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**Urine, beyond the waste: a source for diagnostic and therapeutic
applications in the Extracellular Vesicles field**

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Abstract

Urine production by the kidneys allows the human body to eliminate waste products from blood, while maintaining the homeostasis of ions, water, and pH. Several types of cells can be found in urine, including kidney-derived cells and epithelial cells. Urine contains also genetic material, proteins and extracellular vesicles (EVs). Due to the easy collection, its rich content of biological compounds and noninvasive nature, urine has various applications in medical research. In the present work, I will describe two different projects, both involving the use of urine samples as platform in the renal regeneration field.

In the first project we investigated urinary EVs (uEVs) as source for renal disease biomarkers, focusing on improving the technical aspects of EV analysis using cytofluorimetric platforms. As non-invasive approach that could provide accurate early detection of kidney injury, uEVs represents a promising source for novel renal disease biomarkers. Herein, we aimed at identifying a clinically translatable method to detect surface biomarkers on uEVs. For this purpose, we compared two different flow cytometry (FC) bead-based assays, performed directly on small volume of pre-cleared urine samples. The samples were collected by UPO biobank (Novara, Italy) from a cohort of acute kidney injury (AKI) patients and from a cohort of kidney transplanted (KT) patients. We compared markers expression on uEV surfaces using a commercial kit, MACSPlex, and a novel FC assay, which exploits beads bound to a membrane sensing peptide (FC-MSP-beads assay). MSPs recognize and bind to the specific curvature of small EVs (< 200 nm), allowing a capture not depending on marker expression. MACSPlex analysis revealed a downregulation of CD133-1 and CD24, renal progenitor markers, and an upregulation of CD326, CD29, SSEA-4 and HLA-DR in AKI patients compared with non-AKI controls. More interestingly, SSEA-4 resulted upregulated in AKI patients which showed an improved renal function during the follow-up. This marker, which also correlates with the renal recovery, appeared to be a potential prognostic biomarker to monitor AKI-to-chronic kidney disease (CKD)-transition. With FC-MSP-beads assay the downregulation of CD24 in AKI patients was confirmed, while SSEA-4 resulted to be almost undetectable, underling the need of further technical optimization. Concerning the cohort of transplanted patients, MACSPlex analysis revealed several inflammatory markers to be expressed in KT patients' uEVs, as compared with healthy subjects. KT patients were subdivided in two groups, considering their eGFR one year after the urine analysis (1-year eGFR), and using 45 ml/min as cut-off. CD69 resulted to be the only marker to be differentially

expressed in the two groups, being higher in the group of KT patients with a 1-year eGFR higher than 45 ml/min. Interestingly, this data was confirmed with FC-MSP-beads.

The second project proposes the use of stem cells-derived EVs as pro-regenerative treatment in renal disease and was published in the “Journal of translational medicine” in August 2024. We tested the effect of neonatal kidney progenitor cells (nKPC)-derived EVs on patients-derived podocytes. In this project urine played a central role too. In fact, nKPC were isolated from the urine of preterm neonate and subsequently immortalized. Moreover, the podocytes were isolated from the urine of four patients with characterized genetic mutations affecting proteins of the glomerular filtration barrier. With an *in vitro* permeability assay, we demonstrated that nKPC-EV treatment improved the permselectivity of patient-derived podocytes. In addition, commonly used drugs for treating renal diseases were also tested on podocytes, which however appeared unresponsive, except for one line, in alignment with the patient's clinical response at 48 months. Afterwards, through RNA sequencing, we identified two genes, small ubiquitin-related modifier 1 (SUMO1) and Sentrin-specific protease 2 (SEN2), both involved in the SUMOylation pathway, to be upregulated after EV treatment in all four podocyte cell lines. Moreover, SUMO1 and SEN2 downregulation in healthy podocytes, obtained with siRNA transfection, increased podocyte permeability, confirming the involvement of the pathway. Herein, we demonstrated the positive effect of nKPC-EVs in improving the function of genetically altered podocytes and identified the effect of EVs on the regulation of SUMOylation pathway.

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List of abbreviations

AKI - Acute Kidney Injury
APC - Allophycocyanin
AS - Alport Syndrome
AS-POD - Alport syndrome podocytes
BRISQ - Biospecimen Reporting for Improved Study Quality
BSA - Bovine serum albumin
CD - Cluster of Differentiation
CD2AP - Cluster of Differentiation 2-Associated Protein
CKD - Chronic Kidney Disease
CTL-tPOD - Control tissue Podocytes
CTL-uPOD - Control urine Podocytes
CYCLO - Cyclosporin
dUC - Differential Ultracentrifugation
eGFR - Estimated Glomerular Filtration Rate
EpCAM - Epithelial Cell Adhesion Molecule
ESRD - End-Stage Renal Disease
EV - Extracellular Vesicle
FC - Flow Cytometry
FC-MSP-beads assay – Flow Cytometry Membrane Sensing Peptide bead-based assay
FCS - Foetal Calf Serum
FDA - Food and Drug Administration
FITC - Fluorescein isothiocyanate
GBM - Glomerular Basement Membrane
GFB - Glomerular Filtration Barrier
HLA-DR - Human Leukocyte Antigen - DR isotype
IGFBP7 - Insulin-Like Growth Factor-Binding Protein 7
ISO - International Organization for Standardization
KDIGO - Kidney Disease: Improving Global Outcomes
KT – Kidney Transplant
LP - Lipofectamine
MFI - Median Fluorescence Intensity
mIgG1 - Mouse Immunoglobulin G1
MPB - MACSPlex Buffer
M-PR - Methylprednisolone
MSCs - Mesenchymal Stromal Cells
MSP – Membrane Sensing Peptide
NGAL - Neutrophil Gelatinase–Associated Lipocalin
nKPCs - Neonatal Kidney Progenitor Cells

nMFI - Normalized Median Fluorescence Intensity
NS – Nephrotic Syndrome
NS-POD - Nephrotic Syndrome Podocytes
NTA - Nanoparticle Tracking Analysis
PBS - Phosphate Buffered Saline
PE - Phycoerythrin
REA - Recombinant Epitope Antibody
RITUX - Rituximab
RT-PCR - Real-time PCR
RPC - Renal Progenitor Cells
sCR – Serum Creatinine
SCR - Scramble sequence
SEN2 - Sentrin-specific Protease 2,
SSEA4 - Stage-Specific Embryonic Antigen-4
SUMO1 - Small Ubiquitin-related Modifier 1
TAC - Tacrolimus
TEM - Transmission Electron Microscopy
uEV - Urinary Extracellular Vesicle

Chapter 1: Introduction

Urine in medical research

Urine is produced by the kidneys to allow the human body to eliminate waste products from blood, while maintaining the homeostasis of ions, water, and pH (Decramer *et al.*, 2008). Urine is primarily composed of water (about 95%), and contains nitrogenous waste (urea, creatinine, uric acid), electrolytes, genetic material, hormones, proteins, and extracellular vesicles (Pisitkun, Shen, and Knepper 2004). Moreover, several types of cells can be found in the urine, including kidney-derived cells, epithelial cells and blood cells (Chen *et al.*, 2020). Finally, urine can also contain bacteria and viruses (Santiago-Rodriguez *et al.*, 2015). Currently, urine analysis is a noninvasive diagnostic tool used to assess either kidney function, metabolic disorders (eg. diabetes) or urinary tract infections (Wei and Teece, 2006; Subcommittee on Urinary Tract Infection, Steering Committee on Quality Improvement and Management and Roberts, 2011). However, thanks to its ease of collection, abundance of biological compounds, and noninvasive nature, urine has gained interest in medical research for other numerous possible applications.

Indeed, as mentioned, in the diagnostic field, urine is frequently used for the identification of biomarkers, especially for disease affecting the kidneys, the bladder and the prostate (Gámez-Valero *et al.*, 2015; Togneri *et al.*, 2016; Rzhavskiy *et al.*, 2019). Biomarkers can consist mainly in proteins, metabolites, or extracellular vesicles (EVs) (Erdbrügger *et al.*, 2021). EVs consist of lipid nanovesicles secreted by cells and containing various types of molecules, carrying precious information about the physiopathological state of the analyzed subject. EVs within urine are called “urinary EVs” (uEVs) and have been shown to derive primarily from cells of the urogenital tract. For their properties, uEVs are gaining interest as source for renal disease biomarkers (Erdbrügger *et al.*, 2021).

Urine has also been studied as potential source of stem cells for regenerative medicine. Indeed, among cells present in the urine, researchers had identified a population of adult stem cells, called “urine-derived stem cells (USCs)”, with strong proliferation ability, capable of self-renewal and multidirectional differentiation (Zhang *et al.*, 2008). Moreover, another population of progenitor cells, isolated from preterm neonatal urine, has been reported to express embryonic kidney transcription factors and to protect kidney cells from hypoxic injury in a model of kidney graft perfusion (Arcolino *et al.*, 2016). Interestingly, neonatal kidney

progenitor cells, shown to act in a paracrine way, may act through pro-regenerative EVs secretion (Arcolino *et al.*, 2016).

In addition to stem cells, in some cases urine can represent a non-invasive source of podocytes, glomerular cells which can detach and be shed into the urine. Indeed, while in healthy non-pregnant subjects urine podocytes are limited in number and viability, in certain physiopathological conditions, such as nephrotic syndrome or pregnancy, is possible to isolate and grow them in culture (Ni, Saleem and Mathieson, 2012; Oliveira Arcolino *et al.*, 2015). Indeed, patient-derived urine podocytes represents an interesting tool for personalized medicine (Oliveira Arcolino *et al.*, 2015).

Extracellular vesicles classification and biogenesis

Extracellular vesicles (EVs) are lipid-enclosed nanoparticles secreted by all cell types and playing an important role in intercellular communication. In fact, EVs transport proteins, nucleic acids and lipids, which can be transferred to target cells or can activate signaling cascades through receptor-ligand interactions without being internalized (Blijdorp *et al.*, 2022; Liu *et al.*, 2022). EVs can be classified into three types based on their biogenesis: exosomes, microvesicles, and apoptotic bodies (van Niel, D'Angelo and Raposo, 2018). Exosomes, generally ranging from 30 to 150 nm in diameter, are formed within the endosomal system (Figure 1). This involves the invagination of the plasma membrane, resulting in the creation of early-sorting endosomes containing a combination of cell-surface proteins and extracellular components. Subsequently, these endosomes mature into late-sorting endosomes, which then generate multivesicular bodies containing intraluminal vesicles, which ultimately become exosomes (van Niel, D'Angelo and Raposo, 2018; Kalluri and LeBleu, 2020). At variance, microvesicles, ranging from 100 to 1000 nm, are formed from outward budding of the plasma membrane. Apoptotic bodies, the largest vesicles, ranging from 500 to 2000 nm, arise during cell death and contain cellular debris and nuclear fragments (Théry *et al.*, 2018). However, the term “extracellular vesicle” commonly refers to the first two subtypes.

For many years, these terms were erroneously employed interchangeably to denote EVs isolated solely based on their size. Indeed, contemporary techniques for EV isolation often result in co-isolation regardless of their cellular origin, primarily guided by size considerations (Welsh *et al.*, 2024). Therefore, the most recent ISEV guidelines discourage the use of the term “exosomes” or “microvesicles”, unless their subcellular origin has been clearly proven. At

variance, a definition based on the size is preferable. Indeed, generally “small-EVs” in literature refer to EVs with a diameter <200 nm, while “large-EVs” with diameter >200 nm. However, there is still not strict consensus on the size cut-offs (Welsh *et al.*, 2024). EVs share a remarkable ability to transport diverse molecular cargo, facilitating cellular communication. These cargos, including proteins, lipids, mRNAs, and microRNAs, mirrors the state of the originating cells, making them powerful non-invasive biomarkers for diagnosing diseases like cancer, neurodegenerative disorders, and kidney disease (Théry *et al.*, 2018; van Niel, D’Angelo and Raposo, 2018; Kalluri and LeBleu, 2020). As our understanding of EVs grows, new subtypes like mitovesicles and oncosomes reveal their diverse functions, further expanding their diagnostic potential (Théry *et al.*, 2018). Notably, surface markers CD9, CD63, and CD81 (commonly called “tetraspanins”) are important for the identification of EVs, playing a key role in their biogenesis and function (Théry *et al.*, 2018).

EVs can be isolated using several techniques, each with its own advantages and limitations. Common methods include differential centrifugation, ultrafiltration, immunoaffinity capture, and size-exclusion chromatography (Théry *et al.*, 2018). However, the lack of standardized protocols for uEV separation and quantification challenges quality and complicates assay development. Standardizing these methods is crucial for enhancing accuracy, reliability, and comparability in uEV research (Erdbrügger *et al.*, 2021).

Chapter 2: uEV as source of renal disease biomarkers

Overview

The incidence of kidney disease is on the rise, presenting a substantial healthcare challenge on a global scale (Lozano *et al.*, 2012). Various kidney diseases, such as acute kidney injury (AKI), nephrotic syndrome, and diabetic nephropathy, frequently progress to end-stage renal disease (ESRD), often accompanied by numerous complications (Chawla and Kimmel, 2012). Currently, there is no effective treatment for ESRD, with renal replacement therapy being the primary option. In clinical practice, commonly used markers of kidney disease such as serum creatinine, estimated glomerular filtration rate (eGFR), blood urea, proteinuria, and albuminuria often fail to reflect early functional changes in the kidney and do not predict the occurrence of renal disease (Masaoutis *et al.*, 2020). Consequently, kidney biopsy still represents the gold standard for the diagnosis of renal disease, despite its invasiveness nature (Schnuelle, 2023). Thus, there is a pressing need to identify novel biomarkers, either alone or in combination with conventional ones, to overcome these limitations, better define kidney pathophysiology, and enable personalized therapeutic approaches.

In this perspective, a promising alternative to conventional tissue biopsy is represented by liquid biopsy. The latter consists in a technique which allows clinicians to screen body fluids such as urine, blood or saliva, searching for biomarkers reflecting the specific disease state (Kilgour *et al.*, 2020; Nikanjam, Kato and Kurzrock, 2022). Due to their non-invasive or minimally invasive nature, the use of liquid biopsy can reduce the risks associated with tissue biopsy collection and allow for serial sampling throughout disease progression (Rodríguez *et al.*, 2017).

Extracellular vesicles (EVs), which are nano-sized bilayer particles secreted by nearly all cell types, represent a promising avenue for liquid biopsy. Acknowledged as crucial mediators of intercellular communication across diverse physiological and pathological scenarios, EVs play a pivotal role in cell-to-cell signaling (Quesenberry *et al.*, 2015). In fact, EVs transport proteins, nucleic acids and lipids, which can be transferred to target cells or can activate signaling cascades through receptor-ligand interactions without being internalized (Blijdorp *et al.*, 2022; Liu *et al.*, 2022). Cells secrete an heterogeneous population of EVs into the extracellular milieu, which are, consequently, abundantly found in various body fluids (Wiklander *et al.*, 2019; Erdbrügger *et al.*, 2021). Studying EVs in biofluid can provide precious information for biomarker discovery, since both their cells of origin and their cargo change depending on the

physiopathological condition of the subject (Mathieu *et al.*, 2019). Moreover, molecules transported from EVs are shielded by enzymatic degradation, which represents a further advantage in the biomarker research field (Heidarzadeh *et al.*, 2023).

Among body fluids, urine contains a significant amount of EVs, both in physiological and pathological conditions, which can mirror alterations in specific cellular compartments (Gonzales *et al.*, 2009). Due to the glomerular architecture, circulating EVs normally cannot cross the Glomerular Filtration Barrier (GFB) in physiological conditions (Petrakka *et al.*, 2002; Longmire, Choyke and Kobayashi, 2008). Consequently, urinary EVs primarily originate from epithelial and parenchymal cells facing the urinary space (Borges, Reis and Schor, 2013). Hence, they have been proposed as promising non-invasive source for liquid biopsies for various kidney diseases (Abbasian *et al.*, 2018). In this project, we focused on uEVs as source of prognostic biomarkers for Acute kidney injury and renal transplantation failure.

Acute Kidney Injury (AKI) and Chronic Kidney Disease (CKD)

Acute kidney injury (AKI), affecting about 15% of hospitalized patients, is characterized by a rapid decline in kidney function, often within hours to days, triggered by factors such as ischemia, nephrotoxins, sepsis, or infections (Ronco, Bellomo and Kellum, 2019). Clinically, it is marked by a sharp rise in serum creatinine (sCr) or reduced urine output, reflecting decreased glomerular filtration rate (GFR) (Chawla and Kimmel, 2012). AKI is common among high-risk groups associated to hospitalization, like those undergoing major surgeries, exposed to nephrotoxic drugs, or experiencing critical illnesses (Hsu *et al.*, 2013). AKI can be classified into three types: pre-renal due to decreased kidney perfusion, intrinsic, resulting from direct damage to kidney tubular cells and post-renal, occurring from urinary tract obstructions (Makris and Spanou, 2016). Moreover, the KDIGO (Kidney Disease: Improving Global Outcomes) classification system categorizes AKI in three stages based on sCr levels and urine output. Notably, AKI is recognized as a risk factor for CKD. Indeed, maladaptive repair processes after AKI can trigger chronic inflammation and progressive fibrosis, resulting in gradual loss of kidney function and CKD progression (Chawla *et al.*, 2014). CKD is defined by an eGFR below 60 mL/min for over three months and can progress to end-stage renal disease (ESRD), requiring dialysis or transplantation. The early, often asymptomatic stages of CKD can lead to significant kidney damage before diagnosis (Levey *et al.*, 2005; Coresh *et al.*, 2007) (Table 1). Risk factors

for AKI and its progression to CKD include advanced age, diabetes, hypertension, cardiovascular diseases, and genetic predispositions (Sawhney *et al.*, 2017).

	AKI	CKD
Duration	≤7 days	>3 months
Functional criteria	Increased creatinine level (>0.3 mg/dl within 48 hours; Increased creatinine level > 1.5 times baseline within the prior 7 days; Urine output <0.5 ml/kg/h for more than 6 hours	eGFR < 60mL/min for more than 3 months
Structural criteria	Not defined	Elevated marker of kidney damage (albuminuria, eGFR and creatinine)
Outcomes	Potentially reversible if treated early; can lead to CKD if injury persists	Progressive, often irreversible; may lead to end-stage renal disease (ESRD)
Pathophysiology	Inflammation, oxidative stress, and cellular damage.	Chronic inflammation, interstitial fibrosis, and microvascular injury.
Diagnostic Methods	Serum creatinine levels, eGFR, urine output	Serum creatinine, eGFR, kidney biopsy (advanced stages)
Complications	- Electrolyte imbalances - Fluid retention - Acute systemic effects	- End-stage renal disease (ESRD) - Cardiovascular complications - Renal replacement therapy
Therapy	Prompt intervention (hydration, treating cause, dialysis in severe cases)	Managing underlying conditions (e.g., hypertension, diabetes), renal replacement in ESRD

Table 1. Comparison of AKI and CKD. Features of AKI and CKD, including duration, functional criteria, structural criteria, long-term outcomes, pathophysiology, diagnostic methods, complications, and therapy are reported.

Kidney transplantation (KT)

When CKD arrive to the fourth stage, before reaching end stage renal disease, usually patients start a replacement therapy, which consists in two main options: dialysis and transplantation (Andrews, 2002). While dialysis is an artificial replacement of the excretory kidney function, transplantation is the natural replacement of the whole kidney function, including excretory and incretory functions. Transplantation presents various challenges: patients must face surgery, need to assume immunosuppressive drugs, which have many side effects, and risk organ

rejection. However, transplanted patients can have a better quality of life compared to dialysis patients, which are completely dependent on health care unit and feel less healthy due to lack of erythropoietin synthesis. Moreover, the mortality is higher in case of dialysis. Kidney transplantation (KT) is an allograft, so it's made with the organ from an individual of the same species, but with different genetic heritage, and it's non-life-saving, since there are alternatives such as dialysis. Moreover, KT is heterotopic, meaning that the new kidney is not located in the same place of the native organ, but in the iliac fossa, and the native kidney usually is not removed. As mentioned, to avoid rejection and to prolong graft function, immunosuppressive therapy is needed (Szumilas *et al.*, 2023). However, kidney graft can stop functioning effectively leading to graft failure, which can be caused by either immunological or non-immunological factors. Immunological causes include mainly acute or chronic rejection, while examples of non-immunological causes are recurrence of the original disease, ischemia reperfusion injury, delayed graft function, drug toxicity and infections (Andrews, 2002; Lubetzky *et al.*, 2021; Anfaiha-Sanchez *et al.*, 2023).

Limitations of current diagnostic methods for kidney disease

Current diagnostic markers for kidney disease, including serum creatinine, eGFR, and microalbuminuria, have notable limitations (Inker *et al.*, 2012; Masaoutis *et al.*, 2020). Serum creatinine, a byproduct of muscle metabolism filtered by the kidneys, is used to determine eGFR, which is calculated also based on the subject's sex, age and weight (Masaoutis *et al.*, 2020). However, serum creatinine levels rise only after substantial kidney damage has occurred, which can impair early detection of kidney impairment (Inker *et al.*, 2012; Masaoutis *et al.*, 2020). Additionally, its levels are influenced by factors like muscle mass and hydration, which can lead to inaccurate eGFR calculations (Inker *et al.*, 2012). Another common renal disease parameter is albuminuria, which consists in the amount of albumin in the urine (Inker *et al.*, 2012). Albumin, a small molecule produced by the liver, filtered by glomeruli and reabsorbed by proximal tubular cells, is excreted in higher than normal amounts (> 30 mg/L) in urine when kidney function is impaired (Heerspink and Gansevoort, 2015). However, albuminuria is not always a reliable predictor of kidney disease, as impairment can occur without elevated albumin levels (Inker *et al.*, 2012). Lastly, while kidney biopsy remains the gold standard for diagnosing renal pathology, its invasive nature and associated risks limit its routinary use. Given these

limitations, there is an urgent need for more sensitive, non-invasive methods that provide accurate early detection of kidney injury and disease progression (Schnuelle, 2023).

EVs for liquid biopsy in kidney disease

To overcome the limitations of traditional kidney biopsies, liquid biopsy has emerged as a promising non-invasive diagnostic tool for renal pathologies. By analyzing biomarkers in body fluids such as blood, serum, saliva, and urine, liquid biopsy offers a less intrusive alternative to tissue extraction. This method enables repeated and safe collection of samples, making it valuable for monitoring disease progression and treatment response, enhancing kidney disease management (Kilgour *et al.*, 2020; Nikanjam, Kato and Kurzrock, 2022). EVs are crucial in liquid biopsy for their role in intercellular communication and involvement in various physiological and pathological processes. In particular, EVs within urine (urinary EVs), due to their origin and stable biomolecular content, are gaining interest as source for urogenital disease biomarkers. Indeed, changes in uEV cargo may precede traditional renal function parameters like eGFR, highlighting their potential as early indicators of renal disease (Erdbrügger *et al.*, 2021).

Urinary extracellular vesicles – origins

EVs within urine, called “urinary extracellular vesicles” (uEVs), originate from various cells in the urogenital tract, including glomerular, tubular, prostate, and bladder cells (Øverbye *et al.*, 2015; Erdbrügger *et al.*, 2021). Indeed, researchers utilized omics analyses to explore the origin and composition of uEVs, demonstrating that most proteins are characteristic of urogenital cells (Pisitkun, Shen and Knepper, 2004). Although renal cells are the predominant source, a small portion of uEV proteins may originate from kidney-infiltrating cells or other sources, reflecting their complex nature (Blijdorp *et al.*, 2022). However, *in vitro* studies, such as those conducted by Barreiro *et al.* reinforce the kidney as the primary source of uEVs, detailing contributions from specific kidney areas (Barreiro *et al.*, 2023). In fact, uEV content includes a range of proteins deriving from specific nephron segments, providing information in their origin, aiding in the identification of the affected areas (Pisitkun, Shen and Knepper, 2004; Erdbrügger *et al.*, 2023). For instance, aquaporins (AQP1 and AQP2) serve as markers for the collecting ducts and proximal tubules, respectively, while cotransporters and sodium-hydrogen exchangers

(NHE3) marked ion transport in the proximal tubules (Pisitkun, Shen and Knepper, 2004; Tonneijck *et al.*, 2019). Additionally, cubilin and megalin are linked to endocytosis in these tubules (Hogan *et al.*, 2014; Nielsen, Christensen and Birn, 2016; Adam *et al.*, 2020). Concerning the glomeruli, nephrin and podocin are specific of the podocytes, while podocalyxin marks glomerular endothelial cells (Hogan *et al.*, 2014). uEVs from Henle's loop cells express the type 2 Na-K-2Cl cotransporter (NKCC2) and uromodulin (Pisitkun, Shen and Knepper, 2004; Erdbrügger *et al.*, 2023). Markers such as CD133, SSEA4, and CD24, associated with stem cell properties and regeneration, are also present on uEVs (Dimuccio *et al.*, 2014; Burrello *et al.*, 2023). Renal progenitor cell (RPC) marker CD133, typically higher in healthy individuals, resulted downregulated in uEVs from renal disease patients with delayed graft function (Dimuccio *et al.*, 2014). Additionally, uEVs facilitate glomerular-tubular communication by transferring biomolecules like microRNAs between nephron segments, thus actively participating in renal pathophysiology (Svenningsen, Sabaratnam and Jensen, 2020).

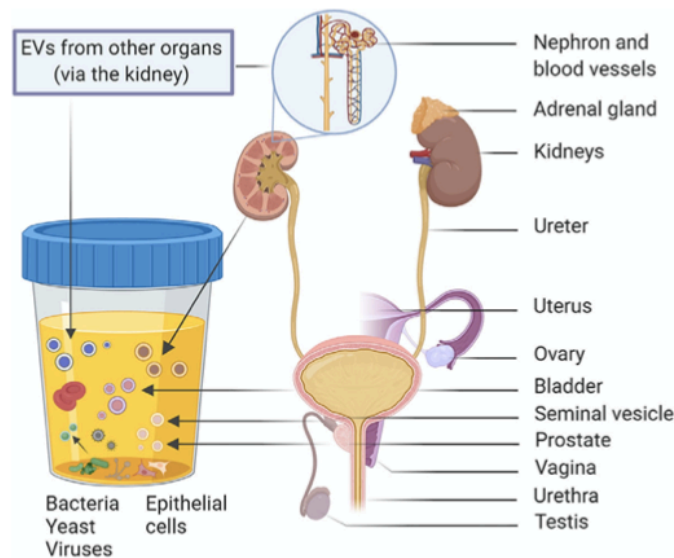


Figure 1. Representative image of uEV origins (Erdbrügger *et al* 2021)

Challenges in urinary extracellular vesicle biomarker discovery

For urine biomarker discovery, adopting a proper normalization process is crucial, considering the highly variability of uEV concentration, and the lack of standardized methods across different laboratories (Erdbrügger *et al.*, 2021). The two main categories of normalization approaches consist in the calculation of a relative or an absolute excretion rate. Erdbrügger *et al.* described in detail the various normalization methods used in uEV field. Briefly, relative

excretion rate defines the abundance of the uEV marker in relation to another marker, while absolute excretion rate describes the rate in which a uEV marker is excreted. Concerning the high variability, it must be considered that uEV content can be affected by various factors also at physiological conditions, such as diet and exercise, underscoring the importance of standardized protocols in clinical settings to ensure reliable results (Grange *et al.*, 2023). Indeed, when working with uEVs, the methods for urine collection, processing, and storage are crucial, as they can influence data quality and reproducibility (Erdbrügger *et al.*, 2021). While frameworks such as the Biospecimen Reporting for Improved Study Quality (BRISQ) and ISO 20387:2018 standards provide guidelines for urine biobanking, maintaining consistency in handling and processing is essential for obtaining accurate results (Erdbrügger *et al.*, 2021; *Biospecimen reporting for improved study quality (BRISQ) - PubMed*, no date; *ISO 20387:2018 - Biotechnology - Biobanking - General requirements for biobanking*, no date). Despite existing guidelines, studies often use different protocols for urine collection and storage due to specific research designs or logistical constraints, leading to inconsistencies in uEV analysis. To overcome this issue, adopting standard procedures and reporting pre-analytical details in the EV-TRACK knowledgebase, following ISEV guidelines for biofluid sample handling, is essential (Théry *et al.*, 2018; Erdbrügger *et al.*, 2021).

Another main challenge in using EVs for clinical diagnostics is the detection of surface markers due to their small size (30-200 nanometers). Conventional flow cytometry, designed for larger particles, lacks the sensitivity needed to distinguish EV event from background noise (Gul *et al.*, 2022). Therefore, both high sensitivity single-EV flow cytometers and bead-based methods have emerged. The latter could be more adaptable for clinical translation, being more straightforward. Among these, a well-known commercial kit, using 39 antibody-coated beads, analyzes 37 surface markers at once, allowing analysis directly from a small volume of biofluid samples, without the need of prior isolation (Wiklander *et al.*, 2018). However, the inability of this technology to customize antibody panels and its high cost limits its use (Wiklander *et al.*, 2018; Gul *et al.*, 2022). In this project we aim at identifying possible alternative bead-based method for uEV surface marker flow cytometry analysis.

Membrane sensing peptides for EV capture

Membrane sensing peptides are peptides which recognize and bind to high curved lipid nanovesicles, enabling the capture of small EVs (Gori *et al.*, 2020). These peptides create

electrostatic and hydrophobic interactions with the lipid components of the EV membrane, such as phosphatidylserine. Its amphipathic structure enables it to approach and insert into lipid-packing defects on highly curved membranes. Once integrated, the peptide folds within the membrane, stabilizing the interaction (Gori *et al.*, 2020). This method effectively distinguished the membrane curvature of small EVs, typically those with a maximum diameter of 200 nm (Gori *et al.*, 2020). Thanks to these properties, MSPs have been designed as EV baits and are under investigation for various applications. Immuno-based capturing, which is typically used for EV capture, introduces bias in the analysis, due to the selection of a particular subpopulation. This capture is mainly based on anti-tetraspanin antibodies; however, recent studies demonstrated a lower tetraspanins prevalence than it was thought to be (Mizenko, no date). In addition, in case of EV mimetics or tetraspanin-lacking EVs (red blood cell-derived EVs), this approach is not functional (Gori *et al.*, 2024). For this reason, targeting the lipid membranes of EVs with MSP results to be a promising alternative. Moreover, its use is quite versatile, and at date has been tested for various application. Indeed, an MSP derived from bradykinin protein, was linked onto chip suitable for single particle interferometric reflectance imaging sensor (SP-IRIS) by the ExoView™ platform and demonstrated a higher binding capacity compared to standard tetraspanin antibodies-capture (Gori *et al.*, 2020). The same peptide was adapted for Single Molecule Array (SiMoA) use, also in this case substituting the immune-capture with an MSP-based capture (Gori *et al.*, 2024). Moreover, when conjugated with specific magnetic beads, it has been shown to allow an efficient isolation of EV from serum (Gori *et al.*, 2024). Recently, the same MSP has been conjugated to specific beads for a bead-based flow cytometry (FC) assay. The protocol was set up on plasma samples of heart transplanted patients, without the previous EV isolation, using a membrane dye as normalizer and a mixture of detection antibodies for the markers of interest (Prof. Lucio Barile group, study under peer-review). However, this FC assay has never been tested on urine samples. Therefore, in this project the protocol has been adapted and tested for uEV surface markers detection.

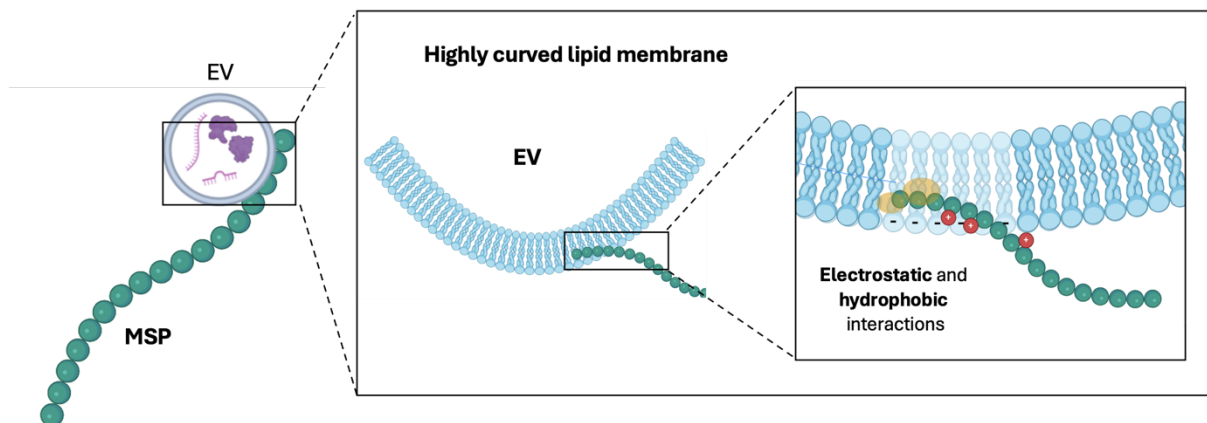


Figure 2. Mechanisms of Membrane Recognition by MSP. The image depicts a molecular design based on a modified bradykinin peptide (RPPGFSPFR). The peptide was designed to interact with phospholipid membranes, specifically leveraging electrostatic and hydrophobic interactions. In the image, negatively charged phospholipids (such as phosphatidylserine) interact with positively charged peptides through electrostatic forces. Additionally, hydrophobic regions within the peptide sequence (yellow spots) further enhance membrane interaction, allowing for the effective capture of small EVs. Created with Biorender.

Aim of the Study

In this project, we aimed at identifying a clinical translatable approach to monitor kidney regeneration process after kidney injury. Due to the limitations of current standard methods for renal disease detection, there is a huge need for novel early diagnostic and prognostic biomarkers which could detect kidney pathological condition to enable timely interventions. In this perspective, urinary EVs represent a promising avenue for liquid biopsy, which consists in analyzing molecules present in biofluid, as non-invasive alternative to tissue biopsy. However, due to technical reasons, studying EV content in an easy and reproducible way is challenging. Herein, we focused on surface markers, and aimed at identifying a flow cytometry bead-based assay which could be in the future used by clinicians for kidney disease screening. Apart from technical aspects, we also intended to identify potential biomarkers to monitor AKI-to-CKD transition and kidney failure during the post-transplantation phase.

Chapter 3: Comparison of two flow cytometry bead-based assays in detecting uEV biomarkers

Manuscript under preparation.

Materials and Methods

AKI and KT Patients and Sample Collection

This study involved one cohort of 49 hospitalized AKI patients (Table 2), one cohort of 58 KT patients (Table 3) and 20 controls, comprising 14 healthy subjects and 6 hospitalized patients without renal pathologies. AKI patients presented multiple comorbidities, such as diabetes, hypertension, cardiovascular disease, and a history of cancer (Table 2). For each AKI patient, key clinical parameters, including serum creatinine levels (sCR) and eGFR, were tracked both at hospital discharge and three months post-discharge. At variance, for KT patients, the clinical parameters were collected both during requested biopsy and after one year. KT patients were classified by clinicians in four groups: functional worsening (group 1, n=25), delayed graft function or incomplete functional recovery (group 2, n=9), no AKI but histological damage (group 3, n=18), no AKI and no histological damage (group 4, n=6). Moreover, the histological analysis highlighted different types of damages in different patients: acute/chronic rejection, nephrotoxicity, sclerotic damage, glomerulonephritis (Table 3).

All urine samples were collected by UPO Biobank (Novara, Italy). The volume of collected urine varied among subjects (1-4 ml). After collection, the samples were centrifuged at 300 x g to remove cellular debris and then stored at -80°C to maintain the stability and integrity of the EVs within the urine. At use, to better eliminate cell debris and larger particles, the urine samples were further pre-cleared by centrifugation at 3,000 × g for 15 minutes and the pellet was discarded.

Table 2. Hospital data and clinical information of AKI patients

Patient N°	SEX	AGE	BMI	DIABETES	CVD	HTN	CKD	TUMOR	SMOKING	DIURETIC	Diagnosis	Sepsi	STAGE	AKI type	RRT	Outcome	3 months outcome	Basal sCr	Discharge sCr	Discharge NGAL	3 months NGAL	BASAL eGFR	Discharge eGFR	3 months eGFR
697	M	54	<30	0	0	0	1	1	0	0	AKI	1	3	parenchymal; post-renal	0	2	0	1.64	1.41	1070		38	56	
699	M	72	<30	0	0	0	0	0	1	0	LPL	0	3	parenchymal; pre-renal	0	1	1	1.00	3.45	<25		61	17	
704	M	70	>30	1	1	1	1	0	1	1	AKI su CKD	0	3	parenchymal	1	2	1	2.47	3.87	187		26	15	
708	F	59	<30	0	0	0	0	0	0	0	diarrhea	0	3	pre-renal	0	1	0	0.59	0.92	2566		100	68	
709	F	60	>30	0	1	0	0	1	0	0	CAN	0	1	pre-renal	0	2	0	0.67	1.01	57		97	60	
713	M	49	<30	0	0	1	0	1	0	1	anemia	1	2	pre-renal	0	0	0	0.7	1.78			113	44	
714	M	80	>30	0	1	0	1	0	1	1	sepsi	1	3	parenchymal	0	0		1.18	3.2	1387		45	17	
715	M	87	<30	0	1	1	1	1	1	1	sepsi	1	1	pre-renal; parenchymal	0	1	3	0.7	0.91	84		58	75	
717	M	81		0	1	1	1	0	1	1	CAN	0	3	pre-renal	0	1	2	1.29	2.33	50	<25	49	25	
722	F	70	>30	1	0	1	0	0	1	0	diarrhea	0	3	pre-renal	0	1	1	0.91	1.13			66	49	
734	M	81	<30	0	0	1	1	0	0	0	pneumonia	0	1	parenchymal	0	1	1	1.82	1.97	2195	740	35	31	
738	M	80	<30	0	1	1	0	0	1	1	sepsi	1	1	pre-renal; parenchymal	0	1	1	1.86	0.7	63	154	34	89	
745	M	88	<30	0	1	1	1	0	1	1	dehydration	0	1	pre-renal	0	2	2	1.35	2.44	252		41	23	
753	F	78	<30	1	1	1	1	0	0	1	MGUS	0	1	parenchymal	0	1	2	0.91	0.73	80		53	79	
758	F	87	<30	1	1	1	1	0	0	1	AKI	0	3	pre-renal	0	1	2	1.23	1.71	235		38	27	
763	F	51	<30	0	1	0	0	0	0	0	ACS	1	2	pre-renal	0	2	3	0.59	0.9	887		107	74	
764	M	82	<30	0	1	1	1	0	1	1	CRS	0	3	pre-renal; parenchymal	0	1	1	/	0.9	176	<25	/	79	47
767	M	69	<30	1	1	1	1	0	1	1	SCA	1	2	pre-renal; parenchymal	0	2	2	1.56	3.6	1326		40	16	
770	M	81	<30	0	0	1	0	1	1	1	LLC	0	3	parenchymal	0	1	1	1.34	1.7	71	<25	64	37	
771	F	47		1	1	1	0	0	0	1	SCC	0	3	pre-renal; parenchymal	1	1	0	0.8	2.5	457		89	22	
774	F	79	<30	1	1	1	1	0	0	1	anemia	0	3	pre-renal	0	1	1	1.66	2.4	412	100	30	19	
775	F	83	>30	1	1	1	1	0	0	1	pneumonia	0	1	parenchymal	0	1	1	1.41	1.24	128		35	40	
780	M	70	<30	1	1	1	1	0	1	1	TBI	1	2	pre-renal; parenchymal	0	2	3	1.3	1.5	1848		54	46	
789	M	79	<30	0	1	1	0	1	0	1	AKI	0	2	pre-renal	0	1	2	0.8	1.17	309	146	78	59	
790	M	60	<30	0	0	1	1	1	0	1	AKI	0	3	parenchymal	1	1	1	1.4	2	4244	<25	54	35	
791	F	80	<30	0	1	1	1	0	0	1	SCC	1	2	parenchymal	0	1	1	1.8	2.2	90		29	21	
792	F	82	<30	0	1	1	0	1	0	1	AFib	0	2	pre-renal	0	1	1	1.61	2.5	129	37	32	17	
794	M	58	>30	1	0	1	1	0	0	1	SCA	0	2	pre-renal	0	1	1	0.73	1.31	283	247	102	56	
796	F	77	>30	0	1	1	1	0	1	0	syncope	0	3	pre-renal	0	1	1	1.06	2	65		48	24	
814	F	84	<30	0	1	1	0	0	0	0	anemia	0	2	pre-renal	0	1	0	1.56	1.4	80		32	34	
816	F	72	<30	0	1	1	0	0	0	0	IVU	1	3	parenchymal	0	1	1	0.65	0.73	421		89	82	
1441	F	71	<30	0	0	1	0	1	0	0	AKI	0	3	pre-renal	0	1	1	0.81	1.29	72	60	74	42	
1454	M	74	<30	0	0	0	0	0	0	0	AKI	1	3	parenchymal	0	1	1	0.9	1.71	80	28	85	39	
1479	M	64	<30	0	1	1	0	0	1	0	AKI	0	3	parenchymal obstructive	0	1	1	0.83	0.9	74	214	93	90	
1508	M	71	<30	1	1	1	1	0	0	1	AKI	0	3	pre-renal	0	1	1	/	1.47	223	114	/	47	44
1532	F	70	<30	0	0	1	0	0	0	1	AKI	1	2	pre-renal; parenchymal	0	1	1	/	0.66		56	/	90	68
1534	M	78	<30	0	0	1	0	0	0	0	necrotizing pancreatitis	1	3	pre-renal; parenchymal	0	0		0.85	2.32		69	29		
1541	F	56	<30	1	1	1	1	0	0	1	AKI	1	3	obstructive	0	1	1		1.62		133		35	22
1543	M	57	<30	0	1	1	0	0	0	0	AKI	0	2	pre-renal	0	1	3		0.84	27			97	
1544	F	65	<30	1	0	0	0	0	0	0	intestinal ischemia	1	3	pre-renal; parenchymal	1	1	1	0.6	0.52		<20	96	101	
1546	F	59	<30	0	0	1	1	1	0	0	AKI on CKD	0	2	pre-renal	0	1	1	1.06	1.35	244	185	58	43	
1548	M	90	<30	0	0	1	1	0	0	0	AKI on CKD	0	2	pre-renal	0	0	0	1.98	2.71	114		29	22	
1549	M	58	<30	0	0	0	0	0	0	0	Polytrauma	1	3	pre-renal; parenchymal	1	0			1.52				54	
1551	F	71	<30	0	0	1	0	0	0	0	AKI	0	3	parenchymal	0	1	1	0.55	0.95	166	62	95	61	
1555	F	70	<30	0	0	0	0	0	0	1	AKI	0	2	pre-renal	0	1	1	0.67	0.79			90	76	
1564	M	62	<30	1	1	0	1	0	1	0	AKI on CKD	1	3	pre-renal	0	1	3	0.8	5.4			96	11	
1565	M	86	<30	1	1	1	1	0	0	1	AKI on CKD	0	1	pre-renal	0	1	2	1.41	1.66			41	35	
1567	F	68	<30	0	1	1	1	0	0	0	AKI	0	3	pre-renal; parenchymal	1	2	3		1.84				28	
1569	F	68	<30	0	0	1	0	1	1	1	AKI	0	3	pre-renal	0	1	1	0.51	1.59			102	33	

Table 2. Hospital data and clinical information of 49 AKI patients. Each patient is listed with their unique ID number (from UPO Biobank), followed by demographic details including sex and age. For each patient, body mass index (BMI) is reported. (BMI < 30 normal weight, BMI > 30 obesity). The table reports comorbidities such as diabetes, cardiovascular diseases (CVD), hypertension (HTN), CKD, cancer history, smoking status, and diuretic use (1 = yes, 0 = no). It also includes the diagnosis at hospital admission, AKI stage (1 = mild, 2 = moderate, 3 = severe), and AKI type (prerenal, post-renal, parenchymal, or mixed). The need for renal replacement therapy (RRT) is marked (1 = yes, 0 = no), along with patient outcomes (0 = deceased, 1 = alive, 2 = readmitted, 3 = lost to follow-up). Outcomes three months post-discharge are also noted (0 = deceased, 1 = discharged, 2 = transferred). Lab data include serum creatinine (sCR) and NGAL levels at baseline, discharge, and three months, as well as eGFR values to track renal function over time. Missing data are due to loss to follow-up, death, or patient transfer. Abbreviations: BMI: body mass index; CVD: cardiovascular diseases, HTN: hypertension; CKD: chronic kidney disease; RRT: renal replacement therapy; sCR: serum creatinine; NGAL: Neutrophil gelatinase-associated lipocalin.

Table 3: Hospital data and clinical information of KT patients

Sample code	AGE	HOSPITAL	SAMPLE DATE	BIOPSY DATE	Months after Tx	BIOPSY REASON	BASAL scr	BASAL eGFR	sCR_Bx	eGFR_Bx	KDIGO stage	1 YEAR scr	1 YEAR eGFR	Tacrolimus	CsA	mTORi	MMF	HISTOLOGY	REJECTION TYPE	GROUP
KT44	39	Novara	23/02/19	22/02/19	3	PROTOCOL	1.80	50	1.88	44		5.03	13	1	0	0	1	Acute rejection/ Chronic rejection	Antibody-mediated	3
KT42	31	Novara	19/04/16	19/04/16	11	FUNCTIONAL WORSENING	0.90		1.22	59	1	1.64	42	1	0	0	1	/	/	1
KT24	76	Novara	06/05/16	06/05/16	24	RAS XXIV MONTH	1.85	35	1.48	43		1.49	47	1	0	0	1	Sclerosis	/	3
KT35_03	71	Novara	13/05/16	16/05/16	12	RAS XII MONTH	1.30	55	1.24	59		1.3	55	1	0	0	1	Sclerosis	/	3
KT54	60	Novara	19/07/16	19/07/16	60	ART LX MONTH	2.70	28	2.93	22		1.43	49	1	0	1	0	Chronic nephrotoxicity/Acute rejection/Chronic rejection	Mixed	3
KT8	36	Novara	16/10/19	16/10/19	6	PROTOCOL	1.46	61	1.50	60		1.73	50	1	0	0	1	Acute rejection	Antibody-mediated	3
KT18_01	38	Novara	19/06/17	20/06/17	13	PROTOCOL	1.60	50	1.63	53		2.35	34	1	0	0	1	GN recurrence	/	3
KT18_02	42	Novara	12/01/21	12/01/21	56	FUNCTIONAL WORSENING	1.70	34	2.53	30	1	1.87	44	1	0	0	1	Sclerosis/Acute rejection	Cell-mediated	1
KT33	42	Novara	01/02/19	31/01/19	155	FUNCTIONAL WORSENING	1.85	44	2.10	39	1	4.6	10	1	0	0	1	Sclerosis/Chronic rejection	Antibody-mediated	1
KT45_01	55	Novara	09/05/16	11/05/16	12	FUNCTIONAL WORSENING	0.90	69	1.80	30	2	1.87	30	1	0	0	1	Sclerosis/Chronic nephrotoxicity	/	1
KT45_02	55	Novara	07/06/16	08/06/16	13	RAS XXIV MONTH	1.80	30	1.79	30		2.08	26	1	0	0	1	Sclerosis	/	3
KT2	22	Novara	09/09/20	08/09/20	8	INCOMPLETE FUNCTIONAL RECOVERY	1.90	50	1.9	50		1.55	62	1	0	0	1	Acute rejection	Cell-mediated	2
KT13	56	Novara	26/11/19	26/11/19	7	FUNCTIONAL WORSENING	1.37	50	2.00	35	1	2.35	30	1	0	0	1	/	/	1
KT37	58	Novara	09/05/16	09/05/16	58	FUNCTIONAL WORSENING	1.30		1.60	45	1	1.9	30	1	0	0	1	Sclerosis /Chronic nephrotoxicity/Acute rejection	Antibody-mediated	1
KT36	63	Novara	26/06/17	27/06/17	61	ART LX MONTH	1.30		1.50	49		1.55		1	0	0	1	Chronic nephrotoxicity	/	3
KT31	22	Novara	26/03/18	23/03/18	4	INCOMPLETE FUNCTIONAL RECOVERY	1.60	60	1.60	60		1.46	66	1	0	0	1	Sclerosis/Papillary adenomas	/	2
KT34	72	Novara	10/04/19	08/04/19	25	FUNCTIONAL WORSENING	2.70	25	3.67	16	1	3.3	18	1	0	1	0	/	/	1
KT9	51	Novara	11/11/19	11/11/19	1	FUNCTIONAL WORSENING	1.31	63	4.78	13	3	0.97	90	1	0	0	0	Acute rejection	Mixed	1
KT4	75	Novara	10/04/19	08/04/19	3	PROTOCOL	1.80	35	1.8	35		1.87	35	1	0	0	1	Sclerosis	/	3
KT22	51	Novara	XXX	02/10/20	17	FUNCTIONAL WORSENING	1.50		2.16	34	1	1.56	50	1	0	0	1	/	/	1
KT16	48	Novara	XXX	02/10/20	17	INCOMPLETE FUNCTIONAL RECOVERY	2.20	37	2.20	37		4.33	15	1	0	0	1	Acute nephrotoxicity	/	2
KT21	30	Novara	17/09/20	17/09/20	65	FUNCTIONAL WORSENING	1.40	66	1.7	53	1	1.73		1	0	0	1	/	/	1
KT23	54	Novara	13/02/19	11/02/19	1	PROTOCOL	1.70	47	1.90	38		1.8		1	0	0	1	/	/	4
KT46	39	Novara	27/01/16	29/01/16	24	RAS XXIV MONTH	1.40		1.06	62		1.99	40	1	0	0	1	/	/	4
KT47	52	Novara	05/03/18	06/03/18	6	PROTOCOL	1.20	53	1.00	65		1.11	57	1	0	0	1	Acute rejection	Antibody-mediated	3
KT6	38	Novara	26/11/19	26/11/19	2	FUNCTIONAL WORSENING	1.60	41	2.45	28	1	1.57	41	1	0	0	1	Sclerosis	/	1
KT43	48	Novara	27/04/16	02/05/16	7	FUNCTIONAL WORSENING	2.50		2.93	23	1	2.70	30	1	0	0	0	Sclerosis	/	1
KT3	63	Novara	23/05/19	23/05/19	120	FUNCTIONAL WORSENING	1.50	26	2.00	26	1			1	0	0	1	Sclerosis/Acute nephrotoxicity /Chronic nephrotoxicity /Chronic rejection	Antibody-mediated	1
KT19	22	Novara	08/02/19	06/02/19	3	INCOMPLETE FUNCTIONAL RECOVERY	1.50	62	2.00	42		2.17	42	1	0	0	1	Acute nephrotoxicity	/	2

Table 3: Cont.

KT25	39	Novara	03/04/19	02/04/19	6	PROTOCOL	1.50	61	1.50	61	1.79	46	1	0	0	1	/	/	4
KT17	45	Novara	05/05/20	05/05/20	8	FUNCTIONAL WORSENING	1.70	45	2.50	30	1		1	0	0	1	Sclerosis/Chronic rejection	Antibody-mediated	1
KT26	60	Novara	13/09/16	11/09/18	207	FUNCTIONAL WORSENING	1.70		2.80	24	1	2.50	1	0	0	0	Sclerosis/Chronic rejection	Antibody-mediated	1
KT52	53	Novara	20/06/17	12/06/17	2	INCOMPLETE FUNCTIONAL RECOVERY	3.50		3.30	17		3.8	17	1	0	1	Sclerosis	/	2
KT11_02	71	Novara	22/10/19	22/10/19	9	PROTOCOL	2.20	23	2.01	24		1.82	27	1	0	1	Acute rejection	Antibody-mediated	3
KT11_01		Novara	21/03/19																3
KT38_01	21	Novara	09/05/16	10/05/16	25	PROTOCOL	2.20		2.40	28		3.3	23	1	0	1	Sclerosis/Acute rejection	Borderline	3
KT38_02	23	Novara	04/04/18	04/04/18	47	FUNCTIONAL WORSENING	3.00	23	3.50	19	1	5.51	10	1	0	1	Sclerosis/Acute rejection	Cell-mediated	1
KT1	52	Novara		31/07/17	4	FUNCTIONAL WORSENING	1.68	44	2.60	27	1	2.00		1	0	1	Sclerosis/Acute nephrotoxicity	/	1
KT39	41	Novara	30/07/17	02/03/17	3	FUNCTIONAL WORSENING	2.20		4.31	16	3	5.00	13	1	0	1	Acute rejection	Mixed?	1
KT29	48	Novara	30/01/20	30/01/20	104	FUNCTIONAL WORSENING	2.00		2.40	35	1	2.93	24	1	0	1	GN recurrence/Acute rejection	Antibody-mediated	1
KT55	42	Novara	03/04/17	03/04/17	5	FUNCTIONAL WORSENING	1.70	47	2.30	34	1	1.6		1	0	1	Sclerosis	/	1
KT40	71	Novara	11/09/15	21/09/15	0	DGF	6.20		9.14			10.27	5				Sclerosis	/	2
KT32	43	Novara	07/07/16	07/07/16	3	PROTOCOL	0.78	85	0.86	83		0.8	90	1	0	1	/	/	4
KT41	63	Novara	15/02/17	16/05/17	7	PROTOCOL	1.80	30	1.78	30		2.35	21	1	0	0	Sclerosis	/	3
KT30	42	Novara	21/03/17	10/07/17	9	FUNCTIONAL WORSENING	1.10	59	2.13	26	2	1.64	37	1	0	1	Acute nephrotoxicity/Acute rejection	Antibody-mediated	1
KT5_02	50	Novara	20/06/17	22/06/17	8	PROTOCOL	1.50	43	1.37	45		1.18	53	1	0	1	Sclerosis/Acute rejection/Chronic rejection	Antibody-mediated	3
KT5_03	52	Novara	24/06/18	25/06/18	21	PROTOCOL	1.30	45	1.18	53		1.3		1	0	1	Chronic rejection	Antibody-mediated	3
KT10	66	Novara	18/10/19	15/10/19	16	FUNCTIONAL WORSENING	1.15	50	1.47	50	1	1.4	50	1	0	1	De novo GN/Acute rejection	Antibody-mediated	1
KT15	56	Novara	01/12/20	01/12/20	147	FUNCTIONAL WORSENING	2.00		2.50	32	1	2.5		1	0	1	Sclerosis/Chronic nephrotoxicity/Chronic rejection	Antibody-mediated	1
KT50	49	Novara	04/05/17	05/05/17	72	PROTOCOL	1.00		1.26	67		1.56	52	1	0	1	Sclerosis/Acute rejection/Chronic rejection	Antibody-mediated	3
KT49	61	Novara	17/05/16	19/05/16	12	RAS XII MONTH	1.60	46	1.60	46		1.5	84	1	0	1	/	/	4
KT14	43	Novara	02/05/18	02/05/18	2	INCOMPLETE FUNCTIONAL RECOVERY	1.30		1.69	46		1.76	46	1	0	1	GN recurrence	/	2
KT27	54	Novara	25/02/19	25/02/19	0	DGF	1.70		2.14	25		0.7		1	0	1	Acute nephrotoxicity	/	2
KT56	45	Novara	07/02/18	06/02/18	0	FUNCTIONAL WORSENING	1.60		3.40	32	2	1.8		1	0	1	Acute rejection	Cell-mediated	1
KT48	36	Novara	04/05/18	04/05/18	2	FUNCTIONAL WORSENING	3.00	24	3.50	21	1	4.27	16	1	0	1	/	/	1
KT12	19	Torino	09/09/20	08/09/20	65	FUNCTIONAL WORSENING	1.70		2.81	32	1	4.63	16	0	1	1	Acute rejection	Antibody-mediated	1
KT51	32	Novara	20/09/17	20/09/17	11	PROTOCOL	1.30	70	1.45	63		1.17	82	1	0	1	GN recurrence	/	3
KT20	55	Novara	27/02/19	26/02/19	3	PROTOCOL	1.15	71	1.20	70		1.2		1	0	1	/	/	4
KT7	40	Novara	01/02/19	31/01/19	0	PROTOCOL	1.00	78	1.00	78		0.86	101	1	0	1	Acute rejection	Cell-mediated	3
KT28	70	Novara	02/12/20	02/12/20	2	INCOMPLETE FUNCTIONAL RECOVERY	2.41	27	2.64	24		2.53	25	1	0	1	/	/	2

Table 3. Hospital data and clinical information of 58 KT patients. Each patient is listed with their code (from UPO Biobank), followed by age, the hospital where they had their kidney transplantation, the urine sample collection date, the number of months passed after their kidney transplantation. Information about their biopsy is reported, comprising the date, the reason, and the histology results. Lab data include serum creatinine (sCR), at basal level, during biopsy (Bx), and one year later (1-year sCR), as well as correspondent eGFR values (basal eGFR, eGFR_Bx, 1-year eGFR), to track renal function over time. Missing data are due to loss to follow-up, death, or patient transfer. Abbreviations: Tx: transplant; sCR: serum creatinine; eGFR: estimated glomerular filtration rate; CsA: Cyclosporine; mTORi: mTOR inhibitors; MMF: Mycophenolate mofetil.

uEV isolation by ultracentrifugation

First morning urine samples from 3 healthy volunteers were pooled and subjected to centrifugation at $3,000 \times g$ for 15 minutes to remove cell debris and larger particles. Following this pre-clearing step, the supernatant was transferred to ultracentrifuge tubes and subjected to ultracentrifugation at $100,000 \times g$ (38,000 rpm) for 2 hours using an OPTIMA L-100 K Ultracentrifuge (Beckman Coulter, Brea, CA, USA) with a Type 70.1 Ti fixed-angle titanium rotor and 26.3 mL polycarbonate centrifuge bottles. This high-speed ultracentrifugation was performed at 4 °C to ensure the integrity and proper isolation of the EVs. After ultracentrifugation, the supernatant was carefully removed, and the pellet containing the EVs was resuspended in serum-free RPMI medium supplemented with 1% dimethyl sulfoxide (DMSO) to preserve EV integrity. Parallely, to rule out any buffer effects associated with MSP-bead uEV isolation, EVs were resuspended in 0.5 M $MgCl_2$. The re-suspended EVs were then aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Similarly, urine samples from 16 AKI patients (patient codes: 697, 704, 709, 714, 715, 738, 758, 763, 764, 780, 792, 814, 974, 1543, 1548 and 1564) and from 11 controls (patient codes: 417, 418, 423, 700, 705, 961, 979, 997, 818, 836, 1571) were pre-cleared and pooled, obtaining respectively an AKI urine pool and a control urine pool of 12.5 mL each. EVs were then purified by a 2 h-ultracentrifugation at $100,000 \times g$ (28,000 rpm) at 4 °C (Beckman Coulter, Brea, CA, USA), with a SW 40 Ti Swinging-Bucket Rotor and 13.2 mL open-top tubes. The pellets were resuspended in serum-free RPMI 1% DMSO and used fresh for super-resolution analysis.

Membrane-sensing peptides (MSP)-Beads for uEV isolation

Beads conjugated with a modified bradykinin peptide (RPPGFSPFR), from now on generally termed as “membrane sensing peptide” (MSP), were kindly provided by Dr. Marina Cretich (CNR, Milan). The MSP was synthesized using solid-phase peptide synthesis and purified by high-performance liquid chromatography (HPLC). Afterwards, it was conjugated to agarose magnetic beads, adapted for maximizing uEV capture. 100 µl of the provided suspension with isolation agarose MSP-beads were transferred into a tube and placed on a magnetic support (Thermo fisher) to remove the supernatant. Subsequently, 10 mL of urine, previously pre-cleared, was added to the beads. The mixture was vortexed to ensure proper mixing and then placed on a rotor at 450 rpm for 1 hour at room temperature to allow uEV capture. After the incubation, the tube was placed on the magnetic support to remove the supernatant. The beads were then subjected to three consecutive washes with filtered PBS. For each wash, 50 µL of PBS was added, the mixture was vortexed and placed on the magnetic support to remove the PBS. To release the captured EVs from the beads, 100 µL of PBS 0.5 M MgCl₂ was added. The tube was vortexed and then placed on a RotoFlex at 450 rpm for 15 minutes. Following this incubation, the tube was placed on the magnetic support, and the supernatant containing the released EVs was carefully collected and used fresh.

EV enrichment using ultrafiltration

Ultrafiltration of urine samples from healthy volunteers was performed using the AU-15 filter system (Ultracel-PL PLTK, 30 kDa, UFC903024) to concentrate EVs. Pre-cleared urine was transferred into the AU-15 filter device, and the unit was securely sealed. To concentrate, the urine was centrifuged at 4,000 x g for 30 minutes, resulting in a final volume of approximately 250 µL, reflecting a 50-fold concentration. After centrifugation, the filter device was carefully removed from the centrifuge, and the collection tube was emptied. The remaining sample was collected from the base of the filter device, yielding a highly concentrated protein and EV preparation, ready for further analysis.

MACSPlex exosome kit analysis

Pre-cleared urine samples (250 µL each) were analyzed using MACSPlex Exosome Kit (human, Miltenyi Biotec, CA, USA 130-108-813) according to the manufacturer’s instructions.

Briefly, 15 μL of MACSPlex Exosome Capture Beads were added. These beads contained 39 distinct antibody-coated bead subsets targeting various markers: CD3, CD4, CD19, CD8, HLA-DR, CD56, CD105, CD2, CD1c, CD25, CD49e, ROR1, CD209, CD9, SSEA-4, HLA-ABC, CD63, CD40, CD62P, CD11c, CD81, MCSP, CD146, CD41b, CD42a, CD24, CD86, CD44, CD326, CD133-1, CD29, CD69, CD142, CD45, CD31, REA control, CD20, CD14, and mIgG1 control. Afterwards, the samples were incubated overnight (14–16 hours) on an orbital shaker at 450 rpm at +4 °C, protected from light. Following incubation, the beads were washed by adding 500 μL of MACSPlex Buffer (MPB), followed by centrifugation at $3,000 \times g$ for 5 minutes and removal of 500 μL of supernatant. For counterstaining, 5 μL each of APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies (provided in the kit) were added to the samples. The samples were then incubated on an orbital shaker at 450 rpm, protected from light, for 1 hour at room temperature. After incubation, the beads were washed with 500 μL of MPB, centrifuged at $3,000 \times g$ for 5 minutes, and the supernatant was removed. This wash step was repeated with 500 μL of MPB, followed by a 15-minute incubation on an orbital shaker at 450 rpm, protected from light, at room temperature. After the final incubation, the samples were centrifuged at $3,000 \times g$ for 5 minutes, leaving approximately 200 μL of sample in each tube. The samples were then analyzed using flow cytometry with FACS Celesta (BD Biosciences, Franklin Lakes, NJ, USA). Samples were analyzed by the instrument, resulting in approximately 5000 events being recorded for each sample. MACSPlex Exosome Setup Beads were used to set up the flow cytometer: measurements were confined to capture beads by setting a trigger for side scatter (SSC) and forward scatter (FSC); single beads were gated to exclude doublets and non-bead events. FITC and PE voltage were adapted to optimize the discrimination of the 37 bead subsets; single bead subsets were each gated to allow the measurement of the APC median fluorescence signal intensity. APC median fluorescence intensity (MFI) was corrected by subtracting the respective value of blank control, and the respective isotype controls (control REA or mIgG1 control), normalized by the average MFI of all detectable markers, and expressed as normalized MFI (nMFI).

Nanoparticle Tracking Analysis

Following isolation, the concentration of uEVs in each sample was measured using Nanoparticle tracking analysis (NTA) instrument called Nanosight NS300 (Malvern Instruments Ltd., Malvern, UK), equipped with a 488 nm laser module. This system utilizes Brownian motion and refractive index to determine particle concentration and size. Nanosight

detects particles ranging from 10 nm to 1000 nm, with optimal detection in the 70–300 nm size range. It tracks particle motion by measuring the scattered light at a 90° angle to the laser beam, which enables accurate observation of the Brownian motion of extracellular vesicles (EVs). Isolated uEVs were diluted 1:100 in physiological solution. For each sample, three 30-second videos were recorded at camera level 15 and a threshold of 5, using a syringe pump. The particle size and concentration were analyzed using NTA 3.2 Analytical Software, with the NTA settings kept constant across all samples to ensure consistency. For particle number measurement of pre-cleared urine samples, another NTA instrument was used, Zeta View (Particle Metrix, Inning am Ammersee, Germany), its functioning is similar to Nanosight, but more adaptable for biofluid samples, which are less pure than EV preparations. In this case samples were diluted 1:50 in physiological solution.

MSP-Beads conjugation for flow cytometry analysis

HisPur Ni-NTA magnetic beads (Thermo Fisher) were conjugated with the MSP, following the protocol provided by Dr. Marina Cretich group. Briefly, 0.5 mg of beads (40 µl) were placed in a 2 mL-microcentrifuge tube and 160 µl of equilibration buffer (600 mM NaCl, 100 NaH₂PO₄, 30 mM Imidazole, 0.05 % Tween, PH 8) were added. After vortexing 10 seconds, the tube was placed on a magnetic separator (Thermo Fisher) and supernatant was discarded. 400 µl of equilibration buffer were added, vortexed for 10 seconds and the supernatant was discarded. Then, the lyophilized peptide extract (8 mg) was resuspended in 270 µl of PBS, and subsequently 200 µl of the resulting solution was diluted with an equal volume of equilibration buffer. Then, 400 µl of the prepared protein extract were added to the beads, vortexed 10 seconds and mixed on an end-over-end rotator for 30 minutes at room temperature. Magnetic separation was performed, and the supernatant was discarded. Afterwards, two washing steps were performed, adding 400 µl of equilibration buffer, vortexing, and separating magnetically. Finally, beads (FC-MSP-beads) were resuspended in 400 µl of PBS Sodium azide 0.05% and used freshly or stored at 4 °C. Any conjugation reaction was used for no more than 45 consecutive days.

Flow cytometry MSP-beads assay

For each sample, 3 μ l of FC-MSP-beads were added to 1×10^8 particles (estimated by NTA) of pre-cleared sample, using PBS to reach 200 μ l as final volume. After gentle vortexing for few seconds, samples were incubated for 2 hours over 900 rpm shaking to allow the capture of small EVs to the beads. Afterwards, 500 μ l of PBS were added to wash, then a magnetic separator (Thermo fisher) was used to separate beads from the supernatant, which was discarded. Beads were then diluted in 160 μ l of PBS and split in three conditions: addition of a mixture of fluorophore-conjugated antibodies (final concentration of each antibody 0.5 μ g/ml), the mixture of the correspondent isotope controls antibodies (final concentration of each antibody 0.5 μ g/ml) and the addition of the membrane dye MemGlow 700 (final concentration 50 nM, Cytoskeleton, Inc.). The mixtures of antibodies were previously prepared diluting the antibodies in PBS 5% BSA, 1 mg/ml human IgG, while MemGlow was diluted in PBS. For AKI cohorts, the following panel of antibodies was used: Brilliant Violet 785 anti-human CD63 (Biolegend, San Diego, U.S.; 353043), Brilliant Violet 421 anti-human CD24 (Biolegend, 311121), FITC anti-human Klotho (LS Bio, LS-B6625), PE anti-human CD133-1 (Miltenyi Biotec, Bergisch Gladbach, Germany; 130-080-801), Alexa Fluor 647 anti-human SSEA-4 (Biolegend, 330407). For the KT cohort, the following antibody panel was used: Brilliant Violet 605 anti-human CD63 (Biolegend, 352048) Brilliant violet 421 anti-human CD24 (Biolegend, 311121), PE anti-human CD133-1 (Miltenyi, 130-080-801), FITC anti-human CD142 (Miltenyi, 130-115-683), Brilliant Violet 785 anti-human CD69 (Biolegend, 310931). The correspondent Isotype controls were used for each antibody, and the value of MFI obtained was subtracted, as representative of the aspecific signal antibody-beads. After 1 h of incubation (staining), 500 μ l of PBS were added to wash. After magnetic separation, beads were resuspended in 200 μ l of PBS and samples were acquired by flow cytometry (A5 FACSymphony, BD).

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on EVs placed on 200-mesh nickel formvar carbon-coated grids (Electron Microscopy Science) for 20 min to promote adhesion. The grids were then incubated with 2.5% glutaraldehyde plus 2% sucrose. EVs were negatively stained with NanoVan (Nanoprobes, Yaphank, NY, USA) and observed using a Jeol JEM 1400 Flash electron microscope (Jeol, Tokyo, Japan).

Super-Resolution Microscopy

Super-resolution microscopy acquisition of uEVs were obtained using Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging, Oxford, UK) equipped with a 100x, 1.4NA oil immersion objective, an XYZ closed-loop piezo 736 stage, and triple emission channels split at 640, 488 and 555 nm. EV profiler Kit (ONI) was used to perform the experiments following manufacturer's protocol. The Kit contains fluorescent antibodies anti CD9-488 and CD81-647 and the imaging buffer. Moreover, antibody anti CD133-1-PE (Miltenyi, 130-113-670) and CD24-FITC (Miltenyi, 130-112-655) were used. Images were taken in dSTORM mode acquired sequentially in total reflection fluorescence (TIRF) mode. Single-molecule data was filtered using NimOS software (v.1.18.3, ONI). Data has been processed with the Collaborative Discovery (CODI) online analysis platform www.alto.codi.bio from ONI and the drift correction pipeline version 0.2.3 was used.

Statistical analyses and data generation

Data are shown as mean $\pm$ SD. Statistical analysis was carried out with Graph Pad Prism 7.4 (GraphPad Software, Inc., San Diego, CA, USA), by using multiple t-test or Pearson's correlation test, where appropriate. A p value < 0.05 was considered significant. Significant differences were denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Samples with undetectable fluorescent signals were excluded from the analysis. Infographics were created using BioRender (<https://www.biorender.com>).

Results

MACSPlex analysis of AKI patients' urine samples

MACSPlex kit was employed to characterize surface markers of EVs present in urine samples. The study included 49 samples from hospitalized patients with varying stages of AKI, ranging from stage 1 to stage 3, and a control group consisting of 6 non-AKI hospitalized patients and 14 healthy volunteers. MACSPlex analysis revealed both shared and distinct expression patterns between AKI patients (n=49) and controls (n=20) (Figure 3A). Indeed, while tetraspanins (CD9, CD81, and CD63) nMFI remained consistent across all patients (Figure 3A), other markers displayed relevant differences. Notably, CD133-1 and CD24, typical uEV markers, were significantly reduced in the uEVs from AKI patients (Figure 3A). In contrast, the expression of CD326 (EpCAM) an epithelial marker, CD29 (integrin β 1), involved in cell adhesion and signaling, SSEA-4 (stage-specific embryonic antigen-4), indicative of embryonic stem cell presence, and HLA-DR reflecting immune activation, were more elevated in the AKI group (Figure 3A).

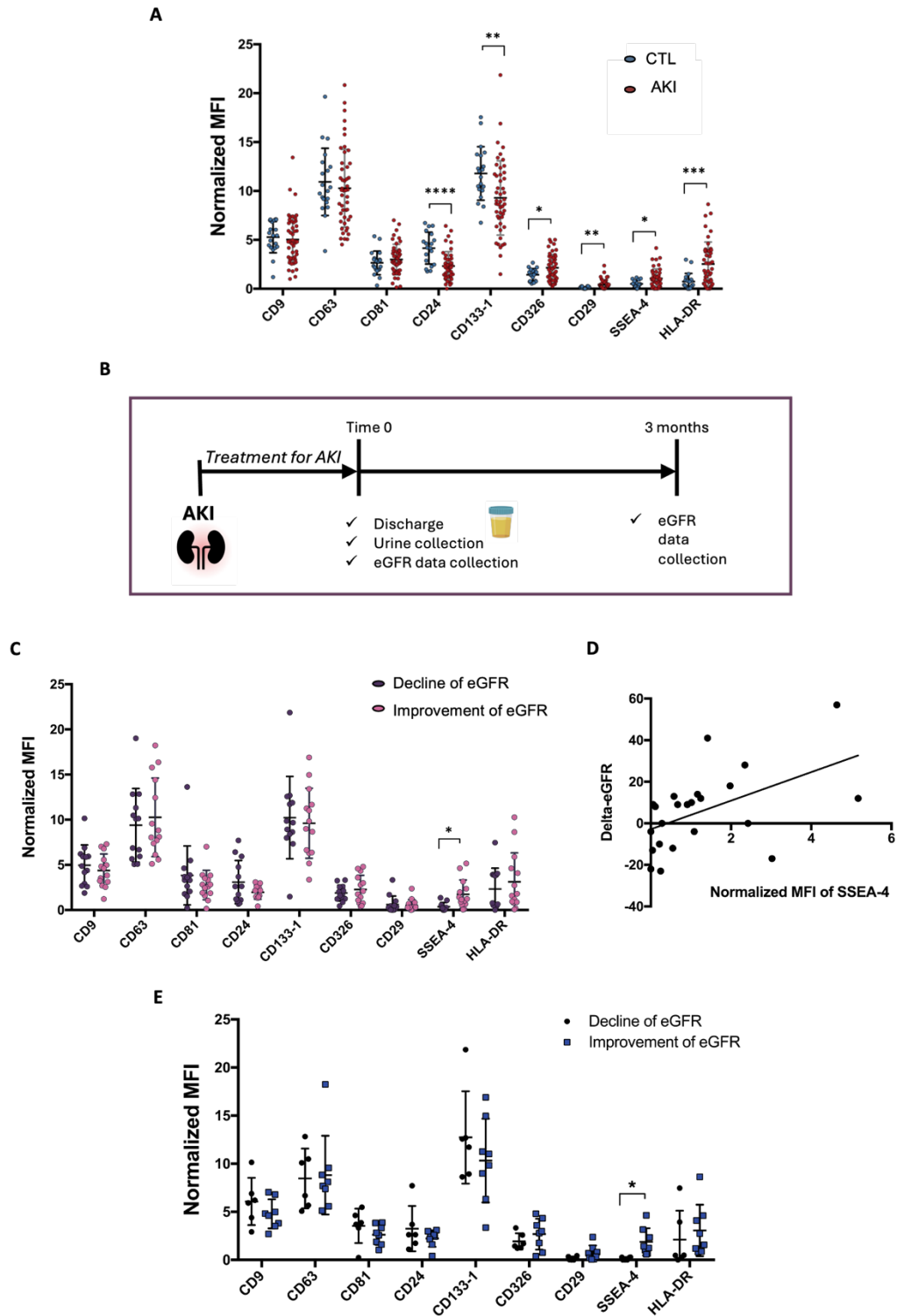


Figure 3. Results of MACSplex analysis for AKI cohort. (A) Expression levels of tetraspanins (CD9, CD63, CD81) and positive surface markers (CD24, CD133-1, CD326, CD29, SSEA4, HLA-DR) were compared between healthy controls (blue, $n=20$) and AKI patients (red, $n=49$). Data were normalized to the mean MFI of all detectable markers (nMFI). Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **(B)** Graphical representation of timeline of AKI patients' follow-up. The study cohort comprised 49 AKI patients who were evaluated at discharge (Time 0, and

3 months post-discharge. Patients were stratified based on changes in eGFR into two groups: those with persistent renal dysfunction (n=14; decreased eGFR 3 months post-discharge) and those with improved renal function (n=14; increased eGFR). **(C)** Comparison of uEV marker expression levels in AKI patients with persistent renal dysfunction and those with improved renal function. Data were normalized against the mean MFI of all detectable markers. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **(D)** Correlation between SSEA-4 nMFI and Δ eGFR was assessed using Pearson's test. Moreover, linear regression analysis yielded an R^2 value of 0.2629 and a statistically significant p-value of 0.0124. **(E)** Comparison of uEV marker expression levels in AKI patients with persistent renal dysfunction and those with improved renal function, after excluding AKI-on-CKD patients. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The second part of the analysis aimed at identifying prognostic markers that could distinguish AKI patients at higher risk of progressing to CKD from those with a renal function recovery. For this purpose, the renal function parameter eGFR was collected during the discharge from the hospital, when urine was collected, and three months after; then the values were compared.

(A) AKI Patients with improved renal functions							
Patient Number	Age	Sex	AKI-on-CKD	Basal eGFR	Discharge eGFR	3 months eGFR	Δ eGFR
722	70	F	0	66	49	90	41
745	88	M	1	41	23	41	18
758	87	F	1	38	27	36	9
767	69	M	1	40	16	30	14
774	79	F	1	30	19	28	9
794	58	M	0	102	56	68	12
796	77	F	1	48	24	36	12
816	72	F	0	89	82	91	9
1454	74	M	0	85	39	67	28
1479	64	M	0	93	90	100	10
1544	65	F	0	96	101	96	-5
1546	59	F	1	58	43	51	8
1551	71	F	0	95	61	74	13
1569	68	F	0	102	33	90	57

(B) AKI Patients with declined renal functions							
Patient Number	Age	Sex	AKI-on-CKD	Basal eGFR	Discharge eGFR	3 months eGFR	Δ eGFR
699	72	M	0	61	17	36	19
717	81	M	1	49	25	8	-17
734	81	M	1	35	31	21	-10
770	81	M	0	64	37	37	0
775	83	F	1	35	40	28	-12
789	79	M	0	78	59	63	4
790	60	M	1	54	35	41	6
791	80	F	1	29	21	17	-4
792	82	F	1	32	17	17	0
1441	71	F	0	74	42	38	-4
1532	70	F	0	/	90	68	-22
1541	56	F	1	69	35	22	-13
1555	70	F	0	90	76	53	-23

Table 4. Classification of patients based on improved or declined renal function The table categorizes AKI patients into two groups based on changes in eGFR three months post-discharge: those with an improvement and those with a decline in renal function. It includes baseline eGFR, eGFR at discharge, and eGFR three months after discharge, along with each patient's age and sex. The delta eGFR, representing the difference between eGFR at three months and at discharge, is used to generally determine functional improvement or decline. However, some patients displayed a delta eGFR non consistent with their overall course: patient 1544, despite an initial negative delta eGFR, shows overall renal recovery when baseline eGFR is considered. Conversely, patients 699, 789, and 790, with positive delta eGFR values, exhibit a progressive decline in renal function when factoring in baseline eGFR. Highlighted in blue, these patients were excluded from the SSEA4 correlation analysis. eGFR values are presented in mL/min.

Therefore, patients were categorized into two groups based on renal function progression: those with improved function, reflected by an increased eGFR three months post-discharge compared to discharge, and those with worsened function, marked by a decrease in eGFR during the same period. However, to obtain a more comprehensive understanding of each patient's renal recovery trend, also baseline eGFR, which had been collected within a year before AKI occurred, was considered during patients' classification. Indeed, for instance, patient 1544, categorized as improved, actually had a slight drop in eGFR at three months, but maintained a high overall eGFR respect to baseline eGFR, justifying its inclusion in the improved group (Table 4A). On the other hand, patients 699, 789, and 790, classified as declined, had a slight eGFR increase at three months compared to discharge but remained quite below their baseline eGFR, indicating ongoing kidney dysfunction (Table 4B). This approach enabled the classification of 14 patients as improved (Table 4A) and 14 as declined (Table 4B). While the initial cohort comprised 49 patients, only 28 were included in this classification due to insufficient follow-up data or patients' death (Table 2). SSEA-4 showed to be more expressed in uEVs from patients with improved eGFR compared to those who progressed to CKD (p-value = 0.0103; Figure 3C). No other significant differences were identified between the two groups.

To provide a quantitative measure of kidney function recovery, we calculated Δ eGFR as the difference between eGFR at three months post-discharge and at discharge, with positive Δ eGFR values indicating improvement and negative values signifying a decline. By using Δ eGFR, we aimed at investigating the relationship between kidney recovery and SSEA-4 expression. For this analysis, the patients with a Δ eGFR inconsistent with their overall eGFR trends (highlighted in blue, Table 4) were excluded from the analysis. Nonparametric correlation results showed a p-value of 0.0038, while linear regression analysis yielded an R^2 value of 0.2629 with a p-value of 0.0124 (Figure 3D).

Finally, the differences in marker expression between the improved and declined renal function groups were evaluated also after excluding AKI-on-CKD patients. Although the low group numerosity (6 non-CKD AKI with decline renal function *versus* 8 non-CKD AKI with improved renal function), the analysis confirmed SSEA-4 to be more expressed in uEVs from patients with improved eGFR (p-value= 0.0112; figure 3E). The exclusion of AKI-on-CKD patients was meant to eliminate the impact of a pre-existing CKD condition on the recovery ability following an AKI event. These results empowered the hypothesis of SSEA-4 on uEVs as potential positive prognostic biomarker to monitor repair after kidney injury.

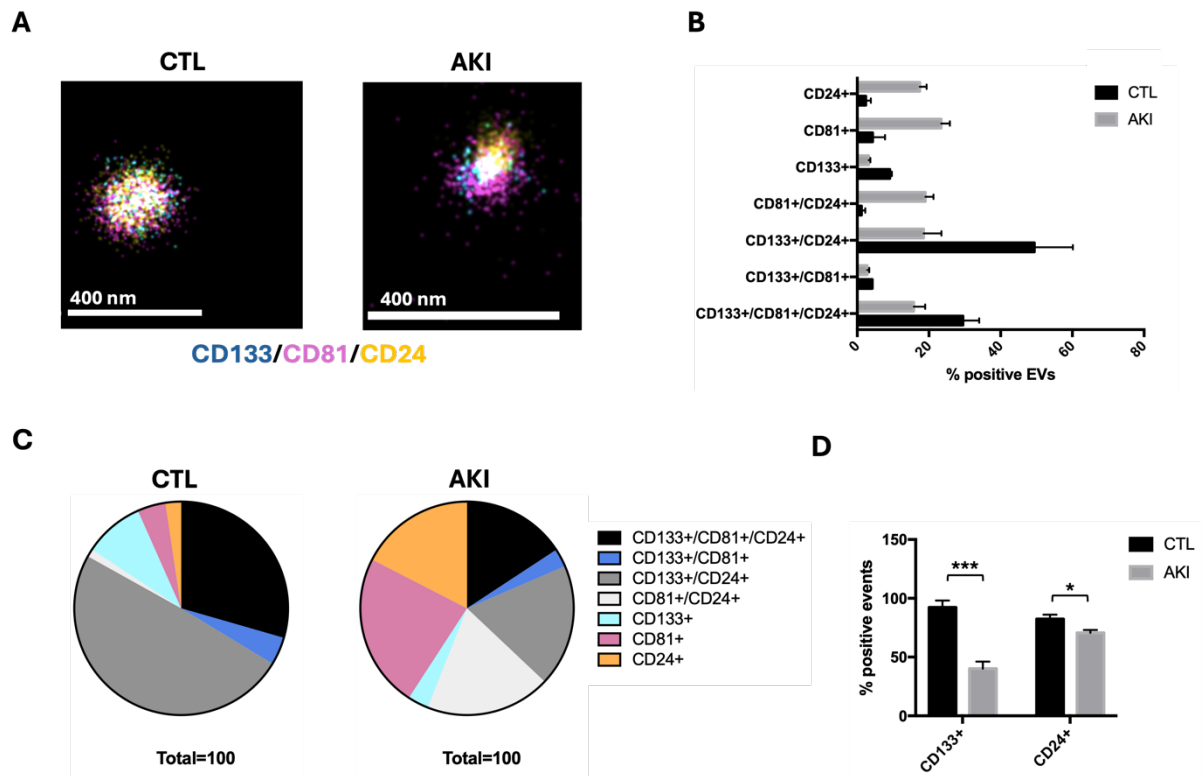


Figure 4. Super-resolution microscopy analysis of AKI pool-uEVs compared with CTL pool-uEVs. (A) Super-resolution microscopy images (at scales of 400 nm) of AKI pool-uEVs and CTL pool-uEVs. The uEVs are labeled with fluorescent antibodies against CD24-FITC (yellow), CD81-647 (pink), and CD133-1-PE (blue), showing triple-marker staining. Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging) equipped with 405 nm/150 mW, 473 nm/1 W, 560 nm/1 W, 640 nm/1 W lasers and dual emission channels split at 640 nm was used. (B) Graph showing the positivity percentage of single, double, and triple positive EV fractions expressing CD133-1, CD24 and CD81. Three acquisitions were performed and analyzed using a CODI software; the graph shows the mean $\pm$ SD of a cumulative analysis of 3 fields for each acquisition. (C) Pie chart illustrating the overall distribution of marker expression across the two groups, considering a total of 100% uEVs. The proportions of uEVs co-expressing CD133, CD81, and CD24 (black), CD133 and CD81 (blue), CD133 and CD24 (dark grey), CD81 and CD24 (light grey), only CD133 (light blue), only CD81 (pink) and only CD24 (orange) are represented. (D) Graphs showing the total positive percentage of CD133-1- and CD24-positive EVs, respectively, in AKI pool-uEV and CTL pool-uEV samples. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Super-resolution microscopy analysis of AKI-uEVs

Pre-cleared urine samples from 16 AKI patients were pooled together and ultracentrifuged to isolate AKI pool-uEVs. Similarly, pre-cleared urine from 11 control subjects were pooled and CTL pool-uEVs were isolated. Super-resolution microscopy was performed on AKI pool- and CTL pool-EVs to study the surface marker expression at single EV level (Figure 4). In particular, we analyzed the co-expression of CD133-1 and CD24, together with the tetraspanin

CD81. The three markers resulted to be expressed in various combinations with different expression profiles between the two groups (Figure 4B-C). In case of CTL pool-uEVs, the double positive CD133-1+CD24+ EVs resulted to be the most prevalent population, while single CD81+ EVs were more prevalent in case of AKI pool-uEVs (Figure 4B-C). Moreover, the total percentage of CD133-1+ EVs, as well as of CD24+ EVs, resulted significantly reduced in AKI pool-uEVs compared with CTL pool-uEVs, in line with MACSPlex results (Figure 4D).

MACSPlex analysis of KT patients' urine samples

The second cohort was composed by 58 kidney transplanted patients, whose urine samples were collected during requested biopsy. The samples were analyzed with MACSPlex, and first compared to urine from 20 controls - the same used for AKI – which were non-transplanted and non-AKI subjects. This analysis revealed the expression of a high number of inflammatory markers on KT subjects not expressed in controls, such as CD3, CD2, CD56, CD25, CD45, CD69 (Figure 5A).

KT patients were classified by clinicians in four group, depending on their renal functional and histological features: patients with decreased renal function (group 1), patients with delayed graft function or incomplete functional recovery (group 2), patients with no alterations in eGFR but with histological damage (group 3), patients with no alterations in eGFR and without histological damage (group 4). To identify alterations in uEV protein expression profile associated to transplantation per se rather than renal injury, the expression profile of group 4, including KT which did a protocolar check and did not present graft injury, was compare with controls' one. As expected, many inflammatory markers resulted to be higher in group 4 compared with controls (Figure 5B). Moreover, CD133-1 was lower, while CD24 higher in KT patients' group 4 compared with controls (Figure 5B).

On the other hand, when comparing group 1 with group 4 the immunological markers CD3, CD8, CD86, CD69, CD142 were more expressed in group 4, while ROR1 was higher in group 1 (Figure 5C). Similarly, when considering together the patients of the first three groups, comprising all “non-healthy KT patients” with graft injuries, with group 4, which are the “healthy” KT patients, CD3, CD8, CD86, CD69, CD142 and CD31 resulted to be upregulated in group 4, while CD133-1 to be downregulated (Figure 5D).

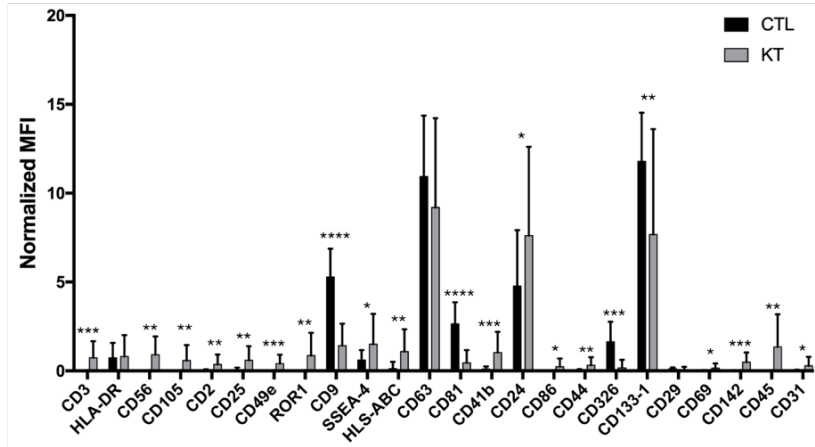
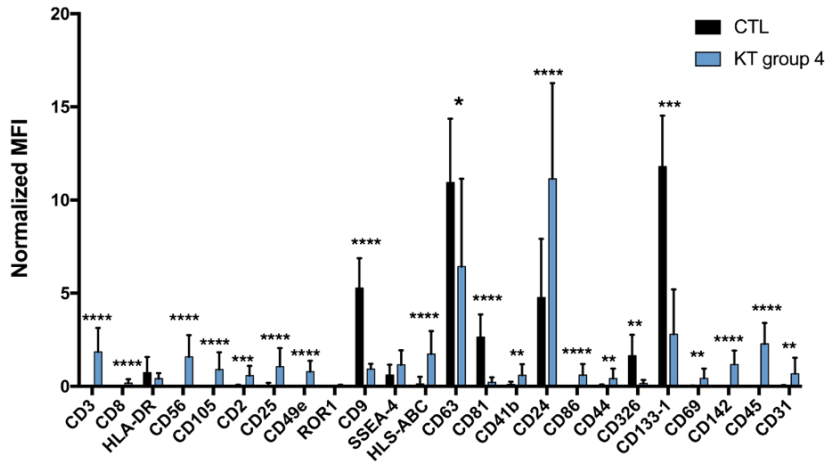
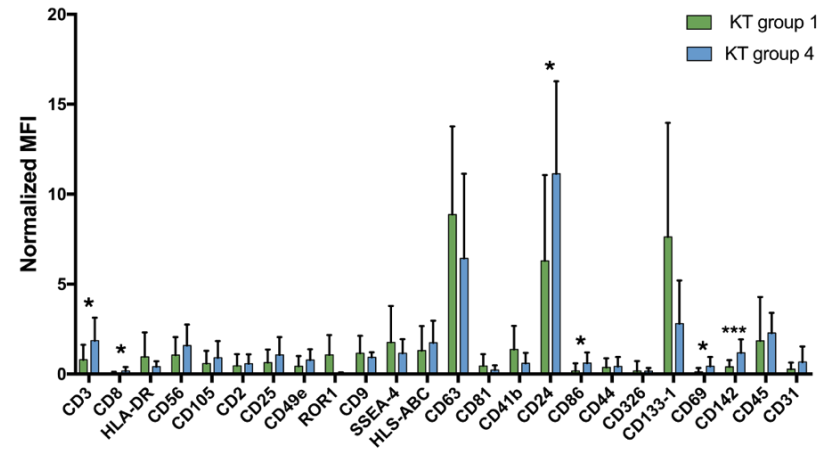
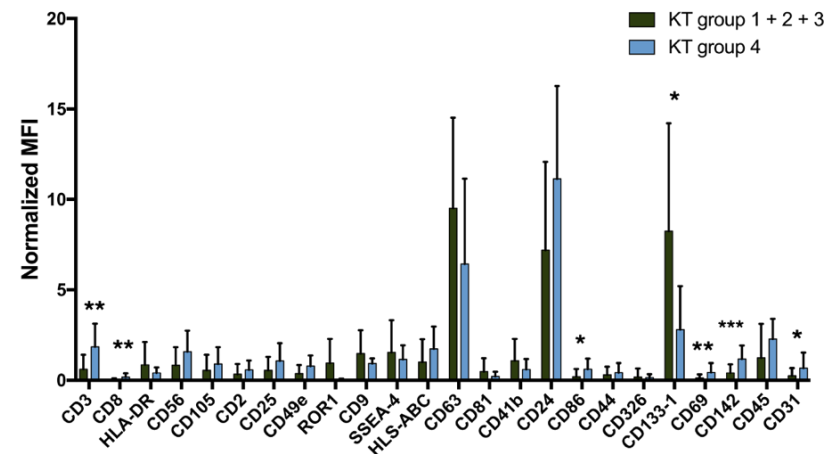
A**B****C****D**

Figure 5. Results of MACSPlex analysis for KT cohort. Expression levels of tetraspanins (CD9, CD63, CD81) and positive surface markers (CD3, HLA-DR, CD56, CD105, CD2, CD25, CD49e, ROR1, SSEA-4, HLS-ABC, CD41b, CD24, CD86, CD44, CD326, CD133-1, CD29, CD69, CD142, CD45, CD31) compared between: (A) KT patients (grey, n=58) and controls (black, n=20). (B) KT group 4 patients (blue, n=6) and controls (black, n=20), (C) KT group 1 patients (green, n=25) and group 4 patients (blue, n=6). (D) KT patients from group 1, 2 and 3 (dark green, n=52) respect with group 4 patients (blue, n=6). Data were normalized to the mean MFI of all detectable markers (nMFI). Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

KT patients were then monitored and eGFR data were collected 1 year after biopsy (Figure 6A). Therefore, patients were classified in two groups: patients with a 1-year eGFR lower than 45 ml/min and patients with a 1-year eGFR higher than 45 ml/min, to distinguish subjects with a persistent renal dysfunction or a renal recovery. In this comparison, CD69 resulted to be more expressed in patients with a 1-year eGFR higher than 45 ml/min (Figure 6B).

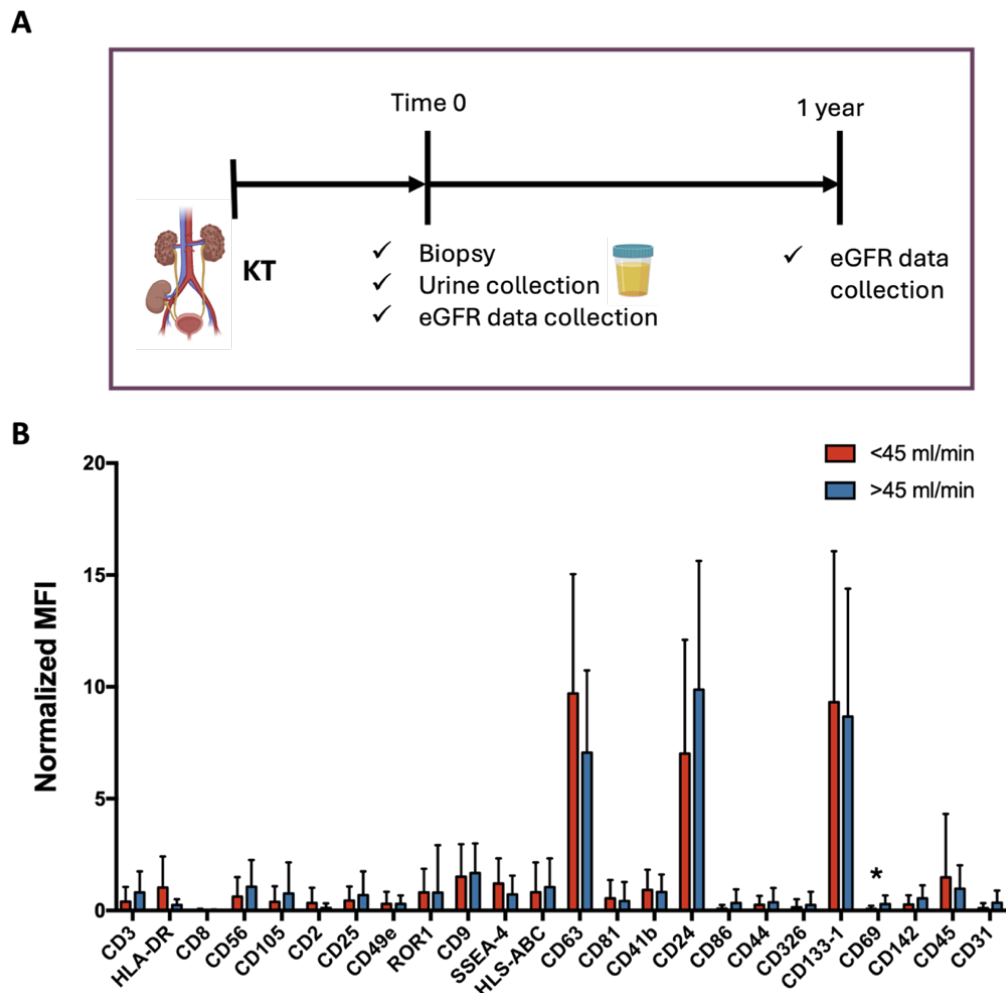


Figure 6. MACSPlex analysis of KT patients' follow-up. (A) Graphical representation of KT patients' follow-up timeline. The study cohort comprised 58 KT patients who were evaluated at the moment of the biopsy and after 1 year. Patients were stratified based on 1-year eGFR into two groups: those with persistent renal dysfunction (n=19; 1-year eGFR < 45 ml/min) and those with improved renal function (n=12; 1-year eGFR >45 ml/min), to identify potential prognostic biomarkers for the

post-transplantation damage monitoring. **(B)** Expression levels of tetraspanins (CD9, CD63, CD81) and positive surface markers (CD3, HLA-DR, CD56, CD105, CD2, CD25, CD49e, ROR1, SSEA-4, HLS-ABC, CD41b, CD24, CD86, CD44, CD326, CD133-1, CD29, CD69, CD142, CD45, CD31) were compared between the group of KT patients with persistent renal dysfunction ($n=19$; 1-year eGFR < 45 ml/min) and the group with improved renal function ($n=12$; 1-year eGFR > 45 ml/min). Data were normalized against the mean MFI of all detectable markers. Statistical significance was determined using multiple *t*-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** < 0.001 .

Optimizing the protocol for the flow cytometry MSP-bead assay

The MACSPlex assay, while beneficial for its ability to use low urine volumes and to analyze 37 markers at the same time, has drawbacks, like high cost and limited antibody panel customization. To address this, we tested an alternative method using FC-MSP-beads (flow cytometry MSP-beads assay), which selectively captures small EVs by targeting their curved membranes. As mentioned in Chapter 2, this assay, already tested on plasma samples (manuscript under review) should allow a selective unbiased capture of EVs. Moreover, with this method is possible to customize the panel of antibodies, focusing on relevant biomarkers. Herein, we investigated its possible use for EV within urine. To set the protocol, we conducted some experiments using urine samples from three healthy subjects that were pooled together. This pre-cleared pool was in part used to isolate uEVs by ultracentrifugation (“dUC”), in part concentrated through ultrafiltration (“concentrated urine”), while the remaining part was not processed (“pre-cleared urine”) (Figure 6). First, we estimated the EV number in the three different conditions by Nanoparticle Tracking Analysis (NTA) (Table 4). Subsequently, we performed the MSP-beads assay, using an increasing particle number, starting from 1×10^5 in all cases, up to 5×10^9 in case of ultracentrifuged uEVs, to 1×10^{10} in case of concentrated urine, and to 1×10^8 in case of pre-cleared urine. The final volume was kept fixed, as well as the total number of FC-MSP-beads. Therefore, the maximum number of particles used in each condition depended on the concentration of the sample. With this experiment we quantify the fluorescent intensity level (median florescent intensity, MFI) associated to CD63 marker expression, across varying particle concentrations. Filtered PBS with beads and anti-CD63 antibody was used to confirm the absence of aspecific signal (Figures 6A-C). As expected, CD63 MFI exhibited a proportional increase corresponding to the number of particles (Figures 6A-C). Results showed that ultracentrifuged urine achieved the maximum MFI at 5×10^9 particles (Figure 6A). However, this method, while achieving a high MFI, requires a large initial urine volume and it is impractical for clinical applications. On the other hand, results from

concentrated urine suggested potential contaminants. Indeed, while the highest CD63 signal was observed at 10^8 particles, a subsequent unexpected reduction in signal intensity was detected when increasing the particle numbers (Figure 6B). This may be due to increased uromodulin—a urinary protein that forms polymers and traps EVs—emphasizing the need to investigate contaminants impact on EV quantification. Thus, this option was excluded. Results with pre-cleared urine showed that the CD63 MFI increased consistently with higher particle concentrations, in line with findings from ultracentrifuged uEVs (Figure 6A). Although the sample was limited to a maximum of 1×10^8 particles due to technical limits, this quantity still produced a detectable signal (Figure 6C). In parallel, we evaluated the performance of MemGlow, a membrane-specific dye expected to increase proportionally in signal with the number of EVs (Figures 6D-F). MemGlow was intended to serve as an indirect indicator of EV concentration. However, contrary to our expectations, the MemGlow signal did not correlate with the particle count across the analyzed conditions, while gave aspecific signal, rendering it unsuitable as a normalizer for our analysis (Figures 6D-F). Based on these results, we decided to use a fixed number of particles for each pre-cleared urine sample, to obtain data which could be considered normalized for particle number. Therefore, the protocol was optimized introducing the step of particle measurement by NTA. Indeed, for each sample 1×10^8 particles were used in a final volume of 200 μ L, achieving a final concentration of 5×10^8 particles/mL (Figure 6G).

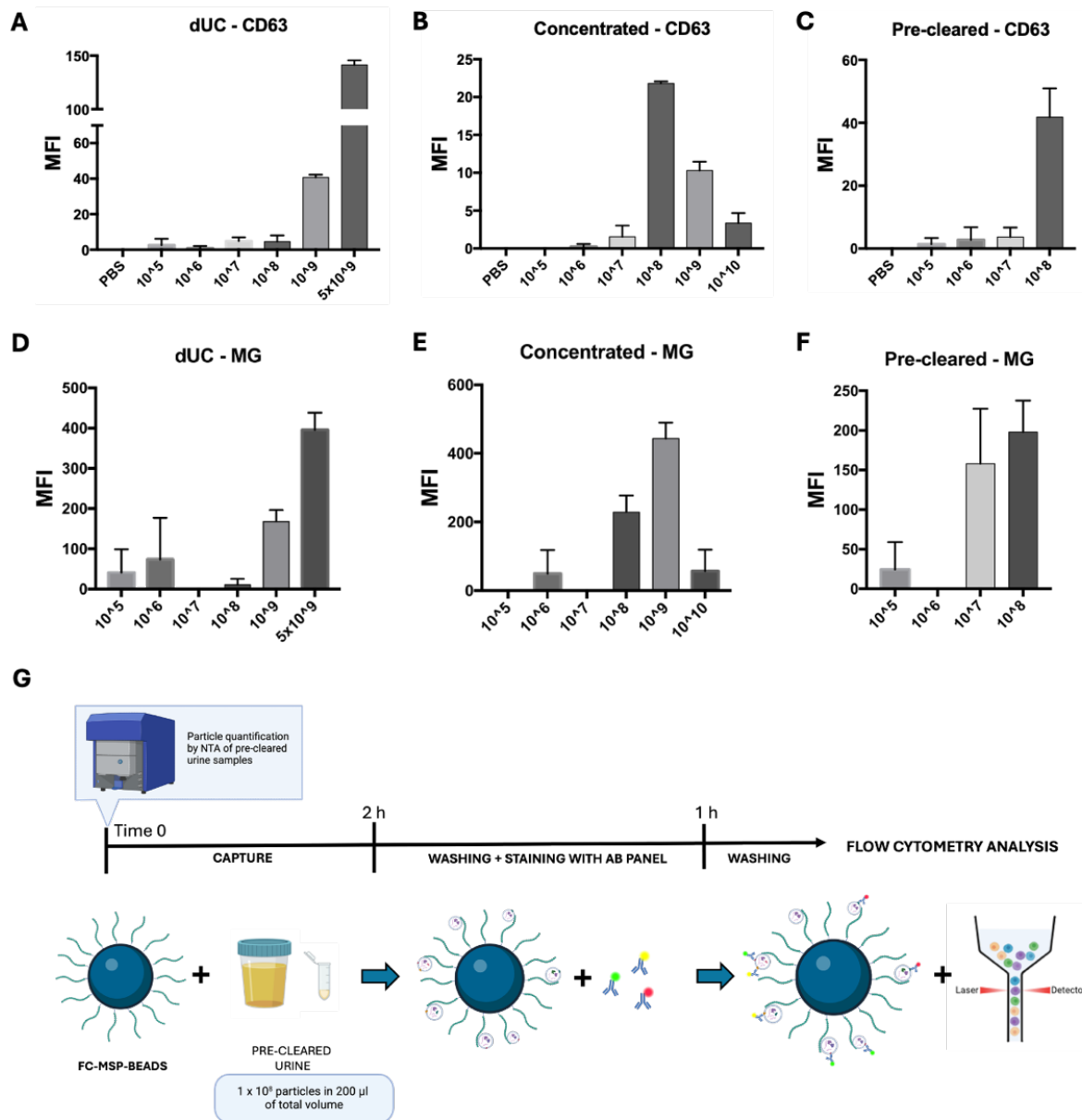


Figure 6. Optimization for FC MSP-Beads in uEVs. (A) Urine pooled from three healthy volunteers was processed using ultracentrifugation (dUC) to isolate uEVs. The MFI for CD63 was measured across increasing particle number, from 10^5 to 5×10^9 particles in a fixed final volume of 200 μ l. (B) The same urine pool was concentrated through ultrafiltration, and CD63 MFI was measured at particle number ranging from 10^5 to 10^{10} particles in a fixed final volume of 200 μ l. (C) Urine was also used without further processing (pre-cleared), with CD63 MFI measured at particle number ranging from 10^5 to 10^8 particles in a fixed final volume of 200 μ l. Filtered PBS served as a negative control for all methods. (D) The ultracentrifuged sample (dUC) was also analyzed using MemGlow (MG), a membrane dye. MemGlow MFI was evaluated across particle number from 10^5 to 5×10^9 particles in a fixed final volume of 200 μ l. (E) The concentrated urine sample was analyzed using MemGlow across particle number from 10^5 to 10^{10} particles in a fixed final volume of 200 μ l. (F) Precleared urine was assessed for MemGlow at particle number from 10^5 to 10^8 in a fixed final volume of 200 μ l. (G) Graphical representation of FC-MSP-bead-based assay protocol. Urine samples were collected and processed by centrifugation at 3'000 x g for 15 minutes to remove contaminants and cellular debris. After particle measurement by NTA, the MSP-bead assay was directly applied to a fixed number of particles of the pre-cleared urine samples. uEVs were captured using beads, washings were performed after

magnetic separation, then a mixture of detection antibodies was used for the staining. After final washing, conventional flow cytometer was used for the detection.

FC-MSP-beads assay on AKI patients' urine samples

After particle measurement, we performed the FC-MSP-beads assay on a fixed number of particles of AKI and control pre-cleared urine samples. For AKI patients, the antibody panel was specifically designed to target markers linked to AKI and its progression to CKD, including Klotho, SSEA-4, CD24, CD63, and CD133. The choice of fluorophores was carefully made to prevent fluorescence signal overlap.

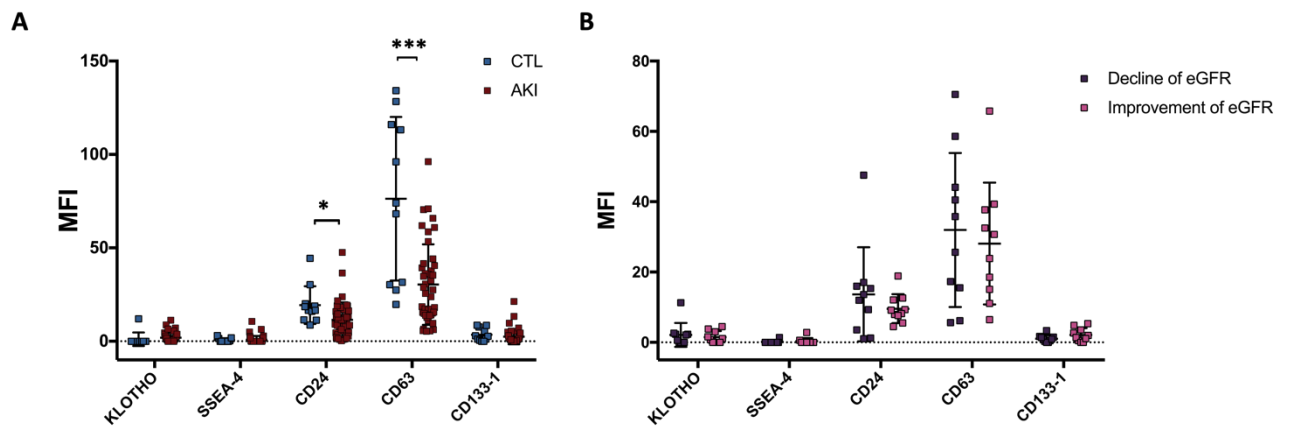


Figure 7. Detection of surface antigens in AKI cohort using MSP-beads method. (A) MFI of KLOTHO, SSEA4 CD24 CD63 and CD133-1 were compared between AKI patients (n=36) and healthy controls (n=17). Respective iso controls were subtracted to remove aspecific signal. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (B) MFI of KLOTHO, SSEA-4, CD24, CD63, and CD133-1 compared between AKI patients with declined or improved eGFR. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$.

While the initial cohort included 20 controls and 49 AKI patients, the final assay was adjusted to 43 AKI patients and 17 controls due to signal absence in some samples. Isotope controls were used to correct non-specific signals and background noise. With this assay AKI patients' uEVs exhibited reduced levels of CD24 and CD63 compared to controls' uEVs (Figure 7A). To identify potential AKI-to-CKD transition prognostic biomarkers, we analyzed differences in marker expression among AKI patients subdividing them as we did for the MACSPlex assay. After accounting for excluded patients due to lack of detectable signal, each group included 10

patients (Table 5A-B). Unfortunately, our analysis did not reveal significant differences in marker expression between the two groups, indicating the need for further research.

(A) AKI Patients with improved renal functions							(B) AKI Patients with declined renal functions						
Patient Number	AGE	SEX	Basal eGFR	eGFR Discharge	eGFR 3 months	Δ eGFR	Patient Number	Age	Sex	Basal eGFR	eGFR Discharge	eGFR 3 months	Δ eGFR
722	70	F	66	49	90	41	717	81	M	49	25	8	-17
745	88	M	41	23	41	18	734	81	M	35	31	21	-10
774	79	F	30	19	28	9	770	81	M	64	37	37	0
794	58	M	102	56	68	12	775	83	F	35	40	28	-12
796	77	F	48	24	36	12	789	79	M	78	59	63	4
1454	74	M	85	39	67	28	790	60	M	54	35	41	6
1544	65	F	96	101	96	-5	791	80	F	29	21	17	-4
1546	59	F	58	43	51	8	1441	71	F	74	42	38	-4
1551	71	F	95	61	74	13	1532	70	F	/	90	68	-22
1569	68	F	102	33	90	57	1555	70	F	90	76	53	-23

Table 5. Classification of patients based on improved or declined renal function, considered for FC-MSP-beads assay. AKI patients' classification based on renal function changes three months after discharge: improved renal function (A) and declined renal function (B) Baseline eGFR, eGFR at discharge, and eGFR three months post-discharge, along with each patient's age and sex are represented. The Δ eGFR, calculated as the difference between eGFR at three months and at discharge, distinguishes improvement or decline. eGFR is expressed in mL/min. Compared with table 4, some patients are excluded since resulted undetectable with FC-MSP-beads assay.

FC-MSP-beads assay on KT patients' urine samples

Based on the data obtained with MACSPlex, we designed the antibody panel for FC-MSP-beads assay choosing six markers of interest: CD3, CD142, CD133-1, CD24, CD63. Unfortunately, CD3 was then excluded due to aspecific signal, probably due to the stickiness of this antibody to the beads. When comparing KT patients with controls, CD142 resulted lower, while CD63 and CD69 higher, in KT (Figure 8A). When comparing patients considering their 1-year-eGFR, CD69 resulted more expressed in patients with a 1-year-eGFR higher than 45 ml/min, in line with MACSPlex results (Figure 8B).

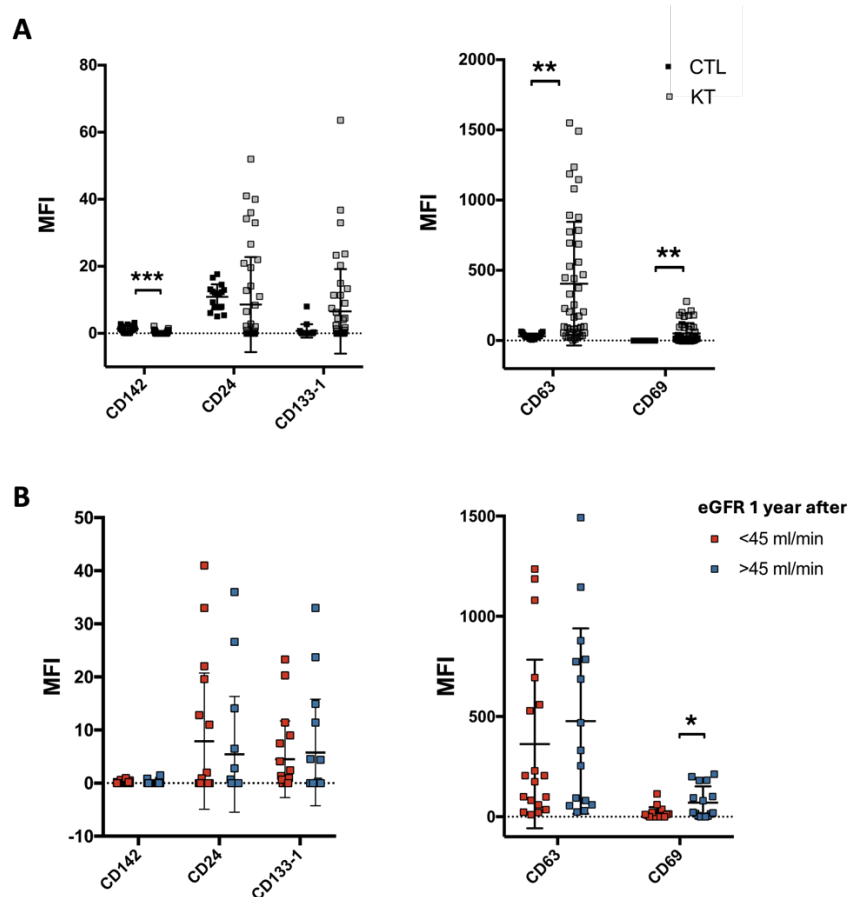


Figure 8. Detection of surface antigens in KT cohort using MSP-beads method. (A) MFI of CD142, CD24, CD133-1, CD63, CD69 were compared between KT patients (n=45) and controls (n=17). Respective iso controls were subtracted to remove aspecific signal. Statistical significance was determined using multiple t-tests with a significance threshold set at $p < 0.05$. Significant differences in marker expression levels were denoted as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (B) MFI of CD142, CD24, CD133-1, CD63, CD69 were compared between KT patients with persistent renal dysfunction (n=18; 1-year eGFR < 45 ml/min) or renal recovery (n=16; 1-year eGFR > 45 ml/min). Statistical significance was determined using multiple t-tests with a significance threshold set at p value < 0.05.

Characterization of uEVs isolated with agarose MSP-beads

In this study, another possible application of MSP in EV field is explored, consisting in uEV isolation. For this purpose, we used another type of beads, which are agarose magnetic beads covalently bound to the MSP (agarose-MSP beads), designed specifically for EV isolation from urine, and supplied by Dr. Cretich (CNR, Milan) (Cheruvanky *et al.*, 2007; Gori *et al.*, 2020). Using these beads allowed us to study which uEV subpopulation was targeted by the MSP, thus, theoretically, which subpopulation is analyzed with FC-MSP-beads assay. Therefore, urine samples from three healthy volunteers were collected and pooled. After pre-clearing, the pool was divided in three conditions, using a fixed initial volume for all conditions to ensure comparability. Then, EVs were isolated either using agarose MSP-beads, using standard

ultracentrifugation protocol, or using ultracentrifugation and resuspending the pellet with the same separation buffer used for the MSP-bead isolation protocol. uEVs isolated with agarose MSP-beads, after the capture and the washing, were separated from the beads, and characterized using NTA and TEM. NTA analysis revealed a mean uEV size of approximately 137 nm (Figure 9A). TEM images confirmed intact vesicles, displaying their distinct morphology and a small size (Figure 9B). Afterwards, the marker expression profiles across the different conditions were compared using super-resolution microscopy and MACSPlex assay. By super-resolution microscopy we also confirmed that MSP-beads-isolated uEVs had a median size of approximately 100 nm, while uEVs obtained through ultracentrifugation averaged around 150 nm (Figure 9C).

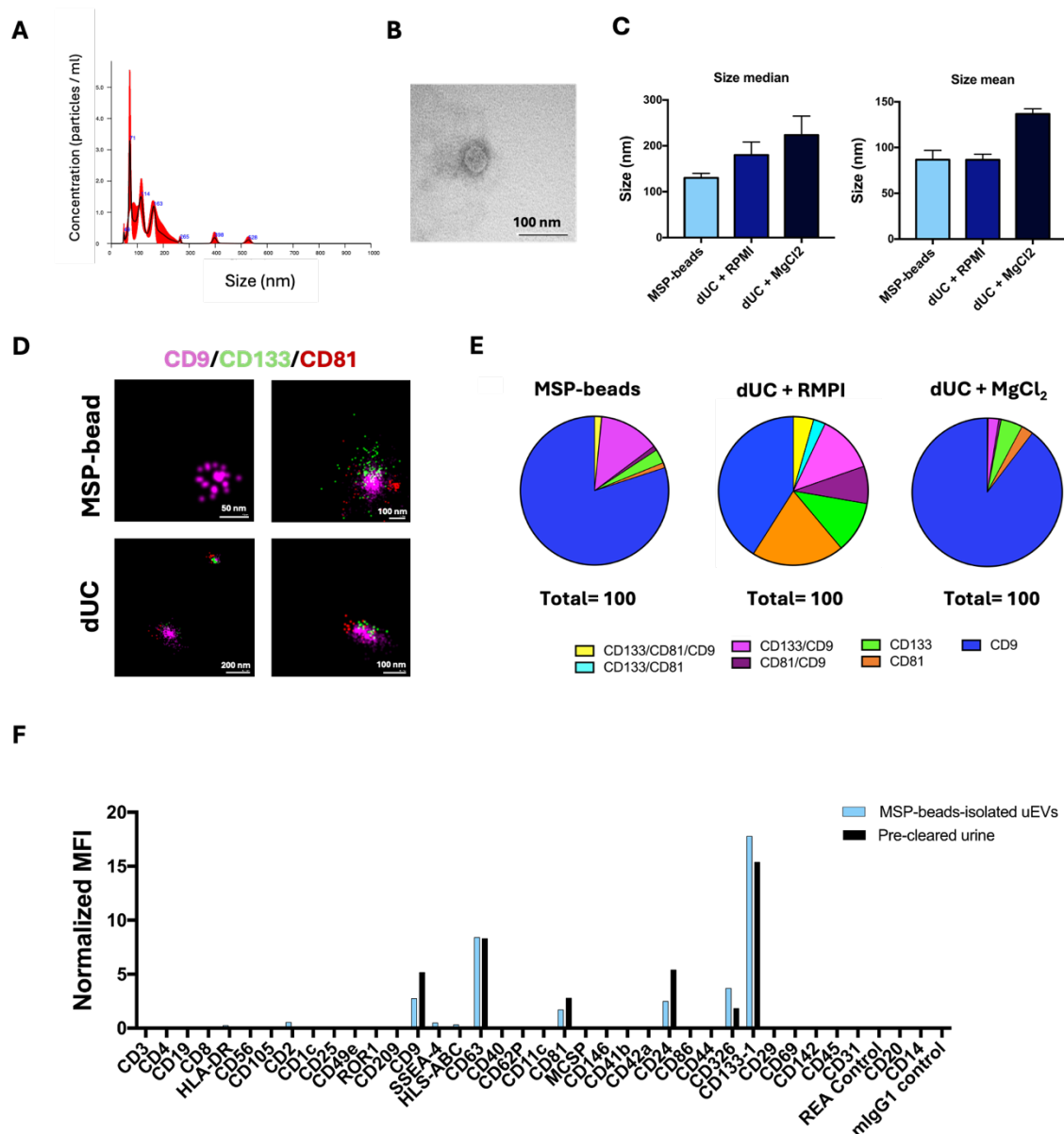


Figure 9. Characterization of healthy subjects uEVs isolated with agarose MSP-beads. (A) Size distribution profile of uEVs isolated with MSP-beads determined by NTA (mean particle size 137.0 ± 0.5 nm); (B) TEM images of isolated uEVs to visualize morphology and size. Results confirm the presence of intact vesicles within a 100 nm scale. (C) Median and mean size (expressed in nm) of uEVs isolated by MSP-beads and ultracentrifugation, estimated by super-resolution microscopy. (D) Super-resolution microscopy images (at scales of 50 nm, 100 nm, and 200 nm) of uEVs isolated with MSP-beads or ultracentrifugation. uEVs are labeled with fluorescent antibodies against CD9-488 (pink), CD81-647 (red), and CD133-1-PE (green), showing triple, dual, and single-marker staining. Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging) equipped with 405 nm/150 mW, 473 nm/1 W, 560 nm/1 W, 640 nm/1 W lasers and dual emission channels split at 640 nm was used. (E) Pie chart illustrating the overall distribution of marker expression across the two isolation methods, considering a total of 100% uEVs. The proportions of uEVs co-expressing CD133, CD81, and CD9 (yellow); CD133 and CD81 (light blue); CD133 and CD9 (pink); as well as those expressing only CD9 (blue), CD81 and CD9 (purple), CD133 (green), and CD81 (orange) are expressed. (F) MACSplex analysis of EV marker expression profiles of MSP-bead isolated uEVs compared with pre-cleared urine samples.

Moreover, super-resolution microscopy analysis demonstrated the expression of CD9, CD81, two tetraspanins, and of CD133-1, a typical uEV marker, in various combinations, with single positive CD9 EV being the most prevalent (Figure 9E). Notably, the surface marker expression profile of MSP-bead-isolated uEVs was similar, though not identical to that of uEVs isolated via ultracentrifugation and resuspended in the detachment buffer (Figure 9E), while partially distinct from uEVs resuspended with the standard protocol. MACSPlex assay was also used to compare EV marker expression among the different conditions. However, ultracentrifuge-isolated uEV MACSPlex analysis was not possible, due to undetectable signal, probably associated to low EV yield. On the other hand, the analysis showed an quite analogous expression levels of tetraspanins, CD133, and CD24 (Figure 9F) when comparing MSP-beads-isolated uEV and pre-cleared urine. These findings suggest that while the MSP-bead method isolates EVs within a specific size range and achieves a higher yield compared to traditional approaches, it maintains a quite similar marker expression profile.

Discussion

Early detection of kidney diseases is critical for improving patient outcomes. uEVs represent a promising source of biomarkers due to their non-invasive collection, abundance, and reflection of their parent cell molecular content. uEVs contain markers from various nephron segments and the urogenital tract (Erdbrügger *et al.*, 2021). Currently, kidney and urinary tract diseases are primarily diagnosed using non-specific biomarkers like serum creatinine, that allows to value estimated GFR (eGFR). These methods detect kidney dysfunction only at advanced stages, limiting their effectiveness (Masaoutis *et al.*, 2020). While biomarkers such as tissue inhibitor of metalloproteinase 2 (TIMP2) and insulin-like growth factor-binding protein 7 (IGFBP7) have received FDA clinical approval, they have some limitations, including high false-positive rates, as seen with the Nephrocheck test (Kashani *et al.*, 2013; Fan *et al.*, 2019). This underscores the pressing need for more reliable and accurate disease markers. Moreover, a significant challenge in uEV research is the lack of standardized protocols for urine collection, uEV separation, and measurement. Variability in pre-analytical procedures can compromise the quality and reliability of uEV preparations, complicating the development of consistent, clinically translatable assays. In addition, also urine signal normalization is an issue due to its dynamic nature. To address this, there is an urgent need for standardized reference materials and methods which could be translated into the clinic.

Various studies have reported promising candidate biomarkers for kidney disease in uEVs. For instance, few years ago, uEV-ATF3 was proposed as an early EV biomarker in a cohort of 79 patients with sepsis-induced AKI (Panich *et al.*, 2017). In another recent work, the transcript levels of 39 genes were analyzed in 127 renal transplanted patients uEVs, revealing differences in expression among different types of kidney graft injury (Harada *et al.*, 2023). However, most uEV biomarker studies are performed on isolated uEVs often analyzed with either Western blot, in case of proteins, or PCR/sequencing, in case of RNAs. Although of scientific interest, for a biomarker to be clinically useful, feasible and affordable techniques should be considered. Indeed, isolation processes are either time consuming and/or expensive. Moreover, methods such as Western blot, but also nucleic acid analysis (PCR, sequencing etc.), requires a huge starting volume of urine, often not available in case of kidney disease patients. In the present study we aimed at identifying a possible clinically translatable method to detect surface biomarkers on uEV starting from a small volume of urine.

When considering EV surface proteins, the commercial kit MACSPlex is gaining more and more interest among researchers, since, coupled with conventional FC, it allows the analysis of

37 surface markers starting from a small volume of biofluid, without the need of previous isolation. The kit includes an immunocapturing process, since the capture beads are conjugated to antibodies against the markers of interest, and the use of detection antibodies against tetraspanins; therefore, only in case of an EV bound to a certain bead (for instance anti-CD24) and to a detection antibody (either anti-CD9 or -CD63 or -CD81), we have a signal for that marker of interest (in this case CD24). While this principle ensures the detection of EVs, at the same time it introduces bias in the analysis. Indeed, although MACSPlex is very efficient and eliminates the need both for large initial volume and the isolation process, it has some limitations. First, as mentioned, the process introduces bias for the enrichment of certain subpopulations and excluding EVs poorly expressing tetraspanins. Secondly, while capable of simultaneously analyzing multiple markers, the method is constrained by its inflexibility in customizing the antibody panel. This represents a limit particularly in case of urine, since the MACSPlex panel of markers is designed for blood samples, and most of them are not expressed by uEVs. Lastly, MACSPlex is quite expensive, limiting further the clinical translatability.

For these reasons, we herein also explored another FC bead-based assay, which allows a capture of EVs not dependent on marker expression. Indeed, this method exploits a MSP, which recognizes and bind to high curved lipid membranes, allowing the capture of small EVs (<200 nm of diameter)(Gori *et al.*, 2020). The use of MSP in EV field appears to be very versatile and could substitute immunocapturing in various applications. In this study we showed two different examples of MSP applications: bead-based flow cytometry assay and uEV isolation. In both cases, an MSP is covalently linked to beads. However, in one case we used agarose beads, ideal to maximize the capture, and detachment can then be performed with a specific solution. This part will be discussed later. In the other case, smaller beads, suitable for FC, were used; instead of detachment, detection antibodies are then used, and FC analysis is performed. Therefore, FC-MSP-beads assay permits to customize the antibody panel as desired.

Herein, we analyzed the uEV surface markers of two cohorts of renal disease patients (AKI and KT) with both MACSPlex and MSP-beads assay. We aimed at identifying prognostic biomarkers to monitor AKI-to-CKD transition in case of acute kidney injury (AKI) patients, while prognostic biomarkers to monitor renal injury in the post-transplantation phase in case of kidney transplanted (KT) patients.

MACSPlex analysis of AKI patients revealed similar levels of tetraspanins but significant differences in markers associated with kidney regeneration, cellular communication, and immune responses, when compared to controls. Notably, CD133-1 and CD24, markers for renal progenitor cells (RPCs), resulted to be downregulated in AKI patients. Of note, we confirmed

the downregulation of CD133-1 and CD24 in AKI patients by super-resolution microscopy performed on uEVs isolated from pool of urine samples. In 2005 our group demonstrated the existence of a resident stem cell population expressing CD133 in adult human normal kidney (Bussolati *et al.*, 2005). These cells, capable of expansion and self-renewal, were then isolated from different nephron segments, all expressing certain renal markers such as PAX2, WT1 and CD24 (Sagrinati *et al.*, 2006; Bussolati *et al.*, 2012; Smeets *et al.*, 2013). In a subsequent study, our group observed CD133+ EVs to be present in urine of normal subjects, while not in patients with end-stage renal disease. More specifically, CD133+ uEVs seemed to reflect the activity of RPCs in renal homeostasis (Dimuccio *et al.*, 2014). Our analysis comparing AKI patients to controls also revealed an increase in several key markers: CD326 (EpCAM), CD29 (integrin β 1), stage-specific embryonic antigen-4 (SSEA-4), and HLA-DR. Various speculations can be made regarding these markers expression. For instance, the elevated expression of HLA-DR, a marker of immune activation, could reflect the higher inflammatory response associated with AKI. While a strong immune response is necessary for initial injury management, excessive inflammation can lead to further kidney damage and fibrosis (Robson *et al.*, 2018). Moreover, elevated SSEA-4 levels, associated with pluripotent stem cells, could indicate a higher pro-regenerative activity in AKI patients, crucial for kidney repair following injury (Bose, Nihad and P, 2023). Since we were interested in prognostic biomarkers, AKI patients were followed-up after their discharge. Indeed, during AKI, patients were treated and subsequently discharged from the hospital, moment in which both eGFR data and urine samples were collected. Afterwards, patients were monitored after three months, and eGFR data were collected. This allows us to classify the patients in two groups: patients which underwent through CKD, with a decrease in eGFR, and patients with renal recovery, with an improvement of eGFR. To better classified the patients, also baseline eGFR – collected within one year before AKI - was considered. MACSplex analysis underline a significant higher SSEA-4 expression in patients with an improvement of eGFR after 3 months compared to patients with a decrease. Moreover, Δ eGFR, calculated as eGFR at the moment of the discharge subtracted to the eGFR at 3 months, correlated with SSEA-4 nMFI, suggesting SSEA-4 as potential prognostic biomarker for renal recovery. Elevated SSEA-4 expression in uEVs may reflect a greater regenerative capacity for tissue repair, positioning it as a promising marker for predicting renal recovery and identifying patients at risk of progression to CKD. To eliminate the possible impact of a pre-existing CKD condition on the recovery ability following an AKI event, we analyzed the two AKI groups with decline or improved renal function after excluding the AKI-on-CKD patients. In fact, kidneys already affected by CKD are compromised in terms of fibrosis, inflammation, and a

reduced functional reserve, making them more vulnerable to additional insults and more susceptible to maladaptive repair mechanisms (Chawla and Kimmel, 2012). Despite the small sample size, this analysis confirmed the SSEA-4 upregulation in the group of non-AKI-in-CKD patients which experienced an improvement of renal function in respect to non-AKI-in-CKD patients with a decrease in renal function 3 months post-discharge. While these findings are promising, further validation with larger patient cohorts is necessary to confirm the utility and reliability of SSEA-4 as a biomarker in clinical settings.

When analyzing AKI samples with MSP-beads assay, we obtained distinct findings compared to the MACSPlex approach. Of the markers analyzed (Klotho, CD133-1, CD24, SSEA-4, CD63), CD24 downregulation in AKI patients was the only difference consistent with MACSPlex results. With FC-MSP-beads we also observed a downregulation of CD63 in AKI compared with controls. At variance, no differences were observed in prognostic biomarkers related with AKI-to-CKD progression. Notably, SSEA-4, which was identified using the MACSPlex approach, was almost not detected with MSP-beads. This lack of differentiation may be attributed to several factors, above all the use of antibody with low brightness of fluorophore, which was chosen for practical reason, but revealed to be improper for the assay. Another limit might be the exclusion of large EVs, which could be necessary for comprehensive biomarker discovery. Moreover, the analysis includes 10 patients per group, which limits the statistical power. Future research should aim to enhance performance by employing antibodies conjugated to brighter fluorophores, improving signal detection, and increasing the overall sensitivity.

Concerning the cohort of KT patients, MACSPlex analysis revealed numerous markers to be expressed in uEVs from KT patients, not expressed in CTL-uEVs. Most of these markers are related to the immune system: CD3, T cell marker, CD56, natural killer cell marker, CD2, CD25 and CD69, associated with T cell activation, HLA-ABC, marker of MHC Class I molecules, CD45, common leukocyte marker, CD86, co-stimulatory molecule mainly expressed on antigen presenting cells, and CD44, involved in cell adhesion and migration (Altin and Sloan, 1997; Lindsey *et al.*, 2007; Qu and Zhao, 2007; Malyszko *et al.*, 2008; García-Ruano *et al.*, 2010; Binder *et al.*, 2020; de Nattes *et al.*, 2022). Moreover, in KT uEVs also the platelet markers CD41b and CD142 resulted to be expressed, possibly associated to vascular injury and thrombosis (Balbi *et al.*, 2021; Burrello *et al.*, 2023). Finally, three endothelial markers are expressed in KT-uEVs and not in CTL-uEVs: CD105, CD31 and CD49e, which can be involved in angiogenesis and vascular remodeling, both repair processes activated after ischemic injury during transplantation (Figueiredo *et al.*, 2019; Li *et al.*, 2023). When

comparing controls only with KT patients which are “healthy”, with no signal of kidney damage (KT group 4), most of the described differences remain consistent, suggesting a huge change in expression profile due to the presence of the graft per se, independent on graft injuries. On the other hand, when comparing the KT group 4 with all the other KT patients (“non-healthy KT”), either with functional decline (KT group 1), delayed graft function (KT group 2) and histological damage (KT group 3), CD3, CD8, CD86, CD69, CD142 and CD31 resulted to be upregulated, while CD133-1 downregulated, in group 4. However, it is difficult to explain the possible reasons underlying these differences. Moreover, the numerosity of the “healthy KT” is quite limited (n=6) compared to the “non-healthy KT” patients (n=52). From a clinical point of view, in fact, protocolar renal biopsy, when no symptoms are detected, is rare, due to its invasive nature. Anyway, regardless of their group affiliation, KT patients were monitored one year after biopsy and urine collection. Indeed, we aimed at identifying markers which could distinguish patients with persistent renal dysfunction (1-year eGFR < 45 ml/min) from patients with renal recovery (1-year eGFR > 45 ml/min). Comparing these two groups, we identified CD69 to be significantly higher in patients with a 1-year eGFR > 45 ml/min.

In a previous study, our group analyzed the surface antigen profile of uEVs from KT patients, using MACSplex kit as well. Moreover, also in this case the outcome was defined as persistent renal dysfunction or renal recovery after 1 year from transplantation, in case of eGFR less or higher to 45 mL/min, respectively. However, while in the present study we analyzed urine collected during requested biopsy (different timepoints among patients, during the post transplantation phase) in this previous work the patients’ urines were analyzed before, and at 10–14 days, 3 months and 1 year after transplant. The analysis showed CD1c, CD105, CD133, SSEA-4 in uEVs to be progressively increased after transplant in patients which experienced renal recovery at follow-up, and these markers to be associated with KT outcome. However, given the differences in study design, it is unsurprising to observe divergent results among the two studies.

In case of FC-MSP-beads assay, the antibody panel chosen for KT cohort was different from the one used for AKI cohort, since the markers of interest partially changed, including CD142, CD24, CD63, CD69, CD133. Of note, when comparing KT patients with controls, CD142 resulted to be downregulated in KT, contrarily to MACSplex analysis. At variance, CD63 and CD69 resulted to be upregulated in KT patients. When subdividing KT patients considering their 1-year eGFR, patients with eGFR higher than 45 mL/min showed higher expression of CD69 compared with other patients. This result was in line with MACSplex analysis and highlighted a potential role of CD69 as prognostic biomarkers. It’s not straightforward to

understand the possible underlying role of CD69 in uEVs of KT patients. CD69 is a type II C-lectin transmembrane protein that is mainly present as an early activation or inducible marker on T cells. However, it also plays a regulatory role in immune responses. Indeed, various studies demonstrated CD69 modulation activity in adaptive immunity, through mechanisms such as reinforcement of T regulatory (T_{reg}) suppressive activity, maintenance of the balance between T helper 17 (T_{h17}) and T_{regs} by prevention of T_{h17} differentiation and downregulation of pro-inflammatory cytokines (Feng *et al.*, 2002; Radulovic *et al.*, 2013; Gorabi *et al.*, 2020). Further studies should investigate the possible association between CD69+ uEVs and improved renal function in KT patients.

FC-MSP-beads assay, despite promising, presents some challenges. As mentioned, in this study a protocol designed for plasma EVs was adapted for uEVs. In case of plasma, a volume of 50 μ l was enough to detect 14 markers, normalizing data with the membrane dye MemGlow. To assess the protocol for urine samples, we made some experiments starting from urine of healthy subjects, we processed the pre-cleared pool in three different conditions and quantified the number of particles. This allows us to perform the assay with increasing number of particles, to study the signal detectable with an anti-CD63 antibody and with the membrane dye MemGlow. In ultracentrifuged uEVs and in pre-cleared urine CD63 signal increases with the increasing number of particles. At variance, in case of concentrated urine the signal reached a peak at 1×10^8 particles and then decreases. A possible explanation could be the concomitant concentration of uromodulin protein, which forms polymers that can entrap EVs and possibly interfering with their binding with either beads or detection antibodies. This finding underscores the complexity of working with uEVs and highlights the importance of considering potential contaminants that may affect accurate quantification. Moreover, differently from CD63, MemGlow in urine samples displayed aspecific signals, for this reason was excluded as candidate normalizer. Consequently, the particle quantification step happened to be essential to use a fixed number of particles for each sample and to obtain normalized data. Moreover, while ultracentrifuged uEVs were used as control, using directly pre-cleared urine samples represents the best option for a clinically translatable method. Therefore, a fixed number of particles for each pre-cleared patient-derived urine samples was used for the FC-MSP-beads assay. Thus, the protocol used for plasma EVs was here preceded by the quantification step, partially hindering its translatability. However, since the EV concentration within urine varies markedly also at physiological conditions, the normalization process is crucial. Moreover, EVs are less concentrated in urine compared with plasma, probably limiting the sensitivity of the assay. Indeed, some markers, such as CD133-1 and SSEA-4, positive with MACSPlex, resulted

almost absent with FC-MSP-beads assay. However, it must be reminded that MACSPlex is more sensitive probably since the capture process works by enriching EV subpopulations, introducing bias on the analysis. In addition, MSP-beads capture only small EVs, potentially excluding biomarkers of interest present in larger EVs. Finally, further studies should explore the use of brighter fluorophore-conjugated antibodies, particularly the one against SSEA-4, which was conjugated with AlexaFluor-700, a fluorophore emitting at the same wavelength of Allophycocyanin (APC), more stable but less bright, chosen for practical reasons. However, while needing further technical optimization and validation, FC-MSP-beads assay appears promising in case of markers expressed mainly on small-uEVs.

Finally, as previously mentioned, during my PhD I explored another application of MSP in EV field. Indeed, we also tested agarose beads, conjugated with the same MSP, but designed for uEV isolation. Agarose MSP-beads were used to isolate EVs from healthy subjects' urine and compared with other common isolation methods. This experiment was aimed at characterizing the subpopulation of uEVs captured by the MSP, as well as investigating another MSP application. Following EV capture and washing, the beads were separated from the EVs using a specific detachment solution. MSP-beads-isolated uEVs were then characterized with various techniques, confirming their small size, and characterizing the surface markers profile. Notably, super-resolution microscopy revealed that the expression profile of uEVs isolated with MSP-beads closely resembled that of ultracentrifuged uEVs resuspended in the detachment solution, with CD9 single-positive EVs being the most abundant. At variance, ultracentrifuged uEVs resuspended in the classical resuspension medium displayed a slightly different expression profile, suggesting potential interference of the detachment solution in the analysis. Therefore, alternative methods for detaching EVs from the beads are now under study. On the other hand, MACSPlex analysis of agarose MSP beads-isolated uEVs showed a similar expression profile to that obtained with pre-cleared urine. At variance, EVs isolated via ultracentrifugation from an equivalent starting volume were insufficient for detection with MACSPlex, highlighting the higher yield achieved with agarose-MSP beads respect to ultracentrifuge.

Conclusions

Current methods for renal disease detection are limited, thus finding new early biomarkers is crucial to improve kidney pathologies management. We here propose uEVs as possible source for prognostic biomarkers in renal disease. Nevertheless, technical constraints pose various limitations in studying EV content in an easy and reproducible way. In this study, we aimed at developing a clinically translatable approach to detect surface biomarkers on uEVs. To achieve this goal, we evaluated and compared two bead-based FC assays. The first one, MACSPlex, is a commercial kit, which is very efficient, but with same disadvantages. On the other hand, the other method, FC-MSP-beads assay is a novel and promising approach, while needing technical validations. Both approaches were used on urine samples from two kidney disease cohorts. Indeed, apart from technical aspects, in the present study we also aimed at identifying potential uEV biomarkers to monitor AKI-to-CKD transition and kidney failure during the post-transplantation phase. Therefore, the first cohort consists in patients treated for AKI and monitored for three months after their discharge; in this case we aimed at identifying prognostic biomarkers for AKI-to-CKD transition. MACSPlex analysis of AKI patients revealed downregulation of CD133-1 and CD24, markers of RPCs, and upregulation of CD326, CD26, SSEA-4 and HLA-DR in AKI patients uEVs respect with control uEVs. More interestingly, SSEA-4 was more expressed in uEVs from patients with improved renal function after three months. Its levels correlated with renal function recovery, making it a promising positive prognostic biomarker for monitoring kidney recovery following AKI. With FC-MSP-beads assay, CD24 resulted to be downregulated in AKI patients respect with controls, in line with MACSPlex results. At variance, SSEA-4 was barely detected with FC-MSP-beads assay, highlighting the need of further technical optimization. Concerning the second cohort, it consists of KT patients, whose urine was collected during a medical visit concomitant with a biopsy and then followed-up for one year; in this case, we aimed at identifying biomarkers to monitor kidney injury in the post transplantation phase. When compared with controls, MACSPlex analysis revealed a very different expression marker profile, with several inflammatory markers being upregulated in KT uEVs. As promising positive prognostic biomarker we identified CD69, a cell T activation marker. Interestingly, this was confirmed with FC-MSP-beads assay.

We also explored another potentiality application of MSP in uEV field. In fact, we here showed that MSP can also be conjugated with another type of beads for uEV isolation, reaching a high

yield. However, other solutions to detach EVs from the beads are under investigation to improve the method.

The present study underlines the potential of uEV surface biomarkers in providing early insights into kidney health, surpassing traditional diagnostic methods. SSEA-4 and CD69 appeared as potential prognostic biomarkers in case of AKI and KT, respectively. However, more patients will be enrolled to validate the present data. Moreover, various technical challenges constraints clinical translatability, needing further studies to optimize a standardized feasible method to detect uEV biomarkers through flow cytometry.

In the future, ideally, such a method might be translated in clinical settings to detect validated biomarkers starting from a small volume of urine sample. Therefore, renal disease development could be easily assessed either in patients which underwent AKI or with renal graft injury. The possibility to customize the antibody panel would allow to adapt the assay according to the disease-associated biomarker. Thus, once the method has been validated, it could be applied to different cohorts of patients, to which correspond different biomarkers of interest. In case of KT patients, for instance, a screening test based on CD69 expression levels on uEVs may be used to identify patients risking renal graft dysfunction during an early stage of the pathological process, when stopping the course of the disease is still possible. This could allow to avoid unnecessary renal biopsy, as well as to promptly contrast the disease.

Future perspectives in the clinical use of EV biomarkers

Traditional approaches to analyze disease-associated EV biomarkers are often subjected to inherent drawbacks such as low throughput, large biofluid volume required, and lack of automation. Indeed, while standard methods like Western Blot and omic approaches are useful to identify novel biomarkers, they are unsuitable for clinical translation. Therefore, with the advancement of technology, new methods are now actively tested to identify a sensitive, specific and straightforward method for biomarker detection (Chiriaco et al., 2018). We herein described a novel FC bead-based assay to analyze uEV biomarkers starting from a small volume of urine, as potential translatable method.

Currently, other innovative methodologies are under investigation to fulfill this necessity. For instance, the incorporation of microfluidics, which consists in the manipulation of small amounts of fluids in microscale channels, could facilitate the enrichment of biomarkers for detection. Indeed, microfluidic systems offer several advantages, including rapid processing, minimal sample requirements, high sensitivity, automation (Chiriaco et al., 2018). Microfluidic devices are studied both for protein and nucleic acid EV content analysis. Innovations in protein detection include optical transduction, such as photonic crystal-enhanced fluorescence platforms for sensitive assays, and label-free electrochemical methods, such as those using conductive polymers and graphene-based materials, which simplify analysis and lower costs. Advanced tools for nucleic acid detection include platforms with optical detection systems, such as microfluidic devices using LEDs cameras (Chiriaco et al., 2018). Examples include μ BAR, which integrates isothermal amplification and real-time fluorescence readout, and droplet microfluidics for high-throughput digital PCR (Ramshani et al., 2019). Another promising area for developing fast and reproducible assays suitable for clinical applications in EV biomarker analysis is biosensing technology. Biosensors are analytical devices that detect and quantify biomarkers by generating signals associated to the concentration of the analyte of interest (De Sousa et al., 2023). These devices are composed of a biological receptor, which ensures specificity, and a transducer which converts biological signals into electrical data. Biosensors are generally classified based on the type of transducer employed: electrochemical, fluorescence, or optical (De Sousa et al., 2023). For instance, an electrochemical sensor-based system, named integrated magneto-electrochemical sensor (iMEX) was reported to quickly isolate and quantify exosomes from μ l of plasma (Jeong et al., 2016). Moreover, electrochemical sensors can also be utilized in the characterization of the nucleic acid content of EVs. For instance, a microfluidic platform called the immuno-magnetic exosome RNA

(iMER) system was developed, which combines immunomagnetic EV separation, RNA purification, and real-time PCR (Shao et al., 2015; De Sousa et al., 2023).

However, both microfluidic systems and biosensors present some limitations for the translatability into the clinic. Key factors for clinical translation of biomarker detection include standardization, automation and easiness, low cost and being approved by regulatory bodies for use in human diagnostics. Both microfluidic and biosensor platforms require specialized materials and complex manufacturing techniques, which could increase costs and limit their scalability and the reproducibility (De Sousa et al., 2023). Moreover, an EV isolation process is often required before the analysis, which can slow down the process and increase the costs. Therefore, these methodologies need extensive optimization and validations, even more in case of urinary EV analysis, since most studies had been performed on plasma-EVs (De Sousa et al., 2023). Nonetheless, their potential remains highly promising for advancing innovative diagnostic tools. Indeed, in the future, following the necessary optimizations, these platforms could be employed in the development of point-of-care (POC) devices aimed at detecting the expression of a disease-associated EV biomarker. In this context, the detection of a single biomarker, as well as the use of a qualitative rather than quantitative approach, appears to be the more probable outcome.

On the other hand, FC-based methods present numerous advantages upon microfluidic systems or biosensors. Indeed, FC is a widely used technology with established protocols and support from a large scientific community. This makes it easily accessible in many clinical and research laboratories, promoting standardization and the adoption of shared methodologies. Moreover, its versatility allows to customize the protocol depending on the markers of interest. Modern flow cytometry systems are highly automated, allowing for the analysis of multiple samples in sequence and increasing the throughput. As a result, it is particularly useful for large-scale studies, such as clinical trials or the analysis of large patient cohorts (Welsh et al., 2023). Moreover, FC is already integrated into various clinical contexts, such as monitoring hematological diseases. Therefore, the technology is familiar and approved by regulatory authorities, which represents an advantage for its clinical translation in EV analysis. When concerning EVs, high sensitivity flow cytometers overcome the issues associated with the small particle size, however, requires an isolation step and the throughput results limited (Welsh et al., 2023). In this context, FC bead-based assays represent an interesting alternative for EV surface markers detection, since do not require previous isolation steps, may allow a quicker analysis, and allow to reach a higher sensitivity (Welsh et al., 2024). Consequently, we believe

that bead-based FC assays, as the ones discussed in this thesis, may represent the most promising methodology in the study of EV surface biomarkers to be translated into the clinic.

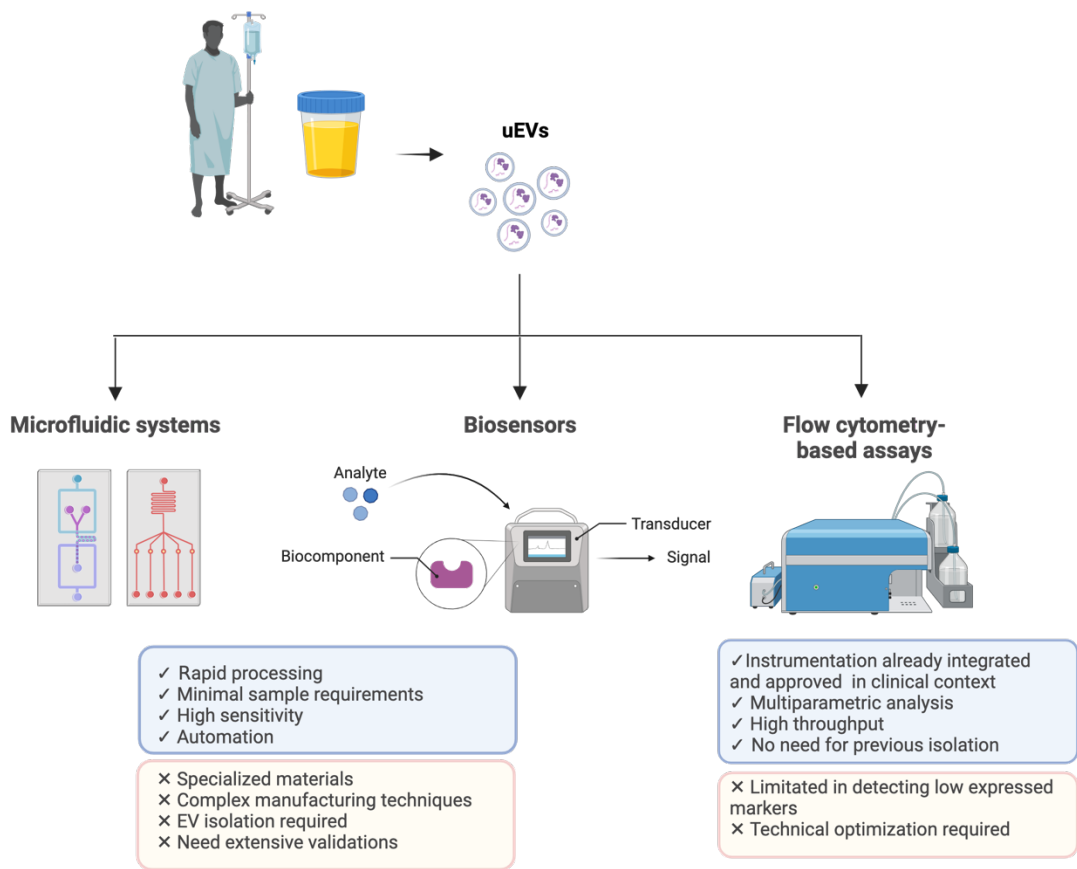


Figure 10. Promising techniques to detect EV biomarkers in the clinical context. Infographic representing the most promising techniques for the detection of EV biomarkers to be translated into the clinic, including their main advantages (blue boxes) and limitations (red boxes). Microfluidic systems and biosensors present similar advantages and limitations.

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Chapter 4: Urine-derived podocytes from steroid resistant nephrotic syndrome patients as a model for renal-progenitor derived extracellular vesicles effect and drug screening

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Introduction

The nephron represents the functional unit of the kidneys, and is composed by a renal corpuscle, which filters blood, and a renal tubule, in which reabsorption, secretion and excretion take place. The renal corpuscle is composed by the glomerulus, a network of filtering capillaries which is located in the Bowman’s capsule (1). The main function of the glomeruli is to filter fluids and electrolytes from the blood, while retaining plasma proteins (1). This activity happens at the level of the glomerular filtration barrier (GFB), and it is coordinated by the interaction of two highly specialized glomerular cells, the fenestrated endothelium, and the podocytes. These two cell types are separated by a thin layer of glomerular basement membrane (GBM). Podocytes are highly dynamic and terminally differentiated cells that interact with the GBM and communicate through signaling at the slit diaphragm (2). The glomerular slit diaphragm represents the junction structure that links the foot processes from neighboring podocytes and consists of a transmembrane-bridging proteins networking with a juxtaposed cytoplasmic platform of protein complexes, which in turn is linked to the actin cytoskeleton (3). Podocytes serve as the final barrier to protein loss in the kidney during filtration of blood by the glomerulus. Hence, the podocyte plays a pivotal role in maintaining the GFB and preventing

protein loss into the urine (1). Therefore, podocyte damage is a causal factor in the progression of multiple variants of kidney diseases (Figure 12).

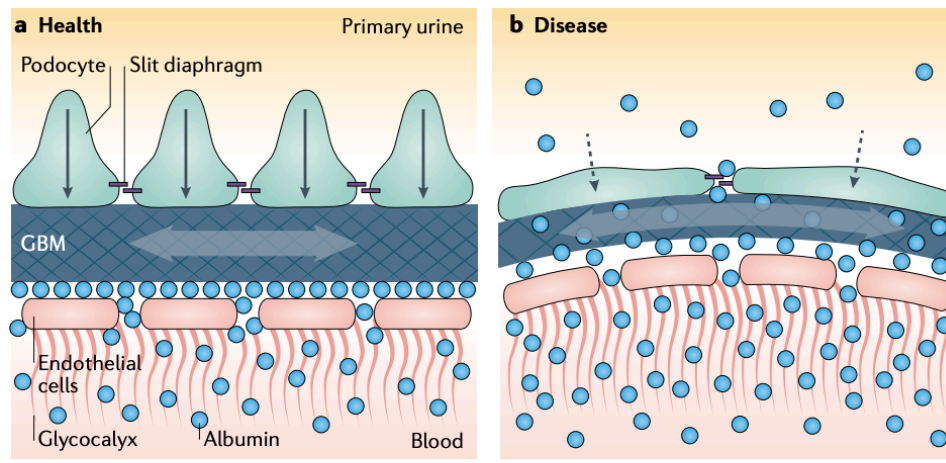


Figure 12. Mechanisms coupling slit diaphragm density and proteinuria. In the extended permeation model, podocyte foot processes are required to compress the glomerular basement membrane (GBM), so that it is impermeable to albumin. In disease states, podocytes and foot processes may be lost and the GBM is therefore distended by the hydrostatic pressure so that it becomes permeable to albumin (Moeller and Chia-Gil, 2020).

Glomerular genetic variants affecting structural or secreted podocyte proteins can disrupt multiple podocyte functions. The main mutations involve genes encoding for slit diaphragm proteins (NPHS1 coding for nephrin, and NPHS2 coding for podocin), and for mesangial matrix synthesis (among which COL4A3, COL4A4 and COL4A5, coding for collagen IV, and LAMB2 coding for laminin subunit beta) (4-5). These disruptions can lead to high-grade proteinuria and severe nephrotic syndrome (NS) that often progress to a rapid decline in kidney function within a few years (6).

NS consists of a heterogeneous disease characterized by increased permeability of the GFB for macromolecules, characterized by proteinuria, hypoalbuminemia, and edema (7). Many diseases and conditions can cause glomerular damage and lead to NS. Indeed, secondary NS is associated to systemic disease such as systemic lupus erythematosus and diabetes mellitus. On the other hand, primary NS, with an annual incidence of 2-7 cases per 100,000 children, can be due to immune deposition, such as membranous nephropathy, but can also be congenital, manifesting within the first 3 months of life (7). Congenital NS is typically caused by genetic mutations that affect either the podocytes or the GBM (7). Indeed, an increasing number of genes have been identified as essential for unique structures and functions of podocytes, including those encoding nephrin, podocin and cluster of differentiation 2-associated protein (CD2AP). Mutations in different podocyte proteins can target the function of the podocyte

through distinct pathologic mechanism by affecting the structure of the slit diaphragm, by breaking cell-matrix interactions, or by blocking important signaling pathways (8) . Abnormalities of cytoskeleton structural proteins can adversely affect cytoskeletal dynamics. For example, hereditary autosomal dominant focal segmental glomerulosclerosis is caused by mutation in α -actinin-4, increasing the affinity for actin and resulting in a change in cytoskeletal fluidity of the podocyte (3). All these mechanisms result in a common final disease pathway characterized by podocyte foot processes effacement, proteinuria, and ultimately disruption of the GFB (7). In case of primary NS, the standard treatment involves steroid therapy, but around 10% of patients are resistant (steroid-resistant nephrotic syndromes), and eventually progress to end-stage kidney disease, requiring replacement therapy (7). Therefore, there is an urgent need for novel effective therapeutic approaches.

Another pathological condition affecting the GFB is Alport syndrome (AS), a genetic disorder caused by pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes encoding collagen IV α 3, α 4 or α 5 chains, resulting in the impairment of the GBM (9). AS can lead to persistent glomerular haematuria, progressive kidney failure, sensorineural hearing loss, and ocular abnormalities. In AS patients, alterations of the GBM affect the function of podocytes, which present foot process effacement, resulting in impaired filtration and passage of blood and/or proteins into the urine (9).

The advent of reliable genetic testing has facilitated the identification of causative genetic mutations (4). Rate of progression can be therefore forecasted basing on the expected impact of the variant on the protein expression but still, several clinical modifiers (hypertension, response to supportive therapy, other clinical additional risk factors) can influence the individual slope of kidney function decline (10). For these reasons, *in vitro* tools to evaluate the response of the single subjects to treatments are highly warranted, with the aim to avoid the multiple attempts to induce proteinuria reduction and the burden of adverse events and toxicity. Focal detachment of podocytes is a phenomenon observed in cases of massive proteinuria in both experimental (11) and human diseases (12). Given this, isolating podocytes from urine samples has acquired interest for understanding their pathological characteristics and identifying potential therapeutic responses (13). In line with this, we recently isolated and characterized podocytes from the urine of patients with Alport syndrome (9).

Extracellular vesicles (EVs), cell-released vesicles involved in cell-to-cell communication, are gaining attention as biological tools with regenerative potential (14). In renal pathology, EVs derived from stem cells of different sources exert anti-apoptotic, anti-inflammatory, and pro-

angiogenic effects, possibly through the transfer of mRNAs, miRNAs, and proteins to renal cells (15-18). We recently demonstrated the beneficial effect of mesenchymal stromal cell-derived EVs (MSC-EVs) on podocyte injury in an *in vitro* microfluidic model of the glomerular filtration barrier (19). Similarly, EVs derived from endothelial cells were shown to inhibit complement-induced podocyte apoptosis, prevent nephrin shedding, and maintain permselectivity during inflammatory damage (20). However, the potential effect of stem cell-derived EVs in treating genetically altered podocytes remains unexplored.

As mentioned in chapter 1, urine has also been studied as a potential source of stem cells for regenerative medicine. Indeed, researchers have identified a specific population of adult stem cells among the cells present in urine, called “urine-derived stem cells (USCs)”, with strong proliferation capacity, ability of self-renewal and multidirectional differentiation (21). Herein, another urine-derived stem cell population has been explored, called neonatal kidney progenitor cells (nKPCs). These cells are isolated from preterm neonatal urine and were previously reported to express embryonic kidney transcription factors, including SIX2 and CITED1 (22). nKPCs are collected before nephrogenesis is complete, and thus could serve as a tissue-specific and highly potent source of progenitors with greater potential than adult cells. Arcolino et al. showed nKPCs to protect renal cells from hypoxic damage in a model of kidney graft perfusion (22). Of note, these stem cells have been shown to act in a paracrine way, thus may act through pro-regenerative EVs secretion (22).

Aim of the study

In this study, we hypothesized that EVs derived from nKPCs (nKPC-EVs) could offer specific benefits to podocytes, given their renal origin. To explore this hypothesis, we used podocytes with genetic alterations obtained from the urine of children with steroid-resistant nephrotic syndrome and we evaluated the effects and potential mechanisms of nKPC-EV treatment. These were compared with common drugs known to modulate podocyte permeability. Additionally, we employed urinary conditionally immortalized podocytes from an Alport syndrome patient as a model for genetically diseased podocytes with altered permeability.

Methods

Ethical statement

All the enrolled subjects provided informed written consent. The study protocol was approved by the Bioethics Committee of the A.O.U. Città della Salute e della Scienza Hospital (protocol no. 0021671). The study was conducted according to the principles expressed by the Declaration of Helsinki of 1975, as revised in 2013.

Generation of podocyte cell lines

A total of three patients diagnosed with steroid-resistant nephrotic syndrome were recruited in this study. Steroid resistance was defined according to the global consensus (24) as persistence of proteinuria in nephrotic range without significant reduction from onset after a course of steroid of 4 weeks prednisone 60mg/m²/day, followed by 3 methylprednisolone pulses of 10 mg/kg each on three consecutive days and two more weeks of observation. If proteinuria is not modified by this treatment the child is classified as "steroid resistant" and is addressed to genetic study. At the time being a panel of 70 genes associated to steroid resistant nephrotic syndrome encoding for proteins involved in podocyte functions is explored (24). Genetic and clinical features of patients are described in Table 1. ACMG classification refers to the time of genetic diagnosis. During follow up patients 1 and 2 did not respond to all the treatments attempted, progressed to CKD 5 (chronic kidney disease, stage 5) and were successfully transplanted without recurrence. Patient 3, which initially proved to be steroid resistant, had a late satisfactory response to steroids combined with angiotensin-converting enzyme inhibitors and one single dose of Rituximab, and after 48 months of follow up has a normal kidney function and minimal proteinuria (Table 1). As control, we used urine from a healthy pregnant woman, a valuable source for control podocytes due to pregnancy-associated podocyturia (25).

Urine samples (~50 ml) were freshly centrifuged at 200 g for 10 min. The pellet was resuspended in DMEM/F-12 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal calf serum (FCS; Invitrogen), 50 IU/ml penicillin, 50 g/ml streptomycin, 5 mM glutamine, 5 g/ml insulin, 5 g/ml transferrin, and 5 mg/ml selenium (all from Sigma-Aldrich, St Louis, MO, USA). Subsequently, primary cells were grown at 37 °C up to the third passage and characterized as podocytes. Three lines of steroid resistant nephrotic syndrome podocytes (NS-POD1-3) and a control urine podocyte line (CTL-uPOD) were generated.

In addition, a conditionally immortalized podocyte cell line (AS-POD) from urine of an Alport syndrome patient, previously isolated and characterized in our laboratory, was used (9). Finally, tissue-derived conditionally immortalized podocytes, which were obtained from a nephrectomy of a healthy subject, were kindly gifted by MA Saleem and used as control for selected experiments (CTL-tPOD) (26). AS-POD and CTL-tPOD were maintained in culture as previously described (27). Briefly, cells were grown at a permissive temperature of 33 °C and, 8 -10 days before experiments, moved to 37°C, to ensure growth arrest and differentiation. The culture medium for AS-POD and CTL-tPOD was the same of primary podocyte cell lines.

Generation of nKPC cell line

Urine sample (500 mL) was collected from a newborn (born at 32 gestational weeks) at day 1 after birth from a catheter bag, as described (22). The sample was centrifugated at 200 g for 10 min and the cell pellet was resuspended in α -MEM Medium (Gibco, Thermo Fisher Scientific) supplemented with 20% Chang Medium B (Irvine Scientific, Santa Ana, California, USA) and 2% Chang Medium C (Irvine Scientific), 20% FCS (Invitrogen), 50 IU/mL penicillin, 50 g/mL streptomycin, 5 mM glutamine (all from Sigma-Aldrich). At passage 2, 1×10^4 primary nKPCs were infected in DMEM F12 20% FCS with a retrovirus containing a pBABE-puro-hTERT plasmid (Addgene plasmid #1771) (28). The day after the infection, the medium was replaced with their growth medium. From passage 3, cells were selected in their growth medium containing 1 μ g/mL puromycin (Gibco) for four weeks, until passage 7.

Non-renal cell lines

Primary human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (ATCC-PCS-100-010, Manassas, VA, USA). Human corneal endothelial cells were previously isolated from discarded cornea patients undergoing corneal transplantation or enucleation and characterized in our laboratory (29). For HUVEC and corneal endothelial cells, EndoGRO-VEGF (Merck Millipore, Burlington, MA, USA) supplemented with 5% FCS (Invitrogen), was used as culture media. Breast cancer cells were previously isolated, grown in DMEM-F12 supplemented with 10 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 5 μ g/mL insulin, and 0.4% bovine serum albumin (all from Sigma-Aldrich), and characterized in our laboratory (30). Human bone marrow-derived MSCs were purchased from Lonza (Switzerland) and grown in mesenchymal stem cells basal medium (MSCBM, Lonza) (29).

EV isolation

nKPC-EVs and MSC-EVs were obtained respectively from the supernatant of nKPCs and MSCs cultured overnight in serum free conditions (RPMI 0% FCS), as previously described (19, 29). Serum-EVs were obtained from a total of 100 mL of serum isolated from a blood pool of five healthy donors, as previously mentioned (31). Informed consent was obtained by the Blood Bank of “Città della Salute e della Scienza di Torino” from all the donors. Culture supernatant and serum were centrifuged for the removal of cell debris and apoptotic bodies at 3,000 g for 20 min. EVs were then purified by a 2 h-ultracentrifugation at 100,000 g at 4 °C (Beckman Coulter, Brea, CA, USA) and used fresh or stored at –80 °C after resuspension in RPMI without FCS and supplemented with 1% dimethyl sulfoxide. Analysis of the size distribution and particle quantification were performed using NanoSight NS300 (NanoSight Ltd, Malvern, UK) equipped with a 405 nm laser and the Nanoparticle Tracking Analysis (NTA) 2.3 software (NanoSight Ltd).

Protein extraction and Western Blot

For protein analysis, different EV preparations (about 5) were pooled to obtain 10^{11} particles and further ultracentrifuged. Subsequently, the EV pellet was resuspended in lysis buffer, composed as follow: 1:100 Phosphatase Inhibitor Cocktail 2, 1:100 Phosphatase Inhibitor Cocktail 3, 1:100 Phenylmethanesulfonyl fluoride (PMSF), 1:100 Protease Inhibitor Cocktail (all from Sigma-Aldrich), in RIPA buffer (20 mM Tris·HCl, 150 mM NaCl, 1% deoxycholate, 0.1% SDS 1% Triton X-100, pH 7.8, Sigma-Aldrich). After 20 minutes of incubation at 4 °C, the protein extract was centrifuged for 15 minutes at 14'000 g and the supernatant was used. Podocyte and nKPC pellets were similarly resuspended in lysis buffer for protein extraction. Protein concentration of podocyte lysate was determined by Bradford solution, according to the manufacturer's procedures (Bio-Rad Inc, Berkeley, CA, USA). At variance, total protein concentration of nKPCs and nKPC-EVs was determined spectrophotometrically using a micro-BCA™ Protein Assay Kit (Thermo Fisher Scientific), as previously described (32). Either 30 µg (for podocytes) or 8 µg (for nKPCs and nKPC-EVs) of proteins were electrophoresed through 4-12% Mini-Protean TGX Stain-Free Gels (Bio-Rad). Using the iBLOT2 system (Life Technologies, Carlsbad, CA, USA), gels were blotted onto PVDF membrane filters according to the manufacturer's procedures. Each membrane was immersed in blocking solution, consisting in 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS, for 1 h before overnight incubation with primary antibodies at the indicated dilutions. After rinsing in wash buffer (0.1% Tween in PBS), horseradish peroxidase-conjugated secondary antibodies were used for 1 h at

1:3000–1:5000 dilutions. After final washings, membranes were incubated with ECL chemiluminescence reagent (Bio-Rad, Milan, Italy). Images were acquired using a ChemiDoc™ XRS+ System (Bio-Rad). For podocytes analysis, the following antibodies were used: rabbit monoclonal anti-Podocin (Cat. No. sc-21009; Santa-Cruz, Dallas, TX, USA), and mouse monoclonal anti-CD2AP (Cat. No. sc-25272, Santa-Cruz). Goat monoclonal anti-Vinculin (Cat. No. sc-7648, Santa-Cruz) and mouse monoclonal mouse anti-PCNA (Cat. No. sc-56, Santa-Cruz) were used as housekeeping. In the case of nKPC and nKPC-EV protein analyses, mouse monoclonal anti-CD63 (Cat. No. sc-5275, Santa-Cruz), rabbit monoclonal anti-Calreticulin (Cat. No. 2891, Cell signalling, Milan, Italy) and mouse monoclonal anti-TSG101 (Cat. No. sc-7964, Santa-Cruz) antibodies were used. The protein bands were detected using either rabbit, mouse, or goat peroxidase-labeled secondary antibodies.

Immunofluorescence

Immunofluorescence on podocytes was performed as follows: cells were plated at a density of 4×10^4 cells/cm² and the next day fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with PBS 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at 4 °C. PBS 1.5% BSA (Sigma-Aldrich) was used to block non-specific sites for 20 min at room temperature. Subsequently, Texas Red-X Phalloidin (Cat. No. T7471, Thermo Fisher Scientific) was incubated for 1 h. Fixed cells were washed with PBS 0.1% BSA before nuclear staining with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 8 min. After the final wash, coverslips were mounted with Fluoromount (Sigma-Aldrich). Images were acquired by the videoconfocal system ViCo microscope Nikon Eclipse 80i (Nikon, Japan).

Transmission Electron Microscopy

The transmission electron microscopy (TEM) was performed on EVs fixed in glutaraldehyde placed on 200-mesh nickel formvar carbon-coated grids (Electron Microscopy Science) for 20 min to promote adhesion. The grids were then incubated with 2.5% glutaraldehyde plus 2% sucrose. EVs were negatively stained with NanoVan (Nanoprobes, Yaphank, NY, USA) and observed using a Jeol JEM 1400 Flash electron microscope (Jeol, Tokyo, Japan) (33).

Super-resolution microscopy

Super-resolution microscopy was performed with Nanoimager S Mark II microscope from ONI (Oxford Nano-imaging, Oxford, UK) equipped with a 100x, 1.4NA oil immersion objective, an

XYZ closed-loop piezo 736 stage, and triple emission channels split at 640, 555 and 488 nm on nKPC-EV. EV profiler Kit (EV-MAN-1.0, ONI) was used for the experiments following manufacturer's protocol. The Kit contains fluorescent antibodies, anti CD9-488, CD63-568 and CD81-647, washing buffer and the imaging buffer. Images were acquired sequentially in dSTORM mode in total reflection fluorescence (TIRF). Single-molecule data was filtered using NimOS software (v.1.18.3, ONI). Data analysis was conducted using Collaborative Discovery (CODI) online analysis platform www.alto.codi.bio from ONI and the drift correction pipeline version 0.2.3 was used (34).

RNA Isolation, Real Time PCR and RNA sequencing analysis

Total RNA of patient-derived podocytes untreated or treated for 24 h with nKPC-EVs or MSC-EVs (5×10^4 EVs/cell) was isolated using Trizol Reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA of CTL-tPOD untransfected or transfected with siRNAs was extracted with Trizol Reagent (Ambion). At variance, RNA of nKPCs and of nKPC-EVs was extracted using miRNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was then quantified spectrophotometrically (Nanodrop ND-1'000, Wilmington, NC, USA). For the gene expression analysis, quantitative real-time PCR (RT-PCR) was performed. Briefly, one-strand cDNA was produced from 200 ng of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). RT-PCR experiments were performed in a 20 μ L-reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech, Eurofins Scientific, Brussels, Belgium), and the Power SYBR Green PCR Master Mix (Applied Biosystems). GAPDH mRNA was used to normalize the RNA inputs. The fold change expression with respect to the control was calculated for all the samples. Primer list can be found in supplementary data (Supplementary table I). For the RNA sequencing analysis, libraries for RNA-seq were generated using a TruSeq RNA stranded sample preparation kit v2 (Illumina Inc, San Diego, CA, USA) following the manufacturer's instructions, using 1 μ g of total RNA as input material. Libraries were pooled and sequenced with a NextSeq 500 sequencer (Illumina Inc) generating 75-bp paired-end sequences. Further analyses were performed using transcript per million (TPM) tables and genes with an average $|\log_2 \text{Fold Change}| \geq 1$ were considered for further analysis using Expression Suite and Funrich V3 Software (Bundora, Australia).

Flow cytometry

After puromycin selection, nKPCs were detached using a nonenzymatic cell dissociation solution, resuspended in PBS 0.1% BSA (Sigma-Aldrich) and incubated with antibodies. Cells were incubated with either phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, or allophycocyanin (APC)-conjugated antibodies against CD90 (Cat. No. 130-114-859, Miltenyi Biotec, Bergisch Gladbach, Germany) CD73 (Cat. No. 550257, BD Bioscience, Franklin Lakes, NJ, USA), CD146 (Cat. No. 550315, BD Bioscience) and CD29 (Cat. No. 130-101-256, Miltenyi Biotec) CD133-1 (Cat. No. 130-090-826, Miltenyi Biotec) and appropriate isotype control. Stained cells were then analyzed using FACSCalibur machine using CellQuest software (Becton Dickinson Bioscience Pharmingen).

MACSPlex Analysis

nKPC-EVs were subjected to bead-based multiplex EV analysis by flow cytometry (MACSPlex Exosome Kit, human, Miltenyi Biotec) as previously described (34). Briefly, 5×10^9 EVs were diluted with a MACSPlex buffer (MPB) to a final volume of 120 μ L and 15 μ L of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each sample. The samples were then incubated on an orbital shaker overnight (14–16 h) at 450 rpm at 4 °C protected from light, followed by several washings with MPB using centrifugations (3'000 g, 5 min). For EV counterstaining, 5 μ L of each APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies were added to each sample and incubated on an orbital shaker at 450 rpm for 1 h at room temperature. After additional washings, samples were subjected to flow cytometric analysis using FACS Celesta (BD Biosciences, New Jersey, USA)

Permeability assay

Permeability assays were performed in a 24 well plate using cell culture inserts with a pore size of 0.4 μ m (Corning, New York, USA). Four $\times 10^4$ podocytes were plated on the insert and left adhere overnight. Subsequently, podocytes were treated for 24 h with nKPC-EVs ($2-5 \times 10^4$ EVs/cell), methylprednisolone (Urbason, Sanofi, 40 μ g/mL), cyclosporin (Sandimmun, Novartis, 25 μ g/mL), tacrolimus (Prograf, Panacea Biotec, 1 ng/mL) or rituximab (Mabthera, Roche, 12 μ g/mL). Subsequently, FBS-free medium (500 μ L) containing or not FITC-BSA (1 mg/mL, Sigma-Aldrich) was placed in the lower compartment and upper podocyte compartments, respectively. After 6 h, BSA filtration from the lower to the upper compartment

was measured, to evaluate the podocyte filtration ability in basal to apical direction. Therefore, 100 µl of medium was taken from the upper compartment and the passage of FITC-BSA was determined by fluorimetry in triplicate, using Promega™ GloMax® Plate Reader (Promega Italia S.r.l., Milano, Italy). Data are expressed as the mean amount of filtered BSA-FITC of four different experiments using at least three inserts for each condition in each experiment.

Podocyte transfection

Transfection of CTL-tPOD was performed using Lipofectamine RNAiMAX Reagent (Invitrogen). Briefly, 4×10^4 CTL-tPOD/well were plated in cell culture inserts, as described for the permeability assay, left adhere overnight at 33 °C and therefore moved at 37 °C, to promote differentiation. After 8 days at 37 °C, cells were transfected with 1.2 µl of Lipofectamine and 10 picomol of the specific MISSION esiRNAs (SUMO1 and SENP2; Merck), according to the manufacturer's instructions. The day after transfection, fresh growth medium was replaced and 48 h later the cells were used for the permeability assay. In parallel, transfected CTL-tPOD were lysed, RNA was extracted, and RT-PCR was performed to verify downregulation of SUMO1 and SENP2 mRNAs. Cells transfected with MISSION negative control (Merck) were used as control.

Statistical analysis

Data are shown as mean ± SD. Statistical analysis was carried out on Graph Pad Prism (GraphPad Software, Inc., San Diego, CA, USA) by using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test, or by unpaired t-test, where appropriate. A p value < 0.05 was considered significant.

Results

Podocyte isolation and characterization

Podocytes were obtained from freshly collected urine derived from three different patients presenting steroid-resistant nephrotic syndrome (NS-POD). Genetically characterized mutations are described in Table I. Podocytes isolated from healthy pregnant woman's urine were used as control (CTL-uPOD). Additionally, conditionally immortalized podocytes, isolated from the urine of a patient with Alport syndrome and previously characterized in our laboratory (9), were included in the study (AS-POD) (Table I).

Table 1. Genetic and clinical features of patients.

Patient	Sex	Gene	Variant	Zygoty	ACMG classification	Coded protein	Clinical follow-up after 48 months
NS-POD1	M	NPHS2	c.479A>G	Compound Heterozygous	C4	Podocin	Progression to CKD stage 5 and transplant
			c.855_856del AA	-	C5	Podocin	
NS-POD2	M	PLCE1	c.1405T>A	Compound Heterozygous	C1	Phospholipase C epsilon 1	Progression to CKD stage 5 and transplant
			C.3281G>C	-	C3		
NS-POD3	F	LAMB2	c.4874_4876delTCC	Heterozygous	C3	Laminin beta-2 subunit	Clinical remission with proteinuria/creatinuria: <0.3 mg/mg
AS-POD	F	COL4A3	c.2777del	Heterozygous	C4	Collagen alpha 3	Progression to CKD stage 5 and transplant

Abbreviations: NS-POD: nephrotic syndrome podocytes; F: female; M: male; NPHS2: Stomatin Family Member, Podocin gene; PLCE1: Phospholipase C epsilon 1 gene; LAMB2: Laminin beta-2 subunit gene; COL4A3: Collagen alpha 3 gene; CKD: chronic kidney disease. ACMG: American College of Medical Genetics and Genomics, ACMG classification refers to the time of genetic diagnosis.

Urine-derived cells were characterized by the presence of podocyte markers such as podocin and CD2AP (Fig. 1A). By morphology, cells showed an organized cytoskeletal structure similar to already well-characterized podocytes (Fig. 1B) (9). Non-renal cell appeared negative for podocytes' markers (Supplementary Fig. 1).

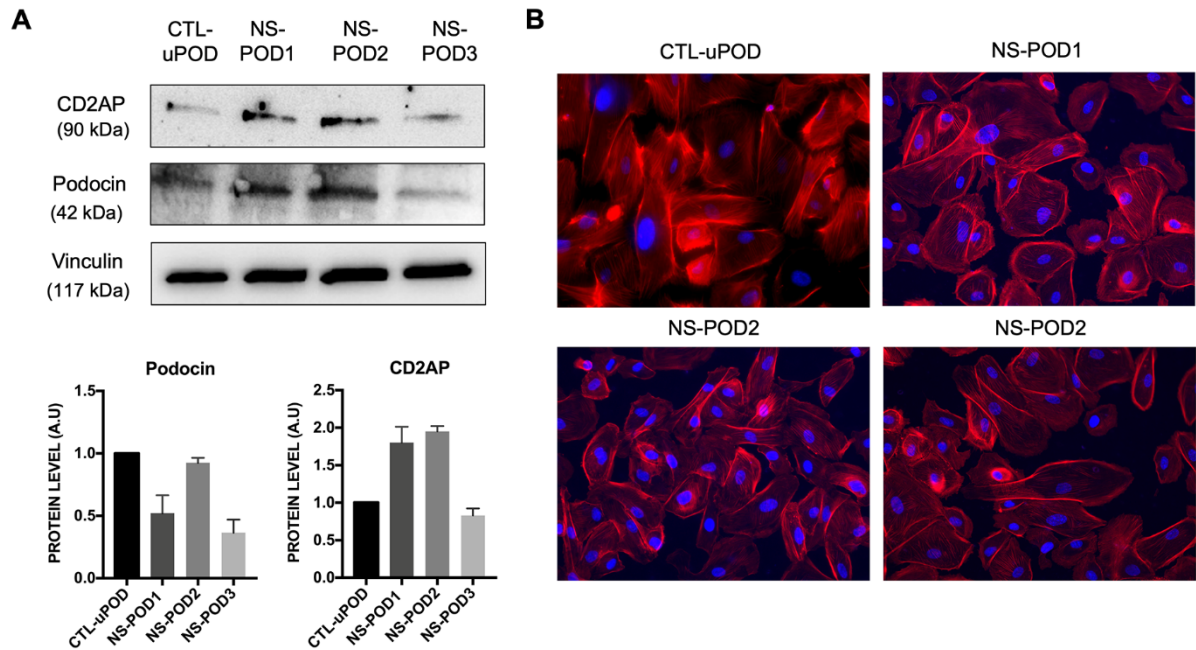


Fig 1. Isolation and characterization of podocytes from urine. *A. Western Blot analysis (representative images and quantification) of podocytes derived from urine of three different patients (NS-POD1, NS-POD2, NS-POD3) and of a healthy pregnant woman (CTL-uPOD) positive for CD2AP and podocin. Vinculin was used as housekeeping. Data are expressed as mean of two experiments $\pm$ SD. B. Representative micrographs of podocytes deriving from urine of CTL-uPOD, NS-POD1, NS-POD2 and NS-POD3 stained with phalloidin (red) and blue nuclear stain DAPI. Original magnification: X20.*

The podocyte phenotype was also assessed by analyzing the expression of a specific signature composed of 68 podocyte-typical genes (Supplementary Fig. 2), as previously outlined by Lu et al., (9, 35). All three podocyte lines expressed the typical podocyte genes, thereby confirming the podocyte phenotype (Supplementary Fig. 2).

Kidney progenitor-derived EV isolation and characterization

EVs were isolated by differential centrifugations from immortalized nKPCs. These cells were originally obtained from the urine of a preterm infant and characterized as previously described (22). The size distribution of nKPC-derived EVs was analyzed using nanosight tracking analysis (NTA), revealing a mean size distribution of approximately 142.7 nm (Fig. 2A). Western Blot analysis confirmed the presence of the EV-specific markers CD63 and TSG101, while the absence of the cytoplasmic marker calreticulin indicated the absence of cellular contamination (Fig. 2B). TEM image revealed the typical cup-shaped morphology of EVs (Fig. 2C). Additionally, super-resolution microscopy demonstrated that nKPC-EVs expressed CD9, CD63, and CD81, typical EV markers, in various combinations, predominantly being CD63 and CD9 single positive (Fig. 2D-E).

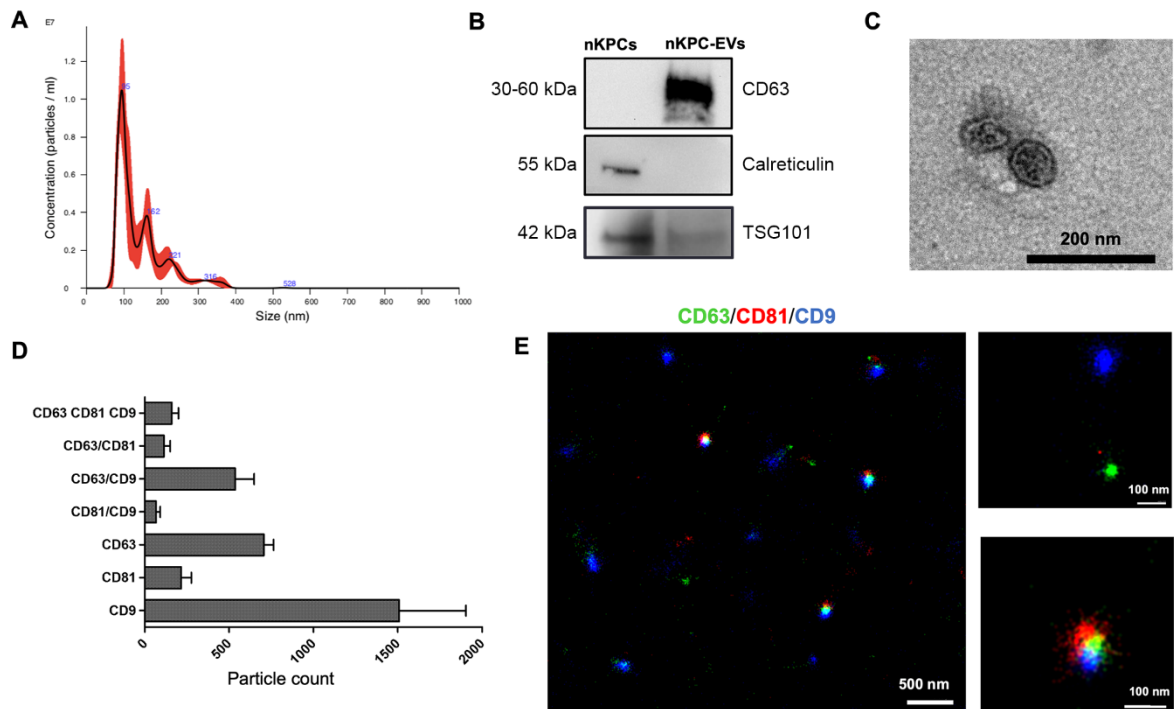


Fig. 2. Characterization of nKPC-EVs. *A. Representative NTA analysis showing the EV size distribution; EV mean size is about 142.7 nm. B. Representative Western Blot images showing the presence in nKPC-EVs of CD63 and TSG101, and the absence of calreticulin. C. Representative micrograph of TEM of nKPC-EVs (scale bar: 200 nm). D. Clustering analysis of super-resolution microscopy images shows the single, double, and triple positive EV fractions expressing the tetraspanins (CD9, CD63, CD81). The analyses were performed in 3 nKPC-EV preparations using a CODI software; the graph shows the mean $\pm$ SD of a cumulative analysis of 3 fields for each preparation. E. Representative super-resolution microscopy*

images of nKPC-EVs showing expression of CD63 (green), CD81 (red) and CD9 (blue). The scale bars are below each EV image.

The expression of mesenchymal and renal progenitor markers in nKPCs and deriving EVs was also evaluated. Notably, nKPCs exhibited a mesenchymal phenotype, expressing CD90, CD73, CD146, and CD29 (Fig. 3A), along with the cytoplasmic nephron progenitor marker SIX2 and stromal progenitor marker FOXD1 (Fig. 3B), as previously reported (22). Conversely, nKPCs were negative for markers associated with adult renal progenitor cells, such as CD133 (Fig. 3A) (36). Similarly, using a bead-based immunocapture assay, nKPC-EVs were found to express mesenchymal stromal markers CD146, CD29, and CD44, in addition to the typical EV markers (CD9, CD63, and CD81). At variance, no expression of CD133 or stage-specific embryonic antigen-4 (SSEA-4) was detected (Fig. 3C). Furthermore, nKPC-EVs expressed SIX2 and FOXD1 mRNAs, as demonstrated by RT-PCR, as the originating cells (Fig. 3D).

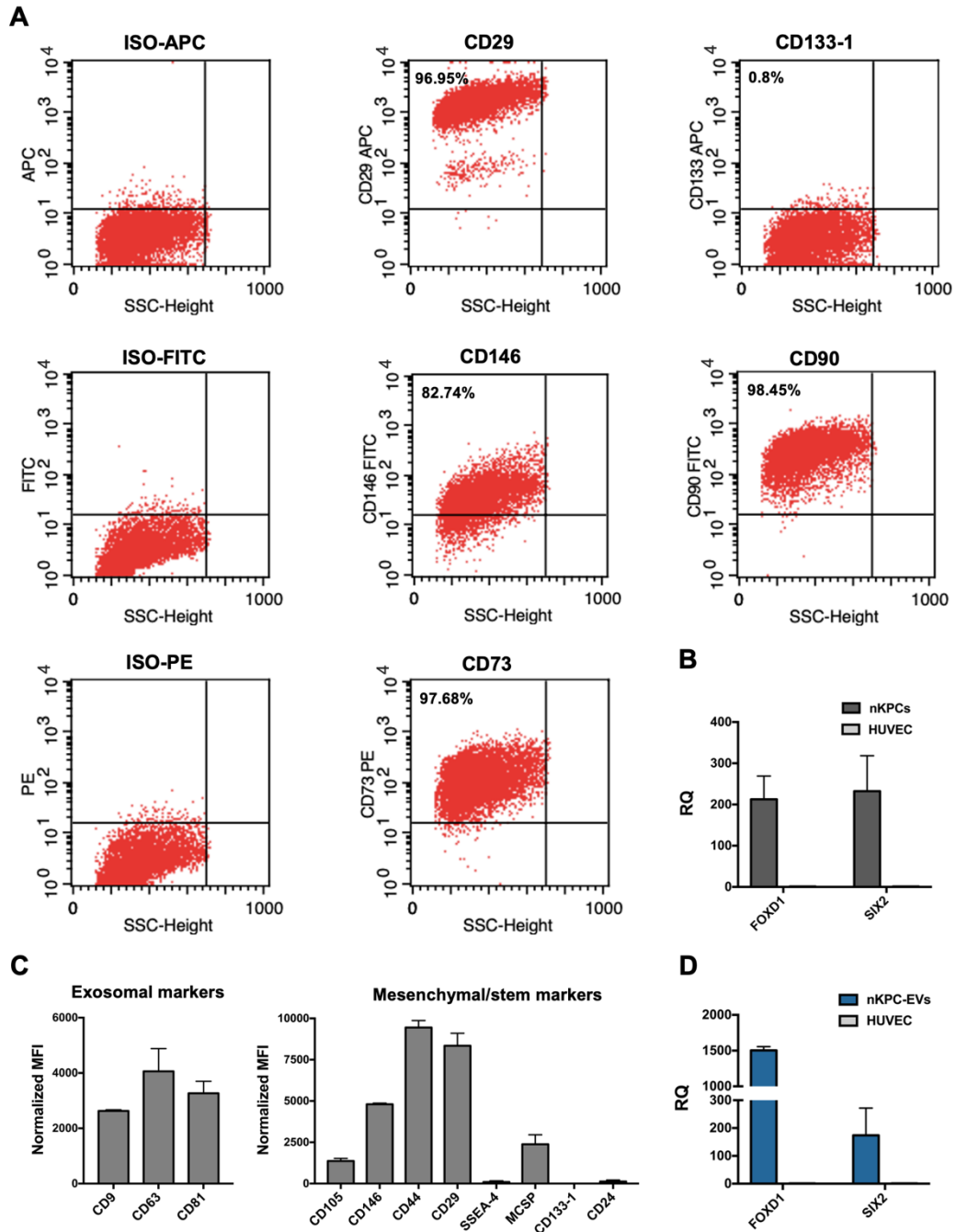


Fig. 3. nKPC and nKPC-derived EV characterization of intracellular and surface markers expression. **A.** Representative flow cytometry dot plots of nKPCs showing positive expression of CD73, CD90, CD29 and CD146; nKPCs resulted negative for CD133 marker. **B.** RT-PCR analyses showing the expression of FOXD1 and SIX2 in nKPCs. GAPDH was used as endogenous normalizer. Data were further normalized to HUVEC cells, used as a negative control for each experiment. The graphs show the RQ average ($2^{-\Delta\Delta C_t}$) of three independent experiments $\pm$ SD. **C.** Quantification of the median APC fluorescence for each bead population after background correction of exosomal and mesenchymal/stem cell markers in nKPC-EVs.

The fluorescence intensity of each marker was normalized to the mean fluorescence intensity of all detectable markers to 1000. Data are expressed as the average of two technical replicates $\pm$ SD. **D.** RT-PCR analysis showing the expression of *FOXD1* and *SIX2* in nKPC-EVs. *GAPDH* was used as endogenous normalizer. Data were further normalized to HUVEC cells, used as a negative control for each experiment. The graphs show the RQ average ($2^{-\Delta\Delta C_t}$) of two independent experiments $\pm$ SD.

Permeability analysis of podocyte cultures

To evaluate whether nKPC-EVs could modulate permselectivity of the patient-derived podocytes, we used an *in vitro* permeability assay. Podocytes were seeded on the upper side of inserts and permeability was assessed by measuring the transit of FITC-BSA from the lower compartment to the upper podocyte compartment as described in the graphical representation (Fig. 4).

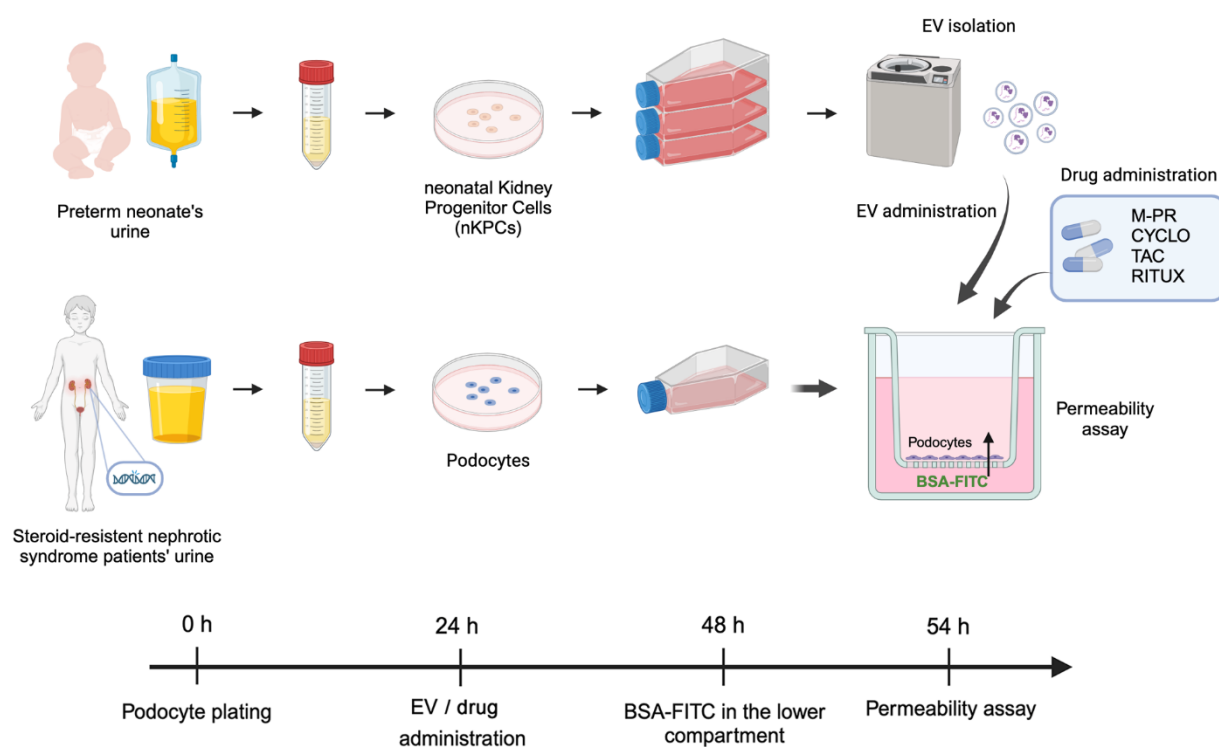
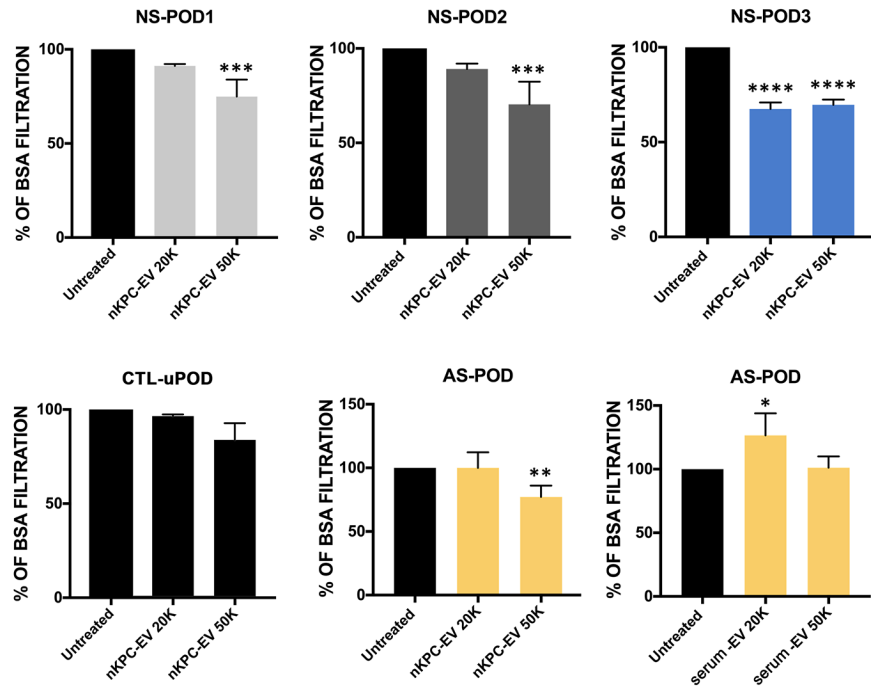


Fig. 4. Schematic experimental design and graphic representation of culture set-up. Graphical representation of nKPC and patient-derived podocyte isolation and EV production. Podocytes were plated in the upper compartment of transwell inserts and treated with EVs or drugs. For the permeability assay, the BSA-FITC filtration was measured. Abbreviations: NS-

POD: nephrotic syndrome podocytes; AS-POD: Alport syndrome podocytes; CTL-uPOD: control urinary podocytes; M-PR: methylprednisolone; CYCLO: cyclosporin; TAC: tacrolimus; RITUX: rituximab. The figure was created with Biorender.com.

To evaluate the efficacy of nKPC-EV administration in the modulation of podocyte permeability, experiments were performed using two different EV doses (2×10^4 and 5×10^4 EVs/cell). A significant reduction of permeability was observed using the 5×10^4 EVs/cell dose on all NS-POD lines, as well as on AS-POD (Fig. 5A). No significant changes were observed on control urine podocytes (CTL-uPOD) (Fig. 5A). The reduction of permeability was lacking when serum-EVs were used on AS-POD. Moreover, nKPC-EV effect was compared to that of commonly used drugs for treating renal diseases: methylprednisolone (M-PR), cyclosporin (CYCLO), tacrolimus (TAC), and rituximab (RITUX). Indeed, all these drugs have been reported to exert a direct anti-proteinuric action on podocytes, beside immunomodulation (37–39). No effect on podocyte viability was observed after drugs treatment (Supplementary Fig. 3). No modulation of permeability by pharmacological treatments was observed in the genetically altered podocytes, except for NS-POD3 (Fig. 5B). Interestingly, the patient generating NS-POD3 cells clinically responded to treatment with steroids, cyclosporin and rituximab, showing at 48 months a clinical remission (see methods and Table I). Permeability of normal podocytes (CTL-uPOD) was significantly lower in respect to diseased podocytes (Fig. 5B), as described (9).

A



B

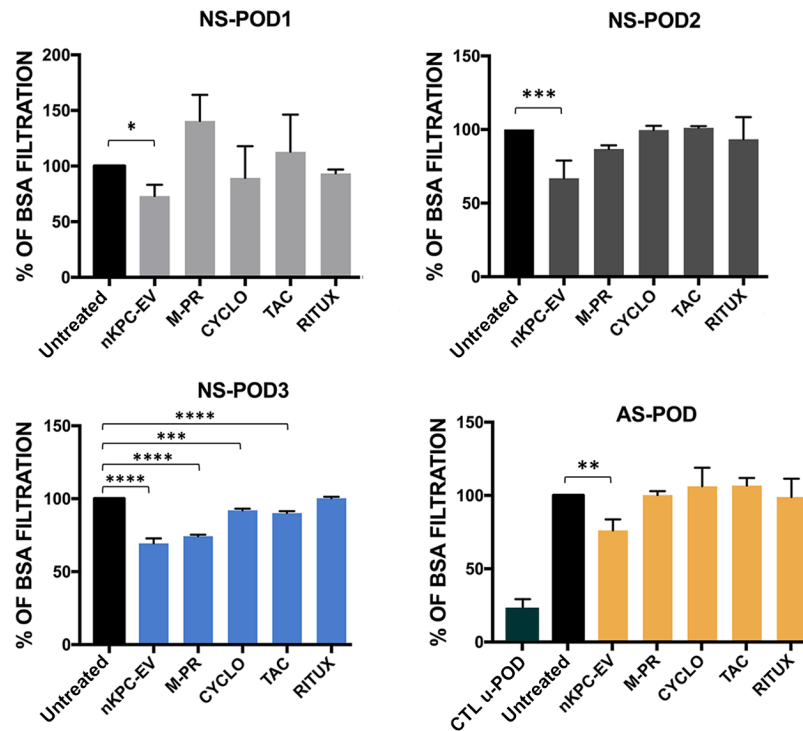


Fig. 5. Permeability assay in podocyte cultures treated with nKPC-EVs and different drugs.
A. Podocytes were treated with different doses of nKPC-EVs (20K: 2×10^4 EVs/cell, 50K: 5×10^4 EVs/cell). Serum-EVs were used as control. Untreated cell condition was used as control

for each experiment, set as BSA filtration rate of 100%. Data are expressed as the mean amount of filtered BSA-FITC of four different experiments using at least three inserts for each condition in each experiment $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was performed after the normalization of each experiment to untreated podocytes; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, vs the respective untreated condition. **B.** Podocytes were treated with nKPC-EVs (5×10^4 EVs/cell) or the different drugs methylprednisolone (MPR, 40 $\mu\text{g/mL}$), cyclosporin (CYCLO, 25 $\mu\text{g/mL}$), tacrolimus (TAC 1 ng/mL) and rituximab (RITUX 12 $\mu\text{g/mL}$). Untreated cell condition was used as control for each experiment, set as BSA filtration rate of 100%. Data are expressed as the mean amount of filtered BSA-FITC of four different experiments using at least three inserts for each condition in each experiment $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was performed after the normalization of each experiment to untreated podocytes; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, vs the respective untreated condition.

Transcriptomic analysis of urine-derived podocytes treated with EVs

To explore the mechanism involved in the observed effect on permeability, we analyzed the change in the RNA profile of the NS-POD and AS-POD after treatment with 5×10^4 nKPC-EVs/cell. The transcripts per kilobase million (TPM) normalization method was employed to compare changes in the gene expression profile between the samples. Our analysis revealed a total of 2876 upregulated genes ($\log_2$ fold change >1) and 2574 downregulated genes ($\log_2$ fold change <-1) in response to EV treatment (Fig. 6A-B). Gene Ontology analysis highlighted cell-to-cell adhesion as the primary upregulated biological activity in treated podocytes (Fig. 6C-D). Only two genes, SUMO1 and SENP2, resulted to be commonly upregulated in all four samples post nKPC-EV treatment (Fig. 6F), showing a $\log_2$ fold change ranging between 3.07 and 3.42 (Fig. 6E-F). At variance, no gene was consistently downregulated in all patient-derived podocyte cell lines (not shown). Interaction network analysis demonstrated a direct interaction between these two genes in the SUMOylation pathway (40-41).

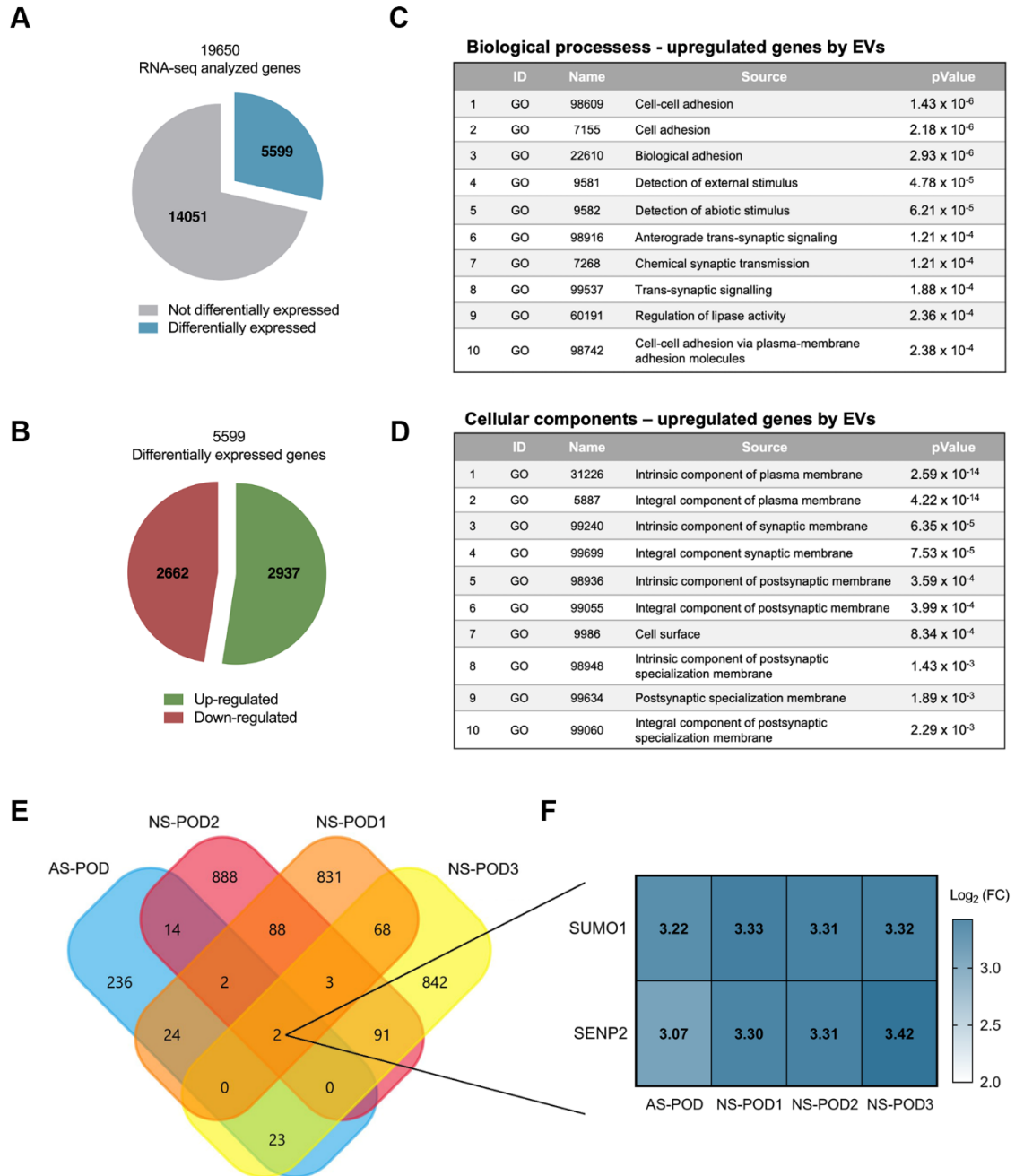


Fig. 6. Cross-analysis for the identification of regulated genes common to the four patient-derived podocytes. **A.** Pie chart representing the sum of all the genes differentially expressed in the four different podocyte lines after the treatment with nKPC-EVs (in blue), compared with the non-differentially expressed genes (grey). **B.** Pie chart representation of up-regulated (green) and down-regulated (red) differentially expressed transcripts in the four different podocyte lines untreated and treated with EVs. **C and D.** Gene Ontology analysis of differentially expressed genes in the four different podocyte lines untreated and treated with EVs. In each table, the identification (ID) number, the name, and the P value associated with the GO are given. **E.** Representative Venn diagram showing the numbers of the genes that

resulted up-regulated after the treatment of the podocytes with nKPC-EVs by total RNA sequencing analysis. Data were analyzed using Expression Suite and Funrich V3 Software. **F.** Heatmap showing the levels of expression regulation of the two genes which were upregulated in all the four podocyte lines.

The nKPC-EV-induced up-regulation of both SUMO1 and SENP2 in urine-derived podocytes was confirmed by RT-PCR, where the up-regulation reached significance in three podocytes lines from nephrotic syndrome patients (Fig. 7A-B). No effect on SUMO1 or SENP2 modulation was observed using MSC-EVs (not shown). Subsequently, we aimed at confirming the relevance of SUMO1 and/or SENP2 on podocyte function. For this purpose, we performed the permeability assay using CTL-tPOD that had been downregulated for either SUMO1 or SENP2 genes by transfection with siRNA (Fig. 7C). Permeability assay revealed an increased BSA-FITC permeability in CTL-tPOD transfected with SENP2 siRNA either alone or combined with SUMO1 siRNA (Fig 7D).

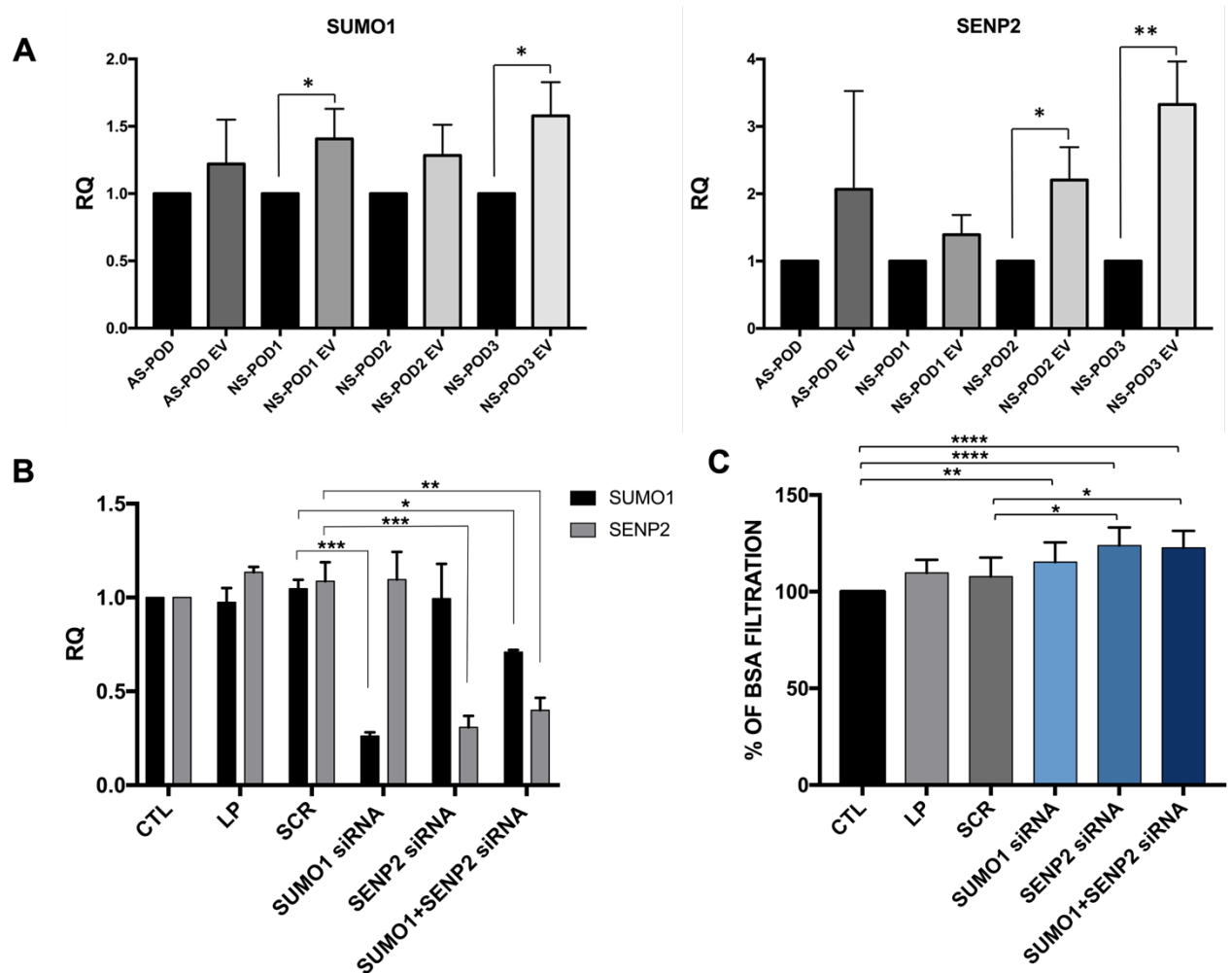


Fig. 7. Role of SUMO1 and SENP2 in podocyte permeability. A-B. Validation of differentially expressed genes in urine-derived podocytes treated with EVs. mRNA expression of SUMO1 (A) and SENP2 (B) genes in urine-derived podocytes treated or not with nKPC-EVs. Data are shown as relative quantification, normalized to GAPDH and to each untreated control respectively set as 1. The graphs show the RQ average ($2^{-\Delta\Delta C_t}$) of at least three independent experiments $\pm$ SD. Unpaired t-test was performed after the normalization of each experiment to its untreated condition (AS-POD, NS-POD1, NS-POD 2, NS-POD3, respectively); * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ vs AS-POD, NS-POD1, NS-POD 2, NS-POD3, respectively.

C. RT-PCR analysis of SUMO1 and SENP2 genes in CTL-tPOD after siRNA transfection. Data are shown as relative quantification, normalized to GAPDH and to the untransfected control (CTL), set as 1. The graphs show the RQ average ($2^{-\Delta\Delta C_t}$) of two independent experiments $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was performed; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs SCR.

D. Permeability assay using CTL-tPOD transfected with siRNA targeting SUMO1 and/or SENP2 genes. Data are expressed as the mean amount of filtered BSA-FITC of at least six different experiments for each condition. One-way ANOVA with Dunnett's multiple comparisons test was performed after the normalization of each experiment to untransfected podocytes (CTL); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, vs SCR. Abbreviations: CTL: untransfected CTL-tPOD; LP: CTL-tPOD treated only with Lipofectamine reagent; SCR: CTL-tPOD transfected with a scramble sequence; SUMO1 siRNA: CTL-tPOD transfected with siRNA targeting SUMO1; SENP2 siRNA: CTL-tPOD transfected with siRNA targeting SENP2; SUMO1+SENP2 siRNA: CTL-tPOD transfected with the combination of siRNA targeting SUMO1 and siRNA targeting SENP2.

Discussion

The current study provides a novel approach to directly assess the potential of regenerative therapies on patient-derived podocytes with genetic alterations. We demonstrated the efficacy of EVs derived from neonatal renal progenitor cells in improving permeability in podocytes obtained from patients with steroid-resistant nephrotic syndrome. Furthermore, we identified the SUMOylation pathway as a possible common mechanism in all patient-derived cell lines treated with nKPC-EVs.

The availability of human podocytes that accurately replicate human pathologies in culture is currently limited. Recent advancements have allowed the derivation of podocyte-like cells from induced pluripotent stem cells from patients, offering a promising avenue (42). However, obtaining a purely homogeneous population of podocytes is challenging; typically, only 30-50% of cells in induced pluripotent stem cell cultures exhibit podocyte-like morphology (43). Another potential source for modeling diseases is the differentiation of urinary renal progenitor cells into podocytes, although this process may involve cell culture and differentiation stimuli (44).

Previous studies have utilized urine as a non-invasive and valuable source to obtain podocytes (12). In patients with active glomerular diseases, podocytes are shed from the glomerulus in response to local environmental factors. Urine patient-derived podocytes have been demonstrated to be positive for podocyte markers, viable, and capable of growth in culture (12). Conversely, due to their limited number and limited replication potential in culture, podocytes cannot be obtained from normal subjects' urine (12). Of interest, increased podocyturia is observed in the urine of uncomplicated pregnant women (25), making it a valuable source for obtaining healthy control podocytes.

In this context, our *in vitro* model of glomerular permeability utilizing podocytes directly obtained from patients' urine holds significant promise for personalized medicine. This study includes podocytes with mutations affecting a slit diaphragm protein (podocin), matrix synthesis components (collagen IV and laminin) and a calcium balance regulator (Phospholipase C epsilon 1). We here showed the feasibility of a patient-specific permeability model that could be used in personalized medicine to evaluate the impact of drugs commonly administered to patients with nephrotic syndrome. Notably, podocytes derived from steroid resistant patients, unresponsive to therapies, also did not respond to the drugs previously reported to modulate permeability (37–39). An effect on permeability was only observed on the

podocyte line from a patient who, despite initially clinically steroid-resistant, exhibited subsequent clinical improvement with minimal proteinuria after 48 months (NS-POD3). In the future, a personalized approach could potentially set the stage for tests capable of predicting patient responses, guiding clinicians in treatment decisions, and avoiding unnecessary immunosuppressive interventions in the management of nephrotic syndrome. It must be however underlined that the short survival of those primary lines only allows a restricted number of tests.

Another potential application of the primary model of the glomerular filtration barrier is the evaluation of innovative biological therapeutics. We here assessed the effect of nKPS-EVs on genetically mutated patient-derived podocytes. Our findings indicate that nKPCs could serve as a valuable source of EVs with regenerative potential. Interestingly, no effect on healthy podocytes was observed, further supporting the importance of disease models to assess therapeutic approaches. Moreover, in consideration to the variability of single line response, the use of different primary cell lines is instrumental to identify a possible nKPC-EV specific mechanism of action.

EVs, in particular those obtained from MSCs, have been previously studied in *in vitro* and *in vivo* experimental models of kidney disease (19,45-49). Using a millifluidic model of glomerular filtration barrier, we previously demonstrated that MSC-EVs could traverse the basal membrane, reach podocytes, and transfer their RNA cargo, resulting in protection against doxorubicin-induced injury (19). Other studies have underscored the protective roles of MSC-EVs on mouse podocyte lines using a model of diabetic nephropathy (48,49). However, the identification of specific mechanisms involved in the effect of EVs on target cells appears quite challenging, in consideration to the multitude of EV cargoes and to the patient-derived cell heterogeneity.

In our approach, we searched for common nKPC-EV-regulated genes at the transcriptomic level. Of interest, SUMO1 and SENP2, its modulator, were identified as the only common genes concordantly regulated in all patient-derived podocyte lines. At variance, no SUMO or SENP2 modulation was detected using MSC-EV treatment. Although we did not identify the factors involved in the specific effect of nKPS-EVs on podocytes due to the complexity of EV cargo, it might be speculated that factors linked to their renal progenitor origin, such as SIX2 expression, could promote SUMO1 and SENP2 expression, known to be relevant in epithelial cell differentiation (23). This could be supported by data obtained using the EV originating cells, nKSPCs, administered to human kidneys discarded for transplantation.

In this study, the *de novo* expression of SIX2 in proximal tubular cells and upregulated regenerative markers, including SOX9, was observed (23).

SUMOylation pathway is considered to be involved in slit diaphragm protein stabilization, thus playing a possible role in the control of glomerular permeability (50). Further studies will be necessary to better elucidate this point. The activity of SUMO, or SUMOylation, consists in a post-translational modification that alters the function of target proteins and modulates cell processes such as protein stability, localization, and activity (50,51). Since SENP2 is a known regulator of SUMO1 (39), it may be indirectly involved in slit diaphragm stabilization as well. This was also confirmed by experiments of SUMO1 and SENP2 downregulation, demonstrating their involvement on podocyte permeability in our *in vitro* model. Concordantly, Gene Ontology analysis highlighted cell-to-cell adhesion as the primary upregulated biological activity in nKPC-EV treated podocytes. Therefore, the potential effect of EVs derived from kidney progenitors on genetically altered podocytes represents a promising strategy for addressing genetic pathologies currently lacking a therapeutic solution. While this strategy was tested on three podocyte lines in this study, further validation and expansion to a larger patient cohort are warranted.

Conclusion

In conclusion, we here set up a human *in vitro* functional model for the analysis of drug response on albumin permeability, thanks to combined effort of clinical, genetic and basic science. Our findings demonstrated the positive impact of nKPC-EVs in improving the function of genetically altered podocytes and identified the effect of EVs on the regulation of SUMOylation, an important pathway for stabilizing podocyte slit diaphragm proteins.

Our data may pave the road for establishing a standard non-invasive *in vitro* model for the screening of regenerative compounds directly on patient-derived podocytes.

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Thesis conclusions

In conclusion, during my PhD I had the opportunity to investigate different roles of urine that extend beyond its conventional definition as waste. Indeed, urine stands as a precious source either for uEV biomarkers, renal progenitor cells and patient-derived podocytes.

Concerning the first part, two flow cytometry-based methods were tested to detect surface biomarkers on uEVs: MACSPlex (effective but with some limitations) and FC-MSP-bead assay (promising but requiring further optimization). Both methods were tested on two cohorts of patients. In acute kidney injury (AKI) patients, SSEA-4 emerged as a potential positive prognostic biomarker for kidney recovery after acute damage, as shown with MACSPlex. In kidney transplanted (KT) patients, CD69 was identified as a potential marker for monitoring renal injury in the post-transplantation phase, as confirmed with both assays.

In the second part, neonatal renal progenitor derived-EVs (nKPC-EVs) were tested in vitro on patient-derived genetically altered podocytes. The EV treatment was shown to improve the podocytes permeability function and to regulate SUMOylation, a key pathway for stabilizing podocyte slit diaphragm proteins. These results could contribute to establishing a standardized, non-invasive in vitro model for screening regenerative compounds, as stem cell-derived EVs, directly on patient-derived podocytes.

The innovative and promising applications I explored in EV field aimed at addressing the urgent need for new therapies and diagnostic approaches for kidney diseases. Given their innovative nature, these applications demand considerable effort and rigorous validation to ensure their efficacy and reproducibility, in order to be translated into the clinic.

Manuscript publications

Tanzi A*, Buono L*, Grange C, Iampietro C, Brossa A, Arcolino FO, Arigoni M, Calogero R, Perin L, Deaglio S, Levtchenko E, Peruzzi L, Bussolati B. Urine-derived podocytes from steroid resistant nephrotic syndrome patients as a model for renal-progenitor derived extracellular vesicles effect and drug screening. *J Transl Med.* 2024 Aug 14;22(1):762. doi: 10.1186/s12967-024-05575-z. PMID: 39143486; PMCID: PMC11323595.

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