Supplemental Methods

Inclusion and Exclusion Criteria

Nonsmokers

Inclusion criteria

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

Exclusion criteria

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

Healthy Smokers

Inclusion criteria

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

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

Exclusion criteria

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

COPD Smokers

Inclusion criteria

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

Exclusion criteria

- Unable to meet the inclusion criteria

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

Human Subjects and Clinical Phenotypes

All 138 subjects enrolled in this study underwent thorough screening including medical history, complete physical exam, blood studies, urinalysis, chest X-ray, electrocardiograms and pulmonary function tests, including forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV1), FEV1/FVC, total lung capacity (TLC) and diffusion capacity (DLCO), all carried out under ATS guidelines[1-3]. If the FEV1 was <80% predicted and/or the FEV1/FVC <0.7, spirometry was retested after standard bronchodilators. Measurement of the DLCO was carried out 2 to 4 times in all subjects; the average of the best 2 trials was used. The GOLD criteria, based on post-bronchodilator FEV1/FVC ratio <0.7, were used to define and stage COPD[4]. The diameter of the main pulmonary artery was assessed by chest CT scans as a correlate to the pulmonary artery pressure. In all subjects, the pulmonary artery diameter was ≤ 30 mm, indicating normal estimated pulmonary pressure[5].

Chest high resolution computed tomography (HRCT) scans were used to determine the percentage of lung affected by emphysema in each subject. Percentage emphysema was evaluated with the EmphylxJ software application (EmphylxJ, Vancouver, BC, Canada) allowing automated quantitative analysis of transverse chest CT scans[6-8]. The lung was divided into quartiles by lung volume, and the top and bottom quartiles were compared for % emphysema at attenuation -950 Hounsfield Units (HU). Emphysema was defined as >5% lung volume, value derived from analyses of HRCT in normal nonsmoking individuals with normal lung function.

Smoking status of all subjects was defined by self-reported smoking history and verified by urinary levels of nicotine and cotinine. Both nicotine and cotinine were used to define non-smoking status but only cotinine levels were used to define a current smoker as it has a longer half-life than nicotine. Since subjects vary in the last time they smoked a cigarette prior to the visit, nicotine that has a short half-life, and is only detected in urine for 2 to 8 hr after smoking[11],

might not be detected in urine at collection time though the subject is a current smoker. Cotinine that has a half-life in urine of 8 to 24 hr[12] provides a more accurate assessment of the smoking status.

Nonsmokers were defined as self-reported never smokers with undetectable urine nicotine (<2 ng/ml) and cotinine (<5 ng/ml)[9]. Current smokers were defined as self-reported current smokers with urine cotinine level ≥ 104 ng/ml, a level based on our previous study of low level smoke exposure[10], where 104 ng/ml was calculated as the induction half maximal level (ID_{50}) at which the small airway epithelium, the initial site of smoking-related pathology, showed abnormal response.

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

Characterization of Plasma Endothelial Microparticles

Endothelial microparticles were quantified as previously described[13]. Blood was collected and processed within 1 hr to prepare platelet-rich plasma. The supernatant was further processed within 5 min to obtain platelet-poor plasma that was stained for 3 antibodies: the constitutive endothelial marker PECAM (CD31); the constitutive platelet-specific glycoprotein Ib (CD42b)

and E-selectin (CD62E), an adhesion molecule expressed on activated endothelium. Anti-human CD45-PECy5 (leukocyte marker, clone HI30, BD PharMingen) was also used to monitor leukocyte MP contamination. To assess the presence of relative contribution of pulmonary capillary endothelium to the elevated EMP levels, $CD42b^{-}CD31^{+}$ EMPs were co-stained with anti-human angiotensin converting enzyme (ACE) based on the knowledge that ACE is abundantly expressed on pulmonary capillary endothelium[14]. The optimized condition for each antibody was determined by serial dilutions. EMP measurements were performed twice to ensure that the measurements were reproducible. $CD42b^{-}CD31^{+}$ and $CD42b^{-}CD62E^{+}$ microparticle levels were corrected for correlating isotype control antibodies. Regarding apoptotic EMPs, relating to $CD31^{+}$ and $CD62E^{+}$, the data is displayed as ratio; following the methods of Jimenez et al.[15], we have chosen to calculate it as the ratio of $CD42b^{-}CD62E^{+}/CD42b^{-}CD31^{+}$, with a lower ratio identifying the apoptotic EMPs.

Smoking Cessation

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

Statistical Analysis

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

References

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Supplemental Figure Legends

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

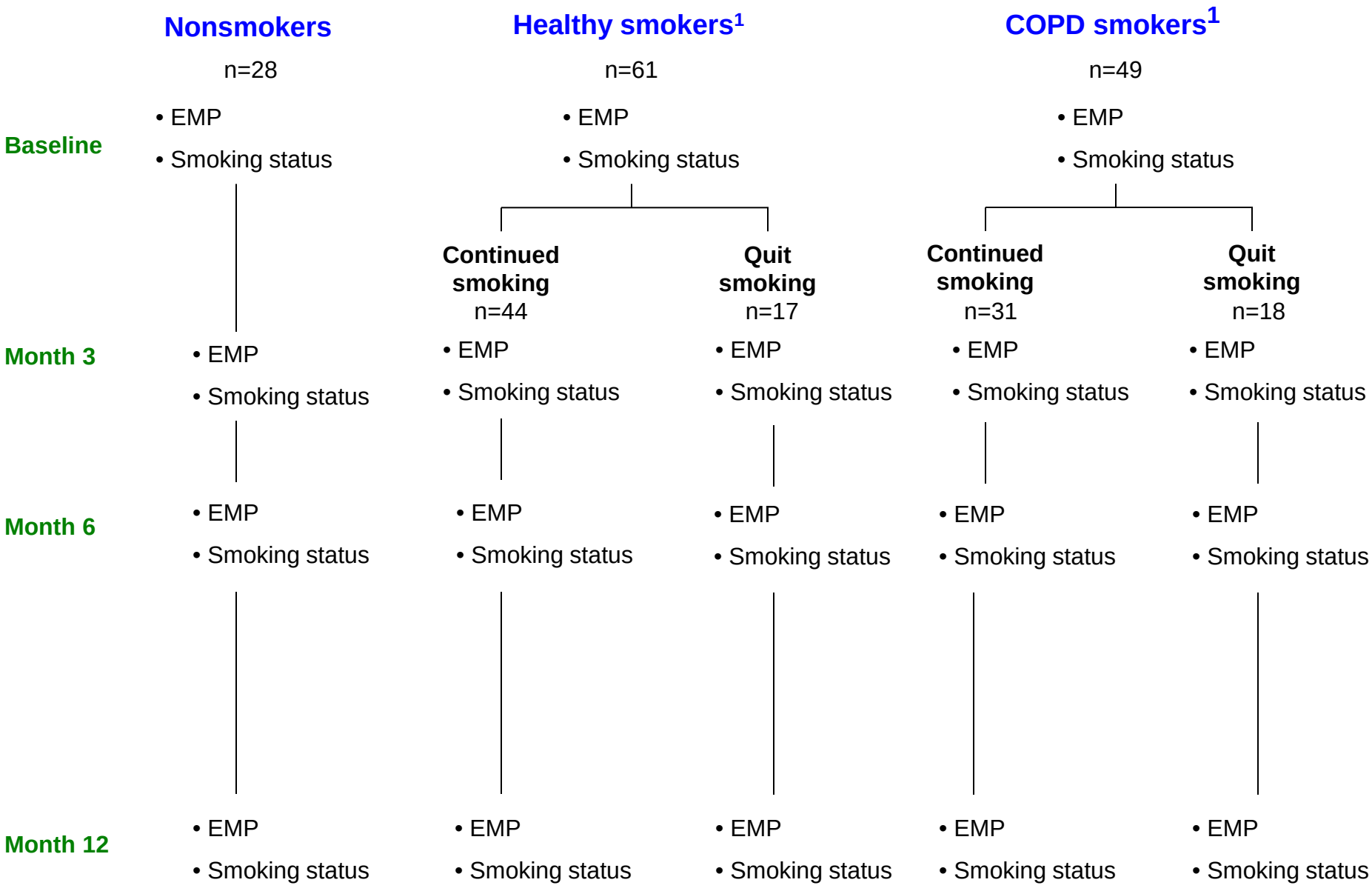
Supplemental Figure 2. Relationship between total $CD42b^{-}CD31^{+}$ EMPs and smoking-related, demographic, and lung function parameters of the study population. The data includes levels of $CD42b^{-}CD31^{+}$ EMPs from plasma of nonsmokers (n=28, green circles), healthy smokers (n=61, yellow circles);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

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

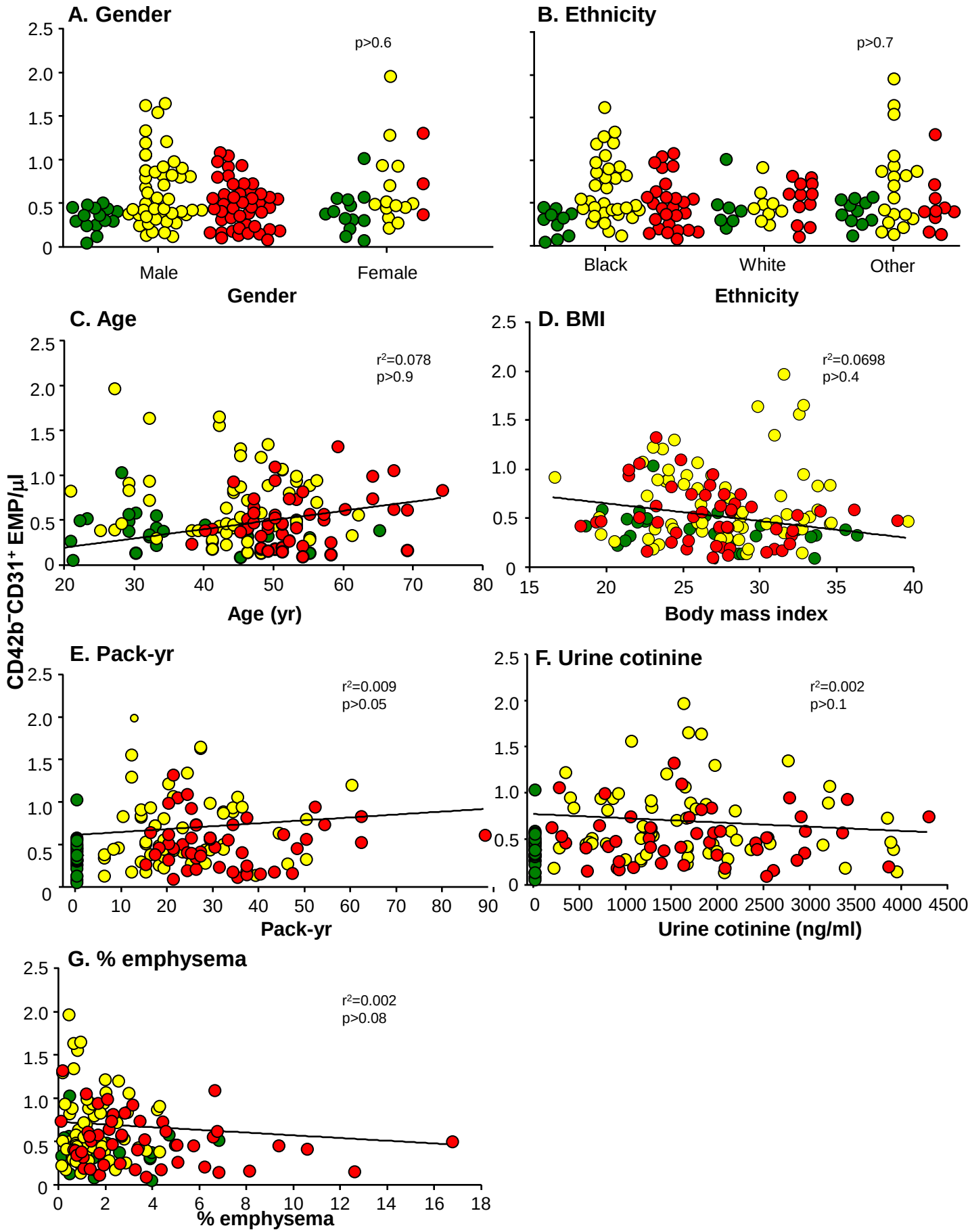
Pack-yr. **F.** Urine cotinine. **G.** % emphysema. **H.** FEV1. **I.** FVC. **J.** FEV1/FVC. **K.** TLC.

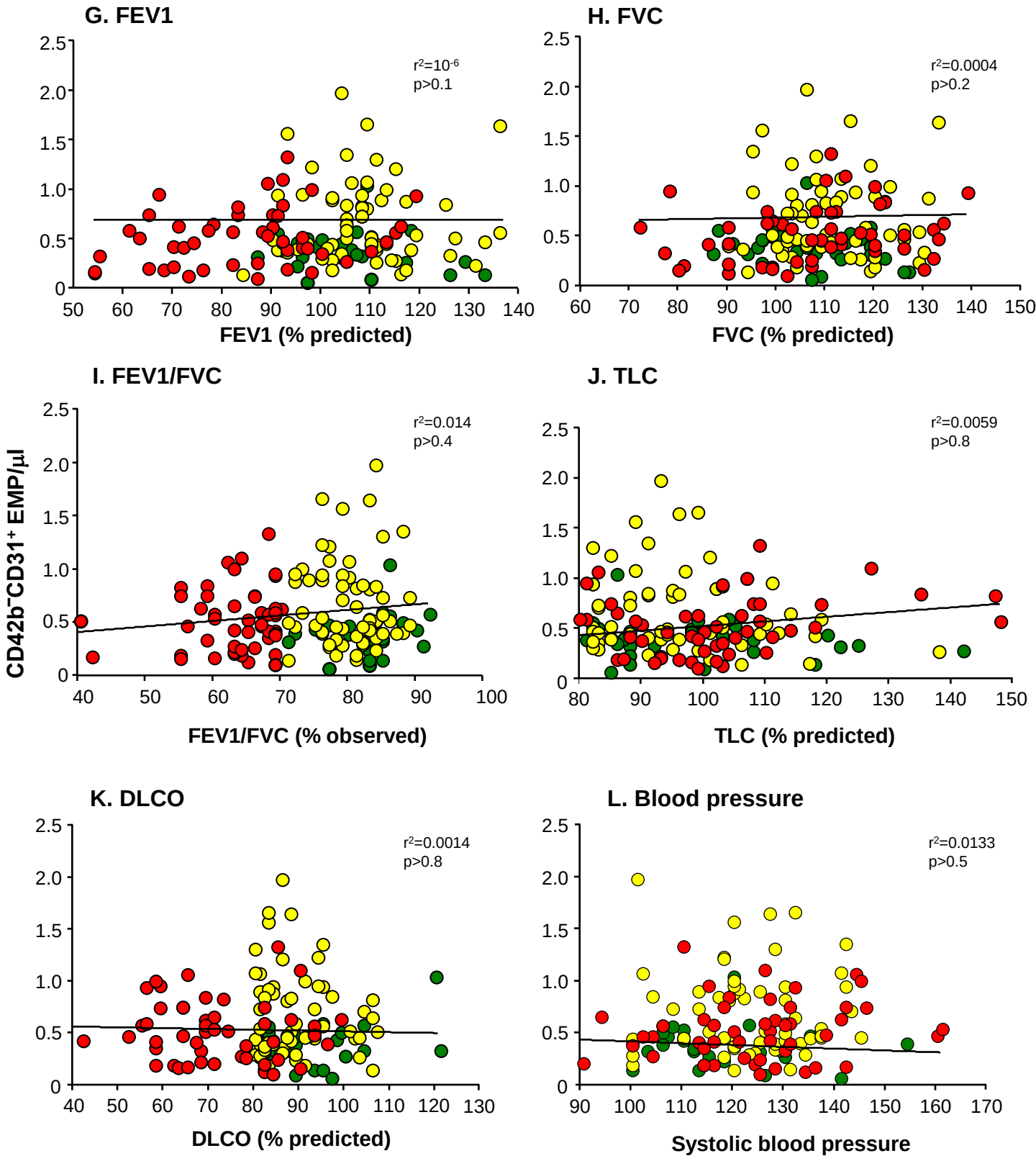
L. Systolic blood pressure. The correlation coefficient and p values are indicated.

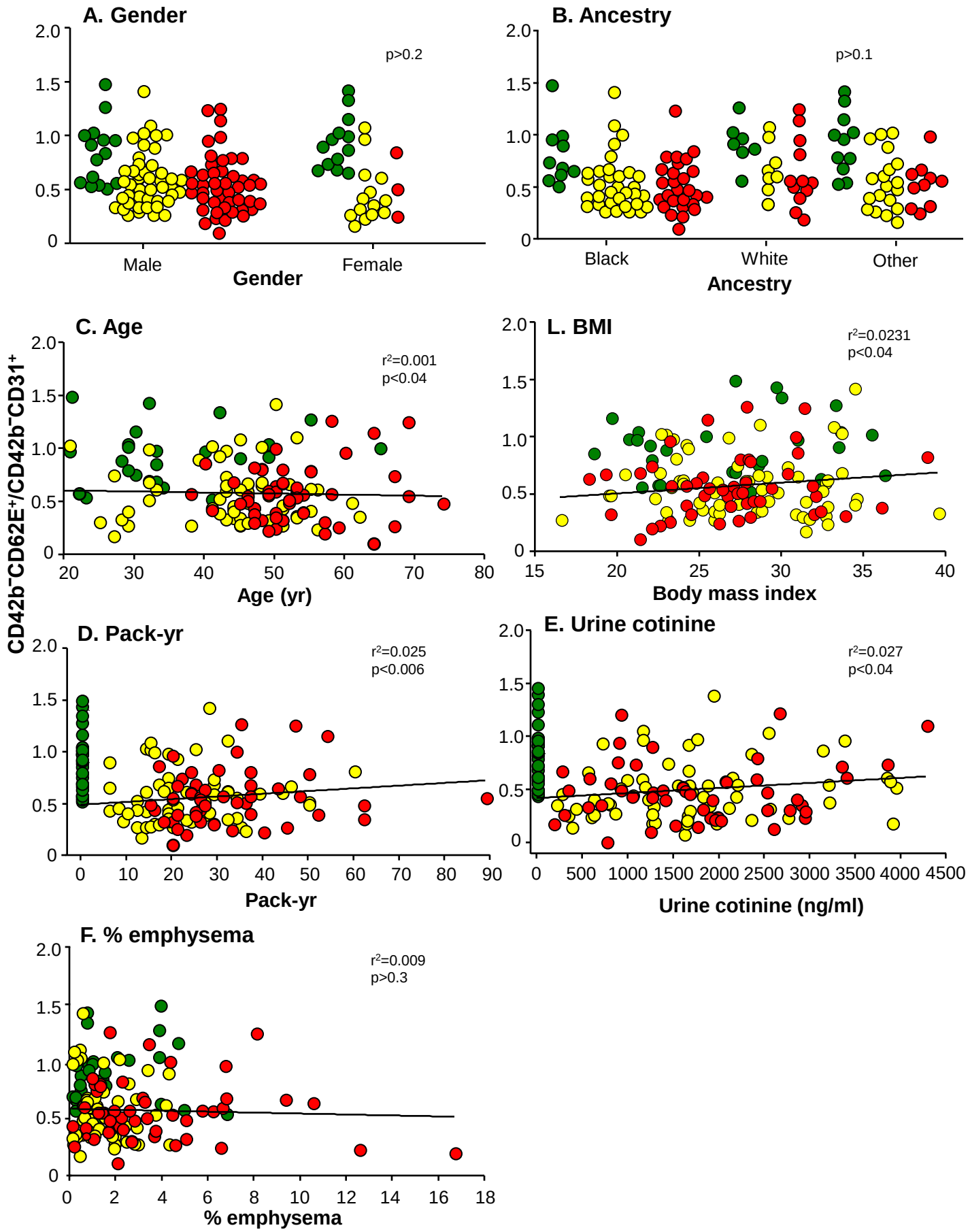
Supplemental Figure 1

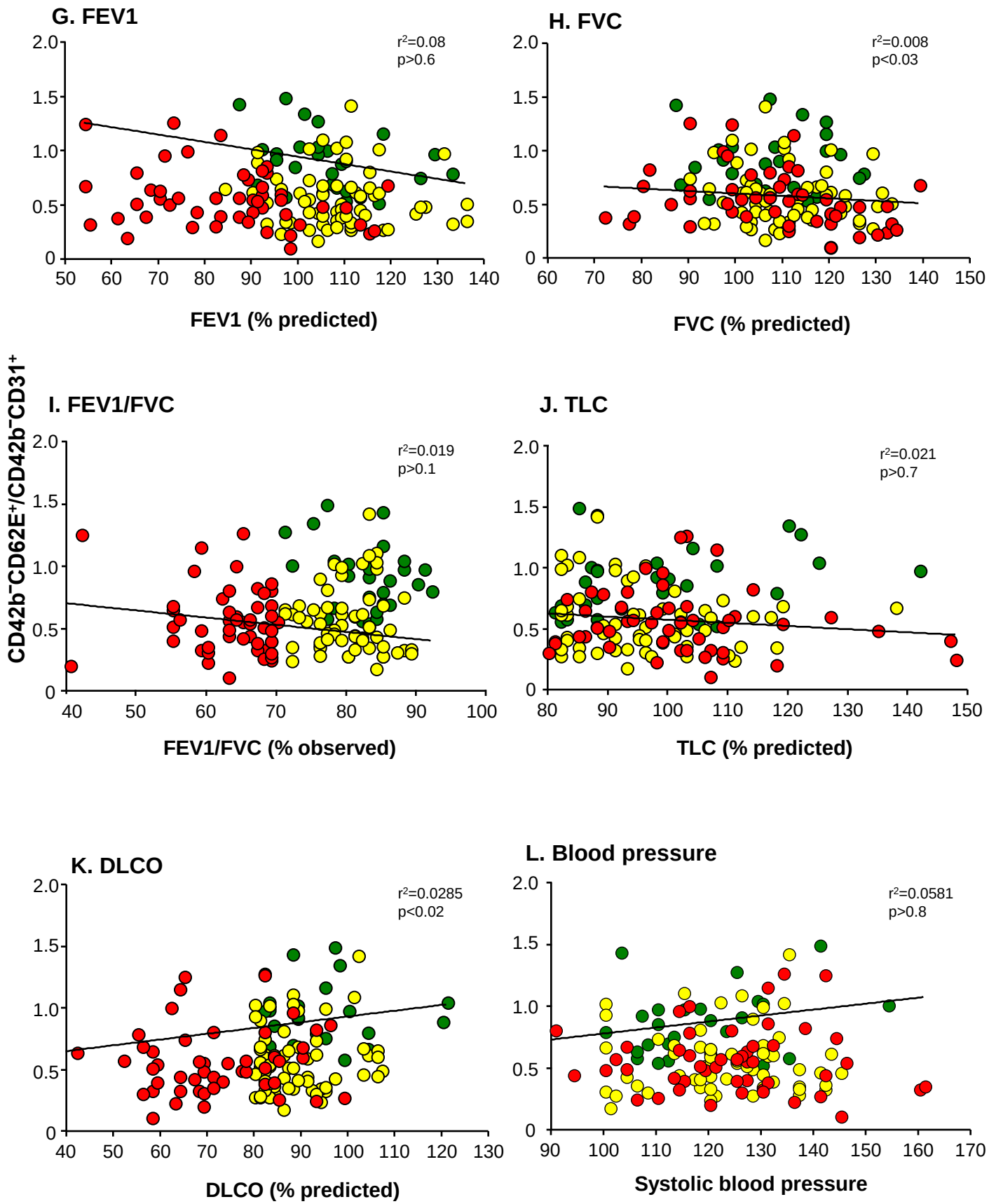


Supplemental Figure 2A-F




G. FEV1
r²=10⁻⁶
p>0.1
FEV1 (% predicted)
H. FVC
r²=0.0004
p>0.2
FVC (% predicted)
I. FEV1/FVC
r²=0.014
p>0.4
FEV1/FVC (% observed)
CD42b⁻CD31⁺ EMP/µl
J. TLC
r²=0.0059
p>0.8
TLC (% predicted)
K. DLCO
r²=0.0014
p>0.8
DLCO (% predicted)
L. Blood pressure
r²=0.0133
p>0.5
Systolic blood pressure


A. Gender
p>0.2
Male
Female
Gender
B. Ancestry
p>0.1
Black
White
Other
Ancestry
C. Age
r²=0.001
p<0.04
Age (yr)
L. BMI
r²=0.0231
p<0.04
Body mass index
D. Pack-yr
r²=0.025
p<0.006
Pack-yr
E. Urine cotinine
r²=0.027
p<0.04
Urine cotinine (ng/ml)
F. % emphysema
r²=0.009
p>0.3
% emphysema
CD42b⁻CD62E⁺/CD42b⁻CD31⁺


G. FEV1
r²=0.08
p>0.6
FEV1 (% predicted)
H. FVC
r²=0.008
p<0.03
FVC (% predicted)
CD42b⁻CD62E⁺/CD42b⁻CD31⁺
I. FEV1/FVC
r²=0.019
p>0.1
FEV1/FVC (% observed)
J. TLC
r²=0.021
p>0.7
TLC (% predicted)
K. DLCO
r²=0.0285
p<0.02
DLCO (% predicted)
L. Blood pressure
r²=0.0581
p>0.8
Systolic blood pressure