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/A6	0.29	N/A	0.88
Ancestry3	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/A6	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/A6	0.32	N/A	0.73
Ancestry3	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=21, 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 μl 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 (combining asymptomatic smokers, n=13, tan circles and symptomatic smokers, n=8, tan triangles) 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.