Pathology (February 2024) **56(1)**, pp. 1–10

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.

Received 21 December 2022, revised 26 September, accepted 19 October 2023
Available online 23 November 2023

## 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

Print ISSN 0031-3025/Online ISSN 1465-3931 
DOI: https://doi.org/10.1016/j.pathol.2023.10.004

delivery, with applications in neurology,[28] oncology[29] 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] lung[41] 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 transplantation[46] 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, Wolf[50] 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<br>Intraluminal vesicles (from vesicular bodies) that fuse with plasma membrane | Dependent on:<br>• Endosomal sorting complex required for transport<br>• Tetraspanin<br>• Ceramide<br>• Stimuli | Enriched cholesterol and ceramide<br>Expose phosphatidylserine with lipid rafts |
| Microvesicles | 100–1,000 nm<br>Outward blebbing of plasma membrane with cellular content | Dependent on:<br>• Ca2+<br>• Stimuli and cell dependent | Enriched in cholesterol and diacylglycerol<br>Expose phosphatidylserine with lipid rafts |
| Apoptotic bodies | 1–5 μm<br>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

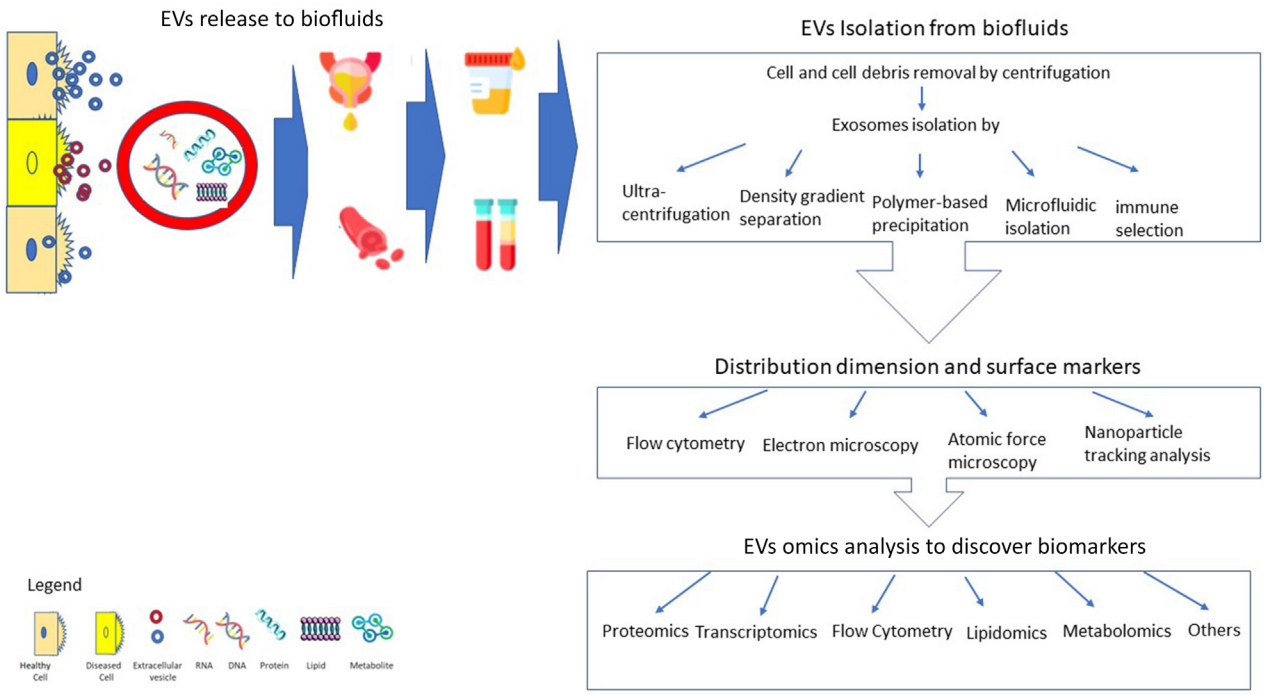


**Fig. 1** Illustration of the process of isolating and characterising extracellular vesicles (EVs).

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

| Method | Advantages | Limitations |
|---|---|---|
| Isolation | | |
| Precipitation | • Fastest turnarounds<br>• Straightforward workflow<br>• No need for special equipment<br>• Retains vesicle morphology<br>• Process many samples | • Polymer contamination<br>• Proteins, aggregates, viruses and lipoproteins can interfere |
| Ultrafiltration | • Concentrates samples<br>• Isolates EVs from dilute biofluids<br>• Simple and straightforward protocol<br>• Processes many samples<br>• No limit on sample volume<br>• Purity of EVs | • Sample loss in filtration<br>• Filter clogging<br>• Some loss of vesicle morphology<br>• Possible contamination with protein aggregates |
| Size-exclusion chromatography | • Customisable<br>• Scalable<br>• Preserves vesicle integrity<br>• Reproducible<br>• High sample recovery | • Low sample volume<br>• Single sample<br>• Low resolution of similarly sized molecules |
| Ultracentrifugation | • Large-volume samples<br>• No added chemicals | • Special equipment<br>• Damage to exosomes as a result of high speeds<br>• Hard to scale up<br>• Sample viscosity impacts sedimentation |
| Density gradient centrifugation | • Vesicle morphology intact<br>• High purity EVs | • Time consuming<br>• Hard to scale up<br>• Special equipment |
| Immunoaffinity | • High purity EVs<br>• High selectivity | • Low sample volume<br>• Lack of specific markers for isolation<br>• Antigen-antibody interaction difficult to dissociate |
| Detection and characterisation | | |
| Transmission electron microscopy | • EV size determination<br>• Biogenesis visualisation<br>• Discrimination from other small particles | • Special equipment<br>• Cost<br>• Time consuming<br>• EVs can lose shape during preparation |
| Atomic force microscopy | • High-resolution images of EVs<br>• Information about size and morphology | • Special equipment<br>• Cost<br>• Time consuming |
| Flow cytometry | • Single vesicle resolution<br>• Identification of multiple surface markers | • Reagent interference due to debris<br>• Special cytometer calibration |
| Nanoparticle tracking analysis (NTA) | • Simultaneous detection, counting, sizing and phenotyping of EVs<br>• Antibody-mediated fluorescent labelling possible<br>• Selectively determine and analyse specific types of particles in complex samples<br>• High throughput | • Small volume samples<br>• Limited in multiplexing capability versus flow cytometry<br>• Special equipment |
| Resistive pulse sensing | • Alternative to NTA for concentration and size distribution measurements | • Heterogeneous populations require the combination of several nanopore membranes |
| Dynamic light scattering | • Less time-consuming<br>• No specific expertise required | • Less appropriate for heterogeneous EV populations<br>• Analysis software prone to give inaccurate results if they are not properly calibrated or if the sample is not prepared correctly |
| Western blot | • Ideal for detection of protein markers associated with EVs<br>• Ideal for confirming correct isolation of EVs | • Not suitable for determining EV quantity<br>• 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;<br>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+<br>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; cABMR 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 hemopexin) 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)-κB 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*, *TNFα* 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<br>Ultracentrifugation | 7 subjects receiving purified EVs,<br>6 subjects receiving mesenchymal stem cell medium,<br>3 subjects of control isograft | EVs imply ≠ pattern of graft infiltrating lymphocytes (↑ T and B cells, ↓ NK cell)<br>EVs imply ≠ pattern of cytokines (↓TNF alpha expression) | 131 |
| Graft survival due to Treg-derived EVs | Conditioned medium<br>Ultracentrifugation | 10 subjects receiving dnIKK2-Treg-EVs with or without cyclosporin,<br>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<br>Ultracentrifugation | 20 subjects receiving mature or immature dendritic cell-derived exosomes,<br>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.

**Conflicts of interest and sources of funding:** The authors state that there are no conflicts of interest to declare. This research was funded by project grant DSAIPA/DS/0117/2020 and PTDC/EQU-EQU/3708/2021, supported by Fundação para a Ciência e a Tecnologia, Portugal; and by the project grant NeproMD/ISEL/2020 financed by Instituto Politécnico de Lisboa.

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