arTs Università degli Studi di Trieste
Archivio della ricerca – postprint

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ARTICLE HISTORY

Accepted: October 22, 2021

Abstract: ***Background*:** Extracellular vesicles (EVs) are a heterogeneous family of small vesicles released by donor cells and absorbed by recipient cells, which represent important mediators with fundamental roles in both physiological and pathological conditions. EVs are present in a large variety of biological fluids and have a great diagnostic and prognostic value. They have gained the interest of the scientific community due to their extreme versatility. In fact, they allow us to hypothesize new therapeutic strategies since, in addition to being cell signal mediators, they play an important role as biomarkers, drug vehicles, and potential new therapeutic agents. They are also involved in immunoregulation, have the ability to transmit resistance to a drug from one cell to a more sensitive one, and can act as drug delivery systems.

***Objective*:** The main reciprocal interactions between EVs and immunosuppressive drugs will be presented.

***Results*:** The known interactions between EVs and immunosuppressive drugs, in particular cyclosporin, glucocorticoids, rapamycin, methotrexate, cyclophosphamide, eculizumab, infliximab, certolizumab, etanercept, glatiramer acetate, and fingolimod are presented.

***Conclusion*:** This review provides relevant information on the links between EVs and immunosuppressive drugs with a focus on EVs' role as tools to assess the effects of immunosuppressants, suggesting innovative properties and new possible therapeutic uses.

Keywords: Extracellular vesicles, immunosuppressants, biomarkers, therapeutic efficacy, adverse effects, therapy personalization.

1. INTRODUCTION

Intercellular communication is an essential hallmark to guarantee proper coordination among different cell types within tissues and can be mediated in different ways, such as direct cell-cell contact and secretion of soluble factors [1]. In the past few decades, the release of specific extracellular vesicles (EVs) from cells has been identified as another mechanism for cell-to-cell communication (Fig. **1**) [2]. EVs belong to a heterogeneous group of small lipid bilayer-enclosed spherical structures [3, 4] secreted in the extracellular space by both eukaryotic and prokaryotic cells [5].

EVs discovery can be traced back to studies concerning blood coagulation. Initially, EVs have been considered as procoagulant platelet-derived particles and were described in 1967 as "platelet dust" by Wolf [3, 6]. Over the years, EVs have been increasingly studied and have gained the interest of the scientific community, especially for their potential use as diagnostic biomarkers and drug delivery systems (Fig. **1**). It was also demonstrated that EVs are secreted in most biological fluids such as blood, urine, saliva, milk, semen, amniotic fluid, and bile (Fig. **1**) [1, 3], and samples derived from these biofluids contain an EVs mix

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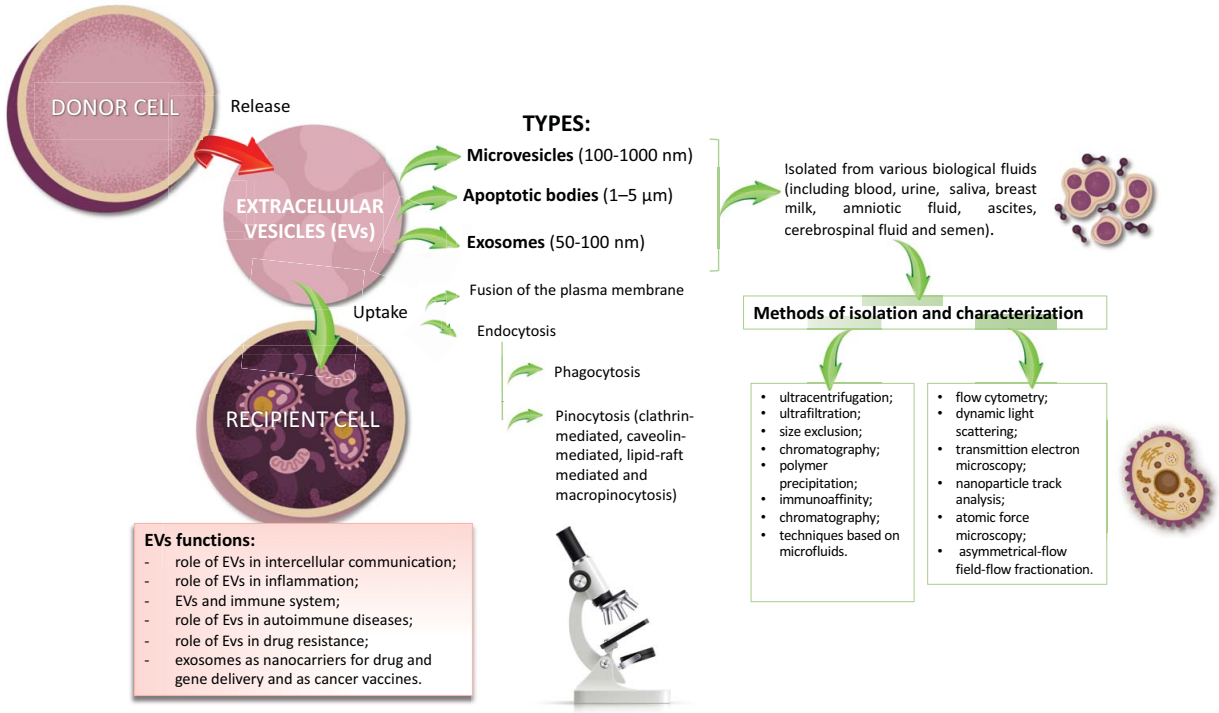

Fig. (1). Summary of the described peculiar characteristics of extracellular vesicles. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

of different cellular origins. A significant obstacle to research in this area is that, nowadays, no fully specific isolation, purification, and characterization techniques are available, and optimization of the current separation protocols is still necessary [5]. EVs are a family of different membrane vesicles, with a nomenclature universally shared that allows to classify these vesicles according to their chemical and physical characteristics, such as size, density, lipid composition, main protein markers, morphology, molecular cargoes, origin, and release mechanism [3]. Among EVs, apoptotic bodies, microvesicles, and exosomes, which are described in Table **1**, are of particular clinical importance [7]. Apoptotic bodies have a diameter of approximately 1-5 µm (similar to platelets) and are blebs released by cells undergoing apoptosis [8]. Microvesicles are 100 -1000 nm in diameter and are formed by budding/blebbing of the plasma membrane, induced by the activation of cell surface receptors, the subsequent increase of intracellular Ca^{2+} and by other cellular processes such as apoptosis [9, 10]. The main protein markers of microvesicles are tetraspanins such as CD29, CD40 and selectins and are characterized by the presence of cholesterol-rich domains, called lipid rafts [11]. Exosomes are the smallest EVs, ranging from 50 to 100 nm. They are surrounded by a phospholipid membrane that contains high levels of cholesterol, sphingomyelin, ceramide, lipid rafts and carry typical markers such as CD63, CD81, and ALG-2-interacting protein X [12]. Exosomes are formed by the budding of the endosomal membrane to form multivesicular bodies; during this process, the endosomal membrane invaginates to generate intraluminal vesicles [13, 14]. Multivesicular bodies are then recycled to endosomes, delivered for degradation or fused with the plasma membrane, releasing intraluminal vesicles, referred to as exosomes, into the extracellular space [7].

Several studies performed in the last decade have demonstrated that, during their biosynthesis and before being released, EVs are loaded with proteins, lipids, and nucleic acids, depending on the type and functional state of the producing cells [4, 15]. In 2007 it was discovered that EVs may represent a vehicle by which one cell communicates with another, and it was shown that they are able to deliver microRNA (miRNA) and messenger RNA (mRNA) [16]. This transfer plays a central role in cell-cell communication in different contexts and pathologies [17]. Indeed, one of the most interesting roles is their ability to transfer nucleic acids into target cells and to influence gene expression, modulating recipient cell protein production [18].

Table 1. Extracellular vesicles: peculiar characteristics.

-	Apoptotic Bodies	Microvesicles	Exosomes
Size	1–5 μm	100 – 1000 nm	50 – 100 nm
Origin	Membrane of apoptotic cells	Plasma membrane	Multivesicular bodies
Main protein markers	Histones	CD29, CD40, selectins	CD63, CD9, CD81, ALIX, TSG101
Flotation density	1.16 – 1.28 g/mL	Not known	1.10 – 1.21 g/mL
Morphology	Heterogeneous	Various shapes	"cup-shaped"
Mode of extracellular release	Cell shrinkage and death	Budding/blebbing of the plasma membrane	Exocytosis of Multivesicular bodies
Composition	Proteins, DNA, miRNA, RNA	Proteins, miRNA, mRNA	Proteins, miRNA, mRNA

Researches have demonstrated that EVs can inhibit or activate immune cells, playing a key role in the regulation of the immune system [19]. For instance, EVs released by CD8+ activated T cells could have immunosuppressive effects when being absorbed by dendritic cells (DCs), resulting in reduced major histocompatibility complex expression and induction of apoptosis in these cells [20]. Besides, regulatory T (Treg) cells release exosomes involved in the reduction of the immune response, suggesting a potential role in autoimmune disease or transplantation [21].

EVs have intrinsic targeting activities on the immune system, biocompatibility, stability, low immunogenicity and toxicity and biological barrier permeability and thus could be considered good candidates for both the treatment and the monitoring of inflammatory and autoimmune diseases [22]. Immunosuppressants are largely used to attenuate the immune response in autoimmune disorders and to prevent the rejection of transplanted tissues and organs [23]. These agents can, however present several problems, among which inter-individual variability in response, drug interactions and adverse effects [24]. There is therefore a need for effective biomarkers to monitor treatment and stratify patients according to the probability of adverse effects. EVs are a promising tool that could be used for these purposes.

The focus of this review is to analyze the reciprocal interactions between EVs and some immunosuppressive drugs: cyclosporin, glucocorticoids, rapamycin, methotrexate, cyclophosphamide, eculizumab, infliximab, certolizumab, etanercept, glatiramer acetate and fingolimod. The main results are summarized in Table **2**.

1.1. EVs and Immunosuppressant Drugs

1.1.1. Calcineurin Inhibitors

Calcineurin inhibitors, among which cyclosporin and tacrolimus, are immunosuppressant drugs used in several diseases such as rheumatoid arthritis, psoriasis, Crohn's disease and in organ transplants to prevent rejection [23, 25]. The main side effects related to the use of these molecules are nephrotoxicity, neurotoxicity, hyperglycemia and hypertension [25].

A consequence of the treatment with calcineurin inhibitors could be the development of sporadic hemolytic uremic syndrome (also called atypical, aHUS), characterized by hemolytic anemia, thrombocytopenia and acute renal damage [26]. Compared to infective HUS, the sporadic form is characterized by a worse prognosis and is more likely to relapse. The aHUS can also derive from dysregulation of the complement pathway, but the mechanisms are still unknown [27]. Recently, research works have hypothesized that endothelial EVs could interfere with the regulation of the complement system: endothelial EVs can promote intrarenal complement activation, causing or aggravating aHUS [28]. A study conducted by Renner *et al.*, both *in vitro* and *in vivo* on animal models or transplanted patients treated with calcineurin inhibitors, demonstrated that these immunosuppressants increase the levels of endothelial EVs and alter their composition [29]. In particular, results indicated that, after treatment with calcineurin inhibitors, endothelial EVs showed high levels of the complement protein C3 and could inappropriately activate the complement pathway. Therefore, these endothelial EVs are able to trigger complement-dependent renal disease.

Calcineurin inhibitors are also able to induce hypertension in about 70-90% of kidney transplant recipients [30]. Calcineurin inhibitors can overexpress the thiazide-sensitive NaCl cotransporter in renal tubular cells, leading to an increase in salt retention and thus to hypertension, usually treated with thiazide diuretics [31]. Since it is known that the urinary EVs reflect the composition of the renal tubule epithelial cells [32], the evaluation of proteins in urinary EVs could be useful to monitor changes during calcineurin inhibitors treatment in order to avoid adverse effects. Esteva-Font *et al.* have found a positive correlation between

plasma cyclosporine levels and NaCl and Na-K-2Cl co-transporters abundance in urinary EVs of cyclosporine-treated kidney transplant recipients [33]. These results were confirmed in tacrolimus-treated hypertensive kidney male transplant patients by Rojas-Vega *et al.* [34].

Recently, Tutakhel and collaborators investigated the effects of cyclosporine and tacrolimus on NaCl co-transporter abundance in urinary EVs [35]. In kidney transplant recipients treated with cyclosporine or tacrolimus, the level of the cotransporter in urinary EVs was 4-5 times higher than in untreated kidney transplant recipients or in healthy volunteers. This led to an increase in sodium reabsorption and could be at the basis of the pathogenesis of hypertension observed in these patients.

Table 2. Schematization of main reciprocal interactions between extracellular vesicles and immunosuppressive drugs.

Immunosuppressant Drug	Interaction with EVs	Refs.
Calcineurin Inhibitors	Cyclosporin and tacrolimus treatment enhance NaCl and Na-K-2Cl co-transporters levels in urinary EVs, leading to hypertension. The evaluation of these transporter levels in urinary EVs can be used as biomarker for monitoring this side effect.	[28, 33, 34]
	After cyclosporin treatment, cells secrete PrP by EVs and this could lead to the spreading of toxic prions among cells and across tissues with the development of neurodegenerative disorders.	[36]
Glucocorticoids	flTRL2 on EVs surface interacts with Pam_3CSK_4, an activator of the pro-inflammatory NF-κB, that in turn enhances the release of endogenous glucocorticoids, improving the immunosuppression induced by glucocorticoids.	[38]
	Osteonecrosis of the femoral head is a severe adverse effect of glucocorticoids. Urine-derived stem cells-EVs deliver pro-angiogenic factor, in particular the deleted in malignant brain tumors 1 protein and anti-apoptotic tissue inhibitor of metalloproteinases 1, that can induce the apoptosis of bone marrow and trabecular bone cells, rescuing the impaired angiogenesis. Therefore, urine-derived stem cells-EVs could be used as a novel nano-sized agent to avoid this adverse effect.	[39]
Rapamycin	The combination of immature DCs-derived exosomes and low-dose rapamycin induces long-term cardiac allograft survival and induces donor specific allograft tolerance.	[42]
	In colorectal cancer cell lines, EV-miRNAs profiles show a significant upregulation of miR-6127, miR-6746-5p, and miR-6787-5p under rapamycin treatment and gene expression analysis identified 22 downregulated genes involved in chromatin organization, DNA packaging, and cell cycle.	[46]
Methotrexate	Salazosulfapyridine and/or methotrexate treatment modify the exosomal proteome, enhancing immunosuppressive and anti-oxidant proteins levels, and inhibit the effects of IL-1β on exosomal proteins, suggesting a role of exosomes in rheumatoid arthritis treatment.	[49]
	The evaluation of uOat5 in urinary exosomes could be a potential non-invasive early biomarker of methotrexate-induced renal injury.	[50]
Cyclophosphamide	The association of exosomes, cyclophosphamide and poly I:C represents an effective combination for suppressing tumour growth.	[51]
	TF - positive EVs can increase the occurrence of venous thromboembolism and cancer and metastasis development: the co-administration of dabrigan etexilate and cyclophosphamide reduces these vesicles levels, avoiding these consequences.	[54]
	Camel milk exosomes can be used to reduce the oxidation and to improve the immune response in cyclophosphamide-treated animals.	[55]
Eculizumab	EVs of platelet origin present pro-coagulant activity and their levels decrease after eculizumab treatment, leading to a reduction of thrombotic effects. Moreover, these EVs levels could be used as biomarkers able to predict thrombotic risk in paroxysmal nocturnal hemoglobinuria patients.	[58, 60]
	TF- positive EVs have a role on the development of thrombosis in paroxysmal nocturnal hemoglobinuria patients. Eculizumab treatment decreases both TF-positive EVs and plasma markers of thrombin generation levels, reducing the occurrence of thromboembolic events.	[61]
	Proteome profile of plasma exosomes of paroxysmal nocturnal hemoglobinuria patients under treatment with eculizumab shows an increase of Ig heavy chain V-1 region HG3 levels: these variations could be used for monitoring, in real-time, the effect of eculizumab treatment.	[63]

(Table 2) contd….

Immunosuppressant Drug	Interaction with EVs	Refs.
Infliximab	In Crohn's disease patients there are high levels of circulating EVs, whose release is mediated by Th1: infliximab leads to a reduction of EVs levels, through the downregulation of Th1-mediated cytokine production.	[67]
	Infliximab treatment reduces both endothelial and platelet EVs levels in the plasma of Crohn's disease patients: these vesicles levels could be used as biomarkers in anti-TNFα therapies.	[68]
	In infliximab-treated patients with bipolar disorders, a reduction of TNFR1 level in NEVs occurs, leading to an improvement of depressive symptoms as well as an increased global cortical thickness. NEVs, used as neuronal biomarkers, allow to reveal that infliximab engaged the TNFR/NF-κB neuroinflammatory pathway in individuals with bipolar disorders in a trauma-dependent manner and in association with brain structural changes.	[69]
Certolizumab	Endothelial EVs can be involved in the generation of cardiovascular disease in rheumatoid arthritis patients. Certolizumab treatment reduces EV levels: endothelial EVs could be so used as vascular damage biomarkers.	[73]
Etanercept	In rheumatoid arthritis patients EVs expose on their surface sTNFα that is involved in the inflammation: etanercept interacts with this portion, block its interaction with the receptor and thus reduces the disease progression.	[76]
Glatiramer Acetate	miR-9-5p, miR-35-3p, miR-155-5p, miR27a-3p, miR-126a-5p, miR-350-3p, present in urine exosomes of multiple sclerosis patients, decrease after glatiramer acetate administration: urine miRNAs expression profile can be used as a non-invasive approach for multiple sclerosis-screening.	[80]
Fingolimod	Endothelial EVs levels are corrected and reduced by fingolimod treatment, improving multiple sclerosis pathogenesis.	[83]
	EVs could be used as biomarkers for early multiple sclerosis-fingolimod treatment monitoring, for assessing either the disease state and the efficacy of the treatment.	[87]
	Multiple sclerosis-fingolimod treatment reduces EVs levels that could be used as biomarkers for monitoring the development of the disease.	[85]

DCs: dendritic cells; EVs: extracellular vesicles; NEVs: EVs of neuronal origin; flTR2: full-length toll-like receptor 2; Oat5: organic anion transporter 5; poly I:C: polyinosinic–polycytidylic acid sodium salt; PrP: prion protein; sTNFα: TNFα expressed on the surface; TF: tissue factor.

Recent results indicate that cyclosporine-treated cells, due to the inhibition of cyclophilins, can release misfolded prion protein (PrP) molecules in exosomes [36]. PrP is an aggregation-prone membrane-anchored glycoprotein associated with the onset of neurodegenerative diseases. No data on EVs and cyclosporine induction of neurodegeneration is available yet, nevertheless further studies are needed to investigate the mechanisms that lead to the secretion of exosomes with the accumulation of misfolded PrP after treatment with cyclosporine.

1.1.2. Glucocorticoids

Glucocorticoids are a class of steroid hormones with an important immunosuppressive action. In addition, these agents are highly effective in suppressing inflammation in a variety of diseases (such as rheumatoid arthritis, inflammatory bowel disease or autoimmune diseases) [25, 37]. The use of these drugs can lead to adverse effects such as avascular bone necrosis, osteopenia, increased risk of infection, delayed wound healing, hyperglycemia and hypertension and, in pediatric patients, growth retardation.

A recent study conducted by Hoppstädter and colleagues has highlighted the anti-inflammatory potential of toll-like receptor 2 (TLR2)-containing EVs released by primary human alveolar macrophages during treatment with the synthetic glucocorticoid dexamethasone plus lipopolysaccharide [38]. Dexamethasone stimulated full-length TLR2 (flTLR2) and soluble TLR2 (sTRL2) upregulation in alveolar macrophages. TLR2-containing EVs were able to bind the TLR2 ligand Pam3CSK4, a synthetic triacylated lipopeptide and potent activator of the pro-inflammatory transcription factor NF-κB [39]. To examine the functional implications of TLR2-EVs production on Pam3CSK4-induced inflammatory responses, TLR2-responsive HEK cells expressing a luciferase reporter gene under the control of the CXCL8 chemokine promoter were used. The authors found that TLR2-containing EVs derived from dexamethasone plus lipopolysaccharide-treated cells were able to inhibit Pam3CSK4-induced luciferase production, suggesting that these EVs can exert decoy functions.

Glucocorticoid treatment can induce osteonecrosis of the femoral head; Chen and collaborators recently in-

vestigated the role of EVs from urine-derived human stem cells, a kind of mesenchymal stem cell that can be isolated from urine, in attenuating this adverse effect [40]. In a rat model of methylprednisolone-induced severe osteonecrosis, the treatment with EVs obtained from urine-derived stem cells alleviated the disease manifestations, with a reduction in destruction of trabecular bone and bone marrow in the femoral heads. In particular, *in vitro* and *in vivo* experiments demonstrated that these EVs are able to deliver pro-angiogenic factors, such as the deleted in malignant brain tumors 1 protein and anti-apoptotic tissue inhibitor of metalloproteinases 1. The consequence was the suppression of apoptosis in bone marrow and trabecular bone cells and the rescue of impaired angiogenesis.

1.1.3. Rapamycin

Rapamycin, also known as sirolimus, is a macrolidic compound structurally related to tacrolimus that belongs to a new class of immunosuppressive drugs, the proliferation signal inhibitors [41]. Rapamycin has been used in organ transplantation to avoid immune rejection and, being much less nephrotoxic, is a good alternative to calcineurin inhibitors [25]. However, both long-term use and high dosage of the drug can induce various adverse effects such as myelosuppression, hepatotoxicity, diarrhea, pneumonia and headache. A new approach for inducing transplant immune tolerance, using low-dose rapamycin, was proposed by Li and collaborators [42]: the effect of the combined treatment with exosomes derived from immature donor DCs and low-dose rapamycin on tolerance induction was tested in a mouse cardiac transplantation model. Immature DCs are able to induce immune tolerance, but cannot be used as therapeutic agents due to their long time of production, storage instability and their maturation *in vivo,* which stimulates T cells and thereby initiate immune responses [43]. To overcome these limits, immature DCs-derived exosomes have been proposed as alternative therapeutic agents and the association with rapamycin administered to recipients before and after transplantation induced long-term cardiac allograft survival and a donor specific allograft tolerance.

Patients who have undergone solid organ transplantation, in particular kidney transplant, have a higher probability of developing colorectal cancer, due to the long-term immunosuppression [44]. Rapamycin and cyclosporine have opposite oncogenic properties: rapamycin presents anti-angiogenic and anti-proliferative effects, while cyclosporine promotes tumor formation and progression [45]. Tubita and collaborators investigated the effects of rapamycin and cyclosporine on miRNAs content of EVs from metastatic (HCT116) and non-metastatic (SW480) colorectal cancer cell lines [46]. Rapamycin treatment induced an increased release of EVs from HCT116 cells; on the contrary, cyclosporine determined a decrease of EVs production. Furthermore, a differential miRNA expression profile of EVs between the two treatments was evident. In particular, rapamycin induced an upregulation of miR-6127, miR-6746-5p and miR-6787-5p, while cyclosporine caused their downregulation. The effects of these miRNAs were investigated: a reduction of transcripts involved in epigenetic regulation, including chromatin assembly and organization, DNA replication, and cell cycle regulation, which may lead to a reduction in cancer progression, was observed. These data support the hypothesis that these EVs-miRNAs, overexpressed under rapamycin treatment and downregulated by cyclosporine, can reflect the rapamycin anti-neoplastic proprieties and the cyclosporine tumorigenic activity.

1.1.4. Methotrexate

Methotrexate is a cytotoxic antimetabolite agent. The compound has chemotherapeutic and immunosuppressive properties and is used in the treatment of several tumors, such as acute lymphoblastic leukemia and osteosarcoma, or autoimmune diseases, such as rheumatoid arthritis or psoriasis [47]. Structurally, it is an analogue of folic acid and its pharmacological actions are related to the antimetabolite effect [25], with disruption of nucleotides synthesis, leading to accumulation of adenosine, affecting cellular proliferation and cytokine expression. The most common side effects associated with methotrexate use are nausea, diarrhea, myelosuppression, nephrotoxicity, liver and lung toxicity.

Rheumatoid arthritis is an autoimmune chronic disease that leads to synovial inflammation; exosomes probably play an important role in both its pathogenesis and therapy. Indeed, exosomes from synovial fibroblasts stimulated with the pro-inflammatory cytokine interleukin-1β (IL-1β) are able to enhance synovial metalloproteinases expression in chondrocytes, increasing inflammation [48]. In order to investigate EVs role in rheumatoid arthritis therapy, Tsuno *et al.* have evaluated two anti-rheumatic drugs, methotrexate and sulfasalazine: their effects on the protein profiles of exosomes derived from a synovial sarcoma cell line (SW982), with and without IL-1β stimulation, were analyzed [49]. Sulfasalazine and methotrexate co-treatment enhanced in exosomes the immune system-related protein Rab-7b, which downregulates inflammatory responses in macrophages.

One of the main adverse effects of methotrexate is renal injury. The gold standard for assessing this problem is monitoring serum creatinine, but this parameter does not always give reliable and precise results. A recent work, performed by Severin and colleagues, analyzed the time course of methotrexate-induced renal damage by measuring, in rats treated with intraperitoneal methotrexate, the presence of the organic anion transporter 5 (Oat5), a marker of proximal tubular damage in urinary exosomes and supernatants [50]. Interestingly, after the second day of methotrexate treatment, when the renal structure and function are still preserved, Oat5 levels were already high in exosomes, suggesting that this protein could be a useful marker for the prediction of renal damage.

1.1.5. Cyclophosphamide

Cyclophosphamide is an alkylating non-phase specific cytotoxic agent used as anti-cancer and immunosuppressant drug; treatment with high doses of this agent can lead to pancytopenia and hemorrhagic cystitis [25]. Guo and colleagues have studied the anti-tumoral effects of the association of DCs-derived exosomes, cyclophosphamide and polyinosinic–polycytidylic acid sodium salt (poly I:C) *in vitro* and *in vivo* [51]. Poly I:C was used as a TLR ligand that promotes the generation of type-1 polarizing DCs and enhances their maturation. Mature DC-derived exosomes stimulated the proliferation of spleen cells and enhanced their cytotoxic effects when incubated with L1210 mouse lymphocytic leukemia cells *in vitro*. The effect of the combination of DCs-derived exosomes, poly I:C and cyclophosphamide on tumor growth in DBA2 mice was also assessed, showing prolongation of survival compared with cyclophosphamide alone and poly I:C plus cyclophosphamide.

The use of cyclophosphamide is among the factors that can induce venous thromboembolism in cancer patients [52]. In addition, EVs shed by tumor cells expose on their surface phosphatidylserine and tissue factor (TF), which can bind initiators of blood coagulation. Thrombin may also support cancer progression and metastasis by fibrin deposition, platelet activation, and cleavage of protease-activated receptors-1, which stimulate growth factors and chemokines, resulting in angiogenesis [53]. Alexander and his team associated dabigatran etexilate, a direct thrombin inhibitor, with cyclophosphamide. The efficacy of this co-administration and the number of TF-positive EVs were assessed in a murine model of breast cancer [54]. In particular, female Balb/c mice were orthotopically injected, in the mammary fat pad, with 4T1 mouse mammary carcinoma cells. Co-treatment with dabigatran etexilate and cyclophosphamide reduced tumor and metastasis development in comparison to the single administration of each drug; the association also significantly lowered circulating TF-positive EVs and platelet activation, thus reducing the incidence of tumor induced venous thromboembolism.

Ibrahim *et al.* demonstrated that camel milk, which contains several proteins and exosomes, can exert anti-oxidant and immune-modulatory effects, useful to counteract the immuno-toxicity and oxidative stress induced by cyclophosphamide in albino rats [55]. In this study, exosomes were isolated from camel milk, obtained from healthy lactating female camels at the mid-lactation period. Co-treatment of cyclophosphamide with camel milk exosomes, increased serum levels of total proteins, albumin and globulin, reduced the lipid peroxidation marker malondialdehyde levels, enhanced anti-oxidant enzymes activities and normalized cyclophosphamide related effects on IL-6, TNF-α and IFN-γ expression.

1.1.6. Eculizumab

Eculizumab is a humanized monoclonal antibody that prevents the cleavage of human complement component C5 into pro-inflammatory components C5a and C5b; C5b in turn leads to the generation of the terminal complement complex C5b-9 that mediates cell lysis [25, 56].

Eculizumab is approved for the treatment of both aHUS [57] and paroxysmal nocturnal hemoglobinuria (PNH), characterized by the susceptibility of blood cells to be attacked by the complement system. In PNH patients, genetically determined loss of glycosylphosphatidylinositol on platelets' surface leads to the loss of two important complement regulators, decay accelerating factor (DAF or CD55) and membrane inhibitor of reactive lysis (MIRL or CD59), by impairment of their attachment to the cell membrane. Consequently, red blood cells are more vulnerable to the action of complement, leading to complement-mediated intravascular hemolysis, EV production and thromboembolism [25, 57, 58]. EVs of platelet origin express important elements of coagulation (phosphatidylserine and TF), thus providing support to the coagulation processes [58]. Several groups have therefore studied the involvement of EVs in PNH, although with conflicting results. Wannez and colleagues have conducted the first pilot, longitudinal, clinical study on 6 PNH-patients in treatment with eculizumab in order to measure the effect of this drug on the procoagulant activity of EVs [59]. A reduction of platelet EVs was demonstrated under eculizumab treatment, increasing

clotting time and thus reducing coagulation. Furthermore, thrombin generation assay revealed a reduction in the procoagulant profile induced by EVs after eculizumab treatment. These results were further confirmed by Devalet *et al.*, a decreased production of platelet EVs and of the phospholipid-dependent procoagulant activity in the plasma of PNH patients treated with eculizumab was observed, that may in part explain the strong reduction of thrombotic risk reported [60].

Weitz and colleagues have evaluated the thromboembolic events in PNH patients with a history of thrombosis [61]. In the blood of these patients, they found elevated levels of EVs from leukocyte expressing TF; authors hypothesized that the thromboembolic events were a result of complement mediated leukocyte activation and TF expression [62]. A prospective study on the effect of complement C5 inhibition with eculizumab, on EV plasma markers of hemostatic activation (thrombin generation), TF expression, total microparticle factor Xa generation, and red cell hemolysis was, therefore, performed [61]. For this analysis, 11 PNH patients have been included and blood was obtained prior to eculizumab treatment and at the beginning of therapy and until day 90. Complement inhibition by eculizumab decreased the markers of thrombin generation, as well as the total number of procoagulant EVs. This study identified a significant correlation between the reduction of plasma IL-6 and markers of thrombin generation, further supporting a link between inflammation and thrombosis in PNH patients.

Teruel-Montoya and colleagues evaluated the ability of eculizumab to modify the protein content of exosomes in patients with PNH, by analyzing the proteomic profile of plasma exosomes of PNH patients under treatment with eculizumab. An increase of Ig heavy chain V-1 region HG3 levels in eculizumab-treated in comparison to untreated patients was observed. Therefore, it is possible to hypothesize that Ig heavy chain V-1 region HG3 levels variations could be used to monitor, in real-time, the effects of eculizumab treatment in PNH patients [63].

1.1.7. Infliximab

Infliximab is a chimeric monoclonal antibody that can bind and neutralize the pro-inflammatory cytokine TNFα [64]. Infliximab is composed of the variable regions of a mouse anti-human TNFα monoclonal antibody joined to the constant region of a human IgG1 [65]. Since TNFα plays a central role in the development of autoimmune diseases, among which Crohn's disease, ulcerative colitis, psoriasis and rheumatoid arthritis, the use of antibodies directed against this pro-inflammatory cytokine has led to a revolution in the treatment of these pathologies [66]. Chamouard *et al.* compared the variations of EVs concentrations in Crohn's disease after treatment with infliximab. Results indicated that EVs concentrations were reduced after treatment with infliximab, probably due to a downregulation of Th1 cells-mediated cytokine production and function [67].

Pelletier and colleagues tried to explore the effects of infliximab on platelet EVs and endothelial EVs in psoriasis [68]. The analysis was performed in 26 patients with severe psoriasis, 6 of them treated with infliximab. After 3 months of treatment, a reduction of 75% in disease activity, evaluated by the Psoriasis Area Severity Index score, occurred. The ability of infliximab to reduce the number of endothelial EVs and of platelet EVs suggests a possible role of these vesicles as promising biomarkers in the follow-up of patients treated with anti-TNFα.

Several studies have highlighted that inflammation is a relevant pathophysiological mechanism for a subset of individuals with bipolar disorder and thus infliximab was used in order to reduce the inflammation state in these subjects [69]. Since it is known that EVs are able to cross the blood brain barrier and contain a cargo that reflects the cell from which they are derived, their use for probing intracellular signalling processes in neuroinflammatory pathways was proposed. A recent innovation is the use of blood EVs enriched for neuronal origin (NEVs) as a source of biomarkers that directly reflect neuronal-specific processes of several neurologic/psychiatric diseases such as Alzheimer's disease or schizophrenia. A clinical study conducted by Mansur and colleagues [69], has tried to identify biomarkers of infliximab response in patients affected by bipolar disorder and with a history of childhood abuse. The study was focused on the TNFα receptors (TNFR) 1 and 2 and the NF-κB pathway, considered in this paper as neuroinflammatory biomarkers in NEV, and their levels were compared to those of the established EV marker ALG-2-interacting protein X, as normalizer. NEVs were obtained from the blood of patients, collected at baseline and at weeks 2, 6 and 12 of infliximab therapy. Results indicated that infliximab did not modify ALG-2-interacting protein X levels, suggesting that the drug does not alter the circulating NEVs levels. Infliximab decreased TNFR1 and NF-κB pathway signalling biomarkers in NEVs, whereas TNFR2 was not altered; these findings were more pronounced in patients who had suffered physical abuse.

1.1.8. Certolizumab Pegol

Certolizumab pegol is a pegylated humanized Fab′ fragment of an anti-TNFα monoclonal antibody with a high affinity for TNFα. It is used for the treatment of Crohn's disease and rheumatoid arthritis [70]. Since certolizumab does not contain an Fc portion, it does not induce complement activation, antibody-dependent cytotoxicity or apoptosis, unlike other monoclonal antibodies, such as infliximab and adalimumab [71]. Accurate screening of several publications conducted by Aviña-Zubieta *et al.*, demonstrated that at least half of the observed deaths in rheumatoid arthritis were due to manifestations of cardiovascular disease (CVD), because of increased endothelial cell activation [72]. TNFα plays a central role in the development of rheumatoid arthritis and can contribute to CVD risk; for this reason, the use of TNFα inhibitors, among which certolizumab pegol, is effective in the treatment of this condition. Rheumatoid arthritis patients affected by CVD present high levels of endothelial EVs and a study was conducted to explore whether certolizumab pegol has beneficial effects on endothelial cell function [73]. E-selectines, such as VCAM1 and ICAM1, are expressed only on endothelial cells activated by cytokines, which stimulate the recruitment of leukocytes to the site of injury [74]. In this study, human aortic endothelial cells were cultured *in vitro* and exposed to TNFα alone, in order to mimic the inflammation state in rheumatoid arthritis patients, or to TNFα plus certolizumab pegol [73]. Results indicate that the administration of TNFα alone leads to an upregulation of E-selectine genes, while the co-administration of TNFα plus certolizumab pegol prevents the upregulation, with a gene expression pattern comparable with the untreated control. The treatment with TNFα induced higher levels of endothelial EVs in comparison to untreated cells or cells treated with TNFα plus certolizumab pegol.

1.1.9. Etanercept

Etanercept is a recombinant fusion protein formed by the extracellular ligand-binding domain of the 75 kDa human receptor for TNFα and the Fc portion of human IgG1. It acts as a competitive inhibitor of TNFα, mimicking the activity of soluble TNFα receptors and preventing TNFα from binding to its receptor. Moreover, etanercept, as well as infliximab, is also able to recognize the TNFα membrane-related portion [75, 76]. The drug is approved for several inflammatory diseases such as psoriasis and rheumatoid arthritis [77].

Barbati and colleagues investigated the role of EVs in rheumatoid arthritis patients [76]. The presence of TNFα on the membrane of human EVs was demonstrated: etanercept could recognize membrane TNFα, avoiding its binding to the receptor. Moreover, after 4 months of treatment with etanercept, the number of total and endothelial EVs decreased. In addition, the authors evaluated apoptosis and autophagy in EA.hy926 cells, an immortalized endothelial cell line. The evaluation was carried out after treating EA.hy926 cells with EVs purified from patients both before treatment and after 4 months of therapy with etanercept. EVs purified before treatment expressed TNFα on their surface and significantly increased both apoptosis and autophagy levels in endothelial cells, in a dose-dependent manner. Furthermore, after 4 months of etanercept treatment, the TNFα expression significantly decreased in EVs and their ability to increase apoptosis and autophagy was not observed.

1.1.10. Glatiramer Acetate

Glatiramer acetate is a mixture of synthetic polypeptides composed by four amino acids (L-glutamic acid, L-lysine, L-alanine and L-tyrosine) with immunomodulating properties, used for the treatment of multiple sclerosis [25, 78]. Several studies of this drug in multiple sclerosis have been conducted using the *in vivo* B6 mouse model with experimental autoimmune encephalomyelitis. These studies have shown that aberrant miRNAs expression is a common characteristic of demyelinating and neuroinflammatory diseases [79]. Therefore, in order to assess a possible correlation between these miRNA alterations and multiple sclerosis, Singh and colleagues performed a targeted RT-PCR-based high-throughput miRNA array analysis on miRNAs isolated from urine exosomes as well as from the plasma and spinal cord at pre-onset, onset and peak stages of experimental autoimmune encephalomyelitis in the mouse model [80]. The changes of miRNAs during treatment with glatiramer acetate were examined and a decreased expression of miRNAs in experimental autoimmune encephalomyelitis mice urinary (miR-9-5p and miR-35-3p) and plasma (miR-155-5p, miR27a-3p, miR-126a-5p, and miR-350-3p) exosomes was observed.

1.1.11. Fingolimod

The immunomodulator fingolimod is a prodrug [81], structural analogue of sphingosine, and is the first oral treatment approved for multiple sclerosis. The compound can induce bradycardia, arrhythmias and prolongation of the QT interval, requiring cardiac monitoring in the first 6 hours after the initial administration [25]. Since fingolimod is used in the treatment of multiple sclerosis, several studies have tried to investigate its activity in this disease and to identify the possi-

ble role of EVs. Fingolimod can inhibit acid sphingomyelinase (A-SMase), an enzyme involved in the regulation of EVs production [82]. Zinger *et al.* have conducted a study in order to evaluate the association between EVs levels and multiple sclerosis disease progression in 19 patients treated with fingolimod [83]. Fingolimod treatment corrected EVs levels, reducing endothelial EVs numbers, while increasing the release of EVs from B cells. Moreover, human brain endothelial cells (HBEC) treated in vitro with TNFα as a pro-inflammatory agent, released high EVs levels that were reduced after treatment with fingolimod. In another study conducted by Sàenz-Cuesta *et al.*, the effects of fingolimod on EVs in the blood of 11 multiple sclerosis patients were analyzed [82]. EVs quantification was performed using nanoparticle tracking analysis that showed an increase 5 hours after fingolimod administration: this result was unexpected since fingolimod, being an analogue of natural sphingosine, plays a role in the inhibition of vesicular trafficking [84]. Fingolimod modulation on EVs miRNAs content was also analyzed, demonstrating the dysregulation of several miRNAs, such as miR-223-3p, miR-28-5p and miR-708-5p. Furthermore, authors have incubated EVs isolated from the blood of multiple sclerosis patients blood with lymphocytes obtained from a healthy donor. EVs inhibited lymphocyte activation and this ability was reduced 5 hours since the administration of the first dose of fingolimod.

Monocyte-derived EVs play a central role in multiple sclerosis progression and in demyelinating activity [85]. A study conducted by Bianco and colleagues has shown that A-SMase, a key enzyme involved in EVs formation, acts as an ATP receptor P2X7 (P2X7R) effector, downstream of p38 phosphorylation, leading to its activation [86, 87]. This A-SMase activity is necessary for EVs release from glial cells. A study conducted by Amoruso *et al.* analyzed the effect of fingolimod on the release of EVs in P2X7R-stimulated monocytes from multiple sclerosis patients and healthy donors [85]. In this study, elevated EVs levels in multiple sclerosis patients compared to healthy donors were identified. Fingolimod was able to reduce the ATP-induced EV shedding in monocytes after 12 months of treatment, compared to untreated multiple sclerosis patients, and inhibited A-SMase activity.

CONCLUSION

Over the years, EVs have gained the interest of the scientific community since they participate in both physiological and pathological processes such as inflammation, immune responses, cancer, angiogenesis, metastasis, blood coagulation, and viral infections. Furthermore, since EVs reflect the physio-pathological state of their parental cells, they can be used as diagnostic biomarkers. From this review, the importance of EVs as tools to assess adverse effects of immunosuppressant drugs has emerged, suggesting innovative proprieties and new possible therapeutic uses. Although, over the years, the research has been increasingly refined, there is still a long way to establish EVs as useful tools for diagnostic, prognostic, and therapeutic applications in the near future. In particular, techniques to isolate specific EVs subpopulations should be improved, and also knowledge on molecular mechanisms underlying their biology, particularly after drug therapy, should be increased.

LIST OF ABBREVIATIONS

A-Smase	=	Acid Sphingomyelinase
aHUS	=	Atypical Hemolytic Uremic Syndrome
CVD	=	Cardiovascular Disease
DCs	=	Dendritic Cells
EVs	=	Extracellular Vesicles
flTRL2	=	Full-length toll-like Receptor 2
HUS	=	Hemolytic Uremic Syndrome
IFNγ	=	Interferon γ
IL	=	Interleukin
miRNA	=	microRNA
MSCs	=	Mesenchymal Stem Cells
NEVs	=	EVs of Neuronal Origin
NF-kB	=	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NTA	=	Nanoparticle Track Analysis
Oat5	=	Organic Anion Transporter 5
P2X7R	=	Ionotropic ATP Receptor P2X7
PNH	=	Paroxysmal Nocturnal Hemoglobinuria
Poly I:C	=	Polyinosinic–polycytidylic Acid Sodium Salt
PrP	=	Prion Protein
TF	=	Tissue Factor
TNFR	=	TNFα Receptors
Treg	=	Regulatory T Cell

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This work was supported by the Institute for Maternal and Child Health "Burlo Garofolo," Trieste, Italy (grant number RC 1/17 and RC 10/19).

CONFLICT OF INTEREST

Dr. Stocco Gabriele is the Editorial Advisory Board Member of the journal Current Medicinal Chemistry CMC.

ACKNOWLEDGEMENTS

Declared none.

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