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ABSTRACT

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

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

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

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

What is already known about this subject

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

What this study adds

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

What impact this may have on practice or policy

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

Keywords

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

Introduction

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

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

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

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

Materials and Methods

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

Patient recruitment and sampling strategy

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

EV characterization

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

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

Statistics and diagnostic modelling

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

Results

Patient characteristics

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

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

Quantitative evaluation of serum- and urine- EVs

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

Comparing serum and urine samples, the number of serum EVs was higher than urine EVs (2.4e12 *vs.* 5.6e9/mL; $p<0.001$), whereas particle diameter was similar (183 *vs.* 181 nm; Figure S1A-D; Table S2), independently from the renal outcome and the evaluated time point. Of interest, the number of serum EVs, but not urine EVs, significantly correlated to the corresponding creatinine level (Figure S1H-L). EVs concentration was re-evaluated after stratification for time points (Table S4). Serum EV number per mL significantly decreased after kidney transplant, while a similar but not significant trend was observed for urine EVs (Figure S2).

Characterization of serum- and urine- EV surface antigens

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

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

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

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

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

EV signature of kidney graft dysfunction

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

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

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

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

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

Prediction of renal recovery after kidney transplantation

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

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

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

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

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

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

EV signature of graft rejection

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

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

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

Discussion

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

both receiver (serum) and graft (urine). In particular, serum EV signature was mainly characterized by endothelial cells and platelets markers, probably reflecting the cardiovascular profile of the recipient. Conversely, urine EV signature was mainly characterized by markers of mesenchymal progenitor cells, which may mirror the repairing/ regenerative features of the graft.

EVs and their content have been extensively studied in the context of kidney transplant. Different EV subpopulations in biological fluids, deriving from different cell types and characterized on the basis of EV surface marker expression, have been previously profiled using conventional cytofluorimetric-based analyses[14,19]. However, this technique implies several limitations in terms of detection threshold (exclusive characterization of larger EVs, so called microparticles), possible identification of multiple vesicles as a single event, and non-specific nanoparticle detection of protein/antibody aggregates. Alternatively, bead-based cytofluorimetric assays have been used to characterize bead-absorbed isolated EVs for single markers[26]. This procedure, however, requires EV isolation, and appears time-consuming and poorly standardized. In our study, we were able to analyze serum- and urine- EVs using a commercially available cytofluorimetric kit[21,22], which allow the fast and reproducible profiling of a standardized panel of 37 EV surface antigens including markers from endothelium, platelets, immune cells, and mesenchymal/stem progenitor cells. According to a previously validated protocol[11], we directly characterize EVs after immuno-capture without other pre-isolation steps. Of note, we did not perform any vesicle pre-enrichment steps, in the effort to implement and standardize an assay, which was developed for an application on isolated EVs[21,22], to be directly applied as point-of-care tool for EV analysis in complex biofluids. This approach has further relevance, as it can be achieved avoiding time-consuming protocols and without sophisticated instrumentation, and therefore it could be easily translated to clinical practice.

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

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

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

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

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

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

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

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

Conflict of interest: nothing to declare.

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Data Availability Statement: Data that support findings of the present study are available on reasonable request from the corresponding author. Data are not publicly available due to privacy and ethical restrictions.

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Authors contributions: All authors contributed extensively to the work presented in this manuscript. J.B., and L.B., and B.B. designed the study. C.P.C., G.C. and V.C. recruited patients and collected clinical information and blood samples. J.B., S.M., S.B., and C.G., performed EV isolation and characterization. J.B., and A.B. performed statistics and diagnostic modelling. J.B., S.M., L.B., and B.B. wrote the manuscript with inputs from all authors. G.O., M.B., and G.L.M. interpreted data and 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
Donor parameters				
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
Receiver parameters				
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
Cause of kidney insufficiency				
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)	
Transplant and treatment				
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
Kidney function/damage at Baseline				
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.

Legends to Figures

Figure 1. Study design and protocol

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

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

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

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

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

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

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

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

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

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

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