SUPPLEMENTAL FILE

Acute and Sustained Increase in Endothelial Biomarkers in COVID-19

Raúl Méndez[1,2]*, Paula González-Jiménez[1,2,3], Ana Latorre[2,4], Mónica Piqueras[3,5], Leyre Bouzas[1,2,3], Katheryn Yépez[1,2,3], Ana Ferrando[1,2,3], Enrique Zaldívar-Olmeda[1,2,3], Antonio Moscardó[4], Ricardo Alonso[5], Soledad Reyes[1,2], Rosario Menéndez[1,2,3,6]*

[1]Pneumology Department. La Fe University and Polytechnic Hospital. Valencia, Spain.

[2]Respiratory Infection Research Group. Health Research Institute La Fe. Valencia, Spain.

[3]University of Valencia. Valencia, Spain.

[4]Platelet Function Unit. Health Research Institute La Fe. Valencia, Spain.

[5]Laboratory Department. La Fe University and Polytechnic Hospital. Valencia, Spain.

[6]Centre for Biomedical Research Network in Respiratory Diseases (CIBERES). Madrid, Spain.

*Equal contribution

Corresponding author: Raúl Méndez

Servicio de Neumología. Hospital Universitario y Politécnico La Fe,

Avda. Fernando Abril Martorell 106, 46026 Valencia. Spain,

e-mail: mendez_rau@gva.es

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Methods

Design

The study was approved by the Biomedical Research Ethics Committee Hospital La Fe (2020-282-1 and 2020-280-1). The informed consent was waived during the acute phase of the disease, as remaining samples from the emergency department laboratory were used. A second approval was acquired by the Biomedical Research Ethics Committee Hospital La Fe from patients with post-hospitalisation follow-up data. In this case, written informed consent was obtained during the follow-up visit at the outpatient clinic. Individuals in the control group were enrolled for analysis if they had presented with a negative antibody serology for SARS-CoV-2 and were without signs or symptoms of respiratory infection within the last month. These individuals were recruited from volunteer workers and relatives of the Health Research Institute La Fe via local adverts and word of mouth.

Biomarkers

Peripheral venous blood was drawn from patients at emergency department visit (T1) and post-hospitalisation follow-up visit (T2) and stored in ethylenediaminetetraacetic acid (EDTA) tubes. EDTA tubes were centrifugated (2500 rpm) for 10 minutes to obtain plasma and later aliquoted for storage at -80 ºC until analysis was performed by two biologists (Ana Latorre and Antonio Moscardó). Once all the samples were collected and frozen, they were analysed in batches simultaneously. Processing was conducted by two laboratory clinicians (Ricardo Alonso and Mónica Piqueras) and two biologists (Ana Latorre and Antonio Moscardó) for three consecutive work days. Proadrenomedullin (proADM) (nmol/L) and proendothelin (pmol/L) were determined by immunofluorescent assays according to manufacturer's instructions (Thermo Scientific BRAHMS through TRACE technology in KRYPTOR Compact

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Plus). The same reagent kit lots were used on all samples. The analyses were carried out in duplicate to improve assay quantification precision; the mean of both analyses was obtained. We used BRAMS Dilcup Cont Plates and React Cont Plates supplied by Thermo Scientific BRAHMS, and all the plates included came from the same batch. The laboratory followed the established quality control protocols at all times. Two levels of controls, one low and the other high, were carried out every day for both techniques used before sample processing.

Follow-up

In the follow-up visit, a routine clinical evaluation was performed, including physical examination, questionnaire of symptoms, pulmonary function test (including spirometry and diffusing capacity), blood test (including C-reactive protein [CRP], D-dimer [DD], troponin T and pro b-type natriuretic peptide [proBNP]) and chest x-ray. Additional tests were ordered per attending physician's decision.

Outcomes and definitions

The main outcome for the initial analysis of biomarkers (T1) was in-hospital death. Outcomes for the follow-up analysis of biomarkers (T2) were radiologic resolution, cardiac damage biomarkers (troponin T and proBNP), systemic inflammation (CRP), fibrin degradation (DD) and diffusing lung capacity for carbon monoxide (D_{LCO}).

Initial intensive care unit admission was defined as admission within the first 24 hours. Sustained elevation of endothelium-derived markers at follow-up was defined as the persistence of levels of proADM and/or proendothelin above the 97.5th and 99th percentiles of normal persons (according to the manufacturers, these thresholds were at 0.55 nmol/L and 73.9 pmol/L, respectively). Presence of comorbidity was defined as binary variable in the Cox analysis if the patients had one or more comorbidities listed as

follows: arterial hypertension, history of smoking, diabetes, dyslipidaemia, and chronic heart disease.

Statistical analysis

Data was summarised as number (%) or median (interquartile range) as appropriate. Correlations between analytical parameters and studied biomarkers were estimated using Spearman's rank correlation. Comparisons between biomarker levels were made using the Mann-Whitney U test. A survival analysis was performed with the Kaplan-Meier test, while multivariable analyses were done with Cox proportional hazards regression model. Somers' D_{XY} with correction for optimism was reported for each model (Somers' D is the percentage difference between concordant and discordant pairs, including ties). The cut-off points for biomarkers were obtained using Youden's Index. Additionally, we performed mixed linear regression models for proADM and proendothelin to evaluate evolution over time, including time difference between T1 and T2, and individuals as a random intercept. Statistical analyses were performed using R (version 4.0.2). GraphPad Prism 9 was used for graphics creation.

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Results

e-Table 1. Included and excluded patients. Baseline characteristics.

Characteristic	**Patients with biomarkers at Day 1 (n= 210)**	**Patients without biomarkers (n= 190)**	**P value**
Age in years, median (IQR)	64 (51-76)	59 (49-74)	0.267
Female sex, no. (%)	99 (47.1)	81 (42.6)	0.365
Current or former smokers, no. (%)	52 (24.8)	59 (31.1)	0.161
Coexisting conditions, no. (%)			
Any	142 (67.6)	122 (64.2)	0.472
Hypertension	92 (43.8)	86 (45.3)	0.770
Diabetes	48 (22.9)	36 (18.9)	0.338
Dyslipidaemia	64 (30.5)	67 (35.3)	0.308
Chronic heart disease	26 (12.4)	28 (14.7)	0.491
Chronic renal disease	26 (12.4)	21 (11.1)	0.680
Chronic liver disease	6 (2.9)	21 (11.1)	0.198
Neurological disease	25 (11.9)	16 (8.4)	0.251
Chronic respiratory disease	23 (11)	27 (14.2)	0.325
Days since onset of symptoms, median (IQR)	7 (5 - 10)	7 (5-10)	0.741
SpO2/FiO2 at admission (IQR)	452.4 (429-461.9)	452.4 (433-461.9)	0.928
Radiologic data at admission			
Bilateral infiltrates, no. (%)	117 (55.7)	123 (64.7)	0.066
Severity			
PSI score, median (IQR)	68 (48-92)	62 (48-87)	0.565
SOFA score, median (IQR)	1 (0-2)	1 (0-2)	0.040
Respiratory support			
HFNC/CPAP/NIV, no. (%)	22 (10.6)	28 (14.7)	0.326
MV, no. (%)	16 (7.6)	27 (14.2)	0.034
Median length of MV, days, median (IQR)	14 (11-17)	17 (10-36)	0.302
Treatment			
Remdesivir, no. (%)	1 (0.5)	0 (0)	0.341
Steroids, no. (%)	74 (35.2)	85 (44.7)	0.053
Hydroxychloroquine/chloroquine, no. (%)	178 (84.8)	173 (91.1)	0.055
Lopinavir/ritonavir, no. (%)	39 (18.6)	89 (46.8)	<0.001
Tocilizumab, no. (%)	35 (16.7)	40 (21.1)	0.262

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Statins (chronic treatment), no. (%)	51 (24.3)	49 (25.8)	0.729
Aspirin (chronic treatment), no. (%)	20 (9.5)	17 (8.9)	0.842
Outcomes			
Length of hospitalisation, days, median (IQR)	12 (9-20)	13 (9-23)	0.252
In-hospital mortality, no. (%)	27 (12.9)	22 (11.6)	0.697

IQR: interquartile range; HFNC/CPAP/NIV: high-flow nasal cannula/continuous positive airway pressure/non-invasive ventilation; MV: mechanical ventilation; NA: not applicable; PSI: Pneumonia Severity Index; SOFA: Sequential Organ Failure Assessment; SpO2/FiO2: peripheral blood oxygen saturation/fraction of inspired oxygen.

e-Table 2. Biomarkers levels in patients with COVID-19 and control group individuals.

Biomarker	**COVID-19**		**Control**	**P value***	**P value****
	Day 1	**Follow-up**			
Proadrenomedullin (nmol/L)	0.79 (0.58-1.19)	0.48 (0.39-0.58)	0.41 (0.36-0.45)	<0.0001	0.0003
Proendothelin (pmol/L)	57.5 (38.14-87.91)	50.61 (42.72-57.04)	46.72 (41.53-51.67)	0.0057	0.0456

Median (interquartile range). *COVID-19 day 1 vs Control. **COVID-19 follow-up vs Controls.

e-Table 3. Biomarkers levels in survivors and non-survivors of COVID-19 at day 1.

Biomarker	**Survivors**	**Non-survivors**	**P value**
Proadrenomedullin (nmol/L)	0.73 (0.54-1.06)	1.48 (1.19-1.83)	<0.0001
Proendothelin (pmol/L)	50.05 (35.48-77.7)	103.26 (70.47-193.4)	<0.0001

Median (interquartile range).

e-Table 4. Cox analysis evaluating the association between biomarkers and mortality.

Model 1					
Variable	**HR (95% CI)**	***P* value**	**Variable**	**HR (95% CI)**	***P* value**
Age, *yrs*	1.1 (1.04-1.13)	<0.001	Age, *yrs*	1.08 (1.04-1.13)	<0.001
Male	0.66 (0.25-1.75)	0.401	Male	0.75 (0.26-2.15)	0.598
Comorbidity	0.87 (0.22-3.48)	0.840	Comorbidity	0.93 (0.23-3.79)	0.920
SpO2/FiO2	0.99 (0.99-0.99)	<0.001	SpO2/FiO2	0.99 (0.99-0.99)	<0.001
ProADM (nmol/L)	1.47 (1.18-1.82)	<0.001	Proendoethelin (pmol/L)	1.01 (1.00-1.02)	0.003
Model 2					
Variable	**HR (95% CI)**	***P* value**	**Variable**	**HR (95% CI)**	***P* value**
Age, *yrs*	1.06 (1.02-1.11)	0.009	Age, *yrs*	1.09 (1.05-1.14)	<0.001
Male	0.64 (0.24-1.67)	0.359	Male	0.56 (0.20-1.55)	0.262
Comorbidity	0.89 (0.23-3.53)	0.871	Comorbidity	0.92 (0.23-3.75)	0.909
SpO2/FiO2	0.99 (0.99-0.99)	<0.001	SpO2/FiO2	0.99 (0.99-0.99)	<0.001
ProADM ≥ 1.16 nmol/L	3.96 (1.26-12.47)	0.019	Proendothelin ≥ 75 pmol/L	1.54 (0.59-4.00)	0.377

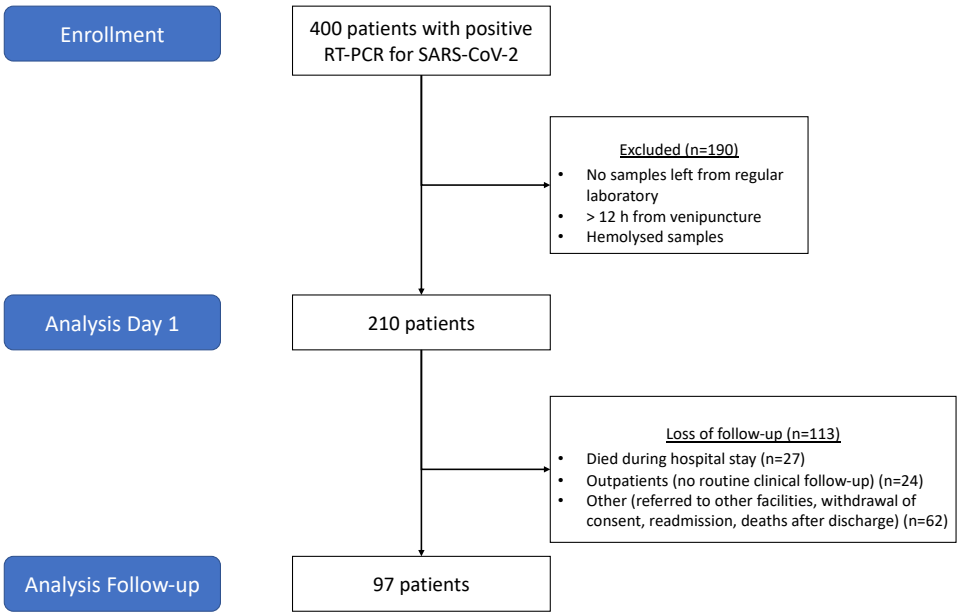
Model 1: Biomarkers (proADM and proendothelin) as a continuous variable adjusted for age (continuous variable), sex (binary variable), comorbidity (binary variable) (arterial hypertension, smoking history, diabetes, dyslipidaemia or chronic heart disease) and SpO2/FiO2 (continuous variable). Model 2: Biomarkers as a dichotomous variable (proADM >1.16 nmol/L and proendothelin >75 nmol/L) according to Youden index adjusted for age (continuous variable), sex (binary variable), comorbidity (binary variable) (arterial hypertension, history of smoking, diabetes, dyslipidaemia or chronic heart disease) and SpO2/FiO2 (continuous variable). CI: confidence interval; HR: hazard ratio; P: percentile; ProADM: Proadrenomedullin; SpO2/FiO2: peripheral blood oxygen saturation/fraction of inspired oxygen.

e-Table 5. Sustained elevation of endothelium-derived markers according to D_{LCO} and radiologic resolution.

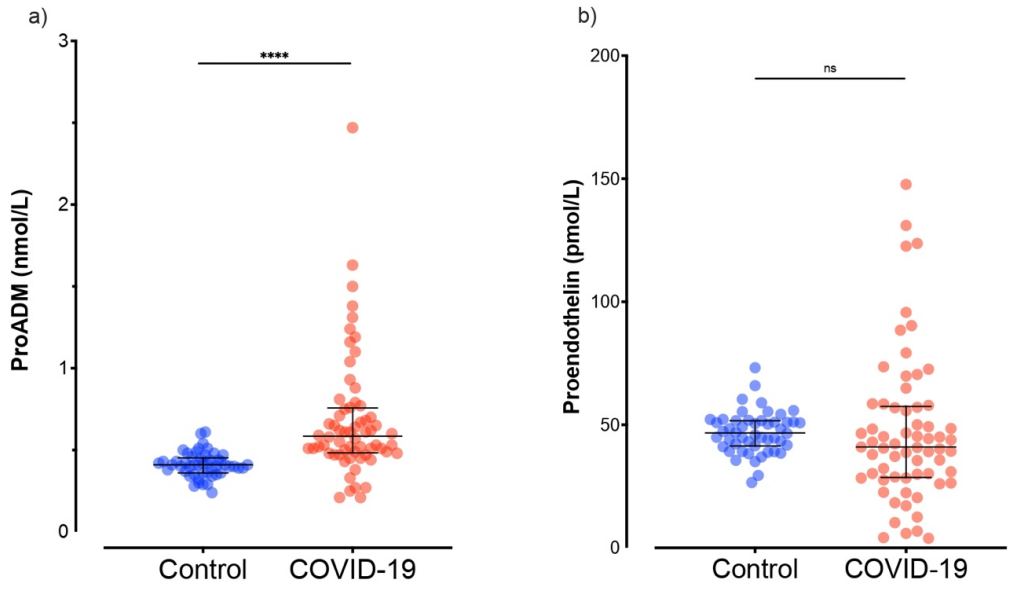
Biomarker	**Sustained elevation of endothelium-derived markers**	**Non-sustained elevation of endothelium-derived markers**	**P value**
Altered D_{LCO} (<80% predicted)*	11 (47.8)	12 (19.7)	0.010
Lack of radiologic resolution**	13 (46.4)	22 (31.9)	0.176

N (%). D_{LCO}, diffusing lung capacity for carbon monoxide. *Data available in 84 patients. ** Data available in 97 patients.

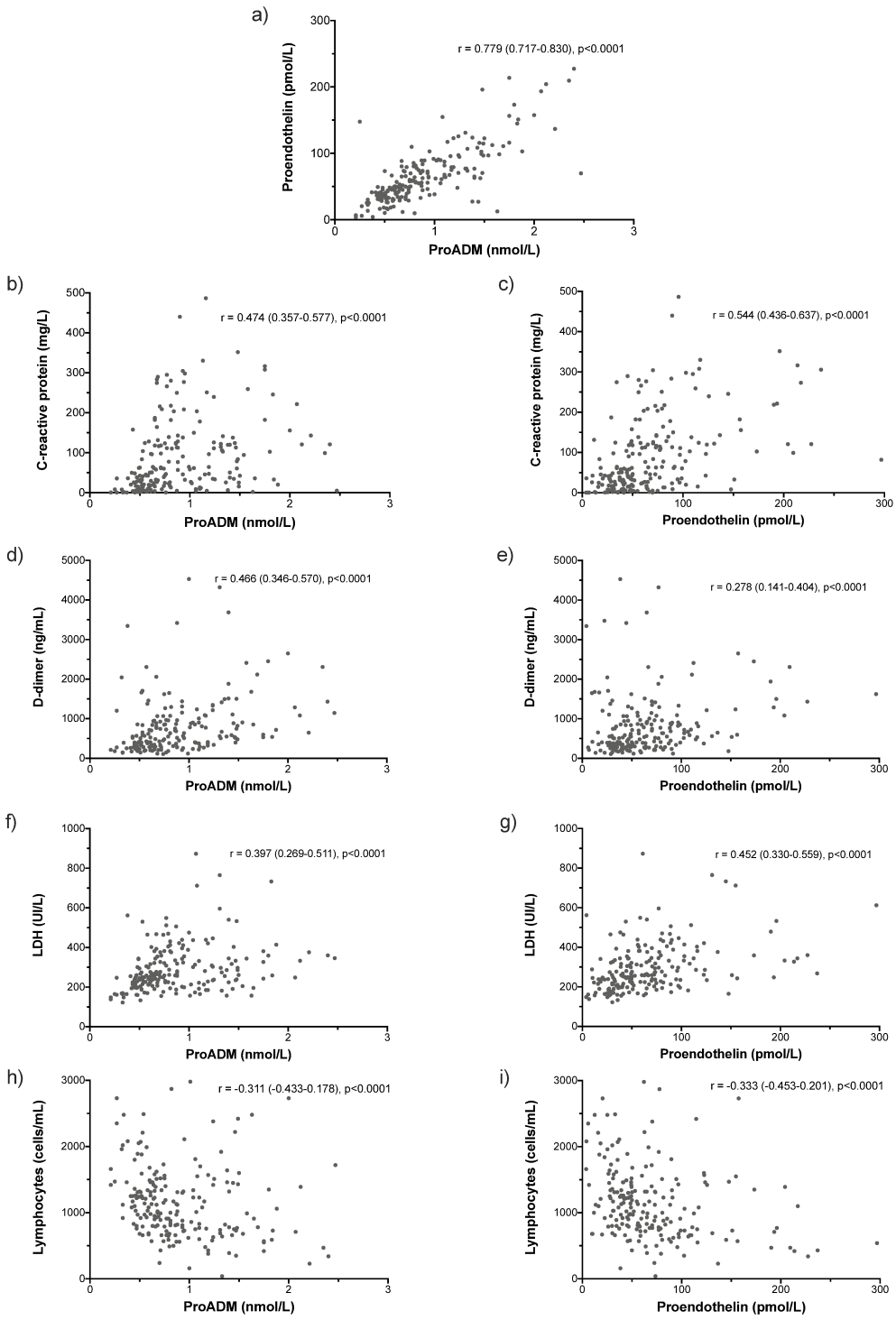
Supplemental Figures



e-Figure 1. Flowchart.



e-Figure 2. Proadrenomedullin (proADM) and proendothelin levels at day 1 after excluding patients with probable, previous endothelial damage (arterial hypertension, history of smoking, diabetes, dyslipidaemia or chronic heart disease). a) Higher proADM levels in patients with COVID-19 than in those belonging to the control group. b) No differences in proendothelin levels were found between those with COVID-19 and those belonging to the control group. ****p<0.0001; ns: non-significant.



e-Figure 3. Correlation plots between endothelial markers and severity-recognised markers in COVID-19 at day 1. a) Proadrenomedullin (proADM) and proendothelin. b) ProADM and CRP. c) Proendothelin and CRP. d) ProADM and DD. e) Proendothelin

and DD. f) ProADM and lactate dehydrogenase (LDH). g) Proendothelin and LDH. h) ProADM and lymphocyte count. i) Proendothelin and lymphocyte count.

Points outside the axis: 5 in a); 7 in b); 3 in c); 11 in d); 9 in e); 7 in f); 3 in g); 6 in h); 2 in i).

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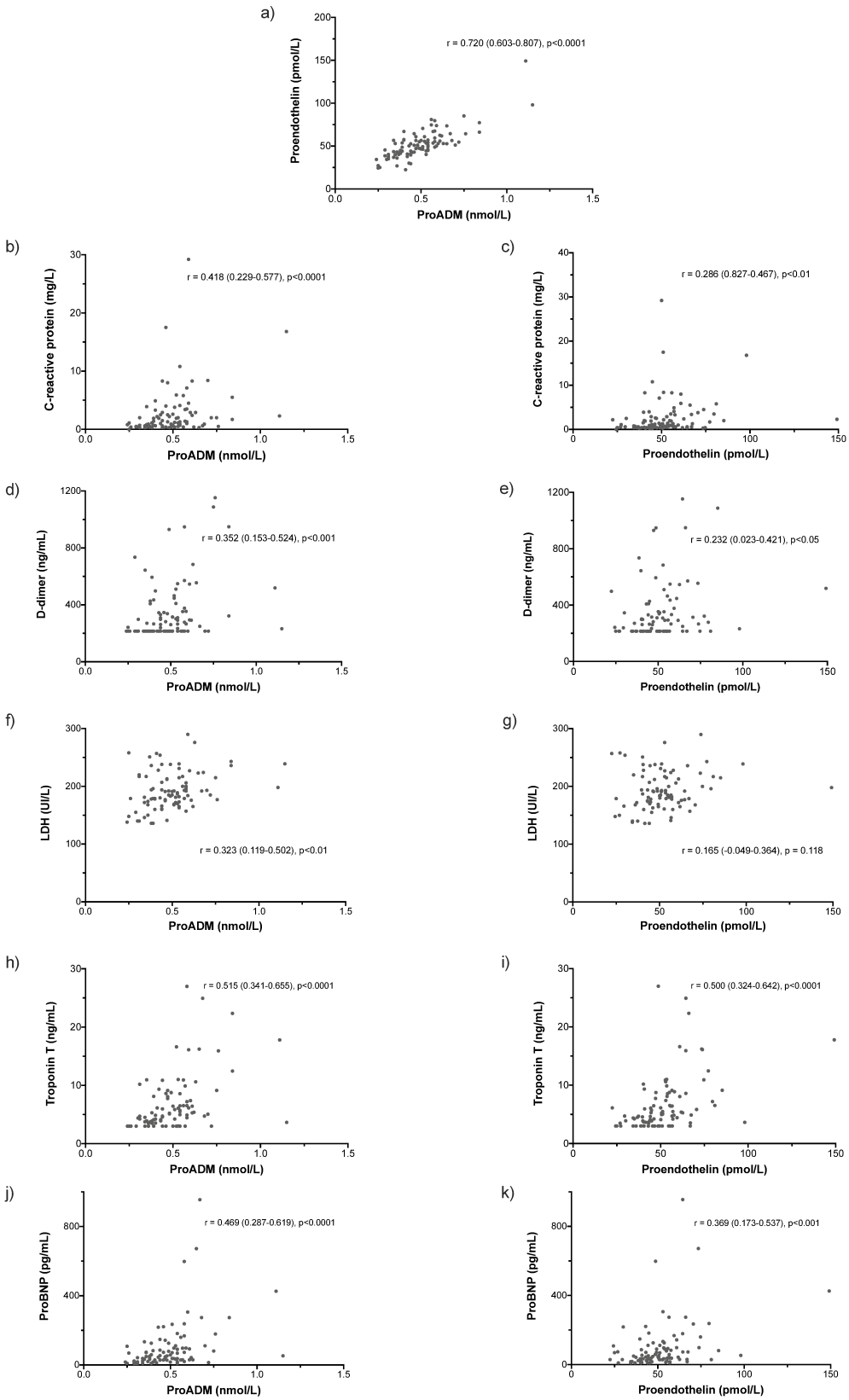
a)
Proendothelin (pmol/L)
ProADM (nmol/L)
r = 0.720 (0.603-0.807), p<0.0001
b)
C-reactive protein (mg/L)
ProADM (nmol/L)
r = 0.418 (0.229-0.577), p<0.0001
c)
C-reactive protein (mg/L)
Proendothelin (pmol/L)
r = 0.286 (0.827-0.467), p<0.01
d)
D-dimer (ng/mL)
ProADM (nmol/L)
r = 0.352 (0.153-0.524), p<0.001
e)
D-dimer (ng/mL)
Proendothelin (pmol/L)
r = 0.232 (0.023-0.421), p<0.05
f)
LDH (UI/L)
ProADM (nmol/L)
r = 0.323 (0.119-0.502), p<0.01
g)
LDH (UI/L)
Proendothelin (pmol/L)
r = 0.165 (-0.049-0.364), p = 0.118
h)
Troponin T (ng/mL)
ProADM (nmol/L)
r = 0.515 (0.341-0.655), p<0.0001
i)
Troponin T (ng/mL)
Proendothelin (pmol/L)
r = 0.500 (0.324-0.642), p<0.0001
j)
ProBNP (pg/mL)
ProADM (nmol/L)
r = 0.469 (0.287-0.619), p<0.0001
k)
ProBNP (pg/mL)
Proendothelin (pmol/L)
r = 0.369 (0.173-0.537), p<0.001

e-Figure 4. Correlation plots between endothelial markers and systemic inflammation, cardiac damage and fibrin degradation markers at follow-up visit. a) Proadrenomedullin (proADM) and proendothelin. b) ProADM and CRP. c) Proendothelin and CRP. d) ProADM and DD. e) Proendothelin and DD. f) ProADM and LDH. g) Proendothelin and LDH. h) ProADM and troponin T. i) Proendothelin and troponin T. j) ProADM and pro b-type natriuretic peptide (proBNP). k) Proendothelin and proBNP.

Points outside the axis: 1 in d); 1 in e); 1 in j); 1 in k).

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