Airway Epithelium and Alveolar Macrophage Gene Expression Analysis

For the microarray data, the MAS5 algorithm (GeneSpring version 7.3, Affymetrix Microarray Suite Version 5) was used to normalize the data per array to the median expression value of each sample. Genome-wide analysis was used to compare the expression in waterpipe smokers to nonsmokers and define SAE and AM waterpipe-responsive genes lists using the criteria: (1) P call “Present” in $\geq 20\%$ of the samples in any of the groups (12); (2) ≥ 1.5 fold-change in average expression for waterpipe smokers vs nonsmokers (6); and (3) $p < 0.05$ calculated using a Student’s t-test, with Benjamini-Hochberg correction (9). Principal component analysis of all probe sets present in at least 20% of the samples was captured using Partek software (Partek Genomics Suite 6.6, St. Louis, MO). The influence of SAE and AM gene expression variation contributed by age differences between waterpipe smokers and nonsmokers was calculated using a signal to noise ratio (F test) across all probes sets. The average F ratio was the same as the error term (1.0) in the SAE and slightly higher for AM (1.4), consistent with there being no age-related differences as a function of age (see also Supplemental Figure 1). The probe sets found to be differentially expressed between the groups were converted into unique and annotated genes using the Affymetrix site (www.affymetrix.com) and GeneCards (www.genecards.org) and functionally annotated using Gene Ontology (GO) and the Human Protein Reference Data Base (www.hprd.org). For each subject, the number of waterpipe-responsive genes expressed outside the normal range (defined as the nonsmoker mean expression level ± 2 standard deviations divided by the total number of waterpipe-responsive genes) was calculated separately for the SAE and AM and presented as % (“SAE and AM waterpipe-transcriptome response score”, respectively), as previously described (13). The raw data is publically available at the Gene Expression Omnibus (GEO) site (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE67143.

Endothelial Microparticles Processing

Endothelial microparticles (EMPs) were processed from blood drawn from all 19 non-smokers and 20 of the 21 waterpipe smokers (a sample was not available for one waterpipe smoker) using a 21-gauge needle collected in 5 ml sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) within 1-hr from collection, as previously described (14). Platelet-rich plasma was separated by a 10 min, 160 x g, 23°C centrifugation and within 5 min, the supernatant was further centrifuged for 8 min, at 1000 x g, 23°C to obtain platelet-poor plasma. Within 5 min, 50 µl aliquots of platelet-poor plasma were incubated for 45 min in 4°C with fluorescent-labeled antibodies: (1) to identify EMPs derived from endothelium, using fluorescein-conjugated anti-human PECAM (4 µl, CD31-FITC, clone WM59, BD PharMingen, San Diego, CA); (2) to identify EMPs derived from apoptotic endothelium using phycoerythrin-conjugated anti-human E-selectin (5 µl, CD62E-PE, clone 68-5H11, BD PharMingen); (3) to exclude platelets conjugated anti-human platelet-specific glycoprotein Ib (4 µl, CD42b -APC, clone HIP1); (4) to exclude leukocytes MP contamination CD45 conjugated anti-human PECy5 (5 µl, CD45, clone HI30, BD PharMingen) and (5) to identify pulmonary capillary endothelium using conjugated anti-human angiotensin converting enzyme (ACE, CD143, clone 171417, R&D, Minneapolis, MN) (15). EMP phenotype analysis was carried out within 15 min based on size and fluorescence.

Events <1.5 µm were identified in forward (size) and side (density) light scatter plots using polystyrene size calibration microspheres (0.2 to 10 µm, Molecular Probes, Invitrogen, Eugene, OR).

Total EMPs were defined as CD42b⁻CD31⁺. Angiotensin converting enzyme, an enzyme abundantly expressed in the pulmonary capillary endothelium, was used to assess the contribution of pulmonary capillary endothelial cells, with pulmonary-originated EMPs defined as CD42b⁻CD31⁺ACE⁺ (14). EMPs with a high ratio of CD42b⁻CD62⁺ to CD42b⁻CD31⁺ were defined as “activated EMPs” and those with a ratio less than the nonsmoker mean -2 SD as “apop-

otic EMPs". The data were analyzed using FlowJo software (Tree Star, OR) and measurements were performed twice in each sample to ensure reproducibility.

Global Index Calculations

A global index score summarizing the abnormalities observed in waterpipe smokers compared to nonsmokers a global index score was calculated for each subject as follows: each subject was scored "1" for each parameter in an abnormal level and "0" for each parameter in a normal level. Abnormal levels were defined as follows: (1) sum of cough and sputum scores >2 ; (2) DLCO $<80\%$ predicted; (3) sum of SAE principal components (PCs) outside the range of nonsmoker mean PC sum ± 2 standard deviations (SD); (4) SAE transcriptome-response score outside the range of nonsmoker mean score ± 2 SD; (5) Sum of AM PCs outside the range of nonsmoker mean PC sum ± 2 SD; (6) AM transcriptome-response score outside the range of nonsmoker mean score ± 2 SD; (7) plasma apoptotic EMP level $<$ nonsmoker mean -2 SD; and (8) for SAE cell types (ciliated, secretory, basal and intermediate) a subject was scored "0.25" if the % cell type was outside the normal range defined as nonsmoker % mean ± 2 SD for each ciliated, secretory and intermediate cell types, or below the lowest % level in a nonsmoker (basal cells), for a maximum abnormal score of "1" (SAE cell types considered as one parameter). The global index score was calculated for each subject as the number of abnormal parameters $\times 100$, divided by the total number of parameters available for that subject, presented as % compared in waterpipe smokers vs nonsmokers.

Supplemental Results

Global assessment comparing abnormalities in cough and sputum scores, DLCO % predicted level, SAE and AM transcriptomes, on a genome-wide basis (converted to PCs) and on waterpipe-responsive gene basis (waterpipe-transcriptome response score), apoptotic EMP level and SAE cell differentials showed a distinct separation of waterpipe smokers and nonsmokers

(Supplemental Figure 4). A global index score (summarizing the number of abnormal parameters) was higher for all waterpipe smokers (i.e., more abnormal parameters) than for the non-smokers on the individual basis (waterpipe smoker index score range 25-81%, nonsmoker index score range 0-13%) and on the average, as a group (waterpipe smoker index score mean $55\pm 16\%$, nonsmoker index score mean $3\pm 6\%$, $p < 10^{-15}$). Within the waterpipe smoker group, the SAE waterpipe-transcriptome response score was significantly higher in waterpipe smokers with low DLCO compared to those with normal DLCO ($p < 0.007$, Supplemental Table IV). The AM waterpipe-transcriptome response score was also significantly higher in waterpipe smokers with low DLCO than in waterpipe smokers with normal DLCO ($p < 0.04$, Supplemental Table IV).

Supplemental Discussion

Small Airway Epithelium

The set of genes significantly up-regulated in the SAE of waterpipe smokers included numerous regulatory factors, including: transcription factors [DEAD box polypeptide 3, X-linked (DDX3X), prospero homeobox 1 (PROX1), and twisted gastrulation homolog 1 (TWSG1)]; transcriptional repressors [Kruppel-like factor 12 (KLF12) and GDNF-inducible zinc finger protein 1 (GZF1)]; and epigenetic regulatory factors [enhancer of polycomb homolog 1 (EPC1), nuclear factor I/B (NFIB), metastasis-associated lung adenocarcinoma transcript (MALAT1), and argonaute RISC catalytic component 1 (AGO1)]. Although specific roles of these genes in airway biology are unknown, these factors have been implicated in regulation of development and postnatal differentiation of various organs and tissues, cell proliferation and carcinogenesis (16-18). Among the genes up-regulated in the SAE by waterpipe smoking was also integrin beta 1 (ITGB1), a receptor for type IV collagen, the major component of the basement membrane, to which basal cells, the stem/progenitor cells of the airway epithelium, attach in order to maintain normal epithelial architecture (19). Interestingly, except for MALAT1, which has been found to

be up-regulated also in the SAE of cigarette smokers, genes up-regulated in the SAE of waterpipe smokers were either down-regulated or unchanged in the SAE of cigarette smokers (13). Further, apart from the cytochrome CYP1B1, a xenobiotic metabolism-related gene induced by cigarette smoking and implicated in smoking-associated lung carcinogenesis (20), no other classic components of smoking-associated oxidative stress genes were up-regulated in the SAE of waterpipe smokers, suggesting that the passage through water may remove many of the oxidants in waterpipe smoke. Together, these data suggest that, although the number of genes up-regulated in the human SAE of waterpipe smokers is relatively small compared to those induced by cigarette smoking, waterpipe smoking generates a unique SAE transcriptome pattern, distinct from that activated by cigarette smoking, and, thus, may result in the SAE pathologic phenotypes different from those that characterize classic smoking-induced diseases.

By contrast, the set of genes down-regulated in the SAE of waterpipe smokers included a number of genes known to be involved in regulation of the airway epithelial biology, which, based on their function, could be divided into 3 major groups. The first group included various components of the apical junctional complex (AJC), a multiprotein structure that constitutes the tight junctions (TJ) and adherens junctions between adjacent differentiated epithelial cells necessary for the maintenance of a stable epithelial barrier (21). Among the AJC-related genes down-regulated by the waterpipe smoking in the SAE were claudin 3 (CLDN3), the central TJ component in the human SAE (22), Y box binding protein 3 (YBX3), which codes for ZONAB, a TJ-associated transcription factor that regulates epithelial cell differentiation and proliferation (23), scaffold attachment factor B (SAFB), a DNA-binding protein that mediates TJ signaling via interaction with the TJ protein 2 (TJP2), and Rho family GTPase 2 (RND2), another component of the “TJ signalosome” that mediates cytoskeletal organization in response to TJ-derived signals (24). Consistent with this data, clinical studies in chronic waterpipe smokers have demonstrated

measured airway epithelial permeability. The second group includes genes relevant to early airway epithelial differentiation phenotypes normally expressed by the early/intermediate progenitors derived from the airway basal cell (BC) stem/progenitor cells during the natural course of differentiation or in response to injury (25). This group included tumor protein p73 (TP73), a transcription factor that regulates generation of early BC-derived progenitors and implicated in ciliated cell differentiation (26), keratin 8 (KRT8), an intermediate filament associated with transition of BC to intermediate undifferentiated and eventually to luminal differentiated (ciliated and secretory) cells (25), and integrin beta 5 (ITGB5), which mediates the response of airway BC to injury (27). The third group includes genes with features of the differentiated SAE, including lipocalin 2 (LCN2) and mucin 5B (MUC5B), both enriched in the normal human SAE signature and implicated in pulmonary host defense against respiratory pathogens (28-30). Together, these data suggest that chronic waterpipe smoking may have significant impact on the SAE transcriptional programs that control normal airway epithelial differentiation, barrier integrity and host defense.

Alveolar Macrophages

Similar to its effect on the SAE transcriptome, waterpipe smoking induced a unique gene expression pattern in the AM, not previously reported to be evoked by cigarette smoking or other known modulators of the macrophage phenotype (29, 31, 32). One remarkable feature of the waterpipe smoking-induced AM transcriptional pattern is that it was dominated by down-regulation of genes (74%), including genes known to be critical for various key aspects of macrophage biology such as inflammation and host defense. In contrast, the up-regulated features, which constituted the minority (26%) of differentially expressed genes, included genes related to non-classic macrophage phenotypes, yet distinct from well-known alternative activation programs (33).

The top up-regulated gene in the AM of waterpipe smokers was the four-and-a-half LIM domain protein 2 (FHL2) (34), a transcriptional co-factor known to suppress the pro-inflammatory NF-kB-dependent pathway and implicated in fibrogenesis and regulation of osteoclastogenesis (35). Another waterpipe smoking-up-regulated gene N-deacetylase/N-sulfotransferase 3 (NDST3) is a downstream target of Runx-related transcription factor 2 (RUNX2), the master transcription factor of osteoblast proliferation and differentiation (34), which was significantly up-regulated in AM of waterpipe smokers. Members of the NDST family are involved in the Runx2-regulated fibroblast growth factor (FGF)/proteoglycan axis relevant to regulation of the extracellular matrix homeostasis and FGF signaling (34). The FGF13 gene, known to modulate lung fibroblast function (36), was also up-regulated in the AM of waterpipe smokers. The list of waterpipe smoking-induced AM genes also included echinoderm microtubule-associated protein-like 4 (EML4), a microtubule-associated gene, whose fusion with the gene coding for anaplastic lymphoma receptor tyrosine kinase (ALK) is found in ~5% of non-small cell lung cancers (NSCLC) (37). Among the genes induced in AM by waterpipe smoking

were 2 kinases known to play important role in macrophage activation, including ceramide kinase (CERK), which is down-regulated during the classic (M1) polarization in response to Toll-like receptor (TLR) signaling (38), and spleen tyrosine kinase (SYK) implicated in the regulation of TLR-induced inflammatory response in macrophages (39). Together, the unique pattern of waterpipe smoking-induced genes in AM suggests that a distinct microenvironment is likely generated in the lung as a result of chronic exposure to diverse components present in the waterpipe smoke that shifts the macrophage activation status to a phenotype distinct from both classic (M1) and previously described alternative (M2) polarization programs (33) but, similar to the latter, is relevant to tissue remodeling and immunoregulation.

Among the genes down-regulated in the AM of waterpipe smokers were a number of central regulators of macrophage function, including the nuclear factor erythroid derived 2-like 2 (NRF2), a transcription factor that coordinates expression of antioxidant genes in AM and mediates lung antimicrobial host defense (40). Decreased AM NRF2 expression has been observed in association with aging and COPD (40), i.e., waterpipe smoking may result in decreased antioxidant and antimicrobial defense via suppression of NRF2. Another down-regulated gene is Jagged1 (JAG1), a Notch ligand known to interact with the TLR signaling relevant to the classic (M1) macrophage activation (41). Decreased Notch signaling in macrophages has been linked to the tumor-associated macrophage phenotype (39). Further, AM of waterpipe smokers demonstrated reduced expression of CD44, a receptor for hyaluronic acid implicated in regulation of host-microbe interactions, inflammation and tissue repair (42), myristoylated, alanine-rich C-kinase substrate (MARCKS), a protein kinase C (PKC) substrate regulated by TLR signaling that mediates macrophage migration and phagocytosis (43), components of the integrin signaling pathway integrin ITGB5 and integrin-linked kinase (ILK), also activated by TLR signaling and involved in antimicrobial defense (44), suggesting that various aspects of TLR signaling might

be altered in the AM by waterpipe smoking. Among the down-regulated genes were also CD58, a ligand for the T-cell antigen CD2 (45), and interleukin-2 receptor gamma chain (IL2RG), which mediates sensing of T-cell-derived IL-2 during the classic (M1) macrophage activation (46), suggesting that waterpipe smoking may alter the capacity of AM to cross-talk with T cells necessary for effective immune responses in the lung.

Supplemental Tables

Supplemental Table I.	Lower Respiratory Tract Epithelial Lining Fluid Metabolites
Supplemental Table II.	Small Airway Epithelium Waterpipe-responsive Genes
Supplemental Table III.	Alveolar Macrophage Waterpipe-responsive Genes
Supplemental Table IV.	Correlation of Small Airway Epithelial and Alveolar Macrophage Waterpipe-transcriptome Response Scores with Clinical Parameters

Supplemental Table I. Metabolites Significantly Differentially Abundant in the Lower Respiratory Tract Epithelial Lining Fluid of Waterpipe Smokers Compared to Nonsmokers

Molecular formula	Natural mass (Da)	Retention time (min)	Molecular identity	Fold-change	P value
Positive ions:					
C5 H10 O5	150.038	2.75	Arabinose	2.76	0.04
C11 H13 N O3	207.069	2.56	N-acetylphenylalanine	2.65	0.04
C9 H17 N O5	219.096	2.51	Pantothenic acid	1.98	0.02
C5 H4 N4 O	136.028	2.75	Hypoxanthine	1.71	0.02
C24 H40 O5	408.287	3.13	Cholic acid	1.58	0.002
C5 H5 N O2	111.030	2.78	2,6-dihydroxypyridine	1.58	0.001
C8 H15 N O3	173.094	2.56	N-acetyl-L-leucine/hexanoylglycine	1.33	0.01
C10 H12 N2 O	176.083	3.44	Cotinine	1.27	0.01
C19 H19 N7 O6	441.148	7.48	Folic acid	-1.75	0.003
C5 H12 N2 O2	132.083	24.55	Ornithine	-1.48	0.04
C8 H8 O	120.054	2.84	Phenylacetaldehyde	-1.42	0.03
C5 H12 N2 O3 S	180.024	10.02	Methionine sulfoximine	-1.35	0.004
Negative ions					
C6 H14 O6	182.078	4.51	Sorbitol/mannitol/galacticol	21.53	0.0009
C H4 N2 O	60.021	3.21	Urea	1.83	0.02
C5 H4 N4 O	136.040	3.45	Hypoxanthine	1.39	0.02
C10 H15 N5 O11 P2	443.035	8.88	Guanosine 5'-diphosphate (GDP)	1.18	0.04
C6 H10 O5	162.016	6.29	3-hydroxymethylglutaric acid	-8.33	0.0002
C6 H13 N O2	131.089	7.57	Leucine/isoleucine/norleucine	-6.79	0.0003
C12 H22 O11	342.114	5.69	Lactose	-6.31	4x10 ⁻⁶
C5 H11 N O2	117.077	10.26	5-aminopentanoate	-1.96	0.04
C3 H6 O4	106.026	3.38	Glyceric acid	-1.77	0.04
C8 H9 N O4	183.049	2.03	4-pyridoxic acid	-1.51	0.02
C7 H6 O2	122.035	1.46	Benzoic acid	-1.51	0.03
C2 H4 O3	76.017	3.37	Glycolic acid	-1.45	0.03
C6 H10 O7	194.032	5.83	Glucuronic acid	-1.45	0.002
C5 H4 N4 O3	168.030	3.19	Uric acid	-1.43	0.02
C5 H5 N O	95.037	2.84	2-hydroxypyridine	-1.34	0.0005
C10 H15 N2 O8 P	322.047	8.87	Thymidine 5'-monophosphate (dTMP)	-1.15	0.02
C9 H14 N3 O8 P	323.047	8.85	Cytidine 5'-monophosphate (5'-CMP)	-1.14	0.03
C8 H13 N O6	219.091	8.88	O-succinylhomoserine	-1.12	0.03
C4 H8 N2 O3	132.053	6.7	Asparagine	-1.09	0.02

Supplemental Table II. Genes Significantly Differentially Expressed in the Small Airway Epithelium of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
202437_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	37	67	3.70	0.03
1558120_at	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	5	24	2.86	0.006
1561939_at	DYNC2H1	dynein, cytoplasmic 2, heavy chain 1	0	57	2.72	0.01
230609_at	CLINT1	clathrin interactor 1	21	86	2.62	0.04
207401_at	PROX1	prospero homeobox 1	11	38	2.61	0.02
228260_at	ELAVL2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	11	57	2.57	0.04
1552779_a_at	SLC44A5	solute carrier family 44, member 5	5	48	2.44	0.01
232751_at	RBBP9	retinoblastoma binding protein 9	11	33	2.37	0.04
220254_at	LRP12	low density lipoprotein receptor-related protein 12	5	29	2.36	0.01
234682_at	BTBD9	BTB (POZ) domain containing 9	16	52	2.25	0.02
203293_s_at	LMAN1	lectin, mannose-binding, 1	47	71	2.24	0.03
213472_at	HNRNPH1	heterogeneous nuclear ribonucleoprotein H1 (H)	63	86	2.24	0.007
228948_at	EPHA4	EPH receptor A4	16	43	2.23	0.03
238795_at	FAM208B	family with sequence similarity 208, member B	11	33	2.21	0.02
215028_at	SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	5	43	2.20	0.04
210790_s_at	SAR1A	SAR1 homolog A (<i>S. cerevisiae</i>)	11	57	2.15	0.02
242571_at	REPS2	RALBP1 associated Eps domain containing 2	0	24	2.12	0.02
242034_at	FBXL17	F-box and leucine-rich repeat protein 17	0	24	2.08	0.04
235540_at	GNRH1	gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	37	86	2.07	0.01
1552438_a_at	ANKAR	ankyrin and armadillo repeat containing phosphatidylinositol binding clathrin assembly protein	11	67	2.00	0.03
215236_s_at	PICALM	ankyrin and armadillo repeat containing phosphatidylinositol binding clathrin assembly protein	42	76	1.98	0.04
209754_s_at	TMPO	thymopoietin	100	100	1.92	0.0003
207626_s_at	SLC7A2	solute carrier family 7 (cationic amino acid transporter, y ⁺ system), member 2	16	52	1.91	0.03
227510_x_at	MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)	100	100	1.91	0.004
1558014_s_at	FAR1	fatty acyl CoA reductase 1	100	100	1.88	0.01
202742_s_at	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	68	100	1.87	0.01
222413_s_at	KMT2C	lysine (K)-specific methyltransferase 2C	100	100	1.86	0.001
224044_at	RHOT1	ras homolog family member T1	100	100	1.85	0.0009
1553148_a_at	SNX13	sorting nexin 13	100	100	1.84	0.003
1559094_at	FBXO9	F-box protein 9	100	100	1.84	9x10 ⁻⁶
236696_at	U2SURP	U2 snRNP-associated SURP domain containing	100	100	1.82	0.002
229881_at	KLF12	Kruppel-like factor 12	11	67	1.81	0.004
234055_s_at	GZF1	GDNF-inducible zinc finger protein 1	26	19	1.80	0.03
211090_s_at	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	100	100	1.80	0.003
229530_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3	74	95	1.79	0.01
224170_s_at	TULP4	tubby like protein 4	53	86	1.79	0.03
206925_at	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-	79	100	1.78	0.003

Supplemental Table II. Genes Significantly Differentially Expressed in the Small Airway Epithelium of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 2)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
		sialyltransferase 4				
1565661_x_at	FUT6	fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	5	43	1.78	0.03
214043_at	PTPRD	protein tyrosine phosphatase, receptor type, D	21	67	1.76	0.04
209750_at	NR1D2	nuclear receptor subfamily 1, group D, member 2	79	100	1.76	0.03
223875_s_at	EPC1	enhancer of polycomb homolog 1 (Drosophila)	100	100	1.75	0.003
1556277_a_at	PAPD4	PAP associated domain containing 4	100	100	1.75	0.03
223161_at	KIAA1147	KIAA1147	100	100	1.75	0.0009
218036_x_at	NMD3	NMD3 homolog (S. cerevisiae)	100	100	1.75	0.006
220735_s_at	SENP7	SUMO1/sentrin specific peptidase 7	95	100	1.74	0.004
239903_at	TPBG	trophoblast glycoprotein	42	81	1.74	0.006
202278_s_at	SPTLC1	serine palmitoyltransferase, long chain base subunit 1	58	95	1.74	0.009
235202_x_at	IKBIP	IKBKB interacting protein	47	86	1.73	0.04
212641_at	HIVEP2	human immunodeficiency virus type 1 enhancer binding protein 2	95	100	1.73	0.004
222576_s_at	AGO1	argonaute RISC catalytic component 1	5	38	1.73	0.02
211478_s_at	DPP4	dipeptidyl-peptidase 4	100	100	1.72	0.02
215248_at	GRB10	growth factor receptor-bound protein 10	37	81	1.72	0.04
222399_s_at	TM9SF3	transmembrane 9 superfamily member 3	100	100	1.72	0.0001
202062_s_at	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	89	95	1.72	0.0001
206098_at	ZBTB6	zinc finger and BTB domain containing 6	89	100	1.72	0.006
241789_at	RBMS3	RNA binding motif, single stranded interacting protein 3	100	100	1.67	0.002
222562_s_at	TNKS2	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	100	100	1.67	0.01
235341_at	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	95	100	1.67	0.0003
214593_at	PIAS2	protein inhibitor of activated STAT, 2	26	62	1.66	0.03
222558_at	RPRD1A	regulation of nuclear pre-mRNA domain containing 1A	95	95	1.65	0.0009
202118_s_at	CPNE3	copine III	100	100	1.65	0.02
212362_at	ATP2A2	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2	74	100	1.65	0.006
241784_x_at	HELQ	helicase, POLQ-like	32	76	1.64	0.02
224642_at	FYTTD1	forty-two-three domain containing 1	74	86	1.63	0.008
204213_at	PIGR	polymeric immunoglobulin receptor	100	100	1.63	0.03
223079_s_at	GLS	glutaminase	47	81	1.62	0.03
232064_at	FER	fer (fps/fes related) tyrosine kinase	100	100	1.62	0.007
211466_at	NFIB	nuclear factor I/B	100	100	1.62	0.02
210282_at	ZMYM2	zinc finger, MYM-type 2	100	100	1.61	0.04
1553678_a_at	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	100	100	1.61	0.0001
1555097_a_at	PTGFR	prostaglandin F receptor (FP)	100	100	1.61	0.01
211812_s_at	B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1 (globoside blood group)	32	76	1.60	0.04
208765_s_at	HNRNPR	heterogeneous nuclear ribonucleoprotein R	100	100	1.60	0.006
225054_x_at	LINC00674	long intergenic non-protein coding RNA 674	21	62	1.59	0.04
235331_x_at	PCGF5	polycomb group ring finger 5	95	100	1.59	0.007
1562836_at	DDX6	DEAD (Asp-Glu-Ala-Asp) box helicase 6	47	86	1.58	0.01

Supplemental Table II. Genes Significantly Differentially Expressed in the Small Airway Epithelium of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 3)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
236293_at	RHOH	ras homolog family member H	95	100	1.58	0.04
235114_x_at	HOOK3	hook homolog 3 (Drosophila)	100	100	1.58	0.0009
1554692_at	SLC23A2	solute carrier family 23 (nucleobase transporters), member 2	37	67	1.58	0.04
211494_s_at	SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	42	90	1.57	0.02
1554029_a_at	TTC37	tetratricopeptide repeat domain 37	95	100	1.57	0.01
224582_s_at	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	100	100	1.57	0.009
207605_x_at	ZNF117	zinc finger protein 117	95	95	1.57	0.02
219201_s_at	TWSG1	twisted gastrulation homolog 1 (Drosophila)	100	100	1.56	0.006
224600_at	CGGBP1	CGG triplet repeat binding protein 1	100	100	1.56	0.0009
1559142_at	KAT6A	K(lysine) acetyltransferase 6A	53	90	1.56	0.02
216657_at	ATXN3	ataxin 3	74	95	1.56	0.04
228250_at	FNIP1	folliculin interacting protein 1	89	100	1.56	0.04
213742_at	SRSF11	serine/arginine-rich splicing factor 11	100	100	1.55	0.03
201711_x_at	RANBP2	RAN binding protein 2	100	100	1.54	0.005
1553575_at	ND6	NADH dehydrogenase, subunit 6 (complex I)	100	100	1.53	0.002
1554433_a_at	ZNF146	zinc finger protein 146	58	76	1.52	0.02
213983_s_at	PDS5A	PDS5, regulator of cohesion maintenance, homolog A (S. cerevisiae)	79	90	1.52	0.04
204107_at	NFYA	nuclear transcription factor Y, alpha	0	29	1.52	0.009
208200_at	IL1A	interleukin 1, alpha	74	100	1.52	0.01
226762_at	PURB	purine-rich element binding protein B	100	100	1.52	0.0008
227818_at	CEP85	centrosomal protein 85kDa	79	90	1.52	0.01
241946_at	ZDHHC21	zinc finger, DHHC-type containing 21	63	100	1.52	0.03
214843_s_at	USP33	ubiquitin specific peptidase 33	100	100	1.51	0.001
232591_s_at	TMEM30A	transmembrane protein 30A	100	100	1.51	0.03
232919_at	AFG3L2	AFG3 ATPase family member 3-like 2 (S. cerevisiae)	37	81	1.51	0.04
229673_at	GPATCH2L	G patch domain containing 2-like	100	100	1.51	0.004
233924_s_at	EXOC6	exocyst complex component 6	95	100	1.50	0.005
231918_s_at	GFM2	G elongation factor, mitochondrial 2	100	100	1.50	0.04
1562080_at	LINC00424	long intergenic non-protein coding RNA 424	32	5	-2.48	0.02
230126_s_at	KDM4B	lysine (K)-specific demethylase 4B	37	5	-2.45	0.03
228520_s_at	APLP2	amyloid beta (A4) precursor-like protein 2	100	100	-2.45	1x10 ⁻¹⁰
231211_s_at	YIF1B	Yip1 interacting factor homolog B (S. cerevisiae)	47	19	-2.43	0.005
201525_at	APOD	apolipoprotein D	100	95	-2.28	0.04
207419_s_at	RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	26	10	-2.13	0.02
214021_x_at	ITGB5	integrin, beta 5	100	71	-2.07	0.004
203691_at	PI3	peptidase inhibitor 3, skin-derived	100	81	-2.05	0.004
211799_x_at	HLA-C	major histocompatibility complex, class I, C	100	100	-2.03	0.002
219529_at	CLIC3	chloride intracellular channel 3	74	24	-1.91	0.01
222487_s_at	RPS27L	ribosomal protein S27-like	100	100	-1.91	0.0009
214041_x_at	RPL37A	ribosomal protein L37a	100	100	-1.88	0.008
213432_at	MUC5B	mucin 5B, oligomeric mucus/gel-forming	100	100	-1.88	0.04
244485_at	HLA-DPB1	major histocompatibility complex, class II, DP beta 1	100	86	-1.87	0.02
204133_at	RRP9	ribosomal RNA processing 9, small subunit (SSU) processome component, homolog	26	10	-1.82	0.0009

Supplemental Table II. Genes Significantly Differentially Expressed in the Small Airway Epithelium of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 4)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
		(yeast)				
238367_s_at	C1orf228	chromosome 1 open reading frame 228	53	24	-1.82	0.04
223925_s_at	MTPN	myotrophin	89	24	-1.82	0.004
212484_at	FAM89B	family with sequence similarity 89, member B	63	19	-1.80	0.0009
213736_at	COX5B	cytochrome c oxidase subunit Vb	47	10	-1.80	0.03
215649_s_at	MVK	mevalonate kinase	58	19	-1.77	0.02
205384_at	FXYD1	FXYD domain containing ion transport regulator 1	100	100	-1.77	0.0009
234344_at	LOC100288675	uncharacterized LOC100288675	26	14	-1.72	0.02
202145_at	LY6E	lymphocyte antigen 6 complex, locus E	100	95	-1.72	0.04
227400_at	NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	100	100	-1.69	0.008
228634_s_at	YBX3	Y box binding protein 3	21	24	-1.68	0.01
211528_x_at	HLA-G	major histocompatibility complex, class I, G	100	100	-1.65	0.02
224329_s_at	CNFN	cornifelin	32	0	-1.65	0.04
220804_s_at	TP73	tumor protein p73	47	10	-1.65	0.04
225779_at	SLC27A4	solute carrier family 27 (fatty acid transporter), member 4	32	24	-1.63	0.03
237189_at	HOXB-AS1	HOXB cluster antisense RNA 1	100	86	-1.62	0.03
233362_at	ZNF341	zinc finger protein 341	53	52	-1.61	0.004
220387_s_at	HHLA3	HERV-H LTR-associating 3	100	100	-1.61	0.009
213490_s_at	MAP2K2	mitogen-activated protein kinase kinase 2	79	52	-1.60	0.01
1567081_x_at	CLN6	ceroid-lipofuscinosis, neuronal 6, late infantile, variant	26	33	-1.59	0.006
213467_at	RND2	Rho family GTPase 2	89	62	-1.58	0.01
216563_at	ANKRD12	ankyrin repeat domain 12	100	100	-1.57	0.002
1555894_s_at	MTSS1L	metastasis suppressor 1-like	95	81	-1.57	0.02
235329_at	NOXO1	NADPH oxidase organizer 1	89	57	-1.57	0.02
202326_at	EHMT2	euchromatic histone-lysine N-methyltransferase 2	100	100	-1.56	0.002
203954_x_at	CLDN3	claudin 3	100	100	-1.56	0.01
212525_s_at	H2AFX	H2A histone family, member X	21	5	-1.56	0.02
1563318_s_at	MAGIX	MAGI family member, X-linked	100	100	-1.55	0.001
37996_s_at	DMPK	dystrophia myotonica-protein kinase	100	100	-1.54	0.01
226079_at	FLYWCH2	FLYWCH family member 2	74	14	-1.54	0.02
228203_at	B3GNT1	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1	21	24	-1.54	0.03
227716_at	UBXN11	UBX domain protein 11	100	100	-1.52	0.009
228193_s_at	RGCC	regulator of cell cycle	37	29	-1.52	0.004
213635_s_at	SAFB	scaffold attachment factor B	100	95	-1.52	0.005
1558412_at	LOC113230	uncharacterized LOC113230	100	95	-1.52	0.004
225294_s_at	TRAPPC1	trafficking protein particle complex 1	100	100	-1.51	0.03
222327_x_at	OR7E156P	olfactory receptor, family 7, subfamily E, member 156 pseudogene	89	62	-1.51	0.006
222814_s_at	ZNHIT2	zinc finger, HIT-type containing 2	79	43	-1.51	0.009
204083_s_at	TPM2	tropomyosin 2 (beta)	100	100	-1.50	0.03
209008_x_at	KRT8	keratin 8	100	100	-1.50	0.006
212531_at	LCN2	lipocalin 2	100	100	-1.50	0.008

¹ P=Present call.

² Benjamini-Hochberg corrected (9).

Supplemental Table III. Genes Significantly Differentially Expressed in the Alveolar Macrophages of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
202949_s_at	FHL2	four and a half LIM domains 2	0	32	3.18	0.02
1557558_s_at	MATN1-AS1	MATN1 antisense RNA 1	5	21	2.91	0.04
226905_at	FAM101B	family with sequence similarity 101, member B	89	100	2.69	0.03
220429_at	NDST3	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	5	21	2.53	0.03
226829_at	AFAP1L2	actin filament associated protein 1-like 2	0	32	2.47	0.03
210281_s_at	ZMYM2	zinc finger, MYM-type 2	84	100	2.34	0.02
236293_at	RHOH	ras homolog family member H	26	74	2.16	0.04
223503_at	TMEM163	transmembrane protein 163	100	100	2.12	0.04
1560391_at	PIGL	phosphatidylinositol glycan anchor biosynthesis, class L	21	74	2.07	0.03
242705_x_at	LRPAP1	low density lipoprotein receptor-related protein associated protein 1	37	79	2.04	0.03
205110_s_at	FGF13	fibroblast growth factor 13	68	100	2.01	0.02
220016_at	AHNAK	AHNAK nucleoprotein	74	100	1.97	0.008
1554878_a_at	ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	100	100	1.90	0.002
240859_at	ZFYVE16	zinc finger, FYVE domain containing 16	95	100	1.88	0.04
200906_s_at	PALLD	palladin, cytoskeletal associated protein	100	100	1.88	0.04
238983_at	NSUN7	NOP2/Sun domain family, member 7	89	100	1.79	0.04
231866_at	LNPEP	leucyl/cystinyl aminopeptidase	100	100	1.74	0.03
1554237_at	SDCCAG8	serologically defined colon cancer antigen 8	100	100	1.72	0.01
1556323_at	CELF2	CUGBP, Elav-like family member 2	84	95	1.71	0.04
224046_s_at	PDE7A	phosphodiesterase 7A	32	68	1.71	0.04
208798_x_at	GOLGA8A	golgin A8 family, member A	100	100	1.69	0.02
230085_at	PDK3	pyruvate dehydrogenase kinase, isozyme 3	84	100	1.66	0.04
218421_at	CERK	ceramide kinase	100	100	1.65	0.01
215268_at	KIAA0754	KIAA0754	95	100	1.65	0.02
224454_at	ETNK1	ethanolamine kinase 1	74	89	1.62	0.02
232094_at	KATNBL1	katanin p80 subunit B-like 1	100	100	1.60	0.03
217625_x_at	LINC00963	long intergenic non-protein coding RNA 963	100	100	1.56	0.03
221830_at	RAP2A	RAP2A, member of RAS oncogene family	100	100	1.59	0.02
200899_s_at	MGEA5	meningioma expressed antigen 5 (hyaluronidase)	100	100	1.56	0.004
213116_at	NEK3	NIMA-related kinase 3	74	100	1.56	0.03
241696_at	CNTLN	centlein, centrosomal protein	100	100	1.56	0.03
231003_at	SLC35B3	solute carrier family 35, member B3	95	100	1.55	0.03
223498_at	SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	89	100	1.55	0.02
226438_at	SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	100	100	1.54	0.01
1557754_at	LOC401068	uncharacterized LOC401068	68	89	1.54	0.03
241360_at	CCDC15	coiled-coil domain containing 15	95	100	1.54	0.02
1556732_at	EML4	echinoderm microtubule associated protein like 4	100	100	1.53	0.02
213822_s_at	UBE3B	ubiquitin protein ligase E3B	100	100	1.53	0.003
215322_at	LONRF1	LON peptidase N-terminal domain and ring finger 1	100	100	1.53	0.03
228084_at	PLA2G12A	phospholipase A2, group X1IA	100	100	1.53	0.01
212651_at	RHOBTB1	Rho-related BTB domain containing 1	100	100	1.52	0.04
235482_at	PCBP1-AS1	PCBP1 antisense RNA 1	95	100	1.52	0.02
215013_s_at	USP34	ubiquitin specific peptidase 34	47	79	1.52	0.04
201795_at	LBR	lamin B receptor	100	100	1.52	0.02

Supplemental Table III. Genes Significantly Differentially Expressed in the Alveolar Macrophages of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 2)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
244023_at	SYK	spleen tyrosine kinase	100	100	1.51	0.04
229871_at	SAMD4B	sterile alpha motif domain containing 4B	95	100	1.51	0.004
230389_at	FNBP1	formin binding protein 1	100	100	1.50	0.03
210985_s_at	SP100	SP100 nuclear antigen	100	100	-1.50	0.007
213201_s_at	TNNT1	troponin T type 1 (skeletal, slow)	32	0	-4.98	0.02
242594_at	BOD1L1	biorientation of chromosomes in cell division 1-like 1	26	5	-2.87	0.02
223861_at	HORMAD1	HORMA domain containing 1	79	53	-2.73	0.03
240572_s_at	LOC374443	C-type lectin domain family 2, member D pseudogene	100	100	-2.60	0.03
202421_at	IGSF3	immunoglobulin superfamily, member 3	26	5	-2.46	0.04
216252_x_at	FAS	Fas cell surface death receptor	100	89	-2.40	0.01
205994_at	ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	58	58	-2.35	0.02
214041_x_at	RPL37A	ribosomal protein L37a	100	100	-2.32	0.002
215071_s_at	HIST1H2AC	histone cluster 1, H2ac	100	100	-2.30	0.03
220387_s_at	HHLA3	HERV-H LTR-associating 3	89	89	-2.30	0.04
217566_s_at	TGM4	transglutaminase 4	42	16	-2.27	0.04
209599_s_at	PRUNE	prune exopolyphosphatase	53	21	-2.19	0.04
209880_s_at	SELPLG	selectin P ligand	58	16	-2.18	0.02
209758_s_at	MFAP5	microfibrillar associated protein 5	100	100	-2.10	0.04
202627_s_at	SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	95	95	-2.08	0.04
238319_at	LOC644090	uncharacterized LOC644090	100	95	-2.08	0.03
236859_at	RUNX2	runt-related transcription factor 2	79	26	-2.05	0.02
216971_s_at	PLEC	plectin	58	32	-2.03	0.04
1567013_at	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	100	100	-1.99	0.01
240574_at	DNAJC3-AS1	DNAJC3 antisense RNA 1 (head to head)	89	79	-1.98	0.02
211300_s_at	TP53	tumor protein p53	84	47	-1.97	0.04
1554574_a_at	CYB5R3	cytochrome b5 reductase 3	100	100	-1.96	0.01
219859_at	CLEC4E	C-type lectin domain family 4, member E	100	100	-1.94	0.03
1558404_at	LINC00622	long intergenic non-protein coding RNA 622	100	95	-1.94	0.02
212003_at	SZRD1	SUZ RNA binding domain containing 1	100	100	-1.93	0.008
201212_at	LGMN	legumain	100	100	-1.93	0.04
244413_at	CLECL1	C-type lectin-like 1	100	89	-1.89	0.02
207389_at	GPIBA	glycoprotein Ib (platelet), alpha polypeptide	89	84	-1.88	0.04
219841_at	AICDA	activation-induced cytidine deaminase	89	74	-1.87	0.04
214021_x_at	ITGB5	integrin, beta 5	100	100	-1.86	0.01
209198_s_at	SYT11	synaptotagmin XI	100	100	-1.86	0.002
205687_at	UBFD1	ubiquitin family domain containing 1	100	100	-1.84	0.003
208748_s_at	FLOT1	flotillin 1	84	47	-1.83	0.04
1554761_a_at	HEATR2	HEAT repeat containing 2	63	68	-1.83	0.04
202017_at	EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	68	26	-1.82	0.02
214318_s_at	FRY	furry homolog (Drosophila)	84	63	-1.82	0.04
201669_s_at	MARCKS	myristoylated alanine-rich protein kinase C substrate	100	100	-1.81	0.04
214001_x_at	RPS10	ribosomal protein S10	100	100	-1.79	0.001
242131_at	ATP6	ATP synthase F0 subunit 6	100	100	-1.76	0.02
213112_s_at	SQSTM1	sequestosome 1	95	63	-1.75	0.02
209098_s_at	JAG1	jagged 1	100	95	-1.73	0.03
214149_s_at	ATP6V0E1	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1	100	100	-1.72	0.004
200615_s_at	AP2B1	adaptor-related protein complex 2, beta 1 subunit	100	100	-1.72	0.002
225295_at	SLC39A10	solute carrier family 39 (zinc transporter),	100	100	-1.71	0.04

Supplemental Table III. Genes Significantly Differentially Expressed in the Alveolar Macrophages of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 3)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
		member 10				
222386_s_at	COPZ1	coatamer protein complex, subunit zeta 1	100	100	-1.71	0.002
221156_x_at	CCPG1	cell cycle progression 1	100	100	-1.70	0.01
1553967_at	ADAT3	adenosine deaminase, tRNA-specific 3	32	42	-1.70	0.04
1557984_s_at	RPAP3	RNA polymerase II associated protein 3	100	100	-1.70	0.03
206498_at	OCA2	oculocutaneous albinism II	26	5	-1.70	0.04
239546_at	LOC100131053	uncharacterized LOC100131053	89	84	-1.69	0.02
222693_at	FNDC3B	fibronectin type III domain containing 3B	100	100	-1.68	0.002
214121_x_at	PDLIM7	PDZ and LIM domain 7 (enigma)	95	68	-1.68	0.04
216602_s_at	FARSA	phenylalanyl-tRNA synthetase, alpha subunit	100	100	-1.68	0.02
203729_at	EMP3	epithelial membrane protein 3	100	100	-1.68	0.04
220264_s_at	GPR107	G protein-coupled receptor 107	100	42	-1.66	0.009
210916_s_at	CD44	CD44 molecule (Indian blood group)	100	100	-1.66	0.01
239888_at	UBQLN2	NULL	100	100	-1.66	0.001
205352_at	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1	100	100	-1.65	0.01
207777_s_at	SP140	SP140 nuclear body protein	100	100	-1.65	0.02
213261_at	TRANK1	tetratricopeptide repeat and ankyrin repeat containing 1	100	79	-1.65	0.002
201234_at	ILK	integrin-linked kinase	100	100	-1.64	0.01
204426_at	TMED2	transmembrane emp24 domain trafficking protein 2	100	100	-1.63	0.03
210285_x_at	WTAP	Wilms tumor 1 associated protein	100	100	-1.63	0.02
218537_at	HCFC1R1	host cell factor C1 regulator 1 (XPO1 dependent)	95	74	-1.63	0.02
210317_s_at	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	100	100	-1.62	0.04
201503_at	G3BP1	GTPase activating protein (SH3 domain) binding protein 1	100	100	-1.62	0.0006
205552_s_at	OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa	100	100	-1.61	0.02
218832_x_at	ARRB1	arrestin, beta 1	100	100	-1.61	0.04
205351_at	GGCX	gamma-glutamyl carboxylase	100	100	-1.61	0.002
244050_at	PTPLAD2	protein tyrosine phosphatase-like A domain containing 2	100	100	-1.61	0.03
200787_s_at	PEA15	phosphoprotein enriched in astrocytes 15	100	100	-1.60	0.02
209729_at	GAS2L1	growth arrest-specific 2 like 1	100	100	-1.60	0.007
209208_at	MPDU1	mannose-P-dolichol utilization defect 1	100	100	-1.60	0.009
218540_at	THTPA	thiamine triphosphatase	100	100	-1.59	0.003
1553992_s_at	NBR2	neighbor of BRCA1 gene 2 (non-protein coding)	100	95	-1.59	0.02
1553158_at	CEP19	centrosomal protein 19kDa	79	42	-1.59	0.009
1554966_a_at	FILIP1L	filamin A interacting protein 1-like	100	100	-1.59	0.03
204133_at	RRP9	ribosomal RNA processing 9, small subunit (SSU) processome component, homolog (yeast)	37	5	-1.58	0.009
219596_at	THAP10	THAP domain containing 10	100	100	-1.58	0.04
224484_s_at	BRMS1L	breast cancer metastasis-suppressor 1-like	100	100	-1.58	0.008
204116_at	IL2RG	interleukin 2 receptor, gamma	100	100	-1.58	0.04
1554462_a_at	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	100	100	-1.58	0.01
200964_at	UBA1	ubiquitin-like modifier activating enzyme 1	100	100	-1.58	0.009
202545_at	PRKCD	protein kinase C, delta	100	100	-1.58	0.01
208916_at	SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5	100	100	-1.57	0.04
201490_s_at	PPIF	peptidylprolyl isomerase F	100	100	-1.57	0.04
202205_at	VASP	vasodilator-stimulated phosphoprotein	100	100	-1.57	0.01

Supplemental Table III. Genes Significantly Differentially Expressed in the Alveolar Macrophages of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 4)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
205264_at	CD3EAP	CD3e molecule, epsilon associated protein	95	68	-1.57	0.02
216322_at	CD58	CD58 molecule	100	100	-1.57	0.01
222532_at	SRPRB	signal recognition particle receptor, B subunit	100	100	-1.57	0.002
202910_s_at	CD97	CD97 molecule	100	100	-1.57	0.03
235234_at	PATL1	protein associated with topoisomerase II homolog 1 (yeast)	89	42	-1.57	0.02
228786_at	PTCHD3P1	patched domain containing 3 pseudogene 1	100	100	-1.56	0.004
1554482_a_at	SAR1B	SAR1 homolog B (<i>S. cerevisiae</i>)	100	100	-1.56	0.002
202865_at	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	95	89	-1.56	0.009
235117_at	CHAC2	ChaC, cation transport regulator homolog 2 (<i>E. coli</i>)	100	100	-1.56	0.01
1555736_a_at	AGTRAP	angiotensin II receptor-associated protein	100	100	-1.56	0.01
215450_at	SNRPE	small nuclear ribonucleoprotein polypeptide E	100	100	-1.55	0.008
218058_at	CXXC1	CXXC finger protein 1	100	100	-1.55	0.02
1555815_a_at	L3MBTL2	l(3)mbt-like 2 (<i>Drosophila</i>)	95	95	-1.55	0.003
203289_s_at	NPRL3	nitrogen permease regulator-like 3 (<i>S. cerevisiae</i>)	26	0	-1.54	0.04
225401_at	C1orf85	chromosome 1 open reading frame 85	100	100	-1.54	0.01
201282_at	OGDH	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	100	100	-1.54	0.02
206553_at	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	95	100	-1.54	0.04
1559052_s_at	PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	100	100	-1.54	0.01
215100_at	ADTRP	androgen-dependent TFPI-regulating protein	100	100	-1.54	0.04
203790_s_at	HRSP12	heat-responsive protein 12	100	100	-1.53	0.02
212275_s_at	SRCAP	Snf2-related CREBBP activator protein	58	21	-1.53	0.02
230623_x_at	USP28	ubiquitin specific peptidase 28	100	100	-1.53	0.009
210458_s_at	TANK	TRAF family member-associated NFKB activator	100	100	-1.53	0.02
227687_at	HYLS1	hydrolethalus syndrome 1	100	100	-1.53	0.007
244641_at	MALSU1	mitochondrial assembly of ribosomal large subunit 1	84	63	-1.53	0.01
1554201_at	CABP4	calcium binding protein 4	89	79	-1.53	0.04
200751_s_at	HNRNPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	100	100	-1.52	0.007
208926_at	NEU1	sialidase 1 (lysosomal sialidase)	100	100	-1.52	0.008
235271_s_at	ZNF397	zinc finger protein 397	42	11	-1.52	0.01
200643_at	HDLBP	high density lipoprotein binding protein	100	100	-1.52	0.004
209509_s_at	DPAGT1	dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminophosphotransferase 1 (GlcNAc-1-P transferase)	100	100	-1.52	0.01
1558508_a_at	C1orf53	chromosome 1 open reading frame 53	100	100	-1.52	0.01
216230_x_at	SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	58	5	-1.52	0.04
227716_at	UBXN11	UBX domain protein 11	53	16	-1.52	0.04
1563222_at	TMBIM4	transmembrane BAX inhibitor motif containing 4	47	16	-1.51	0.02
209497_s_at	RBM4B	RNA binding motif protein 4B	100	100	-1.51	0.007
211622_s_at	ARF3	ADP-ribosylation factor 3	100	100	-1.51	0.01
212102_s_at	KPNA6	karyopherin alpha 6 (importin alpha 7)	100	100	-1.51	0.01
212595_s_at	DAZAP2	DAZ associated protein 2	100	100	-1.51	0.04
240486_at	HELZ	helicase with zinc finger	89	95	-1.51	0.03
57715_at	CALHM2	calcium homeostasis modulator 2	100	100	-1.51	0.04
215684_s_at	ASCC2	activating signal cointegrator 1 complex subunit 2	100	100	-1.50	0.01
233880_at	RNF213	ring finger protein 213	53	32	-1.50	0.04

Supplemental Table III. Genes Significantly Differentially Expressed in the Alveolar Macrophages of Waterpipe Smokers Compared to Nonsmokers (Waterpipe-responsive Genes; cont., page 5)

Probe set ID	Gene symbol	Gene title	P calls ¹ (%)		Waterpipe smokers vs nonsmokers	
			Non-smokers	Waterpipe smokers	Fold-change	p value ²
209026_x_at	TUBB	tubulin, beta class I	100	100	-1.50	0.03
224615_x_at	HM13	histocompatibility (minor) 13	100	100	-1.50	0.02
214647_s_at	HFE	hemochromatosis	100	100	-1.50	0.04

¹ P=Present call.

² Benjamini-Hochberg corrected (9).

Supplemental Table IV. Correlation of Small Airway Epithelial and Alveolar Macrophage Waterpipe-transcriptome Response Scores with Clinical Parameters¹

Parameters	Small airway epithelial waterpipe-transcriptome response score			Alveolar macrophages waterpipe-transcriptome response score		
	Normal	Abnormal	p value	Normal	Abnormal	p value
Apoptotic EMPs ²	34 ± 12	34 ± 13	>0.9	13 ± 5	10 ± 5	>0.1
Cough score ³	33 ± 13	38 ± 10	>0.4	12 ± 5	11 ± 6	>0.6
Sputum score ³	33 ± 12	43 ± 11	>0.1	12 ± 5	14 ± 5	>0.5
DLCO ⁴	29 ± 11	44 ± 8	<0.007	10 ± 5	15 ± 4	<0.04

¹ Small airway and alveolar macrophage waterpipe-transcriptome response scores calculated based on the number of abnormally expressed waterpipe-responsive genes out of the total number of waterpipe-responsive genes, see Methods and Supplemental Methods for details. Data presented as average ± standard deviation, p value calculated using a 2-tailed Student's t-test.

² EMP = endothelium microparticles; normal level ≥ nonsmoker mean -2 SD; abnormal level < nonsmoker mean -2 SD.

³ Normal score <2, abnormal ≥2.

⁴ DLCO=diffusion capacity; normal ≥ 80% predicted, abnormal<80% predicted; corrected for carboxyhemoglobin and hemoglobin levels.

Supplemental Figure Legends

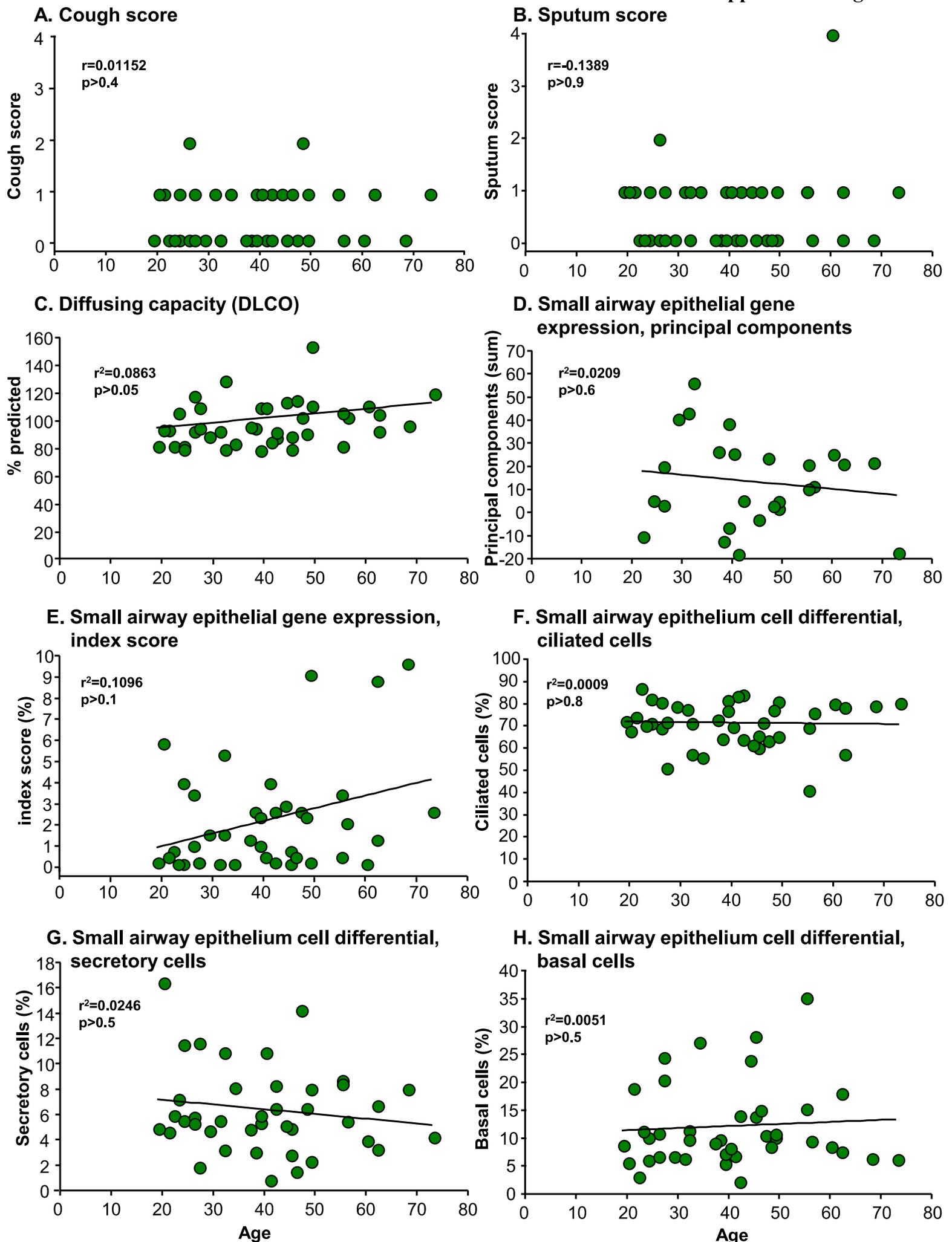
Supplemental Figure 1. Relationship between age and cough and sputum scores, diffusing capacity (DLCO), small airway epithelium (SAE) gene expression, SAE cell differentials, alveolar macrophage (AM) gene expression and endothelial microparticles (EMPs). **A-I.** Data includes cough and sputum scores, SAE gene expression and cell differential for nonsmokers ($n=40^{\text{ref}(47)}$). **J-M.** Data includes AM gene expression for nonsmokers ($n=38^{\text{ref}(48)}$). **K-L.** Data includes total and apoptotic EMP levels for nonsmokers ($n=31^{\text{ref}(14)}$). **A.** Cough score. **B.** Sputum score. **C.** DLCO % predicted. **D.** SAE, genome-wide principal components (PCs). **E.** SAE smoking-related index score. **F.** Ciliated cells in the SAE (%). **G.** Secretory cells in the SEA (%). **H.** Basal cells in the SAE (%). **I.** Intermediate cells in the SAE (%). **J.** AM, NRF2-related PCs. **K.** AM NRF2-related index score. **L.** Total EMP levels. **M.** Apoptotic EMP levels. Each circle represents one nonsmoker. The correlation coefficient and p values are indicated.

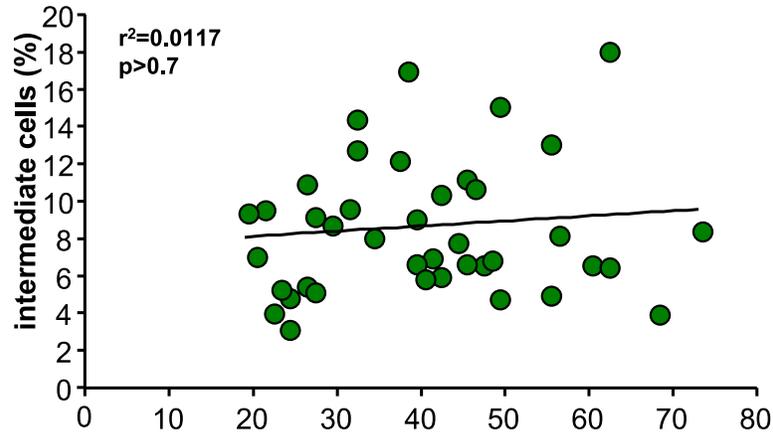
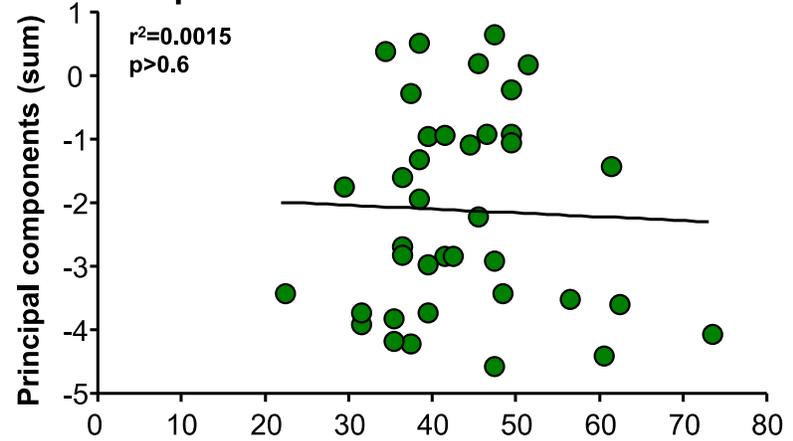
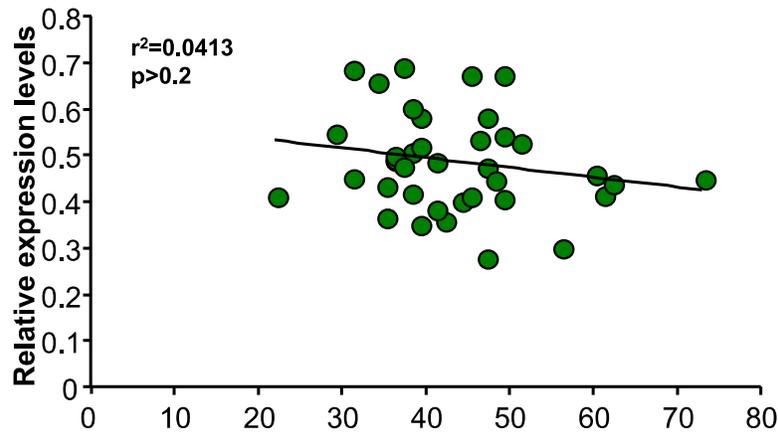
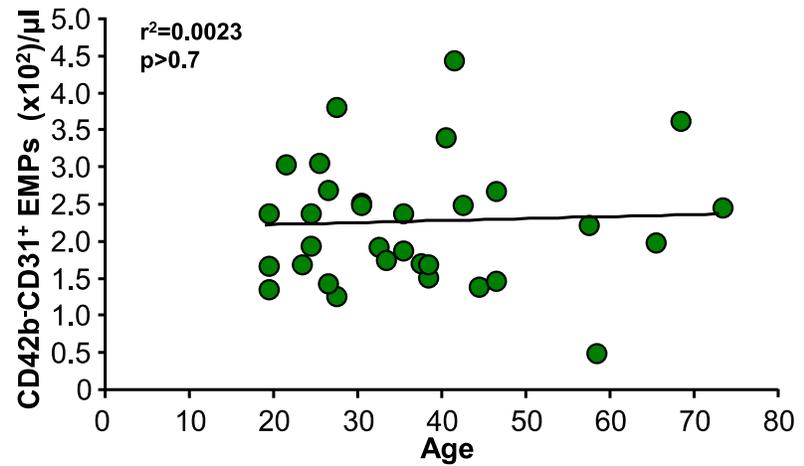
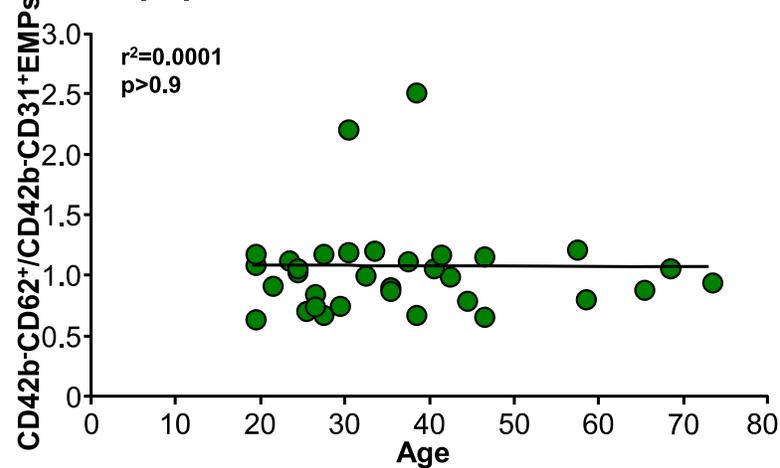
Supplemental Figure 2. Examples of liquid chromatography-mass spectrometry targeted and untargeted metabolite profiling of lower respiratory tract epithelial lining fluid (ELF) of a random subset of waterpipe smokers ($n=8$) compared to nonsmokers ($n=5$). ELF was recovered by bronchoalveolar lavage. NM=natural mass (Da); RT=retention time (min). **A.** NM=181.072, RT=4.2. **B.** NM=280.996, RT=4.6. **C.** NM=260.994, RT=4.5. **D.** NM=182.996, RT=4.5. **E.** NM=527.940, RT=4.5. **F.** NM=217.173, RT=4.4. All panels: green circles = nonsmokers, orange circles = waterpipe smokers; p value calculated using a 2-tailed Student's t-test. *** = $p<0.001$, ****= $p<10^{-3}$. See Supplemental Table I for a list of all structurally identified significantly different metabolites present in waterpipe smoker ELF compared to nonsmoker ELF.

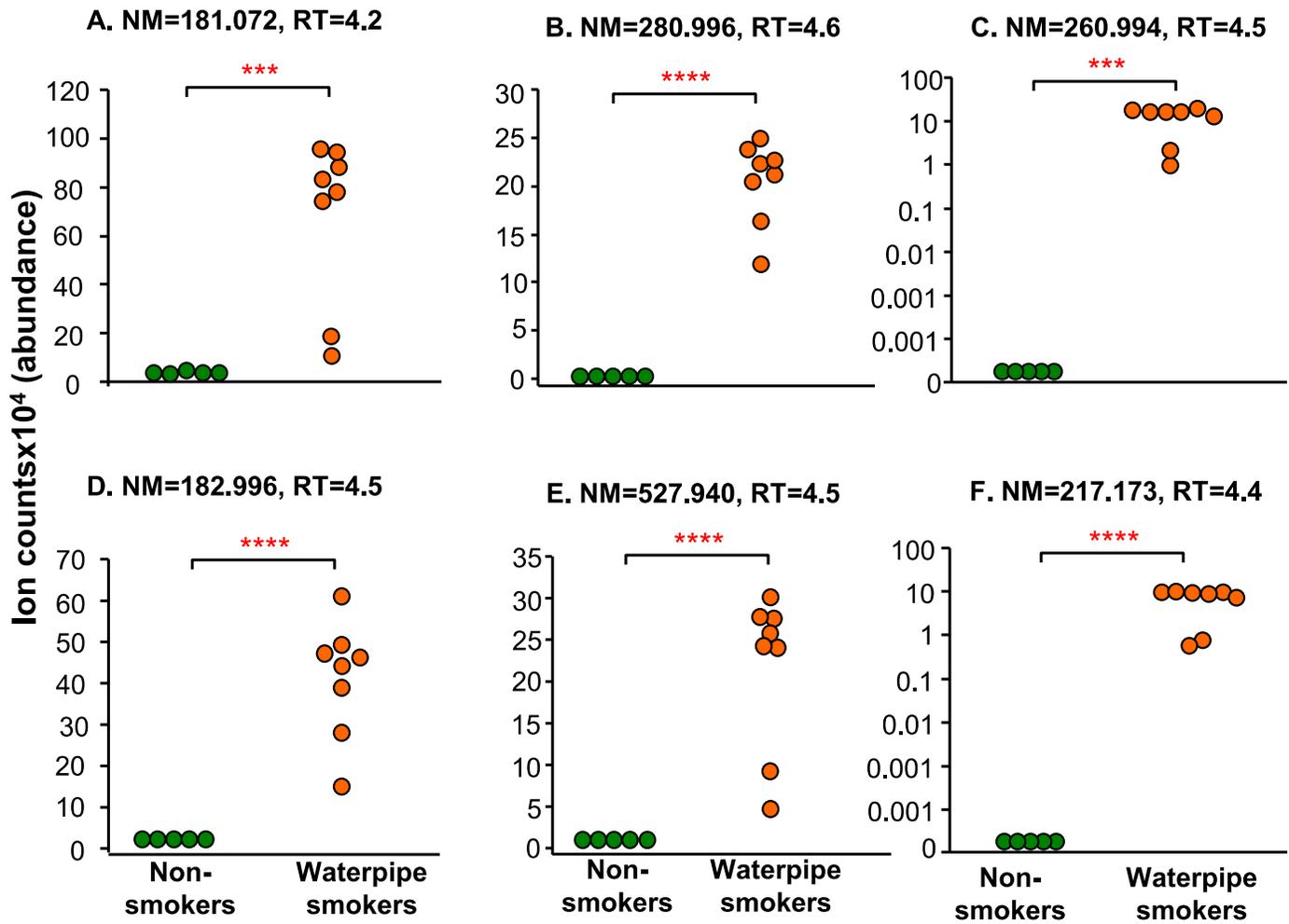
Supplemental Figure 3. Volcano plots assessing all probe sets present in the small airway epithelium in at least 20% of the nonsmokers or waterpipe smokers. Each dot represents a probe set, red dots represent probe sets with a significant difference in expression level (Benjamini-

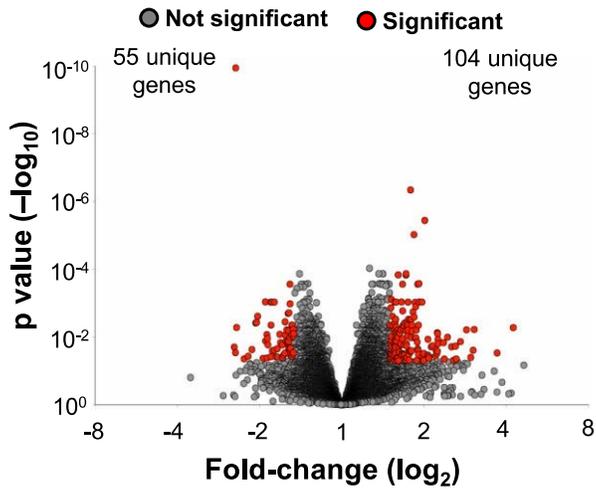
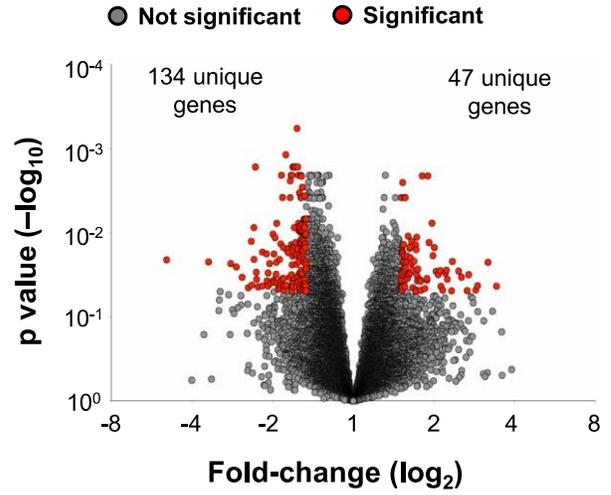
Hochberg (9) corrected $p < 0.05$, fold-change ≥ 1.5) and grey dots represent probe sets not differentially expressed. Genes that are up-regulated in waterpipe smokers are to the top right and those that are down-regulated are to the top left. Fold-change of expression level for waterpipe smokers vs nonsmokers (abscissa) against p values (ordinate). **A.** Small airway epithelium 212 differentially expressed probe sets representing 159 unique, annotated genes (“waterpipe-smoking responsive genes”). **B.** Alveolar macrophages 239 differentially expressed probe sets representing 181 unique, annotated genes (“waterpipe-smoking responsive genes”).

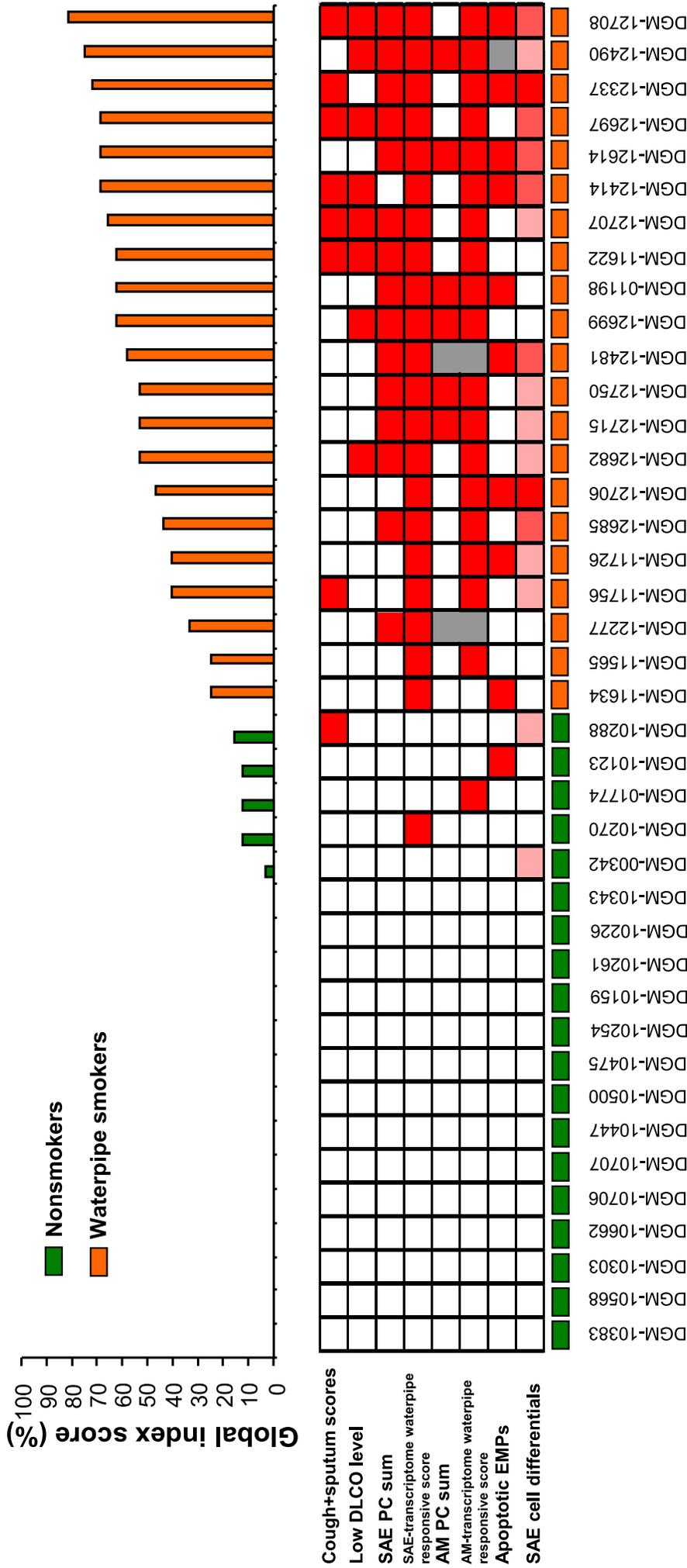
Supplemental Figure 4. Global analysis comparing cough and sputum scores, diffusion capacity (DLCO) % predicted, small airway epithelium (SAE) and alveolar macrophages (AM) transcriptomes, apoptotic endothelial microparticle (EMPs) levels and SAE cell differentials. Each subject was scored “1” (red) for each parameter with an abnormal level, “0” (white) for each parameter with a normal level, or “NA” (grey) for a missing parameter. For cell differentials, each abnormal cell type was scored “0.25” for a total score of 1; colored in gradient between white=0 and red=1. A global index score was calculated for each subject as the number of abnormal parameters divided by the total number of available parameter for that subject and presented as %. Shown is data for all nonsmokers (n=19, green bars) and waterpipe smokers (n=21, orange bars). Each subject is identified using a Department of Genetic Medicine (DGM) randomly assigned number and ordered by index score (from low to high). See Supplemental Methods for criteria of normal and abnormal levels.



I. Small airway epithelium cell differential, intermediate cells**K. Alveolar macrophages, principal components****J. Alveolar macrophages, gene expression****L. Total EMPs****M. Apoptotic EMPs**



**A. Small airway epithelium,
volcano plot****B. Alveolar macrophages,
volcano plot**



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Appendix III – Article 2 Supplemental Methods and Supplemental References

Supplemental Data

Inclusion / Exclusion Criteria for the Study Population

Smokers with normal spirometry and normal diffusion capacity (DLCO)

Inclusion criteria

- Males and females, at least 18 years old
- Capable of providing informed consent
- Willingness to participate in the study
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Females - not pregnant
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium
- Normal serum α 1-antitrypsin level
- HIV1 negative
- Self-reported current daily smokers with >5 pack-yr, validated by urine nicotine >30 ng/ml and/or cotinine >50 ng/ml
- Normal FEV1 ($\geq 80\%$ predicted), FVC ($\geq 80\%$ predicted), FEV1/FVC (≥ 0.7) based on post-bronchodilator spirometry, TLC ($\geq 80\%$ predicted)
- DLCO $\geq 80\%$ predicted

Exclusion criteria

- Unable to meet the inclusion criteria
- Alcohol or drug abuse within the past 6 months
- Evidence of malignancy within the past 5 years
- Current active infection or acute illness of any kind

Smokers with normal spirometry but low DLCO

Inclusion criteria

- Males and females, at least 18 years old
- Capable of providing informed consent
- Willingness to participate in the study
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal physical examination

- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Females - not pregnant
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium
- Normal serum α 1-antitrypsin level
- HIV1 negative
- Self-reported current daily smokers with >5 pack-yr, validated by urine nicotine >30 ng/ml and/or cotinine >50 ng/ml
- Normal FEV1 (\geq 80% predicted), FVC (\geq 80 predicted), FEV1/FVC (\geq 0.7) based on post-bronchodilator spirometry, TLC (\geq 80% predicted)
- DLCO <80% predicted and below the 95% range of normal DLCO calculated for each subject separately based on gender, age and height

Exclusion criteria

- Unable to meet the inclusion criteria
- Alcohol or drug abuse within the past 6 months
- Evidence of malignancy within the past 5 years
- Current active infection or acute illness of any kind

Inclusion / Exclusion Criteria for the Nonsmoker Dataset Population

Inclusion criteria

- Males and females, at least 18 years old
- Capable of providing informed consent
- Willingness to participate in the study
- Good health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Normal PA and lateral chest X-ray
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Females - not pregnant
- No history of allergies to medications used in the bronchoscopy procedure
- Not taking any medications relevant to lung disease or having an effect on the airway epithelium
- Normal serum α 1-antitrypsin level
- HIV1 negative
- Self-reported never-smokers, validated by urine nicotine <20 ng/ml and cotinine <30 ng/ml

- Normal FEV1 ($\geq 80\%$ predicted), FVC ($\geq 80\%$ predicted), FEV1/FVC (≥ 0.7) based on post-bronchodilator spirometry, TLC ($\geq 80\%$ predicted)

Exclusion criteria

- Unable to meet the inclusion criteria
- Alcohol or drug abuse within the past 6 months
- Evidence of malignancy within the past 5 years
- Current active infection or acute illness of any kind

Screening Assessment

Subjects were recruited using advertisements in newspapers and websites. After written informed consent, subjects were evaluated at the Weill Cornell NIH Clinical and Translational Science Center and at the Department of Genetic Medicine Clinical Research Facility under IRB-approved clinical protocols. All individuals had their medical history taken and had a physical exam, complete blood count, biochemical profile, serum α 1-antitrypsin levels, HIV test, urine analysis, chest X-ray, EKG, and pulmonary function tests (PFTs). We excluded HIV positive individuals and subjects with α 1-antitrypsin below normal levels. Smoking status was confirmed by history and urine nicotine and cotinine. A total of 2302 active smokers were screened. After screening, 732 of 2302 (32%) were excluded due to abnormal spirometry or other lung function abnormalities other than low diffusing capacity (DLCO), such as chronic obstructive pulmonary disease (COPD), asthma, restrictive lung disease or lung cancer. Of the remaining 1570 active smokers passing this filter, 397 (17% of the original total individuals) had normal spirometry and normal TLC but low DLCO (referred to as the “normal spirometry/low DLCO” group), and 1173 (51% of the original total individuals) had normal spirometry, normal TLC and normal DLCO (referred to as the “normal spirometry/normal DLCO” group).

In addition, 405 healthy nonsmokers, with a similar distribution of age, gender and ethnicity to the study population, were recruited from the general NY area. Their lung function results were used to calculate the 95% normal range of FEV1/FVC and DLCO % predicted.

Pulmonary Function Tests

Individuals were instructed to refrain from smoking as of the night before the testing. PFTs included spirometry before and after the administration of salbutamol (100 μ g, 4 doses) [1], lung volumes and diffusion capacity of the lung for carbon monoxide (Viasys Healthcare, Yorba Linda, CA). The DLCO test was performed with the individual in the sitting position. Af-

ter tidal breathing, a non-forced expiratory maneuver to residual volume was performed, followed by rapid inhalation to TLC. After breath holding for ~10 sec, the subject was asked to exhale (non-forced), not exceeding 4 sec. The DLCO maneuver was carried out 2 to 4 times; the average of the best 2 trials was used. As an additional quality control measure, PFTs were performed serially in several volunteers during the course of the study. The 95% confidence interval (± 2 standard deviations) for the DLCO was similar to that reported by Hathaway et al [2]. The spirometry and DLCO curves of all PFTs for all subjects were validated based on ATS/ERS guidelines [3]. For DLCO, these included: a stable calculated breath hold for 10 ± 2 sec; no evidence of leaks or Valsalva or Mueller maneuvers in the curves; both inspiration and expiration completed in < 4 sec (and sample collection time < 3 sec), with appropriate clearance of dead-space volume and proper sampling/analysis of alveolar gas as assessed graphically; inspiratory vital capacity $> 85\%$ of the largest expiratory forced vital capacity (from spirometry) in 96% of subjects and $> 80\%$ in 98% of subjects. The DLCO % predicted value was calculated using the Gaensler et al equation [4], and corrected for hemoglobin and carboxy hemoglobin levels using ATS/ETS guidelines [3].

Study Groups and Assessment

Subjects were divided into “normal spirometry/normal DLCO” and “normal spirometry/low DLCO” groups based on their corrected DLCO prediction values. Because the study populations of both the normal and low DLCO groups had similar, but mixed ethnicities (Table I), and because of the lack of definitive, universally accepted correction criteria for DLCO for African-American and other non-European ethnicities [4-6], no correction was made for ethnicity. Instead, in addition to a predicted DLCO of $< 80\%$, a criterion of DLCO level below the 95% range of normal DLCO calculated per subject based on gender, age and height [3,7,8] was required to place a subject in the “normal spirometry/low DLCO” group. Subjects from both

groups were randomly contacted by staff not associated with the study with a goal of recruiting approximately 100 subjects total, equally divided between the 2 groups, to return for subsequent PFT assessment. The final group that returned one or more times included 59 with normal spirometry and normal DLCO and 46 with normal spirometry but low DLCO (Table I). On the average, there were more PFTs performed in the low DLCO group (3 ± 2 , vs normal DLCO 2 ± 1 , $p < 10^{-3}$) with shorter intervals between PFTs (18 ± 20 months vs normal DLCO 33 ± 18 months, $p < 10^{-6}$), but there was no difference in the time of follow-up (normal DLCO group 46 ± 21 months vs low DLCO group, 41 ± 31 months, $p > 0.4$, Table I). The number of PFTs performed, the intervals between them and the follow-up for each subject was dependant of the subject's availability.

Chest High Resolution Computed Tomography

The percentage of the lung affected by emphysema was evaluated at baseline in a random subset of the normal spirometry/normal DLCO ($n=12$) and normal spirometry/low DLCO group ($n=15$) at attenuation -950 Hounsfield Units (HU) using the EmphyxJ software application (EmphyxJ, Vancouver, BC, Canada) allowing automated quantitative analysis of transverse chest CT scans [9-11].

Statistical Analysis

Comparison of demographic parameters among groups was performed by two-tailed Student's t-test or Chi-square test. Progression to COPD between the 2 groups was assessed by Chi-square. A within-between ANOVA test was used to compare lung function at baseline and last visit within the normal spirometry/normal DLCO group and within the normal spirometry/low DLCO group. A 95% normal range for FEV1/FVC and DLCO % predicted was calculated based on the average ± 2 standard deviations of 405 healthy nonsmokers. PFT parameters were converted using a z-score and compared between the normal spirometry/normal DLCO and normal spi-

rometry/low DLCO groups. To assess if DLCO level can predict the development of COPD, a binomial logistic regression model was implemented in which the response was COPD status (“1”=developing COPD, “0” = not developing COPD). In addition, Leave-one-out cross-validation was performed in order to assess the predictive accuracy. Evaluation and fit of the logistic regression model was performed using the "nnet" and "ROCR" packages in the freely available R software [12,13].

Discussion

Low DLCO in Otherwise Healthy Smokers

Several studies have reported decreased DLCO in smokers with normal spirometry. Assessment of 131 healthy Chinese male smokers with normal spirometry found that 21% had low DLCO [14]. Evaluation of 80 Caucasian cigarette smokers with normal spirometry found that 12.5% had low DLCO [15]. Assessment of 80 healthy male adolescents with normal spirometry revealed that 29 passive and 21 active smokers had a lower DLCO than the 30 neither passive nor active smokers [16]. A study of 1612 individuals found lower DLCO in smokers vs non-smokers [17]. A retrospective analysis of 38,095 individuals showed that 179 (0.45%) had normal spirometry but low DLCO. Of these, 27 out of 179 had chest CT revealing a combination of emphysema and fibrosis [18].

Supplemental Table I. Comparison of Smokers with Normal Spirometry and Total Lung Capacity but Low DLCO Who Developed COPD vs Those who Did Not¹

Parameter	Did not develop COPD	Developed COPD	p value
n	36	10	
Gender (male/female)	24/12	7/3	>0.9
Age	45 ± 9	49 ± 5	>0.1
Ethnicity (AA/E/H) ²	29/4/3	8/1/1	>0.8
BMI (kg/m ²)	25 ± 5	23 ± 4	>0.1
Smoking history ³			
Pack-yr	29 ± 16	31 ± 12	>0.7
Pack per day	1.1 ± 0.7	0.8 ± 0.3	>0.2
Age of smoking initiation	17 ± 4	18 ± 6	>0.9
Urine nicotine (ng/ml)	926 ± 1242	1034 ± 1490	>0.8
Urine cotinine (ng/ml)	1223 ± 950	1562 ± 633	>0.2
Cough score ⁴	1.9 ± 1.5	1.1 ± 1.2	>0.1
Sputum score ⁴	1.4 ± 1.4	0.9 ± 1.0	>0.2
MMRC score	0.6 ± 0.7	0.3 ± 0.5	>0.2
% emphysema ⁵	1.2 ± 0.01	3.7 ± 0.05	>0.2
Serology ⁶			
α1-antitrypsin (mg/dl)	143 ± 18	151 ± 31	>0.3
ESR (mm/hr)	12 ± 9	12 ± 14	>0.9
IgE (IU/mL)	180 ± 283	123 ± 122	>0.5
CrP (mg/dL)	0.4 ± 0.2	0.2 ± 0.2	>0.05
Hepatitis C (negative/positive) ⁷	32/3	7/3	>0.2
Lung function ⁸			
VC (% predicted)	108 ± 15	109 ± 13	>0.9
FVC (% predicted)	106 ± 15	109 ± 12	>0.5
FEV1 (% predicted)	105 ± 15	101 ± 10	>0.4
FEV1/FVC (% observed)	80 ± 4	75 ± 3	<0.003
TLC (% predicted)	93 ± 12	97 ± 19	<0.3
RV (% predicted)	86 ± 35	100 ± 44	>0.3
RV/TLC	30 ± 11	33 ± 10	>0.5
DLCO (% predicted)	69 ± 8	66 ± 11	>0.2
DLCO/VA (mL/mHg/min/L)	3.7 ± 0.6	3.3 ± 0.8	>0.05
Assessment over time			
Time of follow-up (month, mean ± SD, range)	37 ± 30 (5-146)	54 ± 32 (17-133)	>0.1
Number of PFTs (mean ± SD, range)	3 ± 2 (2-8)	3 ± 1 (2-6)	>0.8
Interval between PFTs (month, mean ± SD, range)	17 ± 19 (1-127)	23 ± 23 (6-97)	>0.1

¹ A total of 46 active smokers with normal spirometry/low diffusion capacity (DLCO) were followed for 41±31 months with serial PFTs. Of these subjects, 10 developed COPD by the GOLD criteria and 36 did not (Figure 3, Table II). The table compares the baseline characteristics of these 2 subgroups and the timing of their assessment.

² AA – African-American; E - European; H - Hispanic.

³ Current smoking was verified at baseline by urine nicotine and its derivative cotinine; at subsequent visits for lung function testing, active smoking status was verified by questionnaire.

⁴ Cough and sputum scores were each evaluated on a scale of 0-4: 0 = not at all; 1 = only with chest infections; 2 = a few days a month; 3 = several days a wk; 4 - most days a wk [19]. MMRC = Modified Medical Research Council dyspnea scale [20].

⁵ Chest high resolution computed tomography (HRCT); % emphysema at -950 Hounsfield Units (HU).

⁶ All individuals tested negative for HIV and had normal levels of α1-antitrypsin; ESR - erythrocyte sedimentation rate; IgE – immunoglobulin E; CrP – C-reactive protein; hepatitis C – hepatitis C serology.

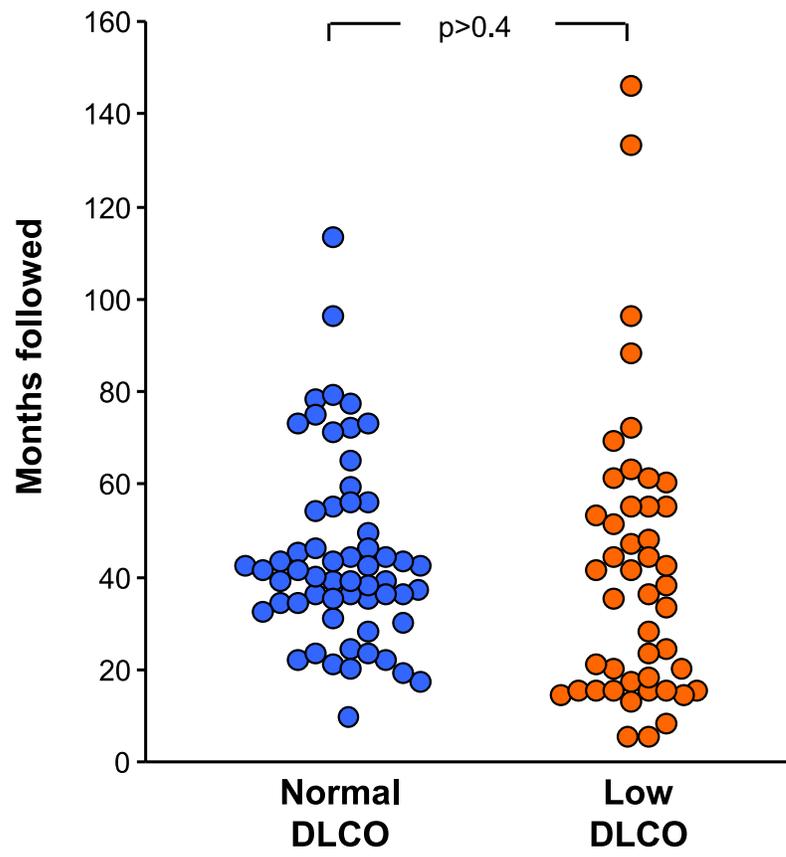
⁷ Data is only available for 35 of 36 low DLCO individuals who did not develop COPD.

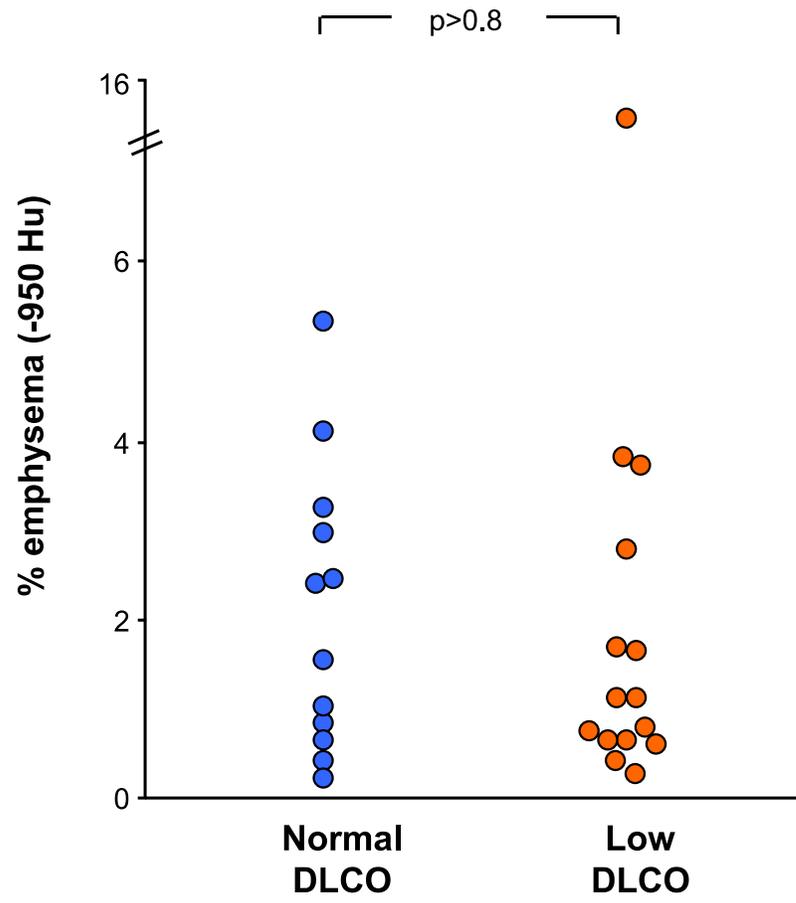
⁸ Lung function parameters are presented as percent predicted except the FEV1/FVC ratio, which is presented as percent observed; VC – vital capacity; FVC - forced vital capacity; FEV1 - forced expiratory volume in 1 second; TLC - total lung capacity; RV - residual volume; DLCO - diffusion capacity; and VA – alveolar volume. The DLCO was corrected for hemoglobin and carboxyhemoglobin.[3]

Supplemental Figure Legends

Supplemental Figure 1. Total number of months each subject was followed, comparing active smokers with normal spirometry and normal diffusion capacity (DLCO) vs active smokers with normal spirometry but low DLCO ($p>0.4$).

Supplemental Figure 2. Percent emphysema (calculated in -950 Hu) in a subset of the active smokers with normal spirometry and normal diffusion capacity (DLCO) vs active smokers with normal spirometry but low DLCO ($p>0.8$).





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Appendix V – Article 3 Supplemental Methods and Supplemental References

Supplemental Methods

Inclusion and Exclusion Criteria

Nonsmokers

Inclusion criteria

- Must be capable of providing informed consent
- Males and females, age 18 or older
- Females - not pregnant
- Never-smokers by history, with current smoking status validated by the undetectable levels of the following metabolites: urine nicotine <2 ng/ml and urine cotinine <5 ng/ml
- Good overall health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease
- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Negative HIV serology
- Normal FEV1 ($\geq 80\%$ predicted), FVC ($\geq 80\%$ predicted), FEV1/FVC (≥ 0.7 predicted) based on pre-bronchodilator spirometry, DLCO ($\geq 80\%$ predicted) and TLC ($\geq 80\%$ predicted)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery ≤ 30 mm in chest CT scans
- Normal chest X-ray (PA and lateral)
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Not taking any medications relevant to lung disease
- Willingness to participate in the study

Exclusion criteria

- Unable to meet the inclusion criteria
- Pregnancy
- Current active infection or acute illness of any kind
- Current alcohol or drug abuse
- Evidence of malignancy within the past 5 years
- Any evidence of interstitial lung disease, pulmonary hypertension, diastolic dysfunction or other disorders associated with a low DLCO
- Subjects with allergies to lidocaine

Healthy Smokers

Inclusion criteria

- Must be capable of providing informed consent
- Males and females, age 18 or older
- Females - not pregnant
- Current daily smokers with pack-yr ≥ 5 , validated by urine cotinine ≥ 104 ng/ml
- Good overall health without history of chronic lung disease, including asthma, and without recurrent or recent (within 3 months) acute pulmonary disease

- Normal physical examination
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Negative HIV serology
- Normal FEV1 ($\geq 80\%$ predicted), FVC ($\geq 80\%$ predicted), FEV1/FVC (≥ 0.7 predicted) based on pre-bronchodilator spirometry, DLCO ($\geq 80\%$ predicted) and TLC ($\geq 80\%$ predicted)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery ≤ 30 mm in chest CT scans
- Normal chest X-ray (PA and lateral)
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- No medications relevant to lung disease
- Willingness to participate in the study

Exclusion criteria

- Unable to meet the inclusion criteria
- Pregnancy
- Current active infection or acute illness of any kind
- Current alcohol or drug abuse
- Evidence of malignancy within the past 5 years
- Any evidence of interstitial lung disease, pulmonary hypertension, diastolic dysfunction or other disorders associated with a low DLCO
- Subjects with allergies to lidocaine

COPD Smokers

Inclusion criteria

- Must be capable of providing informed consent
- Males and females, age 18 or older
- Females - not pregnant
- Current daily smokers with pack-yr ≥ 5 , validated by urine cotinine ≥ 104 ng/ml
- Taking any or no pulmonary-related medication, including beta-agonists, anticholinergics, or inhaled corticosteroids
- Normal routine laboratory evaluation, including general hematologic studies, general serologic/immunologic studies, general biochemical analyses, and urine analysis
- Negative HIV serology and positive HIV serology
- Presence of COPD as defined by the GOLD criteria based on post-bronchodilator FEV1/FVC < 0.7 (observed); stage I-IV but without evidence of respiratory failure
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery ≤ 30 mm in chest CT scans
- Normal chest X-ray (PA and lateral)
- Willingness to participate in the study

Exclusion criteria

- Unable to meet the inclusion criteria

- Individuals in whom participation in the study would compromise the normal care and expected progression of their disease
- Current active infection or acute illness of any kind
- Current alcohol or drug abuse
- Evidence of malignancy within the past 5 years
- Any evidence of interstitial lung disease, pulmonary hypertension, diastolic dysfunction or other disorders associated with a low DLCO
- Individuals with asthma and with recurrent or recent (within three months) acute pulmonary infection
- Individuals with allergies to lidocaine

Human Subjects and Clinical Phenotypes

All 138 subjects enrolled in this study underwent thorough screening including medical history, complete physical exam, blood studies, urinalysis, chest X-ray, electrocardiograms and pulmonary function tests, including forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV1), FEV1/FVC, total lung capacity (TLC) and diffusion capacity (DLCO), all carried out under ATS guidelines¹⁻³. If the FEV1 was <80% predicted and/or the FEV1/FVC <0.7, spirometry was retested after standard bronchodilators. Measurement of the DLCO was carried out 2 to 4 times in all subjects; the average of the best 2 trials was used. The GOLD criteria, based on post-bronchodilator FEV1/FVC ratio <0.7, were used to define and stage COPD⁴. The diameter of the main pulmonary artery was assessed by chest CT scans as a correlate to the pulmonary artery pressure. In all subjects, the pulmonary artery diameter was ≤ 30 mm, indicating normal estimated pulmonary pressure⁵.

Chest high resolution computed tomography (HRCT) scans were used to determine the percentage of lung affected by emphysema in each subject. Percentage emphysema was evaluated with the EmphylxJ software application (EmphylxJ, Vancouver, BC, Canada) allowing automated quantitative analysis of transverse chest CT scans⁶⁻⁸. The lung was divided into quartiles by lung volume, and the top and bottom quartiles were compared for % emphysema at attenuation -950 Hounsfield Units (HU). Emphysema was defined as >5% lung volume, value derived from analyses of HRCT in normal nonsmoking individuals with normal lung function.

Smoking status of all subjects was defined by self-reported smoking history and verified by urinary levels of nicotine and cotinine. Both nicotine and cotinine were used to define non-smoking status but only cotinine levels were used to define a current smoker as it has a longer half-life than nicotine. Since subjects vary in the last time they smoked a cigarette prior to the visit, nicotine that has a short half-life, and is only detected in urine for 2 to 8 hr after smoking¹¹,

might not be detected in urine at collection time though the subject is a current smoker. Cotinine that has a half-life in urine of 8 to 24 hr¹² provides a more accurate assessment of the smoking status.

Nonsmokers were defined as self-reported never smokers with undetectable urine nicotine (<2 ng/ml) and cotinine (<5 ng/ml)⁹. Current smokers were defined as self-reported current smokers with urine cotinine level ≥ 104 ng/ml, a level based on our previous study of low level smoke exposure¹⁰, where 104 ng/ml was calculated as the induction half maximal level (ID₅₀) at which the small airway epithelium, the initial site of smoking-related pathology, showed abnormal response.

The study population included nonsmokers (n=28) - lifelong never smokers with non-detectable urine nicotine (<2 ng/ml) and cotinine (<5 ng/ml), normal pulmonary function tests (PFT; spirometry, TLC, DLCO) and chest X-ray; healthy smokers (n=61) - active smokers with normal pulmonary function tests (PFT; spirometry, TLC, DLCO) and chest X-ray; and smokers with COPD (COPD smokers, n=49), including n=31 GOLD I and n=18 GOLD II. Among the 49 COPD smokers of the initial study population, 7 were on medications for COPD (3 of 31 GOLD I, 4 of 18 GOLD II). The classes of medications included short- and long-acting β -agonists, short- and long-acting anticholinergics, inhaled corticosteroids, systemic corticosteroids and theophylline; several of those treated were on multiple classes of medications. See Supplemental Figure 1 for study design.

Characterization of Plasma Endothelial Microparticles

Endothelial microparticles were quantified as previously described¹³. Blood was collected and processed within 1 hr to prepare platelet-rich plasma. The supernatant was further processed within 5 min to obtain platelet-poor plasma that was stained for 3 antibodies: the constitutive endothelial marker PECAM (CD31); the constitutive platelet-specific glycoprotein Ib (CD42b)

and E-selectin (CD62E), an adhesion molecule expressed on activated endothelium. Anti-human CD45-PECy5 (leukocyte marker, clone HI30, BD PharMingen) was also used to monitor leukocyte MP contamination. To assess the presence of relative contribution of pulmonary capillary endothelium to the elevated EMP levels, CD42b⁻CD31⁺ EMPs were co-stained with anti-human angiotensin converting enzyme (ACE) based on the knowledge that ACE is abundantly expressed on pulmonary capillary endothelium¹⁴. The optimized condition for each antibody was determined by serial dilutions. EMP measurements were performed twice to ensure that the measurements were reproducible. CD42b⁻CD31⁺ and CD42b⁻CD62E⁺ microparticle levels were corrected for correlating isotype control antibodies. Regarding apoptotic EMPs, relating to CD31⁺ and CD62E⁺, the data is displayed as ratio; following the methods of Jimenez et al.¹⁵, we have chosen to calculate it as the ratio of CD42b⁻CD62E⁺/CD42b⁻CD31⁺, with a lower ratio identifying the apoptotic EMPs.

Smoking Cessation

All healthy smokers and COPD smokers were invited to quit smoking using Varenicline 0.5 mg once daily for 3 days, then 0.5 mg twice daily for 4 days, then 1 mg twice daily for 11 weeks, for total treatment time of 12 weeks. Counseling was also carried out by phone once per week for the first 3 weeks, followed by in-person monthly counseling sessions for the first 3 months.

Statistical Analysis

A Pearson correlation was applied to assess the effect of phenotype on EMP variability where covariates were included individually (gender, ethnicity, age, BMI, pack-yr, urine cotinine, FEV1, FVC, FEV1/FVC, TLC, DLCO and blood pressure, Supplemental Figures 1, 2). The effect of the covariates on EMP levels and with each individual phenotype was also assessed with a simple regression approach.

Supplemental Figure Legends

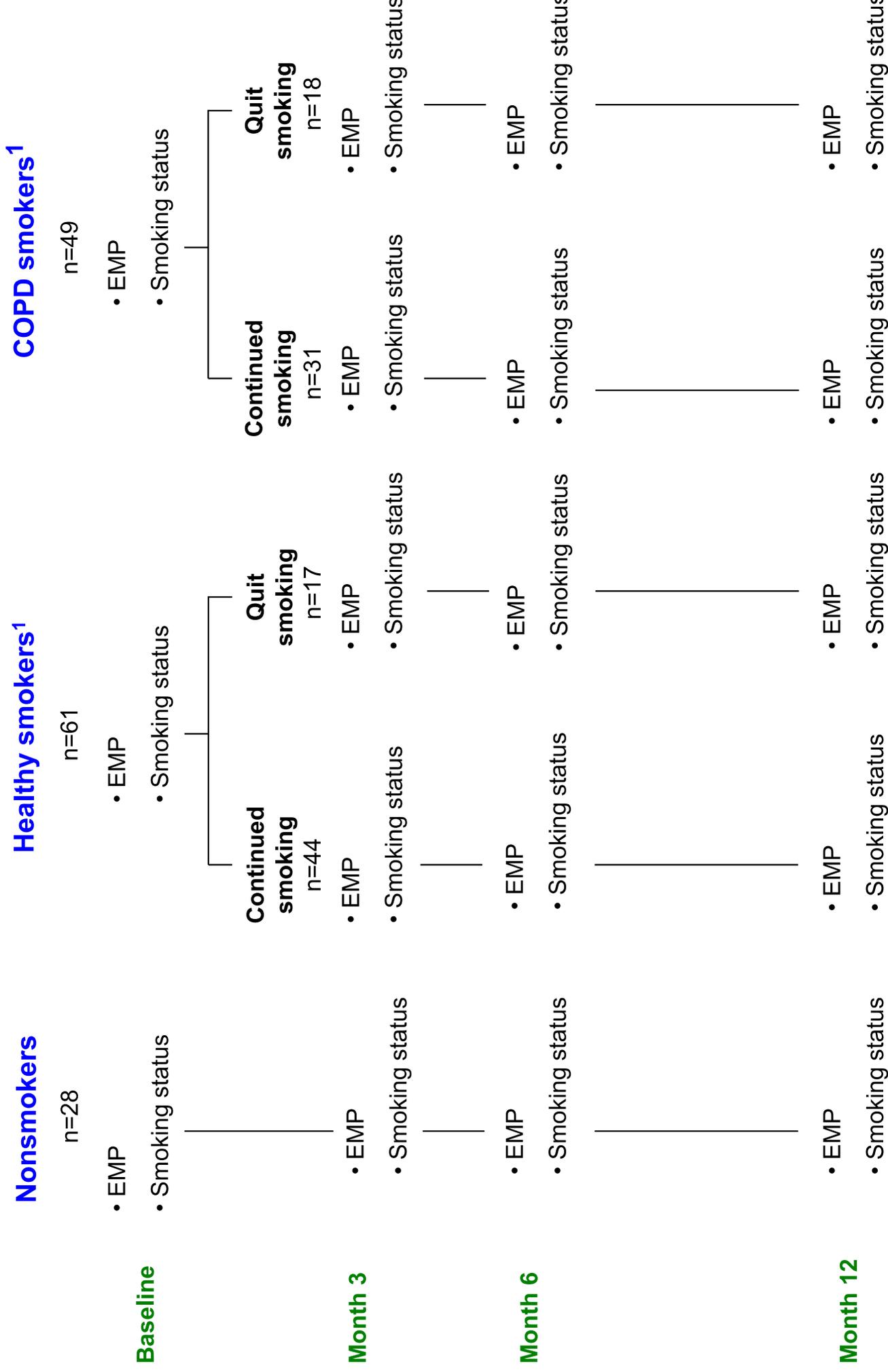
Supplemental Figure 1. Study design. A total of 138 subjects were assessed for circulating total and apoptotic endothelial microparticles (EMP) levels at baseline, 3, 6 and 12 months (28 nonsmokers, 61 healthy smokers and 49 COPD GOLD I/II smokers). Following the baseline visit all healthy smokers and COPD smokers were invited to stop smoking using a combination of varenicline and counseling for 3 months. Seventeen healthy smokers and eighteen COPD smokers quit smoking and remained quitters at each time point as verified by urine nicotine metabolite levels. EMP levels and urine metabolite levels were measured at each time point and compared within a phenotype group between the different time points; between phenotype groups at the same time points; and between smokers who continued smoking and those who quit for the same time point.

Supplemental Figure 2. Relationship between total CD42b⁻CD31⁺ EMPs and smoking-related, demographic, and lung function parameters of the study population. The data includes levels of CD42b⁻CD31⁺ EMPs from plasma of nonsmokers (n=28, green circles), healthy smokers (n=61, yellow circles); and smokers with COPD (n=49, red circles). **A.** Gender (male, female). **B.** Ethnicity (black, white, other). **C.** Age (yr). **D.** BMI. **E.** Pack-yr. **F.** Urine cotinine. **G.** % emphysema. **H.** FEV1. **I.** FVC. **J.** FEV1/FVC. **K.** TLC. **L.** Systolic blood pressure. The correlation coefficient and p values are indicated.

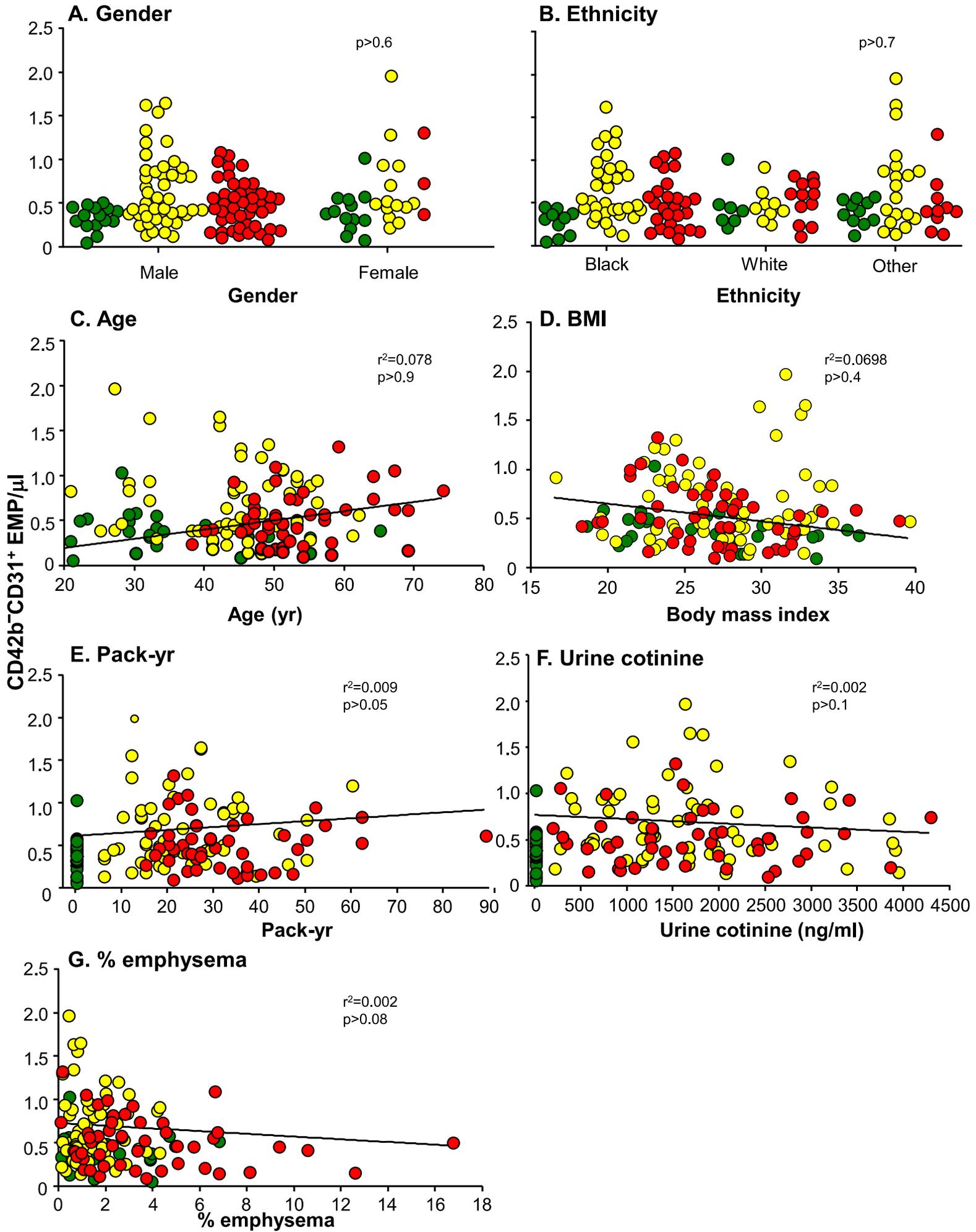
Supplemental Figure 3. Relationship between CD42b⁻CD62E⁺ /CD42b⁻CD31⁺ ratio in EMPs and smoking-related, demographic, and lung function parameters of the study population. The data includes ratio of CD42b⁻CD62E⁺ /CD42b⁻CD31⁺ EMPs from plasma of nonsmokers (n=28, green circles), healthy smokers (n=61, yellow circles); and smokers with COPD (n=49, red circles). **A.** Gender (male, female). **B.** Ethnicity (black, white, other). **C.** Age (yr). **D.** BMI. **E.**

Pack-yr. **F.** Urine cotinine. **G.** % emphysema. **H.** FEV1. **I.** FVC. **J.** FEV1/FVC. **K.** TLC.

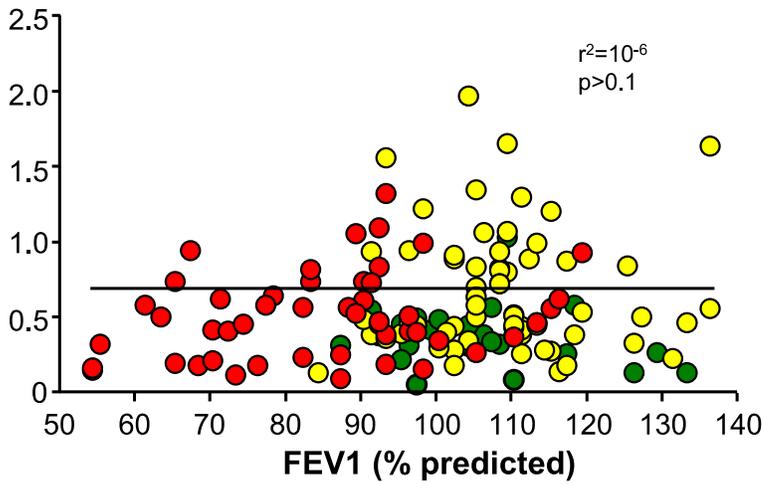
L. Systolic blood pressure. The correlation coefficient and p values are indicated.



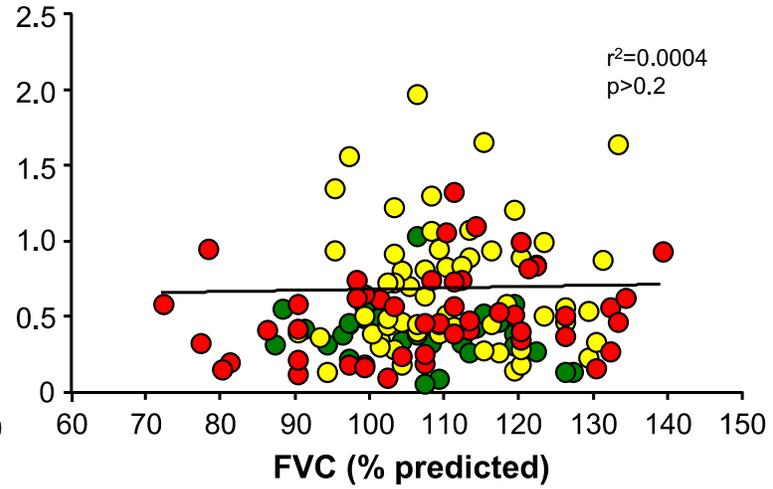
Supplemental Figure 2A-F



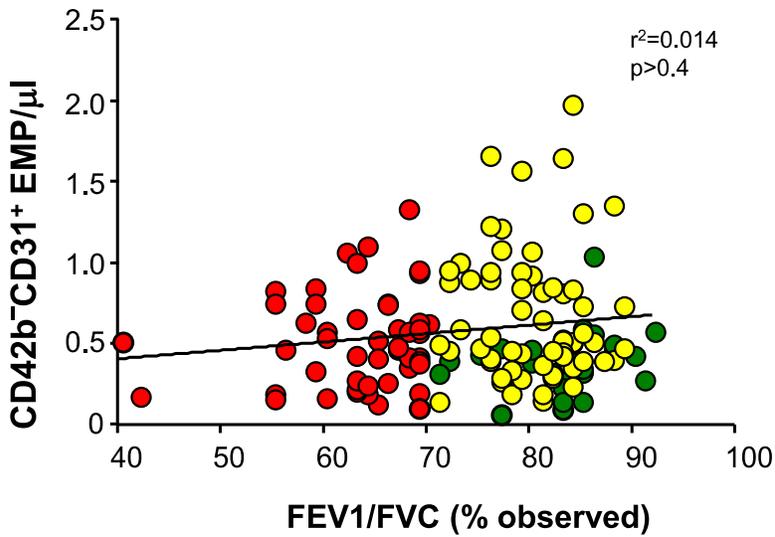
G. FEV1



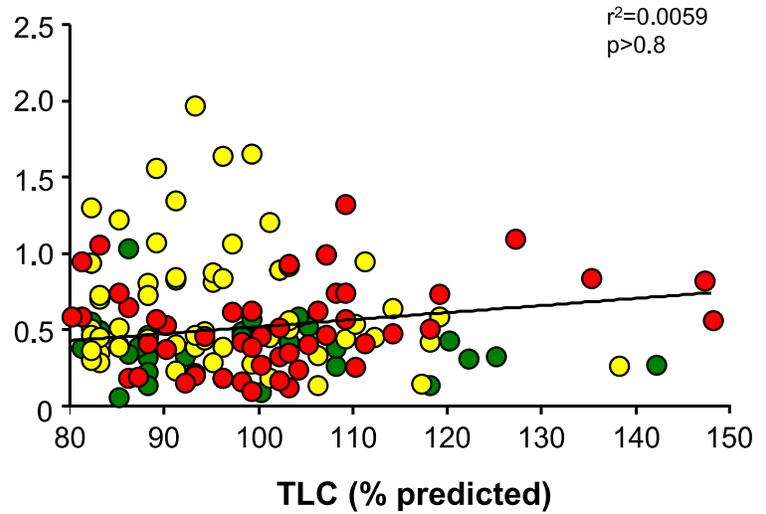
H. FVC



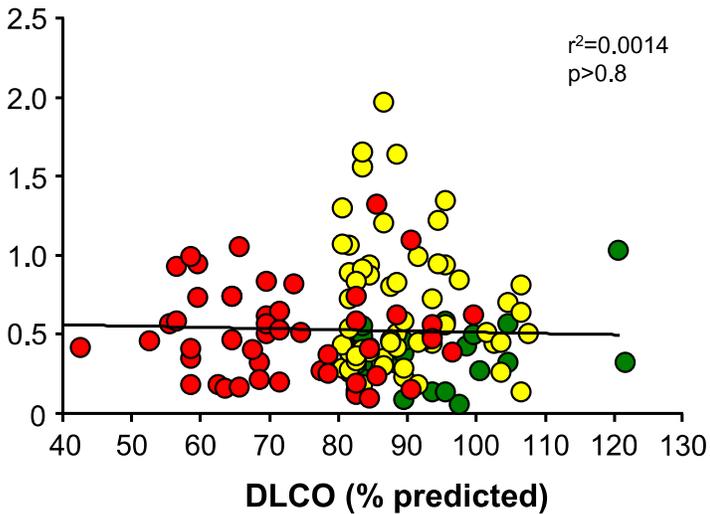
I. FEV1/FVC



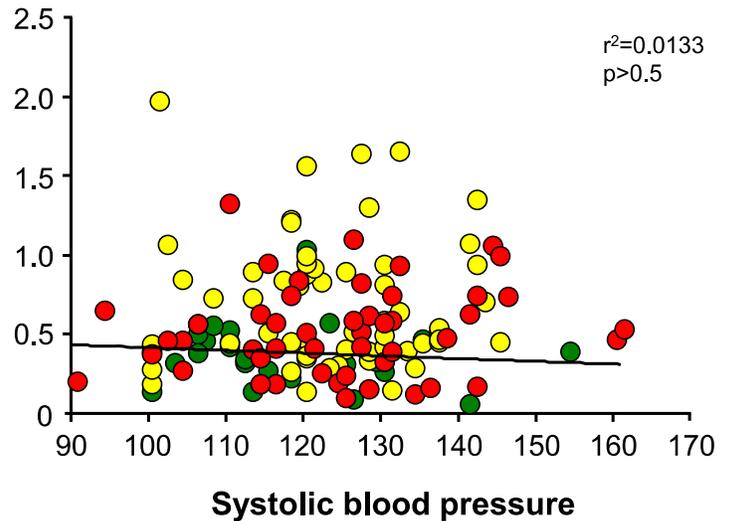
J. TLC

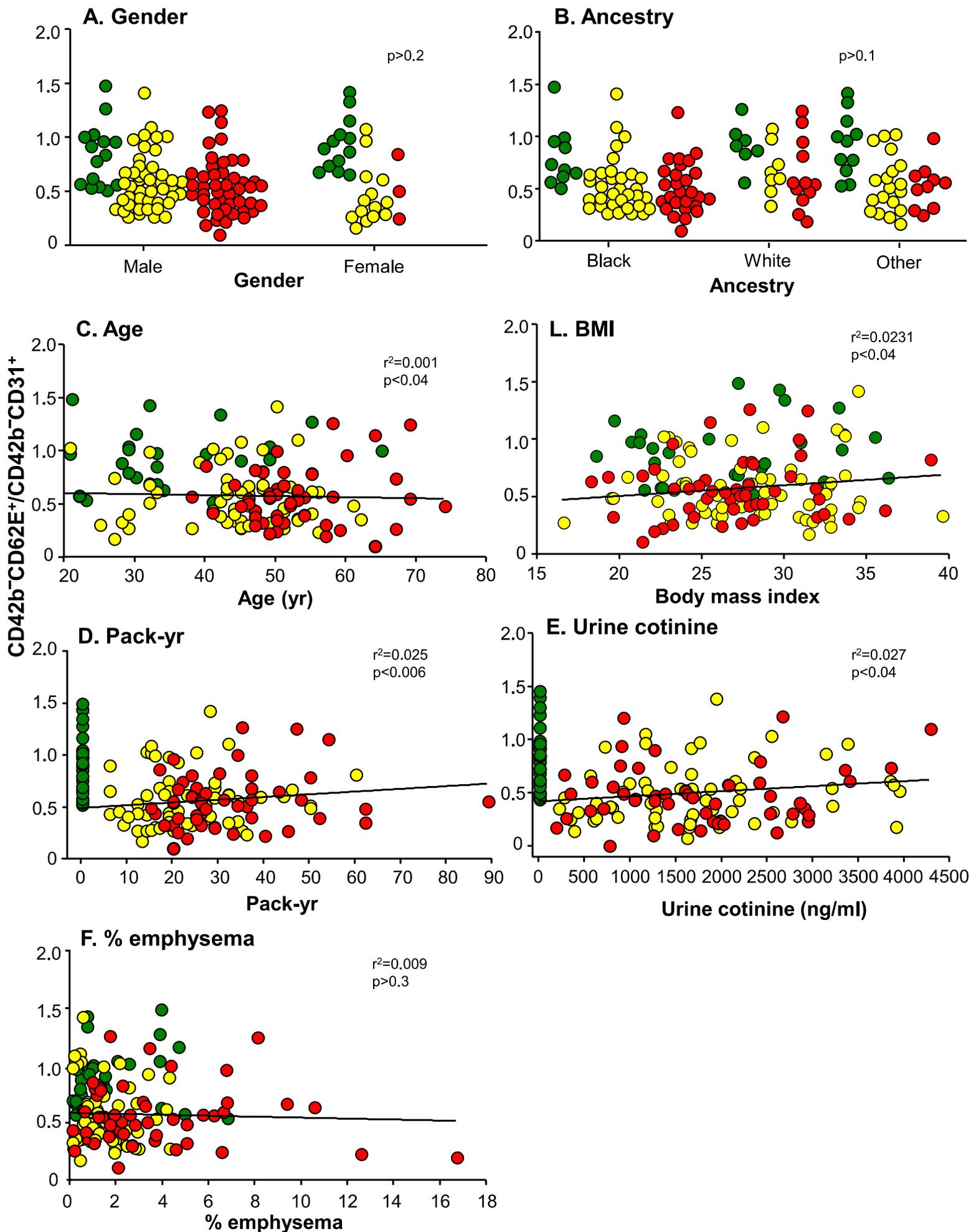


K. DLCO

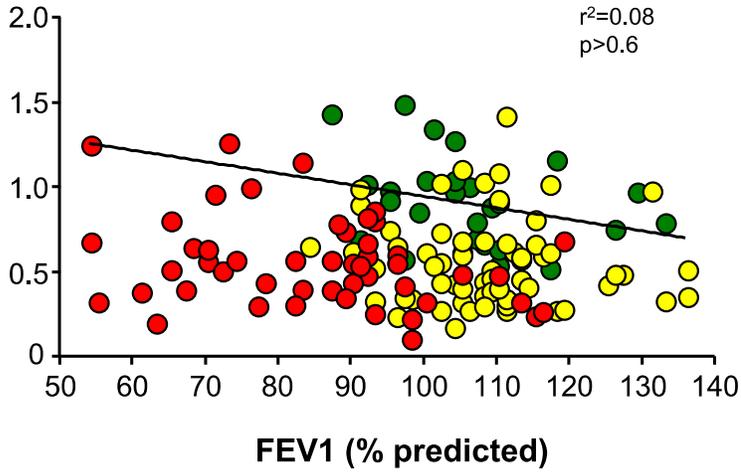


L. Blood pressure

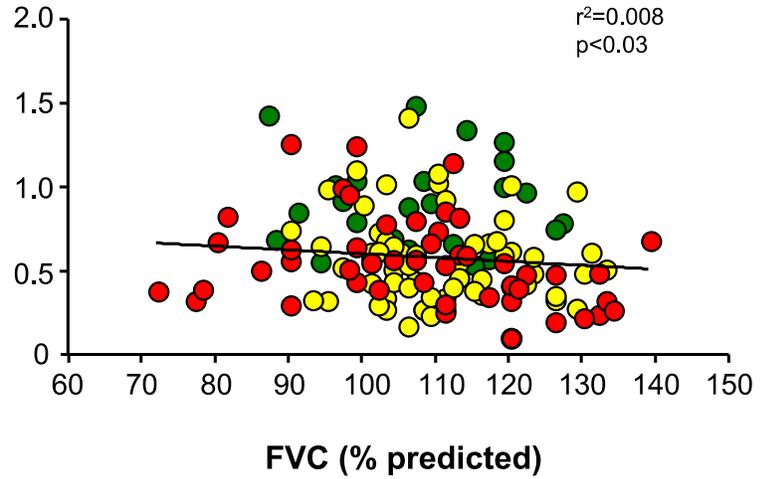




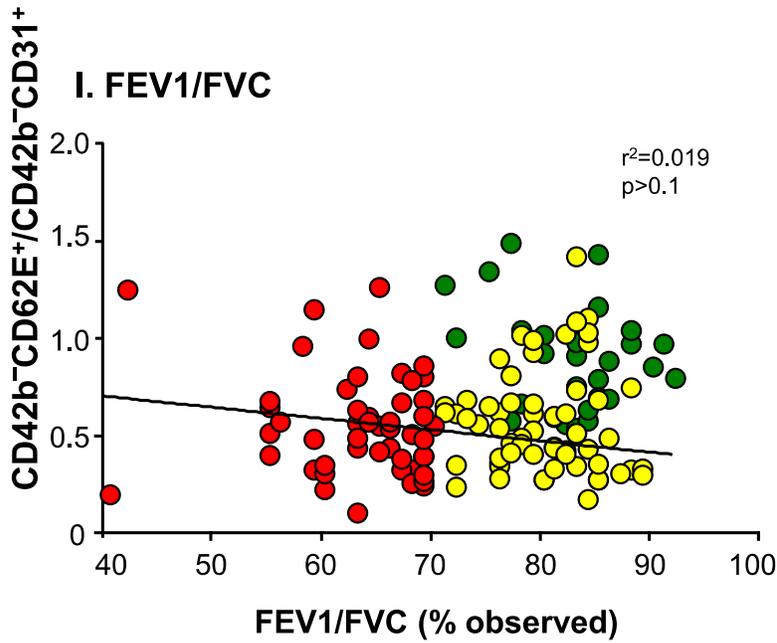
G. FEV1



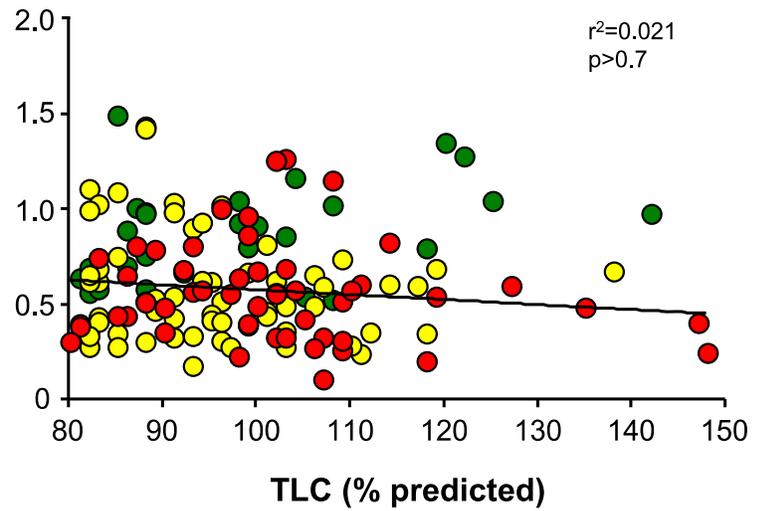
H. FVC



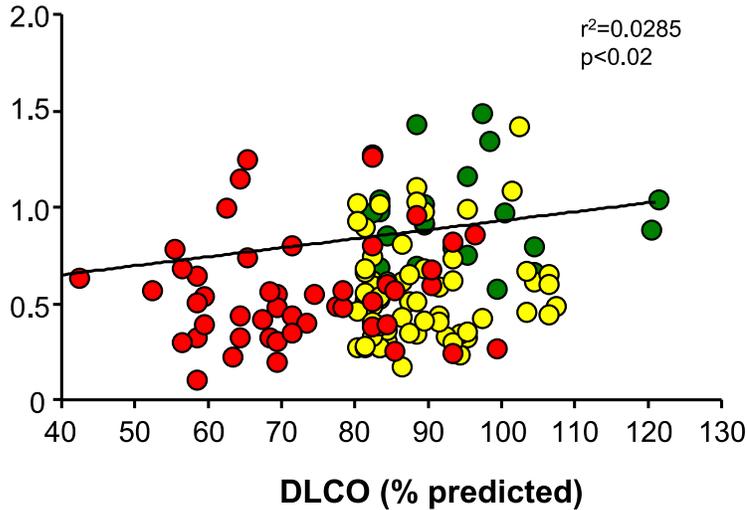
I. FEV1/FVC



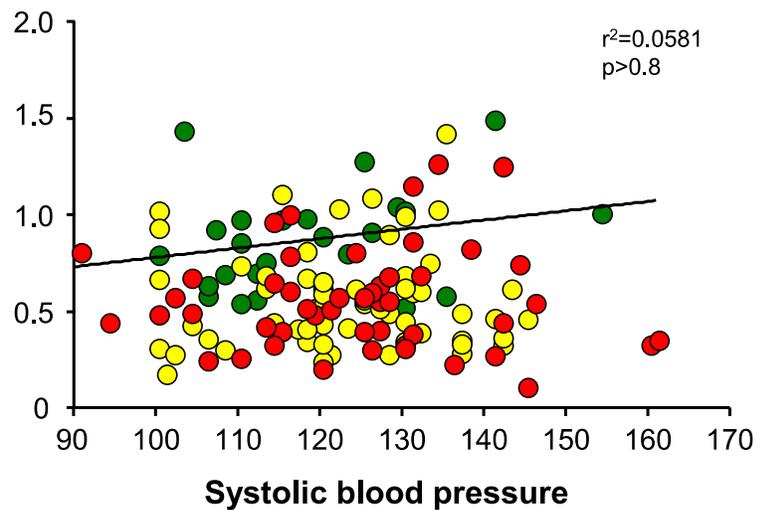
J. TLC



K. DLCO



L. Blood pressure



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Appendix V

Table X. Progression to COPD in Smokers with Normal Spirometry/Low DLCO vs Smokers with Normal Spirometry/Normal DLCO Using Different Methods to Determine Normal Levels*

Parameters		DLCO % predicted level cutoff used to define low DLCO					
		<80%			Below lower limit of normal calculated based on internal database		
		% developed COPD			% developed COPD		
		Normal DLCO	Low DLCO	p value	Normal DLCO	Low DLCO	p value
FEV₁/FVC ratio used to define COPD	GOLD-defined (< 0.7)²	3% (2/59)	22% (10/46)	<0.009	5% (4/74)	26% (8/31)	<0.008
	Below lower limit of normal calculated for each individual based on internal database³	2% (1/58)	22% (10/46)	<0.003	4% (3/73)	26% (8/31)	<0.004
	Below lower limit of normal calculated for each individual using the Quanjer tool^{4,*}	2% (1/59)	16% (7/45)	<0.03	1% (1/74)	23% (7/30)	<0.0007

* Adapted from Harvey B-G et al.¹⁷²

¹ COPD – chronic obstructive pulmonary disease; DLCO – diffusion capacity for carbon monoxide; FEV₁ – forced expiratory rate for 1 sec; FVC – forced vital capacity; GOLD – global initiative lung disease.

² Results detailed or summarized in the published manuscript (Article 2).

³ One normal spirometry/normal DLCO excluded from the study due to baseline FEV₁/FVC ratio below the lower limit of normal.

⁴ One normal spirometry/low DLCO excluded from the study due to baseline FEV₁ % predicted below the lower limit of normal.

* Quanjer et al.¹⁴⁷

Titre : Effets de la Fumée de Nargile sur la Santé du Poumon

Mots clés : nargile, fumée, poumon

Résumé : La Chicha qui sert à fumer du tabac parfumé est utilisée par des millions de personnes. Il y a peu de données sur les effets du chicha sur la santé, peu de régulation et les utilisateurs pensent que la chicha n'est ni addictif ni nocif. Pour évaluer les effets précoces de la chicha sur les poumons nous avons comparé des fumeurs de chicha occasionnels et des non fumeurs pour les paramètres cliniques et biologiques. L'utilisation de la chicha augmentait la toux et les expectorations ainsi que le niveau sanguin de carboxyhémoglobine. Ces modifications étaient associées à des modifications du métabolome des sécrétions pulmonaires, ainsi que de la modification de l'épithélium pulmonaire dans sa composition et son transcriptome. Les fumeurs présentaient une diminution de la capacité de diffusion qui est un marqueur prédictif du développement de la BPCO. Ils avaient également une augmentation du niveau plasmatique des microparticules endothéliales qui sont un marqueur de la destruction alvéolaire. Notre étude démontre que l'utilisation occasionnelle chez les jeunes de la chicha peut avoir des conséquences sur les maladies pulmonaires.

Title : Effects of Waterpipe Smoking on the Human Lung

Keywords : waterpipe, smoking, lung

Abstract : Waterpipe, an instrument for smoking fruit-flavored tobacco, is used by millions worldwide. There is limited data on its health effects, no regulations to its use, and users believe smoking it is not as harmful or addictive as cigarette smoking. To assess the early effects of waterpipe smoking on lung health, light-use waterpipe smokers with normal spirometry were assessed for lung clinical and biologic abnormalities compared to nonsmokers. Waterpipe smokers had increased cough and sputum, increased blood carboxyhémoglobin levels, abnormal lung epithelial lining fluid metabolome profile, abnormal small airway epithelium (SAE) cell composition, and markedly abnormal SAE and alveolar macrophage transcriptomes. They also had reduced diffusion capacity, a lung function marker of high risk for developing COPD in cigarette smokers, and high plasma levels of total and apoptotic endothelial microparticles, biomarkers of alveolar capillary destruction in COPD cigarette smokers that persists despite smoking cessation. These studies suggest that even young, light-use waterpipe smokers are likely at risk for developing lung disease.