Circulating Endothelial Microparticles as a Measure of Early Lung Destruction in Cigarette Smokers

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Rationale: **There is increasing evidence that emphysema is associated with primary loss of pulmonary capillary endothelium. Plasma levels of endothelial microparticles (EMPs), small vesicles released from activated or apoptotic endothelial cells, are elevated in vascular-related disorders.**

Objectives: **To evaluate whether plasma EMP levels are elevated in smokers with early lung destruction as assessed by normal spirometry but reduced diffusing capacity of the lung for carbon monoxide (D_{LCO}).**

Methods: **Lung health was assessed by pulmonary function tests (PFTs: spirometry, total lung capacity, D_{LCO}) and chest X-ray; smoking status was assessed by urine nicotine and cotinine. EMP levels ($CD42b^-CD31^+$ microparticles) were quantified as activated or apoptotic. The initial cohort (n = 92) included healthy nonsmokers (normal PFTs), healthy smokers (normal PFTs), and smokers with early evidence of lung destruction (normal spirometry, low D_{LCO}). Two prospective cohorts were then tested: a group similar to the initial cohort and an $HIV1^+$ cohort.**

Measurements and Main Results: **Healthy smokers had mildly increased levels of EMPs. Strikingly, 95% of smokers with normal spirometry, low D_{LCO} had increased EMPs, with reduced $CD62^+/CD31^+$ ratios ($P < 10^{-4}$) and elevated $CD42b^-CD31^+$ annexin V^+ EMPs ($P < 10^{-4}$), suggesting derivation from endothelial apoptosis. Most elevated EMPs were angiotensin-converting enzyme positive, suggesting derivation from pulmonary capillaries. Both prospective cohorts confirmed the initial cohort data.**

Conclusions: **Plasma EMPs with apoptotic characteristics are elevated in smokers with normal spirometry but reduced D_{LCO}, consistent with the concept that emphysema is associated, in part, with capillary endothelium apoptosis, suggesting that the early development of emphysema might be monitored with plasma EMP levels.**

Keywords: endothelium; apoptosis; endothelium-derived factors; microcirculation; smoking

Gas exchange takes place in the alveoli, fragile structures that bring air and blood in close contact through the alveolar epithelium, interstitial connective tissue, and capillary endothelium (1). When put under the chronic stress of cigarette smoking, alveoli may be destroyed, resulting in emphysema (2–6). The pathogenesis of emphysema is complex and includes the balance of proteases and antiproteases in the lung, tilted toward an excess of unopposed proteases that destroy the connective tissue backbone of the lung parenchyma (2–7). There is increasing evidence, however, that loss of alveolar endothelial cells by apoptosis is also central to the pathogenesis of lung destruction (3, 8–14).

The physiologic correlate of emphysema is a reduction in the diffusion capacity of the lung of carbon monoxide (D_{LCO}), a functional measure of the ability of the alveolar-capillary units to transfer gas from air to blood (15, 16). Eventually, as sufficient numbers of alveolar-capillary units are destroyed, the bronchial tree loses its supporting framework of surrounding alveoli, resulting in limitation to expiratory airflow (3, 17, 18). With this background, and in the context of the evidence that apoptosis of the pulmonary capillary endothelium participates in the pathogenesis of emphysema (8–13), we hypothesized that early in the process of lung destruction, smokers may have fragments of the endothelium in the circulation. This can be measured by quantifying circulating endothelial microparticles (EMPs), 0.1- to 1.5-μm vesicles, shed from the endothelium in response to cell activation, injury, and/or apoptosis (19–21). EMPs, quantified in plasma as particles that are $CD31^+$ (the constitutive endothelial marker PECAM), but $CD42b^-$ (the constitutive platelet-specific glycoprotein Ib), are present in low levels in plasma of healthy individuals and reflecting normal endothelial turnover (19, 21, 22). EMP levels are increased in a variety of vascular-related disorders (21, 23–37). Using CD62 (E-selectin, an adhesion molecule expressed on activated endothelium), activation-induced EMPs have a high $CD42b^-CD62^+/CD42b^-CD31^+$ ratio, and apoptosis-induced EMPs have a low ratio (19–21, 34).

Based on these considerations, we assessed the levels of circulating EMPs in a cohort of 92 subjects, including healthy nonsmokers, healthy and symptomatic smokers with normal lung function, and healthy smokers with normal spirometry but low D_{LCO} (i.e., smokers with early evidence of lung destruction before the development of expiratory airflow limitation). The data in this cohort, as well as in two prospective cohorts with

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary endothelial apoptosis is a mechanism in emphysema development. Increased endothelial apoptosis occurs in the lungs of smokers with emphysema and alveolar destruction may be initiated, in part, by apoptosis of pulmonary capillaries.

What This Study Adds to the Field

Smokers with evidence of emphysema may have elevated plasma levels of endothelial microparticles, released from activated or apoptotic endothelial cells. This study may imply a plasma-based method to identify early onset of smoking-induced emphysema.

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similar physiologic findings, demonstrate that smokers with normal spirometry and normal D_{LCO} have levels of circulating EMPs that are mildly elevated compared with healthy nonsmokers, but that smokers who are normal by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) spirometric criteria for chronic obstructive pulmonary disease (COPD) (38), but have reduced D_{LCO} (a parameter not part of the GOLD criteria), have marked increases in the levels of circulating EMPs. Most of these EMPs have a low $CD42b^-CD62^+/CD42b^-CD31^+$ ratio and elevated $CD42b^-CD31^+$ annexin V^+ levels, suggesting these EMPs arise, at least in part, by apoptosis (19–21, 34). Finally, the majority of the EMPs in the low D_{LCO} smokers are angiotensin-converting enzyme (ACE) positive, suggesting they are derived from the pulmonary capillary endothelium (39). Together, the data suggest that in the early stages of smoking-induced lung destruction, there is apoptosis-mediated loss of endothelium before any spirometric evidence of lung disease.

Some of these results have been previously reported in the form of an abstract (40).

METHODS

Human Subjects and Clinical Phenotypes

All individuals were evaluated at the Weill Cornell National Institutes of Health Clinical and Translational Science Center (CTSC) and Department of Genetic Medicine Clinical Research Facility, under Institutional Review Board–approved clinical protocols. Written informed consent was obtained from each individual before enrollment. Screening included history, complete physical examination, blood studies, urinalysis, chest X-ray, electrocardiogram, and pulmonary function tests (PFTs), including FVC, FEV_1, FEV_1/FVC, total lung capacity (TLC), and D_{LCO}, all performed under American Thoracic Society guidelines (41). If the FEV_1 was less than 80% predicted and/or the FEV_1/FVC less than 0.7, the spirometry was retested after standard bronchodilators (38, 42). Measurement of the D_{LCO} was performed two to four times in all individuals; the average of the best two trials was used. The diameter of the main pulmonary artery was assessed by chest X-rays as a correlate to the pulmonary artery pressure. In all individuals, the PA diameter was less than 30 mm, indicating normal estimated pulmonary pressure. Percentage emphysema was evaluated with the EmphylxJ software application (EmphylxJ; Vancouver, BC, Canada) allowing automated quantitative analysis of transverse chest computed tomography (CT) scans. Emphysema was defined as greater than 3% lung volume with attenuation less than or equal to −950 Hounsfield units (HU) or greater than 16% lung volume with attenuation less than or equal to −910 HU, values derived from analyses of high-resolution CT (HRCT) in normal nonsmoking individuals with normal lung function. Current smokers were defined as self-reported current smokers with verification of current smoking status by urinary levels of nicotine and its derivative cotinine. The last cigarette was more than 12 hours before all testing. All individuals had normal α_1-antitrypsin levels, normal C-reactive protein levels and all were HIV-1 negative (for full inclusion/exclusion criteria, *see* online supplement).

A total of 92 individuals were assessed as an initial study population (Table 1) using the following definitions: "healthy nonsmokers" (n = 32), lifelong never smokers with nondetectable urine nicotine (< 2 ng/ml) and cotinine (< 5 ng/ml), normal PFTs (spirometry, TLC, D_{LCO}) and chest X-ray; "healthy smokers with normal spirometry and normal D_{LCO}" (n = 41): asymptomatic active smokers with normal PFTs and chest X-ray (n = 32) and symptomatic smokers with normal PFTs and chest X-ray (n = 9), but with cough (0–4 scale [42]) and/or sputum production (0–4 scale [43]); and "healthy smokers with normal spirometry but low D_{LCO}" (n = 19): active smokers with normal spirometry and TLC, but reduced D_{LCO}.

In addition, a prospective study population of 60 individuals was assessed using the definitions as described above (Table 2). Prospective cohort 1 included a total of 45 individuals, including healthy nonsmokers (n = 10), healthy smokers with normal spirometry and normal D_{LCO} (n = 20; including asymptomatic active smokers [n = 12] and symptomatic active smokers [n = 8]), and healthy smokers with normal spirometry but low D_{LCO} (n = 15). Prospective cohort 2 assessed a total of 15 individuals classified by serological testing as $HIV1^+$ individuals, including healthy smokers with normal spirometry and normal D_{LCO} (n = 7; including asymptomatic active smokers [n = 5] and symptomatic active smokers [n = 2]) and healthy smokers with normal spirometry but low D_{LCO} (n = 8).

Characterization of Plasma EMPs

To quantify EMPs, a standard operating procedure was established (*see* Figure E1 and Table E1 in the online supplement) based on quality control experiments. Blood was collected in 5-ml sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) using a 21-gauge needle and, within 1 hour, centrifuged 10 minutes (160 × *g*, 23°C) to prepare platelet-rich plasma. Within 5 minutes, the supernatant was further centrifuged 8 minutes (1,000 × *g*, 23°C) to obtain platelet-poor plasma. Within 5 minutes, 50-μl aliquots of platelet-poor plasma were incubated (45 min, 4°C) with 4 μl of fluorescein-conjugated anti-human PECAM (CD31-FITC, clone WM59, optimized condition) and 5 μl phycoerythrin-conjugated anti-human E-selectin (CD62E-PE, clone 68-5H11; BD PharMingen, San Diego, CA; optimized condition). Four microliters of phycoallocyanine-conjugated anti-human CD42b (CD42b-APC, clone HIP1; optimized condition) was added (45 min, 4°C) to each sample to exclude platelet-derived microparticles. Single and double positive $CD42b^-CD31^+$ $CD62^+$ microparticles were determined by simultaneously incubating the plasma with all three specific antibodies. EMP measurements were performed twice to ensure that the measurements were repeatable. $CD42b^-CD31^+$ and $CD42b^-CD62^+$ microparticle levels were corrected for correlating isotype control antibodies. Five microliters of anti-human CD45-PECy5 (leukocyte marker, clone HI30; optimized condition) was also used to monitor leukocyte MP contamination.

To assess the presence of relative contribution of pulmonary capillary endothelium to the elevated EMPs, $CD42b^-CD31^+$ microparticles were costained with 5 μl phycoerythrin-conjugated anti-human ACE (CD143, clone 171417; R&D, Minneapolis, MN; optimized condition) based on the knowledge that ACE is abundantly expressed on pulmonary capillary endothelium (39).

To further evaluate whether the elevated $CD42b^-CD31^+$ EMPs were derived from apoptotic endothelial cells, the EMPs were also assessed by annexin V staining for the presence of phosphatidylserine, a marker linked to apoptosis (32, 33, 37). To accomplish this, the EMPs were labeled using phycoerythrin-conjugated annexin V (BD Pharmingen) in the presence of $CaCl_2$ (5 mM) according to manufacturer's recommendation.

EMP phenotype analysis was performed within 15 minutes based on size and fluorescence. Events less than 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), and analyzed by two- or three-color fluorescence histograms as $CD42b^-CD31^+$, $CD42b^-CD62^+$, $CD42b^-CD31^+$ ACE^+, or $CD42b^-CD31^+$annexin V^+ microparticles. EMP levels were assessed by comparison with calibrator Flowcount beads (10-μm diameter; Beckman Coulter, Miami, FL) with a known concentration, using 30-second stop time, with log gain on forward and sideward light scatter and fluorescence. Single antibody conjugates and compensation fluorochrome beads were used for compensation assessment. Samples were acquired at band pass filters: 530 nm (FITC), 585 nm (PE/PI), and 661 nm (APC) with FL4 option. EMPs were quantified by flow cytometry using Cell Quest-Pro software (FACSCalibur; BD Bioscience, San Jose, CA), by investigators blinded to subject status. The data were analyzed using FlowJo software (Tree Star, OR). A high ratio of $CD42b^-CD62^+$ to $CD42b^-CD31^+$ were defined as "activated" and those with a ratio less than the lowest healthy nonsmoker (< 0.7, *see* RESULTS) as "apoptotic" (19–21, 34). The percentage of annexin V^+ EMPs 2 SDs above that for healthy nonsmokers was considered "apoptotic" (Figure E4C).

Statistical Analysis

We used several linear modeling approaches to test for effects on $CD42b^-CD31^+$ EMP level due to phenotype (healthy nonsmoker, healthy smoker with normal spirometry and normal D_{LCO}, and healthy smoker with normal spirometry but low D_{LCO}) and to each of the measured clinical characteristics (D_{LCO}, FEV_1, FVC, FEV_1/FVC, TLC,

TABLE 1. INITIAL STUDY POPULATION

Parameter	Group A: Healthy Nonsmokers with Normal Spirometry and Normal D_{LCO}	Group B: Healthy Smokers with Normal Spirometry and Normal D_{LCO}*	Group C: Healthy Smokers with Normal Spirometry but Low D_{LCO}
n	32	41	19
Sex, male/female	14/18	31/10	15/4
Age, yr	37 ± 15	40 ± 9	46 ± 8
Ancestry, B/W/O	9/14/9	31/4/6	15/2/2
Smoking history, pack-years	0	19 ± 13	34 ± 19
Urine nicotine, ng/ml	Negative	1,041 ± 1,136	1,500 ± 1,459
Urine cotinine, ng/ml	Negative	1,565 ± 664	1,715 ± 1,132
Pulmonary function†			
FEV_1	106 ± 14	106 ± 12	104 ± 15
FVC	108 ± 14	111 ± 12	108 ± 14
FEV_1/FVC	82 ± 5	79 ± 6	78 ± 5
TLC	100 ± 15	95 ± 10	98 ± 17
D_{LCO}	95 ± 15	91 ± 9	70 ± 7
C-reactive protein (mg/dl)	0.44 ± 0.24	0.51 ± 0.51	0.41 ± 0.26

Definition of abbreviations: B/W/O = black/white/other; D_{LCO} = diffusion capacity of the lung for carbon monoxide; TLC = total lung capacity.

Data are presented as mean ± SD. Normal D_{LCO} value ⩾ 80% predicted. There were no differences between the three groups ($P > 0.05$, all comparisons) except for the low D_{LCO} in group C ($P < 0.05$, compared to groups A and B), and pack-years, smoking metabolites, sex, and ancestry in group A ($P < 0.05$, compared to groups B and C).

* Combined asymptomatic and symptomatic (cough and/or sputum production) smokers, all with normal lung function. There was no significant difference between asymptomatic and symptomatic smokers in any parameter ($P > 0.4$, all comparisons, except urine cotinine $P < 0.04$).

† Pulmonary function testing parameters are given as % of predicted value with the exception of FEV_1/FVC, which is reported as % observed. For healthy nonsmokers and healthy and symptomatic smokers with D_{LCO} ⩾ 80%, FVC, FEV_1 and FEV_1/FVC are prebronchodilator values. For healthy smokers with $D_{LCO} < 80\%$, FVC, FEV_1, and FEV_1/FVC are post-bronchodilator values.

and blood pressure); for the former we considered an analysis of variance coding and for latter a regression coding. We performed these tests without any covariates and when including covariates for age, sex, and pack-years; for each we used a regression coding. Inclusion of these covariates did not alter the significance of tests with phenotype or any of the measured clinical characteristics, so only the analyses without covariates are presented. We also performed these same analyses after removing the individuals with diabetes, hypertension, or both. Again, removing these individuals produced no qualitative effect on the test results or significance of any of the tests, so only the analyses including the entire sample are presented. To guard against deviations from parametric assumptions, a nonparametric permutation test was performed for these models; for each permutation we randomized the $CD42b^{-}CD31^{+}$ EMP values with respect to the samples. The linear model analysis was then applied to each permuted data set and a nonparametric P value was obtained using the ordering of P values

TABLE 2. PROSPECTIVE STUDY POPULATIONS

	Prospective Cohort 1			Prospective Cohort 2	
Parameter	Group D: Healthy Nonsmokers with Normal Spirometry and Normal D_{LCO}	Group E: Healthy Smokers with Normal Spirometry and Normal D_{LCO}*	Group F: Healthy Smokers with Normal Spirometry but Low D_{LCO}	Group G: HIV1+ Smokers with Normal Spirometry and Normal D_{LCO}	Group H: HIV1+ Smokers with Normal Spirometry and Low D_{LCO}
n	10	20	15	7	8
Sex, male/female	5/5	15/5	9/6	4/3	3/5
Age, yr	42 ± 12	44 ± 9	45 ± 10	42 ± 7	47 ± 3
Ancestry, B/W/O	4/3/3	11/3/6	9/3/3	5/0/2	6/1/1
Smoking history, pack-years	0	21 ± 15	23 ± 14	33 ± 29	30 ± 22
Urine nicotine, ng/ml	Negative	1,508 ± 1,710	1,320 ± 1,453	297 ± 301	1,557 ± 1,478
Urine cotinine, ng/ml	Negative	1,593 ± 1,193	1,361 ± 1,041	1,329 ± 881	1,334 ± 704
Pulmonary function†					
FEV_1	105 ± 12	108 ± 13	106 ± 22	99 ± 16	103 ± 16
FVC	108 ± 13	112 ± 12	109 ± 25	103 ± 9	105 ± 15
FEV_1/FVC	81 ± 5	80 ± 6	80 ± 7	79 ± 8	80 ± 7
TLC	101 ± 19	98 ± 15	99 ± 16	85 ± 7	90 ± 10
D_{LCO}	87 ± 10	88 ± 10	66 ± 9	90 ± 14	66 ± 5
C-reactive protein (mg/dl)	0.6 ± 0.2	0.5 ± 0.02	0.6 ± 0.2	0.6 ± 0.3	0.8 ± 1.0

Definition of abbreviations: B/W/O = black/white/other; D_{LCO} = diffusion capacity of the lung for carbon monoxide; TLC = total lung capacity.

Data are presented as mean ± SD. Normal D_{LCO} value ⩾ 80% predicted. There were no differences between groups D, E, and F ($P > 0.05$, all comparisons) except for the low D_{LCO} in group F ($P < 0.05$, compared to groups D and E), and pack-years, smoking metabolites, sex, and ancestry in group D ($P < 0.05$, compared to groups E, F, G, and H). Except for the low D_{LCO} in group H ($P < 0.01$, compared to group G) and the urine nicotine level ($P < 0.02$, comparing group G and H), there were no differences between groups G and H ($P > 0.5$, all comparisons).

* Combined asymptomatic and symptomatic (cough and/or spleen production) smokers, all with normal lung function. There was no significant difference between asymptomatic and symptomatic smokers in any parameter ($P > 0.5$, all comparisons) except urine cotinine ($P < 0.05$).

† Pulmonary function testing parameters are given as % of predicted value with the exception of FEV_1/FVC, which is reported as % observed. For healthy nonsmokers and healthy and symptomatic smokers with D_{LCO} ⩾ 80%, FVC, FEV_1, and FEV_1/FVC are prebronchodilator values. For healthy smokers with $D_{LCO} < 80\%$, FVC, FEV_1, and FEV_1/FVC are post-bronchodilator values.

obtained from 1,000 permutations. The *P* values obtained using the parametric and permutation approach were very close and produced no qualitative difference in the outcomes. We therefore present only the parametric analyses.

RESULTS

EMP Levels

Healthy smokers with normal spirometry and normal D_{LCO} had a mild increase in EMP levels compared with healthy nonsmokers, as did symptomatic smokers compared with healthy nonsmokers ($P < 10^{-4}$ compared with both groups, Figure 1). There was no difference between healthy and symptomatic smokers ($P > 0.4$). In striking contrast, healthy smokers with normal spirometry (i.e., do not have GOLD criteria COPD) but low D_{LCO} had a significant increase in EMP levels ($P < 10^{-4}$ compared with healthy nonsmokers; $P < 10^{-3}$ compared with healthy smokers). A few healthy smokers with normal D_{LCO} and healthy smokers with low D_{LCO} had comorbidities known to be associated with elevated EMPs (systemic hypertension and/or type 2 diabetes); removal of these subjects from the data did not change the results. No individuals had other comorbidities associated with increased circulating EMPs.

When assessed as percent cumulative frequency of subjects in each group with elevated EMPs, the healthy nonsmoker population was distributed between 0 to 500 EMP/μl, whereas 50% of healthy smokers had EMP levels above the normal range of healthy nonsmokers ± 2 SD (Figure E2). In contrast, 95% of healthy smokers with normal spirometry and low D_{LCO} had EMP levels above the range of healthy smokers, with 52% distributed between 500 and 1,250 EMP/μl and 43% greater than 1,250 EMP/μl. Assessed with all groups together, the best correlations of EMP levels with individual clinical parameters

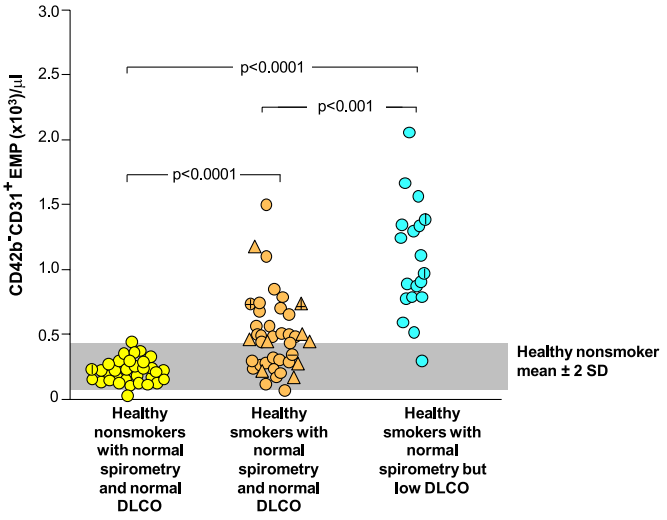

Figure 1. Levels of $CD42b^-CD31^+$ endothelial microparticles (EMPs) per μl in platelet-poor plasma of the study groups. Shown are data for healthy nonsmokers with normal spirometry and normal diffusing capacity of the lung for carbon monoxide (D_{LCO}) (n = 32, *yellow circles*), healthy smokers with normal spirometry and normal D_{LCO} (combining asymptomatic smokers, n = 32, *tan circles*, and symptomatic smokers, n = 9, *tan triangles*), and healthy smokers with normal spirometry and low D_{LCO} (n = 19, *blue circles*). *P* values are indicated. For all groups, a *vertical line* indicates a subject with systemic hypertension, a *horizontal line* indicates a subject with type 2 diabetes mellitus. The *gray shaded area* indicates the mean ± 2 SD of $CD42b^-CD31^+$ EMP/ml platelet of healthy nonsmokers.

were with pack-years, D_{LCO}, FEV_1/FVC, and urine cotinine, with less correlation with urine nicotine, age, blood pressure, or other lung function parameters (Figure E3). Assessed within individual subject groups, there were limited correlations of EMP levels with individual clinical parameters (Table E2). Automated quantification of emphysema levels by transverse chest CT scans also showed a low correlation pattern of emphysema with urine nicotine level, EMPs, or D_{LCO} between all groups (Figure E5) and no differences in emphysema levels between all groups (Figure E6).

None of the covariates were considered significant ($P > 0.1$) except for pack-years. Therefore, *P* values for the analysis of variance test are reported without including additional covariates except those involving comparisons of all smoking groups, in which pack-years as covariate was included. There were no qualitative differences in *P* values obtained from the parametric versus the nonparametric analyses; therefore, the presented results are based on parametric analyses. There was no correlation of EMP levels and age, sex, or ethnicity ($P > 0.1$, all comparisons).

Source of the EMPs

In the context that smoking likely affects multiple vascular beds, the EMPs were assessed for the proportion that were positive for ACE, a surface protein more highly expressed on pulmonary capillary endothelium compared with other endothelial beds (39) (Figure 2). This analysis showed that 55% of the $CD42b^-CD31^+$ EMPs in healthy smokers with normal spirometry and normal D_{LCO} were ACE^+ beyond that observed for healthy nonsmokers ($P < 0.02$ compared with healthy nonsmokers), whereas 76% of the $CD42b^-CD31^+$ EMPs in healthy smokers with normal spirometry but low D_{LCO} were ACE^+ ($P < 0.001$ compared with healthy nonsmokers) (i.e., the majority of the elevated EMPs in the low D_{LCO} group were derived from pulmonary capillary endothelium).

Apoptotic Versus Activated EMPs

Aside from a few outliers, the $CD42b^-CD62^+/CD42b^-CD31^+$ ratio of the healthy nonsmokers was distributed around a mean of 1.09, with the lowest value 0.7 (Figure 3). On the average, all groups of smokers had some $CD42b^-CD62^+/CD42b^-CD31^+$ EMPs less than the lowest level observed in the healthy nonsmokers (39%, mean level 1.09 ± 0.38, $P < 0.05$). By far, however, the highest proportion of EMPs with the lowest $CD42b^-CD62^+/CD42b^-CD31^+$ ratio was observed in the healthy smokers with low D_{LCO} (79%, mean level 0.51 ± 0.22 vs. 1.09 ± 0.38 for healthy nonsmokers, $P < 10^{-4}$).

Replication in Prospective Cohorts

To verify the observations in the initial cohort of elevated EMPs in healthy smokers with normal spirometry but low D_{LCO}, a prospective cohort of 45 individuals was assessed, including healthy nonsmokers, healthy smokers with normal D_{LCO}, and healthy smokers with low D_{LCO} (cohort 1, Table 2, Figure 4). The data in the prospective cohort 1 replicated that in the initial cohort, with significantly increased $CD42b^-CD31^+$ EMPs in healthy smokers with normal D_{LCO} compared with healthy nonsmokers ($P < 10^{-4}$), healthy smokers with low D_{LCO} compared with healthy nonsmokers ($P < 10^{-4}$), and healthy smokers with low D_{LCO} compared with healthy smokers ($P < 0.01$; Figure 4A). Likewise, the prospective cohort also had more apoptotic-derived EMPs in healthy smokers with normal D_{LCO} compared with healthy nonsmokers ($P < 10^{-3}$) and healthy smokers with low D_{LCO} compared with healthy nonsmokers ($P < 10^{-4}$; Figure 4B). By these criteria, 79% of

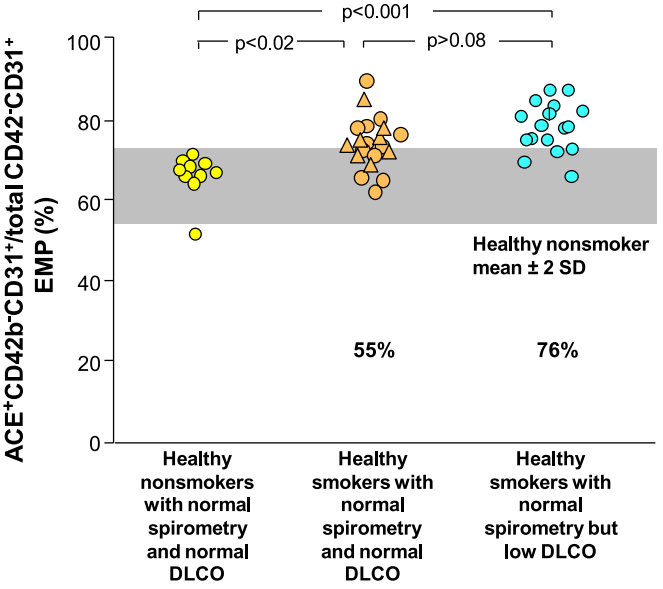

Figure 2. Proportion of $CD42b^{-}CD31^{+}$ endothelial microparticles (EMPs) that express angiotensin-converting enzyme (ACE^{+}). Shown are data for healthy nonsmokers with normal spirometry and normal diffusing capacity of the lung for carbon monoxide (D_{LCO}) (n = 10, *yellow circles*), healthy smokers with normal spirometry and normal D_{LCO} (combining asymptomatic smokers, n = 12, *tan circles*, and symptomatic smokers, n = 8, *tan triangles*), and healthy smokers with normal spirometry and low D_{LCO} (n = 17, *blue circles*). *P* values are indicated. For all groups, a *vertical line* indicates the subject has systemic hypertension. *Gray shaded area* represents range ± 2 SD of healthy nonsmokers. The % values represent the proportion of individuals in that group who had higher levels of $CD42b^{-}CD31^{+}ACE^{+}$ EMPs beyond the level observed for healthy nonsmokers.

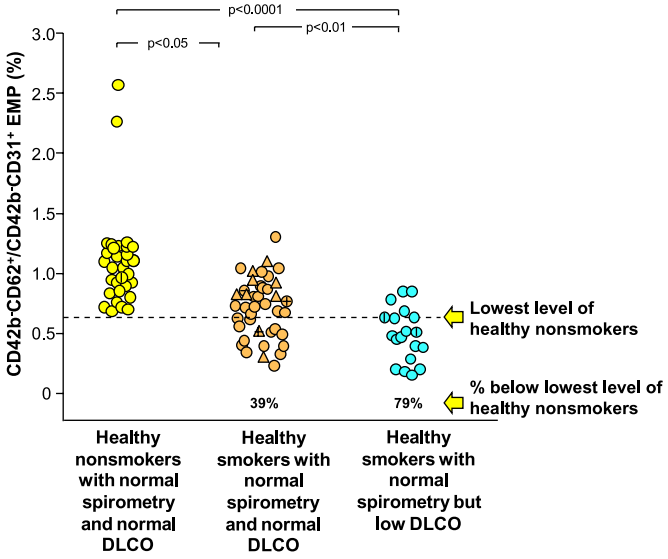

Figure 3. Ratio of circulating $CD42b^{-}CD62^{+}$ to $CD42b^{-}CD31^{+}$ endothelial microparticles (EMPs) in plasma of healthy nonsmokers with normal spirometry and normal diffusing capacity of the lung for carbon monoxide (D_{LCO}) (n = 32, *yellow circles*), healthy smokers with normal spirometry and normal D_{LCO} (combining healthy smokers, n = 32, *tan circles*, and symptomatic smokers, n = 9, *tan triangles*), and healthy smokers with normal spirometry and low D_{LCO} (n = 19, *blue circles*). *P* values are indicated. For all groups, a *vertical line* indicates the subject has systemic hypertension, a *horizontal line* indicates the subject has type 2 diabetes mellitus. The *dashed line* represents the value below any subject in the healthy nonsmoker group. The % values below represent the proportion of individuals in that group below the lowest level of healthy nonsmokers.

the EMPs of the healthy smokers with low D_{LCO} were apoptotic-like, as were 44% of the EMPs of the healthy smokers with normal D_{LCO}. The apoptotic nature of the EMPs was confirmed by annexin V staining, with 50% more $annexinV^{+}$ EMPs in healthy smokers with normal D_{LCO} and 66% more EMPs in healthy smokers with low D_{LCO} compared with healthy nonsmokers ($P < 0.002$ and $P < 10^{-4}$, respectively; Figure E4).

As a further verification that EMPs are elevated in association with early lung destruction in smokers with normal spirometry and low D_{LCO} and based on the knowledge that smokers who are $HIV1^{+}$ have an accelerated form of emphysema (44), we assessed a second prospective cohort, smokers who were $HIV1^{+}$, both those with normal spirometry and normal D_{LCO} and those with normal spirometry and low D_{LCO} (cohort 2; Table 2, Figure 5). Parallel to the initial cohort and the first prospective cohort, the $HIV1^{+}$ low D_{LCO} group had significantly more $CD42b^{-}CD31^{+}$ EMPs than the $HIV1^{+}$ with normal D_{LCO} group ($P < 10^{-3}$; Figure 5A), with 75% of apoptotic-like EMPs in the $HIV1^{+}$ low D_{LCO} group beyond that of the $HIV1^{-}$ nonsmokers (Figure 5B).

DISCUSSION

Based on the knowledge that smoking is the major cause of COPD, that destruction of alveoli is a common component of COPD, and increasing evidence that alveolar destruction may be initiated, in part, by apoptosis of pulmonary capillaries (2–6, 8–14, 38), we hypothesized that smokers with evidence of lung destruction may have elevated plasma levels of EMPs, plasma membrane fragments released when endothelial cells are activated or undergo apoptosis (19–21, 31, 34, 83). As a measure of lung destruction, we used the D_{LCO}, a lung function measure of the functional intactness of the alveolar-capillary bed (15, 16). Healthy smokers and symptomatic smokers with normal spirometry and D_{LCO} had mildly elevated levels of circulating EMPs compared with healthy nonsmokers. Strikingly, however, healthy smokers with normal spirometry but an isolated reduction in D_{LCO} had high levels of circulating EMPs compared with all other groups, with the EMPs likely derived from endothelial cells undergoing apoptosis, and likely mostly from pulmonary endothelium. This observation was replicated in a prospective parallel group of smokers, as well as in $HIV1^{+}$ smokers with low D_{LCO}.

Endothelial Microparticles

Microparticles are submicron membrane vesicles shed from the plasma membranes of different cell types in response to cell activation, injury, and/or apoptosis (19–21, 31, 34, 83). Microparticles in the plasma of healthy subjects are derived from platelets, leukocytes, and endothelial cells (45–47). EMPs are distinguished from microparticles of other cell types by size, constitutive expression of the platelet–endothelial cell adhesion marker CD31, and the absence of the platelet-specific glycoprotein Ib marker CD42b (19, 21, 45). Apoptosis-induced EMPs are more likely to express only CD31 and show the presence of phosphatidylserine (annexin V) as an apoptotic parameter (32, 33, 37), whereas activation-induced EMPs have increased expression of the inducible endothelial marker CD62 (19–21).

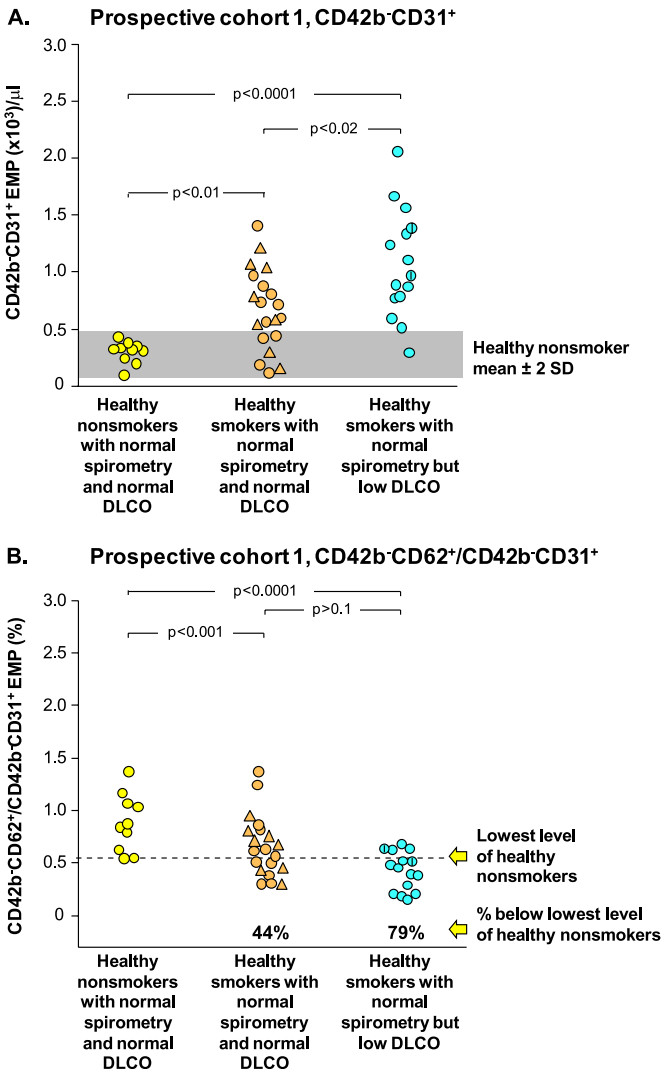

Figure 4. Prospective study cohort 1: plasma endothelial microparticles (EMPs) in a prospective group of healthy nonsmokers with normal spirometry and normal diffusing capacity of the lung for carbon monoxide (D_{LCO}) (n = 10, *yellow circles*), healthy smokers with normal spirometry and normal D_{LCO} (combining healthy smokers, n = 12, *tan circles*, and symptomatic smokers, n = 8, *tan triangles*), and healthy smokers with normal spirometry and low D_{LCO} (n = 15, *blue circles*). *P* values are indicated. For all groups, a vertical line indicates the subject has systemic hypertension. (*A*) Levels of $CD42b^-CD31^+$ EMPs in platelet-poor plasma of the study groups. (*B*) Ratio of circulating $CD42b^-CD62^+$ to $CD42b^-CD31^+$ EMPs in plasma of study groups. The *dashed line* represents the value below any subject in the healthy nonsmoker group; the % values below represent the proportion of that group below the lowest level of healthy nonsmokers.

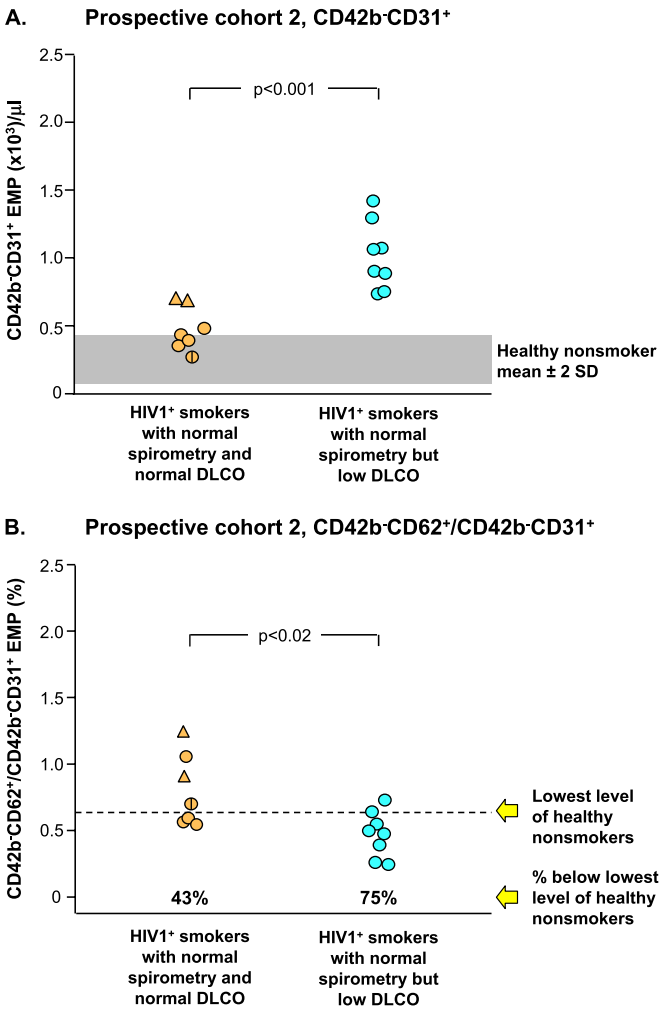

Figure 5. Prospective study cohort 2: endothelial microparticles (EMPs) in a prospective group of $HIV1^+$ healthy smokers with normal spirometry and normal diffusing capacity of the lung for carbon monoxide (D_{LCO}) (combining healthy smokers, n = 5, *tan circles*, and symptomatic smokers, n = 2, *tan triangles*) and $HIV1^+$ healthy smokers with normal spirometry and low D_{LCO} (n = 8, *blue circles*). *P* values are indicated. For all groups, a *vertical line* indicates the subject has systemic hypertension. (*A*) Levels of $CD42b^-CD31^+$ EMPs in platelet-poor plasma of the study groups. (B) Ratio of circulating $CD42b^-CD62^+$ to $CD42b^-CD31^+$ EMPs in plasma of study groups. The *dashed line* represents the value below any subject in the healthy nonsmoker group; the % values below represent the proportion of that group below the lowest level of healthy nonsmokers.

Elevated levels of $CD42b^-CD31^+$ EMPs have been associated with vascular disease and endothelial dysfunction in patients with acute coronary syndromes, severe hypertension, metabolic syndrome, type 2 diabetes, end-stage renal disease, pulmonary arterial hypertension, subclinical atherosclerosis, heart failure, stroke, thrombotic thrombocytopenic purpura, lupus anticoagulant syndrome and other vasculitides, multiple sclerosis, and sickle cell disease (19, 21, 23–37, 46, 48–64).

One of the burdens of smoking is injury to the lung endothelium (10, 65–67). Consistent with this, we observed that, to some extent, all smoking groups (healthy smokers, symptomatic smokers), had elevation of EMPs compared with healthy nonsmokers. Consistent with this, Heiss and colleagues (68) showed that healthy nonsmokers exposed for 30 minutes to low levels of cigarette smoke had increased EMP levels. Together, the data suggest that smoking *per se* causes sufficient endothelial changes to mildly raise plasma EMP levels. Moreover, our comparison of the EMP levels of healthy smokers, symptomatic smokers, and smokers with normal spirometry and low D_{LCO} demonstrates significant variation in EMP levels among these smokers, with the highest, by far, in healthy smokers with normal spirometry and low D_{LCO}. Although there is increasing evidence of alveolar destruction initiated, in part, by apoptosis of pulmonary capillaries (2–6, 8–14, 38), more complementary measures of lung vascular damage in addition to D_{LCO} have to be undertaken to underline the association between EMPs and lung destruction. The data in the present study suggest that

elevated levels of EMP correlate with an early onset of lung destruction (i.e., normal spirometry/low D_{LCO} group) and that the EMPs may confer to a more apoptotic nature of their parental endothelial origin.

Endothelial Apoptosis and Emphysema

The concept of pulmonary endothelial apoptosis as a primary mechanism in the development of emphysema is supported by the observation of endothelial apoptosis in the lungs of humans with emphysema (8–14). Segura-Valdez and colleagues (69) showed increased DNA fragmentation in the pulmonary capillaries and arteriolar endothelium of individuals with COPD, and Kasahara and colleagues (8–10) reported increased septal cell death (endothelial and epithelial cells) in human emphysematous lungs compared with lungs of nonsmokers or smokers without emphysema. Although the mechanisms associated with this endothelial loss are likely complex, there is evidence that reduced levels of alveolar epithelial-derived vascular endothelial growth factor may play a role (9, 10, 65).

Our study provides a plasma-based assessment of this endothelial destruction by measuring the level of plasma EMPs in smokers without and with alveolar loss as measured by decreased D_{LCO}. The presence of increased levels of $CD42b^{-}CD31^{+}$ EMPs with a low $CD42b^{-}CD62^{+}$ to $CD42b^{-}CD31^{+}$ ratio in individuals with normal spirometry and low D_{LCO} further supports the vascular theory of emphysema by suggesting that apoptosis plays a central role in the early destruction of alveolar endothelium.

Early Detection of Lung Destruction

As defined by the GOLD standards, the diagnosis of COPD is based on lung function criteria as a persistent limitation to forced expiratory airflow after treatment with bronchodilators (38). Although this is a useful unified definition, airflow limitation is a relatively crude measure of lung health, as the lung is redundant, and the GOLD COPD minimum criteria of FEV_1/FVC less than 0.7 after bronchodilators occurs only after considerable abnormalities are present (38, 42, 70–73). It has long been recognized that the limitation of forced expiratory airflow observed in COPD can result from intrinsic disease of the airways (chronic bronchitis) and/or destruction of the alveoli (emphysema), with most affected individuals having some contribution of both airway and alveolar disease (2–4, 6, 17, 18). The observation of limitation to forced expiratory airflow after bronchodilators does not indicate whether the cause is intrinsic airway disease and/or alveolar destruction (2–4, 6, 17, 18).

The traditional diagnosis of COPD with emphysema relies on pulmonary function tests demonstrating airflow obstruction and a low D_{LCO} (1, 2, 4, 6, 17, 18, 38, 42). HRCT imaging detects early emphysema by identifying pulmonary tissue with radiologic attenuation below a predetermined threshold, findings that roughly correlate with a low D_{LCO} and pathologic evidence of emphysema (74–80). Although several studies have shown that a significant proportion of asymptomatic smokers have HRCT evidence of emphysema (78, 81–83), early HRCT findings of "emphysema" are not proven to be correlated directly with lung destruction (84–90). Hyperpolarized gas diffusion-weighted magnetic resonance imaging has also been used to identify emphysema, with a correlation of elevated levels of the apparent diffusion coefficient with decreased D_{LCO} (91). We have observed that smokers with normal spirometry and low D_{LCO} are at higher risk for the development of COPD as defined by the GOLD criteria than are smokers with normal spirometry and normal D_{LCO} (92), but there was no direct correlation of emphysema with EMP levels or D_{LCO}. This was not surprising, as healthy smokers with normal spirometry and normal D_{LCO} without any clinical evidence of emphysema showed increased EMP levels as well, indicating that the complexity of the correlation between EMP and smoking-induced early vascular lung endothelium damage may not exclusively rely on the presence of emphysema as detailed by conventional clinical parameters such as D_{LCO} and/or chest HRCT. For future studies it will be of interest to assess measures of endothelial dysfunction to determine if EMP levels are related to early emphysema independent of endothelial dysfunction.

Assessment of EMP levels may provide an early and inexpensive approach to identifying early evidence of emphysema, without the radiation exposure associated with chest HRCT. Interestingly, the smokers with the highest plasma EMP levels are healthy smokers with normal spirometry and isolated low D_{LCO}. This suggests that the vascular-based contributions to the pathogenesis of emphysema may contribute to the early development of emphysema and may identify a point in time where intervention with smoking cessation therapy may prevent the irreversible lung destruction associated with the development of COPD as defined by the GOLD criteria (38). Elevated EMP levels may be a useful biomarker to identify smokers with early emphysema at a stage at which intervention may prevent further permanent lung destruction.

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