

Identification of a serum and urine extracellular vesicle signature predicting renal outcome after kidney transplant

*Original*

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1 **Identification of a serum and urine extracellular vesicle signature predicting renal outcome**  
2 **after kidney transplant**

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34 **ABSTRACT**

35 **Background.** A long-standing effort is dedicated toward identification of biomarkers allowing the  
36 prediction of graft outcome after kidney transplant. Extracellular vesicles (EVs) circulating in body  
37 fluids represent an attractive candidate, as their cargo mirrors the originating cell and its  
38 pathophysiological status. The aim of the study was to investigate EV surface antigens as potential  
39 predictors of renal outcome after kidney transplant.

40 **Methods.** We characterized 37 surface-antigens by flow-cytometry, in serum- and urine- EVs from  
41 58 patients which were evaluated before, and at 10-14 days, 3 months, and 1 year after transplant, for  
42 a total of 426 analyzed samples. The outcome was defined according to estimated glomerular  
43 filtration rate (eGFR) at 1 year.

44 **Results.** Endothelial cells and platelets markers (CD31, CD41b, CD42a and CD62P) in serum-EVs  
45 were higher at baseline in patients with persistent kidney dysfunction at 1 year, and progressively  
46 decreased after kidney transplant. Conversely, mesenchymal progenitor cell marker (CD1c, CD105,  
47 CD133, SSEEA-4) in urine-EVs progressively increased after transplant in patients displaying renal  
48 recovery at follow-up. These markers correlated with eGFR, creatinine and proteinuria, associated to  
49 patient outcome at univariate analysis and were able to predict patient outcome at ROC curves  
50 analysis. A specific EV molecular signature obtained by supervised learning correctly classified  
51 patients according to 1-year renal outcome.

52 **Conclusions.** An EV-based signature, reflecting the cardiovascular profile of the recipient, and the  
53 repairing/regenerative features of the graft, could be introduced as a non-invasive tool for a tailored  
54 management of follow-up of patients undergoing kidney transplant.

55 **What is already known about this subject**

56 Despite progress in understanding processes affecting allograft kidney in transplanted patients, renal  
57 function decline and allograft loss remain significant concerns. Clinical parameters, kidney biopsy,  
58 and instrumental evaluations may guide patient management. To date, a large effort is dedicated to  
59 the identification of prognostic biomarkers of graft dysfunction to direct therapeutic interventions.

60 **What this study adds**

61 We analysed EV surface antigen profile in a longitudinal cohort of transplanted patients. We  
62 identified an EV-based signature comprising endothelial and platelet markers in serum-EVs,  
63 reflecting the cardiovascular profile of the recipient, and mesenchymal/progenitor cell marker in urine  
64 EVs, reflecting the repairing/regenerative features of the graft, and predicting 1-year renal outcome.

65 **What impact this may have on practice or policy**

66 EV profiling may be performed by standardized, low-cost, flow cytometric assays directly applicable  
67 on a small amount of fresh or frozen samples. This approach is minimally invasive, amenable to full  
68 automation, and represent a promising point-of-care testing tool for a tailored management of follow-  
69 up of patients undergoing kidney transplant.

70

71 **Keywords**

72 Kidney transplant, chronic kidney disease, extracellular vesicle, biomarker, machine learning.

## 73 **Introduction**

74 Kidney transplantation is the preferred treatment for patients with end stage renal disease, as it  
75 provides higher survival rates and better quality of life compared to dialysis<sup>1,2</sup>. Despite the progress  
76 in understanding the multiple processes affecting the allograft kidney, renal function decline and  
77 allograft loss remain significant concerns. In fact, while improvements in the immunosuppressive  
78 therapy enabled to mitigate organ function decline in relation to acute rejection, the complex and  
79 multifactorial mechanisms affecting the long-term survival of the kidney graft still need to be  
80 addressed<sup>3</sup>. Overall, renal graft function decline may result from an imbalance between immune and  
81 non-immune mediated organ damage and the organ ability to repair toward functional tissue after  
82 damage, limiting maldifferentiation of fibrotic tissue<sup>3,4</sup>. In this context, clinical parameters of organ  
83 function and immune monitoring, percutaneous allograft biopsy, and instrumental evaluations may  
84 guide the graft management and surveillance. In addition, a large effort is currently dedicated to the  
85 identification of noninvasive diagnostic and prognostic biomarkers of delayed graft function,  
86 rejection, and chronic allograft dysfunction to direct therapeutic interventions<sup>5</sup>.

87 Extracellular vesicles (EVs) are considered promising candidates as disease biomarkers. They are  
88 nanosized vesicles released from multi-vesicular bodies or shed from the surface membranes of  
89 almost all cell types<sup>6,7</sup>. Of interest, surface markers and cargo, including proteins and RNA species,  
90 reflect the originating cell and its physiopathological state<sup>8,9</sup>. In serum, EVs are a heterogeneous  
91 population deriving from the different cells of the bloodstream as well as from the endothelial  
92 layer<sup>9,10</sup>. In particular, serum EVs deriving from platelets, leukocytes and endothelial cells can be  
93 identified through specific surface markers of the originating cell<sup>11</sup>. In urine, EVs are considered to  
94 mainly derive from cells of the nephron, and their marker expression might provide relevant  
95 information on the kidney pathophysiology<sup>12,13</sup>. Data from literature suggest that dynamics changes  
96 of EV markers and content in serum and urine during kidney transplant might mirror recovery of  
97 renal and endothelial functions<sup>14-17</sup>. In particular, our group previously showed that urinary EVs  
98 expressing CD133, a marker of renal progenitor cells involved in tissue repair, progressively  
99 increased in the first week after transplant, and paralleled the graft function<sup>18</sup>. In analogy, the kinetics  
100 of EV serum subpopulations at different timings after graft transplant showed decrease of endothelial  
101 and platelet derived particles, suggesting a decrease of cardiovascular injury after transplant<sup>14,17,19</sup>.  
102 Interestingly, their levels correlated with renal function<sup>20</sup>.

103 In the present study, we aimed to combine the analyses of serum and patient-matched urinary EVs,  
104 before and at different time points after kidney transplant, in order to stratify patients according to  
105 their outcome. We reasoned that data from serum- and urine- EVs, altogether, may provide  
106 information on the status of the graft tissue and, in parallel, on the recipient cardiovascular and

107 immune profile. We took advantage from a previously validated flow cytometric platform which  
108 allowed the simultaneous profiling of several EV surface antigens (including markers from  
109 mesenchymal/stem progenitor cells, platelets, endothelium, and immune cells)<sup>21,22</sup>, and through  
110 supervised learning algorithms, we obtained a specific molecular signature able to predict renal  
111 outcome after kidney transplant.

112

## 113 **Materials and Methods**

114 A detailed description of patient enrollment, EV characterization and statistics is provided as  
115 supplementary material.

116

### 117 Patient recruitment and sampling strategy

118 We consecutively recruited 58 patients who underwent kidney transplant for end-stage renal disease.  
119 All patients who gave written informed consent. Patients were excluded in case of concomitants  
120 infections, acute inflammatory disease, or active cancer. The study complied with the Declaration of  
121 Helsinki. Patient outcome was defined according to glomerular filtration rate estimated by CKD-EPI  
122 equation (eGFR) at 12 months, using a cut-off of 45 mL/min. For each patient, peripheral blood and  
123 urine samples were collected before kidney transplant (baseline, or T0), 10-14 days (T1), 3 months  
124 (T2), and 12 months (T3) after transplant (urine was not available for 38 anuric patients at T0; Figure  
125 1A). Pre-analytical factors for sample handling and storage complied with recommendations of the  
126 International Society for Extracellular Vesicles<sup>23,24</sup>.

127

### 128 EV characterization

129 Venous blood was collected in serum separator tubes; after clot formation a first centrifugation at  
130 1600 g for 15 min at 4°C was performed to separate serum from cellular components. Serum was  
131 transferred in a new clean tube and centrifuged at 3,000 g for 20 min, at 10,000 g for 15 min, and at  
132 20,000 g for 30' to remove intact cells, cellular debris and larger EVs. Second morning urine samples  
133 were collected in parallel; a first centrifugation at 3000 g for 15 min at 4°C was performed to separate  
134 urine from cellular components. Urine was transferred in a new clean tube and centrifuged at 3,000  
135 g for further 15 min; high-speed centrifugation steps were not performed for urine to avoid co-  
136 precipitation of Tamm-Horsfall protein and EVs<sup>24,25</sup>. Samples were processed immediately after  
137 collection and pre-cleared aliquots were then stored at -80°C and never thawed prior to analysis.  
138 Particle concentration and diameter were measured by nanoparticle tracking analysis (NTA). After  
139 EV immuno-capture by beads coated with antibodies against 37 specific EV markers, EV surface  
140 antigenic profile was evaluated by a multiplex flow cytometric (FC) assay (MACSPlex human

141 Exosome Kit; Miltenyi Biotec), as previously described (Figure 1B)<sup>11</sup>. The average levels of  
142 tetraspanins (CD9-CD63-CD81) for each serum and urine sample were used as internal normalizer  
143 of fluorescence levels of all the other 37 markers to allow comparison among samples and correct for  
144 intra- and inter-patient variations of vesicle concentration in the analyzed biofluid<sup>23,24</sup>. Our data  
145 provide an evaluation of specific antigen fluorescence intensity normalized to a standard EV marker  
146 (tetraspanins levels), thus reflecting an EV qualitative profile for a normalized EV concentration  
147 rather than a quantitative EV characterization. To rule out confoundings related to the experimental  
148 protocol, our standard protocol was compared with an alternative protocol including a pre-isolation  
149 step by ultracentrifugation (see Extended Methods). Single vesicle analysis was performed by super-  
150 resolution microscopy using Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging,  
151 Oxford, UK) after EV isolation by ultracentrifugation.

152

### 153 *Statistics and diagnostic modelling*

154 Normally distributed variables are expressed as mean  $\pm$  standard deviation (SD) and analyzed by T  
155 student test. Non-normally distributed variables are expressed as median [interquartile range] and  
156 analyzed by Mann-Whitney test or Wilcoxon test, as appropriated. Categorical variables are  
157 expressed as absolute number (percentage) and compared with chi-square tests. Correlations were  
158 evaluated by Pearson's test. Odds ratio (OR) were calculated by univariate logistic regression.  
159 Receiver operating characteristics (ROC) curves were analyzed to assess area under the curve (AUC).  
160 Machine learning (ML) supervised algorithms were used to train and validate diagnostic models to  
161 predict renal outcome at T3, using nMFI of serum- or urine- EV surface antigens. Four different  
162 machine learning classifiers (linear discriminant analysis, random forest, support vector machine with  
163 linear or gaussian kernel) and 3 algorithms for data imbalance correction were applied, generating  
164 616 different models. After tuning of hyperparameters, best models were validated by a leave-one-  
165 out algorithm (see extended methods).

166

## 167 **Results**

### 168 *Patient characteristics*

169 We enrolled 58 patients who underwent kidney transplant for end-stage renal disease. Baseline  
170 characteristics are reported in Table 1: mean age was 54 years, 44.8% were male, 77.6% received the  
171 transplanted kidney from a deceased donor. Patients were evaluated at baseline (before transplant,  
172 T0), and at 10-14 days (T1), 3 months (T2), and 12 months (T3) after transplant (Figure 1A); 35  
173 patients displayed renal function recovery, while 23 had an eGFR equal or lower to 45 mL/min at T3  
174 and were classified as persistent renal dysfunction. At baseline, no differences were found between

175 patients with renal recovery vs. persistent dysfunction; donor parameters were also similar (Table 1).  
176 At follow-up, eGFR was significantly lower at T2 and T3 in patients with persistent renal dysfunction  
177 compared to those with renal recovery, while creatinine and proteinuria were higher at T3 (Table S1).  
178 No other significant differences were found between patients with renal recovery vs. persistent renal  
179 dysfunction, including prevalence/incidence of delayed graft function, vesical-ureteral reflux,  
180 bacterial and viral infections (urinary tract infections, sepsis, BKV, CMV, and colonization by  
181 *Klebsiella Pneumoniae* carbapenemase-producing bacteria), new-onset diabetes mellitus, graft  
182 rejection, and positivity for donor specific antibodies.

183

#### 184 Quantitative evaluation of serum- and urine- EVs

185 Serum and urine samples were collected at each time point (urine was not available for 38 anuric  
186 patients at T0); overall, we analyzed 426 samples (232 serum and 194 urine). Serum and urine  
187 samples were first directly analyzed by NTA; after immuno-capture, EV surface antigens were then  
188 systematically characterized by a multiplex FC assay (Figure 1, Tables S2-S3).

189 Comparing serum and urine samples, the number of serum EVs was higher than urine EVs ( $2.4 \times 10^{12}$   
190 vs.  $5.6 \times 10^9$ /mL;  $p < 0.001$ ), whereas particle diameter was similar (183 vs. 181 nm; Figure S1A-D; Table  
191 S2), independently from the renal outcome and the evaluated time point. Of interest, the number of  
192 serum EVs, but not urine EVs, significantly correlated to the corresponding creatinine level (Figure  
193 S1H-L). EVs concentration was re-evaluated after stratification for time points (Table S4). Serum EV  
194 number per mL significantly decreased after kidney transplant, while a similar but not significant  
195 trend was observed for urine EVs (Figure S2).

196

#### 197 Characterization of serum- and urine- EV surface antigens

198 Serum and urine EVs, characterized by labelling to typical tetraspanins markers, were further  
199 analyzed using fluorescent-labelled beads coated with antibodies against 37 different surface markers  
200 (Figure S3; Tables S5-S6). We reasoned that serum EVs, deriving from endothelial cells, platelets  
201 and immune cells, could reflect the cardiovascular and immunological features of the recipient,  
202 whereas urine EVs mainly deriving from renal and infiltrating cells, the graft physiopathology.

203 For both serum and urine samples, the levels of expression of EV markers (CD9-CD63-CD81),  
204 correlated with the EV concentration measured by NTA (Figure S1G-I). Moreover, consistently with  
205 the observed EV number, the specific CD9-CD63-CD81 EV expression was higher in serum than in  
206 urine (Figure S1E-F) and decreased after kidney transplant (Figure S2C-F).

207 The average MFI for CD9-CD63-CD81 was then used as internal normalizer of fluorescence levels  
208 of all other 37 markers to enable comparison among the different samples and to exclude non-specific



209 binding such as small debris. A separate pool of samples was analyzed to evaluate whether a pre-  
210 isolation step by ultracentrifugation may affect the profiling of serum- and urine- EV surface antigens.  
211 After ultracentrifugation, as expected, mean MFI for CD9, CD63, and CD81 was higher in samples  
212 underwent EV enrichment compared to standard protocol, whereas EV surface profile, after  
213 normalization by CD9-CD63-CD81, was similar to that obtained by the standard protocol (Figure  
214 S4).

215 Serum- vs. urine- EVs showed a very different profile, being different for 29 of the 37 tested markers  
216 (Figure S3). Of note, CD42a, CD41b, CD62P and HLA-II were highly expressed in serum EVs,  
217 whereas CD105, SSEA-4 and HLA-I in urine EVs. We subsequently analyzed the kinetic of evaluated  
218 EV surface antigens in transplanted patients at different times after transplant. The expression of a  
219 large number of markers varied during the follow up. In particular, 12 out of 37 evaluated surface  
220 antigens of serum EVs showed significant differences during follow-up (Figure S5A), possibly due  
221 to effect of drugs as well as to the normalization of the uremic status. In parallel, 34 out of 37 markers  
222 changed in urine, most of which at T3 (12 months) as compared to T1 or T2 (Figure S5B), in relation  
223 to a large variety of cellular processes occurring in the transplanted graft (Tables S6).

224

#### 225 *EV signature of kidney graft dysfunction*

226 We therefore evaluated the different EV profile according to the transplant outcome, defined as  
227 persistent renal dysfunction, or renal recovery after 1 year, in case of eGFR less/equal or higher to 45  
228 mL/min, respectively (Figure S6).

229 Among serum EV surface antigens, CD62P, CD41b, CD42a, and CD31 (platelet/endothelial markers)  
230 were highly expressed in patients with persistent renal dysfunction compared to those with renal  
231 recovery at both T0 and T1, and their expression was able to predict patient outcome at T3 (Figure  
232 2). CD62P, CD42a, and CD31 appeared higher also at T2 in patients with kidney dysfunction. During  
233 follow-up, the expression of these markers gradually decreased in all patients independently from  
234 renal outcome and CD62P, CD42a, and CD31 were also inversely correlated to eGFR (R ranging  
235 between -0.247 and -0.130; Figure 2). The expression of all EV markers was similar between groups  
236 at T3 (Tables S7-S10). The association of CD62P, CD41b, CD42a, and CD31 with patient outcome  
237 was confirmed by univariate analysis at T0, with ORs ranging between 0.84 and 0.98 (Table S11).  
238 The analysis indicates a 2% to 19% decrease in the likelihood of renal recovery for each 1 unit  
239 increase in nMFI of the considered EV surface antigens.

240 Considering urine EVs, as differences observed from T0 to T1 may be attributable to vesicles secreted  
241 by transplanted kidney (Table S12), we analyzed their profile starting from T1 (Tables S13-S15).  
242 CD105, CD1c, SSEA-4, and CD133/1, characteristic of immune cells and mesenchymal/stem

243 progenitor cells, gradually and significantly increased from T1 to T3 in patients with renal recovery,  
244 but not in those with persistent renal dysfunction at T3 (Figure 3). Noteworthy, at T1 these 4 EV  
245 markers were already significantly higher in patients with renal recovery, and associated to patient  
246 outcome at univariate analysis, with ORs ranging between 1.01 and 1.15 (Table S11), thus indicating  
247 a 1% to 15% increase in the likelihood of renal recovery for each 1 unit increase of their nMFI.  
248 CD105, CD1c, SSEA-4, and CD133/1 were also directly correlated to eGFR (R ranging between  
249 0.187 and 0.384; Figure 3). A pool of urine EVs isolated by control subjects was analyzed using  
250 super-resolution microscopy to assess colocalization of these markers on single vesicles: 48.9%,  
251 21.3%, and 10.6% of EVs expressed CD105, CD133/1 and SSEA-4, respectively. Interestingly,  
252 CD105 appeared as the marker with higher expression levels also in flow cytometric analyses. In  
253 addition, 24.7% of urine EVs co-expressed CD105 and CD133/1, while other combinations were  
254 observed in less than 2% of vesicles (Figure S7).

255 Finally, we correlated serum- and urine- EV surface antigens with creatinine, eGFR and proteinuria  
256 (Table S16). Of note, all urine EV markers were correlated to creatinine and eGFR, while CD31 on  
257 serum EVs and SSEA-4 on urine EVs correlated with proteinuria as index of renal damage (R of  
258 0.264 and -0.206, respectively;  $p < 0.01$ ).

259

#### 260 Prediction of renal recovery after kidney transplantation

261 The diagnostic performance of serum and urine EV surface antigens associated to patient outcome at  
262 univariate analysis (Figure 4A-5A) was assessed by analysis of ROC curves; each EV marker was  
263 evaluated singularly or as a compound EV marker generated by linear weighted combination of all  
264 the others (CD62P-CD41b-CD42a-CD31 for serum EVs; CD105-CD1c-SSEA4-CD133/1 for urine  
265 EVs; Table S17). AUC for serum EV markers ranged between 0.730 and 0.999, with the compound  
266 marker displaying an AUC of 0.836 (95%CI 0.736-0.929; Figure 4B); of note, serum CD42a  
267 displayed an AUC of 0.999 (95%CI 0.995-1.000), correctly discriminating all except one patient. On  
268 the other side, the AUC for urine EV markers was comprised between 0.686 and 0.856, with the  
269 compound marker reaching up to 0.901 (95%CI 0.823-0.978; Figure 5B).

270 Finally, in the attempt to exploit the specific EV signature and develop an advanced diagnostic model  
271 to predict renal outcome at T3, we combined nMFI levels of all EV surface antigens differentially  
272 expressed in patients with persistent renal dysfunction compared to those with renal recovery, at T0  
273 for serum EVs (HLA-II-CD62P-CD41b-CD42a-CD29-CD31; Table S7), or at T1 for urine EVs  
274 (CD19-CD56-CD105-CD2-CD1c-SSEA-4-HLA-I-CD42a-CD133/1-CD45-CD20; Table S13) by  
275 the use of supervised ML algorithms. As detailed in the methods section, 4 ML classifiers and  
276 different algorithms for dataset imbalance correction were applied to levels of EV markers in serum

277 and urine, resulting in 616 different models. Accuracy of prediction models based on serum EV  
278 antigens ranged between 72.4% and 100.0% at training, and between 69.0% and 98.3% at validation;  
279 models based on urine EV antigens displayed an accuracy comprised between 74.1% and 86.2% at  
280 training and between 62.1% and 80.1% at validation (Table S18).

281 The best ML model exploiting a serum EV signature was a RF regressor with synthetic minority over-  
282 sampling technique as correction for data imbalance; confusion matrix and a representative  
283 classification tree are shown in Figure 4C-D. At training, all patients with persistent renal  
284 dysfunction, and 34 of 35 patients with renal recovery were correctly classified (sensitivity 100.0%  
285 and specificity 97.1%), resulting in an overall accuracy of 98.3%. At validation, the model confirmed  
286 a very high performance (98.3% accuracy, 95.7% sensitivity, 100.0% specificity) without any  
287 detected overfitting effect. Of note, only 1 patient with persistent renal dysfunction was misclassified  
288 at validation, thus meaning a negative predictive value of 97.3%.

289 Conversely, a urine EV signature obtained by a linear support vector machine algorithm (see  
290 methods) displayed a lower but still reliable performance, with the correct prediction of 17 of 23, and  
291 32 of 35 patients with persistent renal dysfunction or renal recovery, respectively (84.5% accuracy,  
292 73.9% sensitivity, 91.4% specificity) at training. At validation, we observed a minimum overfitting  
293 bias (4.4%), with a final accuracy of 80.1%, and a sensitivity/specificity respectively of 71.6% and  
294 85.7% (Figure 5C). The plot built on the two best discriminants (SSEA-4 and CD105) confirmed an  
295 excellent discrimination of patients according to their outcome (Figure 5D).

296 Considering donor age and type (explant from deceased vs. living donors) as potentially associated  
297 to graft function, we also performed a multivariate logistic regression analysis to assess their impact  
298 on associations between renal outcome and each single serum- and urine- derived EV marker (Table  
299 S19). All EV antigens which were significantly associated to renal outcome (CD105-CD1c-SSEA4-  
300 CD133/1 from urine, and CD62P-CD41b-CD42a-CD31 from serum) confirmed their association  
301 independently from donor age/type, except the serum EV marker CD42a which was no longer related  
302 to patient outcome after correction for donor age or type. Interestingly, renal outcome was not only  
303 directly associated to CD133/1 (OR 1.09;  $p=0.008$ ), but also inversely related to donor age (OR 0.97;  
304  $p=0.035$ ), thus meaning an increase likelihood of renal recovery at the increase of CD133/1 levels  
305 and at the donor age decrease. Consistently, sensitivity analysis performed on ML models confirmed  
306 a negligible impact of donor age/type on prediction performance, which remains highly reproducible  
307 even when models were applied on the cohort stratified for age tertile, or for deceased vs. living donor  
308 (Figure S8).

309

310 EV signature of graft rejection

311 Finally, we performed a sub-analysis on serum and urine EV profile in patients with or without graft  
312 rejection, diagnosed in transplanted patients by kidney biopsy in 7 cases during a follow-up of 1 year  
313 (6 cellular and 1 humoral acute rejection; Tables S20-21).

314 Serum EV concentration and mean MFI for CD9-CD63-CD81 were respectively 2.4- and 4.2-fold  
315 higher in rejecting patients compared to the others (Figure S9A-C); 15 of the 37 serum EV antigens  
316 (CD3-CD19-CD8-CD25-CD49e-ROR1-CD209-CD9-CD11c-CD86-CD44-CD326-CD69-CD45-  
317 CD20) were highly expressed in case of graft rejection compared to normal follow-up (Figure S9D).  
318 Similarly, urine EV concentration and mean MFI for CD9-CD63-CD81 were respectively 2.6- and  
319 3.6-fold higher in rejecting patients (Figure S9E-G), and 10 EV antigens (CD19-CD56-CD105-  
320 CD1c-ROR1-CD209-CD9-CD42a-CD86-CD14) were more expressed in case of rejection, compared  
321 to non-rejecting patients (Figure S9H). Of interest, both serum and urine EV markers were mainly of  
322 immune origin, and different from those associated with renal outcome.

323 At univariate analysis, we confirmed the association of 9 of the 15 serum EV markers and 7 of the 10  
324 urine EV markers with a diagnosis of graft rejection (Figure S10A-B and Table S22). The diagnostic  
325 performance of EV markers associated to the diagnosis of rejection was assessed by ROC curves;  
326 AUC ranged between 0.720 and 0.834 (Table S23). Serum EV compound biomarker reached an AUC  
327 of 0.857 (95% CI 0.702-1.000), whereas urine EV compound biomarker 0.770 (95% CI 0.578-0.962  
328 - Figure S10C-D). Finally, supervised learning was used to develop and validate diagnostic models  
329 to detect graft rejection. As before, we trained 616 different models based on serum or urine EV  
330 markers differentially expressed in rejecting patients. After tuning, ML models with the highest  
331 accuracy were reported in Table S24: accuracy ranged between 81.5% and 99.1% at training, and  
332 81.0% and 96.1% at validation for models combining serum EV antigens, and between 71.6% and  
333 80.9% at training, and 72.3% and 79.3% at validation for urine EV antigens. The best model was  
334 again a RF regressor based on serum EV markers; confusion matrix and a representative classification  
335 tree are shown in Figure S10E-F. At training the accuracy was 99.1%, with the correct identification  
336 of all cases of rejection (100% sensitivity) and of 223 out of 225 cases of normal follow-up  
337 (specificity 99.1%). Reliability of the models was confirmed by leave-one out validation: accuracy  
338 was 96.1% (3% overfitting), with a sensitivity of 71.4% and a specificity of 96.9%.

339

## 340 **Discussion**

341 We here report for the first time a comprehensive characterization of serum- and urine- EVs in a  
342 cohort of transplanted patients by a standardized multiplex flow cytometric assay. The prospective  
343 longitudinal evaluation of EV profile over 1-year follow-up, allowed us to identify a molecular  
344 signature that appear to predict the outcome of the grafted kidney, related to pre-transplant asset of

345 both receiver (serum) and graft (urine). In particular, serum EV signature was mainly characterized  
346 by endothelial cells and platelets markers, probably reflecting the cardiovascular profile of the  
347 recipient. Conversely, urine EV signature was mainly characterized by markers of mesenchymal  
348 progenitor cells, which may mirror the repairing/ regenerative features of the graft.  
349 EVs and their content have been extensively studied in the context of kidney transplant. Different EV  
350 subpopulations in biological fluids, deriving from different cell types and characterized on the basis  
351 of EV surface marker expression, have been previously profiled using conventional cytofluorimetric-  
352 based analyses<sup>14,19</sup>. However, this technique implies several limitations in terms of detection  
353 threshold (exclusive characterization of larger EVs, so called microparticles), possible identification  
354 of multiple vesicles as a single event, and non-specific nanoparticle detection of protein/antibody  
355 aggregates. Alternatively, bead-based cytofluorimetric assays have been used to characterize bead-  
356 absorbed isolated EVs for single markers<sup>26</sup>. This procedure, however, requires EV isolation, and  
357 appears time-consuming and poorly standardized. In our study, we were able to analyze serum- and  
358 urine- EVs using a commercially available cytofluorimetric kit<sup>21,22</sup>, which allow the fast and  
359 reproducible profiling of a standardized panel of 37 EV surface antigens including markers from  
360 endothelium, platelets, immune cells, and mesenchymal/stem progenitor cells. According to a  
361 previously validated protocol<sup>11</sup>, we directly characterize EVs after immuno-capture without other  
362 pre-isolation steps. Of note, we did not perform any vesicle pre-enrichment steps, in the effort to  
363 implement and standardize an assay, which was developed for an application on isolated EVs<sup>21,22</sup>, to  
364 be directly applied as point-of-care tool for EV analysis in complex biofluids. This approach has  
365 further relevance, as it can be achieved avoiding time-consuming protocols and without sophisticated  
366 instrumentation, and therefore it could be easily translated to clinical practice.

367

368 Using this assay, we systematically characterized surface antigens expressed on serum- and urine-  
369 EVs from 58 patients evaluated at the different time points, for a total of 426 analyzed samples. A  
370 large number of markers appeared to change after transplant. In particular, endothelial- and platelet-  
371 derived EVs from serum samples progressively decreased 3 and 12 months after transplant. This is  
372 in line with prospective studies in transplanted patients evaluating serum endothelial and platelet  
373 microparticles, that were reported to progressively decrease, paralleling renal function  
374 recovery<sup>14,19,20</sup>. The novelty of our findings was the ability of endothelial and platelet EV markers,  
375 namely, CD31, CD41b, CD42a and CD62P, to predict the renal recovery at 1 year. These results  
376 suggest that not only renal function improvement may decrease the uremia-induced cardiovascular  
377 injury, lowering inflammation and oxidative stress, but that, in turn, the recipient pre-transplant  
378 cardiovascular and/or metabolic status may profoundly impact graft vascularization and function at

379 follow-up. The use of serum rather than plasma may have determined the artificial generation of  
380 platelet derived EVs; however, low-speed centrifugation may determine in-vitro cold-induced platelet  
381 activation also in plasma samples<sup>27,28</sup>. EV release by platelets in this circumstance is not fully  
382 standardizable, thus making EV quantitative data less reliable. In vitro platelet activation induced by  
383 serum separator tubes is expected to be similar in all groups, thus avoiding significant biases when  
384 comparing EV surface profiles. Indeed, both plasma and serum have been used in biomarkers  
385 discovery studies, and previous studies did not find any significant difference in EV profiling of  
386 serum and plasma from matched samples<sup>11,29</sup>.

387

388 In analogy, we identified four different markers in urine EVs (CD1c, CD105, CD133, and SSEA-4),  
389 that progressively increased in transplanted patients, and that were able to predict the recovery of  
390 renal function. These markers are characteristic of proliferating mesenchymal/stem cells and immune  
391 cells which may be involved in the reparative ability of the kidney. Of interest, CD133 has been  
392 described as characteristic marker of progenitor cells, with the ability to survive after damage and  
393 proliferate in response to cell injury<sup>30,31</sup>. Accordingly, the levels of urine EVs expressing CD133 were  
394 found elevated in healthy individuals and almost absent in end stage kidney disease<sup>18</sup>. Our group  
395 previously reported the increase in CD133 expressing EVs in the first week following a kidney  
396 transplant associated with early graft function, underlying that EV-carried CD133 might mirror the  
397 regenerative processes occurring in the transplanted kidney after ischemic processes<sup>18</sup>. Indeed, at  
398 graft tissue level, the number of CD133 expressing cells was lower in delayed graft function in respect  
399 to early graft function patients<sup>32</sup>, underlying the ability of EVs to mirror the tissue expression profile.

400

401 Our results on the prominent role of intrinsic pro-regenerative markers to predict long term graft  
402 function underline the concept that the pre-transplant graft status might dictate the gain of functional  
403 *versus* fibrotic tissue after ischemia-reperfusion insults. These findings are also in line with recent  
404 data showing the importance of organ biological age not only on post-transplant function, but also on  
405 risk of rejection, as organ damage may lead to leakage of cellular chromatin and mitochondrial  
406 proteins triggering immune responses in the recipient<sup>33,34</sup>. EVs may also carry information predicting  
407 ongoing or imminent rejection. At this regard, we observed, in a small subset of patients, the increase  
408 of a distinct subset of antigens in case of rejection, either in serum- or urine- EVs, including mainly  
409 markers of T-/B-lymphocytes and of immune system activation. In line with this hypothesis, an  
410 increase of CD3-positive EVs has been observed in urine of patients with acute cellular rejection,  
411 reflecting infiltration of T cells in the graft<sup>35</sup>. Moreover, circulating CD31/CD45 endothelial EVs and  
412 C4d-positive EVs increased in patients with antibody-mediated humoral rejection and may provide

413 information on its severity and response to treatment<sup>36,37</sup>. These data suggest that an EV signature  
414 reflecting immune cell activation may allow the discrimination of rejecting patients<sup>38</sup>, representing  
415 an attractive choice, to be validated in a dedicated study.

416

417 Altogether, we were able to identify a signature of the pre-transplant cardiovascular asset and graft  
418 regenerative ability that might predict the post-transplant graft performance. The molecular signature  
419 was obtained by combination of fluorescence levels of single EV antigens using advanced  
420 computational algorithms. Supervised learning was applied to train and validate the prediction  
421 models, exploiting high-dimensional and non-linear boundaries among data obtained from EV  
422 profiling, allowing an accurate prediction of renal outcome. Accuracy at validation was 98.3% and  
423 80.1% respectively for serum- and urine- EV markers, outperforming previously reported  
424 conventional biomarkers<sup>5,13</sup>.

425

426 The main limitation of our study is the absence of an external validation cohort. Anyway, the  
427 longitudinal design and the use of ML algorithms allowed a robust internal validation, demonstrating  
428 the dynamic consistent change of EV biomarkers over patient follow-up, and a high generalizability  
429 of the proposed models due to the negligible overfitting effect. Second, our experimental approach  
430 including beads-based immunocapture and flow-cytometry does not allow the evaluation of single  
431 vesicles, while the use of pre-clearing steps by low-medium speed centrifugation excludes larger EVs  
432 from the analysis. A third limitation is the absence of kidney specific antigens among EV markers  
433 included in the analysis; on the contrary, we chose to use a validated and high-performing platform  
434 which included the majority of surface markers expressed on vesicles, and we focused on the specific  
435 EV signature, as reflex of the cardiovascular profile of the recipient and of the repairing/regenerative  
436 capability of the graft.

437 In conclusion, we systematically characterized serum- and urine- EVs from a highly selected  
438 longitudinal cohort of patients underwent kidney transplant. We developed the first prediction model  
439 based on the profile of antigens expressed on EV surface; our model was able to predict renal outcome  
440 at 1 year follow-up using EV parameters before or immediately after kidney transplant. EV profiling  
441 has been performed by a standardized, low-cost, flow cytometric platform. This approach is  
442 minimally invasive, amenable to full automation, and represent a promising point-of-care testing tool.  
443 After validation in larger studies, EV profiling could be integrated in the post-transplant clinical  
444 work-up, selecting patients at higher risk of persistent renal dysfunction for a closer follow-up.

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446

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449

450 **Data Availability Statement:** Data that support findings of the present study are available on  
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453

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456

457 **Authors contributions:** All authors contributed extensively to the work presented in this manuscript.  
458 J.B., and L.B., and B.B. designed the study. C.P.C., G.C. and V.C. recruited patients and collected  
459 clinical information and blood samples. J.B., S.M., S.B., and C.G., performed EV isolation and  
460 characterization. J.B., and A.B. performed statistics and diagnostic modelling. J.B., S.M., L.B., and  
461 B.B. wrote the manuscript with inputs from all authors. G.O., M.B., and G.L.M. interpreted data and  
462 critically revised the manuscript.



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552 **Table 1. Baseline characteristics of patients**

Variable	Overall cohort [n=58]	Renal Recovery [n=35]	Persistent renal dysfunction [n=23]	P-Value
<b>Donor parameters</b>				
Age (years)	54 ± 18.1	50 ± 17.5	59 ± 18.1	0.071
Male sex, n (%)	26 (44.8)	17 (48.6)	9 (39.1)	0.479
Hypertension, n (%)	15 (25.9)	8 (22.9)	7 (30.4)	0.519
Diabetes, n (%)	5 (8.6)	3 (8.6)	2 (8.7)	1.000
Deceased donor, n (%)	45 (77.6)	29 (82.9)	16 (69.6)	0.235
Cause of death				
Cerebrovascular, n (%)	31 (68.9)	19 (65.5)	12 (75.0)	0.738
Trauma, n (%)	14 (31.1)	10 (34.5)	4 (25.0)	
eGFR* (mL/min)	98 ± 25.5	98 ± 26.1	97 ± 25.1	0.910
<b>Receiver parameters</b>				
Age at transplant (years)	49 ± 13.5	48 ± 13.6	51 ± 13.4	0.358
Male sex, n (%)	38 (65.5)	25 (71.4)	13 (56.5)	0.243
Hypertension, n (%)	41 (70.7)	24 (68.6)	17 (73.9)	0.662
Diabetes, n (%)	2 (3.4)	0 (0.0)	2 (8.7)	0.153
Months on dialysis prior to transplant	43 [24; 60]	45 [21; 59]	41 [27; 72]	0.956
Peritoneal dialysis, n (%)	19 (32.8)	13 (37.1)	6 (26.1)	0.380
Hemodialysis, n (%)	44 (75.9)	26 (74.3)	18 (78.3)	0.729
<b>Cause of kidney insufficiency</b>				
Unknow, n (%)	19 (32.8)	12 (34.3)	7 (30.5)	
APDKD, n (%)	16 (27.6)	11 (31.4)	5 (21.7)	
Glomerular disease, n (%)	11 (19.0)	8 (22.9)	3 (13.0)	0.198
Diabetes, n (%)	2 (3.4)	0 (0.0)	2 (8.7)	
Vascular, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	
Other*, n (%)	10 (17.2)	4 (11.4)	6 (26.1)	
<b>Transplant and treatment</b>				
HLA mismatches (n)	3 [3; 4]	3 [3; 4]	3 [3; 4]	0.870
Cold ischemia (hours)	11.0 [6.8; 14.0]	11.0 [8.0; 14.0]	9.0 [3.0; 15.0]	0.463
Thymoglobulin, n (%)	17 (29.3)	11 (31.4)	6 (26.1)	0.662
Basiliximab, n (%)	41 (70.7)	24 (68.6)	17 (73.9)	0.662
Steroid, n (%)	58 (100.0)	35 (100.0)	23 (100.0)	1.000
FK-506, n (%)	58 (100.0)	35 (100.0)	23 (100.0)	1.000
Ciclosporin, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	1.000
Mycophenolic acid, n (%)	57 (98.3)	34 (97.1)	23 (100.0)	1.000
M-Tor inhibitor, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	1.000
<b>Kidney function/damage at Baseline</b>				
Creatinine (mg/dL)	8.5 ± 3.06	8.7 ± 2.78	8.2 ± 3.49	0.509
eGFR* (mL/min)	7 ± 2.9	7 ± 2.7	7 ± 3.1	0.580

553

554 Clinical and biochemical characteristics of patients included in the analysis after stratification for  
 555 post-transplant renal outcome at baseline (T0; before kidney transplant): renal recovery (n=35) vs.  
 556 persistent renal dysfunction (n=23; eGFR ≤ 45 mL/min at T3). APDKD, autosomal dominant  
 557 polycystic kidney disease. A p<0.05 was considered significant and shown in bold. \*eGFR:  
 558 glomerular filtration rate was estimated by CKD-EPI equation. \*\*Other includes autoimmune  
 559 diseases, pyelonephritis, and hemolytic-uremic syndrome.

560 **Legends to Figures**

561

562 **Figure 1. Study design and protocol**

563 We analyzed serum and urine at different time points in patients who underwent kidney transplant.  
564 (A) A cohort of 58 patients was included in the study, and evaluated at baseline (before transplant,  
565 T0), 10-14 days after transplant (T1), and at 3 months (T2), or 12 months after transplant (T3).  
566 Patients were discriminated according to creatinine levels at T3 (eGFR  $\leq$  45 mL/min, persistent renal  
567 dysfunction, vs. eGFR  $>$  45 mL/min, renal recovery). A total of 232 serum and 194 urine samples  
568 were analyzed (\*urine were not available for 38 anuric patients at T0). (B) Whole blood and urine  
569 samples underwent serial centrifugation cycles to eliminate cells, cellular debris and larger vesicles.  
570 EVs were immuno-captured using fluorescent-labelled beads (different amount of phycoerythrin, PE,  
571 and fluorescein isothiocyanate, FITC) coated with antibodies against 37 EV surface antigens. The  
572 analysis of EV surface antigens was performed by flow cytometry after incubation with detection  
573 antibodies against CD9, CD63, and CD81, labeled with allophycocyanin (APC). Gating strategy is  
574 described in the extended methods section; representative plots are reported for one serum (above)  
575 and one urine sample (below).

576

577 **Figure 2. Prediction of renal recovery by serum EV surface antigens**

578 Serum extracellular vesicle (EV)- surface antigens were evaluated by flow cytometry in transplanted  
579 patients at different time points (T0, before transplant; T1, 10-14 days after transplant; T2, 3 months  
580 after transplant; T3, 12 months after transplants; left column); median fluorescence intensity (nMFI;  
581 %) was reported after normalization for mean MFI for CD9, CD63 and CD81. The correlation of  
582 each EV antigen with glomerular filtration rate (eGFR; mL/min) was evaluated by Pearson's R test  
583 (central column); regression lines with 95% confidence intervals were shown for each correlation. In  
584 the right column, mean nMFI (with standard error) is shown at the different time points in patients  
585 displaying renal recovery (green line) or persistent renal dysfunction at T3 (red line; eGFR  $\leq$  45  
586 mL/min). \* $p < 0.05$ ; \*\* $p < 0.01$  \*\*\* $p < 0.001$ ; statistics is reported in Tables S5, and S7 to S10. We  
587 reported EV surface antigens associated to renal outcome at univariate logistic regression analysis  
588 (Table S11): CD62P (A), CD41b (B), CD42a (C), and CD31 (D).

589

590 **Figure 3. Prediction of renal recovery by urine EV surface antigens**

591 Urine extracellular vesicle (EV)- surface antigens were evaluated by flow cytometry in transplanted  
592 patients at different time points (T0, before transplant; T1, 10-14 days after transplant; T2, 3 months  
593 after transplant; T3, 12 months after transplants; left column); median fluorescence intensity (MFI;

594 %) was reported after normalization for mean MFI for CD9, CD63 and CD81. The correlation of  
595 each EV antigen with glomerular filtration rate (eGFR; mL/min) was evaluated by Pearson's R test  
596 (central column); regression lines with 95% confidence intervals were shown for each correlation. In  
597 the right column, mean MFI (with standard error) is shown at the different time points for each EV  
598 antigen in patients displaying renal recovery (green line) or persistent renal dysfunction at T3 (red  
599 line; eGFR  $\leq$  45 mL/min). \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; statistics is reported in Tables S6, and  
600 S12 to S15. We reported EV surface antigens associated to renal outcome at univariate logistic  
601 regression analysis (Table S11): CD105 (A), CD1c (B), SSEA-4 (C), and CD133/1 (D).

602

#### 603 **Figure 4. Supervised learning to predict renal recovery using serum EV markers**

604 Supervised learning was used to train and validate a prediction model able to discriminate patients  
605 with renal recovery (n=35) from those with persistent renal dysfunction (Glomerular Filtration Rate,  
606 eGFR  $\leq$  45 mL/min; n=23). Normalized median fluorescence intensity (nMFI) of serum extracellular  
607 vesicle (EV) surface antigens at T0 was used to derive the prediction models. (A) The association of  
608 differentially expressed serum EV antigens with renal outcome was assessed by univariate regression  
609 analysis. Odds ratios (ORs) are reported for each EV antigen together with its 95% confidence  
610 interval; an OR greater than 1 is associated with an increased likelihood of renal recovery; an OR less  
611 than 1 is associated with a decreased likelihood (significant associations were highlighted in red). (B)  
612 Analysis of receiver operating characteristic (ROC) curves for EV surface antigens associated with  
613 renal outcome at univariate analysis. Diagnostic performance was assessed also for a compound EV  
614 marker derived by linear combination of all the others (black line) (C-D) Machine learning algorithms  
615 were used to train and validate 308 different diagnostic models based on serum EV markers.  
616 Confusion matrix and a representative tree are shown for the best model at training and validation: a  
617 random forest regressor with SMOTE correction for dataset imbalance, 10 classification trees and a  
618 maximum split number of 20. Validation is provided by leave-one-out algorithm (see extended  
619 methods). Statistics is reported in Tables S11, S17, and S18.

620

#### 621 **Figure 5. Supervised learning to predict renal recovery using urine EV markers**

622 Supervised learning was used to train and validate a prediction model able to discriminate patients  
623 with renal recovery (n=35) from those with persistent renal dysfunction (Glomerular Filtration Rate,  
624 eGFR  $\leq$  45 mL/min; n=23). Normalized median fluorescence intensity (nMFI) of urine extracellular  
625 vesicle (EV) surface antigens at T1 was used to derive the prediction models. (A) The association of  
626 differentially expressed urine EV antigens with renal outcome was assessed by univariate regression  
627 analysis. Odds ratios (ORs) are reported for each EV antigen together with its 95% confidence

628 interval; an OR greater than 1 is associated with an increased likelihood of renal recovery; an OR less  
629 than 1 is associated with a decreased likelihood (significant associations were highlighted in red). **(B)**  
630 Analysis of receiver operating characteristic (ROC) curves for EV surface antigens associated with  
631 renal outcome at univariate analysis. Diagnostic performance was assessed also for a compound EV  
632 marker derived by linear combination of all the others (black line) **(C-D)** Machine learning algorithms  
633 were used to train and validate 308 different diagnostic models based on urine EV markers. Confusion  
634 matrix and a representative plot are shown for the best model at training and validation: a support  
635 vector machine with linear kernel. Validation is provided by leave-one-out algorithm (see extended  
636 methods). The plot illustrates discriminant performance of 2 of the 11 differentially expressed EV  
637 antigens: if a circle of a defined color (real outcome) falls within a graph area of the same color  
638 (predicted outcome), then the patient is correctly predicted according to its outcome. Statistics is  
639 reported in Tables S11, S16, and S17.