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

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At a Glance: Pulmonary endothelial apoptosis is the primary 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. 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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Running head: Endothelial microparticles and emphysema

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Abstract

Rationale: There is increasing evidence that emphysema is associated with primary loss of pulmonary capillary endothelium. Plasma levels of endothelial microparticles (EMP), 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, reduced DLCO.

Methods: Lung health was assessed by pulmonary function tests (PFT; spirometry, total lung capacity, DLCO) and chest X-ray; smoking status 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 PFT); healthy smokers (normal PFT); 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 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 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.

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Introduction

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 (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 acti-

vated 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 DLCO, i.e., smokers with early evidence of lung destruction prior to the development of expiratory airflow limitation. The data in this cohort, as well as in 2 prospective cohorts with similar physiologic findings, demonstrate that smokers with normal spirometry and normal DLCO have levels of circulating EMPs that are mildly elevated compared to healthy nonsmokers, but that smokers that are normal by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) spirometric criteria for chronic obstructive pulmonary disease (COPD) (38), but have reduced DLCO (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 $CD42b^{-}CD31^{+}$ 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 DLCO smokers are angiotensin converting enzyme positive, suggesting they are dervied 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 prior to 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 NIH Clinical and Translational Science Center (CTSC) and Department of Genetic Medicine Clinical Research Facility, under In-

stitutional Review Board approved clinical protocols. Written informed consent was obtained from each individual prior to enrollment. Screening included history, complete physical exam, blood studies, urinalysis, chest X-ray, electrocardiogram and pulmonary function tests, including forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV1), FEV1/FVC, total lung capacity (TLC) and DLCO, all carried out under ATS gudielines (41). If the FEV1 was <80% predicted and/or the FEV1/FVC <0.7, the spirometry was retested after standard bronchodilators (38, 42). Measurement of the DLCO was carried out 2 to 4 times in all individuals; the average of the best 2 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 <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 CT scans. Emphysema was defined as >3% lung volume with attenuation ≤-950 Hounsfield units (HU) or >16% lung volume with attenuation ≤-910 HU, values derived from analyses of 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 prior to all testing was >12 hr. All individuals had normal α1-antitrypsin levels, normal C-reactive protein levels and (for full inclusion/exclusion criteria, see Supplemental Methods).

A total of 92 individuals were assessed as an initial study population (Table I), using the following definitions: “healthy nonsmokers” (n=32) - life long 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 with normal spirometry and normal DLCO” (n=41), including asymptomatic active smokers with normal PFT and chest X-ray

(n=32) and symptomatic smokers with normal PFT and chest X-ray (n=9), but with cough [0 to 4 scale (42)] and/or sputum production [0 to 4 scale (43)]; and "healthy smokers with normal spirometry but low DLCO" (n=19) - active smokers with normal spirometry and TLC, but reduced DLCO.

Additionally, a prospective study population of 60 individuals was assessed using the definitions as described above (Table II). Prospective cohort 1 included a total of 45 individuals, including "healthy nonsmokers" (n=10), "healthy smokers with normal spirometry and normal DLCO" [n=20; including asymptomatic active smokers (n=12) and symptomatic active smokers (n=8)] and "healthy smokers with normal spirometry but low DLCO" (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 DLCO" (n=7; including asymptomatic active smokers n=5 and symptomatic active smokers, n=2) and "healthy smokers with normal spirometry but low DLCO" (n=8).

Characterization of Plasma EMPs

To quantify EMPs, a standard operating procedure was established (Supplemental Methods, Supplemental Figure 1, Supplemental Table I) 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 hr, centrifuged 10 min, 160 g, 23°C to prepare platelet-rich plasma. Within 5 min, the supernatant was further centrifuged 8 min, 1000g, 23°C to obtain platelet-poor plasma. Within 5 min, 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 µl 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 3 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 μl 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 co-stained with 5 μl phycoerythrin-conjugated anti-human angiotensin converting enzyme (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 manufacturers recommendation.

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 polysterene 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 Coul-

ter, Miami, FL) with a known concentration, using 30 sec 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 two standard deviations above that for healthy nonsmokers was considered "apoptotic" (Supplemental Figure 4C).

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 nornal spirometry and normal DLCO, and healthy smoker with normal spirometry but low DLCO) and to each of the measured clinical characteristics (DLCO, FEV1, FVC, FEV1/FVC, TLC and blood pressure), where for the former we considered an ANOVA coding and for latter a regression coding. We performed these tests without any covariates and when including covariates for age, sex, and pack-years, where 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 is presented. To guard against deviations from parametric as-

sumptions, a non-parametric permutation test was performed for these models, where 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 non-parametric p value was obtained using the ordering of p values obtained from 1000 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 DLCO had a mild increase in EMP levels compared to healthy nonsmokers, as did symptomatic smokers compared to healthy nonsmokers ($p<10^{-4}$ compared to 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 DLCO, had a significant increase in EMP levels ($p<10^{-4}$ compared to healthy nonsmokers; $p<10^{-3}$ compared to healthy smokers). A few healthy smokers with normal DLCO and healthy smokers with low DLCO had co-morbidities 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 co-morbidities associated with increased circulating EMPs.

When assessed as % 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 (Supplemental Figure 2). In contrast, 95% of healthy smokers with normal spirometry and low DLCO had EMP levels above the range of healthy smokers, with 52% distributed between 500-1250 EMP/μl and

43% >1250 EMP/μl. Assessed with all groups together, the best correlations of EMP levels with individual clinical parameters were with pack-yr, DLCO , FEV1/FVC and urine cotinine, with less correlation with urine nicotine, age, blood pressure or other lung function parameters (Supplemental Figure 3). Assessed within individual subject groups, there were limited correlations of EMP levels with individual clinical parameters (Supplemental Table II). Automated quantification of emphysema levels by transverse chest CT scans showed as well a low correlation pattern of emphysema with urine nicotine level, EMPs or DLCO between all groups (Supplemental Figure 5) and no differences in emphysema levels between all groups (Supplemental Figure 6).

None of the covariates were considered significant ($p>0.1$) except for pack-yr. Therefore, p values for the ANOVA test are reported without including additional covariates except those involving comparisons of all smoking groups, where pack-yr as covariate was included. There were no qualitative differences in p values obtained from the parametric versus the non-parametric analyses, therefore the presented results are based on parametric analyses. There was no correlation of EMP levels and age, gender 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 angiotensin converting enzyme (ACE), a surface protein more highly expressed on pulmonary capillary endothelium compared to other endothelial beds (39) (Figure 2). This analysis showed that 55% of the $CD42b^{-}CD31^{+}$ EMPs in healthy smokers with normal spirometry and normal DLCO were ACE^{+} beyond that observed for healthy nonsmokers ($p<0.02$ compared to healthy nonsmokers) while 76% of the $CD42b^{-}CD31^{+}$ EMPs in healthy smokers with normal spirometry but low DLCO were ACE^{+} ($p<0.01$ compared to healthy nonsmokers), i.e., the majority of the elevated EMPs in the low DLCO group were de-

rived from pulmonary capillary endothelium.

Apoptotic *vs* Activated EMPs

Aside from a few outliers, the $CD42b^{-}CD62^{+}/CD42b^{-}CD31^{+}$ ratio of the healthy non-smokers 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 DLCO (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 DLCO, a prospective cohort of 45 individuals were assessed, including healthy nonsmokers, healthy smokers with normal DLCO and healthy smokers with low DLCO (cohort 1, Table II, 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 DLCO compared to healthy nonsmokers ($p<10^{-4}$), healthy smokers with low DLCO compared to healthy nonsmokers ($p<10^{-4}$) and healthy smokers with low DLCO compared to healthy smokers ($p<0.01$; Figure 4A). Likewise, the prospective cohort also had more apoptotic derived EMPs in healthy smokers with normal DLCO compared to healthy nonsmokers ($p<10^{-4}$), healthy smokers with low DLCO compared to healthy nonsmokers ($p<10^{-4}$) and healthy smokers with low DLCO compared to healthy smokers with normal DLCO ($p<0.05$; Figure 4B). By this criteria, 79% of the EMPs of the healthy smokers with low DLCO were apoptotic-like, as were 44% of the EMPs of the healthy smokers with normal DLCO. The apoptotic nature of the EMPs was confirmed by

annexin V staining, with 50% more annexinV^{+} EMPs in healthy smokers with normal DLCO and 66% more EMPs in healthy smokers with low DLCO compared to healthy nonsmokers ($p<0.01$ and $p<10^{-4}$, respectively; Supplemental Figure 4).

As a further verification that EMPs are elevated in association with early lung destruction in smokers with normal spirometry and low DLCO and based on the knowledge that smokers who are HIV1^{+} have an accelerated form of emphysema (44), we assessed a 2nd prospective cohort, smokers who were HIV1^{+}, both those with normal spirometry and normal DLCO and those with normal spirometry and low DLCO (cohort 2, Table II, Figure 5). Parallel to the initial cohort and the 1st prospective cohort, the HIV1^{+} low DLCO group had significantly more CD42b^{-}CD31^{+} EMPs than the HIV1^{+} with normal DLCO group ($p<10^{-3}$; Figure 5A), with 75% of apoptotic-like EMPs in the HIV1^{+} low DLCO 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 DLCO, a lung function measure of the functional intactness of the alveolar-capillary bed (15, 16). Healthy smokers and symptomatic smokers with normal spirometry and DLCO had mildly elevated levels of circulating EMPs compared to healthy nonsmokers. Strikingly, however, healthy smokers with normal spirometry but an isolated reduction in DLCO had high levels of circulating EMPs compared to 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 DLCO.

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, we observed that, to some extent, all smoking groups (healthy smokers, symptomatic smokers), had elevation of EMPs compared to healthy nonsmokers. Consistent with this, Heiss and colleagues (68) showed that healthy nonsmokers exposed for 30 min 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. 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 DLCO have to be undertaken to underline the association between EMPs and lung destruction. The data in the present study suggests that elevated levels of EMP correlate with an early onset of lung destruction (i.e., normal spirometry/low DLCO 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 et al (69) showed increased DNA fragmentation in the pulmonary capillaries and arteriolar endothelium of individuals with COPD, and Kasahara et al (8-10) reported increased septal cell death (endothelial and epithelial cells) in human emphysematous lungs compared with lungs of nonsmokers or smokers without emphysema. While 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 DLCO. 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 DLCO 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). While 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 FEV1/FVC <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 DLCO (1, 2, 4, 6, 17, 18, 38, 42). High resolution CT (HRCT) imaging detects early emphysema by identifying pulmonary tissue with radiologic attenuation below a predetermined threshold, findings that roughly correlate with a low DLCO 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 DLCO (91). We have observed that smokers with normal spirometry and low DLCO are at higher risk for the development of COPD as defined by the GOLD criteria than are smokers with normal spirometry and normal DLCO (92), but there was no direct correlation of emphysema with EMP levels or DLCO. This was not surprising, as healthy smokers with normal spirometry and normal DLCO 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 DLCO 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 DLCO. 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 where intervention may prevent further permanent lung destruction.

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Table I. Initial Study Population[1]

Parameter	Group A - Healthy nonsmokers with normal spirometry and normal DLCO[2,4]	Group B - Healthy smokers with normal spirometry and normal DLCO[2,3]	Group C - Healthy smokers with normal spirometry but low DLCO[2,4]
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)[5]	9/14/9	31/4/6	15/2/2
Smoking history (pack-yr)	0	19±13	34±19
Urine nicotine (ng/ml)	Negative	1041±1136	1500±1459
Urine cotinine (ng/ml)	Negative	1565±664	1715±1132
Pulmonary function[6]			
FEV1	106±14	106±12	104±15
FVC	108±14	111±12	108±14
FEV1/FVC	82±5	79±6	78±5
TLC	100±15	95±10	98±17
DLCO	95±15	91±9	70±7
C-reactive protein (mg/dl)	0.44±0.24	0.51±0.51	0.41±0.26

[1] Data are presented as mean ± standard deviation.

[2] DLCO=diffusion capacity of the lung for carbon monoxide; normal value≥80% predicted.

[3] 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 $p<0.04$).

[4] There were no differences between the 3 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-yr, smoking metabolites, sex and ancestry in group A ($p<0.05$, compared to groups B and C).

[5] B=Black, W=White, O=Other.

[6] Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusion capacity. For healthy nonsmokers and healthy and symptomatic smokers with DLCO ≥80%, FVC, FEV1 and FEV1/FVC are pre-bronchodilator values. For healthy smokers with DLCO <80%, FVC, FEV1 and FEV1/FVC are post-bronchodilator values.

Table II. Prospective Study Populations[1]

Parameter	Prospective cohort 1			Prospective cohort 2	
	Group D - Healthy non-smokers with normal spirometry and normal DLCO[2,3,4]	Group E - Healthy smokers with normal spirometry and normal DLCO[2,3,4]	Group F - Healthy smokers with normal spirometry but low DLCO[2,4]	Group G - HIV1+ Smokers with normal spirometry and normal DLCO[2,5]	Group H - HIV1+ smokers with normal spirometry and low DLCO[2,5]
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)[6]	4/3/3	11/3/6	9/3/3	5/0/2	6/1/1
Smoking history (pack-yr)	0	21±15	23±14	33±29	30±22
Urine nicotine (ng/ml)	Negative	1508±1710	1320±1453	297±301	1557±1478
Urine cotinine (ng/ml)	Negative	1593±1193	1361±1041	1329±881	1334±704
Pulmonary function[7]					
FEV1	105±12	108±13	106±22	99±16	103±16
FVC	108±13	112±12	109±25	103±9	105±15
FEV1/FVC	81±5	80±6	80±7	79±8	80±7
TLC	101±19	98±15	99±16	85±7	90±10
DLCO	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

[1] Data are presented as mean ± standard deviation.

[2] DLCO=diffusion capacity of the lung for carbon monoxide; normal value≥80% predicted.

[3] 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 ($p>0.5$, a ll comparisons) except urine cotinine ($p<0.05$).

[4] There were no differences between the groups D, E and F ($p>0.05$, all comparisons) except for the low DLCO in group F ($p<0.05$, compared to groups D and E), and pack-yr, smoking metabolites, sex and ancestry in group D ($p<0.05$, compared to groups E, F, G and H).

[5] Except for the low DLCO 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)

[6] B=Black, W=White, O=Other.

[7] Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusion capacity. For healthy non-smokers and healthy and symptomatic smokers with DLCO ≥80%, FVC, FEV1 and FEV1/FVC are pre-bronchodilator values. For healthy smokers with DLCO <80%, FVC, FEV1 and FEV1/FVC are post-bronchodilator values.

Figure Legends

Figure 1. Levels of $CD42b^{-}CD31^{+}$ EMPs per µl in platelet-poor plasma of the study groups. Shown is data for healthy nonsmokers with normal spirometry and normal DLCO (n=32, yellow circles), healthy smokers with normal spirometry and normal DLCO (combining asymptomatic smokers, n=12, tan circles and symptomatic smokers, n=8, tan triangles); and healthy smokers with normal spirometry and low DLCO (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 standard deviations of $CD42b^{-}CD31^{+}$ EMP/µl platelet of healthy nonsmokers.

Figure 2. Proportion of $CD42b^{-}CD31^{+}$ EMP that express anigotension converting enzyme (ACE^{+}). Shown is data for healthy nonsmokers with normal spirometry and normal DLCO (n =10, yellow circles); healthy smokers with normal spirometry and normal DLCO (combining asymptomatic smokers, n=12, tan circles and symptomatic smokers, n=8, tan triangles); and healthy smokers with normal spirometry and low DLCO (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 standard deviations of healthy nonsmokers. The % values represent the proportion of individuals in that group that had higher levels of $CD42b^{-}CD31^{+}ACE^{+}$ EMPs beyond the level observed for healthy nonsmokers.

Figure 3. Ratio of circulating $CD42b^{-}CD62^{+}$ to $CD42b^{-}CD31^{+}$ EMPs in plasma of healthy nonsmokers with normal spirometry and normal DLCO (n =32, yellow circles); healthy smokers with normal spirometry and normal DLCO (combining healthy smokers, n=32, tan circles and symptomatic smokers, n=9, tan triangles); and healthy smokers with normal spirometry and low DLCO (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.

Figure 4. Prospective study cohort 1 - plasma EMPs in a prospective group of healthy nonsmokers with normal spirometry and normal 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 - EMPs in a prospective group of $HIV1^{+}$ healthy smokers with normal spirometry and normal 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.

Figure 1

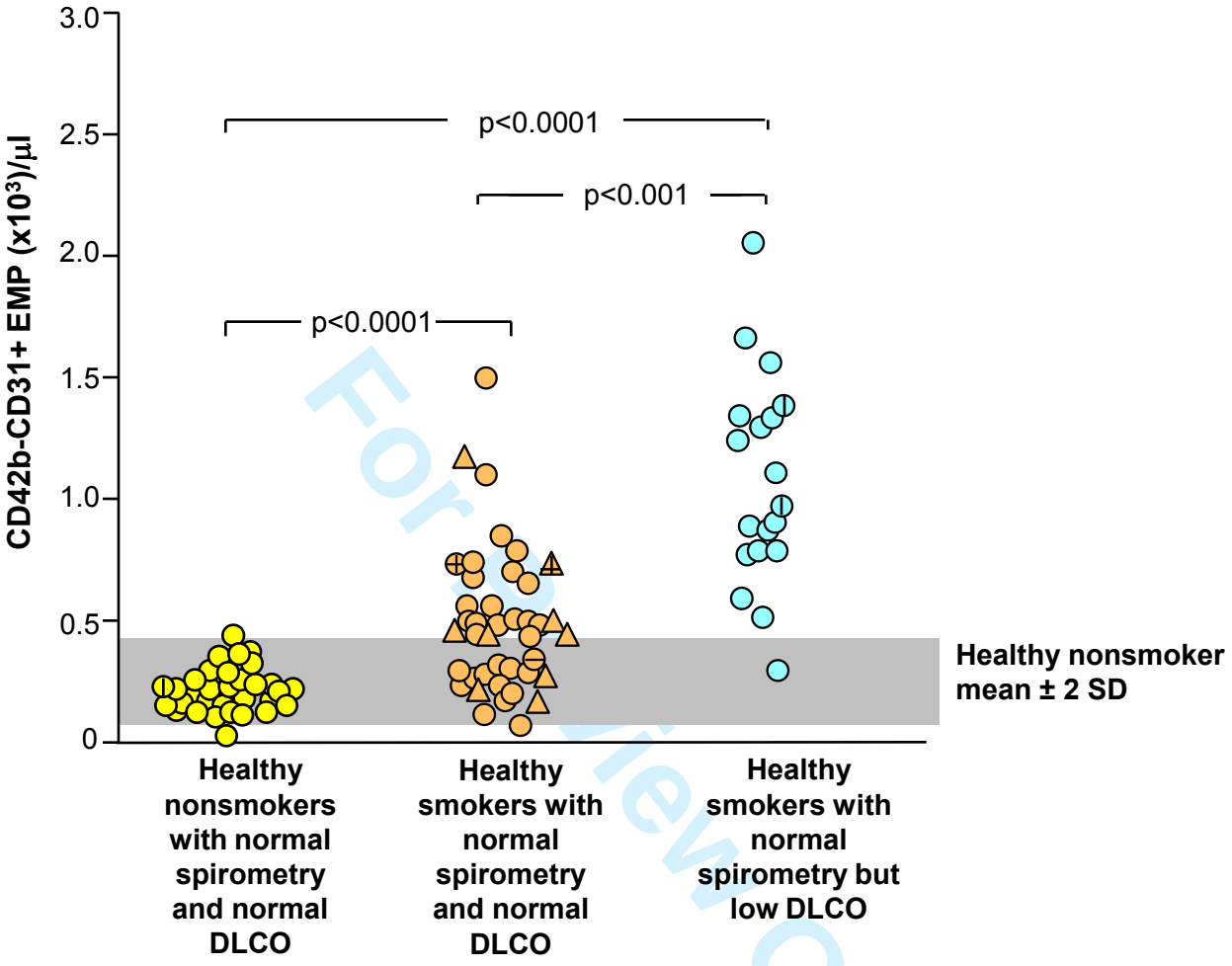


Figure 2

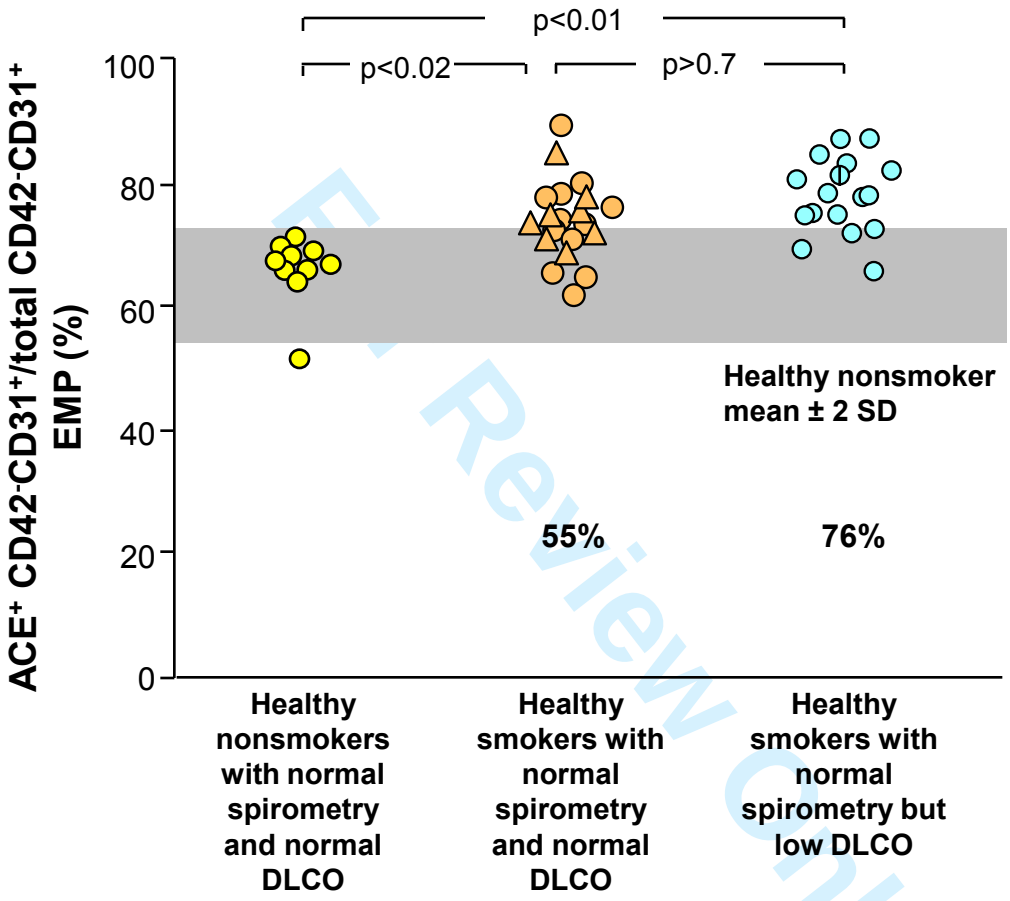


Figure 3

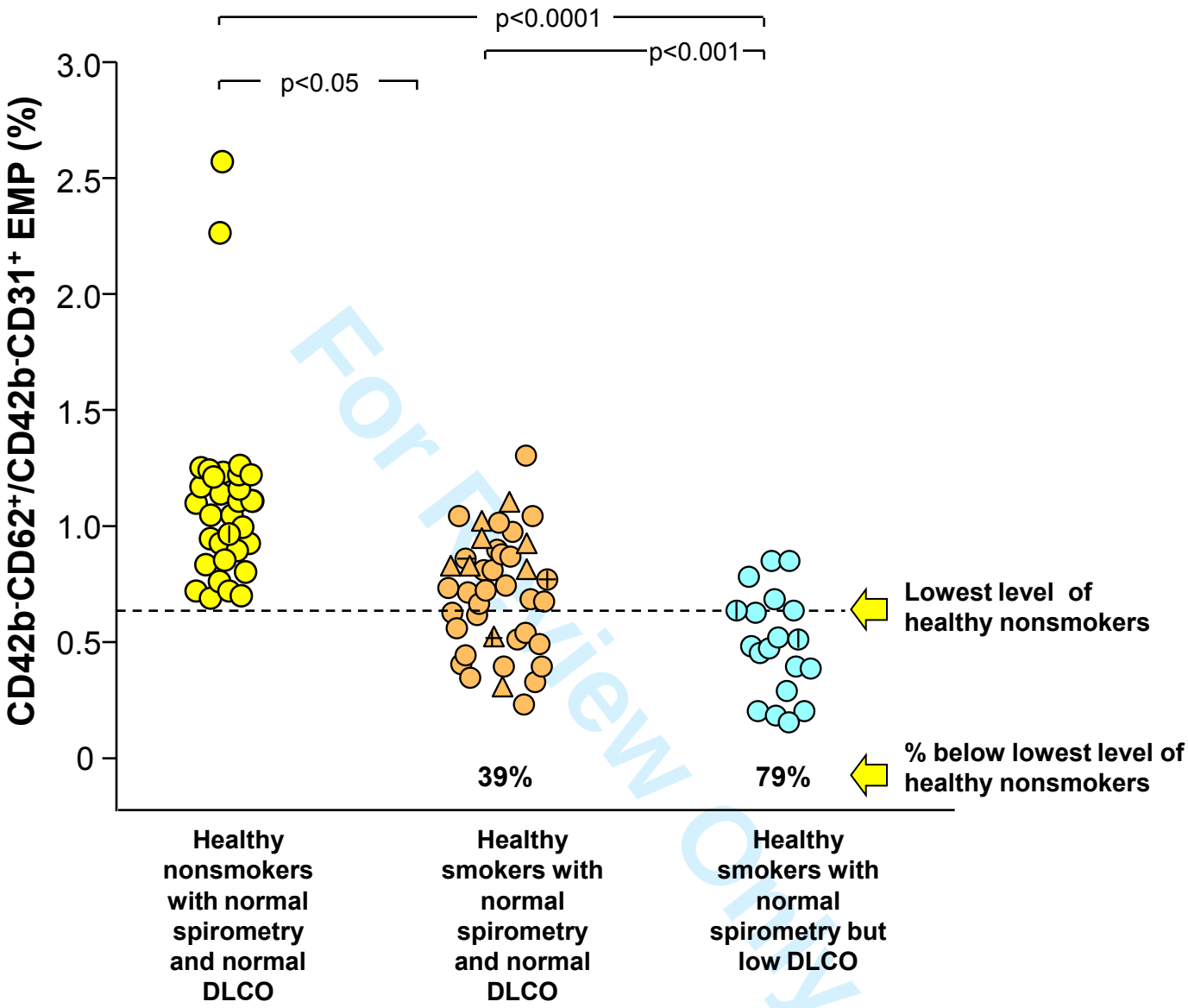


Figure 4

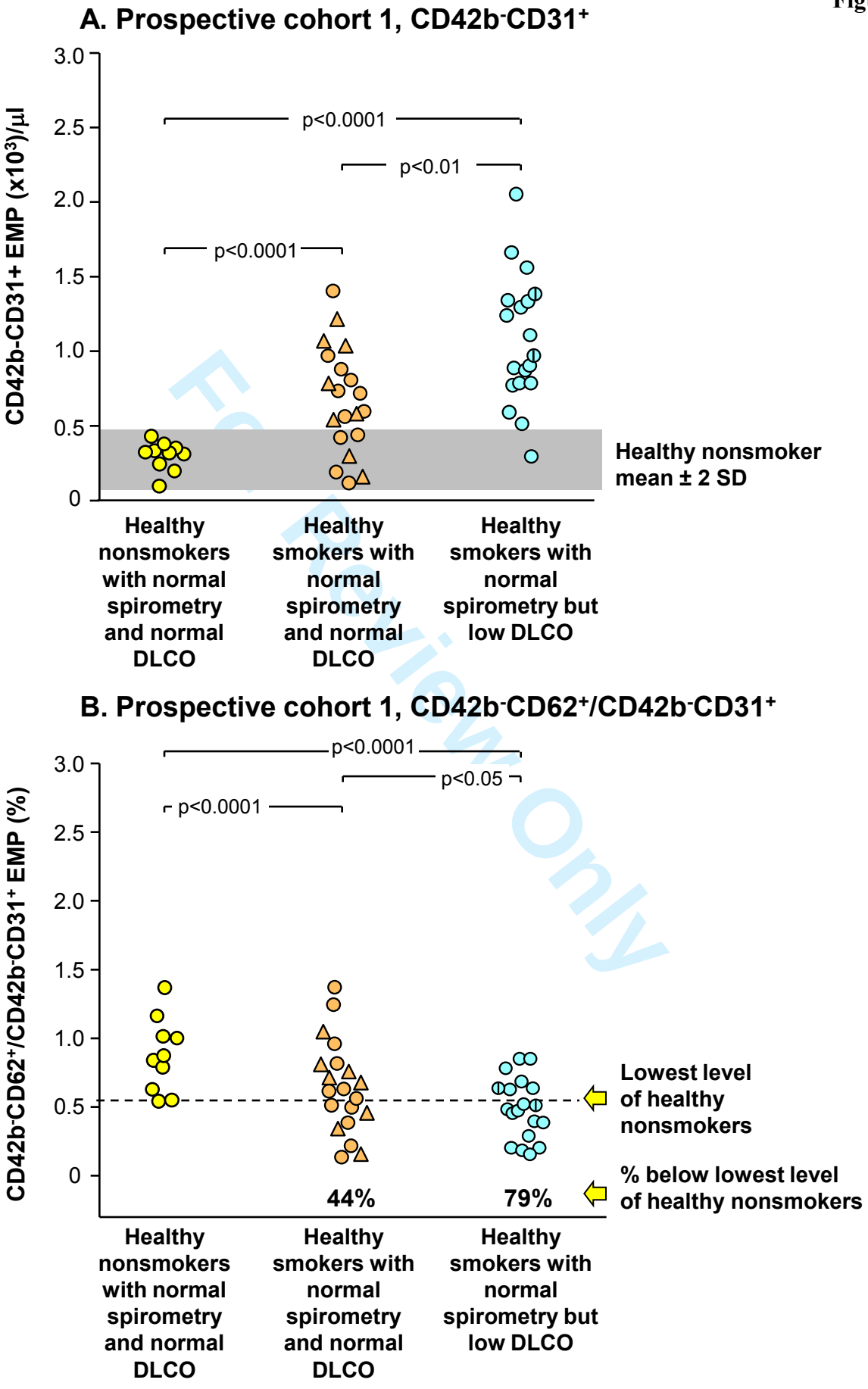
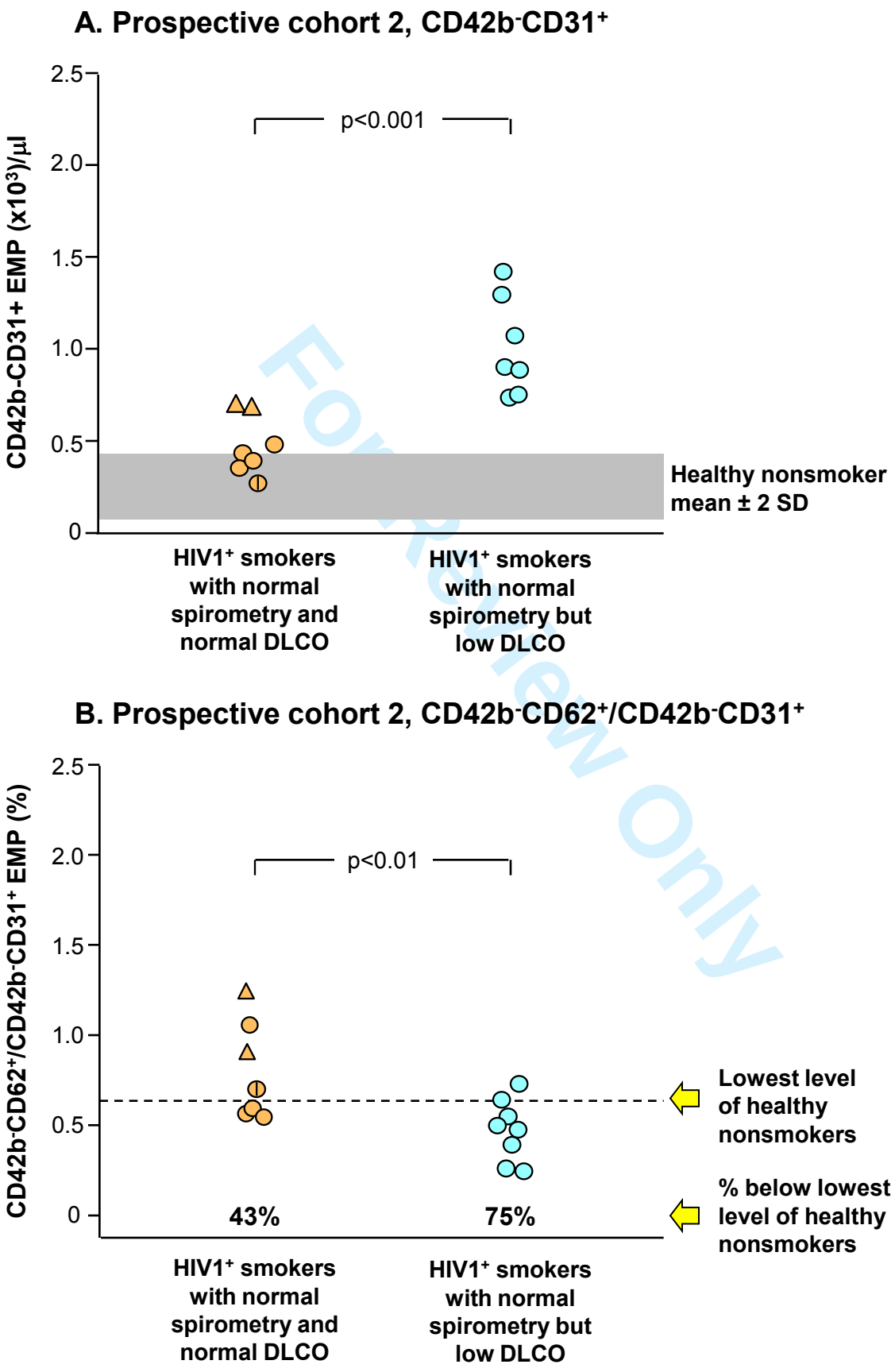


Figure 5



Supplemental Methods

Inclusion and Exclusion Criteria

Healthy nonsmokers (Initial study population and prospective cohort 1)

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 absence of 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 (≥80% predicted), FVC (≥80 predicted), FEV1/FVC (≥0.7) based on pre-bronchodilator spirometry and TLC (≥90% predicted)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery <30 mm in chest X ray.
- 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
- Evidence of co-morbidities associated with increased circulating EMPs (except subjects with systemic hypertension and/or type 2 diabetes that were included; if subjects have either co-morbidity they are indicated in the data)

Healthy smokers (Initial study population and prospective cohort 1)

Inclusion criteria
- Must be capable of providing informed consent
- Males and females, age 18 or older
- Females - not pregnant

- Current daily smokers with any number of pack-yr, validated by any of the following: urine nicotine >2 ng/ml or 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
- Symptomatic smokers with cough (0 to 4 scale) and/or sputum production (0 to 4 scale) can be included if they meet all of the other inclusion/exclusion criteria
- 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 (≥80% predicted), FVC (≥80 predicted), FEV1/FVC (≥0.7) based on pre-bronchodilator spirometry and TLC (≥90% predicted)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery <30 mm in chest X ray.
- 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
- Evidence of co-morbidities associated with increased circulating EMPs (except subjects with systemic hypertension and/or type 2 diabetes that were included; if subjects have either co-morbidity they are indicated in the data)

Smokers with normal spirometry and low DLCO (Initial study population and prospective cohort 1)

Inclusion criteria

- Must be capable of providing informed consent
- Males and females, age 18 or older
- Females - not pregnant
- Current daily smokers with any number of pack-yr, validated by any of the following: urine nicotine >2 ng/ml or urine cotinine >5 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

- Normal FEV1 (≥80% predicted), FVC (≥80 predicted), FEV1/FVC (≥0.7) based on post-bronchodilator spirometry and TLC (≥90% predicted)
- Normal electrocardiogram (sinus bradycardia, premature atrial contractions are permissible)
- Normal FEV1 (≥80% predicted), FVC (≥80 predicted), FEV1/FVC (≥0.7) based on post-bronchodilator spirometry and TLC (≥90% predicted)
- Normal estimated pulmonary artery pressure assessed by diameter of the main pulmonary artery <30 mm in chest X ray.
- All individuals have chest X-ray (PA and lateral) and chest CT
- 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
- Evidence of co-morbidities associated with increased circulating EMPs.(except subjects with systemic hypertension and/or type 2 diabetes that were included; if subjects have either co-morbidity they are indicated in the data)

HIV1$^+$ smokers with normal spirometry and normal DLCO (Prospective cohort 2)

Inclusion and exclusion criteria - identical to that of the healthy smokers, but must be HIV1$^+$ by serologic testing

HIV1$^+$ smokers with normal spirometry and low DLCO (Prospective cohort 2)

Inclusion and exclusion criteria - identical to that of the smokers with normal spirometry and low DLCO, but must be HIV1$^+$ by serologic testing

Quality Control

Quality control experiments were conducted to define the time points with the least variability during the different procedure steps to quantify EMP levels. All procedure steps were performed at room temperature (23°C) if not otherwise noted. To standardize the analysis of EMPs of different subject groups by flow cytometry, calibrator standard beads in sizes 10 μm were used to define the size (Supplemental Figure 1A). EMPs were defined as elements in

platelet poor plasma at a size <1.5 μm in a forward (size)-sideward (density) light scatter (Supplemental Figure 1B), expressing the platelet endothelium adhesion molecule marker CD31 (PECAM-1), but not the platelet-specific glycoprotein Ib marker CD42b. $CD42b^{-}CD31^{+}$ EMPs of healthy nonsmoker with normal spirometry and normal DLCO, healthy smoker with normal spirometry and normal DLCO and healthy smoker with normal spirometry and low DLCO are presented. (Supplemental Figure 1C-E, respectively). To ensure that the time of each analytic step did not influence the quantification of EMPs, the different procedure steps were assessed to ensure minimal variance. To accomplish this, blood of healthy donors (n=4) was assessed for each step in the analytic procedure (Supplemental Figure 1F). Each experiment addressed one procedure step, with the time for all other steps held consistent.

First, the effect of time between blood collection and 1st centrifugation to obtain platelet-rich plasma was assessed. Blood was collected and centrifuged within 0.5, 1, 2, 3 or 4 hr of venipuncture at 160g, 10 min, 23°C (Supplemental Figure 1G). After 5, 30, 60 or 90 min after centrifugation, platelet-rich plasma underwent a 2nd centrifugation (8 min, 1000g, 23°C) to obtain platelet-poor plasma (Supplemental Figure 1H). Platelet-poor plasma was then stained (5, 30, 60, 90 or 120 min) for the presence of $CD42b^{-}CD31^{+}$ EMPs (Supplemental Figure 1J). After incubation with the corresponding antibodies (4°C), the samples were diluted and assessed by flow cytometry after 5, 30, 60 or 90 min (Supplemental Figure 1K). The experiment that addressed the effect of time between blood collection and first centrifugation showed that there was no difference in the EMP levels up to 1 hr after blood draw ($p>0.2$), but there was significant variance from ≥2 hr compared to the 30 min time point ($p<0.03$, Supplemental Figure 1G). No differences were observed by varying the time period between the 1st and 2nd centrifugation (5 to 90 min; $p>0.1$, Supplemental Figure 1H). A >5 min delay between the 2nd

centrifugation to obtain platelet-poor plasma and antibody incubation gave some variability in EMP levels ($p<0.01$; Supplemental Figure 1I). Increasing the time for antibody incubation did not change the EMP levels ($p>0.4$, Supplemental Figure 1J). Finally, varying the time between the antibody incubation and acquisition to >30 min resulted in variability in the EMP levels ($p<0.05$; Supplemental Figure 1K).

Based on these results, a standard procedure was established that included the following steps (Supplemental Table I): (1) after blood collection, platelet-rich plasma was prepared within 1 hr and immediately processed to obtain platelet-poor plasma with <5 min between 1st and 2nd centrifugation. Platelet-poor plasma was then immediately stained with anti-CD42b and anti-CD31 antibodies (<5 min) for a constant time of 45 min and flow cytometry was carried out within 15 min after antibody incubation was terminated.

Supplemental Table I. Standard Operating Procedure for Assessment of Plasma Endothelial Microparticles[1]

Step	Procedure	Time (min)
1	Blood Collection	0
2	Time to 1st centrifugation to obtain platelet-rich plasma	15-60
3	1st centrifugation to obtain platelet-rich plasma (160g, 23°C)	10
4	Time between 1rst centrifugation and 2nd centrifugation to obtain platelet-poor plasma	<5
5	2nd centrifugation to obtain platelet-poor plasma (1000g, 23°C)	8
6	Time between 2nd centrifugation and antibody incubation	<5
7	Time of antibody incubation (4°C)	45
8	Time between antibody incubation and acquisition	<15

[1] The standard operating procedure for isolation of circulating endothelial microparticles was determined experimentally by a time course analysis for steps 2, 4, 6, 7 and 8 as detailed in Methods and Supplemental Figure 1.

Supplemental Table II. Correlation of Plasma Endothelial Microparticle Levels and Demographics, Smoking-related and Pulmonary Function Parameters for Each of the Smoking Groups[1] - Initial Study Population

	Healthy smokers with normal spirometry and normal DLCO[2]		Smokers with normal spirometry but low DLCO	
Smoking parameters	**r^2**	**p**	**r^2**	**p**
Demographics				
Age	0.01	0.73	<0.01	0.99
Gender	N/A[6]	0.29	N/A	0.88
Ancestry[3]	N/A	0.80	N/A	0.72
Smoking-parameters				
Pack-yr[4]	0.39	0.01	0.06	0.33
Urine nicotine (ng/ml)	0.31	0.02	0.03	0.47
Urine cotinine (ng/ml)	0.01	0.84	0.01	0.66
Pulmonary function parameters[5]				
FEV1	0.15	0.12	<0.01	0.93
FVC	0.08	0.29	<0.01	0.70
FEV1/FVC	0.04	0.63	0.09	0.23
TLC	0.01	0.83	0.07	0.27
DLCO	0.02	0.79	0.01	0.63

[1] Correlation between EMP levels of healthy smokers and smoking-related parameters; p values were calculated by ANOVA and r^2 were calculated by linear regression; this analysis is from the primary group (Figure 1).

[2] Combined asymptomatic and symptomatic smokers, see Table I.

[3] Ancestry: black, white and other descents.

[4] Smoking history is indicated in pack-yr.

[5] Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusion capacity. For healthy non-smokers and healthy and symptomatic smokers with DLCO ≥80%, FVC, FEV1 and FEV1/FVC are pre-bronchodilator values. For smokers with DLCO <80%, FVC, FEV1 and FEV1/FVC are post-bronchodilator values.

[6] N/A - Correlation coefficient not applicable.

Supplemental Table III. Correlation of Plasma Endothelial Microparticle Levels and Demographics, Smoking-related and Pulmonary Function Parameters for Each of the Smoking Groups[1] - Prospective Cohort 1

	Healthy smokers with normal spirometry and normal DLCO[2]		Smokers with normal spirometry but low DLCO	
Smoking parameters	**r^2**	**p**	**r^2**	**p**
Demographics				
Age	0.03	0.68	<0.01	0.81
Gender	N/A[6]	0.18	N/A	0.73
Ancestry[3]	N/A	0.72	N/A	0.67
Smoking-parameters				
Pack-yr[4]	0.38	0.04	0.08	0.39
Urine nicotine (ng/ml)	0.31	0.49	0.12	0.68
Urine cotinine (ng/ml)	0.18	0.50	0.02	0.69
Pulmonary function parameters[5]				
FEV1	0.35	0.19	0.02	0.84
FVC	0.16	0.19	0.03	0..67
FEV1/FVC	0.1	0.45	0.02	0.13
TLC	0.08	0.78	0.08	0.39
DLCO	0.09	0.54	0.05	0.56

[1] Correlation between EMP levels of healthy smokers, and smoking-related parameters; p values were calculated by ANOVA and r^2 were calculated by linear regression.

[2] Combined asymptomatic and symptomatic smokers, see Table I.

[3] Ancestry: black, white and other descents.

[4] Smoking history is indicated in pack-yr.

[5] Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusion capacity. For healthy non-smokers and healthy and symptomatic smokers with DLCO ≥80%, FVC, FEV1 and FEV1/FVC are pre-bronchodilator values. For smokers with DLCO <80%, FVC, FEV1 and FEV1/FVC are post-bronchodilator values.

[6] N/A - Correlation coefficient not applicable.

Supplemental Table IV. Correlation of Plasma Endothelial Microparticle Levels and Demographics, Smoking-related and Pulmonary Function Parameters for Each of the Smoking Groups[1] - Prospective Cohort 2

	HIV1$^+$ smokers with normal spirometry and normal DLCO[2]		HIV1$^+$ smokers with normal spirometry but low DLCO	
Smoking parameters	**r^2**	**p**	**r^2**	**p**
Demographics				
Age	0.05	0.36	<0.01	0.81
Gender	N/A[6]	0.32	N/A	0.73
Ancestry[3]	N/A	0.37	N/A	0.67
Smoking-parameters				
Pack-yr[4]	0.27	0.06	0.08	0.39
Urine nicotine (ng/ml)	0.49	0.41	0.12	0.68
Urine cotinine (ng/ml)	0.15	0.59	0.02	0.69
Pulmonary function parameters[5]				
FEV1	0.31	0.21	0.02	0.84
FVC	0.14	0.25	0.03	0.67
FEV1/FVC	0.21	0.48	0.02	0.13
TLC	0.16	0.52	0.08	0.39
DLCO	0.14	0.54	0.05	0.56

[1] Correlation between EMP levels of HIV1$^+$ smokers, and smoking-related parameters; p values were calculated by ANOVA and r^2 were calculated by linear regression.

[2] Ancestry: black, white and other descents.

[3] Smoking history is indicated in pack-yr.

[4] Pulmonary function testing parameters are given as % of predicted value with the exception of FEV1/FVC, which is reported as % observed; FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 sec, TLC - total lung capacity, DLCO - diffusion capacity. For healthy non-smokers and healthy and symptomatic smokers with DLCO ≥80%, FVC, FEV1 and FEV1/FVC are pre-bronchodilator values. For smokers with DLCO <80%, FVC, FEV1 and FEV1/FVC are post-bronchodilator values.

[5] N/A - Correlation coefficient not applicable.

Supplemental Figure Legends

Supplemental Figure 1. Quality control assessment of plasma EMPs. **A.** Calibrator beads (10, 6, 4, 2 and 1 μm) represented on a forward (size)/side (density) light scatter dot plot histogram; **B.** EMPs in platelet-poor plasma of a healthy normal subject analyzed on a forward/side scatter dot plot. EMPs are defined as events at a size of < 1.5 μm particles and gated; **C-E.** Size-selected events plotted in a dual color dot blot histogram as a function of fluorescence for CD42b and CD31. EMPs are defined as $CD42b^{-}CD31^{+}$ events in the lower right quadrant accordingly to the isotype controls for: **C.** Healthy nonsmoker with normal spirometry and normal DLCO; **D.** Healthy smokers with normal spirometry and normal DLCO; and **E**. Healthy smoker with normal spirometry and low DLCO; **F.** Standard operating procedure. **G-K.** Quality control experiments to determine optimal EMP processing time points with the lowest variability: **G.** Time to 1^{st} centrifugation - time points (30-240 min) between blood collection and 1^{st} centrifugation; **H.** Time between 1^{st} and 2^{nd} centrifugation (5-90 min); **I**. Time between 2^{nd} centrifugation and antibody incubation (5-90 min); **J.** Time of antibody incubation (15-60 min); and **K**. Time between antibody incubation and flow cytometry (5-120 min). All experiments represent mean ± standard error of the mean, n=4 healthy nonsmokers.

Supplemental Figure 2. Cumulative frequency distribution of $CD42b^{-}CD31^{+}$ endothelial microparticle (EMP) levels in the study subjects. The percentage of subjects with EMP counts/μl in a given range is shown on the ordinate. Range of EMP counts/μl in batches of 250 are shown on the abscissa. Healthy nonsmokers with normal spirometry and normal DLCO (n=32, yellow circles), healthy smokers with normal spirometry and normal DLCO (combining asymptomatic smokers, n=12, tan circles and symptomatic smokers, n=8, tan triangles); and healthy smokers with normal spirometry and low DLCO (n=19, blue circles). Gray shaded area represents range ±

2 standard deviations of healthy nonsmokers. % of subjects in each group with values >2 standard deviations above that of healthy nonsmokers was 50% for healthy smokers with normal spirometry and 95% for healthy smokers with low DLCO.

Supplemental Figure 3. Correlation between $CD42b^{-}CD31^{+}$ plasma EMPs and smoking-related parameters, demographic parameters, and lung function parameters of the initial study population. The data includes levels of $CD42b^{-}CD31^{+}$ EMPs in platelet-poor plasma of healthy nonsmokers with normal spirometry and normal DLCO (n =32, yellow circles); healthy smokers (combining healthy smokers, n=32, tan circles and symptomatic smokers, n=9, tan triangles) and healthy smokers with normal spirometry and low DLCO (n=19, blue circles). **A.** Age (yr). **B.** Gender (male, female). **C.** Ancestry (black, white, others). **D.** Pack-yr. **E.** Urine nicotine. **F.** Urine cotinine. **G.** FEV1. **H.** FVC. **I.** FEV1/FVC. **J.** TLC. **K.** DLCO. **L.** Blood pressure Correlation coefficient and p values are indicated.

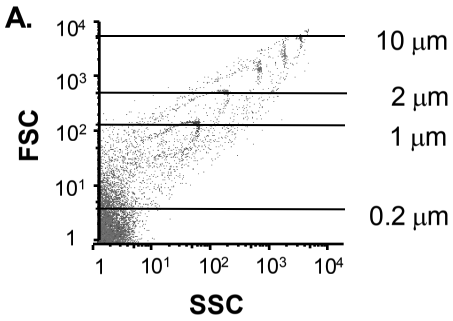
Supplemental Figure 4. Evaluation of $CD42b^{-}CD31^{+}$ EMPs derived from apoptotic endothelial cells using annexin V as the apoptotic parameter. Data is shown from healthy nonsmokers with normal spirometry and normal DLCO (n=8, yellow circles), healthy smokers with normal spirometry and normal DLCO (combining asymptomatic smokers, n=8, tan circles and symptomatic smokers, n=4, tan triangles); and healthy smokers with normal spirometry and low DLCO (n=9, blue circles). **A**. Levels of $CD42b^{-}CD31^{+}$ EMPs per µl platelet poor plasma of study groups. **B.** Ratio of circulating $CD42b^{-}CD62^{+}$ to $CD42b^{-}CD31^{+}$ EMPs in plasma of the same study groups. The % values below represent the proportion of individuals in that group below the lowest level of healthy nonsmokers. **C.** Levels of $CD42b^{-}CD31^{+}$ annexin V^{+} EMPs per µl platelet poor plasma of the same study groups. The % values represent the proportion of individuals in that group that had $CD42b^{-}CD31^{+}$ annexin V^{+} EMP levels beyond that observed

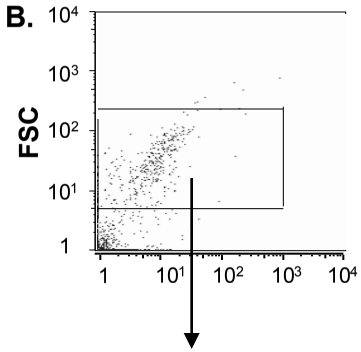
for healthy nonsmokers. p values are indicated. For all groups, a vertical line indicates a subject with systemic hypertension, a horizontal line indicates a subject with type 2 diabetes mellitus. The gray shaded area indicates the mean ± 2 standard deviations of $CD42b^{-}CD31^{+}$ EMP/µl platelet of healthy nonsmokers.

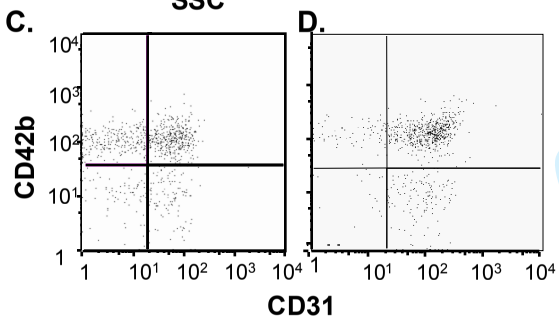
Supplemental Figure 5. Correlation between % emphysema, urine nicotine, $CD42b^{-}CD31^{+}$ EMP and DLCO. The study population included levels of urine nicotine metabolites, $CD42b^{-}CD31^{+}$ EMPs in platelet-poor plasma and DLCO of healthy nonsmokers (n=9, yellow circle), healthy smokers with normal spirometry and normal DLCO (n=20, tan circle) and healthy smokers with normal spirometry but low DLCO (n =6, light blue circle). **A.** Correlation between urine nicotine (ng/ml) and percent emphysema score at -950 HU. **B.** Correlation between CD42b¯CD31+ EMPs per ul platelet-poor plasma and percent emphysema score at -950 HU. **C.** Correlation between DLCO and percent emphysema score at -950 HU. **D**. Correlation between urine nicotine (ng/ml) and percent emphysema score at -910 HU. **E**. Correlation between CD42b¯CD31+ EMPs per µl platelet-poor plasma and percent emphysema score at -910 HU. **F.** Correlation between DLCO and percent emphysema score at -910 HU.
For all groups, a vertical line indicates a subject with systemic hypertension, a horizontal line indicates a subject with type 2 diabetes mellitus, and shaded circle indicates subjects with HIV-1. The gray shaded area indicates the mean ± 2 standard deviations of $CD42b^{-}CD31^{+}$ EMP/µl platelet of healthy nonsmokers.

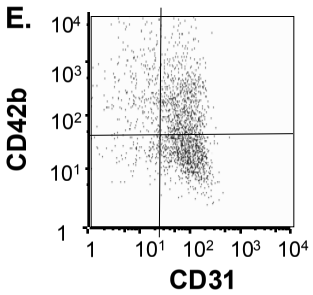
Supplemental Figure 6. Percent of emphysema score in the study groups. Shown is data of healthy nonsmokers (n=9, yellow circle), healthy smokers with normal spirometry and normal DLCO (n=20, tan circle) and healthy smokers with normal spirometry but low DLCO (n =6, light blue circle). **A.** Percent emphysema score at -910 HU. **B.** Percent emphysema score at -950

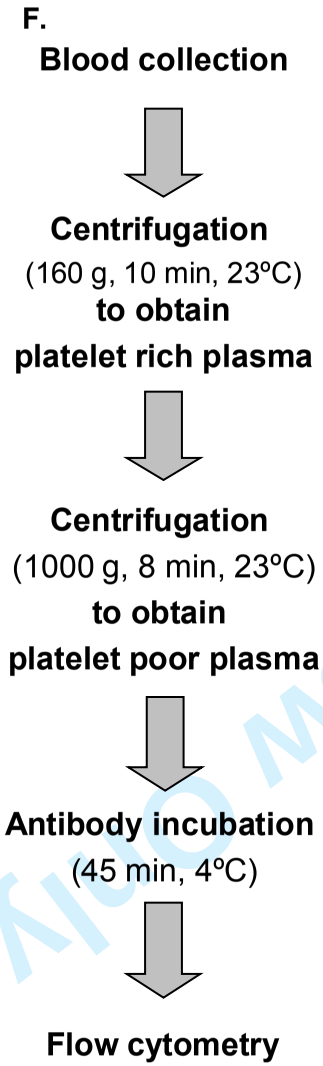
HU. For all groups, a vertical line indicates a subject with systemic hypertension, a horizontal line indicates a subject with type 2 diabetes mellitus. The gray shaded area indicates the mean ± 2 standard deviations of emphysema score of healthy nonsmokers. The % values represent the proportion of individuals in that group that had higher levels of percent emphysema score beyond the level observed for healthy nonsmokers.

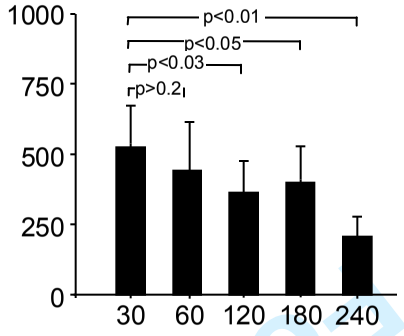


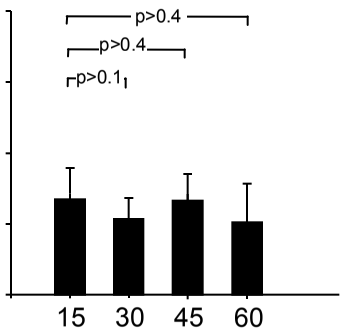


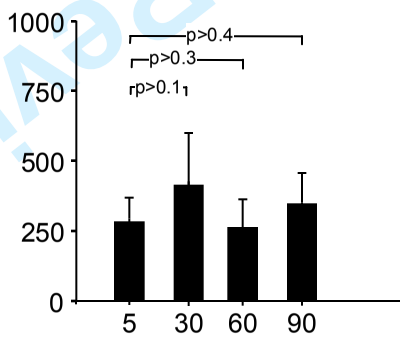


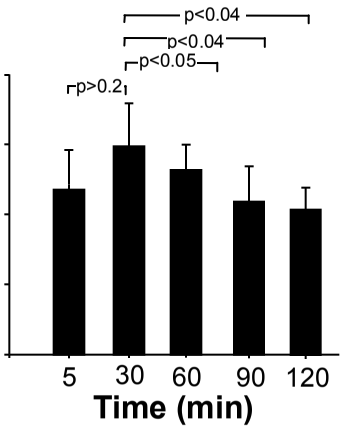


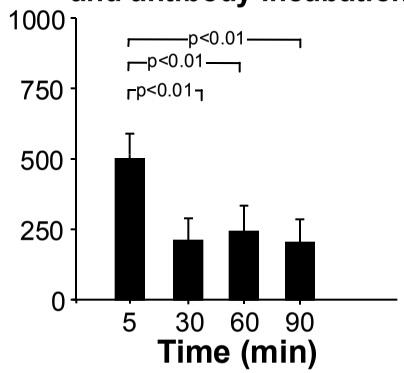




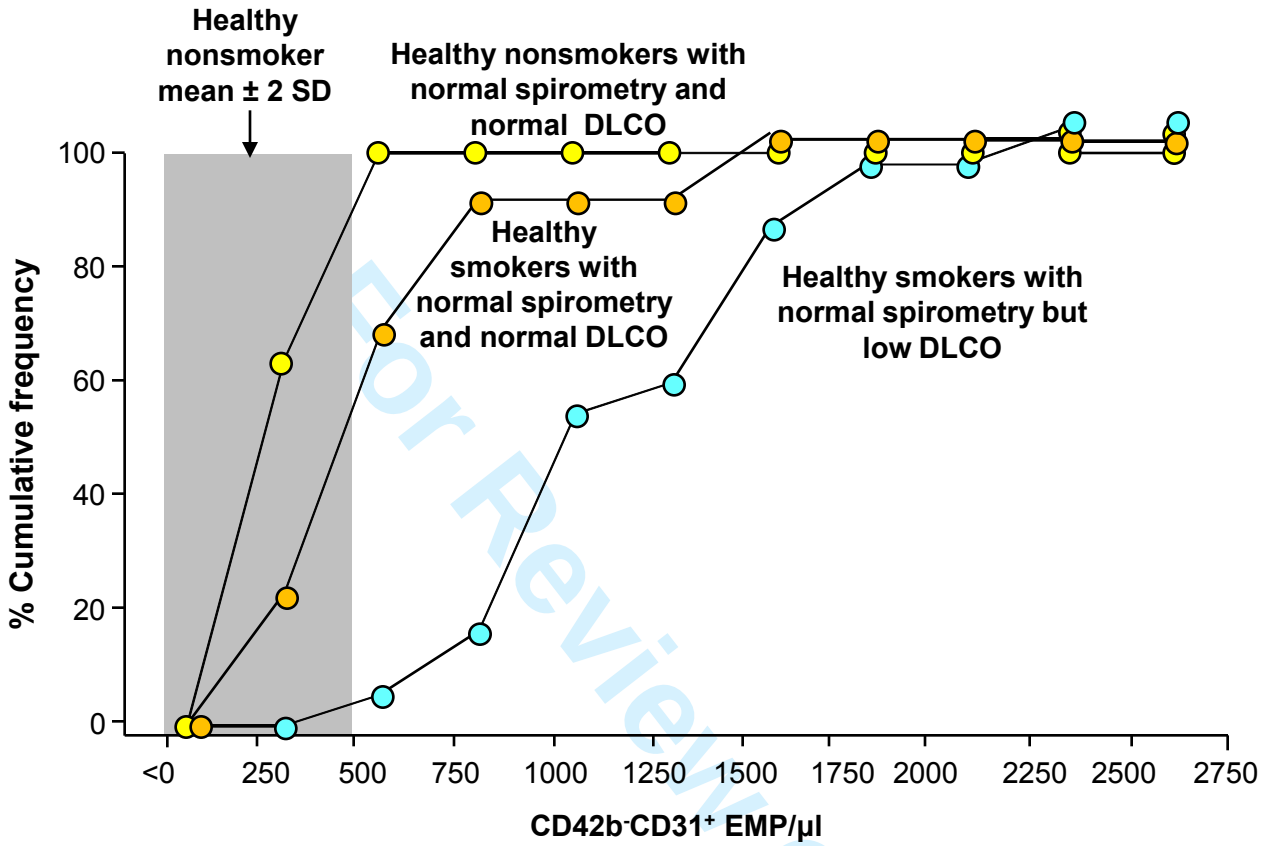




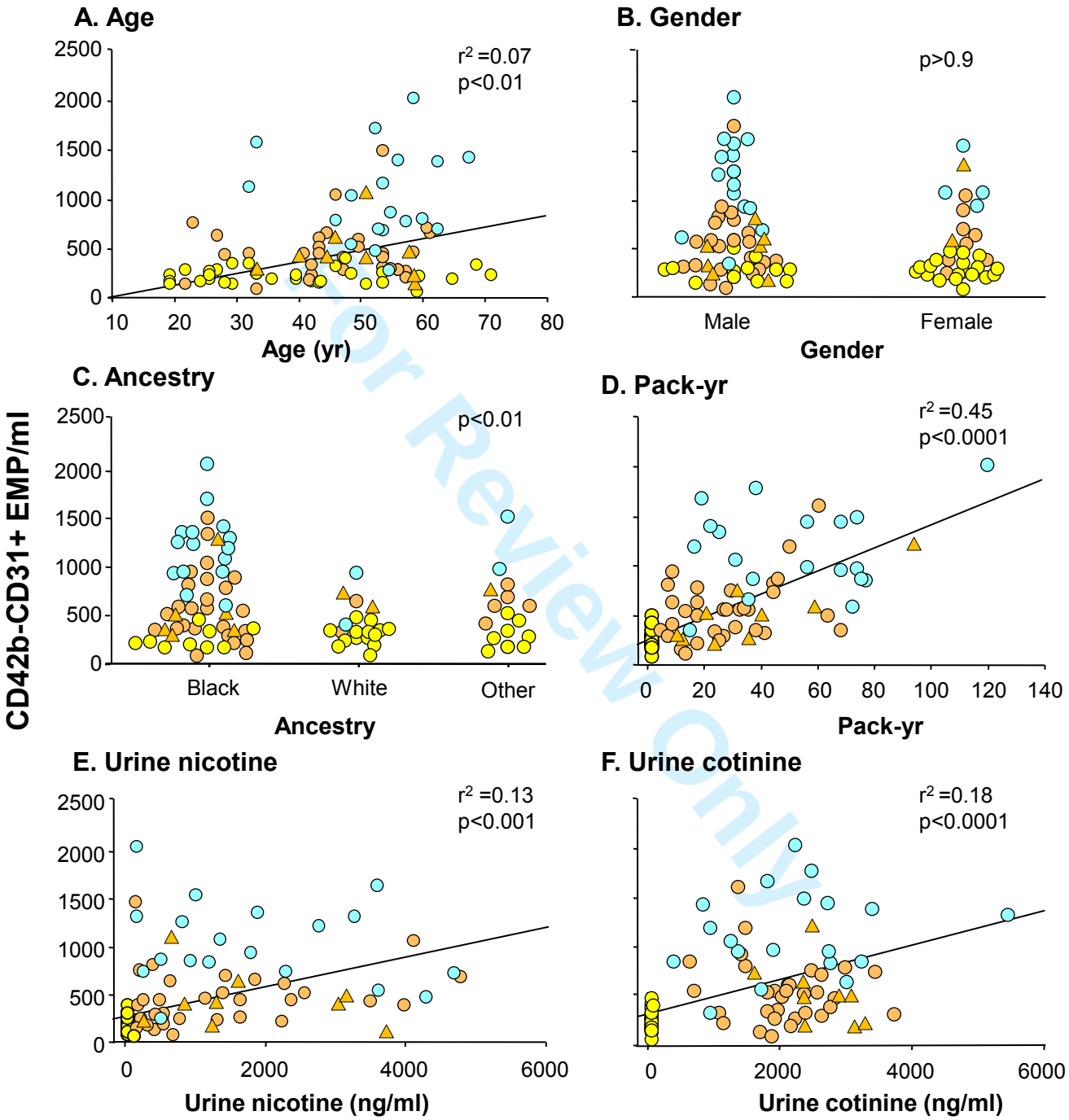




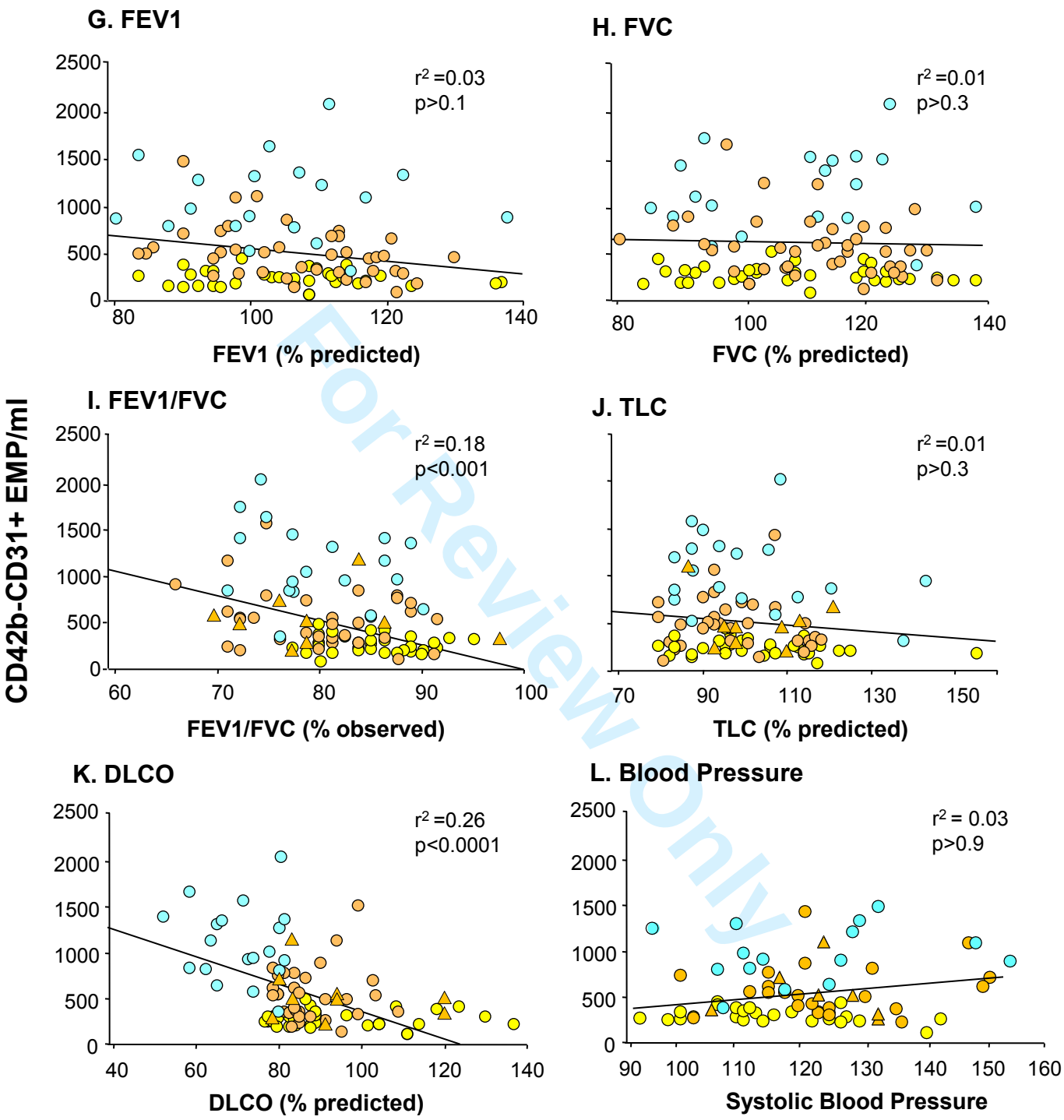
Supplemental Figure 2



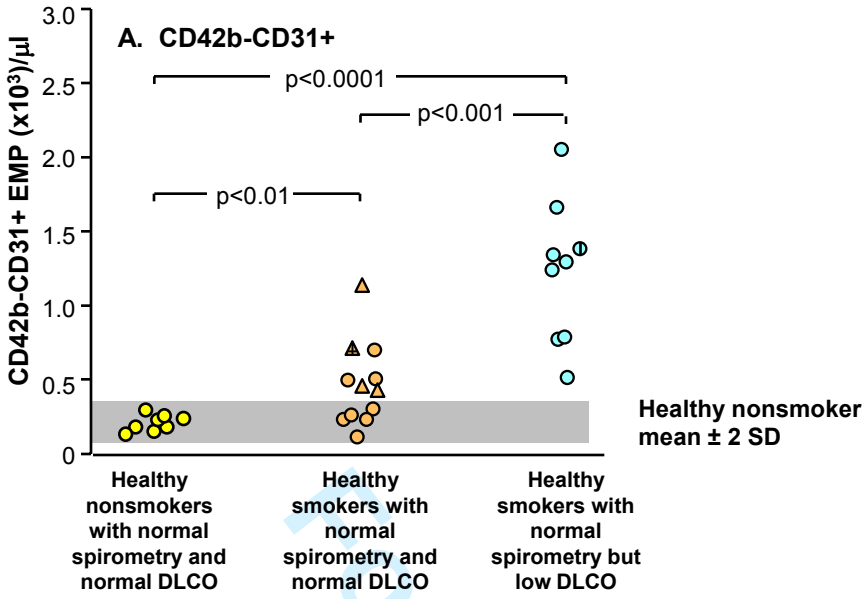
Supplemental Figure 3A-F

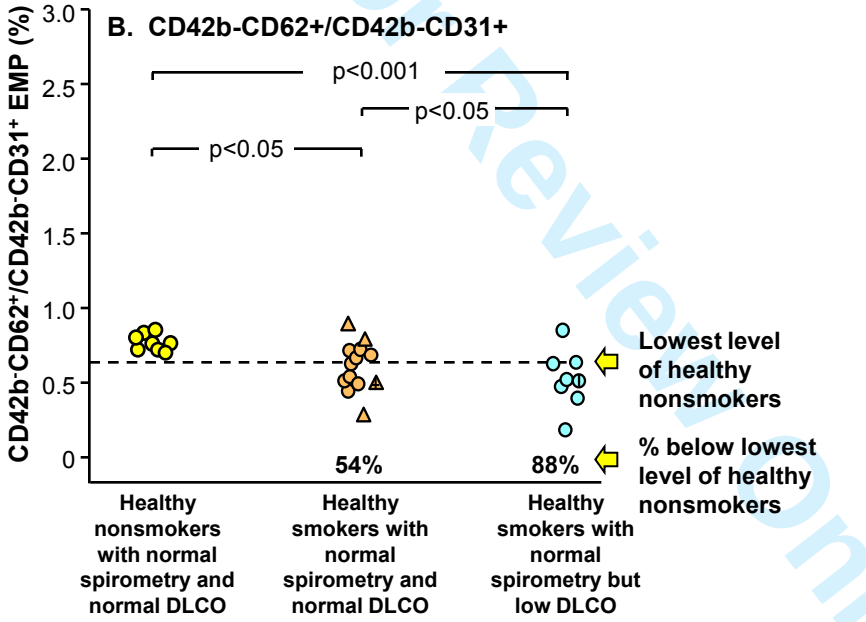


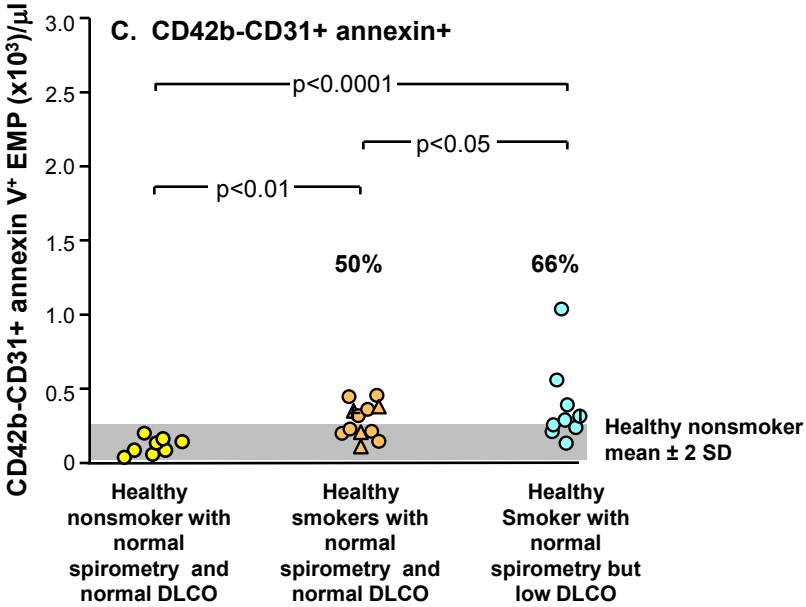
Supplemental Figure 3G-L

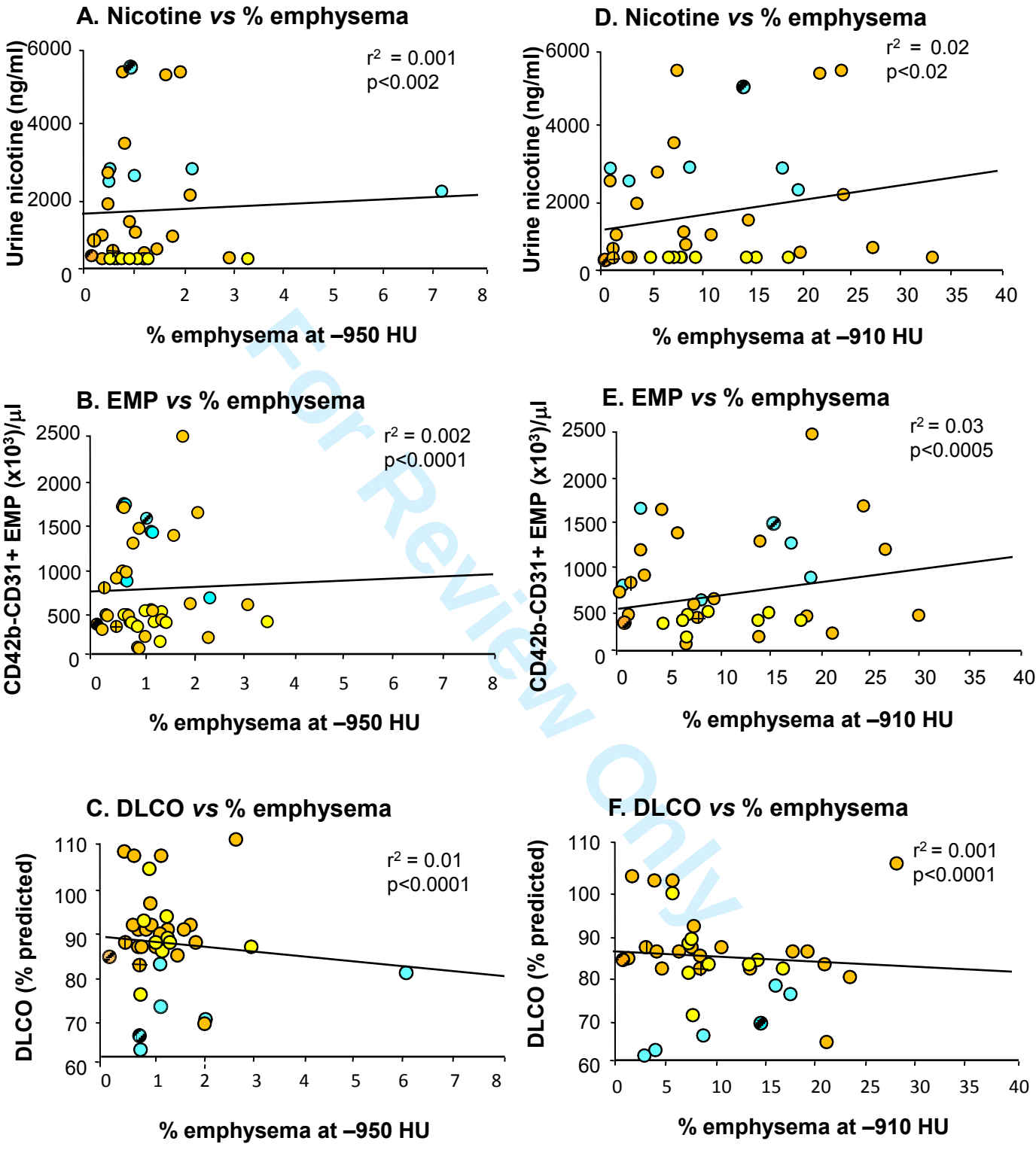


Supplemental Figure 4









Supplemental Figure 6