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(Article begins on next page)

Risk stratification of patients with SARS-CoV-2 by tissue factor expression in circulating extracellular vesicles

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Running title: CD142-EV to predict prognosis in SARS-CoV2

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ABSTRACT

Inflammatory response following SARS-CoV-2 infection results in substantial increase of amounts of intravascular pro-coagulant extracellular vesicles (EV) expressing tissue factor (CD142) on their surface. CD142-EV turned out to be useful as diagnostic biomarker in COVID-19 patients. Here we aimed at studying the prognostic capacity of CD142-EV in SARS-CoV-2 infection.

Expression of CD142-EV was evaluated in 261 subjects admitted to hospital for pneumonia and with a positive molecular test for SARS-CoV-2. The study population consisted of a discovery cohort of selected patients (n=60) and an independent validation cohort including unselected consecutive enrolled patients (n=201). CD142-EV levels were correlated with post-hospitalization course of the disease and compared to the clinically available 4C Mortality Score as referral.

CD142-EV showed a reliable performance to predict patient prognosis in the discovery cohort (AUC=0.906) with an accuracy of 81.7%, that was confirmed in the validation cohort (AUC=0.736). Kaplan-Meier curves highlighted a high discrimination power in unselected subjects with CD142-EV being able to stratify the majority of patients according to their prognosis. We obtained a comparable accuracy, being not inferior in terms of prediction of patients' prognosis and risk of mortality, with 4C Mortality Score. The expression of surface vesicular CD142 and its reliability as prognostic marker was technically validated using different immunocapture strategies and assays.

The detection of CD142 on EV surface gains considerable interest as risk stratification tool to support clinical decision making in COVID-19.

KEYWORDS

CD142, tissue factor, SARS-CoV2, COVID-19, extracellular vesicles

LIST OF ABBREVIATIONS

Extracellular Vesicles, EVs; Immuno-Capture, IC; OroTracheal Intubation, OTI; Median Fluorescence Intensity, MFI; normalized MFI, nMFI; Severe Acute Respiratory Syndrome CoronaVirus 2, SARS-CoV-2; Tissue Factor, TF; Tissue Factor-positive EVs, EV-TF or CD142-EV; Ultra Centrifugation, UC; Western Blot, WB.

1. INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 455 million subjects as of 12 March 2022 (<https://coronavirus.jhu.edu>). The resulting disease (COVID-19) is associated with high hospitalization rates and an increased risk of respiratory failure, thus determining tremendous burden on the healthcare system of several countries and affecting the best possible care for patients.^{1,2} A pragmatic risk score that uses analytic assay to estimate poor outcome from infection may assist medical staff in tailoring management strategies for patients and allocating limited healthcare resources.³ Several prognostic models have been approached in the past two years to meet the urgent need of an efficient and early prognosis in patients with a confirmed diagnosis of COVID-19 for mortality risk, progression to severe disease and intensive care unit admission.³ The most frequently used prognostic factors including age, image features, lymphocyte count and C-reactive protein, showed moderate performance in terms of clinical decision making.^{3, 4} A clinically applicable prediction model with very good discrimination and performance characteristic has recently been validated in large cohort of patients.⁵ The 4C Mortality Score including eight variables at hospital admission, outperformed other risk stratification tools and showed clinical decision making utility.⁵

Over the years, several studies have described the potential value of circulating extracellular vesicles (EVs) as prognostic biomarkers.⁶⁻¹⁰ Molecular profiles of circulating EVs turned out to be useful as early prediction tool of COVID-19 severity.¹¹ Very recently, the total number of tissue factor-positive EVs (EV-TF) as well as their enzymatic activity were significantly associated with an increased severity risk in COVID-19 patients.¹²⁻¹⁴

In line with these studies we have lately showed that the expression of TF onto surface of EV isolated from COVID-19 patients serum was significantly higher than EVs isolated from healthy subjects as well as from those isolated from serum of subjects with pneumonia but different etiology from SARS-CoV-2.¹⁶ Furthermore, the levels of expression of TF-bearing EV (CD142-EV) was significantly correlated with the capacity of EVs to generate factor Xa.¹⁶ Contextually we produced very preliminary evidence showing that TF was significantly more expressed in severe COVID-19 patients undergoing orotracheal intubation (OTI) and/or death. However, we could not assess the performance of such EV marker as prognostic indicator due to the limited number of included patients. This paved the way for exploring the potential of this specific surface antigen as indicator of disease prognosis in a larger cohort of patients. The scientific endeavor of the present paper relies on the inclusion of 261 laboratory-confirmed COVID-19 patients hospitalized for pneumonia, who underwent blood

99 sampling at time of molecular swab test and were longitudinally monitored to assess the
100 clinical progression of disease. The expression of EV-associated TF was then
101 retrospectively correlated with the course of the disease and its clinical performance was
102 evaluated with the incidence of OTI and/or death as indicator of poor prognosis. The 4C
103 Mortality Score was used as gold standard referral trying to put our experimental tool into
104 scale with a widely validated in-use model.⁵ We took advantage from reproducible flow
105 cytometer assay that has been previously standardized and validated for the detection and
106 characterization of EV surface signatures.¹⁷⁻²⁰

107

108 **2. METHODS**

109 Supporting data for the present study are available within the article and the supplementary
110 material. Because of their sensitive nature, additional information and single patient data
111 are available from the corresponding author upon reasonable request.

112

113 **2.1 Patient recruitment**

114 The study was approved by the local ethical. Subjects gave informed consent according to
115 the Declaration of Helsinki. The study population consists of 261 Caucasian white subjects
116 hospitalized for pneumonia and SARS-CoV-2 infection at Internal Medicine Department and
117 Cardiocentro Ticino Institute, Ente Ospedaliero Cantonale, Lugano, Switzerland. All patients
118 were positive for SARS-CoV-2 as for molecular tests (polymerase chain reaction). Serum
119 samples were collected at the time of nasopharyngeal swab sampling. The study population
120 consisted in a discovery cohort (n=60) composed by selected patients admitted to hospital
121 in March 2020, and in a validation cohort (n=201) composed by unselected consecutive
122 patients admitted to hospital between April 2020 and May 2020. Patients were included in
123 the study if they met the following criteria: infection by SARS-CoV-2, diagnosis of pneumonia
124 and admission to hospital. Exclusion criteria were: (1) Age lower than 18 years; (2)
125 Pregnancy; (3) Concomitant acute non-respiratory infection; (4) Cancer (active or recent
126 history); (5) Inappropriateness to invasive emergency treatment (i.e., orotracheal intubation,
127 advanced life support). Patients were classified in terms of outcome in good vs. poor
128 prognosis, the latter was defined as need of orotracheal intubation, OTI, and/or death.

129

130 **2.2 Sample handling**

131 Peripheral venous blood samples were collected in serum separator tubes and maintained
132 30 min at room temperature before centrifugation. After clot formation, blood underwent

133 serial low speed centrifugations at 4°C (1'600 x g for 10 min; 3'000 x g for 20 min; 10'000 x
134 g for 15 min) to separate serum and to remove cellular debris and larger vesicles. Cleared
135 serum was then aliquoted, stored at -80°C and never thawed prior to analysis.

136

137 **2.3 EV characterization**

138 **2.3.1 Bead-based EV surface profiling.** Serum samples underwent bead-based EV-
139 capture and flow cytometric analysis by MACSPlex human Exosome Kit (Miltenyi) without
140 further pre-isolation step, as previously described ^{16, 21}. EVs were isolated using capture-
141 beads coated with antibodies coated with 37 different surface antigens and then analyzed
142 after incubation with a detection reagent (labelled antibodies against CD9-CD63-CD81).
143 Median fluorescence intensity (MFI; expressed as arbitrary unit, a.u.) was measured by
144 MACSQuant Analyzer 10 flow cytometer (Miltenyi). Expression levels for each EV surface
145 antigen were reported after subtraction for the respective fluorescence values of blank
146 control and normalization for mean MFI for CD9/CD63/CD81 (normalized MFI, nMFI;
147 expressed as percentage, %).^{17,18} A reverse flow cytometric assay was also performed by
148 isolating EVs by capture beads coated with antibodies against CD9-CD63-CD81 (EpCam;
149 JSR Micro) and then incubated with fluorochrome-conjugated antibodies against CD142,
150 and CD63 (as normalizator). MFI was measured CytoFLEX (Beckman Coulter).

151 **2.3.2 Western blot.** WB was performed on protein lysate after EV bead-based immuno-
152 capture. Serum aliquots were incubated overnight with MACSPlex capture beads and saline
153 solution. Unbounded fraction was discarded, and samples were lysed in RIPA buffer; total
154 proteins were separated on SDS Page 4-12% gel (BioRad) and signals were detected by
155 Odyssey CLx Detection System (LI-COR Biosciences). Blots for 3 representative samples
156 were incubated with the following primary antibodies: rabbit polyclonal anti-ApoB48, mouse
157 monoclonal anti-GRP94, rabbit monoclonal anti-Alix, rabbit monoclonal anti-CD142, rabbit
158 monoclonal anti-TSG101, rabbit polyclonal anti-Syntenin-1, rabbit monoclonal anti-CD81 (all
159 from Abcam), and rabbit monoclonal anti-Mitofillin (Invitrogen).

160 **2.3.3 Activity assay.** The activity assay for CD142 on EVs was performed with Human
161 Tissue Factor Activity Assay (Abcam), according to manufacturer instructions. The protocol
162 assesses amidolytic activity of TF/FVIIa complex to activate factor X (FX) to factor Xa.

163 **2.3.4 Co-localization assay (ExoView).** Co-localization was assessed by on-chip EV
164 analysis using ExoView® R100 Analyzer, as previously described.^{22, 23} Functionalized chips
165 were spotted with a solution of a mixture of anti-tetraspanin antibodies (CD9-CD63-CD81;
166 Ancell). Serum samples incubated on chips for 2 hours at room temperature; chips were

167 than stained with a mixture of labelled antibodies against CD9-CD63-CD81 (Ancell), and for
168 the CD142 co-localization assay, with antibody anti-TF (Invitrogen).

169

170 **2.4 Statistical analysis**

171 We expressed variables with a normal distribution as mean \pm standard deviation and their
172 analysis was performed by T-student test. We expressed variables with a non-normal
173 distribution as median [interquartile range] and their analysis was performed by Mann-
174 Whitney test. Categorical variables were expressed as absolute number (percentage) and
175 analyzed by Chi square test (or Fisher test, when appropriated). *P*-value of less than 0.05
176 were considered significant. Logistic regression analysis was performed to assess the
177 association of EV surface antigens with the outcome of patients. Hazard ratios (HRs) were
178 evaluated together with their 95% confidence intervals. Receiver Characteristics Operating
179 (ROC) curves were drawn to estimate the area under the curve (AUC) for EV surface
180 antigens, to estimate their prediction performance (patient outcome). Statistics was
181 performed by IBM SPSS Statistics 26 (IBM, New York, USA) and GraphPad PRISM 8.0 (La
182 Jolla, California). For Estimation of study power see detailed method in supplementary file.

183

184 **3. RESULTS**

185 **3.1 Characteristics of the study cohorts**

186 We enrolled a total of 261 subjects with SARS-CoV-2 infection confirmed by PCR molecular
187 test and admitted to hospital with a diagnosis of pneumonia (Table). Mean age was 68 years,
188 65.5% were males, 62.1% displayed bilateral pneumonia, and 1.1% suffered from
189 pulmonary embolism at hospitalization. Patients were stratified according to their outcome:
190 36% needed to be treated with high flow O₂, 13.8% underwent OTI, while the overall
191 mortality was 19.2%. A poor prognosis, defined as needed of OTI or death, was reported in
192 72 patients (27.6%); the median time from hospitalization to OTI/death was 7 days. As
193 expected, the median duration of hospitalization was longer for patients with a poor
194 prognosis compared to those with a good one (14 vs. 8 days).

195 Patients with a poor prognosis showed a higher incidence of bilateral pneumonia, a higher
196 respiratory rate, and a lower peripheral O₂ saturation at admission. Moreover, they suffered
197 from a higher number of comorbidities, and in particular chronic kidney disease, chronic
198 heart failure, coronary artery disease, liver disease, chronic neurological conditions, and
199 dementia. Concerning biochemical parameters, patients with a poor prognosis displayed
200 higher values of lactic acid, C-reactive protein, D-dimer, aPTT, urea, and troponin I, and

201 lower levels of pO₂ at arterial blood gas analysis ($p<0.05$ for all comparisons; Table); no
202 difference was found evaluating levels of haemoglobin, white blood cells and platelets count.
203 The study population consisted in a first cohort of selected patients (discovery cohort; $n=60$),
204 which was used to identify the detection threshold of CD142 expressed as nMFI (see
205 methods) and corresponding to the expression level of such antigen onto surface of EV, that
206 could be used as cut-off value to predict SARS-CoV-2 prognosis. Following the same criteria
207 of inclusion as for the discovery cohort, a second prospective group composed by
208 unselected consecutive patients was included as validation cohort ($n=201$). An overview of
209 study design is depicted in Online Figure 1. Characteristics of discovery and validation
210 cohorts are reported in Online Tables 1-3.

211

212 **3.2 EV profiling and selection of CD142-EV as biomarker to predict patient outcome**

213 The bead-based immunocapture flow cytometric assay used for EV profiling was first
214 validated for its specificity to bind EVs by western blot for specific markers and potential
215 contaminants and flow cytometry for tetraspanins expression on EV surface (Supplementary
216 Results; Online Figure 2). We then evaluated the expression of 37 EV surface antigens in
217 all recruited patients (discovery and validation cohort; $n=261$) and compared their
218 fluorescence levels in patients with SARS-CoV2 infection after stratification for prognosis
219 (Online Tables 4-5) and mortality (Online Tables 6-7). The EV surface signature in patients
220 stratified according to their prognosis is shown in Online Figures 3 and 4.

221 In the discovery cohort ($n=60$), among the differentially expressed surface epitopes in
222 patients with good vs. poor prognosis (CD49e, CD69, CD142, and CD45; see
223 Supplementary Results, Figure 1A and Online Table 4), CD142-EV displayed the strongest
224 association with prognosis with a hazard ratio (HR) of 1.074 (95% CI 1.032-1.119) at
225 regression models, thus meaning that for each single unit of increase in nMFI for this EV
226 marker, the risk of a poor prognosis increased of 7.4% (Figure 1B and Online Table 8). At
227 ROC curve analysis, CD142-EV had an AUC of 0.906 (95% CI 0.833-0.979) with an
228 accuracy of 81.7% (Figure 1C and Online Table 9), using a cut-off value of 33.5 (nMFI, %).
229 After stratification for mortality, CD4, CD142 and CD45 were highly expressed in deceased
230 patients (see Supplementary Results, Figure 1D and Online Table 6). CD142-EV was again
231 di best predictor, with an HR of 1.039 (95% CI 1.018-1.057), thus meaning an increase of
232 3.9% in mortality rate, for each unit of increase in nMFI of the marker (Figure 1E and Online
233 Table 8). The analysis of ROC curve showed an AUC of 0.842 (95% CI 0.727-0.957) with

234 an accuracy of 85% to predict mortality (Figure 1F and Online Table 10), using a cut-off
235 value of 52.8 (nMFI, %).

236

237 **3.3 CD142-EV discriminates SARS-CoV2 patients according to prognosis**

238 In the discovery cohort a cut-off greater than 33.5 (nMFI, %) for CD142-EV correctly
239 identified 18 out of 24 patients with a poor prognosis (sensitivity 75%), while those with an
240 nMFI equal or lower to 33.5 displayed a good prognosis in 31 out of 36 cases (specificity
241 86.1%; Figure 2A and Online Tables 11-12). The potential of CD142-EV as discriminant for
242 subjects belonging to the discovery cohort was further assessed by Kaplan-Meier curves
243 showing a log-rank of 4.75 (95% CI 2.09-10.81; Figure 2B). By applying the same cut-off in
244 the validation cohort (unselected subjects), we were still able to correctly classify 131 out of
245 153 patients with a good prognosis (specificity 85.6%) and a high negative predictive value
246 (86.2%). The overall accuracy was 78.6%, with a negligible overfitting bias (3.1%) when
247 compared with accuracy in the discovery cohort (Figure 2A). Kaplan-Meier curves further
248 confirmed a high discrimination power, with CD142-EV able to correctly stratify 158 out of
249 201 patients according to prognosis (good vs. poor prognosis; log-rank = 2.22 - 95% CI
250 1.23-3.99; Figure 2C).

251

252 **3.4 CD142-EV discriminates SARS-CoV2 patients according to mortality**

253 Considering the outcome of survival as for the ROC analysis, the nMFI value of 52.8% was
254 selected as critical cut-off for CD142-EV (Figure 3A;). Such value allowed the classification
255 of 51 out of 60 patients in the discovery cohort, resulting in an accuracy of 85%, with a
256 sensitivity and specificity of 75.0% and 87.5%, respectively (Online Tables 11-12). Kaplan-
257 Meier curves showed that CD142-EV was able to stratify patient according to their mortality
258 in discovery cohort with a log-rank = 11.30 (95% CI 2.82-45.34 Figure 3B). At validation, we
259 correctly predicted the survival of 152 out of 163 patients (specificity 94.3%), once again
260 with a high negative predictive value (85.9%) and an overall accuracy of 82.1% with an
261 overfitting bias of 2.9% (Figure 3A). The discrimination power according to mortality (Kaplan-
262 Meier curves) was consistent with 165 out of 201 patients correctly predicted (survival; log-
263 rank = 3.37 - 95% CI 1.27-8.93; Figure 3C).

264

265 **3.5 CD142-EV predict prognosis and mortality in SARS-CoV2**

266 Having assessed the performance of CD142-EV as prognostic tool, we applied such
267 unconventional biomarker to the entire population of included patients (discovery plus

validation cohort), to assess patient distribution according to outcome and expression levels of CD142-EV. The likelihood of a poor prognosis, as well as mortality, gradually increased at the increase of nMFI for CD142-EV (Figures 2D-3D). Among patients with lowest score (CD142-EV ≤ 10) 107 out of 117 displayed good prognosis (91.5%), and 108 out of 120 (90.0%) were alive at follow-up. Thus, translating in a very high specificity and negative predictive value (Online Table 13). Conversely, among patients with the highest score (CD142-EV >70), 22 out of 23 (95.7%) displayed a poor prognosis and 15 out of 25 (60%) deceased at follow-up, with a very high sensitivity and positive predictive value (Online Table 13). For each patient, we calculated the 4C (Coronavirus Clinical Characterization Consortium) Mortality score as described in Knight SR et al.⁵ 4C Mortality score was then used as referral to estimate the potential application of our experimental model based on CD142-EV in predicting patient prognosis and mortality (Online Figure 5; Online Table 11). Considering all patients CD142-EV showed a higher accuracy compared to 4C score in predicting patient prognosis (AUC 0.792 vs. 0.705 – $p=0.044$; accuracy 79.3% vs. 67.8%; Figure 2E), whereas the overall accuracy was comparable when predicting mortality (AUC 0.714 vs. 0.786; $p=0.131$; accuracy 82.3% vs. 73.9%; Figure 3E). Diagnostic performance and confusion matrix of CD142-EV and 4C Mortality score to predict either patient prognosis or mortality, are summarized in Online Table 12.

286

287 **3.6 CD142-EV experimental validation as biomarker in SARS-CoV2**

288 We have previously shown that TF expressed on the surface of EV possess enzymatic
289 activity which directly correlate with its level of expression.¹⁶ Here, we assessed whether
290 such activity has also potential as prognostic marker, as further confirmation of CD142-EV
291 as predictor of patient outcome in SARS-CoV2. EVs isolated by ultracentrifugation (UC) and
292 by immuno-capture beads (IC) from serum of 20 randomly selected patients from the
293 validation cohort (10 with a good prognosis and 10 with a poor prognosis) were quantitatively
294 measured for CD142 enzymatic activity. Both, EVs enriched by classical UC or using IC
295 showed an augmented CD142 activity when isolated from serum of patients with poor vs.
296 good prognosis ($p<0.05$; Figure 4A-B). Notably, CD142-EV level of expression measured
297 at flow cytometry directly correlated to CD142 activity measured by ELISA ($R=0.720$;
298 $p<0.001$; Figure 4C).

299 To overcome possible methodological- or instrumental-related biases, the expression of
300 surface vesicular CD142 was also measured by using a reverse immunocapture strategy.
301 Indeed, EV were captured by using beads coated with antibodies direct against tetraspanins

302 and immuno-stained for CD142. We confirmed that the level of expression of CD142 was
303 significantly higher in EV from patients with poor vs. good prognosis regardless the protocol
304 of EV binding ($p<0.01$; Figure 4D).

305 Finally, we further assessed the co-localization of tetraspanins with CD142 by ExoView®
306 Analyzer which allowed the immunocapture of EV onto silico chip and the simultaneously
307 detection of surface antigens CD9, CD63, CD81 and CD142, (Figure 4E). The assay
308 confirmed that EV specific tetraspanins are mainly co-expressed with tissue factor. By
309 quantifying the degree of expression of each marker, we could further confirm that the
310 number of total tetraspanin positive EV as well as the number of CD142-bearing EV, were
311 both increased in patients with more severe disease (FC 1.4 - $p=0.005$, and FC 3.5 -
312 $p=0.002$, respectively).

313

314 **4. DISCUSSION**

315 We have addressed the potential value of CD142-EV as prognostic biomarker in a cohort of
316 patients admitted to hospital for pneumonia and SARS-CoV-2 infection. Both the discovery
317 and the validation cohorts were tailored on reliable estimation of minimum number of
318 subjects to be included on the base of our previous pilot study.¹⁶ By using this prospective
319 cohort of unselected patients consecutively recruited, we obtained an overall accuracy of
320 78.6% and 82.1% in predicting patient prognosis and mortality, respectively. Noteworthy,
321 CD142-EV reached a reliable grade of “generalizability” as prognostic marker since the
322 overfitting bias was negligible when comparing accuracy in the discovery and validation
323 cohorts (ranging between 2.9 and 3.1%).

324 CD142-EV performed well against the clinically applied 4C Mortality Score, which is
325 currently one of the most robustly validated COVID-19 prognostic model.⁵ When considering
326 all patients, we obtained an overall comparable accuracy, being not inferior in terms of
327 prediction of patients’ prognosis (overall accuracy 79.3% CD142-EV vs. 67.8% 4C Mortality
328 Score) and risk of mortality (overall accuracy 82.3%% CD142-EV vs. 73.9% 4C Mortality
329 Score). CD142-EV displayed a very high specificity and negative predictive value (ranging
330 between 83.4 and 93.3%). However, as compared to 4C Mortality score it shows lower
331 sensitivity and positive predictive value (ranging between 34.2-78.2%), thus making CD142-
332 EV mainly suitable to rule out severe cases. CD142-EV also performed well in stratifying
333 patients according to their risk of a poor prognosis. The likelihood of a poor outcome (OTI
334 and/or death) gradually increases with CD142-EV expression and therefore it was suitable
335 for the quantification of a discrete risk. For instance, patients with a CD142-EV nMFI ranging

336 between 20 and 30 display a likelihood of 25% and 13% in terms of poor prognosis and
337 mortality respectively. On the other hand, patients with CD142-EV ranging between 60 and
338 70 will have a poor prognosis in 75% of cases, with a mortality of 42.9%.

339 Although patients for validation have been enrolled by avoiding selection bias, the cohort
340 still suffer of the limited number of enrolled subjects. Such small cohort of validation also
341 represents the main limit for more in-depth comparison with 4C Mortality Score that included
342 more than 22000 subjects in validation. A further limitation includes the fact that the study
343 was temporally and geographically narrowed. Infection rates and patients' characteristics
344 might change by time and geography during a pandemic. Here we could not show
345 robustness of the CD142-EV over time and geography.

346 We do not add substantial advancing in the debate concerning whether it is better to
347 measure levels of TF activity or TF protein as marker of thrombotic risk,^{24, 25} however we
348 clearly show that the TF protein level on the surface of EV consistently predict the severity
349 of COVID-19 disease. We have also shown that CD142-bearing EVs have an augmented
350 enzymatic activity when isolated from serum of patients with severe disease and a poor
351 prognosis, regardless the method of isolation. Finally, we showed that the level of
352 expression of TF strongly correlates with its activity in COVID-19 patients and it is hampered
353 when using specific antibody that causes steric hindrance with the enzymatic site of the
354 TF.¹⁶ It is plausible, and some recently published data come in help supporting this
355 hypothesis, that both parameters are associated with severity of disease in COVID-19
356 patients.^{12, 13, 15} The discrepancy between protein expression and activity, due to the
357 presence of undefined portion of intravascular TF present as inactive or encrypted state,²⁶
358 is negligible when referring to EVs. The cytokines storm²⁷ as well as the hyper-activation of
359 platelets¹² occurring in these patients may dramatically contribute to increase the release of
360 EV with pro-coagulant activity, thus expressing TF in a decrypted state.²⁸ As respect to
361 Guervilly et al. we found significant increase in the total amount of circulating EV in patients
362 with poor versus good prognosis. The apparent discrepancy might be explained by the fact
363 that we only addressed concentration (expressed as nMFI) of CD9; CD81 and CD63 positive
364 EV, while a direct FC assay as in Guervilly et al. can account for enumeration of large
365 vesicles that can be negative for tetraspanins while still expressing TF.¹⁵ A second possible
366 explanation reside in the starting material as EV source: we used serum in stand of plasma.
367 We are aware that this aspect may represent a weakness of the study, however we have
368 previously shown that the profiling of EV from serum has good potential as biomarker,
369 showing consistent diagnostic and prognostic performances, in line with gold-standard

370 biomarkers.²⁹ Both plasma and serum have been used in previous studies; while biobanking
371 of plasma may be preferable for studies involving isolation of EV, RNA or functional in vitro
372 / in vivo assays, serum also has appropriate uses.³⁰ Above all, the prognostic performance
373 of serum CD142-EV is in line with others regardless EV's sources.^{12,14,15} The methodological
374 assessment of the most appropriate biological fluid is beyond the scope of the present study.
375

376 5. CONCLUSIONS

377 The aim of the present study was to give clinical relevance to a biomarker that can be useful
378 to assess the risk of negative outcome and to prompt the adoption of strategies to treat the
379 disease. Indeed, the detection of CD142 on the surface of EV is a cost-effective and rapid
380 test that can be available at time of admission by using conventional flow cytometer. The
381 method used is well standardized from our group^{21, 31} as well as from other independent
382 groups^{17, 18} from sample preparation to data analysis, ensuring that results can be
383 reproducible and shared among different laboratories. We believe that such analysis gains
384 considerable interest as risk stratification tool to support frontline clinical decision making.
385

386

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390 manuscript writing. E.C., L.G.G., E.R., T.F.S., and E.F., patient data collection. E.P., and
391 G.Ma. sample collection. A.B. statistical analysis. C.B., S.B., E.L., V.B., R.F., A.G., M.C.,
392 G.V., and G.Me. data generation and interpretation, critical revision. S.M., and L.B. study
393 design, data interpretation, manuscript writing. All authors read and approved the final
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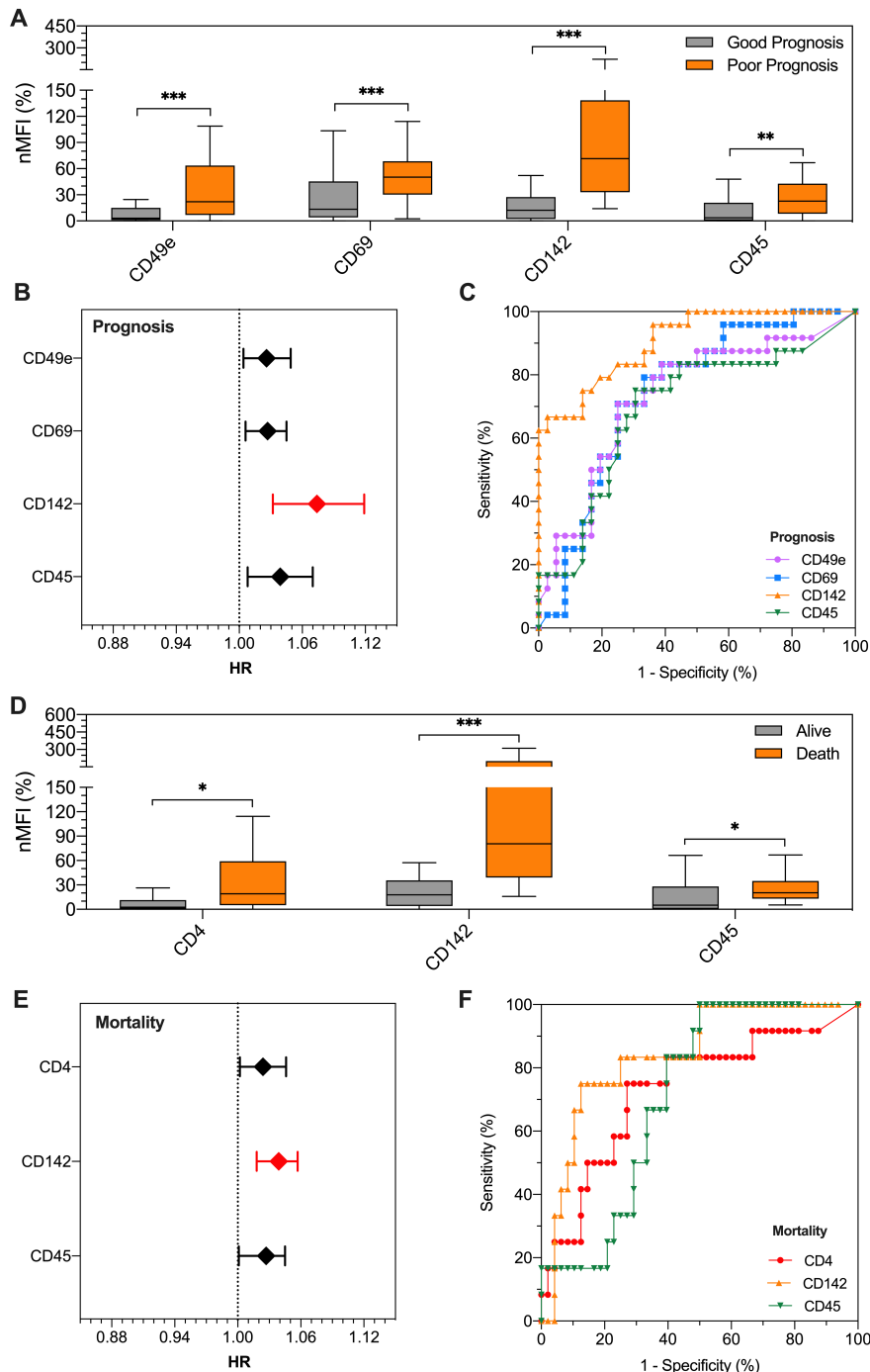
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529 **Table 1. Patient characteristics**

Variable	All Patients [n=261]	Good Prognosis [n=189]	Poor Prognosis [n=72]	P-value
Age (years)	68 ± 13.4	68 ± 13.6	71 ± 12.8	0.100
Sex (Male; %)	171 (65.5)	119 (63.0)	52 (72.2)	0.160
BMI (Kg/sqm)	27.4 ± 5.67	27.0 ± 5.60	28.2 ± 5.78	0.204
Bilateral Pneumonia (%)	162 (62.1)	104 (55.0)	58 (80.6)	<0.001
Pulmonary Embolism (%)	3 (1.1)	2 (1.1)	1 (1.4)	1.000
Respiratory rate (a.p.m.)	22 ± 5.0	20 ± 4.0	25 ± 5.6	<0.001
Peripheral O ₂ saturation (%)	92 ± 4.1	93 ± 3.5	90 ± 4.9	<0.001
GCS (<15; n)	28 (10.7)	16 (8.5)	12 (16.7)	0.056
<i>Anamnesis</i>				
CKD (%)	44 (16.9)	16 (8.5)	28 (38.9)	<0.001
Hypertension (%)	144 (55.2)	101 (53.4)	43 (59.7)	0.362
Chronic Pulmonary Disease (%)	46 (17.6)	30 (15.9)	16 (22.2)	0.229
Diabetes (%)	66 (25.3)	45 (23.8)	21 (29.2)	0.373
Smoking habit (%)	36 (13.8)	20 (10.6)	16 (22.2)	0.015
CHF (%)	20 (7.7)	10 (5.3)	10 (13.9)	0.020
CAD (%)	47 (18.0)	25 (13.2)	22 (30.6)	0.001
Liver Disease (%)	51 (19.5)	29 (15.3)	22 (30.6)	0.006
Chronic Neurological Disease (%)	58 (22.2)	36 (19.0)	22 (30.6)	0.046
Dementia (%)	39 (14.9)	22 (11.6)	17 (23.6)	0.015
Autoimmune Disease (%)	0 (0.0)	0 (0.0)	0 (0.0)	1.000
HIV/AIDS (%)	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Cancer (%)	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Obesity (%)	52 (19.9)	34 (18.0)	18 (25.0)	0.205
Number of Comorbidities (n)	1 [0; 2]	1 [0; 2]	2 [1; 4]	<0.001
<i>Arterial blood gas assay</i>				
pCO ₂ (KPa)	4.5 [4.1; 5.0]	4.5 [4.1; 4.9]	4.7 [4.0; 5.2]	0.171
pO ₂ (KPa)	9.1 [8.1; 10.5]	9.3 [8.5; 10.8]	8.6 [7.7; 10.1]	0.003
Bicarbonate (mmol/L)	23.6 ± 3.10	23.8 ± 2.74	23.0 ± 3.85	0.163
Lactic acid (mmol/L)	1.3 ± 0.80	1.2 ± 0.69	1.5 ± 1.02	0.049
<i>Biochemical parameters</i>				
Haemoglobin (g/L)	139 ± 18.6	141 ± 17.4	135 ± 21.1	0.052
PLTS (*10E9/L)	190 ± 73.3	187 ± 71.4	199 ± 77.7	0.238
WBC (*10E9/L)	6.6 ± 3.30	6.4 ± 3.08	6.9 ± 3.80	0.242
Neutrophils (*10E9/L)	5.3 ± 4.22	5.3 ± 4.74	5.5 ± 3.61	0.806
Lymphocytes (*10E9/L)	1.0 ± 0.92	1.1 ± 0.84	0.9 ± 0.81	0.533
Monocytes (*10E9/L)	0.4 ± 0.23	0.4 ± 0.19	0.4 ± 0.30	0.868
Eosinophils (*10E9/L)	0.06 ± 0.043	0.06 ± 0.034	0.07 ± 0.029	0.780
Basophils (*10E9/L)	0.06 ± 0.051	0.05 ± 0.032	0.06 ± 0.028	0.905
C-reactive protein (mg/L)	47 [21; 98]	40 [18; 86]	69 [35; 137]	0.003
D-dimer (mg/L)	0.76 [0.50; 1.41]	0.67 [0.48; 1.15]	1.08 [0.66; 2.35]	<0.001
PT-INR (a.u.)	1.2 ± 0.62	1.2 ± 0.53	1.4 ± 0.81	0.097
aPTT (sec)	32 ± 7.1	31 ± 6.1	34 ± 8.8	0.031
LDH (U/L)	467 [381; 633]	462 [375; 601]	484 [392; 776]	0.079
Urea (mmol/L)	7.2 ± 5.06	6.4 ± 3.50	9.4 ± 7.38	0.001
Troponin I (ng/L)	14 [8; 29]	12 [6; 19]	25 [13; 66]	<0.001
<i>Outcome</i>				
4C Mortality Score (n)	9 ± 4.0	8 ± 3.7	11 ± 3.8	<0.001
Hospitalization (days)	9 [2; 16]	8 [2; 14]	14 [7; 26]	<0.001
Time to OTI / Death (days)	N.A.	N.A.	7 [4; 12]	N.A.
Low-flow O ₂ Treatment (%)	222 (85.1)	158 (83.6)	64 (88.9)	0.284
High-flow O ₂ Treatment (%)	94 (36.0)	41 (21.7)	53 (73.6)	<0.001
Orotracheal Intubation (%)	36 (13.8)	N.A.	36 (50.0)	N.A.
Death (%)	50 (19.2)	N.A.	50 (69.4)	N.A.

530 **Legend to Table 1. Patient characteristics**

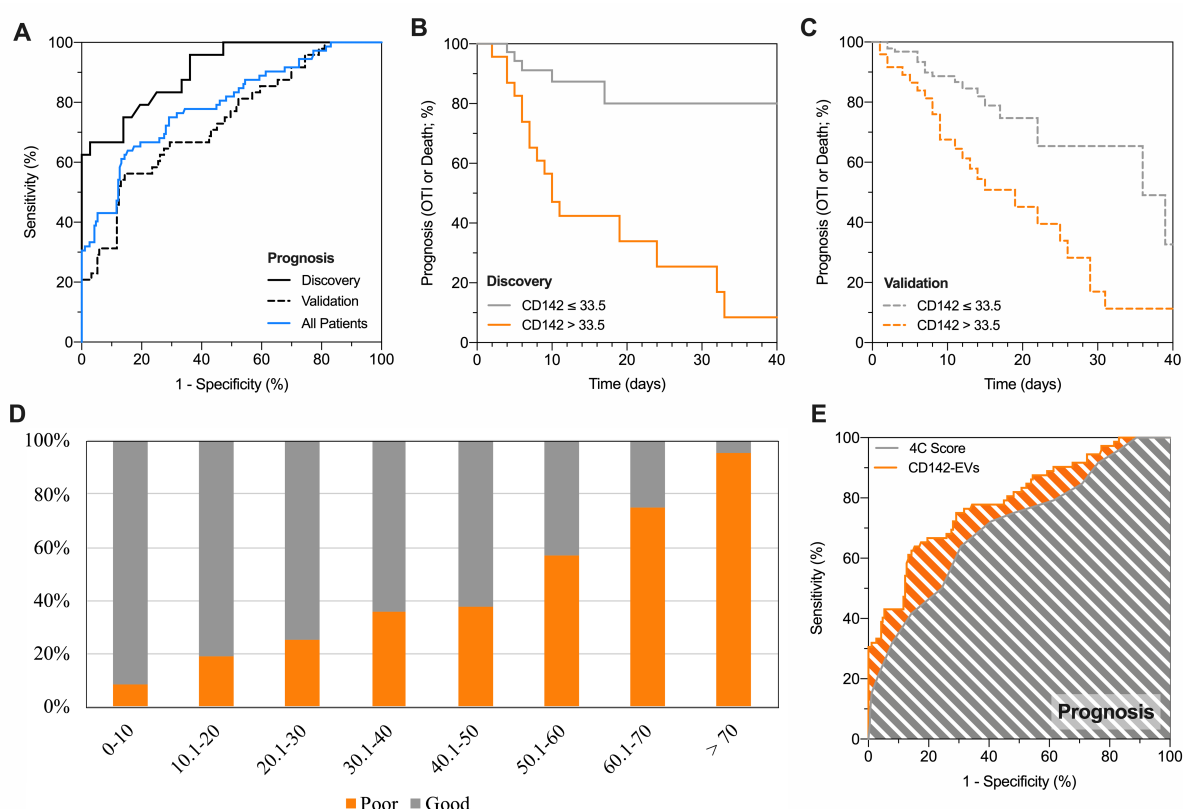
531 Clinical and biochemical characteristics of patients admitted to hospital for SARS-CoV2
532 infection and pneumonia (n=261) stratified according to prognosis; a poor prognosis is
533 defined as need of orotracheal intubation (OTI) or death. GCS, Glasgow Coma Scale; CKD,
534 Chronic Kidney Disease (defined as eGFR < 60 mL/min); CHF, Chronic Heart Failure
535 (defined as ejection fraction < 35%), CAD, Coronary Artery Disease; Liver disease, defined
536 as chronic hepatitis or cirrhosis with or without portal hypertension; Chronic neurological
537 disease, defined as presence of Parkinson disease, Alzheimer disease, history of major
538 cerebrovascular accident; HIV/AIDS, infection by Human Immunodeficiency Virus, Acquired
539 Immunodeficiency Syndrome; WBC, White Blood Cells; PT-INR, Thrombin Time -
540 International Normalized Ratio; aPTT, activated Partial Thromboplastin Time; LDH, Lactate
541 Dehydrogenase; N.A., Not Applicable. 4C Mortality Score was calculated as detailed in
542 Knight SR et al 2020.⁵ Comorbidities were defined using the Charlson comorbidities index.³²
543 A $p < 0.05$ was considered significant and shown in bold.



Legend to Figure 1. EV surface antigens associated to patient outcome

Profiling of EV surface antigens in patients admitted to hospital for SARS-CoV2 infection and pneumonia in the discovery cohort (n=60). Patients were stratified for outcome (good prognosis, grey, vs. poor prognosis, orange; a poor prognosis is defined as need of orotracheal intubation or death) and mortality. Median fluorescence intensity (MFI) was analyzed after normalization by the average MFI of CD9-CD63-CD81 (normalized MFI; nMFI, %). **(A)** Expression levels of EV surface antigens differentially expressed in patients with good vs. poor prognosis; **(B)** Association of EV surface antigens with patient outcome

553 (good vs. poor prognosis; a poor prognosis is defined as need of orotracheal intubation,
 554 OTI, or death). Hazard ratios (HRs) are shown together with their 95% confidence intervals.
 555 (C) ROC curves for EV surface antigens discriminating patients according to prognosis. (D)
 556 Expression levels of EV surface antigens differentially expressed in patients stratified for
 557 mortality. (E) Association of EV surface antigens with mortality. Hazard ratios (HRs) are
 558 shown together with their 95% confidence intervals. (F) ROC curves for EV surface antigens
 559 discriminating patients according to mortality. Statistics is reported in Online Tables 4-6-8-
 560 9-10. * $p < 0.01$; ** $p < 0.01$; *** $p < 0.001$.
 561

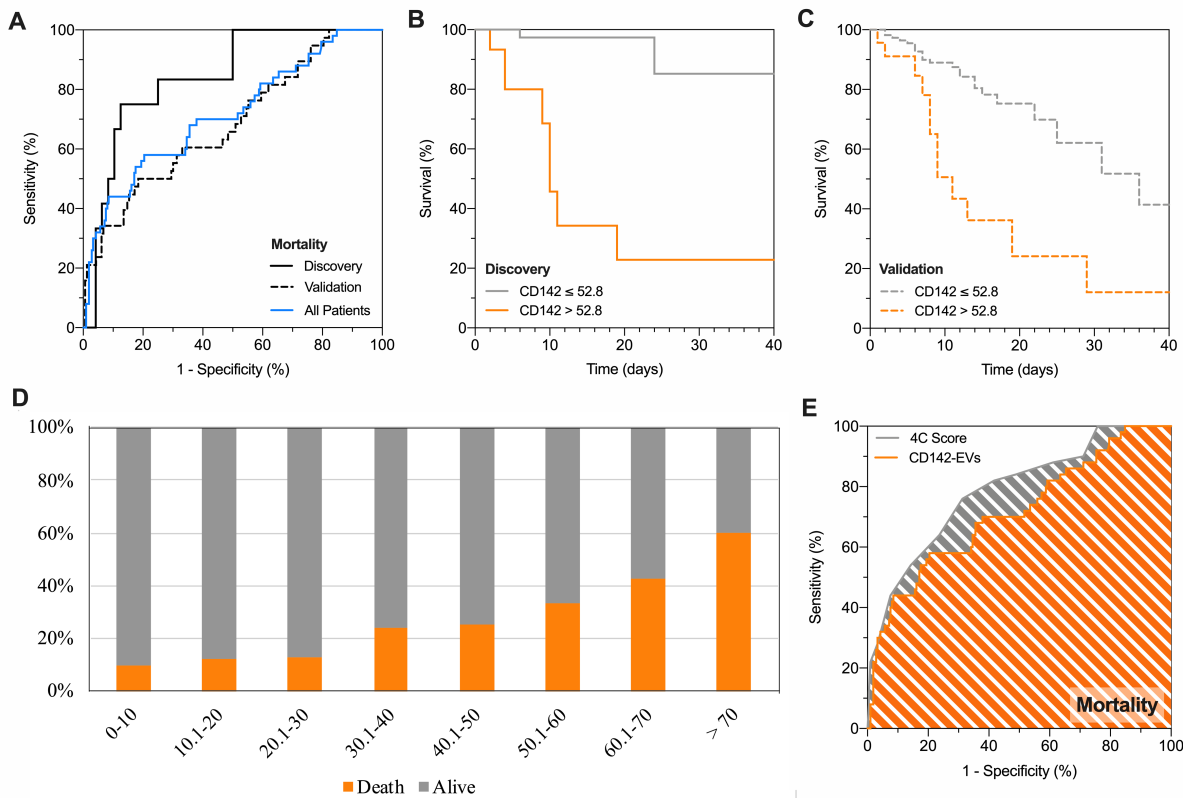


562
 563 **Legend to Figure 2. CD142-EV to predict patient prognosis**

564 Performance of CD142 expressed on EV surface (CD142-EV) to predict outcome (poor
 565 prognosis vs. good prognosis) in patients with SARS-CoV2 infection and pneumonia
 566 (Discovery cohort, n=60; Validation cohort, n= 201; All patients, n=261); a poor prognosis is
 567 defined as need of orotracheal intubation or death. Median fluorescence intensity (MFI) was
 568 analyzed after normalization by the average MFI of CD9-CD63-CD81 (normalized MFI;
 569 nMFI, %) for each EV antigen. (A) ROC curves showing performance of CD142-EV to
 570 predict patient prognosis: AUC at discovery = 0.906 (0.833-0.979); AUC at validation = 0.736
 571 (0.654-0.818); AUC in all patients = 0.792 (0.728-0.855). (B) Kaplan-Meier curves for
 572 CD142-EV; the cut-off (nMFI = 33.5%) to discriminate patient outcome (good vs. poor

573 prognosis; discovery cohort) was defined by analysis of ROC curves. HR (log-rank) = 4.75
574 (95% CI 2.09-10.81). (C) Kaplan-Meier curves for CD142-EV; the cut-off (nMFI = 33.5%) to
575 discriminate patient outcome (good vs. poor prognosis; validation cohort) was defined by
576 analysis of ROC curves. HR (log-rank) = 2.22 (95% CI 1.23-3.99). (D) Stratification of
577 patients according to levels of expression of CD142 on EV surface and patient prognosis
578 (good prognosis, grey; poor prognosis, orange) on the combined discovery and validation
579 cohorts. (E) ROC curve analysis: prediction of patient prognosis; CD142-EV vs. 4C Score⁵.
580 Statistics is reported in Online Tables 11-12-13.

581



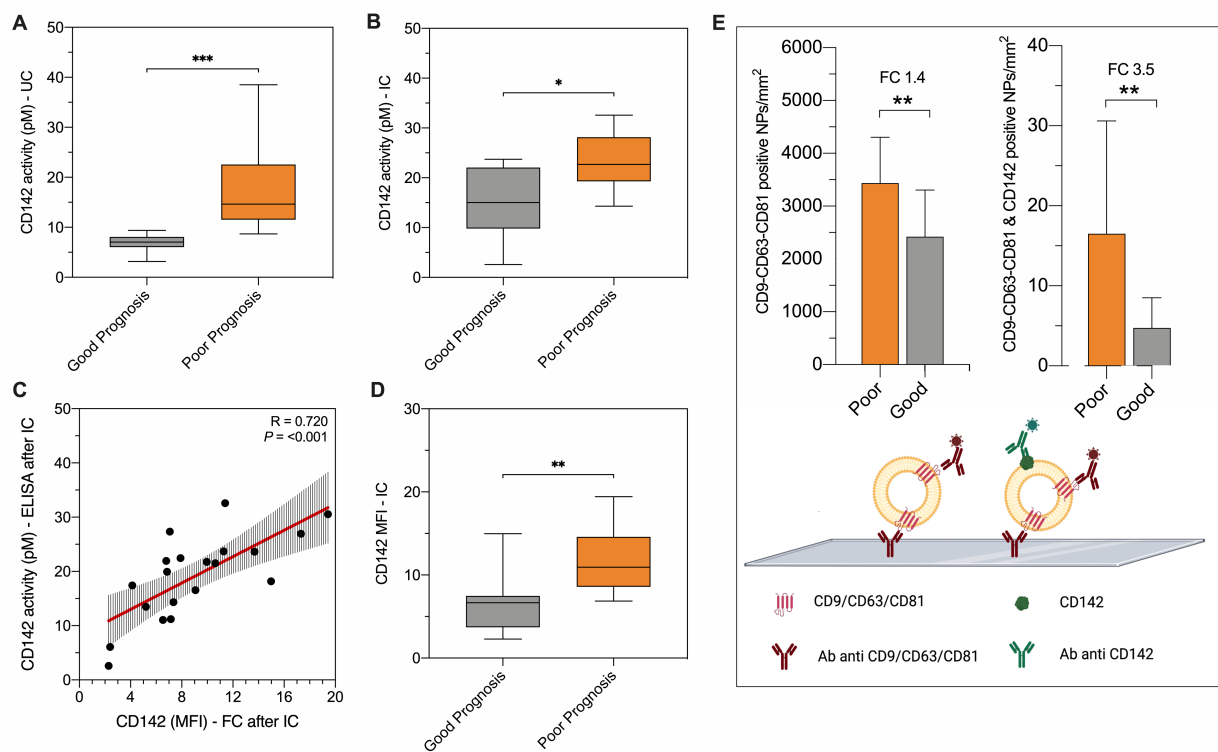
582

583 Legend to Figure 3. CD142-EV to predict patient prognosis and mortality

584 Performance of CD142 expressed on EV surface (CD142-EV) to predict mortality (death vs.
585 alive) in patients with SARS-CoV2 infection and pneumonia (Discovery cohort, n=60;
586 Validation cohort, n= 201; All patients, n=261). Median fluorescence intensity (MFI) was
587 analyzed after normalization by the average MFI of CD9-CD63-CD81 (normalized MFI;
588 nMFI, %) for each EV antigen. (A) ROC curves showing performance of CD142-EV to
589 predict mortality: AUC at discovery = 0.842 (0.727-0.957); AUC at validation = 0.682 (0.585-
590 0.779); AUC in all patients = 0.714 (0.630-0.798). (B) Kaplan-Meier survival curves for
591 CD142-EV; the cut-off (nMFI = 52.8%) to predict patient mortality (discovery cohort) was

592 defined by analysis of ROC curves. HR (log-rank) = 11.30 (95% CI 2.82-45.34). (C) Kaplan-
 593 Meier survival curves for CD142-EV; the cut-off (nMFI = 52.8%) to predict patient mortality
 594 (validation cohort) was defined by analysis of ROC curves. HR (log-rank) = 3.37 (95% CI
 595 1.27-8.93). (D) Stratification of patients according to mortality (alive, grey; death, orange).
 596 (E) ROC curve analysis: prediction of mortality; CD142-EV vs. 4C Score⁵. Statistics is
 597 reported in Online Tables 11-12-13.

598



599

600 **Legend to Figure 4. Experimental validation with different techniques**

601 The discriminant performance of CD142-EV was experimentally validated by different
 602 techniques in patients with SARS-CoV2 infection: good prognosis (grey; n=10) vs. poor
 603 prognosis (orange; n=10). (A-B) CD142 activity per particle measured by ELISA (pM per
 604 10⁹ particles), after EV isolation by ultracentrifugation (UC) or immunocapture (IC using
 605 beads covered by antibodies against CD9-CD63-CD81). (C) Correlation between CD142
 606 activity per particle (pM) and CD142 MFI at flow cytometry after IC. (D) CD142-EV MFI after
 607 IC (direct staining after immuno-capture, using beads covered by antibodies against CD9-
 608 CD63-CD81). (E) Colocalization of tetraspanins (CD9-CD63-CD81) and CD142 was
 609 assessed by ExoView® R100 Analyzer. Data are reported for mean number of
 610 nanoparticles (NPs) per mm² for vesicles labelled with fluorochrome-conjugated antibodies
 611 against CD9-CD63-CD81 and for the double positive for CD9-CD63-CD81 and CD142.