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Immuno-fibrotic drivers of impaired lung function in post-COVID-19 syndrome

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ABSTRACT

Introduction: Subjects recovering from COVID-19 frequently experience persistent respiratory ailments; however, little is known about the underlying biological factors that may direct lung recovery and the extent to which these are affected by COVID-19 severity.

Methods: We performed a prospective cohort study of subjects with persistent symptoms after recovering from acute COVID-19 illness, collecting clinical data, pulmonary function tests, and blood. Plasma samples were used for multiplex profiling of circulating factors associated with inflammation, metabolism, angiogenesis, and fibrosis.

Results: Sixty-one subjects were enrolled across two academic medical centers at a median of 9 weeks (interquartile range 6-10) after COVID-19 illness: n=13 subjects (21%) mild/non-hospitalized, n=30 (49%) hospitalized/non-critical, and n=18 subjects (30%) hospitalized/intensive care ("ICU"). Fifty-three subjects (85%) had lingering symptoms, most commonly dyspnea (69%) and cough (58%). Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and diffusing capacity for carbon monoxide (DLCO) declined as COVID-19 severity increased ($P<0.05$), but did not correlate with respiratory symptoms. Partial least-squares discriminant analysis of plasma biomarker profiles clustered subjects by past COVID-19 severity. Lipocalin 2 (LCN2), matrix metalloproteinase-7 (MMP-7), and hepatocyte growth factor (HGF) identified by the model were significantly higher in the ICU group ($P<0.05$) and inversely correlated with FVC and DLCO ($P<0.05$).

Conclusions: Subjective respiratory symptoms are common after acute COVID-19 illness but do not correlate with COVID-19 severity or pulmonary function. Host response profiles reflecting neutrophil activation (LCN2), fibrosis signaling (MMP-7), and alveolar repair (HGF) track with lung impairment and may be novel therapeutic or prognostic targets.

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INTRODUCTION

As the number of patients who recover from acute coronavirus disease 19 (COVID-19) rises, it is increasingly apparent that a substantial subset displays persistent subjective and objective respiratory ailments. Patients report a high prevalence of lingering respiratory symptoms such as fatigue and dyspnea (1-4), and spirometry and diffusion capacity testing show impaired pulmonary function (3). However, little is known about the biological drivers of long-term respiratory disease after COVID-19, and how acute COVID illness severity impacts the emerging COVID-19 “long-hauler syndrome”. To better understand the relationship between subjective and objective respiratory abnormalities and underlying biological drivers, we measured symptom burden, pulmonary function tests, and plasma biomarkers in subjects that have recovered from COVID-19 infection.

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RESULTS

We enrolled 61 subjects into the post-COVID-19 clinics. Subject demographics for each group are shown in the **Table**. Median (IQR) age was 53 (43-62) years and sex distribution was equitable: 56% males and 44% females. Median (IQR) time from onset of COVID-19 infection to post-COVID follow up was 9 (6-11) weeks. Thirteen subjects (21%) recovered at home ("home" group), 30 subjects (48%) were hospitalized but did not require ICU ("non-ICU" group) and nineteen subjects (31%) were hospitalized and required ICU level care ("ICU" group). Nine of the subjects admitted to the ICU required intubation and mechanical ventilation.

Fifty-three subjects (85%) reported lingering symptoms of COVID-19, the most common being dyspnea (69%), cough (58%), fever (47%), fatigue (29%), chest pain (26%), and headache (23%) (**Figure 1A**). There were no significant differences in symptom burden between the home, non-ICU, and ICU groups (P=NS, **Figure 1B**). Pulmonary function testing was performed on 59 subjects. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and diffusing capacity for carbon monoxide (DLCO) decreased as severity of COVID-19 increased (**Figure 2A**). There was no relationship between pulmonary function and presence of persistent respiratory symptoms, measured by the sum of the scores for fatigue, dyspnea, and cough (**Figure 2B**).

To identify potential biological factors that associate with impaired pulmonary function after COVID-19 infection, we measured levels of circulating inflammatory, metabolic, angiogenic, and fibrotic markers in plasma from a subset of 22 subjects (home group n=7, non-ICU group n=5, ICU group n=10) and performed partial least-squares discriminant analysis (PLS-DA) (**Figure 3**). The score plot (**Figure 3A**) shows that subjects largely cluster in distinct quadrants based on how severe their COVID-19 illness was, even ~9 weeks after infection. In fact, the clustering shows a counter-clockwise rotation around the origin as severity of viral infection increases from home to non-ICU to ICU-level of care. The loadings plot (**Figure 3B**) shows the associations between the clusters of subjects and the measured plasma biomarkers

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(**Supplemental Table**). The top right quadrant shows the biomarkers that directly correlate with critical COVID-19 illness requiring ICU admission. Several of the most impactful biomarkers (i.e. furthest from the point of origin) that associate with ICU level of care are LCN2 (lipocalin-2), MMP-7 (matrix metalloproteinase-7), and HGF (hepatocyte growth factor). Plasma levels of all three factors were significantly higher in the ICU group compared to the home group (**Figure 4A**).

To further determine if specific circulating factors are associated with persistently impaired pulmonary function, we used random-forest feature selection (5) to identify which factors best explain the variability seen in FVC and DLCO (**Supplemental Figures 1-2**). This analysis identified MMP-7 as the marker that was most significantly associated with FVC and DLCO. LCN2, HGF, and leptin, among others, were also significantly associated with FVC, DLCO, or both. In our patient cohort, leptin did not differ by acute COVID-19 severity, but was strongly inversely correlated with post-COVID-19 FVC and also directly correlated with body mass index (**Supplemental Figure 3**). Confirmation of the relationship between LCN2, MMP-7, and HGF and pulmonary function using simple linear regression identified a highly significant, inverse correlation with FVC and DLCO (**Figure 4B**).

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DISCUSSION

Herein we report that respiratory symptoms and impaired lung function are common after acute COVID-19 infection. Pulmonary function in particular worsens as COVID-19 illness severity increases. We further show that plasma biomarker profiles measured at 9 weeks after COVID-19 infection reveal prior COVID-19 severity and cluster patients accurately. Plasma LCN-2, MMP-7, and HGF levels are highest in survivors of severe COVID-19 infection and correlate strongly with pulmonary function impairment.

Patients recovering from acute COVID-19 infection commonly display persistent symptoms such as fatigue and dyspnea (1-4). Our study found a high degree of symptom burden across subgroups (home, non-ICU, ICU) that did not associate by COVID-19 severity (3). Impaired pulmonary function was reported by Townsend et al (1), and similar to our findings, correlated with prior COVID-19 severity. Together these data highlight the discordance of subjective and objective respiratory abnormalities after COVID-19 infection and indicate that subjective symptoms alone cannot discern degree of post-COVID-19 lung function impairment.

Partial least-squares discriminant analysis could reliably identify and separate subjects by COVID-19 illness severity based on their plasma biomarker profiles, even 9 weeks after recovery. Our analysis highlighted a number of important biomarkers that strongly associated with severe/ICU COVID-19 status. We chose to further analyze three of these markers, LCN2, MMP-7, and HGF based on their differing roles in host response. LCN2 is a component of neutrophil secondary granules that was found to strongly associate with acute COVID-19 critical illness and mortality (6-9); however, the role in post-COVID-19 recovery has not been previously described. That higher levels of LCN-2 are found at 9 weeks after severe COVID-19 illness compared with non-severe cases suggests these patients may have ongoing neutrophil activation that could be amenable to targeted therapy.

MMP-7 is a protease that breaks down extracellular matrix deposited in the lung after injury. The role of MMP-7 in COVID-19 has not been reported; however, previous studies of

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non-COVID-19 acute lung injury have shown higher circulating levels of MMPs (10) that associate with worse disease severity and clinical outcomes (11). MMP-7 is also a validated biomarker for idiopathic pulmonary fibrosis severity that correlates with pulmonary function (12), and our findings suggest MMP-7 may be a novel biomarker of COVID-19 ARDS recovery.

HGF is a growth factor secreted by alveolar fibroblasts shown to be elevated in bronchoalveolar lavage fluid of patients with ARDS (13) and in plasma of patients with acute COVID-19 illness (14); however, the plasma levels after COVID-19 recovery have not been previously reported. In vivo and in vitro studies indicate HGF promotes alveolar epithelial and endothelial repair after acute lung injury (15, 16) via induction of counter inflammatory and anti-oxidant gene expression (17-19). Our findings suggest high HGF levels at 9 weeks post-COVID-19 could indicate ongoing alveolar repair proportionate to the degree of acute lung injury; however, further studies are needed to determine the impact of HGF on COVID-19 recovery.

Leptin is a hormone secreted by adipocytes in proportion to obesity, a risk factor for severe acute COVID-19 (20), and may contributes to immune dysregulation during acute COVID-19 (21, 22). In our patient population with post-COVID-19 syndrome, we found leptin was inversely related to FVC, however, it is not clear if this simply reflects underlying obesity, a known cause for reduced FVC, or if it represents ongoing immune dysregulation (or both), and requires further study.

Our study has several limitations. First, we only recruited subjects that had ongoing symptoms after COVID-19 infection, therefore we cannot report the true prevalence of symptoms or pulmonary function derangements in subjects with asymptomatic infection or in subjects with symptom resolution after COVID-19 infection. Second, our plasma biomarker profiles represent a single time point for each subject, and therefore we cannot draw conclusions about trajectory or changes over time. Third, we cannot establish mechanism in this human cohort study; however, our findings are highly associational and lay the foundations to drive further work in this area.

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In conclusion, persistent respiratory symptoms and impaired lung function are common after acute COVID-19 illness, but subjective symptoms alone do not predict lung injury. Partial least-squares discriminant analysis reliably identifies and separates subjects by COVID-19 illness severity based on their plasma biomarker profiles, even 9 weeks after recovery. Circulating factors associated with acute neutrophil activation, fibrosis signaling, and alveolar epithelial repair remain elevated in survivors of acute COVID-19 infection and strongly associate with impaired pulmonary function. Our study provides novel insights into divergent host responses of lung repair after COVID-19 pneumonia and suggest that immunologic and fibrotic drivers of disease severity in the acute COVID-19 illness may regulate the trajectory of recovery in the post-COVID-19 phase. These markers may represent novel therapeutic targets that can be followed during and after COVID-19 illness, and may identify COVID-19 survivors at increased risk for chronic parenchymal lung disease, and will require validation in a larger study.

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METHODS

Subject Enrollment

The studies were approved by the institutional review boards of Yale University (#2000027792 and #1401013259) and Duke University (Pro00105518 and Pro00106151). Subjects with persistent symptoms after recovering from COVID-19 infection were recruited to a post-COVID-19 clinic for further evaluation. Written informed consent was provided by all subjects prior to study activities.

Data Collection

All subjects completed symptom questionnaires (presence or absence of the following: fatigue, fever, headache, dyspnea, cough, chest pain, nausea, diarrhea, anosmia, dysgeusia, sore throat, nasal congestion, red eyes, and vision loss), physical examinations, and pulmonary function testing (spirometry and diffusion capacity) at the time of their follow-up visits. Symptom scores were calculated by the sum of the number of symptoms reported. Severity of COVID-19 infection was defined by need for intensive care unit (ICU) hospital care, non-ICU hospital care (non-ICU), or non-hospitalized home recuperation (home). Whole blood was collected by venipuncture, centrifuged, separated into plasma, and stored at -80°C until use.

Plasma biomarker profiling

Multiplex profiling of plasma was performed on a subset of subjects (n=22) by Eve Technologies (Calgary, Alberta, Canada) using the following assays: Human Cytokine 71-Plex, Human Complement Panel 1, Human Angiogenesis 17-Plex, Human MMP 9-Plex and TIMP 4- Plex, and Human Adipokine 5-Plex. A complete list of biomarkers profiled is provided in the **Supplemental Table**.

Statistical Analysis

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Grouped data are expressed as median (interquartile range) unless specified otherwise. Differences between groups were analyzed by Kruskal-Wallis test with Benjamini-Hochberg post-hoc test to control for false discovery rate or one-way ANOVA with Fisher's LSD post-hoc test (Graphpad Prism, San Diego, CA). Simple linear regression were performed by Prism. Partial least-squares discriminant analysis (PLS-DA) was performed using SIMCA v15 (Umetrics, Umeå, Sweden). The random-forest feature selection (Boruta algorithm) was performed using the Boruta package in R software. All P-values are two-tailed. $P<0.05$ was accepted as statistically significant.

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AUTHOR CONTRIBUTIONS

H.J.C. and B.D.K. wrote the manuscript that was approved by all authors. All authors participated in data collection, analysis, and/or interpretation. The overall study design was conceived by Y.J.C., B.D.K., L.G.Q., A.I.L., and P.J.L.

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Table: Subject Demographics

	Post-COVID-19 Clinic N=61
Age (years), median (IQR)	53 (43-65)
Sex, N (%)	
Female	27 (44%)
Male	34 (56%)
Ethnicity, N (%)	
Hispanic/Latino	10 (16%)
Not Hispanic/Latino	47 (77%)
Unknown	4 (7%)
Race, N (%)	
African American	13 (21%)
American Indian or Alaskan Native	2 (3%)
Asian	2 (3%)
Caucasian	39 (64%)
Two or more races	1 (2%)
Unknown	4 (7%)
Body mass index, median (IQR)	30 (26-37)
COVID-19 Severity, N (%)	
Home	13 (21%)
Hospitalized, non-ICU	30 (49%)
Hospitalized, ICU	18 (30%)
Intubated, N (%)	8 (13%)
Weeks since COVID-19, median (IQR)	9 (6-10)

Abbreviations: COVID-19, coronavirus disease 2019; ICU, intensive care unit; IQR, interquartile range; N, number.

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FIGURE LEGENDS

Figure 1: Post-COVID-19 symptom burden. A) Prevalence of symptoms reported by subjects (n=61) at the time of initial follow up visit. B) Symptom scores (total number of symptoms reported) with median bars and interquartile range error bars for home (n=13), non-ICU (n=30), and ICU (n=18) groups. There were no significant differences between groups using the Kruskal-Wallis test with Benjamini-Hochberg post-hoc test.

Figure 2: Pulmonary function tests and Respiratory Symptom Score. A) Scatter plots show forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and diffusing capacity for carbon monoxide (DLCO) as percentages of predicted values for each subject in the home, non-ICU, and ICU groups for N (number) shown. B) Scatter plots show the sum of respiratory symptoms (1 point each for dyspnea, cough, and fatigue) vs. FVC, FEV1, and DLCO. Bars are medians and error bars are interquartile range. Statistical analysis by Kruskal-Wallis test with Benjamini-Hochberg post-hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure 3: Partial least-squares discriminant analysis (PLS-DA). A) The score plot shows each individual subject in the home (blue circles), non-ICU (purple circles), and ICU (red circles) groups. The grey zone in the score plot depicts outliers with 95% confidence. B) The loadings plot depicts associations between clusters and the measured biomarkers.

Figure 4: Biomarkers. A) Plasma levels of LCN2 (lipocalin-2), MMP-7 (matrix metalloproteinase-7), and HGF (hepatocyte growth factor) in individual subjects (black circles) in the home, non-ICU, and ICU groups. Bars are means and error bars are standard deviation. Statistical analysis by one-way ANOVA with Fisher's LSD post-hoc test. *P<0.05, **P<0.01, ***P<0.001. B). Scatter plots for subjects (black circles) of LCN2 (lipocalin-2), MMP-7 (matrix metalloproteinase-7), and HGF (hepatocyte growth factor) on abscissa versus FVC (forced vital

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capacity) or DLCO (diffusing capacity) on ordinate. Statistical analysis is simple linear regression. Blue shaded area shows 95% confidence intervals.

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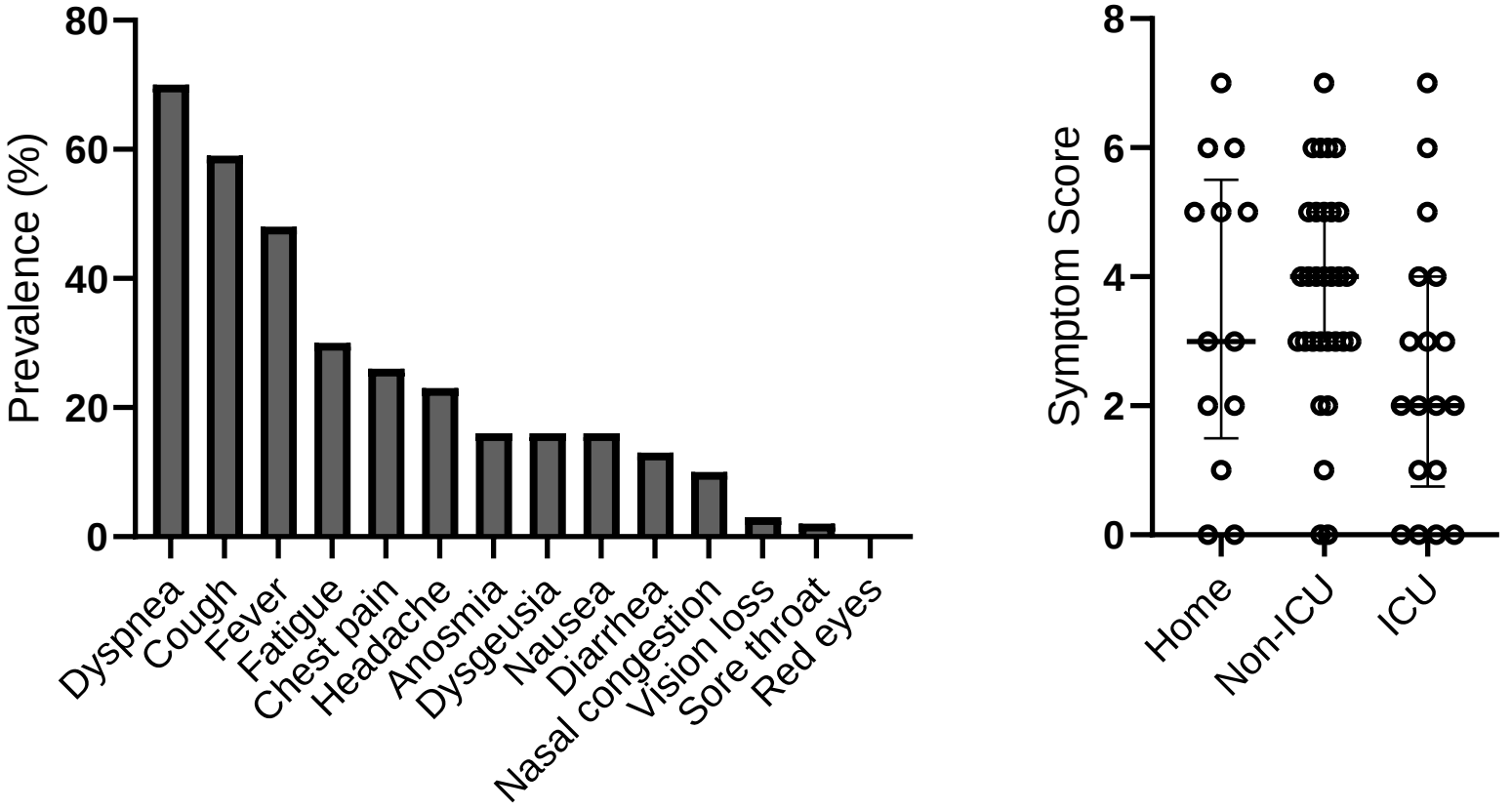

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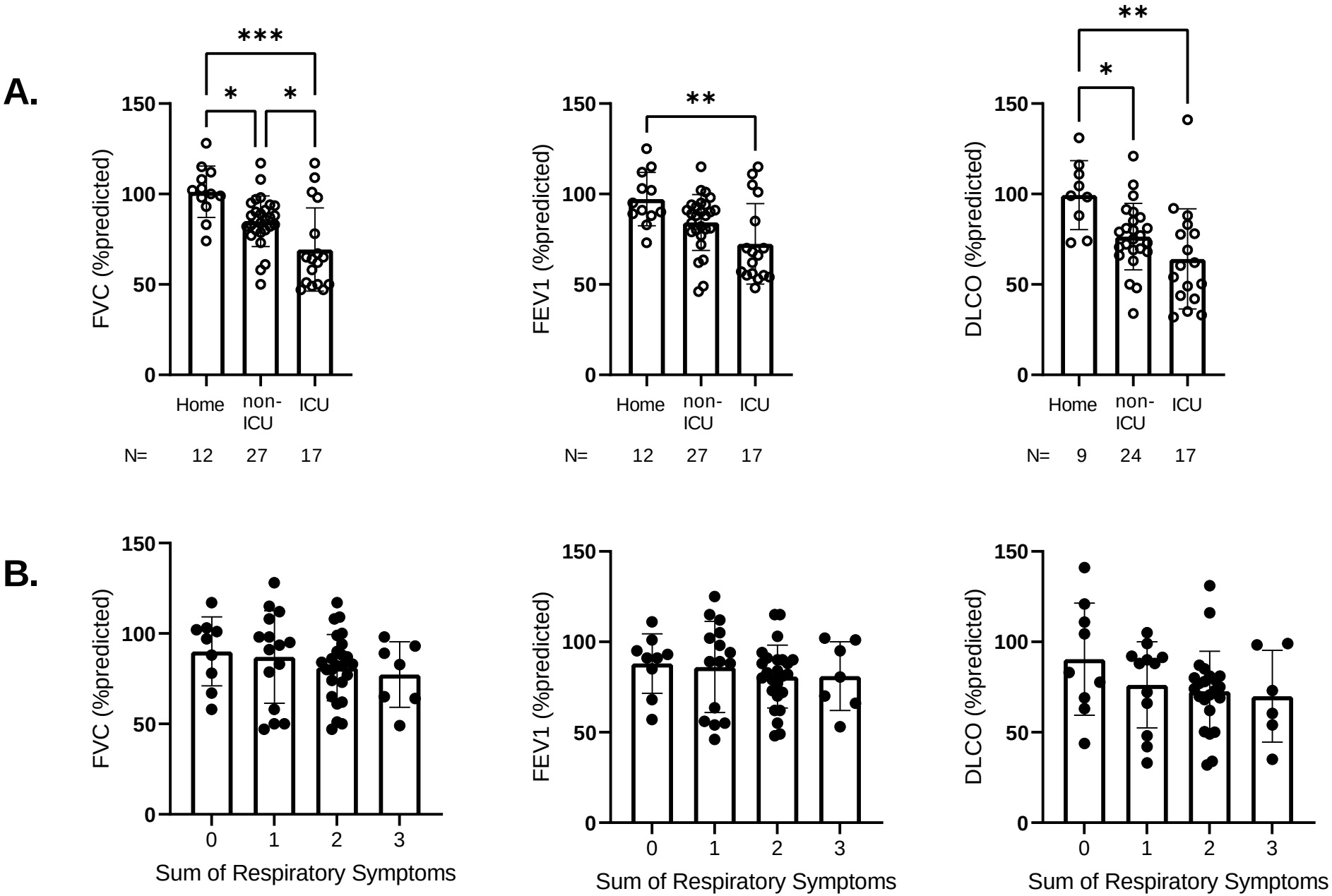

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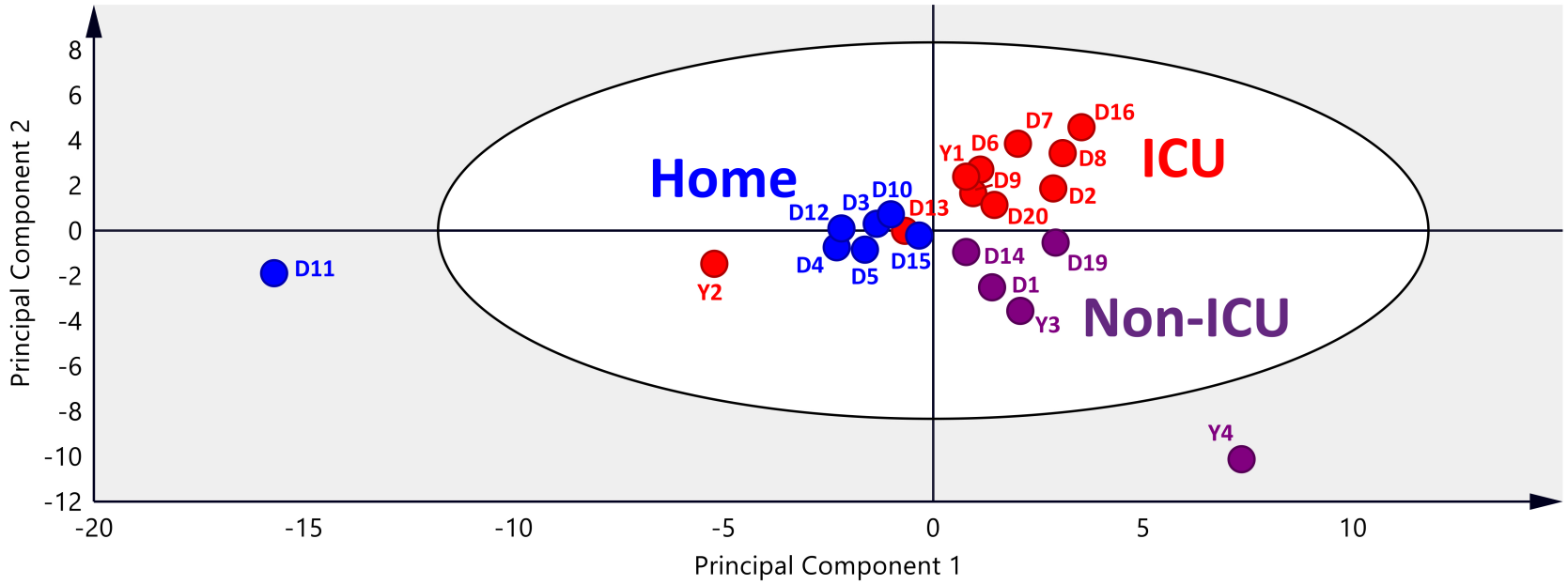
A. Score Plot



B. Loadings Plot

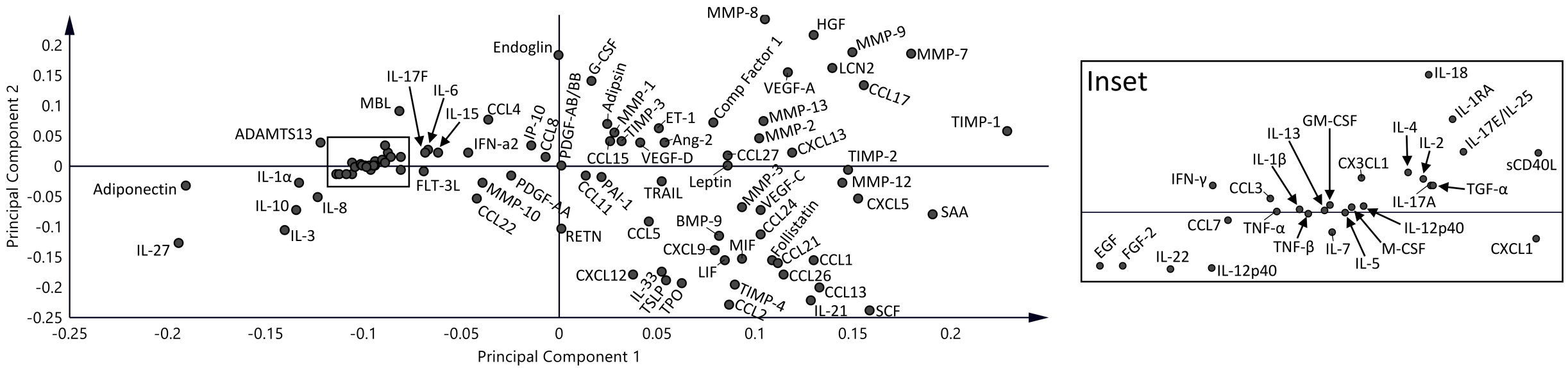


Figure 3: Partial least-squares discriminant analysis (PLS-DA). A) The score plot shows each individual subject in the home (blue circles), non-ICU (purple circles), and ICU (red circles) groups. the grey zone in the score plot depicts outliers with 95% confidence. B) The loadings plot depicts associations between clusters and the measured biomarkers.

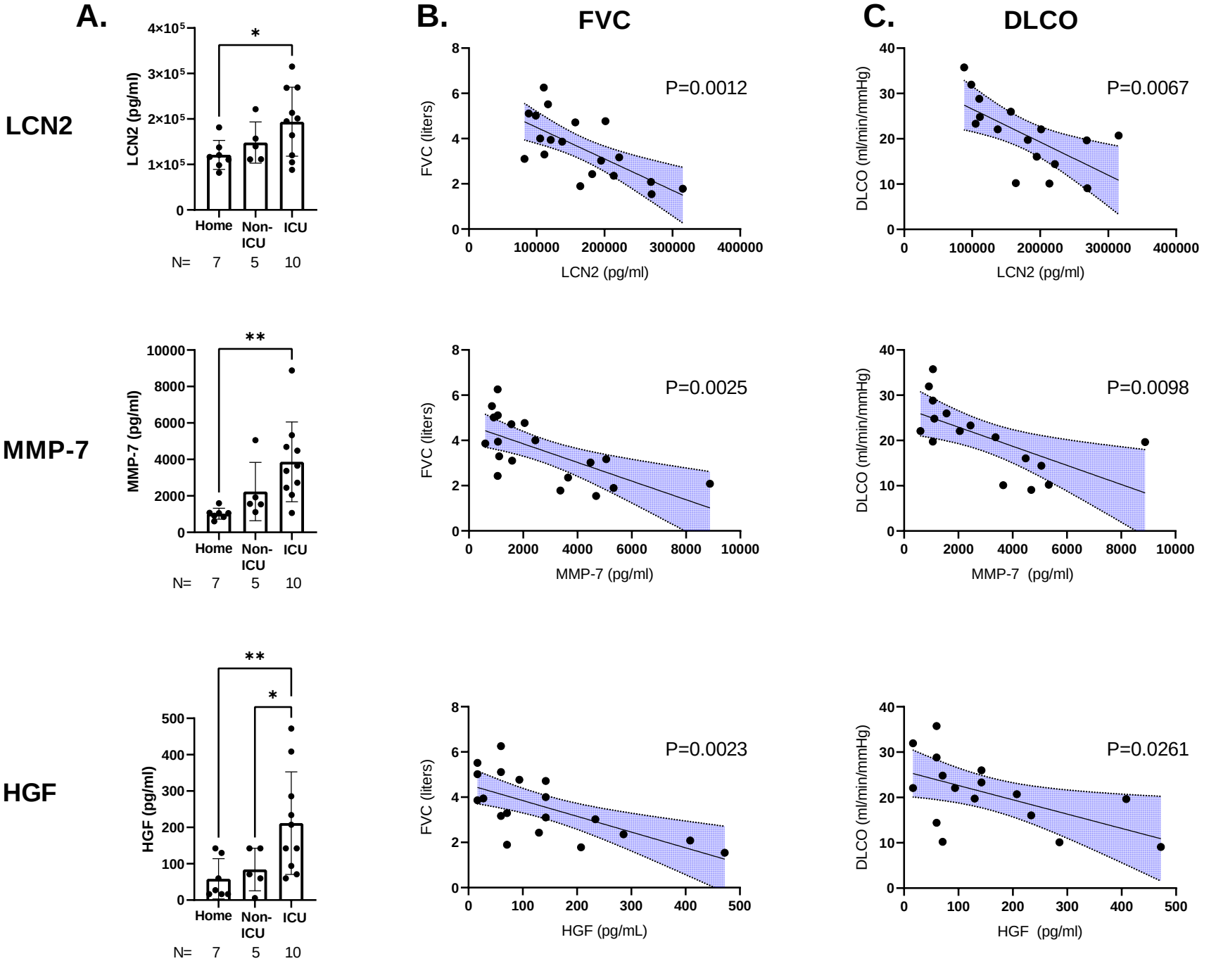


Figure 4: Biomarkers. A) Plasma levels of LCN2 (lipocalin-2), MMP-7 (matrix metalloproteinase-7), and HGF (hepatocyte growth factor) in individual subjects (black circles) in the home, non-ICU, and ICU groups. Bars are means and error bars are standard deviation. Statistical analysis by one-way ANOVA with Fisher's LSD post-hoc test. *P<0.05, **P<0.01, ***P<0.001. B). Scatter plots for subjects (black circles) of LCN2, MMP-7, and HGF on abscissa versus FVC (forced vital capacity) or DLCO (diffusing capacity) on ordinate. Statistical analysis is simple linear regression. Blue shaded area shows 95% confidence intervals.

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Supplemental Table: Plasma Biomarkers
ADAMTS13
Adiponectin
Adipsin
Angiopoietin-2 (Ang-2)
BMP-9
C5a
CCL1
CCL11
CCL13
CCL15
CCL17
CCL2
CCL21
CCL22
CCL24
CCL26
CCL27
CCL3
CCL4
CCL5
CCL-7
CCL8
Complement (Comp) Factor 1
CX3CL1
CXCL1
CXCL12
CXCL13
CXCL5
CXCL9
EGF
Endoglin
Endothelin-1
FGF-1
FGF-2
FLT-3L
Follistatin
G-CSF
GM-CSF
HB-EGF
Hepatocyte Growth Factor (HGF)
IFN-a2
IFNy
IL-10
IL-12p40
IL-12p70
IL-13

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IL-15
IL-16
IL-17A
IL-17E/IL-25
IL-17F
IL-18
IL-1RA
IL-1α
IL-1β
IL-2
IL-20
IL-21
IL-22
IL-23
IL-27
IL-28A
IL-3
IL-33
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IP-10
LCN-2
Leptin
LIF
Mannose Binding Lectin (MBL)
M-CSF
MIF
MMP-1
MMP-10
MMP-12
MMP-13
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
PAI-1
PDGF-AA
PDGF-AB/BB
PLGF
RETN
SAA
sCD40L
SCF

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TGF-α
TIMP-1
TIMP-2
TIMP-3
TIMP-4
TNF-α
TNF-β
TPO
TRAIL
TSLP
VEGF-A
VEGF-C
VEGF-D

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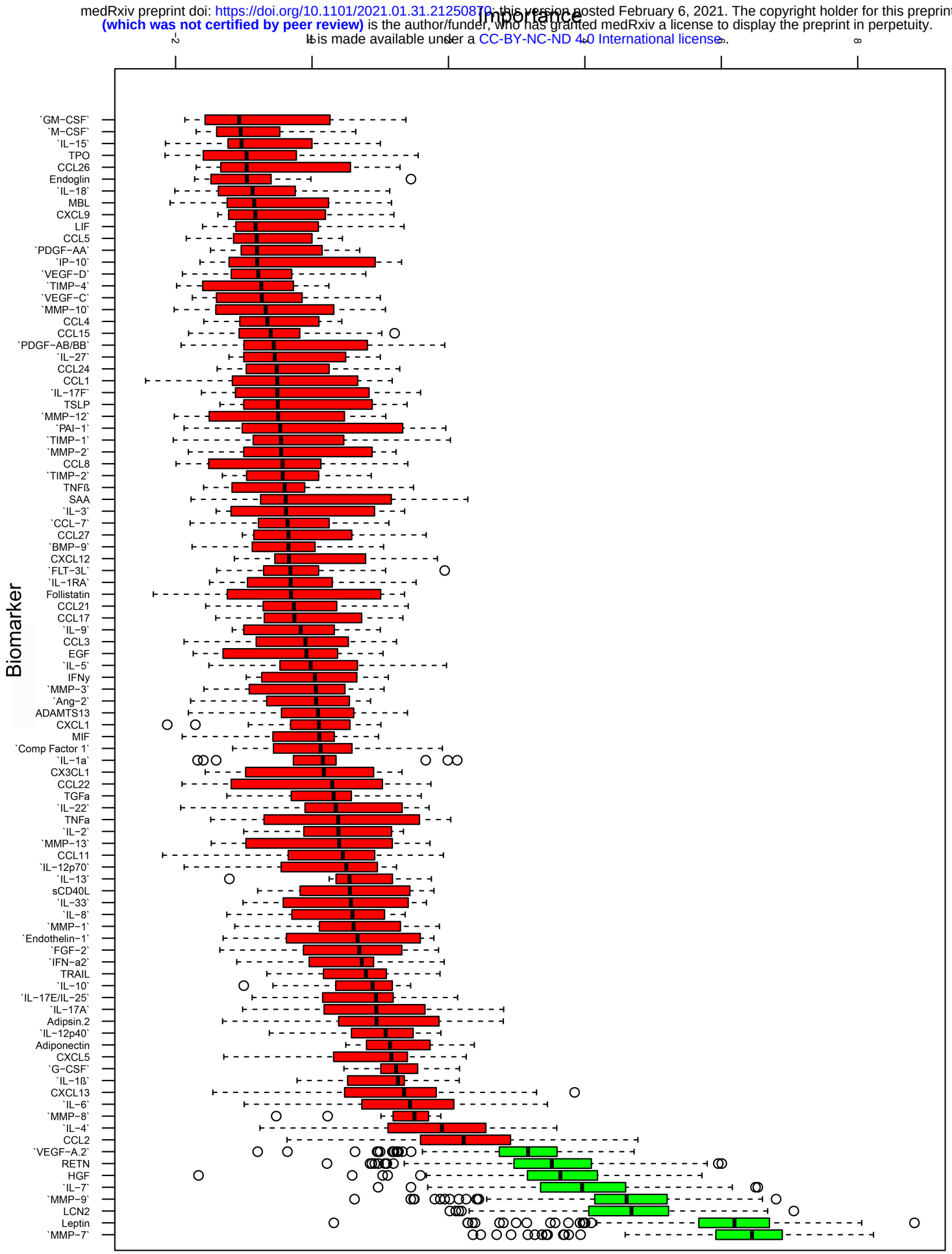

Supplemental Figure 1: Random-forest feature selection Boruta procedure showing the biomarkers in reverse order of importance for explaining variation in forced vital capacity. Green biomarkers are confirmed and red biomarkers are rejected.

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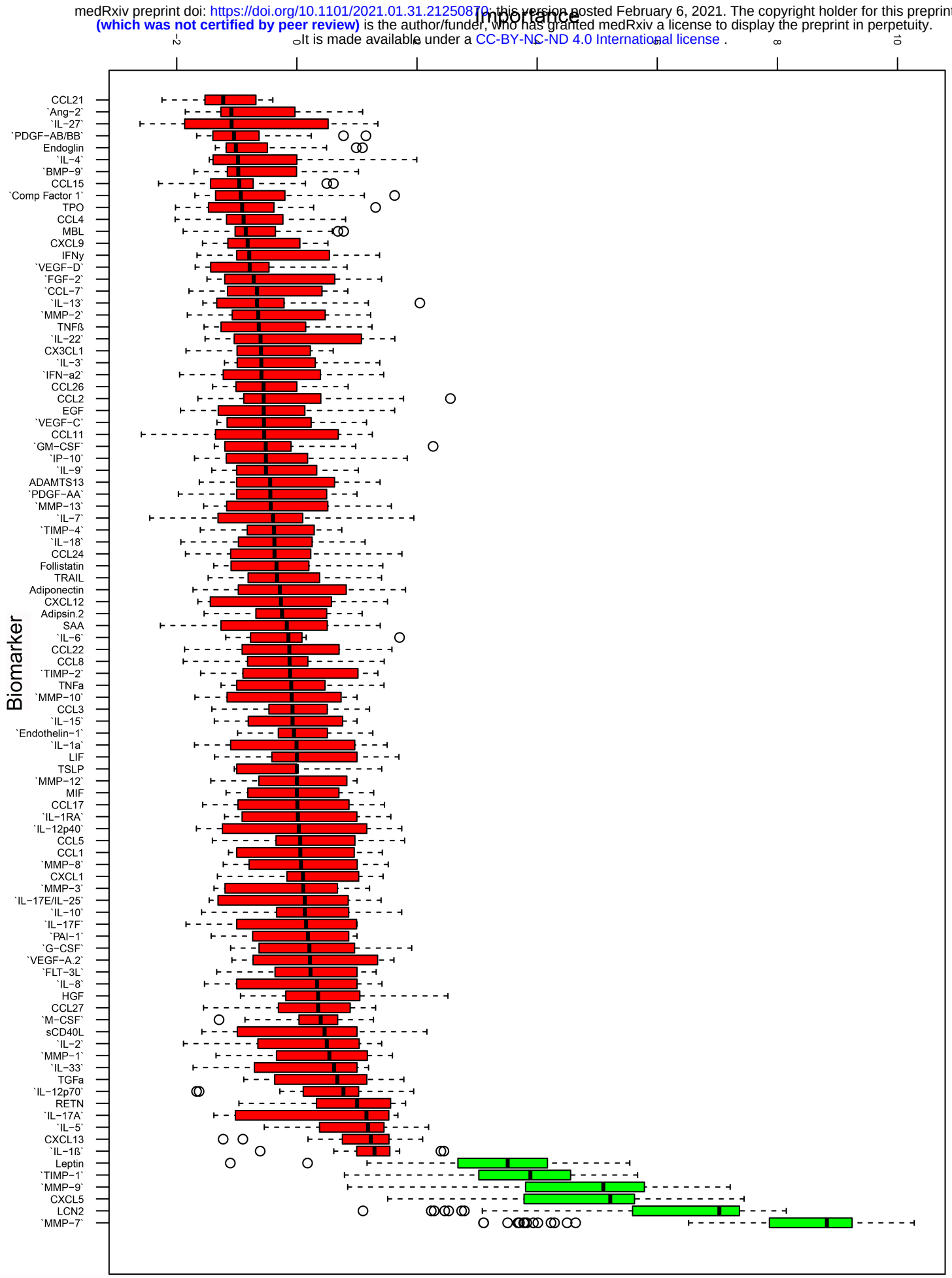
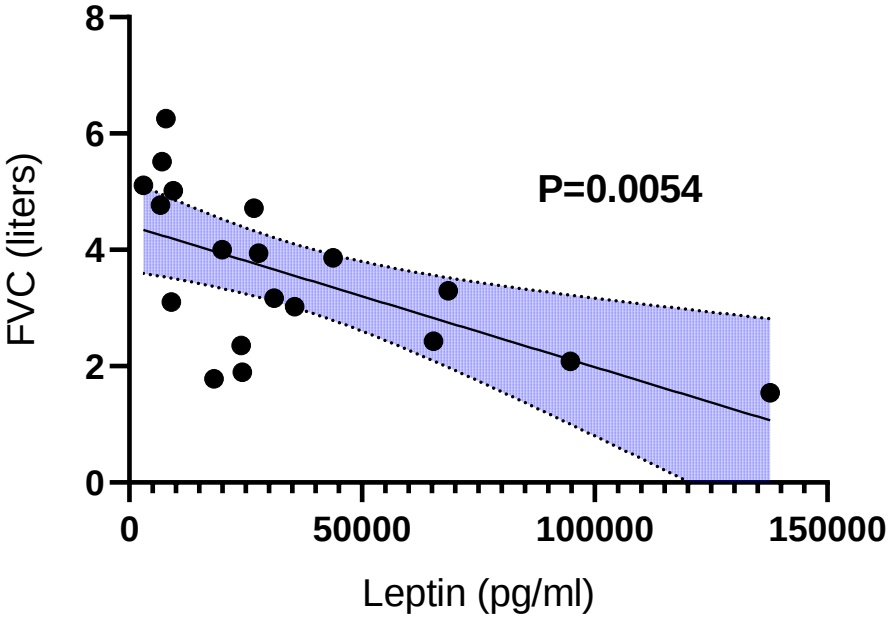
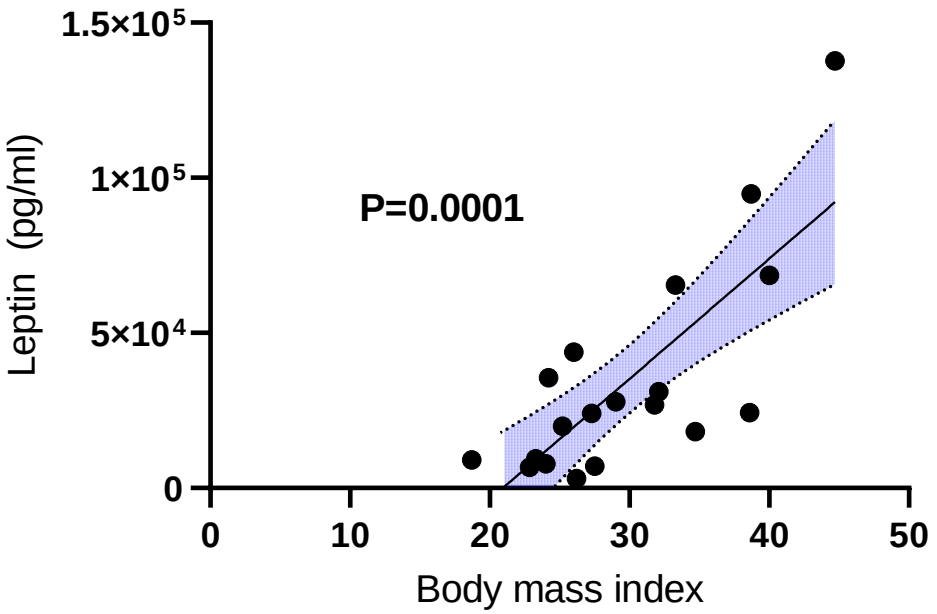


Supplemental Figure 2: Random-forest feature selection Boruta procedure showing the biomarkers in reverse order of importance for explaining variation in diffusion capacity for carbon monoxide. Green biomarkers are confirmed and red biomarkers are rejected.





Supplemental Figure 3: Leptin. *Top*: Scatter plot of plasma leptin levels on abscissa and forced vital capacity (FVC) on ordinate for n=19 subjects. *Bottom*: Scatter plot of body mass index on abscissa and plasma leptin levels on ordinate for same 19 subjects. Statistical analysis is simple linear regression. Blue shaded regions show 95% confidence intervals.