

REVIEW

Exosomes and microvesicles in kidney transplantation: the long road from trash to gold

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Summary

Kidney transplantation significantly enhances the survival rate and quality of life of patients with end-stage kidney disease. The ability to predict post-transplantation rejection events in their early phases can reduce subsequent allograft loss. Therefore, it is critical to identify biomarkers of rejection processes that can be acquired on routine analysis of samples collected by non-invasive or minimally invasive procedures. It is also important to develop new therapeutic strategies that facilitate optimisation of the dose of immunotherapeutic drugs and the induction of allograft immunotolerance. This review explores the challenges and opportunities offered by extracellular vesicles (EVs) present in biofluids in the discovery of biomarkers of rejection processes, as drug carriers and in the induction of immunotolerance. Since EVs are highly complex structures and their composition is affected by the parent cell's metabolic status, the importance of defining standardised methods for isolating and characterising EVs is also discussed. Understanding the major bottlenecks associated with all these areas will promote the further investigation of EVs and their translation into a clinical setting.

Key words: Extracellular vesicles; kidney transplant; rejection; diagnosis; therapeutics.

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INTRODUCTION

Kidney transplantation (KT) is the preferred treatment for end-stage renal disease as it offers patients the potential for longer life expectancy and improved quality of life when compared with alternatives such as dialysis.^{1,2} However, all allografts are vulnerable to immune rejection processes, including acute (AR) and chronic rejection (CR), which can lead to allograft loss.³ To counter these processes, patients are

maintained on immunosuppressive drugs, with the types of drug used dependent on the associated mechanism of rejection. Drug dosage must be adapted to the patient's metabolic profile and should be high enough to reduce the rejection process but also below a defined level so as to minimise long-term toxic side effects.⁴

To predict early events associated with allograft rejection, it is critical that patients are monitored frequently for biomarkers of these events, ideally using non-invasive or minimally invasive processes such as analysis of biofluids. This higher monitoring frequency allows detection of early rejection events and facilitates prompt dose or therapeutic modification, supporting more precise, individualised medicine. To reduce the use of immunosuppressive drugs, it is also necessary to promote new strategies for inducing allograft immunotolerance. For both biomarker discovery and the development of new immune-based therapies, extracellular vesicles (EVs) present in biofluids such blood and urine should be explored.

EVs are a family of particles that, under specific conditions, are released from cells, reaching biofluids such as blood and urine.^{5,6} EVs present specific exterior receptors and have specific molecular contents including genetic material, such as messenger RNA (mRNA) and microRNAs, peptides and proteins, such as cytokines or chemokines, lipids, carbohydrates and other metabolites.^{7–9} Once released from the parent cell and, based on their exterior layer, EVs can recognise specific cells via interaction with their receptor ligands, leading to a cellular stimulus response and/or to material transfer to target cells.^{10,11} Therefore, EVs function as a key player in intercellular communication. Indeed, EVs have been explored as a potential source of biomarkers for diagnosis and prognosis of diverse diseases, such as neurodegenerative diseases,^{12–14} cancers^{15–17} and renal diseases,^{18,19} and for allograft transplantation.²⁰ Likewise, because of their low immunogenicity,²¹ exceptional biocompatibility^{22–26} and high bioavailability,²⁷ EVs have been investigated as therapeutic strategies, including for drug

delivery, with applications in neurology,²⁸ oncology²⁹ and other diseases.^{30–33}

In the field of transplantation, EVs have been identified as one of the major players in the alloimmune response. Their influence in allograft rejection may occur via their ability to deliver donor major histocompatibility complex (MHC) and other molecules that interact with receptors on antigen-presenting cells, which subsequently promote activation of the immune system towards allograft rejection.^{34–36} EVs play an active role in antigen presentation to T cells, by direct or indirect allorecognition pathways,³⁷ and in B cell regulation.³⁸ For example, EVs released from B cells will present MHC class II antigens, leading to CD4+ T-cell responses, while EV proteasome activity can control humoral responses.³⁹

There are diverse works exploring EVs as biomarkers for predicting graft status and rejection processes, as reviewed by Mirzakhani *et al.*,²⁰ including in heart,⁴⁰ lung⁴¹ and liver transplantation.^{42,43} For example, in heart transplantation, Castellani *et al.*,⁴⁰ Sukma Dewi *et al.*⁴⁴ and Kennel *et al.*⁴⁵ developed predictive models of antibody-mediated rejection (ABMR) or acute cellular rejection (ACR) based on EVs present in plasma or serum. Also, with regard to the therapeutic potential of EVs, several studies have highlighted the use of exosomes obtained from mesenchymal stem cells (MSCs) for the treatment of immune diseases – for example, to reduce acute graft-versus-host disease in haematopoietic stem cell transplantation⁴⁶ and to reduce rejection of allograft corneas.⁴⁷

This review focuses on applications for EVs in kidney transplantation, including their use as biomarkers for diagnosis and prognosis of rejection processes and as a therapy-focused tool. The nomenclature, isolation, detection and characterisation of EVs will also be discussed.

NOMENCLATURE

Originally, EVs were classified as ‘garbage bins’, responsible for the removal of cellular waste debris,⁴⁸ or as ‘virus-like particles’.⁴⁹ In the late 1960s and early 1970s, several researchers attributed other functions to these particles. For instance, Wolf⁵⁰ attributed clotting properties to EVs derived from platelets. The functions of EVs relating to cellular communications were elucidated more recently, subsequent to knowledge of their biogenesis.⁵¹ Currently, it is accepted

that the contents of EVs are highly dependent on the parent cells and those cells’ metabolic status, which ultimately depends on the pathophysiological state of the organism as a whole. It is also accepted that EVs act as vehicles for passing information between cells at both near and long distances.^{52,53}

Depending on their biogenesis, release pathways, size and contents, EVs can be classified as exosomes, microvesicles or apoptotic bodies (Table 1). Exosomes range in size from 30–200 nm and are formed in the endosomal system. Their main functions relate to protein processes such as recycling, storage, transport and release,⁵⁴ and to the transport of genetic material such as mRNAs and microRNAs.⁵⁵ Various types of molecules have been identified in exosomes from different cell sources, including 41,860 proteins, 7,540 RNAs, 764 microRNAs and 1,116 different lipids, demonstrating their complexity and functional diversity.^{56,57} Microvesicles are bigger in size, varying from 100–1,000 nm, and are formed by the outward blebbing of the plasma membrane. They are involved in local and distant cell–cell communication.⁵⁸ Apoptotic bodies are large particles, between 1–5 µm in size, originating from cell shrinkage and programmed cell death (apoptosis).⁵⁴ While exosomes and microvesicles mainly carry proteins, mRNA, microRNA, lipids and metabolites, apoptotic bodies include cell remains such as organelle fragments and nuclear fractions.^{11,58}

There are also some differences in the composition of the lipid membrane of EVs. The membrane of exosomes includes cholesterol, ceramide and phosphatidylserine, while the membrane of microvesicles includes phosphatidylserine enriched in cholesterol and diacylglycerol.⁵⁹

ISOLATION, DETECTION AND CHARACTERISATION

A critical bottleneck in developing the potential of EVs is the wide variety of methods currently used for their isolation and characterisation, associated with incomplete descriptions of the methods used and the lack of standardised procedures applied by different laboratories.⁶⁰ For example, even if a specific isolation method is used, such as ultracentrifugation, diverse variations are applied relating to time, speed, temperature and other parameters. As such, and to promote the potential of EVs, it is worth highlighting the Minimal Information for Studies of Extracellular Vesicles (MISEV)

Table 1 Classification of extracellular vesicles according to biogenesis, dimension and membrane characteristics

	Dimension and origin	Pathways	Membrane specifications
Exosomes	30–200 nm Intraluminal vesicles (from vesicular bodies) that fuse with plasma membrane	Dependent on: • Endosomal sorting complex required for transport • Tetraspanin • Ceramide • Stimuli	Enriched cholesterol and ceramide Expose phosphatidylserine with lipid rafts
Microvesicles	100–1,000 nm Outward blebbing of plasma membrane with cellular content	Dependent on: • Ca2+ • Stimuli and cell dependent	Enriched in cholesterol and diacylglycerol Expose phosphatidylserine with lipid rafts
Apoptotic bodies	1–5 µm Plasma membrane and cellular fragments from cell shrinkage and apoptosis	Apoptosis	–

Table adapted from Ståhl *et al.*¹²¹

guidelines released in 2018 by the International Society for Extracellular Vesicles (ISEV), which relate to the isolation and characterisation of EVs.^{61,62} The MIFlowCyt-EV framework, developed by a working group of EV flow cytometry researchers, provides a consensus framework for the minimum information that should be given regarding flow cytometry applied to EVs.⁶⁰ It is intended that these guidelines will be refined and revised periodically. In addition, the EV-TRACK (Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research) initiative provides a centralised open-access platform for documentation associated with EVs, to facilitate standardisation of EV research.⁶³

Processes for isolating EVs are based on their size or density (e.g., ultracentrifugation, centrifugation based on density gradient, filtration and size-exclusion chromatography), on their solubility (e.g., polymeric precipitation) or on their specific interactions with ligands (immunoprecipitation, affinity or ion-exchange chromatography) (Fig. 1, Table 2).^{64–67} All isolation techniques present advantages and limitations. Benefits include the rapid turnaround time observed with precipitation and immunoaffinity techniques, for example, and the high degree of sample purity achieved with immunoaffinity, ultrafiltration or centrifugation with density gradient. Examples of limitations associated with isolation techniques include the low sample volume supported by techniques such as immunoaffinity, the need for special equipment (e.g., for ultrafiltration) and scalability limitations (e.g., with ultracentrifugation) (Table 2).

The isolation method chosen should also consider subsequent processes to which the EVs will be subject. For example, despite the high purity obtained with immunoaffinity, this technique is not suitable for the isolation of large-volume samples; alternatives such as filtration-based processes exist,

although these can result in aggregates of EVs which block the filter pores.^{68,69} The integrated use of isolation methods should also be considered. For example, ultracentrifugation, although simple and one of the most-used methods, is prone to protein aggregates, leading to the lowest degree of purity of EVs in relation to other methods. The question of purity can be partly overcome using density-gradient separation or by complementing ultracentrifugation with other techniques.^{65,70} For instance, Abramowicz *et al.*⁷¹ observed that, although labour intensive, ultracentrifugation combined with iodixanol density gradient centrifugation or with gel filtration provided a superior exosome purity.^{71,72} More recently, in order to increase purity and reduce the purification turnaround time, other methodologies have been introduced, such as affinity-based methods in conjunction with separations relying on magnetic beads.⁷³

Driven by recent microfabrication techniques, the isolation of EVs via microfluidics platforms has also been explored, based on immunoaffinity principles,⁷⁴ enabling isolation directly from plasma or serum in a single step.⁷⁵ These microfluidic platforms may also enable analysis of EVs by incorporating microanalytical devices, for example,⁷⁶ leading to 'lab-on-chip' devices.

Several techniques can be employed to detect and characterise EVs, such as flow cytometry,^{60,77,78} electron microscopy (both transmission electron microscopy and cryogenic electron microscopy),^{79,80} atomic force microscopy,⁸¹ nanoparticle tracking analysis^{82,83} and resistive pulse sensing (an alternative to nanoparticle tracking analysis),⁸⁴ among others (Table 2). Some of these techniques are expensive because they require special equipment (e.g., transmission electron and atomic force microscopy) and some can be time consuming (e.g., transmission electron and atomic force microscopy). Other techniques can lead to interference between reagents (e.g., as observed with secondary antibodies in flow

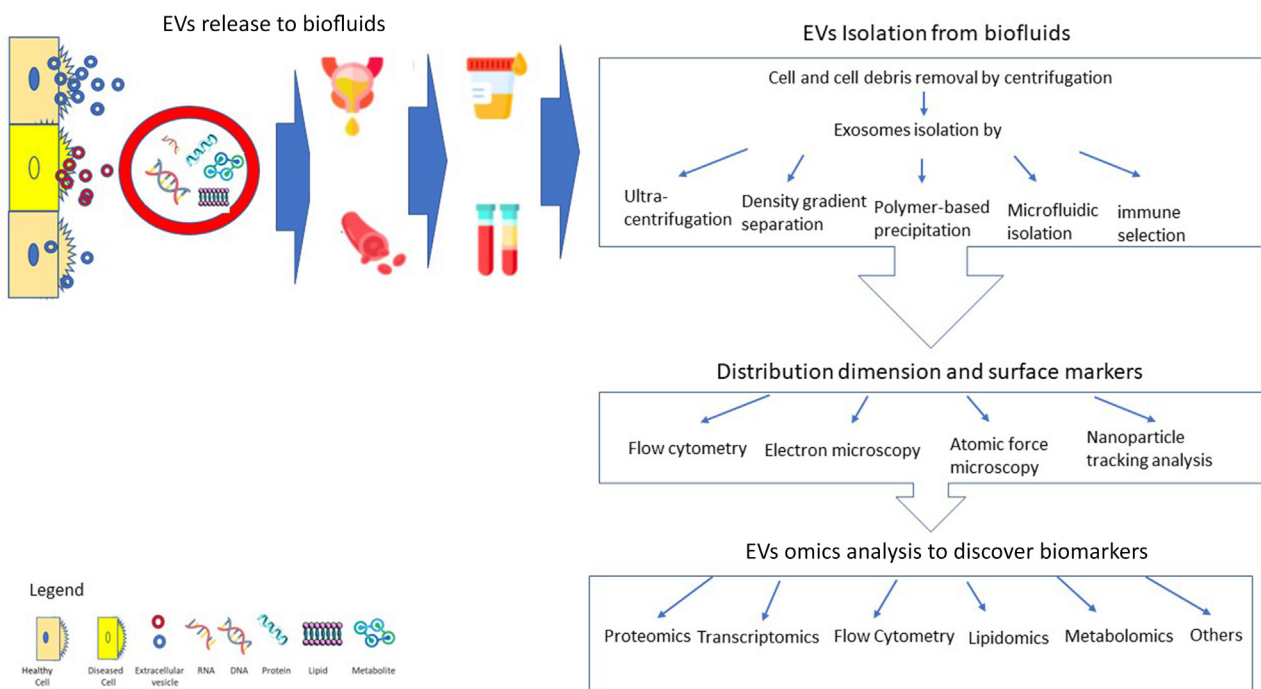


Fig. 1 Illustration of the process of isolating and characterising extracellular vesicles (EVs).

Table 2 Advantages and limitations of the main methodologies for the isolation, detection and characterisation of extracellular vesicles (EVs)

Method	Advantages	Limitations
Isolation		
Precipitation	<ul style="list-style-type: none"> • Fastest turnarounds • Straightforward workflow • No need for special equipment • Retains vesicle morphology • Process many samples 	<ul style="list-style-type: none"> • Polymer contamination • Proteins, aggregates, viruses and lipoproteins can interfere
Ultrafiltration	<ul style="list-style-type: none"> • Concentrates samples • Isolates EVs from dilute biofluids • Simple and straightforward protocol • Processes many samples • No limit on sample volume • Purity of EVs 	<ul style="list-style-type: none"> • Sample loss in filtration • Filter clogging • Some loss of vesicle morphology • Possible contamination with protein aggregates
Size-exclusion chromatography	<ul style="list-style-type: none"> • Customisable • Scalable • Preserves vesicle integrity • Reproducible • High sample recovery 	<ul style="list-style-type: none"> • Low sample volume • Single sample • Low resolution of similarly sized molecules
Ultracentrifugation	<ul style="list-style-type: none"> • Large-volume samples • No added chemicals 	<ul style="list-style-type: none"> • Special equipment • Damage to exosomes as a result of high speeds • Hard to scale up • Sample viscosity impacts sedimentation
Density gradient centrifugation	<ul style="list-style-type: none"> • Vesicle morphology intact • High purity EVs 	<ul style="list-style-type: none"> • Time consuming • Hard to scale up • Special equipment
Immunoaffinity	<ul style="list-style-type: none"> • High purity EVs • High selectivity 	<ul style="list-style-type: none"> • Low sample volume • Lack of specific markers for isolation • Antigen-antibody interaction difficult to dissociate
Detection and characterisation		
Transmission electron microscopy	<ul style="list-style-type: none"> • EV size determination • Biogenesis visualisation • Discrimination from other small particles 	<ul style="list-style-type: none"> • Special equipment • Cost • Time consuming • EVs can lose shape during preparation
Atomic force microscopy	<ul style="list-style-type: none"> • High-resolution images of EVs • Information about size and morphology 	<ul style="list-style-type: none"> • Special equipment • Cost • Time consuming
Flow cytometry	<ul style="list-style-type: none"> • Single vesicle resolution • Identification of multiple surface markers 	<ul style="list-style-type: none"> • Reagent interference due to debris • Special cytometer calibration
Nanoparticle tracking analysis (NTA)	<ul style="list-style-type: none"> • Simultaneous detection, counting, sizing and phenotyping of EVs • Antibody-mediated fluorescent labelling possible • Selectively determine and analyse specific types of particles in complex samples • High throughput 	<ul style="list-style-type: none"> • Small volume samples • Limited in multiplexing capability versus flow cytometry • Special equipment
Resistive pulse sensing	<ul style="list-style-type: none"> • Alternative to NTA for concentration and size distribution measurements 	<ul style="list-style-type: none"> • Heterogeneous populations require the combination of several nanopore membranes
Dynamic light scattering	<ul style="list-style-type: none"> • Less time-consuming • No specific expertise required 	<ul style="list-style-type: none"> • Less appropriate for heterogeneous EV populations • Analysis software prone to give inaccurate results if they are not properly calibrated or if the sample is not prepared correctly
Western blot	<ul style="list-style-type: none"> • Ideal for detection of protein markers associated with EVs • Ideal for confirming correct isolation of EVs 	<ul style="list-style-type: none"> • Not suitable for determining EV quantity • Does not exclude the presence of contaminants

Table adapted from Théry *et al.*,⁶² Witwer *et al.*⁶⁹ and Lucchetti *et al.*¹²²

cytometry and nanoparticle tracking analysis) and others imply complex sample processing (e.g., with heterogeneous EV populations when using resistive pulse sensing).

Due to the complexity of EVs, the ISEV recommends that their characterisation should be performed by at least two different methods, one being transmission electron or atomic force microscopy, in order to perform single vesicle analysis.⁶¹ The visualisation techniques will allow high-resolution observations of single EVs, providing useful information in terms of size distribution and morphology,^{61,62,85,86} while other complementary techniques will enable characterisation of the molecular composition of EVs.^{62,87}

Currently, there is no consensus about the specific markers associated with different types of EVs, as EVs do not express consistent levels of specific markers and biological samples usually present a mixture of EVs. Despite this, EVs have been classified according to their surface markers. For example, the membrane of exosomes contains CD63, CD9, CD81, CD82, HSP70, HSP90 and MHC class I molecules, while that of microvesicles usually contains CD40, selectins and integrins.^{6,88} In characterising EVs, the ISEV recommends that researchers should evaluate both the presence and absence of EV-specific markers in a semi-quantitative manner.⁶¹ This recommendation suggests the analysis of at

least three proteins, especially transmembrane and cytosolic proteins with membrane-binding capacity [e.g., tetraspanins (CD9, CD63, CD81) and integrins] and determination of the presence of unexpected proteins associated with compartments other than the plasma membrane or endosome.⁶¹

'OMICS' IN THE CHARACTERISATION OF EVs

Diverse technologies, including 'omics'-based methodologies, can be used to analyse the surface and contents of EVs in the search for biomarkers. As these contents can be RNA, DNA, proteins and even metabolites, methodologies such as transcriptomics, genomics, proteomics, lipidomics and metabolomics can all be applied.

In the case of transcriptomics, special attention must be paid to obtaining purified RNA, as it is possible to introduce study bias as a result of differences in experimental methodologies, which will subsequently lead to discrepant results between studies. The main analytical methodologies applied have been microarray analysis, quantitative reverse transcriptase-polymerase chain reaction (PCR), next-generation sequencing and digital droplet PCR.^{72,89,90}

Proteomics may present some advantages when compared to genomics or transcriptomics. Because it allows evaluation of factors such as alterations at the transcriptional level (e.g., resulting from alternative splicing) or protein post-translational modification, it more closely represents the phenotype. The most common proteomics techniques are 2D electrophoresis, 2D western blotting and chromatography associated with mass spectrometry-based methods. With these methodologies, special care must be taken when isolating EVs, as some isolation methods may be more suitable for subsequent proteomic analysis than others. As reviewed by Li *et al.*,⁹¹ contaminants present in EV samples may mask the signal of smaller EV proteins in the course of mass spectroscopy analysis, making data analysis more challenging.

Even though lipidomics and metabolomics have been less exploited, various researchers have employed these strategies. Lipids are essential molecular components of EVs, being able to form the bilayer membrane that protects the encapsulated material, while being themselves part of the cargo of EVs. These facts have brought lipidomics to the attention of several researchers.^{92,93} In the case of metabolomics, attention is focused on intermediate and final products of cell metabolism, including, for example, amino acids, redox regulatory molecules or sugars.⁹⁴ Mass spectrometry-based methodologies associated with chromatography have been the most frequently used techniques.

The following sections present some examples of the application of 'omics' science to EVs in the discovery of biomarkers for kidney transplantation.

EVs AS A DIAGNOSTIC AND PROGNOSTIC TOOL IN KIDNEY TRANSPLANTATION

EVs can be an efficient source of biomarkers as they can present a higher concentration of target molecules when compared to the corresponding biofluid.^{95–98} For example, Alvarez *et al.*⁹⁹ observed that neutrophil gelatinase-associated lipocalin (NGAL) in urinary EVs could be used as a biomarker of graft function, whereas NGAL

concentration in urine was negligible and consequently could not be used as a biomarker. Sigdel *et al.*¹⁰⁰ predicted AR based on target proteins present in urinary exosomes, but not in urine. Bruschi *et al.*¹⁰¹ also observed minimal overlapping among the proteome of urine EVs and urine (28% of proteins of the total 959 proteins analysed).

Some approaches based on EV analysis have been focusing on the quantity of EVs. For example, Park *et al.*¹⁰² developed iKEA (integrated kidney exosome analysis), a biosensor platform to predict kidney allograft rejection, which detected exosomes presenting CD3 in urine; this molecule is usually overexpressed by T lymphocytes. In this study, a total of 44 KT patients were enrolled, with 30 used for model training and 14 for model validation. For each model, half of the patients presented an ACR while the other half did not. A predicting model of ACR was developed, with an area under the receiver operating characteristic curve (AUC), sensitivity, specificity and accuracy of 0.911, 0.928, 0.875 and 0.900, respectively.

Other studies focus on 'omics' analysis of EVs, including transcriptomics, proteomics, lipidomics and metabolomics, as reviewed in Table 3. Most of the studies are based on EVs from urine, because this biofluid can be collected non-invasively. In all these studies, 'omics' methodologies were applied to characterise the contents or surface of EVs. Based on the large set of molecules attained, diverse statistical analyses, including supervised classification methods, were applied to define a small number of molecules that could be used to discriminate a phenotype as acute or chronic rejection, humoral or cellular rejection, etc. These subsets of molecules will work as biomarkers of a rejection process.

Most of the studies in Table 3 present promising results, ranging from very good to excellent predictive performances. However, all the studies also present diverse limitations that hinder their clinical translation. Some of the major bottlenecks are: (1) a lack of technical standardisation between studies, which hampers their comparison, as different isolation methodologies and variations of the 'omics' science applied can provide different downstream results; (2) the low number of enrolled patients and little variability in the patients' state or in therapeutic practices, which limit representation of the real clinical environment (usually patients from only one centre are enrolled and are characterised by a low diversity of disease states, e.g., lacking patients with a high diversity of mechanisms of rejection, or with renal and non-renal infections, as well as other pathophysiological states); (3) most of the predictive models are not validated with independent data sets, especially from different hospital centres and including patients with a high diversity of disease states; and (4) a general lack of longitudinal studies that evaluate the condition of the patients over time.

Some of the studies listed in Table 3 are considered in more detail below.

Jung *et al.*¹⁰³ studied proteomics of urinary exosomes from 106 KT patients subdivided into groups with chronic antibody-mediated rejection (CAMR), long-term graft survival and non-rejection. They discovered six proteins (APOA1, TTR, PIGR, HPX, AZGP1 and CP) that enabled discrimination of CAMR from long-term graft survival, with an AUC ranging from 0.73–0.93 for individual proteins, while the combination of APOA1 and TTR provided an AUC of 0.929. These predictive capabilities were higher than those

Table 3 Extracellular vesicle studies in kidney allograft rejection and allograft status conducted on urine samples or plasma or serum samples, highlighting population phenotype and size, analytical techniques used and study outputs

Study aim; population studied and dimension (validation population)	Biofluid; EV extraction and analytical technique	Study outputs ^{a,b}	Ref
Prognosis; ^c TI 6, TCMR 6, ABMR 3, non-specific 2	Urine; differential centrifugation and proteomics (LC-MS/MS)	Metabolic pathways: TI, sodium ion transport; ABMR, acute inflammatory response; TCMR, epithelial cell differentiation ^a	123
Diagnostic DGF; LD 11, DD 4	Urine; ultracentrifugation, proteomics, WB	Biomarker: exosomal NGAL ^a	99
Diagnostic AR; ^c AR 10, non-AR 20	Urine; ultracentrifugation + nanomembrane filtration, proteomics (LC-MS/MS)	Biomarker: CLCA1, PROS1, KIAA053 ^a	100
Prognostic recovery; ^c KT 25, ESRD age-matched controls 20, residual diuresis 5	Urine; ultracentrifugation + magnetic cell sorting, proteomics, WB	CD133+ EVs ^b	124
Diagnostic ACR; ^c B-R 8, ACR 8, STA 14 (B-R 4, ACR 4, STA 6)	Urine; magnetic beads, N/A (biosensor development)	CD3+ EVs ^b	102
Diagnostic TCMR; ^c acute TCMR 25, STA 22	Urine; ultracentrifugation, proteomics, nano-UPLC-MS/MS	Biomarker: tetraspanin-1 and hemopexin ^a	104
Prognosis AR; ^c AR 23 (AR 32)	Urine; UN, transcriptomics, UN	Biomarker: CXCL9, CXCL10, IL17RA	106
Diagnostic AR; KT 108	Urine; UN, transcriptomics, qRT-PCR	Biomarker: hsa-miR-4488, hsa-miR-4532, hsa-miR-30a-3p, hsa-miR-21-5p, hsa-miR-223-3p ^a	125
Diagnostic chronic ABMR; ^c CAMR 26, long-term graft survival 57; STA 10	Urine; ultracentrifugation, proteomics, LC-MS	Biomarker: APOA1, TTR, PIGR, HPX, AZGP1, CP ^a	103
Diagnostic chronic ABMR; ^c IF/TA 9, CAMR 22, CIT 17, STA 20	Urine; commercial kit, proteomics, WB	Biomarker: synaptotagmin-17 ^a	105
Diagnostic TCMR and ABMR; ^c acute ABMR 8, STA 133, chronic ABMR 16, TCMR 35, chronic TCMR 3	Urine; commercial kit, transcriptomics, qRT-PCR	Biomarker: rejection vs non-rejection CXCL11, CD74, IL32, STAT1, CXCL14, SERPINA1, B2M, C3, PYCARD, BMP7, TBP, NAMPT, IFNGR1, IRAK2, IL18BP; TCMR vs ABMR CD74, C3, CXCL11, CD44, IFNAR2 ^a	106
Diagnostic; ^c IF/TA 5, acute TCMR 6, CIT 5, STA 7 (IF/TA 11, acute TCMR 10, CIT 10, STA 10)	Urine; size-exclusion chromatography, proteomics, shotgun LC-MS/MS	Biomarker: vitronectin ^a	126
Diagnostic rejection; ^c KT 227, HC 60	Plasma; flow cytometry, N/A, immunolabelling + flow cytometry	↑ endothelial microparticles in rejection, post-treatment endothelial microparticles ↓	127
Prognostic ABMR; ^c ABMR 12, ABMR + TCMR 6, TCMR 8, STA 38	Plasma; commercial kit, transcriptomics, qRT-PCR	Biomarker: mRNA transcript, gp130, SH2D1B, TNFα, CCL4 ^a	109
Diagnostic ABMR; ^c ABMR 28, non-ABMR 67, HC 23	Plasma; flow cytometry, N/A, flow cytometry	ABMR ↑C4d+ EVs, ↑C4d+/CD144+, C4d+/annexin V+ Post-treatment ↓ C4d+/CD144+ EVs ^b	128
Prognostic; HC 10, KT poor prognosis 10, KT good prognosis 10	Plasma; ultracentrifugation, proteomics, ESI-MS/MS	Protein fingerprint complement activation, coagulation cascades, PPAR, TGF-β, HIF-1 signalling pathways ^a	112
Prognostic DGF; DGF 4, non-DGF 5, HC 5	Plasma; commercial kit, transcriptomics, qRT-PCR	Biomarker: hsa-miR-33a-5p_R-1, hsa-miR-98-5p, hsa-miR-151a-5p ^a	109
Diagnostic ABMR; ^c ABMR 14, non-ABMR 14, STA 14	Serum; commercial kit, N/A, flow cytometry	↑ CD4+CXCR5+CXCR3 EVs ^b	129
Prognosis; KT 22, HC 10 (KT 17, HC 36)	Plasma; exclusion chromatography, transcriptomics, qRT-PCR	Biomarker: miR-21, miR-210 and miR-4639 ^a	108
Allograft quality: donors 43	Donor preservation fluid; ultracentrifugation/precipitation, transcriptomics, real-time qPCR arrays	Brain death donors EVs ≠ circulatory death donors EVs	130

ABMR, antibody-mediated rejection; ACR, acute cellular rejection; AR, acute rejection; B-R, borderline rejection; CAMR, chronic antibody-mediated rejection; CIT, calcineurin inhibitor toxicity; DD, deceased donor; DGF, delayed graft function; ESI-MS/MS, electrospray-tandem mass spectrometry; ESRD: end-stage renal disease; EV, extracellular vesicle; HC, healthy controls; IF/TA, interstitial fibrosis and tubular atrophy; KT, kidney transplant patients; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LD, living donor; N/A, not applicable; nano-UPLC-MS/MS, nano-ultraperformance liquid chromatography-tandem mass spectrometry; NGAL, neutrophil gelatinase-associated lipocalin; qRT-PCR, quantitative real-time polymerase chain reaction; STA, stable graft function; TCMR, T-cell-mediated rejection; TI, tubular injury; UN, unavailable; WB, western blot.

^a EV cargo.

^b EV external marker.

^c Classification based on biopsy.

obtained by Lim *et al.*¹⁰⁴ who, based on proteomics of urinary EVs, identified two proteins (tetraspanin-1 and hemo-pexin) enabling prediction of acute T-cell mediated rejection (TCMR) (AUC=0.74).

Takada *et al.*¹⁰⁵ analysed a subset of genes associated with interleukin (IL)-6 amplifier activation [genes synergistically activated by nuclear factor (NF)- κ B and signal transducer and activator of transcription 3 (STAT3)], resulting in a chemokine hyperexpression associated with chronic inflammation. These authors observed that the SYT17 protein was strongly expressed in the tubular cells of patients with CAMR when compared to other KT groups. The SYT17 protein was undetectable in the urine of KT patients, whereas analysis of urinary CD9+ exosomal SYT17 levels allowed prediction of CAMR with an AUC of 0.82.

Using transcriptomic profiling of urinary exosomes from 175 KT patients with ABMR ($n=24$), TCMR ($n=35$) and no rejection ($n=133$), El Fekih *et al.*¹⁰⁶ identified mRNA discriminating non-rejection from rejection (AUC=0.93).

Despite most studies being based on urinary exosomes, some have explored blood EVs. For example, Zhang *et al.*¹⁰⁷ conducted transcriptomic analysis of plasma exosomes from 64 KT patients (18 with ABMR, 8 with TCMR, 38 without rejection) and discriminated ABMR from TCMR based on mRNA of the genes *gp130*, *SH2D1B*, *TNF α* and *CCL4* (AUC=0.796). Chen *et al.*¹⁰⁸ analysed microRNA in plasma exosomes, based on quantitative real-time PCR, identifying three microRNA predicting glomerular filtration rate (AUC=0.89). Wang *et al.*¹⁰⁹ by conducting high-throughput sequencing of microRNA in plasma exosomes, discovered that delayed graft function in KT patients was associated with upregulation of hsa-miR-33a-5p_R-1, hsa-miR-98-5p, and hsa-miR-151a-5p. Using proteomic analysis of plasma exosomes, Al-Nedawi *et al.*¹¹⁰ observed that several proteins were involved in complement activation and coagulation cascades, suggesting that instead of focusing on individual proteins, perhaps a closer look should be taken at pathways (e.g., hypoxia-inducible factor 1 or coagulation, among others) in order to predict pathophysiological processes of kidney transplantation.

EXOSOME-BASED THERAPY

EVs have been explored as therapeutic agents and as vehicles for drug delivery. Since EVs comprise cell membrane constituents, they are usually well tolerated by the host when compared to synthetic polymers, while presenting prolonged stability in the blood.^{5,6,111,112} Indeed, there are already more than 100 clinical trials investigating therapeutic uses for exosomes (www.clinicaltrials.gov; search term = exosomes+therapeutic; conducted 16 November 2023). Despite this, the introduction of these particles to the clinical scenario presents several challenges that must be overcome, including the need for standardisation over the various steps of the production process.^{112–114} To date, there are no new exosome-derived therapeutics approved by the US Food and Drug Administration.¹¹⁵

One of the main objectives in transplantation medicine is to achieve allograft immunological tolerance, in order to minimise or, ideally, avoid the use of immunosuppressive drugs.¹¹⁶ The application of EVs in the field of kidney transplantation has been explored mainly *in vitro* and in animal-based studies. As an example, Ezzelarab *et al.*¹¹⁷ prolonged renal allograft survival in rhesus monkeys by injecting, before transplantation, dendritic cells obtained from the allograft donor pre-loaded with EVs obtained from peripheral blood mononuclear cells of the allograft donor. Likewise, in a rat-based model and using EVs derived from CD4+, CD25+ regulatory T cells, Yu *et al.*¹¹⁸ were able to postpone allograft rejection, thus prolonging kidney allograft survival. They observed that the autologous transfer of exosomes derived from regulatory T cells had the ability to suppress T-cell proliferation. More recently, Wu *et al.*,¹¹⁹ in a mouse kidney transplant model, observed that specific long non-coding RNA (lnc-RNA) from exosomes of bone marrow mesenchymal stem cells (BM-MSCs) promoted CD4+ T-cell differentiation into regulatory T cells (Treg), resulting in immune tolerance. Furthermore, the authors were able to reverse rejection via injection of lnc-RNA-containing exosomes obtained from BM-MSCs.

Although this approach seems promising, to date, the transplant community has not reached a consensus on the

Table 4 Exosome-based therapeutic applications explored in animal models^a

Study goals	EV origin and isolation	Study subjects	Study outcomes	Ref
Immunomodulatory effects of EVs derived from mesenchymal stem cells	DMEM, Hams-F12 medium Ultracentrifugation	7 subjects receiving purified EVs, 6 subjects receiving mesenchymal stem cell medium, 3 subjects of control isograft	EVs imply \neq pattern of graft infiltrating lymphocytes (\uparrow T and B cells, \downarrow NK cell) EVs imply \neq pattern of cytokines (\downarrow TNF alpha expression)	131
Graft survival due to Treg-derived EVs	Conditioned medium Ultracentrifugation	10 subjects receiving dnIKK2-Treg-EVs with or without cyclosporin, 10 subjects with allografts with or without cyclosporin	dnIKK2-Treg-EV with cyclosporin prevent acute rejection, increase allograft survival, increase stable renal function	132
Treg differentiation and immune tolerance promotion by immature dendritic cell-derived exosomes	DMEM-low glucose medium Ultracentrifugation	20 subjects receiving mature or immature dendritic cell-derived exosomes, 20 subjects as controls	miR-682 in EVs from immature dendritic cells improve allograft survival, decrease IFN- γ , IL-2, IL-17, promote Treg cell differentiation	133

DMEM, Dulbecco's Modified Eagle Medium; EV, extracellular vesicle; IFN, interferon; IL, interleukin; NK, natural killer; TNF, tumour necrosis factor; Treg, regulatory T cell.

^a All based on the rat model.

benefits of EVs and their immunomodulatory capabilities. This is illustrated by Ramirez-Bajo *et al.*,¹²⁰ who observed that administering MSCs had an immunomodulatory effect, but the administration of EVs derived from these cells did not exert the same effects.

In addition to the examples discussed above, Table 4 presents other exosome-based applications designed to achieve a better understanding of kidney allograft dysfunction or for therapeutic rescue.

CONCLUSIONS

As EVs are key players in intercellular communication, especially within the immune system, these particles have the potential to enable a better understanding of the complex pathophysiological processes associated with rejection of kidney allografts. Also, since EVs present higher concentrations of target molecules than their corresponding biofluid, the analysis of EVs in biofluids may facilitate the discovery of biomarkers for diagnosis and prognosis of rejection processes. These biomarkers, obtained from non-invasive or minimally invasive analysis, enable more frequent monitoring of patients and the detection of early rejection events, prompting potential adjustment of drug therapy and thus improving survival of both graft and patient and the patient's quality of life. EVs also represent a very appealing tool for therapeutic purposes, through their use as compatible and stable drug carriers and in inducing immune tolerance.

Despite the untapped potential of EVs, most studies present critical constraints. These include a lack of standardisation of the methods used for the isolation and characterisation of EVs. Additionally, most studies are based on a small number of patients, lack diversity in patient disease types, are not multicentric and therefore do not account for variability in patient states and therapeutic practices. Furthermore, most of the prediction models developed are not validated with independent data sets, including data from other health centres. There is also a general lack of longitudinal studies, which would take into consideration patients' profiles over the course of immunosuppression treatments, enabling risk assessments to be conducted and facilitating the development of personalised surveillance strategies. Despite these challenges, however, the field of biomarker discovery in EVs holds significant promise and potential.

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References

- Djamali A, Premasathian N, Pirsch JD. Outcomes in kidney transplantation. *Semin Nephrol* 2003; 23: 306–16.
- van Dijk PC, Jager KJ, de Charro F, *et al.* Renal replacement therapy in Europe: the results of a collaborative effort by the ERA ± EDTA

- registry and six national or regional registries. *Nephrol Dial Transplant* 2001; 16: 1120–9.
- El-Zoghby ZM, Stegall MD, Lager DJ, *et al.* Identifying specific causes of kidney allograft loss. *Am J Transplant* 2009; 9: 527–35.
- Claeys E, Vermeire K. Immunosuppressive drugs in organ transplantation to prevent allograft rejection: mode of action and side effects. *J Immunol Sci* 2019; 3: 14–21.
- van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018; 19: 213–28.
- Yáñez-Mó M, Siljander PRM, Andreu Z, *et al.* Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015; 4: 27066.
- Veziroglu EM, Mias GI. Characterizing extracellular vesicles and their diverse RNA contents. *Front Genet* 2020; 11: 1–30.
- Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem* 2019; 88: 487–514.
- Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002; 2: 569–79.
- Tetta C, Ghigo E, Silengo L, Deregibus MC, Camussi G. Extracellular vesicles as an emerging mechanism of cell-to-cell communication. *Endocrine* 2013; 44: 11–9.
- Williams C, Palviainen M, Reichardt NC, Siljander PRM, Falcón-Pérez JM. Metabolomics applied to the study of extracellular vesicles. *Metabolites* 2019; 9: 276.
- Thompson AG, Gray E, Heman-Ackah SM, *et al.* Extracellular vesicles in neurodegenerative disease — pathogenesis to biomarkers. *Nat Rev Neurol* 2016; 12: 346–57.
- Kapogiannis D, Mustapic M, Shardell MD, *et al.* Association of extracellular vesicle biomarkers with Alzheimer disease in the Baltimore longitudinal study of aging. *JAMA Neurol* 2019; 76: 1340.
- Eren E, Hunt JF, Shardell M, *et al.* Extracellular vesicle biomarkers of Alzheimer's disease associated with sub-clinical cognitive decline in late middle age. *Alzheimer's Dement* 2020; 16: 1293–304.
- Zhou E, Li Y, Wu F, *et al.* Circulating extracellular vesicles are effective biomarkers for predicting response to cancer therapy. *EBio-Medicine* 2021; 67: 103365.
- Hoshino A, Kim HS, Bojmar L, *et al.* Extracellular vesicle and particle biomarkers define multiple human cancers. *Cell* 2020; 182: 1044–1061.e18.
- Min L, Zhu S, Chen L, *et al.* Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs. *J Extracell Vesicles* 2019; 8: 1643670.
- Erdbrügger U, Le TH. Extracellular vesicles in renal diseases: more than novel biomarkers? *J Am Soc Nephrol* 2016; 27: 12–26.
- Lv LL, Feng Y, Tang TT, Liu BC. New insight into the role of extracellular vesicles in kidney disease. *J Cell Mol Med* 2019; 23: 731–9.
- Mirzakhani M, Mohammadnia-Afrouzi M, Shahbazi M, Mirhosseini SA, Hosseini HM, Amani J. The exosome as a novel predictive/diagnostic biomarker of rejection in the field of transplantation. *Clin Immunol* 2019; 203: 134–41.
- Zhu X, Badawi M, Pomeroy S, *et al.* Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles* 2017; 6: 1324730.
- Saleh AF, Lázaro-Ibáñez E, Forsgard MAM, *et al.* Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale* 2019; 11: 6990–7001.
- Akuma P, Okagu OD, Udenigwe CC. Naturally occurring exosome vesicles as potential delivery vehicle for bioactive compounds. *Front Sustain Food Syst* 2019; 3: 1–8.
- Klyachko NL, Arzt CJ, Li SM, Gololobova OA, Batrakova EV. Extracellular vesicle-based therapeutics: preclinical and clinical investigations. *Pharmaceutics* 2020; 12: 1171.
- Auchincloss H. In search of the elusive holy grail: the mechanisms and prospects for achieving clinical transplantation tolerance. *Am J Transplant* 2001; 1: 6–12.
- Benichou G, Wang M, Ahrens K, Madsen JC. Extracellular vesicles in allograft rejection and tolerance. *Cell Immunol* 2020; 349: 104063.
- Carobolante G, Mantaj J, Ferrari E, Vilasaliu D. Cow milk and intestinal epithelial cell-derived extracellular vesicles as systems for enhancing oral drug delivery. *Pharmaceutics* 2020; 12: 9–11.
- Reed SL, Escayg A. Extracellular vesicles in the treatment of neurological disorders. *Neurobiol Dis* 2021; 157: 105445.
- Walker S, Busatto S, Pham A, *et al.* Extracellular vesicle-based drug delivery systems for cancer treatment. *Theranostics* 2019; 9: 8001–17.
- Li Y, Xiao Q, Tang J, Xiong L, Li L. Extracellular vesicles: emerging therapeutics in cutaneous lesions. *Int J Nanomedicine* 2021; 16: 6183–202.
- Liu S, Wu X, Chandra S, *et al.* Extracellular vesicles: emerging tools as therapeutic agent carriers. *Acta Pharm Sin B* 2022; 12: 3822–42.

32. Cheng YC, Chang YA, Chen YJ, *et al.* The roles of extracellular vesicles in malignant melanoma. *Cells* 2021; 10: 2740.
33. Soltani S, Mansouri K, Emami Aleagha MS, *et al.* Extracellular vesicle therapy for Type 1 diabetes. *Front Immunol* 2022; 13: 865782.
34. Liu Q, Rojas-Canales DM, Divito SJ, *et al.* Donor dendritic cell-derived exosomes promote allograft-targeting immune response. *J Clin Invest* 2016; 126: 2805–20.
35. Morelli AE, Bracamonte-Baran W, Burlingham WJ. Donor-derived exosomes. *Curr Opin Organ Transplant* 2017; 22: 46–54.
36. Gonzalez-Nolasco B, Wang M, Prunevicielle A, Benichou G. Emerging role of exosomes in allorecognition and allograft rejection. *Curr Opin Organ Transplant* 2018; 23: 22–7.
37. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014; 14: 195–208.
38. Raposo G, Nijman HW, Stoorvogel W, *et al.* B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996; 183: 1161–72.
39. Dieudé M, Bell C, Turgeon J, *et al.* The 20 S proteasome core, active within apoptotic exosome-like vesicles, induces autoantibody production and accelerates rejection. *Sci Transl Med* 2015; 7: 318ra200.
40. Castellani C, Burrello J, Fedrigo M, *et al.* Circulating extracellular vesicles as non-invasive biomarker of rejection in heart transplant. *J Heart Lung Transplant* 2020; 39: 1136–48.
41. Ravichandran R, Bansal S, Rahman M, *et al.* The role of donor-derived exosomes in lung allograft rejection. *Hum Immunol* 2019; 80: 588–94.
42. Mastoridis S, Martinez-Llordella M, Sanchez-Fueyo A. Extracellular vesicles as mediators of alloimmunity and their therapeutic potential in liver transplantation. *World J Transplant* 2020; 10: 330–44.
43. Carlson K, Kink J, Hematti P, Al-Adra DP. Extracellular vesicles as a novel therapeutic option in liver transplantation. *Liver Transplant* 2020; 26: 1522–31.
44. Sukma Dewi I, Hollander Z, Lam KK, *et al.* Association of serum MiR-142-3p and MiR-101-3p levels with acute cellular rejection after heart transplantation. *PLoS One* 2017; 12: e0170842.
45. Kennel PJ, Saha A, Maldonado DA, *et al.* Serum exosomal protein profiling for the non-invasive detection of cardiac allograft rejection. *J Heart Lung Transplant* 2018; 37: 409–17.
46. Li KL, Li JY, Xie GL, Ma XY. Exosomes released from human bone marrow-derived mesenchymal stem cell attenuate acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation in mice. *Front Cell Dev Biol* 2021; 9: 617589.
47. Jia Z, Lv Y, Zhang W, *et al.* Mesenchymal stem cell derived exosomes-based immunological signature in a rat model of corneal allograft rejection therapy. *Front Biosci (Landmark Ed)* 2022; 27: 86.
48. Harding CV, Heuser JE, Stahl PD. Exosomes: looking back three decades and into the future. *J Cell Biol* 2013; 200: 367–71.
49. Dalton AJ. Microvesicles and vesicles of multivesicular bodies versus “virus like” particles. *J Natl Cancer Inst* 1975; 54: 1137–48.
50. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol* 1967; 13: 269–88.
51. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987; 262: 9412–20.
52. Naito Y, Yoshioka Y, Yamamoto Y, Ochiya T. How cancer cells dictate their microenvironment: present roles of extracellular vesicles. *Cell Mol Life Sci* 2017; 74: 697–713.
53. Maacha S, Bhat AA, Jimenez L, *et al.* Extracellular vesicles-mediated intercellular communication: roles in the tumor microenvironment and anti-cancer drug resistance. *Mol Cancer* 2019; 18: 55.
54. Borges FT, Reis LA, Schor N. Extracellular vesicles: structure, function, and potential clinical uses in renal diseases. *Braz J Med Biol Res* 2013; 46: 824–30.
55. Narang P, Shah M, Beljanski V. Exosomal RNAs in diagnosis and therapies. *Non-coding RNA Res* 2022; 7: 7–15.
56. Hade MD, Suire CN, Mossell J, Suo Z. Extracellular vesicles: emerging frontiers in wound healing. *Med Res Rev* 2022; 42: 2102–25.
57. Mathivanan S. ExoCarta. Cited 1 Sep 2022. www.exocarta.org/
58. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014; 30: 255–89.
59. Skotland T, Sandvig K, Llorente A. Lipids in exosomes: current knowledge and the way forward. *Prog Lipid Res* 2017; 66: 30–41.
60. Welsh JA, Van Der Pol E, Arksteijn GJA, *et al.* MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. *J Extracell Vesicles* 2020; 9: 1713526.
61. Lötvall J, Hill AF, Hochberg F, *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 2014; 3: 26913.
62. Théry C, Witwer KW, Aikawa E, *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; 7: 1535750.
63. Van Deun J, Mestdagh P, Agostinis P, *et al.* EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat Methods* 2017; 14: 228–32.
64. Zhang M, Jin K, Gao L, *et al.* Methods and technologies for exosome isolation and characterization. *Small Methods* 2018; 2: 1800021.
65. Ayala-Mar S, Donoso-Quezada J, Gallo-Villanueva RC, Perez-Gonzalez VH, González-Valdez J. Recent advances and challenges in the recovery and purification of cellular exosomes. *Electrophoresis* 2019; 40: 3036–49.
66. Momen-Heravi F, Balaj L, Alian S, *et al.* Current methods for the isolation of extracellular vesicles. *Biol Chem* 2013; 394: 1253–62.
67. Talebjeidi B, Tasnim N, Hoorfar M, Mastromonaco GF, De Almeida Monteiro Melo Ferraz M. Exploiting microfluidics for extracellular vesicle isolation and characterization: potential use for standardized embryo quality assessment. *Front Vet Sci* 2021; 7: 620809.
68. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; Chapter 3: Unit 3.22.
69. Witwer KW, Buzás EI, Bemis LT, *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013; 2: 20360.
70. Zhao Z, Wijerathne H, Godwin AK, Soper SA. Isolation and analysis methods of extracellular vesicles (EVs). *Extracell Vesicles Circ Nucleic Acids* 2021; 2: 80–103.
71. Abramowicz A, Widlak P, Pietrowska M. Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation. *Mol Biosyst* 2016; 12: 1407–19.
72. Ramirez MI, Amorim MG, Gadelha C, *et al.* Technical challenges of working with extracellular vesicles. *Nanoscale* 2018; 10: 881–906.
73. Mussack V, Wittmann G, Pfaffl MW. Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—enabling robust and non-invasive biomarker research. *Biomol Detect Quantif* 2019; 17: 100089.
74. Raju D, Bathini S, Badilescu S, Ghosh A, Packirisamy M. Microfluidic platforms for the isolation and detection of exosomes: a brief review. *Micromachines* 2022; 13: 730.
75. Chen C, Skog J, Hsu CH, *et al.* Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab Chip* 2010; 10: 505–11.
76. Rojalin T, Phong B, Koster HJ, Carney RP. Nanoplasmonic approaches for sensitive detection and molecular characterization of extracellular vesicles. *Front Chem* 2019; 7: 279.
77. Robert S, Lacroix R, Poncelet P, *et al.* High-sensitivity flow cytometry provides access to standardized measurement of small-size microparticles—brief report. *Arterioscler Thromb Vasc Biol* 2012; 32: 1054–8.
78. Chandler WL, Yeung W, Tait JF. A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer. *J Thromb Haemost* 2011; 9: 1216–24.
79. Arraud N, Linares R, Tan S, *et al.* Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J Thromb Haemost* 2014; 12: 614–27.
80. Yuana Y, Koning RI, Kuil ME, *et al.* Cryo-electron microscopy of extracellular vesicles in fresh plasma. *J Extracell Vesicles* 2013; 2: 21494.
81. Yuana Y, Oosterkamp TH, Bahatyrova S, *et al.* Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost* 2010; 8: 315–23.
82. Frisken BJ. Revisiting the method of cumulants for the analysis of dynamic light-scattering data. *Appl Opt* 2001; 40: 4087.
83. Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res* 2010; 27: 796–810.
84. Maas SLN, Broekman MLD, de Vrij J. Tunable resistive pulse sensing for the characterization of extracellular vesicles. *Methods Mol Biol* 2017; 1545: 21–33.
85. Parisse P, Rago I, Ulloa Severino L, *et al.* Atomic force microscopy analysis of extracellular vesicles. *Eur Biophys J* 2017; 46: 813–20.
86. Kurland NE, Drira Z, Yadavalli VK. Measurement of nanomechanical properties of biomolecules using atomic force microscopy. *Micron* 2012; 43: 116–28.
87. Sharma S, Gillespie BM, Palanisamy V, Gimzewski JK. Quantitative nanostructural and single-molecule force spectroscopy biomolecular analysis of human-saliva-derived exosomes. *Langmuir* 2011; 27: 14394–400.
88. Cavallero S, Riccobono D, Drouet M, François S. MSC-derived extracellular vesicles: new emergency treatment to limit the

- development of radiation-induced hematopoietic syndrome? *Health Phys* 2020; 119: 21–36.
89. Hill AF, Pegtel DM, Lambertz U, *et al.* ISEV position paper: extracellular vesicle RNA analysis and bioinformatics. *J Extracell Vesicles* 2013; 2: 22859.
 90. Van Deun J, Mestdagh P, Sormunen R, *et al.* The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles* 2014; 3: 24858.
 91. Li J, He X, Deng Y, Yang C. An update on isolation methods for proteomic studies of extracellular vesicles in biofluids. *Molecules* 2019; 24: 3516.
 92. Skotland T, Sagini K, Sandvig K, Llorente A. An emerging focus on lipids in extracellular vesicles. *Adv Drug Deliv Rev* 2020; 159: 308–21.
 93. Chen S, Datta-Chaudhuri A, Deme P, *et al.* Lipidomic characterization of extracellular vesicles in human serum. *J Circ Biomarkers* 2019; 8: 184945441987984.
 94. Dudzik D, Macioszek S, Struck-Lewicka W, *et al.* Perspectives and challenges in extracellular vesicles untargeted metabolomics analysis. *TrAC Trends Anal Chem* 2021; 143: 116382.
 95. Ciferri MC, Quarto R, Tasso R. Extracellular vesicles as biomarkers and therapeutic tools: from pre-clinical to clinical applications. *Biology (Basel)* 2021; 10: 359.
 96. Schiller EA, Cohen K, Lin X, El-Khawam R, Hanna N. Extracellular vesicle-microRNAs as diagnostic biomarkers in preterm neonates. *Int J Mol Sci* 2023; 24: 2622.
 97. Alberro A, Iparraguirre L, Fernandes A, Otaegui D. Extracellular vesicles in blood: sources, effects, and applications. *Int J Mol Sci* 2021; 22: 8163.
 98. Gutiérrez-Fernández M, de la Cuesta F, Tallón A, *et al.* Potential roles of extracellular vesicles as biomarkers and a novel treatment approach in multiple sclerosis. *Int J Mol Sci* 2021; 22: 9011.
 99. Alvarez S, Suazo C, Boltansky A, *et al.* Urinary exosomes as a source of kidney dysfunction biomarker in renal transplantation. *Transplant Proc* 2013; 45: 3719–23.
 100. Sigdel TK, Ng YW, Lee S, *et al.* Perturbations in the urinary exosome in transplant rejection. *Front Med (Lausanne)* 2015; 1: 57.
 101. Bruschi M, Santucci L, Ravera S, *et al.* Human urinary exosome proteome unveils its aerobic respiratory ability. *J Proteomics* 2016; 136: 25–34.
 102. Park J, Lin HY, Assaker JP, *et al.* Integrated kidney exosome analysis for the detection of kidney transplant rejection. *ACS Nano* 2017; 11: 11041–6.
 103. Jung HY, Lee CH, Choi JY, *et al.* Potential urinary extracellular vesicle protein biomarkers of chronic active antibody-mediated rejection in kidney transplant recipients. *J Chromatogr B* 2020; 1138: 121958.
 104. Lim JH, Lee CH, Kim KY, *et al.* Novel urinary exosomal biomarkers of acute T cell-mediated rejection in kidney transplant recipients: a cross-sectional study. *PLoS One* 2018; 13: e0204204.
 105. Takada Y, Kamimura D, Jiang JJ, *et al.* Increased urinary exosomal SYT17 levels in chronic active antibody-mediated rejection after kidney transplantation via the IL-6 amplifier. *Int Immunol* 2020; 32: 653–62.
 106. El Fekih R, Hurley J, Tadigotla V, *et al.* Discovery and validation of a urinary exosome mRNA signature for the diagnosis of human kidney transplant rejection. *J Am Soc Nephrol* 2021; 32: 994–1004.
 107. Zhang H, Huang E, Kahwaji J, *et al.* Plasma exosomes from HLA-sensitized kidney transplant recipients contain mRNA transcripts which predict development of antibody-mediated rejection. *Transplantation* 2017; 101: 2419–28.
 108. Chen Y, Han X, Sun Y, He X, Xue D. A circulating exosomal microRNA panel as a novel biomarker for monitoring post-transplant renal graft function. *J Cell Mol Med* 2020; 24: 12154–63.
 109. Wang J, Li X, Wu X, *et al.* Expression profiling of exosomal miRNAs derived from the peripheral blood of kidney recipients with DGF using high-throughput sequencing. *BioMed Res Int* 2019; 2019: 1759697.
 110. Al-Nedawi K, Haas-Neill S, Gangji A, Ribic CM, Kapoor A, Margetts P. Circulating microvesicle protein is associated with renal transplant outcome. *Transpl Immunol* 2019; 55: 101210.
 111. Dai J, Su Y, Zhong S, *et al.* Exosomes: key players in cancer and potential therapeutic strategy. *Signal Transduct Target Ther* 2020; 5: 145.
 112. Claridge B, Lozano J, Poh QH, Greening DW. Development of extracellular vesicle therapeutics: challenges, considerations, and opportunities. *Front Cell Dev Biol* 2021; 9: 734720.
 113. Canha Pereira MJ, Ramalhete L, Aleixo S, *et al.* Impact of the human mesenchymal stem cells donor on conditional medium composition. In: IEEE 6th Portuguese Meeting on Bioengineering (ENBENG); Lisbon, Portugal, 2019. <https://ieeexplore.ieee.org/document/8692511>
 114. Nelson BC, Maragh S, Ghiran IC, *et al.* Measurement and standardization challenges for extracellular vesicle therapeutic delivery vectors. *Nanomedicine* 2020; 15: 2149–70.
 115. Centers for Disease Control and Prevention. Stem cell and exosome products. Warning about unapproved therapies. 2019; cited 20 Jun 2022. www.cdc.gov/hai/outbreaks/stem-cell-products.html
 116. Kalra H, Adda CG, Liem M, *et al.* Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *Proteomics* 2013; 13: 3354–64.
 117. Ezzelarab MB, Raich-Regue D, Lu L, *et al.* Renal allograft survival in nonhuman primates infused with donor antigen-pulsed autologous regulatory dendritic cells. *Am J Transplant* 2017; 17: 1476–89.
 118. Yu X, Huang C, Song B, *et al.* CD4+CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model. *Cell Immunol* 2013; 285: 62–8.
 119. Wu X, Wang Z, Wang J, *et al.* Exosomes secreted by mesenchymal stem cells induce immune tolerance to mouse kidney transplantation via transporting lncRNA DANCER. *Inflammation* 2022; 45: 460–75.
 120. Ramirez-Bajo MJ, Rovira J, Lazo-Rodriguez M, *et al.* Impact of mesenchymal stromal cells and their extracellular vesicles in a rat model of kidney rejection. *Front Cell Dev Biol* 2020; 8: 10.
 121. Ståhl AL, Johansson K, Mossberg M, Kahn R, Karpman D. Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. *Pediatr Nephrol* 2019; 34: 11–30.
 122. Lucchetti D, Fattorossi A, Sgambato A. Extracellular vesicles in oncology: progress and pitfalls in the methods of isolation and analysis. *Biotechnol J* 2019; 14: 1700716.
 123. Pisitkun T, Gandolfo MT, Das S, Knepper MA, Bagnasco SM. Application of systems biology principles to protein biomarker discovery: urinary exosomal proteome in renal transplantation. *Proteomics Clin Appl* 2012; 6: 268–78.
 124. Dimuccio V, Ranghino A, Praticò Barbato L, *et al.* Urinary CD133+ extracellular vesicles are decreased in kidney transplanted patients with slow graft function and vascular damage. *PLoS One* 2014; 9: e104490.
 125. Cho WH, Lee YH, Kim YH, *et al.* FP699: urinary exosomal microRNA as a non-invasive biomarker for the diagnosis of acute rejection in kidney transplant recipients. *Nephrol Dial Transplant* 2018; 33 (Suppl_1): i281.
 126. Carreras-Planella L, Cucchiari D, Cañas L, *et al.* Urinary vitronectin identifies patients with high levels of fibrosis in kidney grafts. *J Nephrol* 2021; 34: 861–74.
 127. Qamri Z, Pelletier R, Foster J, *et al.* Early posttransplant changes in circulating endothelial microparticles in patients with kidney transplantation. *Transpl Immunol* 2014; 31: 60–4.
 128. Tower CM, Reyes M, Nelson K, *et al.* Plasma C4d+ endothelial microvesicles increase in acute antibody-mediated rejection. *Transplantation* 2017; 101: 2235–43.
 129. Yang J, Bi L, He X, *et al.* Follicular helper T cell derived exosomes promote B cell proliferation and differentiation in antibody-mediated rejection after renal transplantation. *BioMed Res Int* 2019; 2019: 6387924.
 130. Gremmels H, de Jong OG, Toorop RJ, *et al.* The small RNA repertoire of small extracellular vesicles isolated from donor kidney preservation fluid provides a source for biomarker discovery for organ quality and posttransplantation graft function. *Transplant Direct* 2019; 5: e484.
 131. Koch M, Lemke A, Lange C. Extracellular vesicles from MSC modulate the immune response to renal allografts in a MHC disparate rat model. *Stem Cells Int* 2015; 2015: 486141.
 132. Aiello S, Rocchetta F, Longaretti L, *et al.* Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. *Sci Rep* 2017; 7: 11518.
 133. Pang XL, Wang ZG, Liu L, *et al.* Immature dendritic cells derived exosomes promotes immune tolerance by regulating T cell differentiation in renal transplantation. *Aging (Albany NY)* 2019; 11: 8911–24.