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Extracellular vesicles in parasitic diseases

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Parasitic diseases affect billions of people and are considered a major public health issue. Close to 400 species are estimated to parasitize humans, of which around 90 are responsible for great clinical burden and mortality rates. Unfortunately, they are largely neglected as they are mainly endemic to poor regions. Of relevance to this review, there is accumulating evidence of the release of extracellular vesicles (EVs) in parasitic diseases, acting both in parasite–parasite inter-communication as well as in parasite–host interactions. EVs participate in the dissemination of the pathogen and play a role in the regulation of the host immune systems. Production of EVs from parasites or parasitized cells has been described for a number of parasitic infections. In this review, we provide the most relevant findings of the involvement of EVs in intercellular communication, modulation of immune responses, involvement in pathology, and their potential as new diagnostic tools and therapeutic agents in some of the major human parasitic pathogens.

Keywords: *extracellular vesicles*; *microvesicles*; *exosomes*; *parasites*; *protozoa*; *helminths*

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Extracellular vesicles (EVs) are key players implicated in intercellular communication without direct cellular contact. Until very recently virtually unknown and regarded as by-products of cellular metabolism, the current perception about EVs has changed drastically due to their newfound role as mediators in the transmission of biological signals and immune responses (1,2). These vesicles of various origins can carry as their cargo a wealth of bioactive molecules such as proteins, DNA, mRNAs and miRNAs, through which they can regulate their targeted cells.

EVs are small membrane-bound vesicles that are generally classified in two major types, exosomes and microvesicles (MVs), based on their size, biogenesis and composition. Exosomes are 30–100 nm vesicles of endocytic origin that are released after the fusion of multivesicular bodies (MVBs) with the plasma membrane. MVs, also sometimes referred to as microparticles (MPs) or ectosomes, are more heterogeneous in shape, can be bigger in diametrical size (0.1–1 μm) and are shed directly from the plasma membrane (1). The composition of exosomes and MVs does not correspond to a random sampling of their cell of origin, which implies active sorting of a specific subset of components including proteins, mRNAs, miRNAs and lipids. Exosomes, for example, are enriched in proteins of the endosomal pathway and proteins involved in vesicle formation (3), while proteins from organelles such as the nucleus and endoplasmic reticulum are not commonly found in these vesicles (4). The lipid composition of EVs is different from that of the plasma

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membrane. Both exosomes and MVs contain higher levels of amino phospholipids and the asymmetrical distribution of phosphatidylethanolamines is lost (5,6). Exosomes are enriched in ceramide (7) but not in lysobisphosphatidic acid (6,8). Also, the composition and morphology of exosomes are clearly distinct from those of apoptotic bodies (9). However, a clear discrimination between exosomes and MVs in terms of their composition is still difficult as there is an overlap of detected components and physical properties (1,10,11). On-going efforts of EV characterization have demonstrated that besides a common set of components, vesicles also feature cell-type-specific subsets, which complicate their biochemical characterization. Furthermore, confusion on the nomenclature and the origin of EVs in the literature, as well as differences in the isolation methodologies make it difficult to fully differentiate EV types (1).

Parasites have plagued humans since their appearance and migration throughout the world around 150,000 years ago (12). In fact, the existence of parasitism was likely described in ancient papyrus dating 3,000–4,000 years ago, but it was not until the beginning of the renascence period that descriptions of human infections undoubtedly related to parasitism were reported. It is believed that close to 400 species can affect humans, of which around 90 are responsible for great clinical burden and mortality rates. There is accumulating evidence of the release of EVs in parasitic diseases, acting both in parasite–parasite inter-communication as well as in parasite–host interactions (13–15). Production of EVs from parasites or parasitized cells has been described in a number of parasitic infections (Table I). The world of human parasites, however, is so vast that for simplicity, and following the division suggested by Cox (12), we have divided them in 2 large groups: parasitic protozoa and helminths.

EVs and parasitic protozoa

Literarily meaning "first animals," protozoa are a rather complex group of organisms commonly divided into 4 major groups according to their locomotion: amoeba, flagellates, ciliates and sporozoa. With circa 11,000 different species, 70 affecting humans, parasitic protozoa are a diverse group of unicellular eukaryotic organisms displaying complex life cycles often alternating between different hosts. Diseases such as amoebiasis, malaria, African and American trypanosomiasis, as well as leishmaniasis are responsible for hundred millions of clinical cases every year in different countries around the world (Fig. 1). Here, we will concentrate on two major groups from which data on EVs is rapidly growing: apicomplexa and kinetoplastids.

Apicomplexa

The apicomplexa is a large and diverse phylum of unicellular parasites of animals characterized by the presence of an organelle of endosymbiotic origin, the apicoplast, and an apical complex composed of secretory organelles called rhoptries and micronemes involved in cell invasion. The apicomplexa are composed of more than 5,000 named species of which 7 genera infect humans: *Plasmodium*, *Babesia*, *Cryptosporidium*, *Isospora*, *Ciclospora*, *Sarcocystis* and *Toxoplasma* (16,17).

Plasmodium *spp.*

Malaria is a parasitic disease caused by apicomplexan protozoa of the genus *Plasmodium* and is transmitted to humans through the bite of the female mosquito *Anopheles*. Four species of *Plasmodium* account for the disease (*P. falciparum, P. vivax, P. malariae* and *P. ovale*), with a fifth species, *P. knowlesi*, recently identified from a zoonotic phenomenon (18). According to WHO estimates, in 2012 there were close to 207 million clinical cases and an estimated 627,000 deaths (19). In addition, several species of *Plasmodium* exist that can infect other animal species such as birds, reptiles, monkeys and rodents (20–22). In humans, the parasite develops silently in the liver before reaching the blood stage, where malaria parasites invade and replicate within host red blood cells. A proportion of parasites will convert to gametocytes, the sexual stages that are capable of transmitting the infection to the insect vector.

In malaria, the release of EVs from different cells has been described in murine models as well as in human infections. Different human studies have demonstrated that the circulating levels of EVs rise during infections by the human parasites *Plasmodium vivax* (23) and *Plasmodium falciparum* (24–26). In these studies, the levels of circulating EVs correlated with clinical manifestations such as fever and cerebral dysfunctions, suggesting a role of EVs in malaria pathogenesis. Studies in mice models suggest that EVs contributed to the induction of systemic inflammation (27,28). In the murine malaria model of mice infected with *Plasmodium berghei* (ANKA strain), a reference strain from African origin in which mice, *albeit* variably, develop cerebral malaria (CM), EVs isolated from plasma, primarily derived from infected erythrocytes, induced a potent activation of macrophages via toll-like receptors (TLR) when compared to plasma-derived MVs from naïve animals (28). In the same murine model, the abrogation of MV formation in mice knocked out for the gene ABCA1 protected these animals against CM, demonstrating a link between EV production and pathogenesis (29). The ABCA1 transporter is a modulator of the presence of phosphatidylserine (PS) in the outer layer of the plasma membrane, and PS is a major component of the surface of MVs.

Exosomes have also been described in the context of malaria infection in the mice model *Plasmodium yoelii* 17X-BALB/c. In this non-lethal malaria murine model, the parasites present a tropism for reticulocytes, resembling the human malaria infection by *P. vivax*. Exosomes were

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Table I. Extracellular vesicles in parasites

	Parasitic pathogen	Type of vesicle	Characterization	Diameter (mean or range)	References
Protozoa					
Apicomplexa	*Plasmodium falciparum*	Plasma-derived microvesicles	FC	n.d.	(24–26)
		Exosome-like vesicles	EM, AFM	70–120 nm	(31)
		Erythrocyte-derived microvesicles	SG, EM, WB, MS/P	100–400 nm	(32)
	Plasmodium vivax	Plasma-derived microvesicles	FC	n.d.	(23)
	Plasmodium berghei	Plasma-derived microvesicles	EM, FC	75–450 nm	(27)
			EM	150–250 nm	(28)
	Plasmodium yoelii	Exosomes from infected reticulocytes	SC, EM, FCb, MS/P	30–120 nm	(30)
	Toxoplasma gondii	Exosome-like vesicles	EM, WB	60–150 nm	(39)
			EM/m@	80–90 nm	(43)
	Cryptosporidium parvum	Exosomes	EM, WB, NTA	40–100 nm	(44)
Kinetoplastids	*Trypanosoma brucei*	Exosome-like vesicles	SG, EM, MS/P	50–100 nm	(48)
	Trypanosoma cruzi	Plasma membrane-derived vesicles, exosomes, ectosomes	EM	500 nm	(50)
			EM	n.d.	(72)
			EM	20–80 nm	(51)
			EM	n.d.	(49)
			EM, WB	<1,000 nm	(52)
			SG, EM, WB, NTA, MS/P	70–90 nm, 130–140 nm	(87)
			EM, NTA, DS	130–140 nm	(92)
			EM, NTA, MS/L	70–90 nm, 130–140 nm	(94)
			EM, DS	20–200 nm	(91)
			EM, EA	40–500 nm	(95)
		T. cruzi-induced host cell plasma-membrane-derived vesicles	SG, EM, FC	200–500 nm	(89)
	Leishmania spp.	Exosomes	EM, MS/P	50 nm	(97)
			SG, EM, WB	30–70 nm	(98,99)
			SG, EM, MS/P, WB	<100 nm	(103)
		Leishmania-induced macrophage exosomes	SG, EM, MS/P, WB	40–100 nm	(100)
Other	*Trichomonas vaginalis*	Parasite-derived exosomes	SG, EM, WB, NTA	50–100 nm	(106)
	Giardia duodenalis	Extracellular vesicles	FC	n.d.	(107)
Helminth					
Flatworms	*Echinostoma caproni*	Membrane-bound vesicles	EM, MS/P	30–100 nm	(112,113)
	Fasciola hepatica	Exosome-like vesicles	EM	30–100 nm	(122)
	Dicrocoelium dendriticum	Exosome-like vesicles	EM, MS/P, DS	30–100 nm	(127)
Roundworms	*H. polygyrus*	Extracellular vesicles	EM, m@	50–100 nm	(129)

FC: flow cytometry; SG: sucrose gradient; SC: sucrose cushion; EM: electron microscopy; WB: western blotting; NTA: nanoparticle-tracking analysis; AFM: atomic-force microscopy; FCb: flow cytometry of bound-to-beads vesicles; MS/P: mass spectrometry/proteomics; MS/L: MS/lipidomics; DS: deep sequencing or RNA-seq of small RNAs; EA: enzymatic assay; m@: microarrays.

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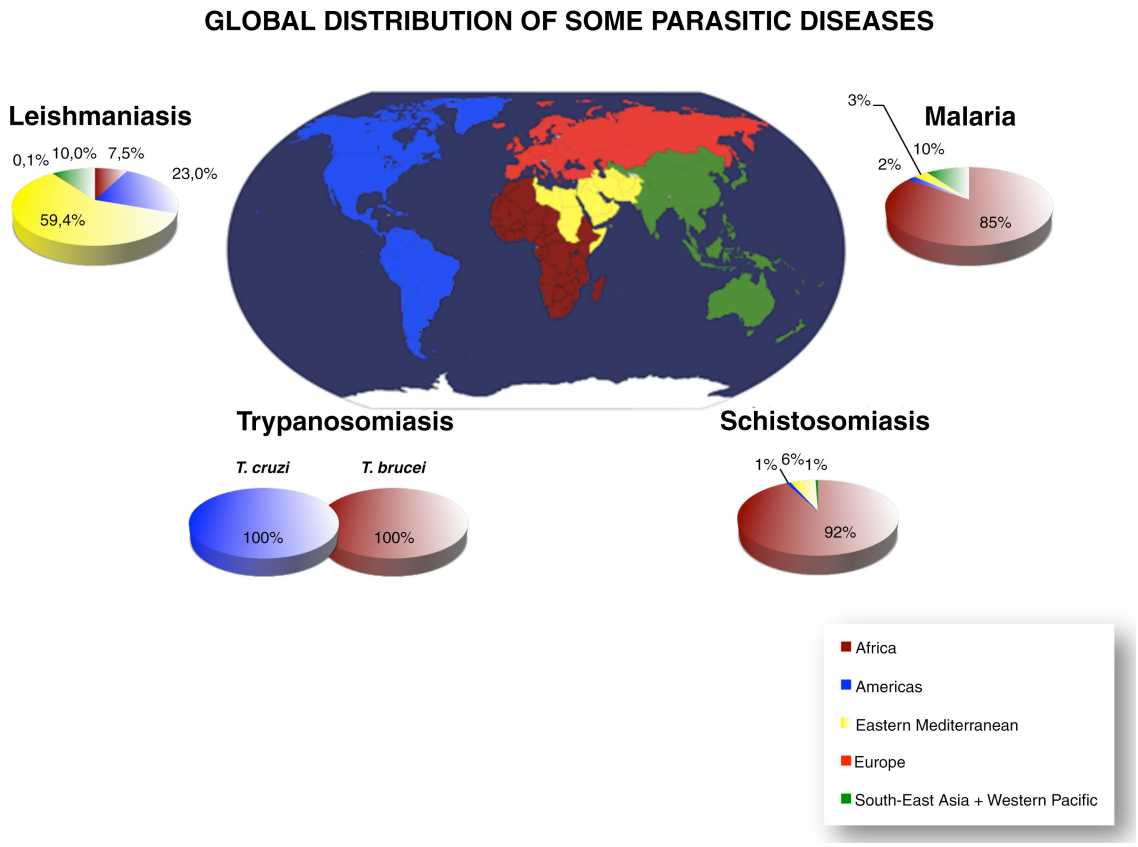

Fig. 1. Endemic geographical distribution of some human parasitic diseases. The map shows 5 different areas of the world according to WHO: Americas, Africa, Europe, Eastern Mediterranean and South-East Asia Western Pacific. The percentages represent the distribution of autochthonous endemic cases (133).

isolated from plasma of mice and from reticulocyte culture by differential centrifugation and their identity was confirmed by electron microscopy, FACS and proteomic analyses. The study demonstrated that exosomes derived from infected reticulocytes contained host and parasite proteins and had a role in modulating immune responses (30). Parasite proteins within the exosomes included several antigens such as merozoite surface proteins 1 and 9, as well as blood stage surface antigens and enzymes related to proteolysis and metabolic processes. Immunizations of BALB/c mice with exosomes from infections, isolated both from peripheral blood and from reticulocyte *in vitro* cultures, elicited IgG antibodies capable of recognizing *P. yoelii*-infected red blood cells (iRBCs), induced reticulocytosis and changed the cell tropism to reticulocytes of the normocyte-prone lethal *P. yoelii* 17XL strain upon infection. Moreover, when combined with CpG-oligodeoxynucleotides, immunizations conferred complete and long-lasting protection against lethal infections in close to 85% of the mice tested. These data thus showed for the first time that exosomes derived from reticulocytes could be explored as a vaccine and platform against malaria infections (30).

More recently, two publications have implicated EVs as mediators of intercellular communication in malaria. Both studies show that *P. falciparum*-infected erythrocytes shed vesicles, that these vesicles contain host as well as parasite-derived proteins and that they are transferred to other iRBCs *in vitro* (31,32). Apart from their overall concurrence, these studies have important distinctions that are worth mentioning before further detailing of their findings. In the work by Regev-Rudzki et al., the authors have used OptiPrep™ followed by atomic force microscopy to describe the release of exosome-like vesicles of 80–120 nm by ring-stage parasites (31), whereas Mantel et al. describe slightly larger vesicles in the range of 100–400 nm, isolated by ultracentrifugation in a sucrose cushion and measured by electron microscopy, with the timing of release of a subpopulation of 150–250 nm coinciding with the moment of schizogony or shortly after (32).

According to Mantel et al., iRBCs produce 10 times more EVs than uninfected RBCs, corroborating previous results that described a 13-fold increase in the production of EVs both in *in vitro* conditions as well as during *P. falciparum* infections (24). Proteomic analysis of these EVs showed no apparent difference in host-derived protein

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content between uninfected and infected RBCs. Immunoblotting with immune sera from malaria patients revealed the presence of parasite antigens in iRBC-derived EVs and mass spectrometry identified over 30 parasite proteins, mainly Maurer's clefts residents and surface proteins, but no markers of the knob complex were identified. The iRBC-derived EVs showed strong inflammatory properties, inducing cytokine production in macrophages and PBMCs, activating neutrophils and increasing their migration rate (32).

In the work by Regev-Rudzki et al., exosome-like vesicles were shown to promote the transference of genetic material between iRBCs, demonstrating a novel mechanism for cell-to-cell communication between parasites. Transgenic *P. falciparum* lines resistant to either blasticidin or WR99210, but not both, were co-cultured in the presence of the 2 drugs and doubly resistant parasites were observed after a few days in culture, whereas the individual lines did not thrive under combined drug treatment. After discarding the possibility of plasmodial DNA uptake from the medium, the involvement of EVs was demonstrated by growing the parasite lines in different compartments of a transwell with 400 nm pores. In this setting, even without direct cell-to-cell contact, doubly resistant parasites were still recovered. Of note, the transference of resistance was unidirectional, always with the selection cassette for blasticidin being transferred as the donor. Deletion of PfPTP2, a protein that localizes to membranous structures budding from Maurer's clefts, significantly reduced both the production of EVs and the capacity of the parasite line to receive a donor plasmid through EVs of other lines, implicating this protein in the mechanism by which iRBCs communicate via exosome-like vesicles (31). Interestingly, Mantel et al. also demonstrated a connection to the Maurer's cleft even though the type of EVs recovered and their stage of production during the asexual cycle were different.

Perhaps, most intriguingly is the fact the 2 studies have independently demonstrated a connection between iRBC-derived EVs and gametocytogenesis. Gametocytogenesis is the process by which a fraction of the circulating parasites abandon asexual replication and differentiate into sexual stages, the gametocytes, that will ensure parasite transmission through the invertebrate host, a female anopheline mosquito (33). In both studies, the rate of formation of sexual stages was positively altered, in a titrable fashion, by the addition of EVs from iRBCs. The switch to sexual commitment responds to environmental cues, and the involvement of parasite-derived factors, capable of diffusing through a membrane with 200 nm pores, has been previously described (34,35). However, the nature of these factors remains unknown and the data on EVs from these 2 studies could provide a plausible explanation. Whether these findings have relevance for the dynamics of asexual/sexual balance in infections remains to be demonstrated (36).

The role of EVs in malaria pathogenesis has also been highlighted very recently in an article using a rodent-malaria model of CM. Previous studies had demonstrated elevated levels of MPs in patients with CM (24) and ablation of a transporter implicated in the release of MPs conferred protection against CM in a murine model (29). These results suggested a role of MPs in the pathogenesis of CM but a direct demonstration of this role was missing. Combes and co-workers studied the fate and distribution of MPs in mice models of CM and non-cerebral malaria (NCM). Firstly, they determined the concentration of MPs in each model and showed that there were elevated levels of MPs in mice developing CM in contrast to NCM mice. Moreover, they showed that MPs production coincides with CM onset, production of cytokines and chemokines up-regulation of adhesins and binding of vascular cells to MVs. Next, they determined the fate of fluorescently labelled MPs in the peripheral blood of animals after intra-venous injection. As expected, MPs were rapidly and mostly cleared from circulation by yet to be identified spleen cells within the red pulp. Of interest, histopathological analysis of brain smears from all recipient mice showed that only MPs from *P. berghei*-infected mice were found within the brain microvessels of CM mice. Most relevant, transfer of TNF generated endothelial cell-derived MPs produced *in vitro* induced CM-like pathology in healthy mice (37). All together, these data show for the first time a connection between MPs and the pathogenesis of CM. Whether these findings will translate into novel prognostic markers and/or therapies for this clinical syndrome remains to be determined.

Toxoplasma *and* Cryptosporidium *species*

In addition to *Plasmodium* spp., studies on EVs and exosomes of other apicomplexa have been reported. *Toxoplasma gondii* is an obligate intracellular parasite that causes a disease called toxoplasmosis that infects humans and domestic animals throughout the world (38). In humans, it causes serious damage during pregnancy and in immunocompromised patients, it can also lead to severe clinical complications including death. Noticeably, pioneering studies on the use of exosomes as therapeutic agents against parasitic diseases were first reported in *T. gondii* (39). Immortalized dendritic cells were pulsed *in vitro* with *T. gondii* antigens and exosomes obtained from culture supernatants. *T. gondii*-tagged exosomes were used in immunizations of mice and then challenged with lethal and sub-lethal doses. Immunized mice elicited potent and specific humoral and cellular immune responses against *T. gondii* and close to 70% of the mice challenged with a lethal dose were protected. Mice challenged with a sublethal dose revealed significantly lower number of

cysts in the brains of the animals examined. These results were thus the first to demonstrate that, similar to DCs primed with tumour antigens (40), DCs pulsed with parasite antigens were capable of eliciting protective immune responses. Stimulation of pro-inflammatory responses was later reported *in vitro* and *in vivo* with exosomes obtained from *T. gondii*-infected macrophages (41) and the protective efficacy of DC-derived tagged exosomes further demonstrated in a mouse model for pregnancy (42). Recently, the presence of exosome-like vesicles obtained from supernatants of *Toxoplasma*-infected human foreskin fibroblasts has also been reported (43). Of interest, unique expression profiles of RNA-cargo of yet unidentified origin and function were observed.

Cryptosporidium parvum is an obligate opportunistic intracellular pathogen, recognized as a major cause of diarrhoea in AIDS and immunodepressed patients often resulting in death. Previous studies had demonstrated that infection by *C. parvum* elicited a TLR-4-dependent pathway for regulating transcription of miRNAs to control parasitism (44). Remarkably, a search for mechanistic insights revealed that infection stimulated the release of host intestinal epithelial exosomes shuttling anti-parasite peptides. Thus, this study represents the first report to implicate the release of exosomes from intestinal luminal cells as an important mechanism to control gastrointestinal parasitic infections.

Kinetoplastids

The kinetoplastids are flagellated unicellular organisms characterized by the presence of the kinetoplast, a network of circular mitochondrial DNA, with many copies of the mitochondrial genome (45). They parasitize virtually all vertebrate species as well as insects and plants, and are transmitted by insects, where they complete their biological cycle. A group of kinetoplastid protozoan parasites, known as trypanosomatids, which are characterized by possessing a single flagellum, affects millions of people worldwide. The major pathogenic trypanosomatids for humans are: *Trypanosoma brucei* or African trypanosomes, which cause sleeping sickness or human African trypanosomiasis (HAT); *Trypanosoma cruzi*, which causes Chagas disease or American trypanosomiasis; several species of the genus *Leishmania*, which provoke a wide spectrum of diseases with different pathological manifestations, collectively known as leishmaniasis; *T. cruzi and Leishmania* spp. have obligatory intracellular cycles in the definitive host, whereas *T. brucei* remains as an extracellular parasite while infecting humans. All trypanosomatids are polymorphic, with different forms that appear during the biological cycle in the insect or in the vertebrate, and undergo a strong physiological adaptation with biological changes in the passage from the insect vector to the blood of the definitive host, during the internalization to the cell cytoplasm or vice versa (45).

Trypanosoma *spp.*

The genus *Trypanosoma* contains many species, most causing major veterinary diseases but two of them, that is, *Trypanosoma cruzi* and *Trypanosoma brucei*, are responsible for two of the most neglected human diseases, Chagas disease and African trypanosomiasis. *T. brucei* and *T. cruzi* are flagellates that share many biochemical pathways and specific subcellular organelles, such as glycosomes and kinetoplast (46,47). Trypanosomes are able to produce and release different types of vesicles into the extracellular milieu that play important roles in the parasite–host interaction, presumably by enabling pathogen survival and replication within the host (48–51). These vesicles can interact directly with host target cells, exert long-distance effects on the host immune system, and promote life-cycle transitions within their own population (52).

Two subspecies of *T. brucei*, that is, *T. b. gambiense* and *T. b. rhodesiense*, cause sleeping sickness or HAT, which is transmitted by tsetse flies (*Glossina* spp.). It is lethal when untreated and is a threat for over 60 million people living in sub-Saharan countries. After a long period of increasing prevalence, now HAT incidence seems to be decreasing (53–55). *T. brucei* spends its entire cycle as an extracellular parasite, fully exposed to the host immune system and, therefore, it has developed survival strategies, antigenic variations, among others.

For a better understanding of the pathogenic process and the survival strategy of *T. brucei,* some proteomic approaches have begun to characterize the proteins secreted by this parasite. In 2010, Geiger et al. provided the first overview of the proteins secreted by bloodstream forms of different strains (i.e. Feo, OK, and Biyamina) of *T. brucei gambiense*. With the use of liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI MS/MS), 444 proteins were identified. Notably, a significant proportion of proteins in the secretome lacked a transit peptide, suggesting they are not secreted through a classical sorting pathway. Moreover, the authors found a proportion of vesicles with the same size and density as exosomes, and with some proteins in common. EVs were isolated and characterized from secreted material as well as from infected rat sera, confirming an active budding process on the plasma membrane (48). In addition, proteomic analysis of the secretome isolated from the procyclic form (tsetse fly) revealed a spectrum of proteins, the majority of which belong to 14 families of proteases. These proteases may be required for every aspect of the parasite's life cycle, from the modification of the physiological environment to the immune evasion (56). The secretion of proteins via this new pathway may have several advantages for trypanosomes, such as delivering an avalanche of new epitopes to overwhelm the host immune system, or establishing a communication link between parasites as a survival strategy. These findings may open

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new approaches to formulate novel strategies for controlling the parasites and the disease.

Trypanosoma cruzi causes Chagas disease, a neglected tropical infection endemic to Latin America, where it is transmitted mostly to humans in the faeces of triatomine bugs, known as "kissing bugs" (46). However, other forms of transmission are vertical transmission (congenital), blood transfusion, organ transplantation, and oral contamination via tainted fluids and foods. Outbreaks of the disease through oral transmission, causing the acute form of Chagas disease, are exponentially increasing (57,58). The disease affects almost 8–10 million people in South America, and an estimated 50,000–200,000 new cases are confirmed every year (59), with an increasing number of cases reported in non-endemic regions, including the United States, Canada, Europe and some Western Pacific countries, due to human migratory currents from endemic countries (60–62). The course of the disease is marked by 2 phases: an acute phase with a mortality rate of approximately 5%, and a chronic phase 3–8 weeks after the onset of the illness, which remains asymptomatic for a long time; in fact only 20–30% of cases ever develop any symptoms related to the disease. In contrast to the other human trypanosomes, *T. cruzi* has an obligate intracellular multiplication phase, the amastigote form, which protects the parasite from the humoral immunity, as well as the trypomastigote form in the bloodstream (63).

In 1979, da Silveira et al. (50) were the first to demonstrate the secretion or shedding of MVs by *T. cruzi*. These vesicles were obtained from non-infective epimastigote forms following parasite incubation with cross-linking reagents or acid pH buffers/solutions, and purification by ultracentrifugation followed by sucrose gradient. Transmission electron microscopy (TEM) and freeze-fracture experiments showed that epimastigote vesicles were derived mainly from the plasma membrane and flagellar pocket. Interestingly, these authors also demonstrated by SDS-PAGE, stained with periodic acid-Schiff, that epimastigote-derived vesicles were rich in glycoconjugates, named bands A, B, C and D, previously described by Alves and Colli (64). Later, these molecules were fully characterized and proven to be major glycoproteins (mucins, bands A–C) (65–67), glycolipids (lipopeptidophosphoglycan-LPPG or glycoinositolphospholipids-GIPLs) (68) and glycopeptides (NETNES) (band D) (69) of the parasite surface.

Plasma membrane-derived vesicles were later shown by Gonçalves et al. to also be spontaneously shed by infective host-cell-derived trypomastigote (TCT) forms of *T. cruzi*. These authors clearly demonstrated that the shedding process was temperature and time-dependent, independent of the presence of proteins in the culture medium, and happened in different parasite strains analysed. Interestingly, that report also described the use of size-exclusion chromatography (in Sepharose-4B) for the purification of [^{35}S]-methionine-labelled parasite EVs (51). Immunoprecipitation experiments of these labelled molecules revealed that Tc-85, a member of the *trans*-sialidase (TS)/gp85 glycoprotein superfamily involved in host-cell adhesion and invasion by the parasite (70,71), was a major component of the shed EVs. TEM results also showed that the TCT-derived EVs ranged from 20 to 80 nm in diameter, resembling therefore exosomes. In fact, the expression of Tc-85 in EVs shed by trypomastigote stage, mainly from its flagellar pocket, had been previously reported by Ouaissi et al. (72). Subsequently, Ouaissi et al. (49) also described that the major *T. cruzi* flagellar 24-kDa antigen, currently known as flagellar calcium-binding protein (FCaBP) (73), was also secreted in EVs derived from the plasma membrane and flagellar pocket of infective trypomastigote forms. Together, the aforementioned reports clearly pointed out the importance of shedding of EVs as a novel mechanism used by the parasite to deliver or present major antigens to host cells.

Recently, preliminary proteomic analysis of the TCT-derived EVs has shown that these EVs are enriched in glycoproteins of the TS/gp85 superfamily, α-galactosyl-containing glycoproteins, proteases (i.e. cruzipain), cytoskeleton proteins, mucin-associated surface proteins (MASP) and others (74,75). Many of these proteins are involved in the processes of adhesion and cell invasion (76,77), some of which are specific proteins of *Trypanosoma cruzi*. Shed vesicles could represent an additional mechanism by which invading *T. cruzi* parasites systematically present their antigens to the host. The TS/gp85 glycoproteins, MASP, and mucins found in EVs are associated with the plasma membrane via glycosylphosphatidylinositol (GPI) anchor, and represent the major antigens expressed on the plasma membrane (74,75,78). GPI-anchored glycoproteins protect parasites against the immune system by modulating the host immune response mediated by cytokines and/or forming a dense glycocalyx coat, which hampers the destruction of the parasite by lytic, protective anti-α-galactosyl antibodies (78–83). *T. cruzi* EVs, which contain bioactive molecules, can also play a role in the interaction with the host cells, determining the susceptibility to infection of mammalian cells.

Trans-sialidases are involved in the transfer of sialic acid from host glycoconjugates to the parasite surface mucins, as well as to gp85 glycoproteins (71). The activity of TS assists the parasite in several functions, including parasite survival, infectivity, and host-cell recognition (70,80). Other members of the gp85/TS glycoprotein family, such as the Tc85 sub-family, are involved in *T. cruzi* adhesion (77,84,85). These molecules and others, like gp82 or gp90, have been proposed to mediate the parasite interaction with host cells, playing a role in host-cell invasion (58). Mucins, the other abundant sugar-containing compounds, induce both cellular and humoral

responses, as well as the activation of cells through the TLR 2 (75,78,82,86).

Experiments injecting EVs into BALB/c mice prior to *T. cruzi* infection showed effects on the course of the infection (52). EVs were obtained through Sepharose CL-4B chromatography and α-galactosyl (α-Gal)-positive fractions were pooled and their biochemical analysis revealed the presence of highly abundant surface glycoproteins, such as TS, Tc85, and cruzipain. Treated animals developed severe heart pathology with an intense inflammatory reaction and a higher number of amastigote nests (52). Thus, EVs can interact with the host-cell surface or be internalized, preparing the microenvironment for the incoming trypanosome, supporting their potential role in virulence and pathogenesis.

Recently, the individual proteomes of 2 EV populations from the non-infective epimastigote and the infective metacyclic trypomastigote forms of *T. cruzi* have been described (87). This study provided evidence supporting the existence of at least 2 mechanisms of vesicle secretion in *T. cruzi*: exocytic fusion of MVBs resulting in exosomes, and budding of vesicles directly from the plasma membrane, resulting in MVs (ectosomes or plasma membrane-derived vesicles). Using a combination of methods, including morphological (TEM), immunochemical, and label-free quantitative proteomic analysis, the authors demonstrated that distinct proteins are enriched in each vesicle population, revealing a rich collection of excreted/secreted molecules involved in trafficking and membrane fusion, metabolism, signalling, nucleic acid binding, and parasite survival and virulence (87). These results provided the first insight in the search for potential protein markers for each population of the EVs.

Bayer-Santos et al. (87) have also shown how EVs may be used by infective metacyclic trypomastigotes to deliver cargo into mammalian (HeLa) cells. EVs from *T. cruzi* infective forms can either be released inside the mammalian cells or endocytosed by cells after their release in the medium. Once *T. cruzi*-derived EVs reach the host-cell cytoplasm, they likely modulate host cells to support parasite survival and replication. Moreover, the release of bioactive molecules in EVs may be an efficient strategy employed by the parasite to protect these biomolecules against extracellular degradation or serving as decoys against the highly abundant lytic anti-α-Gal antibodies (82,83,88). An additional immune evasion mechanism of *T. cruzi* relies on the ability of trypomastigote forms to induce the release of host cell-derived vesicles, which contribute to immune evasion by protecting the parasite from the complement attack, and ultimately resulting in increased parasite infectivity and survival (89).

As mentioned above, both *T. cruzi* and *T. brucei* have complex life cycles. To survive changing environmental conditions, parasites must undergo rapid and significant changes in gene expression, which are achieved essentially at the post-transcriptional level through modulation of mRNA stability and translational control mechanisms. Over the last decade, an expanding family of small regulatory RNAs (miRNAs, small interfering RNAs, and Piwi-interacting RNAs) was recognized as key players in novel forms of post-transcriptional gene regulation in many eukaryotes (90). Notably, stressed epimastigotes shed high levels of vesicles (20 to 200 nm in diameter) to the extracellular medium, which carry small tsRNAs and TcPIWItryp proteins as cargo (91). Moreover, at least a fraction of EV cargo was transferred between parasites and susceptible mammalian cells, but not to non-susceptible cells. These data suggest that EVs shed by *T. cruzi* mediate parasite-parasite communication by promoting metacyclogenesis, thus assuring parasite survival through the emergence of the infective form, as well as increasing the susceptibility of mammalian cells to infection (91). The presence of varying and differentially expressed small RNAs by non-infected epimastigotes and infective metacyclic trypomastigotes has also been recently reported (92). More recently, it has been demonstrated that both *T. cruzi*-derived EVs and tRNA-derived small RNAs found in *T. cruzi* EVs are able to up- or down-regulate a variety of host-cell genes (93). All together, these results indicate that small RNAs play an as yet to be fully defined regulatory role(s) in *T. cruzi* infections.

More recently, a focused lipidomic analysis of all four *T. cruzi* developmental stages revealed a lysophosphatidylcholine (LPC) containing C18:1 fatty acid, which has platelet activating (PAF)-like factor activity (94). Interestingly, the bioactive C18:1-LPC species is also secreted to extracellular medium and can be found in larger EVs (ectosomes) secreted by infective metacyclic trypomastigote forms. The authors proposed that *T. cruzi*-derived C18:1-LPC, but not other parasite LPC species lacking PAF-like activity, could eventually be involved in some key aspects of the pathophysiology of Chagas disease, including increased platelet aggregation related to myocarditis, focal ischemia, and myonecrosis.

Neves et al. (95) have recently shown that *T. cruzi* trypomastigote-derived EVs contain acid and alkaline phosphatase activities, which can increase the parasite capacity to adhere and infect host cells.

Leishmania *spp.*

Leishmaniasis are diseases caused by more than 20 species of parasites of the genus *Leishmania* and transmitted to their host by the bites of sandfly mosquitoes *Phlebotomus* and *Lutzomyia*. It is calculated that around 12 million people from 98 different countries are currently infected. There are 3 main forms of the disease: cutaneous which causes localized skin ulcers, visceral which is the most severe form infecting vital organs, and mucocutaneous which causes irreversible destruction of membranes in the nose, mouth and throat (96).

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The presence of exosome-like vesicles in *Leishmania donovani* was originally suggested after analysing the proteome of supernatants obtained from infected macrophages cultures. The absence of the classical secretion signal in the majority of the parasite proteins identified indicated the use of non-classical targeting mechanisms to direct protein export (97). On the other hand, the presence of proteins previously identified in exosomes led the authors to propose a model in which protein export occurs largely through the release of MVs. Secretion of exosomes was confirmed in the growth medium from cultured *L. donovani, L. mexicana* and *L. major* species. Of interest, exosome release was found to be sensitive both to temperature and pH. Moreover, using fluorescent labelling of *Leishmania* surface proteins (such as leishmanolysin GP63, which was consistently present in *Leishmania* exosomes) as well as *Leishmania* expressing green fluorescent protein (GFP), they found an uptake of fluorescent vesicles by non-infected cells, with accumulation of GFP and parasite proteins in structures consistent with MVBs in the cytosol of infected macrophages. Also, it was shown that this intracellular communication selectively induced secretion of IL-8, thus demonstrating, for the first time in parasitic protozoa, that exosomes constitute cargo machinery for intercellular communication and modulation of immune responses (98).

It has also been demonstrated that exosomes released by *Leishmania* spp. modulate the cytokine production by human monocytes and the phenotype of dendritic cells, having a predominantly immunosuppressive effect promoting IL-10 production and inhibiting and regulating the TNF-α and IFNγ that promote parasite progression (99). In addition, the comparisons of the proteome of uninfected, infected, and the LPS-stimulated cell line of macrophages have corroborated the differential parasite cargo specificity in exosomes derived from these cells, and also identifying for the first time the surface protease GP63, specific from *Leishmania*, in exosomes released by cells infected with the protozoa (100). GP63 is a metalloprotease present on the surface of the promastigote and amastigote forms, which actively participates as a critical virulence factor of *Leishmania*, as well as in immunomodulating the host response. Evidence suggests that exosomes secreted from *Leishmania*-infected cells, which contain the virulence factor GP63, when delivered to hepatocytes, can down-regulate the production of specific host miRNAs, thus facilitating liver infection (101).

Similar to *Toxoplasma gondii* (39), the use of dendritic cells-derived exosomes loaded with *L. major* antigens in combination or not with CpG, conferred protection in experimental infections of Balb/C mice (102). Mice were immunized with DC-derived exosomes i.v. and 1 week later challenged with 5×10^5 stationary-phase promastigotes in the right hind footpad. Protection was defined in terms of size of lesions developed in the right footpad as compared to the non-infected footpad and in terms of parasitized cells in the lymph nodes draining the site of infection. The size of the lesion was significantly smaller and approximately 200-fold less cells were found in BMDC-derived exosomes vaccinated animals in contrast to controls. Of note, CpG was not required for conferring protection. These results further reinforced the value of exosomes as cell-free vaccines against *Leishmania*.

Recently Hassani et al. have demonstrated using WT and KO forms of GP63, that the enzyme present in the exosomes can modulate the macrophage immune response at both signalling and gene-expression levels. WT exosomes seem to regulate gene expression of IFNγ and IL-12 receptors, as well as TLR2 and TLR8, inducing the production of iNOS. The KO exosomes seem to be more pro-inflammatory than their WT counterparts. Furthermore, GP63 seem to participate in exosomal protein sorting in the parasite (103).

Other human parasitic protozoa

Trichomonas vaginalis

The urogenital extracellular parasite *Trichomonas vaginalis* is the causative agent of trichomoniasis, the most prevalent sexually transmitted infection, and affecting 275 million people every year. Early studies of the surface membrane proteome of *T. vaginalis* revealed the presence of at least 3 tetraspanins (Tsps) (104,105). As some Tsps are well known constitutive components of exosomes, it was suggested that this extracellular parasite could secrete Tsps-containing exosomes facilitating colonization of the urogenital tissue. To demonstrate the presence of Tsps-exosomes in *T. vaginalis*, they constructed transgenic parasites expressing HA-tagged Tsp1 and showed their presence in the plasma membrane as well as in the cytoplasm of parasites in MVBs structures (106). The vesicles purified from the parasite growth media exhibited the characteristics of exosomes. The characterization of the proteome of these exosome-like vesicles demonstrated that *T. vaginalis* exosomes contained parasite Tsps as well as proteins previously shown to be involved in pathology. In addition to their protein cargo, *T. vaginalis* exosomes also contain a yet uncharacterized small RNAs ranging from 25 to 200 nt. Noticeably, labelled exosomes were shown to interact with and deliver their content to host cells, and to modulate the production of cytokines IL6 and IL8. Moreover, exosomes from a highly adherent strain induced strong parasite attachment of a less adherent strain to epithelial cells (106). These studies thus convincingly demonstrate that *T. vaginalis* uses exosomes in host cell colonization.

Giardia duodenalis

Giardia duodenalis is an extracellular parasite of the human intestine with high global prevalence ($>20\%$ in

developing countries) causing diarrheal illness in individuals from both developing and industrialized countries. Deolindo et al. have reported an increase in *G. duodenalis* EVs formation in response to different conditions (i.e. pH changes, presence of bile, etc.), suggesting that these vesicles could provide a mechanism to the parasite to adapt to the host changing environment in the course of the infection (107). A recent study has characterized the proteome of the vesicles produced during *G. duodenalis* encystation, a process that seems to be mediated by these vesicles, supporting the formation of a low complexity extracellular matrix (108).

EVs in parasitic helminths

Helminths infections are considered neglected tropical diseases. The high medical, educational, and economic burden of helminths infections, together with their coendemicity with malaria and AIDS, provides an important rationale for launching a global assault on parasitic worms. Helminths can be divided into 2 major groups known as the nematodes (roundworms) and the Platyhelminthes (flatworms), the latter composed by the cestoda (tapeworms) and trematoda (flukes) (109). Together, they are responsible for a large burden of disease and socioeconomic losses, as hundreds of millions of people mostly in areas of extreme poverty are infected, albeit with variable numbers (Fig. 1).

Helminths are a rich source of interesting molecules that could lead to innovation for many aspects of biomedicine. Recent studies of host–parasite interactions have led to important discoveries related to the identification of potential new targets for diagnosis and treatment, as well as new vaccine targets for helminthiasis (109,110). New helminths target molecules consist mainly of those present at the external surface (cuticle in nematodes and tegument in trematodes and cestodes) and the excretory/secretory products (ESP), where cytoskeletal proteins, nuclear proteins and glycolytic enzymes are the most abundant ones (110–112).

Flatworms

Recently, the existence of exosome-like vesicles in the parasitic intestinal trematodes *Echinostoma caproni* and the liver fluke *Fasciola hepatica* has been described (112). This constitutes the first description of exosomes in parasitic helminths, although the existence of "membrane bound vesicles" in *E. caproni* and MVBs in *F. hepatica* were previously reported (113–115). Vesicles extruding from the tegument have been also identified in other trematodes like the causative agent of schistosomiasis, *Schistosoma mansoni* (116).

EVs are present in the worm tegument as well as in insoluble fractions of ESP (112). The first studies on EVs composition in *E. caproni* and *F. hepatica* have identified 51 and 79 parasitic proteins, respectively, containing more than half of the proteins previously identified in the secretome of *E. caproni*, *F. hepatica* and other parasitic trematodes (110,115,117,118). Furthermore, it may explain the presence of atypical proteins lacking classical secretion signal peptides, like enolase, in the helminths secretions (110). More recently, the presence of tetraspanins as well as other typical *exosome* proteins in the *Schistosoma japonicum* and *Schistosoma mansoni* teguments has been described, suggesting a similar protein distribution in membranes of tegumentary vesicles in these parasites (119,120). All together, these data suggests that EVs constitute the primary mechanism for protein export in trematodes, as reviewed here for other parasites.

Trematode EVs also contain constituent host proteins that vary depending on the parasite species. For example, while mucin-2 was found in *E. caproni* EVs, *F. hepatica* EVs contained CD19 and the constant region of the IgA heavy chain (112,115). It is noteworthy to mention that proteomic identification is difficult in most of these organisms due to the lack of available assembled sequenced genomes, and it relies on search engines that use transcriptomic data (121,122). Marcilla et al. have also described that EVs were not only actively released by the trematode *E. caproni*, but also were up taken by intestinal cells in culture (112), suggesting a role for these vesicles in host–parasite communication as well as in the establishment of the infection, not only with this trematode but for a variety of flukes and tapeworms.

The secretion and subsequent uptake of EVs provides a mechanism of cell-to-cell communication and enables RNA transport. Exosomes are considered the major "miRNA transporter" between cells, since they contain most of the extracellular miRNAs that have been identified (123,124). Packaging of RNA (mRNA and miRNA) in vesicles appears to provide stability and resistance to RNAse digestion in body fluids, due to the lipid membrane (125,126). More recently, the presence of miRNAs in vesicles from another parasitic helminths, *Dicrocoelium dendriticum*, have been demonstrated confirming this phenomenon as a common feature in parasitic helminths (127). EVs appear to serve as vehicles for miRNA and other regulatory molecules, such as regulatory sequences of mRNA, and may play an important role in the synchronization of metabolism between the host and its parasites regulating host gene expression. Future studies should focus on the characterization and functional analysis of these molecules in the host.

Roundworms

Little is known about the presence of EVs in parasitic nematodes. A secretion pathway involving MVBs and the release of exosomes at the apical plasma membrane from the non-parasitic model nematode *Caenorhabditis elegans*, has been described (128). Buck and co-workers have confirmed recently the existence of EVs in *H. polygyrus*,

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showing their immunomodulatory effect on a murine model confirming previous observations with ESP from the same nematode (129). The presence of "atypical secreted" proteins, including 14-3-3 and serpin, in the *Ascaris suum* larval proteome have been described, suggesting that they are secreted in EVs (130).

Although the secretion of EVs by parasitic helminths is just beginning to be characterized, the research on secretion vesicles and their involvement in intra- and extracellular signalling will address whether these vesicles constitute good targets for new control strategies in helminthiases, which could be implemented as new diagnostic and treatment tools and vaccines.

Concluding remarks

Initially regarded as by-products of cellular metabolism, EVs are now known to act as mediators involved in the transmission of biological signals. In their approximately 150,000 years of co-evolution (12), human parasites have evolved complex life cycles where intercellular communication is essential. EVs appear to play an important role on many levels. (a) In parasite adaptation to the changing host environment such as drug pressure and pH changes. (b) In infectivity where possible associations with intensity of vesicle shedding of different species have been reported. (c) In immunomodulation where EVs have been proposed to act as messengers for invasion, somehow preparing the host cell for the incoming parasite. (d) In direct regulation of the host transcriptome upon internalization of EVs (Fig. 2). Not surprisingly, even if research on EVs in parasites is just in its infancy, it is already demonstrating that EVs are key players in such intercellular communications thus contributing to chronic infections and pathophysiology.

Research on EVs also holds great promise as new therapeutic agents and diagnostic tools. The role of EVs in modulating immune responses was first described in a pioneering study using exosomes secreted by a human B cell line (131). Since then, the immune modulatory properties of exosomes and EVs from other cells have been demonstrated (2). Remarkably, the use of exosomes in human clinical trials against late-stage cancer patients demonstrated their great potential as cell-free vaccines (40). With regard to parasitic diseases, no clinical trials have yet been reported; however, proof-of-principle of

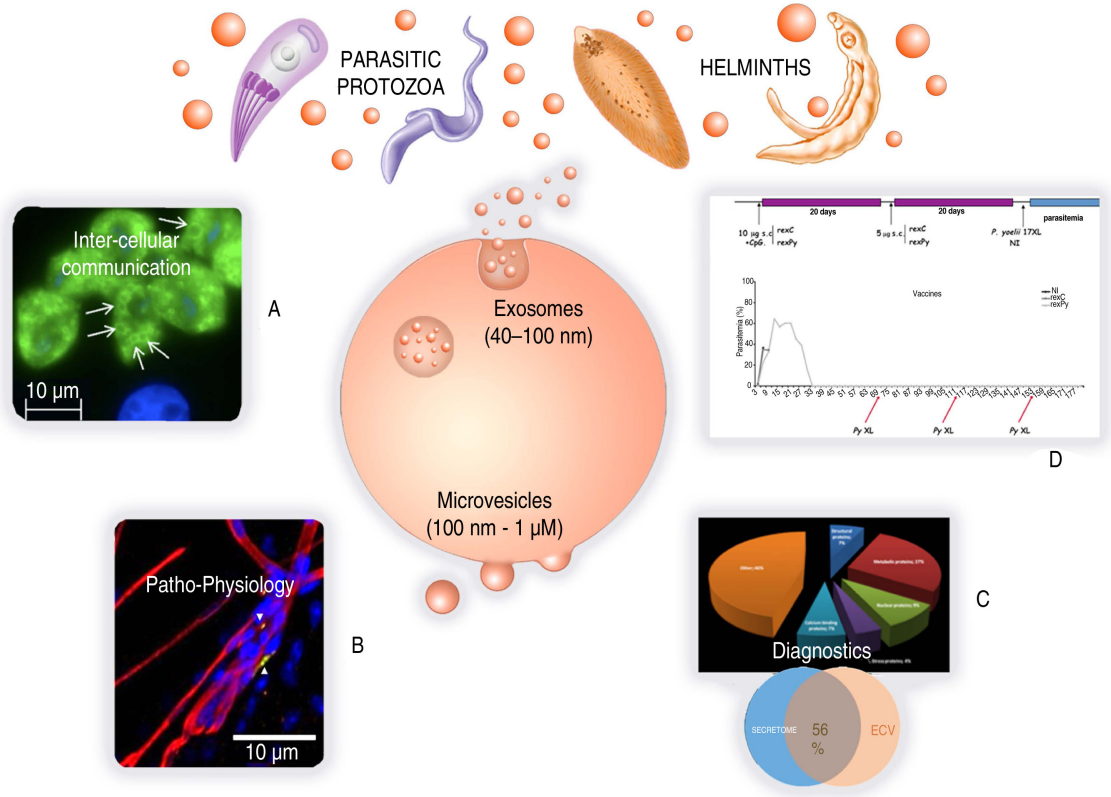

Fig. 2. Extracellular vesicles (EVs) and parasitic diseases. Two major groups of parasitic diseases, protozoa and helminths, as reviewed here actively secrete EVs of endocytic origin (exosomes, 40–100 nm) or membrane budding (microvesicles, 100 nm^{-1} µM). As illustrated here with selected examples, they can act in different biological/pathological processes or potentially used to discover new biomarkers for diagnostics or as cell-free vaccines. (A) Intercellular communication mediated by exosomes in *Trichomonas vaginalis* (106). (B) Induction of cerebral malaria-like histopathology (37). (C) Discovery of new biomarkers for diagnostics in helminths (112). (D) Cell-free vaccines in malaria (30).

their potential as novel vaccines have been shown for *Toxoplasma gondii* (39), *Leishmania* major (102) and *P. yoelii* (30), where exosomes from antigen loaded DCs or from infected cells protect animal models from infection. Last but not least, because EVs are found in all biological fluids (132), the molecular composition of EVs from different origins and pathologies is already demonstrating their great potential as biomarkers. All of these studies, however, are presently confounded by the lack of consensus definitions and protocols for isolation and characterization of EVs from different cell and tissue origins (1). Overcoming these technical difficulties will pave the way to unveil molecular basis of pathophysiology and to use them as novel diagnostic tools and therapeutic agents against parasitic diseases.

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The authors declare there is no conflict of interest.

References

1. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200:373–83.
2. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9:581–93.
3. Simons M, Raposo G. Exosomes – vesicular carriers for intercellular communication. Curr Opin Cell Biol. 2009;21:575–81.
4. Bobrie A, Colombo M, Raposo G, Thery C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic. 2011;12:1659–68.
5. Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology. 2005;20:22–7.
6. Laulagnier K, Motta C, Hamdi S, Roy S, Fauvelle F, Pageaux JF, et al. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. Biochem J. 2004;380:161–71.
7. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science. 2008;319:1244–7.
8. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem. 2003;278:10963–72.
9. Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol. 2001;166:7309–18.
10. Choi DS, Kim DK, Kim YK, Gho YS. Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. Proteomics. 2013;13:1554–71.
11. Kastelowitz N, Yin H. Exosomes and microvesicles: identification and targeting by particle size and lipid chemical probes. Chembiochem. 2014;15:923–8.
12. Cox FE. History of human parasitology. Clin Microbiol Rev. 2002;15:595–612.
13. Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic. 2008;9:871–81.
14. Barteneva NS, Maltsev N, Vorobjev IA. Microvesicles and intercellular communication in the context of parasitism. Front Cell Infect Microbiol. 2013;3:49.
15. Twu O, Johnson PJ. Parasite extracellular vesicles: mediators of intercellular communication. PLoS Pathog. 2014;10:e1004289.
16. Reid AJ. Large, rapidly evolving gene families are at the forefront of host-parasite interactions in Apicomplexa. Parasitology. 2014:1–14.
17. Tulane. Apicomplexa 2010 [cited 2012 Dec 20]. Available from: http://www.tulane.edu/ ~wiser/protozoology/notes/api.html
18. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:1017–24.
19. WHO. Malaria Fact Sheet No 94. Geneva; 2014. Available from: http://www.who.int/mediacentre/factsheets/fs094/en/
20. Garnham PC. Malaria in mammals excluding man. Adv Parasitol. 1967;5:139–204.
21. Escalante AA, Ayala FJ. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proc Natl Acad Sci USA. 1994;91:11373–7.
22. Cox FE. History of the discovery of the malaria parasites and their vectors. Parasites Vectors. 2010;3:5.
23. Campos FM, Franklin BS, Teixeira-Carvalho A, Filho AL, de Paula SC, Fontes CJ, et al. Augmented plasma microparticles during acute *Plasmodium vivax* infection. Malar J. 2010;9:327.
24. Nantakomol D, Dondorp AM, Krudsood S, Udomsangpetch R, Pattanapanyasat K, Combes V, et al. Circulating red cell-derived microparticles in human malaria. J Infect Dis. 2011;203:700–6.
25. Combes V, Taylor TE, Juhan-Vague I, Mege JL, Mwenechanya J, Tembo M, et al. Circulating endothelial microparticles in Malawian children with severe falciparum malaria complicated with coma. JAMA. 2004;291:2542–4.
26. Pankoui Mfonkeu JB, Gouado I, Fotso Kuate H, Zambou O, Amvam Zollo PH, Grau GE, et al. Elevated cell-specific microparticles are a biological marker for cerebral dysfunctions in human severe malaria. PLoS One. 2010;5:e13415.
27. Coltel N, Combes V, Wassmer SC, Chimini G, Grau GE. Cell vesiculation and immunopathology: implications in cerebral malaria. Microbes Infect. 2006;8:2305–16.
28. Couper KN, Barnes T, Hafalla JC, Combes V, Ryffel B, Secher T, et al. Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation. PLoS Pathog. 2010;6:e1000744.
29. Combes V, Coltel N, Alibert M, van Eck M, Raymond C, Juhan-Vague I, et al. ABCA1 gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology. Am J Pathol. 2005;166:295–302.

30. Martin-Jaular L, Nakayasu ES, Ferrer M, Almeida IC, Del Portillo HA. Exosomes from *Plasmodium yoelii*-infected reticulocytes protect mice from lethal infections. PLoS One. 2011;6:e26588.
31. Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, et al. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell. 2013;153:1120–33.
32. Mantel PY, Hoang AN, Goldowitz I, Potashnikova D, Hamza B, Vorobjev I, et al. Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host Microbe. 2013;13:521–34.
33. Alano P, Carter R. Sexual differentiation in malaria parasites. Ann Rev Microbiol. 1990;44:429–49.
34. Dyer M, Day KP. Commitment to gametocytogenesis in *Plasmodium falciparum*. Parasitol Today. 2000;16:102–7.
35. Dyer M, Day KP. Regulation of the rate of asexual growth and commitment to sexual development by diffusible factors from *in vitro* cultures of *Plasmodium falciparum*. Am J Trop Med Hyg. 2003;68:403–9.
36. Del Portillo HA, Chitnis CE. Talking to each other to initiate sexual differentiation. Cell. 2013;153:945–7.
37. El-Assaad F, Wheway J, Hunt NH, Grau GE, Combes V. Production, fate and pathogenicity of plasma microparticles in murine cerebral malaria. PLoS Pathog. 2014;10:e1003839.
38. CDC. Parasites – Toxoplasmosis (*Toxoplasma* infection) 2013 [cited 2013 Jan 10]. Available from: http://www.cdc.gov/parasites/toxoplasmosis/epi.html
39. Aline F, Bout D, Amigorena S, Roingeard P, Dimier-Poisson I. *Toxoplasma gondii* antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. Infect Immun. 2004;72:4127–37.
40. Chaput N, Thery C. Exosomes: immune properties and potential clinical implementations. Semin Immunopathol. 2011;33:419–40.
41. Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response *in vitro* and *in vivo*. Blood. 2007;110:3234–44.
42. Beauvillain C, Ruiz S, Guiton R, Bout D, Dimier-Poisson I. A vaccine based on exosomes secreted by a dendritic cell line confers protection against *T. gondii* infection in syngeneic and allogeneic mice. Microbes Infect. 2007;9:1614–22.
43. Pope SM, Lasser C. *Toxoplasma gondii* infection of fibroblasts causes the production of exosome-like vesicles containing a unique array of mRNA and miRNA transcripts compared to serum starvation. J Extracell Vesicles. 2013;2:22484, doi: http://dx.doi.org/10.3402/jev.v2i0.22484
44. Hu G, Gong AY, Roth AL, Huang BQ, Ward HD, Zhu G, et al. Release of luminal exosomes contributes to TLR4-mediated epithelial antimicrobial defense. PLoS Pathog. 2013;9:e1003261.
45. Tulane. Kinetoplastids. New Orleans: Tulane University; 1999 [cited 2013 Oct 16]. Available from: http://www.tulane.edu/ ~wiser/protozoology/notes/kinet.html
46. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, et al. The trypanosomiases. Lancet. 2003;362:1469–80.
47. Rodrigues JC, Godinho JL, de Souza W. Biology of human pathogenic trypanosomatids: epidemiology, lifecycle and ultrastructure. Subcell Biochem. 2014;74:1–42.
48. Geiger A, Hirtz C, Becue T, Bellard E, Centeno D, Gargani D, et al. Exocytosis and protein secretion in *Trypanosoma*. BMC Microbiol. 2010;10:20.
49. Ouaissi A, Aguirre T, Plumas-Marty B, Piras M, Schoneck R, Gras-Masse H, et al. Cloning and sequencing of a 24-kDa *Trypanosoma cruzi* specific antigen released in association with membrane vesicles and defined by a monoclonal antibody. Biol Cell. 1992;75:11–7.
50. da Silveira JF, Abrahamsohn PA, Colli W. Plasma membrane vesicles isolated from epimastigote forms of *Trypanosoma cruzi*. Biochim Biophys Acta. 1979;550:222–32.
51. Gonçalves MF, Umezawa ES, Katzin AM, de Souza W, Alves MJ, Zingales B, et al. *Trypanosoma cruzi*: shedding of surface antigens as membrane vesicles. Exp Parasitol. 1991;72:43–53.
52. Trocoli Torrecilhas AC, Tonelli RR, Pavanelli WR, da Silva JS, Schumacher RI, de Souza W, et al. *Trypanosoma cruzi*: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. Microbes Infect. 2009;11:29–39.
53. Brun R, Blum J. Human African trypanosomiasis. Infect Dis Clin North Am. 2012;26:261–73.
54. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet. 2010;375:148–59.
55. Kennedy PG. Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). Lancet Neurol. 2013;12:186–94.
56. Atyame Nten CM, Sommerer N, Rofidal V, Hirtz C, Rossignol M, Cuny G, et al. Excreted/secreted proteins from trypanosome procyclic strains. J Biomed Biotechnol. 2010;2010:212817.
57. Alarcon de Noya B, Diaz-Bello Z, Colmenares C, Ruiz-Guevara R, Mauriello L, Zavala-Jaspe R, et al. Large urban outbreak of orally acquired acute Chagas disease at a school in Caracas, Venezuela. J Infect Dis. 2010;201:1308–15.
58. Yoshida N, Tyler KM, Llewellyn MS. Invasion mechanisms among emerging food-borne protozoan parasites. Trends Parasitol. 2011;27:459–66.
59. Tarleton RL, Reithinger R, Urbina JA, Kitron U, Gurtler RE. The challenges of Chagas disease – grim outlook or glimmer of hope. PLoS Med. 2007;4:e332.
60. Coura JR. Chagas disease: control, elimination and eradication. Is it possible? Mem Inst Oswaldo Cruz. 2013;108:962–7.
61. Coura JR, Vinas PA. Chagas disease: a new worldwide challenge. Nature. 2010;465:S6–7.
62. Gascon J, Vilasanjuan R, Lucas A. The need for global collaboration to tackle hidden public health crisis of Chagas disease. Expert Rev Anti Infect Ther. 2014;12:393–5.
63. Alvarez JM, Fonseca R, Borges da Silva H, Marinho CR, Bortoluci KR, Sardinha LR, et al. Chagas disease: still many unsolved issues. Mediators Inflamm. 2014;2014:912965.
64. Alves MJ, Colli W. Glycoproteins from *Trypanosoma cruzi*: partial purification by gel chromatography. FEBS Lett. 1975;52:188–90.
65. Acosta-Serrano A, Almeida IC, Freitas-Junior LH, Yoshida N, Schenkman S. The mucin-like glycoprotein super-family of *Trypanosoma cruzi*: structure and biological roles. Mol Biochem Parasitol. 2001;114:143–50.
66. Buscaglia CA, Campo VA, Frasch AC, Di Noia JM. *Trypanosoma cruzi* surface mucins: host-dependent coat diversity. Nat Rev Microbiol. 2006;4:229–36.
67. Mendonca-Previato L, Penha L, Garcez TC, Jones C, Previato JO. Addition of alpha-O-GlcNAc to threonine residues define the post-translational modification of mucin-like molecules in *Trypanosoma cruzi*. Glycoconj J. 2013;30:659–66.
68. de Lederkremer RM, Colli W. Galactofuranose-containing glycoconjugates in trypanosomatids. Glycobiology. 1995;5:547–52.
69. Macrae JI, Acosta-Serrano A, Morrice NA, Mehlert A, Ferguson MA. Structural characterization of NETNES, a

novel glycoconjugate in *Trypanosoma cruzi* epimastigotes. J Biol Chem. 2005;280:12201–11.
70. Alves MJ, Colli W. Role of the gp85/trans-sialidase superfamily of glycoproteins in the interaction of *Trypanosoma cruzi* with host structures. Subcell Biochem. 2008;47:58–69.
71. Frasch AC. Functional diversity in the trans-sialidase and mucin families in *Trypanosoma cruzi*. Parasitol Today. 2000; 16:282–6.
72. Ouaissi MA, Dubremetz JF, Kusnierz JP, Cornette J, Loyens M, Taibi A, et al. *Trypanosoma cruzi*: differential expression and distribution of an 85-kDa polypeptide epitope by in vitro developmental stages. Exp Parasitol. 1990;71:207–17.
73. Maric D, McGwire BS, Buchanan KT, Olson CL, Emmer BT, Epting CL, et al. Molecular determinants of ciliary membrane localization of *Trypanosoma cruzi* flagellar calcium-binding protein. J Biol Chem. 2011;286:33109–17.
74. Nakayasu ES, Almeida IC. Proteomics studies in *Trypanosoma cruzi*. Bethesda, MD: National Center for Biotechnological Information (US); 2008.
75. Torrecilhas AC, Schumacher RI, Alves MJ, Colli W. Vesicles as carriers of virulence factors in parasitic protozoan diseases. Microbes Infect. 2012;14:1465–74.
76. Magdesian MH, Tonelli RR, Fessel MR, Silveira MS, Schumacher RI, Linden R, et al. A conserved domain of the gp85/trans-sialidase family activates host cell extracellular signal-regulated kinase and facilitates *Trypanosoma cruzi* infection. Exp Cell Res. 2007;313:210–8.
77. Magdesian MH, Giordano R, Ulrich H, Juliano MA, Juliano L, Schumacher RI, et al. Infection by *Trypanosoma cruzi*. Identification of a parasite ligand and its host cell receptor. J Biol Chem. 2001;276:19382–9.
78. Almeida IC, Gazzinelli RT. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: structural and functional analyses. J Leukoc Biol. 2001;70:467–77.
79. Bermejo DA, Jackson SW, Gorosito-Serran M, Acosta-Rodriguez EV, Amezcua-Vesely MC, Sather BD, et al. *Trypanosoma cruzi* trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. Nat Immunol. 2013;14:514–22.
80. Freire-de-Lima L, Oliveira IA, Neves JL, Penha LL, Alisson-Silva F, Dias WB, et al. Sialic acid: a sweet swing between mammalian host and *Trypanosoma cruzi*. Front Immunol. 2012;3:356.
81. Pereira-Chioccola VL, Acosta-Serrano A, Correia de Almeida I, Ferguson MA, Souto-Padron T, Rodrigues MM, et al. Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies. J Cell Sci. 2000;113: 1299–307.
82. Almeida IC, Ferguson MA, Schenkman S, Travassos LR. Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas' disease recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*. Biochem J. 1994;304:793–802.
83. Almeida IC, Milani SR, Gorin PA, Travassos LR. Complement-mediated lysis of *Trypanosoma cruzi* trypomastigotes by human anti-alpha-galactosyl antibodies. J Immunol. 1991;146:2394–400.
84. Giordano R, Fouts DL, Tewari D, Colli W, Manning JE, Alves MJ. Cloning of a surface membrane glycoprotein specific for the infective form of *Trypanosoma cruzi* having adhesive properties to laminin. J Biol Chem. 1999;274:3461–8.
85. Marroquin-Quelopana M, Oyama S, Jr, Aguiar Pertinhez T, Spisni A, Aparecida Juliano M, Juliano L, et al. Modeling the *Trypanosoma cruzi* Tc85–11 protein and mapping the laminin-binding site. Biochem Biophys Res Commun. 2004;325:612–8.
86. De Pablos LM, Osuna A. Multigene families in *Trypanosoma cruzi* and their role in infectivity. Infect Immun. 2012;80: 2258–64.
87. Bayer-Santos E, Aguilar-Bonavides C, Rodrigues SP, Cordero EM, Marques AF, Varela-Ramirez A, et al. Proteomic analysis of *Trypanosoma cruzi* secretome: characterization of two populations of extracellular vesicles and soluble proteins. J Proteome Res. 2013;12:883–97.
88. Almeida IC, Krautz GM, Krettli AU, Travassos LR. Glycoconjugates of *Trypanosoma cruzi*: a 74 kD antigen of trypomastigotes specifically reacts with lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas disease. J Clin Lab Anal. 1993;7:307–16.
89. Cestari I, Ansa-Addo E, Deolindo P, Inal JM, Ramirez MI. *Trypanosoma cruzi* immune evasion mediated by host cell-derived microvesicles. J Immunol. 2012;188:1942–52.
90. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet. 2009;10:94–108.
91. Garcia-Silva MR, das Neves RF, Cabrera-Cabrera F, Sanguinetti J, Medeiros LC, Robello C, et al. Extracellular vesicles shed by *Trypanosoma cruzi* are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. Parasitol Res. 2014;113:285–304.
92. Bayer-Santos E, Lima FM, Ruiz JC, Almeida IC, da Silveira JF. Characterization of the small RNA content of *Trypanosoma cruzi* extracellular vesicles. Mol Biochem Parasitol. 2014;193:71–4.
93. Garcia-Silva MR, Cabrera-Cabrera F, das Neves RF, Souto-Padron T, de Souza W, Cayota A. Gene expression changes induced by *Trypanosoma cruzi* shed microvesicles in mammalian host cells: relevance of tRNA-derived halves. BioMed Res Int. 2014;2014:305239.
94. Gazos-Lopes F, Oliveira MM, Hoelz LV, Vieira DP, Marques AF, Nakayasu ES, et al. Structural and functional analysis of a platelet-activating lysophosphatidylcholine of *Trypanosoma cruzi*. PLoS Negl Trop Dis. 2014;8:e3077.
95. Neves RF, Fernandes AC, Meyer-Fernandes JR, Souto-Padron T. *Trypanosoma cruzi*-secreted vesicles have acid and alkaline phosphatase activities capable of increasing parasite adhesion and infection. Parasitol Res. 2014;113:2961–72.
96. WHO. Leishmaniasis. World Health Organization; 2014. Available from: http://www.who.int/mediacentre/factsheets/fs375/en/
97. Silverman JM, Chan SK, Robinson DP, Dwyer DM, Nandan D, Foster LJ, et al. Proteomic analysis of the secretome of *Leishmania donovani*. Genome Biol. 2008;9:R35.
98. Silverman JM, Clos J, de'Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from *Leishmania* and communication with macrophages. J Cell Sci. 2010;123:842–52.
99. Silverman JM, Clos J, Horakova E, Wang AY, Wiesgigl M, Kelly I, et al. *Leishmania* exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. J Immunol. 2010;185:5011–22.
100. Hassani K, Olivier M. Immunomodulatory impact of *Leishmania*-induced macrophage exosomes: a comparative proteomic and functional analysis. PLoS Negl Trop Dis. 2013;7:e2185.
101. Ghosh J, Bose M, Roy S, Bhattacharyya SN. *Leishmania donovani* targets Dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. Cell Host Microbe. 2013;13:277–88.
102. Schnitzer JK, Berzel S, Fajardo-Moser M, Remer KA, Moll H. Fragments of antigen-loaded dendritic cells (DC) and

DC-derived exosomes induce protective immunity against *Leishmania* major. Vaccine. 2010;28:5785–93.
103. Hassani K, Shio MT, Martel C, Faubert D, Olivier M. Absence of metalloprotease GP63 alters the protein content of *Leishmania* exosomes. PLoS One. 2014;9:e95007.
104. de Miguel N, Lustig G, Twu O, Chattopadhyay A, Wohlschlegel JA, Johnson PJ. Proteome analysis of the surface of *Trichomonas vaginalis* reveals novel proteins and strain-dependent differential expression. Mol Cell Proteomics. 2010;9:1554–66.
105. de Miguel N, Riestra A, Johnson PJ. Reversible association of tetraspanin with *Trichomonas vaginalis* flagella upon adherence to host cells. Cell Microbiol. 2012;14:1797–807.
106. Twu O, de Miguel N, Lustig G, Stevens GC, Vashisht AA, Wohlschlegel JA, et al. *Trichomonas vaginalis* exosomes deliver cargo to host cells and mediate hostratioparasite interactions. PLoS Pathog. 2013;9:e1003482.
107. Deolindo P, Evans-Osses I, Ramirez MI. Microvesicles and exosomes as vehicles between protozoan and host cell communication. Biochem Soc Trans. 2013;41:252–7.
108. Wampfler PB, Tosevski V, Nanni P, Spycher C, Hehl AB. Proteomics of secretory and endocytic organelles in *Giardia lamblia*. PLoS One. 2014;9:e94089.
109. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Investig. 2008;118:1311–21.
110. Toledo R, Bernal MD, Marcilla A. Proteomics of foodborne trematodes. J Proteomics. 2011;74:1485–503.
111. Hewitson JP, Ivens AC, Harcus Y, Filbey KJ, McSorley HJ, Murray J, et al. Secretion of protective antigens by tissue-stage nematode larvae revealed by proteomic analysis and vaccination-induced sterile immunity. PLoS Pathog. 2013;9:e1003492.
112. Marcilla A, Trelis M, Cortes A, Sotillo J, Cantalapiedra F, Minguez MT, et al. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One. 2012;7:e45974.
113. Andresen K, Simonsen PE, Andersen BJ, Birch-Andersen A. *Echinostoma caproni* in mice: shedding of antigens from the surface of an intestinal trematode. Int J Parasitol. 1989;19:111–8.
114. Threadgold LT. The ultrastructure of the "cuticle" of *Fasciola hepatica*. Exp Cell Res. 1963;30:238–42.
115. Wilson RA, Wright JM, de Castro-Borges W, Parker-Manuel SJ, Dowle AA, Ashton PD, et al. Exploring the *Fasciola hepatica* tegument proteome. Int J Parasitol. 2011;41:1347–59.
116. Wilson RA, Barnes PE. The formation and turnover of the membranocalyx on the tegument of *Schistosoma mansoni*. Parasitology. 1977;74:61–71.
117. Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. Mol Cell Proteomics. 2009;8:1891–907.
118. Sotillo J, Valero ML, Sanchez Del Pino MM, Fried B, Esteban JG, Marcilla A, et al. Excretory/secretory proteome of the adult stage of *Echinostoma caproni*. Parasitol Res. 2010;107:691–7.
119. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol. 2012;10:e1001450.
120. Mulvenna J, Moertel L, Jones MK, Nawaratna S, Lovas EM, Gobert GN, et al. Exposed proteins of the *Schistosoma japonicum* tegument. Int J Parasitol. 2010;40:543–54.
121. Garg G, Bernal D, Trelis M, Forment J, Ortiz J, Valero ML, et al. The transcriptome of *Echinostoma caproni* adults: further characterization of the secretome and identification of new potential drug targets. J Proteomics. 2013;89:202–14.
122. Young ND, Hall RS, Jex AR, Cantacessi C, Gasser RB. Elucidating the transcriptome of *Fasciola hepatica* – a key to fundamental and biotechnological discoveries for a neglected parasite. Biotechnol Adv. 2010;28:222–31.
123. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One. 2012;7:e30679.
124. Xu L, Yang BF, Ai J. MicroRNA transport: a new way in cell communication. J Cell Physiol. 2013;228:1713–9.
125. Li H, Huang S, Guo C, Guan H, Xiong C. Cell-free seminal mRNA and microRNA exist in different forms. PLoS One. 2012;7:e34566.
126. Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. Curr Opin Lipidol. 2012;23:91–7.
127. Bernal D, Trelis M, Montaner S, Cantalapiedra F, Galiano A, Hackenberg M, et al. Surface analysis of *Dicrocoelium dendriticum*. The molecular characterization of exosomes reveals the presence of miRNAs. J Proteomics. 2014;105:232–41.
128. Liegeois S, Benedetto A, Garnier JM, Schwab Y, Labouesse M. The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in *Caenorhabditis elegans*. J Cell Biol. 2006;173:949–61.
129. Buck AH, Coakley G, Simbari F, McSorley HJ, Quintana JF, Le Bihan T, et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat Commun. 2014;5:5488.
130. Wang T, Van Steendam K, Dhaenens M, Vlaminck J, Deforce D, Jex AR, et al. Proteomic analysis of the excretory-secretory products from larval stages of *Ascaris suum* reveals high abundance of glycosyl hydrolases. PLoS Negl Trop Dis. 2013;7:e2467.
131. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996;183:1161–72.
132. Properzi F, Logozzi M, Fais S. Exosomes: the future of biomarkers in medicine. Biomark Med. 2013;7:769–78.
133. WHO. 2014. Available from: http://www.who.int/mediacentre/factsheets/en/