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 (DLCO).

Methods: Lung health was assessed by pulmonary function tests (PFTs: spirometry, total lung capacity, DLCO) 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 non-smokers (normal PFTs), healthy smokers (normal PFTs), and smokers with early evidence of lung destruction (normal spirometry, low DLCO). 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 DLCO 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 angiotesin-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 DLCO, 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 (DLCO), 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− 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 DLCO (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

(Received in original form September 6, 2010; accepted in final form March 10, 2011)

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Supported in part by National Institutes of Health grants R01 HL074326, P50 HL084936, and UL1 RR024996.

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


Originally Published in Press as DOI: 10.1164/rccm.201012-2061OC on March 11, 2011

Internet address: www.atsjournals.org

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.
similar physiologic findings, demonstrate that smokers with normal spirometry and normal D L CO have levels of circulating EMPs that are mildly elevated compared with healthy non-smokers, 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 L CO (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 L CO 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 L CO, 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 L CO 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 Emphyx software application (Emphyx; 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 L CO) and chest X-ray; “healthy smokers with normal spirometry and normal D L CO” (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 L CO” (n = 19): active smokers with normal spirometry and TLC, but reduced D L CO.

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 non-smokers (n = 10), healthy smokers with normal spirometry and normal D L CO (n = 20; including asymptomatic active smokers [n = 12] and symptomatic active smokers [n = 8]), and healthy smokers with normal spirometry but low D L CO (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 L CO (n = 7; including asymptomatic active smokers [n = 5] and symptomatic active smokers [n = 2]) and healthy smokers with normal spirometry but low D L CO (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-ul 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 6B-8H11; BD PharMingen, San Diego, CA; optimized condition). Four microliters of phycoerythrin-conjugated anti-human CD42b (CD42b-APC, clone HIPI; optimized condition) was added (45 min, 4°C) to each sample to exclude platelet-derived microparticles. Single and double positive CD42b-CD31” CD62b” 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-CD62b” 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 CD24b-CD3” 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 CaCl2 (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-CD62b”, 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. EMP microparticles were quantified by flow cytometry using CellQuest software (BD Biosciences, San Jose, CA), by investigators blinded to subject status. The data were analyzed using FlowJo software (Tree Star, OR), a high ratio of CD42b”CD62b” 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 L CO, and healthy smoker with normal spirometry but low D L CO) and to each of the measured clinical characteristics (D L CO, FVC, FEV 1, FEV 1/FVC, TLC, D L CO).
TABLE 1. INITIAL STUDY POPULATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A: Healthy Nonsmokers with Normal Spirometry and Normal DLCO</th>
<th>Group B: Healthy Smokers with Normal Spirometry and Normal DLCO*</th>
<th>Group C: Healthy Smokers with Normal Spirometry but Low DLCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>41</td>
<td>19</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>14/18</td>
<td>31/10</td>
<td>15/4</td>
</tr>
<tr>
<td>Age, yr</td>
<td>37 ± 15</td>
<td>40 ± 9</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>Ancestry, B/W/O</td>
<td>9/14/9</td>
<td>31/4/6</td>
<td>15/2/2</td>
</tr>
<tr>
<td>Smoking history, pack-years</td>
<td>0</td>
<td>19 ± 13</td>
<td>34 ± 19</td>
</tr>
<tr>
<td>Urine nicotine, ng/ml</td>
<td>Negative</td>
<td>1,041 ± 1,136</td>
<td>1,500 ± 1,459</td>
</tr>
<tr>
<td>Urine cotinine, ng/ml</td>
<td>Negative</td>
<td>1,565 ± 664</td>
<td>1,715 ± 1,132</td>
</tr>
<tr>
<td>Pulmonary function†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1</td>
<td>106 ± 14</td>
<td>106 ± 12</td>
<td>104 ± 15</td>
</tr>
<tr>
<td>FVC</td>
<td>108 ± 14</td>
<td>111 ± 12</td>
<td>108 ± 14</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>82 ± 5</td>
<td>79 ± 6</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>TLC</td>
<td>100 ± 15</td>
<td>95 ± 10</td>
<td>98 ± 17</td>
</tr>
<tr>
<td>DLCO</td>
<td>95 ± 15</td>
<td>91 ± 9</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>0.44 ± 0.24</td>
<td>0.51 ± 0.51</td>
<td>0.41 ± 0.26</td>
</tr>
</tbody>
</table>

* 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 FEV1/FVC, which is reported as % observed. For healthy nonsmokers and healthy and symptomatic smokers with DLCO > 80%, FVC, FEV1, and FEV1/FVC are prebronchodilator values. For healthy smokers with DLCO < 80%, FVC, FEV1, and FEV1/FVC are post-bronchodilator values.

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

Data are presented as mean ± SD. Normal DLCO value ≥ 80% predicted. There were no differences between the three groups (P > 0.05, all comparisons) except for the low DLCO 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).

TABLE 2. PROSPECTIVE STUDY POPULATIONS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prospective Cohort 1</th>
<th>Prospective Cohort 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group D: Healthy Nonsmokers with Normal Spirometry and Normal DLCO</td>
<td>Group E: Healthy Smokers with Normal Spirometry and Normal DLCO*</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>5/5</td>
<td>15/5</td>
</tr>
<tr>
<td>Age, yr</td>
<td>42 ± 12</td>
<td>44 ± 9</td>
</tr>
<tr>
<td>Smoking history, pack-years</td>
<td>0</td>
<td>21 ± 15</td>
</tr>
<tr>
<td>Urine nicotine, ng/ml</td>
<td>Negative</td>
<td>1,508 ± 1,710</td>
</tr>
<tr>
<td>Urine cotinine, ng/ml</td>
<td>Negative</td>
<td>1,593 ± 1,193</td>
</tr>
<tr>
<td>Pulmonary function†</td>
<td>FEV1</td>
<td>105 ± 12</td>
</tr>
<tr>
<td></td>
<td>FVC</td>
<td>108 ± 13</td>
</tr>
<tr>
<td></td>
<td>FEV1/FVC</td>
<td>81 ± 5</td>
</tr>
<tr>
<td></td>
<td>TLC</td>
<td>101 ± 19</td>
</tr>
<tr>
<td></td>
<td>DLCO</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.02</td>
</tr>
</tbody>
</table>

* 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 < 0.05).
† Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed. For healthy nonsmokers and healthy and symptomatic smokers with DLCO > 80%, FVC, FEV1, and FEV1/FVC are prebronchodilator values. For healthy smokers with DLCO < 80%, FVC, FEV1, and FEV1/FVC are post-bronchodilator values.

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

Data are presented as mean ± SD. Normal DLCO value ≥ 80% predicted. There were no differences between groups D, E, and F (P > 0.05, all comparisons) except for the low DLCO in group G (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 DLCO in group H (P < 0.01, compared to group C) 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).

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 and 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.
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/\( \mu L \), whereas 50% of healthy smokers had EMP levels above the normal range of healthy nonsmokers \( \pm 2 \text{ SD} \) (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/\( \mu L \) and 43% greater than 1,250 EMP/\( \mu L \). Assessed with all groups together, the best correlations of EMP levels with individual clinical parameters were with pack-years, \( D_{LCO} \), \( \text{FEV} \_1/\text{FVC} \), 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 \( \pm 0.38, P < 0.05\) \() \). 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 \( \pm 0.22 \) vs. 1.09 \( \pm 0.38 \) 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 \((P < 10^{-4})\) and healthy smokers with low \( D_{LCO} \) compared with healthy nonsmokers \((P < 10^{-4})\). By these criteria, 79% of
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 annexin V+ 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, 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).
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, Gordon, Gudi, Krause, et al. (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 DLCO demonstrates significant variation in EMP levels among these smokers, with the highest, by far, in healthy smokers with normal spirometry and low DLCO.

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 (DLCO) (n = 10, yellow circles), healthy smokers with normal spirometry and normal DLCO (combining healthy smokers, n = 12, tan circles, and symptomatic smokers, n = 8, tan triangles), and healthy smokers with normal spirometry and low DLCO (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 (DLCO) (combining healthy smokers, n = 5, tan circles, and symptomatic smokers, n = 2, tan triangles) and HIV1+ healthy smokers with normal spirometry and low DLCO (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 EMP correlate with an early onset of lung destruction (i.e., normal spirometry/low DL_{CO} 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 DL_{CO}. 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 DL_{CO} 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 DL_{CO} (1, 2, 4, 6, 17, 18, 38, 42). HRCT imaging detects rough correlation with a low DL_{CO} 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 DL_{CO} (91). We have observed that smokers with normal spirometry and low DL_{CO} are at higher risk for the development of COPD as defined by the GOLD criteria than are smokers with normal spirometry and normal DL_{CO} (92), but there was no direct correlation of emphysema with EMP levels or DL_{CO}. This was not surprising, as healthy smokers with normal spirometry and normal DL_{CO} 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 DL_{CO} 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 DL_{CO}. 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.

**Author Disclosure:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

**Acknowledgment:** The authors thank Ann E. Tilley and Timothy P. O’Connor for helpful discussions; Fadi Zakko for help with data acquisition; and Nahla Mohamed for help in preparing this manuscript.

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