Extracellular vesicles and host-parasite interactions

Review Article

Sherif M Abaza

Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

ABSTRACT

Extracellular vesicles (EVs) are a collection of small membrane-surrounded structures released by nearly all prokaryotic and eukaryotic cells. They contain bioactive molecules such as proteins, lipids, metabolites, and nucleic acids including non-coding micro RNA (miRNA), and transfer RNA (tRNA) in an evolutionarily conserved manner. These bioactive molecules play a crucial role in transmitting information, and genetic cargo without direct contact between cells. In fact, EVs gained significant attention in the last decade due to their contributions to cell-to-cell communication and disease pathogenicity. In parasitic infections, EVs mediate communication during host-parasite interactions through the influence of parasite-derived molecules, and EVs released from the host immune cells that are triggered by the parasitic antigens. Besides, EVs facilitate the transfer of virulence factors with subsequent regulation of both parasite and host gene expressions, and modulation of host immune responses. Understanding the mechanisms regulating EVs biogenesis and functions would certainly lead to identification of diagnostic and prognostic biomarkers, novel therapeutic and protective approaches. To understand the cellular processes governed by EVs system biology, and how infection influences EV biogenesis, several approaches were utilized to isolate EVs and characterize their functions. The objective of the present review is to highlight EVs' impact on the major three eras of Parasitology research namely diagnosis, treatment, and control.

Keywords: cargo transfer; diagnostic marker; exosomes; immunization; prognostic marker; virulence factors.

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Corresponding Author: Sherif M. Abaza, **Tel.:** +20 1005243428, **E-mail:** smabaza@hotmail.com

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Abbreviations: ESCRT: Endosomal sorting complex required for transport; **EVs:** Extracellular vesicles; **HSP:** Heat shock protein; **PG:** Prostaglandin; **miRNA:** Micro RNA; **NF-kB:** Nuclear factor kappa B cells; **TLR:** Toll-like receptor; **TSG101:** Tumor susceptibility gene 101; **TSP:** Tetraspanin.

INTRODUCTION

In humans, EVs are small membrane vesicles derived from the endocytic compartment of different cells commonly existing in blood, urine, saliva, and CSF. According to the origin and size, there are four main types: exosomes, microvesicles, apoptotic bodies, and oncosomes. Exosomes (<150 nm) formation is by interior budding of the endosomal membrane created when intraluminal vesicles (ILVs) are formed by inward budding of the early endosome. Mature ILVs form multi-vesicular bodies (MVBs), while exosomes are released when MVBs fuse with cell plasma membrane. Microvesicles (150-1000 nm) are extracellular membrane shedding particles released directly from the plasma membrane. Apoptotic bodies (1000-5000 nm), and oncosomes (1-10 μm) are EVs released during apoptotic cell death, and migration of large cancer cells, respectively[1]. Exomeres is a new term describing small EVs (<50 nm) that lack an external membrane and are enriched with mechanistic target of rapamycin (mTOR) signaling pathway. They contain fewer lipids and RNAs in comparison to small exosomes (60-80 nm), and large microvesicles (90-120 nm)[2].

Exosome biogenesis and release is mainly dependent on three pathways: endosomal sorting complex required for transport (ESCRT), a ceramide-dependent mechanism, and tetraspanins (TSPs) presence. In the cytosol, there are four multi-protein complexes for ESCRTs that are targeted to the endosome membrane through interaction with ubiquitin. Three ESCRTs (0-II) are able to recognize and bind with ubiquitin, while the fourth ESCRT-III is involved in the final steps of ILVs' formation. It was claimed that ESCRTs, exosome-bound proteins, annexins, and Rab proteins govern membrane transport and fusion, while ALIX, flotillins, and tumor susceptibility gene 101 (TSG101) are critical molecules involved in exosome biogenesis[3]. In contrast, microvesicles are secreted by the plasma membrane by a calcium-dependent mechanism. Notably, ALIX is a protein interacting with several ESCRT proteins to participate in EVs budding and separation processes. While flotillins are evolutionary conserved membrane-associated proteins, TSG101 is a signal-transducing adaptor molecule.

Several critical factors contribute to exosome biogenesis such as originating site, protein cargo, transacting mediators, and physicochemical aspects. Accordingly, exosomes composition varies; however, several components are commonly recognized. Among them, members of Rab proteins possessing

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a GTPase essentially required to regulate vesicle generation, a crucial step in membrane trafficking along the actin and tubulin networks. Besides, Rab proteins cause exosome docking and fusion of the exosome membrane. Lysobisphosphatidic acid-binding protein and annexins participate in vesicle formation, and regulation of membrane cytoskeleton dynamics, respectively. Other common components include adhesion molecules (CD146, CD9, CD18, and CD11), TSP markers (CD9, CD63, CD81, and CD82), thioredoxin peroxidase II, galectin 3, and heat shock proteins (HSPs) 70 and 90. The latter are important characteristic features of exosomes because they promote binding of major histocompatibility complexes (MHC-I, and MHC-II) to peptides of the genetic cargo. Besides, lipids are essential components of exosomal membranes enriched with cholesterol, glycosphingolipids, and different acylated proteins. Messenger, transfer and micro RNAs (mRNAs, tRNAs, miRNAs, respectively), and mitochondrial DNA, as well as small and long non-coding RNAs are also identified[4].

Similar to all pathogens, parasites adopt variable approaches for cell-cell-communications to manipulate their long parasitism in different host species. Therefore, it is important to recognize mechanisms controlling cell-cell communications that influence parasite survival, growth, differentiation, and virulence. In the last decade, EVs discovery opened a new avenue that attracted much attention in researches focusing on cellular crosstalk and the transfer of bioactive molecules and genetic cargo to host-parasite interface[5].

Exosomes identified in several parasites consist of cytoplasm enclosed in a lipid bilayer which is in contact with the extracellular environment by transmembrane proteins. Parasite exosomes provide conserved specific information transferred to the host cells to modulate host immune response, and definitely promote infection. It is worth mentioning that pathogenic mechanisms of several parasitic diseases rely not only on parasite-derived exosomes, but also on exosomes of host cells suffering from stress induced by parasitic invasion. Release of host cell exosomes activates T and B cells as well as dendritic cells, natural killer cells, and macrophages that play anti-inflammatory roles[4].

Several functions are assigned to exosomes; however, immune modulation comes first. Exosomes provide parasitic antigens to antigen-presenting cells modulating both the innate and adaptive immune responses, and regulate expression of bioactivated molecules, e.g. complement factors. Transfer of exosomal miRNA targets host mRNAs inducing epigenetic modifications in intracellular signaling and post-transcriptional regulation of host gene expression. Besides, several tRNA small-derived RNAs (tsRNAs) are uploaded by host cells with subsequent contribution in interference with host defensive barriers[6].

The major obstacle in EVs potential use for diagnostic, therapeutic, and protective applications is their isolation and characterization. For isolation, several approaches were utilized including differential ultracentrifugation-based techniques, size-based techniques (ultra-filtration), immunoaffinity capture-based techniques, exosome precipitation techniques, and microfluidics-based techniques. Characterization techniques include ultrastructural analysis using electron microscopy, nanoparticle-tracking analysis to determine concentration and size distribution of exosomes particles, asymmetric flow field-flow fractionation to identify nanoparticles, and flow cytometry. Methods for molecules analysis included western blotting, ELISA, mass spectrometry, and recently a new generation of biosensors was used[6].

Imaging flow cytometry (IFC) was used to monitor the uptake of *Plasmodium*-derived vesicles by host immune cells through staining different EVs components. The investigators succeeded to track each component internalization over time and evaluate its kinetics delivery. Moreover, they demonstrated IFC ability to analyze cellular dynamics after EVs release on host-parasite communication[7]. A Chinese study proposed a protocol for isolation and characterization of *S. japonicum* EVs using centrifugation combined with a commercial exosome isolation kit[8]. Swiss investigators developed a simple method to label and track EV internalization by endothelial cells using a green cell linker dye. Besides, using PKH67 fluorescent dye labeled with dextran, they succeeded to measure EVs permeability across the endothelial cell monolayer, and investigate their uptake[9].

Although an aqueous two-phase system was previously used because it is a rapid isolation assay, it proved to be inefficient for the minute-sized EVs[10]. Utilizing a high-end flow cytometry system, Dekel *et al*.[11] succeeded in characterizing small EVs (less than 200 nm) with subsequent identification of different parasite-derived EV biomolecules according to their genetic cargo. The investigators used antibody-free labeling to monitor a selective EVs-derived component that carried a specific parasitic DNA cargo. It was concluded that such labeling would enable researchers to analyze EVs cargo components[11]. Using asymmetric flow field-flow fractionation, an AF4 device was developed to separate EVs subpopulations, where small particles with high diffusion coefficients are eluted earlier than larger particles that are accumulated later in its membrane. The AF4 device was used to identify small and large distinct *Plasmodium* EVs differing in size and content[12]. An optimized protocol for isolation of EVs from *S. mansoni* schistosomula and adult worms was developed. The study used iodixanol gradients instead of sucrose as medium for isolation of the small density gradients. Results revealed that iodixanol allowed for simultaneous separation of *Schistosoma* EVs from non-EV contaminants with subsequent sufficient yield[13].

The present review aims to highlight the value of EVs released from parasites and hosts as potential diagnostic and prognostic biomarkers, novel therapeutic approaches and protective strategies in parasitology researches.

***Schistosoma* spp.**

A Chinese study was the first to report isolation and characterization of exosome-like vesicles derived from *S. japonicum* adults. Their research demonstrated the prompting of M1 macrophage polarization with increased production of pro-inflammatory factors such as TNF-α, inducible nitric oxide synthase, and CD16/32[14]. Later, several studies documented the schistosomal EVs essential role in host-parasite interactions, that could be used as potential prognostic marker for hepatic fibrosis grading and vaccine candidate for immunization. A Canadian study identified exosome-like vesicles released from *S. mansoni* adults *in vitro*. Their proteomic analyses revealed common exosomal markers such as HSPs, energy-generating enzymes, and cytoskeletal proteins (actin, tubulin, myosin, and paramyosin). Results also proposed integrins, cell adhesion proteins, glutathione-S-transferase, and TSP-2, as potential protective markers. Quantitative PCR analysis demonstrated the abundance of schistosome-derived miRNAs in exosomes purified from experimentally infected mice sera, i.e., presenting possible diagnostic biomarkers[15].

In 2020, two studies were conducted on *Schistosoma* EVs. The first study performed proteomic analysis of EVs released from male and female *S. japonicum* followed by miRNAs sequencing. Results revealed that miR-750 was significantly rich in female EVs, and its inhibition led to decreased egg production *in vitro*. The investigators suggested that miR-750 within female EV load contributed in ovary development and egg production[16]. The second study demonstrated that glycosylation of *S. mansoni* EVs facilitated the interaction with host immune cells. Additionally, the study showed that *S. mansoni* schistosomula EVs were internalized by human monocyte-derived dendritic cells. Mass spectrometric analysis identified surface N-glycans with terminal motifs and a known legend for CD209. Besides, schistosomula EVs increased expression of IL-12 and IL-10, which supported the essential role played by surface glycans in immune modulation of host immune response[17].

Exosomes cargo transfer also facilitates *Schistosoma* biological processes, i.e., migration, nutrient acquisition, and reproduction. Therefore, each *Schistosoma* life-stage develops its own skills to communicate with the host *via* EVs for the maintenance of its biological functions. German reviewers[18] summarized the role of each life stage-derived exosomes in the following points:

1. Egg-derived exosomes (EDEs) carry lysophosphatidylcholine and prostaglandin D2 (PGD2) that bind with toll-like receptors (TLRs) on eosinophils, i.e. promoting eosinophils activation; an important event in hepatic granuloma formation. Besides, miRNAs (miR-1, miR-2162, and miR-71a) of *S. japonicum* EDEs stimulated hepatic stellate cells by upregulating the expression of fibrosis-related proteins, i.e., collagens. Interestingly, miR-3096 in *S. japonicum* EDEs suppressed hepatoma proliferation and migration, i.e., denied the hypothesis of hepatocellular carcinoma development from liver fibrosis in schistosomiasis *japonicum*.
2. Fluorescence labeling of excretory/secretory (E/S) products of *S. mansoni* cercariae revealed visible vesicles released by their acetabular glands. Proteomic analysis of cercaria-derived exosomes showed that molecules required for skin penetration included proteases such as chymotrypsin-like serine protease [cercarial elastase (CE), and metalloprotease of the leishmanolysin family (PepM8)], paramyosin, and SPO-1 (*Sm*16). Paramyosin (*Sm*23) is an inhibitor of complement membrane attacking complex *via* its binding with complement factors leading to cell lysis. Due to its immunosuppressive properties, *Sm*16 contributes in protecting invading cercariae from host innate immune response.
3. Proteomic analysis of EVs released from schistosomula revealed several molecules that facilitate invasion and migration. They included cytoskeletal proteins (actin, and tubulin), TSPs, HSPs, annexins, Rab 11 proteins, and glycerinaldehyde-3-phosphate.
4. Variety of fatty acid binding proteins, phospholipid, lipids, cholesterol, phosphatidylserine, plasmalogen, sphingomyelin, PGD2, and lysophosphatidylcholine were the major components of adult-derived exosomes (ADEs). They constitute a protective lipid bilayer as an interacting surface against host immune cells. Besides, ADEs are rich in proteases (cathepsin B-like, metallo-, and serine), essentially required for exosome-mediated signaling. In ADEs of *S. mansoni* and *S. japonicum*, miRNAs (bantam, miR-36-3P, miR125b, and miR-10) were identified essential mediators of cell-cell communication to modulate host immune responses[18].

Diagnostic biomarkers: Due to implication of miR-191-5p in the pathogenesis of schistosomiasis *japonicum*, the study suggested its use as a potential prognostic biomarker[19]. A new method was developed to diagnose schistosomiasis utilizing schistosome miR-2c-3p isolated from EVs in sera from patients with low infection intensity[20]. In chronic schistosomiasis, miR-10 from adult-derived exosomes suppressed mitogen-activated protein kinase (MAPK) with subsequent modulation of host Th2 immune response. Therefore, bantam, miR-2c-3p, miR-3488, miR-142-3p and miR-223-3p were proposed diagnostic biomarkers with high sensitivity and specificity. Use of a miRNAs combination improved diagnostic accuracy compared to single miRNA, and proved valuable in grading hepatic fibrosis[18].

Potential therapeutic efficiency: Proteomic analysis of EVs derived from *S. japonicum* adults showed molecules with catalytic activity, and binding affinity and may therefore be considered as possible drug targets[19]. Clinical applications of schistosomal EVs extended to their use as immunosuppressive drugs in prevention of autoimmune diseases development. Intraperitoneal injections with exosomes derived from

dendritic cells treated with *S. japonicum* soluble eggs antigen attenuated the severity of acute inflammatory bowel disease in mice with induced colitis[21]. On the other hand, because neutrophils contribute in clearance of *Schistosoma* by releasing neutrophil extracellular traps (NETs), a recent Chinese study investigated the role of EVs derived from infected liver of *S. japonicum*-infected mice (IL-EVs). The investigators demonstrated that IL-EVs delivered miR-142a-3p to target host Wiskott-Aldrich syndrome-like (WASL) gene, blocking *S. japonicum* development. Notably, WASL is a gene encoding WAS proteins family involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton. Therefore, they directly or indirectly associate with the small GTPases known to regulate formation of actin filaments, and the cytoskeletal organizing complex. The investigators observed IL-10 expression downregulation, and significant reduction of NETs formation in WASL knockout mice. Accordingly, the study proposed IL-EVs and WASL as potential therapeutic target in schistosomiasis[22].

Potential vaccine candidate: The first proteomic analysis of EVs from *S. haematobium* adults revealed schistosomal vaccine candidates, e.g., TSPs, glutathione S-transferase, saponins and aminopeptidases. Because EVs were enriched with TSPs, the study immunized mice with recombinant TSPs against *S. mansoni* challenged infection. Results revealed significant reductions in both hepatic and intestinal egg burdens[23]. A Chinese review tabulated all bioactive molecules identified in *Schistosoma*- and host-derived exosomes. The reviewers discussed the functional roles for each as potential vaccine candidates. They included glutathione-S-transferase (GST), TSP-2, calpain, fatty acids and sterols. It was observed that both eggs and adults of *S. japonicum* and *S. mansoni* contribute in releasing host-derived exosome either directly through schistosomal antigens or indirectly *via* their EVs miRNAs cargo[6]. An Egyptian study evaluated the protective efficacy of egg-derived EVs against murine schistosomiasis *mansoni*. Subcutaneous three doses of 20 μg EVs, with or without alum adjuvant were administered two weeks prior to challenged infection. Results showed significant reduction of hepatic and intestinal egg burdens with remarkable amelioration of the hepatic granulomas[24].

Hepatic flukes

***Fasciola* spp.:** Early in the last decade, a study reported the first research recognizing *F. hepatica*-derived exosome-like vesicles. Spanish investigators identified released E/S proteins (HSPs, and detoxifying enzymes), and nuclear molecules (histones, and elongation factors)[25]. Characterization of large EVs released from cells lining *F. hepatica* gastrodermus, and small exosome-like vesicles originating from the tegumental proteins was described. The small exosomes were found to carry immunomodulatory molecules that were delivered into host cells to facilitate migration of juvenile flukes[26]. Later, a report was published in which the reviewers discussed several miRNAs and their potential immune-regulatory functions in EVs released from *F. hepatica*, and *D. dendriticum*[27].

***Clonorchis sinensis*:** Recently, a study observed enrichment of *C. sinensis* EVs (*Cs*EVs) with let-7a-5p miRNA. Mice administered *Cs*EVs exhibited severe biliary injuries with significant activation of M1 macrophages polarization, proving EVs pathological effects[28].

***Echinococcus* spp.**

Host immune response modulation: In 2019, Co-culture of EVs released from protoscolices (PSCs) with murine peripheral blood mononuclear cells showed their significant role in regulation of T lymphocyte functions[29]. In the same year, an Argentinean study isolated an exosome-like enriched EV fraction from infected mice and showed that PSC-EVs were internalized by murine dendritic cells that induced PSCs maturation with increased CD86 and down-regulation in the expression of MHC-II molecules. The investigators identified several echinococcal antigens, i.e., antigen 5, P29 and endophilin-1, as well as TSG101, ALIX, TSPs and 14-3-3 proteins[30]. While antigen 5 and P29 are specific for *Echinococcus* spp., endophilin-1 is a Taeniidae metacestodes antigen playing critical roles in the maintenance of membrane endocytosis, the 14-3-3 proteins are a family of highly conserved proteins that play a key role in cellular proliferation, i.e., signal transduction, cell-cycle control, cell differentiation and survival.

In 2022, two studies were conducted where the first study co-cultured plasma EVs released from mouse experimentally infected with *E. granulosus* with spleen mononuclear cells. Results showed a relative increase of regulatory T (Treg) cells and IL-10, and the investigators demonstrated EVs internalization with $CD4^+$ and $CD8^+$ T cells, B cells, and myeloid-derived suppressor cells[31]. A Chinese study demonstrated that PSC-EVs were internalized by bone marrow-derived dendritic cells to deliver miR-277a-3p producing pro-inflammatory cytokines such as IL-6, IL-12, TNF-α, and IFN-γ. The study suggested that miR-277a-3p enhanced dendritic cells maturation and differentiation to modulate host immune responses, i.e., presenting a possible therapeutic approach for prevention and treatment[32].

Potential diagnostic biomarkers: To identify potential diagnostic biomarkers, a cohort study was conducted. Serum pools from patients infected by active or inactive hydatid cysts, as well as from controls, were processed to isolate host-derived exosomes for proteomic analysis. Results revealed that Src and Lyn kinases were potential biomarkers for active hydatid cysts[33]. It is worth noting that Src and Lyn, members of the Src family kinases, are signaling intermediaries in several cellular processes such as proliferation, differentiation, metabolism, and apoptosis. Using liquid chromatography-mass spectrometry, a study profiled serum-derived EVs of infected mice, and identified three *E. multilocularis* proteins, thioredoxin peroxidase 1 (TPx-1), endoplasmic reticulum ATPase (ER ATPase), and 14-3-3. The study showed that TPx-1 and ER ATPase were promising

diagnostic markers for early infections, and suitable for prognostic evaluation[34].

Role of miRNAs: The miR-4989-3p encapsulated into *E. multilocularis* EVs was identified in the sera of infected mice. It significantly inhibited nitric oxide (NO) production, increased expression of TNF-α, and induced the dysregulation of several key components in lipopolysaccharide (LPS)/TLR4 signaling pathway, compared to the control. Accordingly, the investigators proposed *E. multilocularis* miR-4989-3p a virulence' factor encapsulated in *Em*EVs with a possible potential role in hydatid cyst pathogenesis[35]. Another study demonstrated that miR-4989-3p and miR-277a-3p expression in PSC-EVs regulated encystation[36].

Hydatid cyst fertility: To characterize exosome-like EVs isolated from hydatid fluids of fertile cysts, a study conducted a proteomic analyses that revealed several host- and parasite-derived molecules. Host-derived molecules included those contributing in host immune response, e.g. peroxiredoxin, triose-phosphate isomerase, and immunogenic antigens. Peptidases essential for parasite survival and cyst fertility were the main components of the parasite-derived molecules[37]. Similar results were obtained in a previous study when Brazilian investigators demonstrated abundance of exosomes in fertile cysts due to the significant content of virulence factors in EVs[38]. Using high-throughput sequencing, followed by genomes pathway analyses, long non-coding and circular RNA (lncRNA, and circRNA) profiles of EVs isolated from fertile cysts hydatid fluid, and PSC-EVs were compared. Results revealed higher expression levels of lncRNA, and circRNA in PSC-EVs than in hydatid fluid EVs. Functional analysis revealed that miR-125-5p and miR-10a-5p shared identical sites with host miRNAs suggesting their roles in mediating collagen catabolic process, i.e., inflammatory responses during echinococcosis[39].

Brugia malayi

In 2015 was the first study identifying exosome-like vesicles (30-120 nm) secreted from the third stage larvae of *B. malayi* with abundance of miRNAs in comparison to those released by adults. There was a significant increased expression of *B. malayi* miRNAs including let-7, miR-9, miR-1, miR-92, and miR-100b. Since the investigators observed J774A.1, a murine macrophage cell line, internalizing exosome-like vesicles, they suggested a miRNA-dependent pathway that stimulated M1 macrophage polarization for host immune response immunomodulation[40]. Utilizing nanoparticle tracking analysis, the same investigators demonstrated release of EVs from microfilariae excretory-secretory pore. They also showed sex-specific quantitative and qualitative differences in the immunomodulatory bioactive molecules[41]. Interestingly, an American study demonstrated that Ivermectin inhibited EVs secretion across all nematode species and life cycle stages[42]. Later, another American study showed that miR100 released in EVs of *B. malayi* microfilaria caused inhibition of the mechanistic target of rapamycin (mTOR). Inhibition of mTOR was associated with dendritic cell dysfunction leading to modulation of host immune response during infection. The study also confirmed internalization of microfilaria-derived EVs by host dendritic cells and monocytes, and accordingly it was suggested that miR100 downregulated mTOR phosphorylation *via* dendritic cells dysfunction[43].

Recently, a third American study characterized two *B. malayi* galectins (LEC1, and LEC2) in EVs released from adults. Sequence analysis of both galectins showed high homology to mammalian galectin-9. Functional analysis showed that LEC-2 selectively induces apoptosis in Th1 cells causing immunomodulation of host response, and modified type 2 response characteristic of chronic infection. Therefore, the study proposed *Bm*LEC2 a possible novel drug target in treatment of lymphatic filariasis[44].

Intracellular protozoa

Not only do EVs mediate intracellular communications between protozoa and their mammalian hosts, but they also extend this to their vectors. A collaborative review published by American and Czech scientists claimed that the mechanisms involved in EVs secretion are evolutionarily conserved among blood-feeding arthropods. It was shown that EVs released from the vector interact with host cells through endocytosis or receptor-mediated processes. It was hypothesized that membrane integrins in vector-derived exosomes might regulate their interaction with specific host receptors[45]. An inter-kingdom co-evolution for EVs functional role was suggested. Phylogenetic comparative evidence showed that the evolutionary effects of parasite- and host-derived EVs were transmitted to vectors to maintain parasite survival, virulence and transmissibility. The reviewers hypothesized that both derived EVs might regulate vector gene expression or trigger signaling pathways leading to receptors activation, i.e., increased attraction to infected mammalian hosts[46].

An interesting review discussed the role of the human vascular endothelium and parasite-derived exosomes of intracellular protozoa in diseases pathogenesis. Both elements regulate proliferation, invasion, signaling for parasite-host communications, as well as spread to other host organs. Both also contribute to modulate innate and adaptive host immune responses and maintain the inflammatory process integrity. The reviewers discussed the essential roles played by vascular epithelial cells, and drew a schematic diagram describing the cellular and molecular mechanisms of both elements during infection[47]. In another report, American reviewers confirmed that each intracellular protozoon creates its own strategy to transfer bioactive molecules from their intravacuolar niche into the host cell cytosol or cell organelles. Cargo molecules released into the cell cytosol were involved in triggering host exosome biogenesis machinery. Meanwhile, EVs released by phagocytic cells infected by intracellular protozoa kept

specific molecular cargo to contribute in host immune response, and disease pathogenesis[4].

***Plasmodium* spp.**

It was claimed that EVs released from *Plasmodium* spp. as well as from host cells, e.g., vascular endothelium, reticulocytes, hepatocytes, monocytes, lymphocytes, and platelets, are elevated during severe infections. The strong correlation of EVs number with disease severity suggested their essential contribution in development of inflammatory processes and disease pathogenesis. Besides, released EVs trigger several host immune cells including macrophages, dendritic cells, and natural killer cells. On the induction of increased stress conditions, host released EVs that orchestrate parasite population level and initiate gametocytes maturation for vector transmission. Reduction of EVs number was observed after drug treatment suggesting that EVs can be used as biomarkers to predict the severity of the disease[48].

***Plasmodium* EVs populations:** The membrane ability of small (30-70 nm, EV fraction 3) and large (70-300 nm, EV fraction 4) *P. falciparum* EVs populations to fuse with early and late endosome liposomes of RBCs plasma membrane was investigated. Due to their fusion capacity, protein content, and host cells' biophysical properties, small EVs significantly fused to early endosome liposomes much more than large EVs. In contrast, late endosomal membrane showed reduced fusion for both fractions. Proteomic analysis of each fraction revealed enrichment of host immune system cells suggesting that *P. falciparum* utilized its own EV pool employing a certain population with specific membrane and molecular content to target different host cells or pathways, i.e., cell-cell communication[12]. Since all intra-erythrocytic stages, have distinct metabolic, morphological, and biochemical phenotypes, Canadian investigators hypothesized difference in the molecular content of *P. falciparum*-derived EVs. To separate between microvesicles released from host cells, and those released from *P. falciparum*-infected RBCs, the study used differential ultracentrifugation protocol. According to EVs size (below or above 200 nm), two EVs populations were detected, small (exosomes) and large EVs (microvesicles). While ring forms showed both EVs populations, the other stages released exosomes. All EV populations expressed CD81, flotillin 2, and TSPs, whereas CD63 was only detected in EVs of rings and trophozoites. This preliminary characterization of EVs released from each stage is a crucial step to characterize *P. falciparum* EV markers in malaria pathogenesis[49].

Potential prophylactic candidates: It is established that mature RBCs lack the required endosomal machinery, and instead they shed microvesicles essentially involved in homeostatic RBCs aging process. In contrast, reticulocytes and hepatocytes are nucleated and possess the biosynthetic exosome machinery. A study demonstrated increased uptake of EVs derived from plasma of *P. vivax* patients with high parasitemia, by human spleen fibroblasts as compared to their uptake from controls. The investigators also demonstrated specific up-regulation of intercellular adhesion molecule-1 (ICAM-1) associated with translocation of nuclear factor kappa B cells (NF-kB). Infected reticulocytes showed specific adhesion properties to spleen fibroblasts, reversed by inhibiting NF-kB nuclear translocation[50]. Using bioinformatics and phylogenetic reconstruction, a recent study identified potential *P. falciparum* molecules with similar sequences to host proteins, carried by EVs released from infected RBCs. Eight molecules were identified including actin-1, elongation factor 1-alpha, the 20s proteasome alpha subunit, the 40S small ribosomal subunit-3 (RPS3), HSP90, 14-3-3 chaperon protein, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase. The investigators discussed their functional roles and proposed them as key modulators in host-parasite interaction[51].

A new approach was developed to improve enrichment of reticulocyte-specific EV molecules in the plasma of *P. vivax*-infected patients that enabled the investigators to identify several antigens, as potentially recognized vaccine candidates. Among them were merozoite surface proteins (MSPs 1, 3, 7, and 9), serine-repeat antigen 1 (SERA1), HSP70, *Plasmodium* helical interspersed sub-telomeric cytosolic protein (*Pvx*-088830), and four members of the *Plasmodium* exported proteins (*Pvx*-094300, *Pvx*-081830, *Pvx*-110835, and *Pvx*-083560). Since all are immunogenic during infections, the study discussed their potential protective role against *vivax* malaria. Besides, CD71, also known as transferrin receptor protein 1 (TfR1), was also identified in all patient's plasma. Notably, TfR1 is required for iron import from transferrin into RBCs by endocytosis, and is encoded by *tfrc* gene in human. This result suggested that immune-captured $CD71^+$ EVs were enclosed in exosomes[52]. Therefore, all the previous studies suggested use of exosomes derived from infected reticulocytes as a prophylactic vaccine.

Potential diagnostic biomarkers: In fact, because it is not possible to diagnose asymptomatic cases with dormant hypnozoites using current diagnostic methods, proteomic analysis of plasma-derived exosomes isolated from *P. vivax* infected chimeric mice (with a liver partially repopulated with humanized hepatocytes) was conducted. The investigators identified a conserved *P. vivax*-derived protein (*Pvx*-110940) in exosomes isolated from the infected chimeric mice. Three additional proteins were identified in hepatocytes-derived exosomes after 16 and 21 days of infection, and the study recommended further studies to investigate their diagnostic potentiality[53]. Later, the same group of investigators treated *P. vivax*-infected chimeric mice with a schizonticidal drug to achieve a pure non-hypnozoite infected experimental model, and to confirm schizonts removal from the chimeric mice livers. Proteomic analysis of EVs derived from mice livers proposed a putative filamin domain-containing protein (*Pv*P01-0915600) diagnostic biomarker for

asymptomatic hypnozoite infection associated with *P. vivax*[54]. Notably, filamin proteins in mammals are involved in actin binding of cell membranes to regulate cell shape and migration.

In a case-control study, researchers investigated human miRNAs isolated from exosomes released from two groups of Thailand patients infected with *P. vivax* and *P. falciparum* compared to healthy controls. According to a previous *in vitro,* infected-animal model, and clinical studies, only five human miRNAs were selected for their investigation: miR-451a, miR-150-5p, miR-15b-5p, let-7a, and miR-16-5p. Results revealed relative increased expression of miR-150-5p, and miR-15b-5p in *P. vivax*, while let-7a-5p was up-regulated in both species. The study recommended further validation in a large cohort study to assess miR-150-5p and miR-15b-5p potentiality to differentiate between *Plasmodium* spp.[55].

Potential therapeutic efficacy: Release of 20S proteasome complexes encapsulated within EVs (EV-20S) was observed from *in vitro* cultured *P. falciparum*. On treatment of EV-20S with *P. falciparum*-derived exosomes, the investigators observed degradation of the cytoskeletal network of naïve surrounding RBCs. The secreted 20S proteasome degraded phosphorylated cytoskeletal proteins, β-adducin, ankyrin-1, dematin, and erythrocyte membrane protein band 4.1, all were involved in growth and invasion through altering membrane rigidity. The study concluded that *Plasmodium* EVs were utilized to reshape surrounding uninfected RBCs to facilitate parasite invasion. Based on the obtained results, the study proposed EV-20S a novel avenue for therapeutic intervention[56]. Recently, a Thailand study conducted a comparative proteomic analysis of EVs (microvesicles and exosomes) among 4 different *P. falciparum* strains. It was observed that all parasite-derived molecules were detected in both microvesicles and exosomes, while human-derived molecules were detected either in microvesicles or exosomes. Molecules involved in RBCs invasion were the most detected proteins including merozoite surface protein (MSP1), apical merozoite antigen (AMA1), ring-infected erythrocyte antigen (RESA), and knob-associated histidine-rich protein (KAHRP). It is well known that *in vitro* growth has limited maximum growth capacity; however, invasion efficiency of cultured RBCs by all strains was attenuated on addition of microvesicles to *in vitro* cultures. Although the study included two limitations: *in vitro* cultures lack host immune response, and the absence of a drug-resistant strain among *P. falciparum* investigated strains, the investigators claimed the role of microvesicles in regulating *P. falciparum* growth density to avoid host cellular death. Therefore, they suggested combined therapy using current antimalarial drugs combined with bioengineered microvesicles carrying sensing molecules[57].

On the other hand, *P. falciparum* EVs biogenesis requires an alternative recruitment pathway involving the action of *Pf*Bro1, and *Pf*Vps32/*Pf*Vps60 proteins that activate ESCRT-III. Previous *in silico* studies showed possession of putative proteins from the ESCRT-III complex (Vps32 and Vps60), and AlIX which is a Bro1-domain protein that binds to Vps proteins to trigger ESCRT-III machinery formation. Distributed ESCRT-III components with EVs count reduction was demonstrated after RNA interference of the gene encoding *Pf*Vps60. The investigators drew a diagram showing *Pf*Bro1 export from parasitophorous vacuole to host cytosol *via Plasmodium* translocon of exported proteins, where it recruits *Pf*Vps proteins to initiate microvesicle formations. Therefore, the study suggested ESCRT-III machinery as a possible potential drug target against *falciparum* malaria[58].

Cerebral malaria (CM): In order to investigate the uptake of *Plasmodium*-derived exosomes by human microglia and their cytokine response in CM, Mbagwu *et al*.[59] isolated exosomes from *P. falciparum* cultures The study demonstrated exosomes internalization by human microglia with significant TNFα increased, and IL-10 reduced levels leading to neuroinflammation. Additionally, microglial morphological changes including cytoplasmic granulations, pseudopodia formation, and cellular body swellings were demonstrated[59]. Later, a recent study observed increased EVs release in CM patients, and Australian investigators compared the lipid profile derived from plasma of CBA mice infected with *P. berghei* that causes CM, to those from *P. yoelii* that does not. Using mass spectrometry, platelet-free plasma fractions enriched with EVs were assayed in both groups in comparison to non-infected mice. In spite of identical parasitemia, a dramatic difference was observed in mice with CM. With CM development, the study recorded significant increase of glycerolipids and phospholipids, and decrease of lysophosphatidyl-ethanolamine. The investigators suggested that circulating EVs with their lipid profile might have a role in CM pathophysiology[60].

Other EVs functional roles: Studies using transgenic parasites expressing a drug resistance marker showed that DNA packaged in EVs could be exchanged by parasites in an infected cell resulting in the spread of the resistance marker[61]. In Ghana, two studies were conducted, where in the first study the investigators demonstrated *P. falciparum*-derived EVs contribution in cellular homeostasis of infected RBCs under artemisinin pressure[62]. In the second study, they identified *P. falciparum*-derived EVs associated with artemisinin resistance[63]. Atovaquone and Tafenoquine when loaded into EVs derived from *P. falciparum*-infected RBCs exhibited significant *in vitro* antimalarial efficiency against *P. falciparum* growth in comparison to free drug administration. It was concluded that EVs potentially increased the efficacy of antimalarial drugs that possess hydrophobic properties[64]. Recently, French reviewers claimed that *Plasmodium*-derived microvesicles were involved in not only differentiation to sexual forms, parasite virulence,

and dissemination within the host, but also in evading host autophagy for parasite clearance. They discussed possible mechanisms contributing in the crosstalk between *Plasmodium*-derived microvesicles and host programmed cell death machinery. They also drew a schematic hypothesis of astrocytes regulatory events that determine either regulation or exacerbation of neuroinflammation in CM[65].

Toxoplasma gondii

A proteomic study was conducted in Brazil to compare molecular exosomes components released from cultured tachyzoites (RH strain) in a human cell line. The study identified 346 proteins related to *T. gondii* EVs, 69 proteins in *T. gondii*-infected human cell lines (I-EVs), and 15 proteins in non-infected human cell lines (N-EVs). Due to strong similarity between the molecular components released from I-EVs, and N-EVs cultured cells, the investigators performed a gene ontology study for all identified proteins in the parasites' *Tg*EVs and I-EVs cell line. Results revealed similarity (20-60%) in cellular components (nuclear, mitochondrial, and cytoskeleton), activity functions (binding, catalytic, and anti-oxidant), and biological processes (localization, and cellular metabolic processes)[66].

Potential vaccine candidate: In 2016, Dlugonska and Gatkowska[67] reviewed the potential protective role of *T. gondii* exosomes. They claimed that exosomal immunization produced significant increase in synthesis of Th1 cytokines by splenocytes, and a pronounced decrease in secretion of Th2 cytokines. Although it did not completely prevent *T. gondii* placental transmission, it led to reduced cyst burden in the offspring (65% lower than non-vaccinated infected pregnant mice). A collaborative work from China and Canada demonstrated that *T. gondii* exosomes modulated macrophage activation with increased production of IFN-γ, IL-12, and TNF-α *in vitro*. In addition, BALB/c mice immunized with parasite exosomes exhibited humoral and cellular immune responses with prolonged survival time[68]. Similar results were obtained when a Brazilian study immunized mice with *T. gondii* EVs prior to challenge of infection with a virulent strain. Parasitemia, survival time, humoral and cellular immune response in brain, liver, and spleen were determined. In comparison to chronically infected, and non-infected mice as controls, immunized mice showed reduced parasitemia and increased survival time. Furthermore, they had higher IgG1 levels than IgM or IgG2a, while their brain and spleen expressed high levels of IFN-γ, IL-10 and TNF-α[69].

Host behavioral changes: It was observed that EVs are responsible for the most striking observation of the parasite-induced changes in host behavior. Hakimi and Bougdour[70] showed that the effectors released from rhoptries and dense granules induced host behavioral changes e.g., there is a loss of aversion toward felids smell. Chronic toxoplasmosis was associated with increased expression of host miR-146a and miR-155 in brain tissue. The investigators demonstrated that both miRNAs were regulated, in part, by parasite rhoptry kinase (ROP16). The study also observed that miR-146a-deficient mice showed significant control of gut *T. gondii* burden with subsequent lower dissemination rate and brain cysts colonization associated with a higher survival rate than control mice[71]. Moreover, a Korean study demonstrated that *in vitro* incubation of exosomes secreted by *T. gondii*-infected cells attenuated the proliferation of L6 cells, a rat myoblast cell line. The investigators observed transient attenuation of L6 proliferation that disappeared two days after exosome treatment. It was concluded that *T. gondii* exosomes influenced host cell cycle and cell proliferation through involvement of *T. gondii* miRNAs in regulating host target genes related to cell division[72].

***Cryptosporidium* spp.**

It was demonstrated that cryptosporidiosis stimulated host intestinal epithelium to release exosomes. The investigators observed that released exosomes up-regulated splenic inflammatory genes in neonatal mice through NF-kB activation triggering inflammatory gene transcription in isolated splenocytes. Besides, the study identified a subset of *Cryptosporidium* miRNAs in the released exosomes that activated host immune response[73].

Pathogenic trypanosomatids

All proteomic studies focused on EVs molecules of the pathogenic trypanosomatids', and ignored the non-EV exoproteome (vesicle-depleted exoproteome; VDE) although it represents a significant portion of the total exoproteome. Therefore, Portuguese reviewers discussed VDE importance and its biological relevance, and hypothesized that studying VDE can provide relevant targets for diagnosis, treatment, and control. They claimed that exoproteome includes actively secreted and non-secreted proteins released from surface shedding or cell lysis. Since exoproteome describes the extracellular protein content, its results revealed not only conventional secretome, but also all proteins in the extracellular space such as those released from the surface or cell lysis or EVs rupture. These studies apparently focused on molecules utilized for infection establishment, host immune response modulation and virulence, and ignored secretome value. While the flagellar pocket is a highly specialized organelle in all pathogenic trypanosomatids and secretes the variant surface glycoprotein (VSG), promastigote secretory gel (PSG) is secreted in the vector and is mainly composed of phosphoglycans. Both VSG and PGS play essential roles in parasite survival and proliferation in both its vectors and mammalian hosts. Besides, acid phosphatases and proteo-phosphoglycans secreted from amastigotes and promastigotes were suggested as possible potential virulence factors. The reviewers discussed two main technical problems, namely the suitability of the

cultivation medium and the isolation protocol for characterization. They claimed that both challenged VDE recovery approaches to compare between EV and VDE fractions in exoproteome studies[74].

***Leishmania* spp.**

Infection establishment: A Brazilian study demonstrated that EVs isolated from *L. amazonensis* promastigotes stimulated macrophages and B-1 lymphocytes to express IL-6 and TNF-α with significant increase of parasite burden and polarization to Th2 response[75]. Notably, B-1 cells are an innate-like B cell population participating in an effective innate and adaptive responses to pathogens. They produce immunoglobulins, and cytokines that migrate to the inflammatory sites, and differentiate into mononuclear phagocyte-like cells. Another Brazilian study evaluated the production of myeloid and lymphoid lineage commitment factors, TLRs, NO, and reactive oxygen species (ROS) in murine peritoneal B-1 cells collected after *L. amazonensis* infection. Results revealed substantial increase in the expression of myeloid restricted transcription factors, significant decrease in NO, and ROS production as well as significant increased expression of TLRs 2, 6, and 9. The study confirmed the *Leishmania* EVs modulatory role in B1-cells activation and differentiation[76].

On the other hand, it is established that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has a crucial role in macrophage cell signaling and function. A recent Indian study demonstrated the role played by *Lm*GAPDH in cross talk with host immune response. The investigators showed enrichment of *Lm*GAPDH within EVs released during infection, and demonstrated that it inhibited TNF-α expression in infected macrophages *via* post-transcriptional repression[77]. Another recent Indian study hypothesized that EVs released from *Leishmania*-infected cells transfer *Leishmania*- and host-derived molecules to target certain pathways in neighboring non-infected cells to establish infection and propagation. During infection, host miRNAs, as an epigenetic signal, change gene expression of several cytokines in neighboring cells to restrict the inflammatory process. The investigators observed that *L. donovani* amastigotes adopt dual strategy for survival. First, amastigotes EVs downregulated the intercellular transport of host miRNAs *via* which caused alteration of host mitochondrial function preventing the entry of liver cell derived EVs containing miR-122 into infected macrophages. Second, amastigotes EVs upregulated the export of host miR-146a from infected macrophages which decreased miR-122 production in hepatocytes[78].

Finally, an American study demonstrated an interesting issue; EV composition and functions differ among intracellular pathogens. While exosomes released by *Leishmania*-infected macrophages exhibited gene expression towards M2 polarization, those released by *Salmonella*-infected macrophages showed genes transcription associated with M1 polarization[79].

Virulence factors: In 2015, a Canadian study demonstrated that the major *Leishmania* virulence factor, surface metalloprotease (glycoprotein 63, GP63), altered host macrophage nuclear envelope through degradation of its nucleoporins. Proteomic analysis showed that GP63 directly modified macrophages nuclear translocation of the transcription factors [NF-κB and activator protein 1 (AP-1)][80]. In fact, *Leishmania* spp. release virulence factors to modulate host immune response, while infected host macrophages secrete exosomes affecting amastigotes growth and survival. It was demonstrated that promastigotes, amastigotes, and macrophages infected with *L. major* expressed a phosphatase (*Lm*PRL-1) *via* exosomes release that was associated with increased amastigotes proliferation in macrophages[81]. In an attempt to compare exosomes content of GP63 in *L. infantum* promastigotes in their avirulent procyclic, virulent stationary, and metacyclic stages, American investigators observed that *Li*GP63 was highly expressed from exosomes of metacyclic stages followed by stationary ones, and the lowest level was observed in procyclic promastigotes[82]. In contrast, a Brazilian study demonstrated that cultured *L. i. chagasi* procyclic promastigotes showed marked increase in protein content and several virulence factors, (GP63, elongation factor-1α, oligopeptidase, and peroxidoxin)[83].

Since *L. mexicana* causes severe progressive cutaneous leishmaniasis, a Mexican study evaluated the immunomodulatory effect exhibited by exosomes released from *L. mexicana* amastigotes on infected bone marrow-derived macrophages. Two major virulence factors were identified, GP63 and protein phosphatase 2C (PP2C). The latter is a metal-dependent protein phosphatase that dephosphorylates serine/threonine substrates with subsequent downregulation of protein kinase cascade. The study demonstrated co-localization of *L. mexicana* exosomes with CD63 in macrophages endosomal membrane. There was a significant decrease in nitric oxide production, and expressions of antigen-presenting cells, associated with reduction of MHC-1 and CD86, and subsequent alteration of T cell activation. The investigators concluded that virulence factors released by *L. mexicana* exosomes immunomodulated host immune response in favor for the progressive inflammatory cutaneous lesions[84]. In contrast, another study attributed the severity of cutaneous leishmaniasis caused by *L. amazonensis* to EVs role, not to the virulence factors. To compare macrophage activation using EVs released from promastigotes of *L. infantum*, *L. braziliensis*, and *L. amazonensis*, the investigators demonstrated that only those released from *L. amazonensis* were able to induce pro-inflammatory response through macrophage activation. Furthermore, EVs modulated levels of NO, TNF-α, IL-6, and IL-10 *via* TLR4 and TLR2. It was demonstrated that the promastigotes' EVs showed

higher possession of translocated NF-κB[85]. Later, enrichment of GP63 expression in *L. amazonensis* EVs was recorded, and the investigators demonstrated its crucial role in exacerbating the inflammatory cutaneous response *in vivo*[86].

Macrophages infected with *L. donovani* amastigotes release exosomes containing a mixture of host and parasite proteins contributing to the pathogenic processes. In EVs released by infected macrophages, 59 *Leishmania* proteins were identified including nucleoside transporter 1, kinesin, DNA directed RNA polymerase II subunit 2, dynein heavy chain, and putative protein kinases. Comparing *Leishmania*-derived exosome molecules from promastigotes with those released from amastigote-infected macrophages, several overlapping of molecules were observed. These included elongation factor-1α, serine/threonine-protein kinase, kinesin, calpain-like cysteine peptidase, and the *Leishmania* homolog of vasohibins (Vash). Since Vash promotes angiogenesis in mammals, the investigators developed a transgenic *L. donovani* expressing endogenous Vash to investigate its role. They demonstrated Vash enriched expression in the EVs released from infected macrophages. In addition, the study observed that *Ld*Vash increased production of angiogenesis promoting mediators to increase vascularization in *Leishmania* infections. Accordingly, it was suggested that protein expression in EVs is differentially regulated between *Leishmania* life cycle stages. In other words, amastigotes or promastigotes translocate necessary molecules into the host cytosol according to their requirements for survival, growth, proliferation, and infection establishment[87].

In a recent report, Canadian and Brazilian reviewers discussed the contribution of endosymbiotic virus *Leishmania* RNA virus 1 (LRV1) with *Leishmania* exosomes to manipulate host immune response. The causative agent of mucocutaneous leishmaniasis (*L. guyanensis*) is naturally infected with LRV1. The study observed LRV1 release within *Leishmania* EVs to enhance virus transmission and influence progression. In infected macrophages, LRV1 triggered TLR3 activation leading to increased production and autophagy induction of interferons (IFNs). Moreover, LRV1-induced autophagy inhibited inflammasome activation with increased replication and disease exacerbation. The reviewers hypothesized that LRV1 within *Leishmania* EVs modulate host RNA sensors and NLRP3 inflammasome network[88]. The NLRP3, a nucleotide oligomerization domain (NOD)-like receptor (NLR), is a specialized intracellular protein that plays a critical role regulating host innate immune response.

Potential therapeutic efficacy: A Canadian study proposed a novel approach for developing a potential platform for drug delivery in treating leishmaniasis. Since *L. tarentolae* is non-pathogenic to humans, the investigators compared EVs contents released from *L. tarentolae* and *L. major*. Using dot blot analysis, similar GP63 content was observed in both species and the study detected *Leishmania*-derived GP63 in cultured macrophages treated with *L. tarentolae* EVs. Moreover, there was a significant increase in IFN-γ, TNF-α, and IL-1β production, followed by a significant decrease in *Leishmania* burden. It was concluded that *L. tarentolae* released EVs with potential ability to control leishmaniasis in human macrophages[89].

Drug resistance: It was shown that the mechanism of drug resistance in *L. infantum* induced changes in released EVs size, and distribution. The investigators hypothesized that EVs released by drug resistant isolates carry enriched proteins involved in survival according to its drug resistance mechanism. Size, and contents of EVs released from three drug resistant cell lines (against Antimony, Miltefosine, and Amphotericin) and a wild-type strain were determined. Results revealed several virulence factors, transcription factors, and molecules encoded by drug-resistance genes. The study suggested that discovery of EV-based profiles might lead to identified novel therapy for treatment of resistant *L. infantum* strains[90]. Later, the same group of investigators confirmed that EVs facilitated transmission of drug-resistance genes. Using next-generation sequencing and PCR assays, the study demonstrated the enrichment of amplicons carrying drug-resistance genes associated with EVs. It was hypothesized that these amplicons exploited *Leishmania* EVs to guarantee efficient propagation and rapid emergence of drug-resistant subpopulations for better control of oxidative stress conditions. Utilizing three different approaches, the investigators demonstrated that EVs released by drug-resistant strains efficiently altered the drug-sensitivity phenotype of recipient parasites after a single exposure[91].

American trypanosomiasis (*T. cruzi*)

In 2013, the first comprehensive study on *T. cruzi* EVs was conducted when Brazilian investigators characterized three EV populations using morphological and proteomic analyses. The study purified EVs from epimastigotes (Es) and metacyclic trypomastigotes (MTs), as well as from the flagellar pocket of both forms (FP). The three fractions; ectosome, exosome, and vesicle-free fractions were characterized in Es, MTs, and FP with a mean diameter of 143, 87, and >50 nm, respectively. Proteomic analysis showed that EVs released from Es and MTs shared 70.4% of the molecular cargo. Although 29.5% of protein composition were Es-specific, and only 6.44% were MT-specific, label-free quantitative proteomic analysis revealed enrichment of MT EVs with molecules essentially involved in metabolism and signaling required for survival, proliferation, and virulence. The investigators concluded that MTs utilized their EVs to deliver cargo into host cells[92].

Brazilian reviewers described the interaction between *T. cruzi* EVs and host cells as a bidirectional phenomenon where EVs contributed to host immune evasion utilizing the modulation of lipid metabolism for the production of host PEG2. It was observed

that trypomastigotes EVs (TEVs) had a lipid bilayer containing cytoplasmic lipid bodies (LBs) among EVs molecules. These LBs are intracellular sites of enzymes involved in the synthesis of biological active lipid mediators, e.g., PEG2. For the sake of parasite survival, TEVs and EVs from infected macrophages were recognized by uninfected macrophages *via* TLR2 that induced LBs formation with subsequent host PGE2 synthesis. Therefore, PGE2 reduces host Th1 immune response and activates NK-κB to modulate cytokines synthesis[93].

Host immune response modulation: In an attempt to evaluate immune modulation by *T. cruzi* EVs, a Brazilian study demonstrated that bone marrow-derived macrophages were stimulated on direct exposure to EVs *in vitro*. The *in vivo* study showed increased parasitemia due to decrease NO production by spleen cells, with elevated cardiac parasitism. The investigators also observed reduction of TNF-α and IL-6 cytokine production, a result that was explained by the EVs role to induce lipid body formation and PGE2 production by infected macrophages[94]. To investigate EVs role in macrophage response in progressive Chagas' disease (CD), an American study purified *T. cruzi* EVs released from trypomastigotes in axenic cultures, and EVs of *T. cruzi*-infected cells and plasma of acutely and chronically infected mice. On exposure to both EVs, cultured and bone marrow-derived macrophages showed increased TNF-α, IL-6, and IL-1β production. The study demonstrated that both EVs carried *T. cruzi,* and host DNA that were captured by macrophages with subsequent signaling of NF-κB-mediated pro-inflammatory cytokine production. Genetic deletion of PARP1 (a DNA repair enzyme) in infected mice significantly decreased EVs-induced transcriptional and translational activation of pro-inflammatory macrophages response. Since PARP1-mutant mice showed significant decrease (<80%) in cardiac inflammation in chronically infected mice, the study suggested use of PARP1 inhibitors to modulate macrophage pro-inflammatory signaling[95].

Virulence factors: A multigene family composed of trypomastigote, alanine, serine, and valine (TASV) was identified with conservative homology in *T. cruzi* lineages (I and VI) with no orthologues in other trypanosomatids. Argentinean investigators demonstrated *Tc*TASV-C localization on trypomastigotes surface, and its secretion through EVs with high expression level in blood stream trypomastigotes. Since its absence in some *T. cruzi* lineages and its involvement in infection establishment, the study proposed *Tc*TASV-C a potential virulence factor expressed in *T. cruzi* EVs[96]. A proteomic study was conducted to compare EVs released by tissue-cultured *T. cruzi* trypomastigotes and epimastigotes. Results revealed marked differences in EVs surface molecular cargos of EVs. There was expansion of all trans-sialidases in trypomastigote's EVs with higher adhesion properties in comparison to epimastigotes. Notably, trans-sialidases, highly polymorphic surface protein family are established virulence factors in *Trypanosoma* spp. involved in host cell cytoadherence and invasion[97]. A recent collaborative report published by Spanish and Brazilian reviewers tabulated all virulence factors reported in EVs released from epimastigotes and metacyclic trypomastigotes of four *T. cruzi* strains. For each virulence factor, they also described its stage, and its essential role(s). They included cruzipain, trans-sialidase, TASV, mucin-like glycoproteins, mucin-associated surface proteins (MASPs), and synaptotagmin-like mitochondrial-lipid-binding domain proteins. The latter are evolutionarily conserved proteins in all eukaryotes localized between the endoplasmic reticulum and either the plasma membrane or other organelles. It induces calcium signaling and lysosome mobilization to facilitate parasitophorous vacuole formation. Due to their essential roles in host immune modulation, the reviewers proposed TASV, and MASPs potential vaccine candidates. They also suggested use of anti-MASPs IgG potential diagnostic biomarker to predict disease outcome[98].

Diagnostic biomarkers: Brazilian investigators analyzed exosomes in serum of three groups of patients: CD with heart failure, asymptomatic CD, and healthy controls. The study used zymography, and probe anti-archaeal DNA to determine the content of anti-archaemetzincin-1 antibody (AMZ 1, archaea collagenase), and archaeal DNA activity, respectively. While sera of asymptomatic patients had higher AMZ1, and archaea collagenase, those from heart failure patients had higher free archaeal DNA content. Archaeal DNA activity was suggested as a possible potential diagnostic biomarker for heart failure in CD[99]. To determine the role of circulating EVs as predictive biomarkers to evaluate therapeutic response and disease outcome in CD, a Spanish study collected pretreatment and post-treatment plasma samples and characterized their EVs content in comparison to negative controls. Using proteomic and secretomic analyses, the investigators identified only one *T. cruzi* enzyme (pyruvate phosphate dikinase, PPDK), in pretreatment samples. The study proposed *Tc*PPDK as a possible diagnostic biomarker for follow up treatment. Notably, PPDK plays a central role in the metabolism of *T. cruzi* glycosomes[100]. Later, another Brazilian group of investigators concluded that decreased concentration of circulating EVs associated with increased production of IFN-γ and IL-17 in patients with chronic CD was significantly linked with establishment of chronic disease. This result was considered a possible biomarker for monitoring disease progression[101].

Novel immunotherapy: In an attempt to investigate the role of *T. cruzi* trypomastigotes EVs on cultured bone-marrow-derived dendritic cells (DCs), the investigators observed that EVs downregulated DCs immunogenicity *in vitro* and *in vivo*. It was demonstrated that DCs interacted with *T. cruzi* EVs became easily captured by unstimulated-DCs, in comparison to EVs released

from DCs cultured without *T. cruzi* trypomastigotes. Moreover, mice treated with stimulated DCs showed partial protection after *T. cruzi* challenge infection. Accordingly, the study proposed *T. cruzi*-DCs derived EVs a novel strategy for immunotherapy against CD[102].

African trypanosomiasis (*T. brucei*)

As known, human is innately immune to African trypanosomes utilizing circulating trypanosome lytic factor (TLF). However, pathogenic *T. b. rhodesiense* evades TLF activity through expression of serum-resistance associated protein (SRA), a virulence factor. An American study demonstrated membrane nanotubes budding from the flagellar membrane, and EVs. Proteomic analysis of these EVs showed their enrichment with molecules involved in virulence and long persistence within the host. Among them are major surface proteases (MSPs), variant surface glycoprotein (VSG), and phospholipase-C (PLC). The investigators observed EVs fusion with the lipid bilayers of the flagellar pocket of neighboring trypanosomes resulting in transfer and internalization of their cargo including SRA. Fusion of EVs with host RBCs membrane, and alteration of the RBCs physical properties and subsequent phagocytosis by macrophages was also observed. The study attributed this observation to the occurrence of anemia during African sleeping sickness[103]. To understand the exact role of EVs released from *T. b. brucei* in host immune response, a collaborative Portuguese and Brazilian research examined *Tb*EVs effects on cultured mouse macrophages and T lymphocytes. The investigators demonstrated that *Tb*EVs induced differentiation of both M1- and M2-polarization that elicited expansion of macrophage subpopulations of MHCI, and MHCII. In T lymphocytes, they upregulated expression of cell-surface CD3 and the nuclear factor FoxP3 leading to the differentiation of regulatory $CD4^+$ and $CD8^+$ T cells. Notably, FoxP3 is a regulator of gene expression that suppresses other nuclear factors to avoid expression of the genes encoding pro-inflammatory cytokines. It was postulated that *Tb*EVs displayed a complementary balance between parasite growth and replication and host immune cells during the early phase of infection. In other words, *Tb*EVs established a direct communication within cells of innate and adaptive immunity[104].

Giardia lamblia

Since *G. lamblia* underwent a reduced evolution, it lacks both ESCRT machinery and TSPs, and it is unable to synthesize ceramide *de novo*. It seems that EVs biogenesis occurs in peripheral vacuoles that play the role of endosome and lysosome simultaneously[105]. Proteomic analysis revealed enrichment of microvesicles with virulence factors (variant surface proteins, HSP70, and cysteine proteases), and molecules involved in cytoskeletal adhesion and attachment (giardin, ankyrin, and α- and β-tubulins). The study also detected anti-oxidative stress response proteins, enzymes expressed to escape host NO production as defense mechanism against trophozoites [arginine deaminase (ADI), and ornithine carbamoyl transferase (OCT)], and enolase. While ADI and OCT are enzymes expressed to escape host NO production as defense mechanism against trophozoites, enolase participates in the glycolysis pathway, crucial for survival. *In vitro* studies showed that large EVs increased trophozoites adhesion to *Caco-2* cells and activated immature dendritic cells. The investigators evaluated the effect of inhibitors of EV release, e.g., peptidylarginine deiminase (PAD), and cannabidiol (CBD), and both efficiently reduced EVs release. However, PAD specifically inhibited microvesicles release with subsequent reduction in attachment to host cells *in vitro*[106].

In 2021, a group of Chinese investigators conducted two studies to investigate the role of *G. lamblia* EVs on cultured mouse peritoneal macrophages. Results revealed that EVs were captured by macrophages with increased production of pro-inflammatory cytokines, and activation of TLR2 and NLRP3 inflammasome signaling pathway. The latter signaling pathway is a multi-protein complex playing an essential role in innate immune system regulation. Upon activation by pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively), NLRP3 activates caspase-1 to initiate release of pro-inflammatory cytokines. Pre-treatment with a pan-caspase inhibitor, significantly inhibited NLRP3 signaling pathway, with abolishment of expression of pro-inflammatory cytokines[107]. In the second study, the investigators explored the role of *Giardia* EVs in induction of pro-inflammatory response of cultured mouse macrophages utilizing other signaling pathways. They included signaling pathways of protein kinases (PKs), namely mitogen-activated PK (MAPK), and PKB (AKT), as well as NF-κB. It is known that AKT serine/threonine kinase family includes three isomers of PKB (α, β, and γ). The study used real-time quantitative PCR to measure levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), while the phosphorylation levels of MAPK, AKT, and NF-κB signaling pathways were determined using Western blotting, and immunofluorescence assays. Results showed that *Giardia* EVs significantly induced cytokines transcription and secretion from mouse macrophages, and upregulated the phosphorylation levels of MAPK, AKT and NF-κB signaling pathways. In addition, inhibition of both PKs, and NF-κB signaling pathways significantly downregulated expression of pro-inflammatory cytokines[108].

In 2015, American investigators demonstrated localization of lipid raft-like structures in the plasma membrane of *Giardia* trophozoites and on the periphery of their ventral discs, whereas they were localized in membranes beneath the cyst wall. Since glycosphingolipid-enriched lipid rafts (LRs), small collections of cholesterol, sphingolipids, and proteins, are involved in transducing extracellular signals through plasma membrane participating in host-pathogen interactions. The study showed LRs involvement in encystation and use of the LR inhibitors,

Nystatin and Filipin III, blocked the biogenesis of encystation-specific vesicles and cyst production[109]. Recently, the same group of investigators isolated *G. lamblia* EVs, and LRs. Proteomic analysis identified several potential virulence factors (the major cysteine protease giardin, variant surface proteins, elongation factors, and arginine deaminases) in LRs encapsulated in microvesicles, and exosome-like vesicles. An *in vitro* study showed that trophozoites treated with LRs inhibitors (nystatin and oseltamivir) decreased expression of virulence factors in both vesicles. However, a significant result was recorded in exosome-like vesicles, indicting the significant decreased expression of virulence factors in exosome-like vesicles more than microvesicles. The *in vivo* part of the study showed that experimentally infected mice treated with Oseltamivir exhibited significant reduction in parasite burden[110].

Another recent study proposed *Giardia* EVs for treatment of inflammatory bowel disease (IBD). Prior to dextran sulfate sodium-induced experimental murine colitis, Korean investigators administered EVs to the mice. Reduced clinical signs, colon histopathology, and expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) were recorded. The study concluded that *Giardia* EVs ameliorated clinical and histological features of colitis, and exhibited protective effects against IBD[111].

Trichomonas vaginalis

The first report describing *T. vaginalis*-derived exosomes was in the year 2013. American investigators demonstrated presence of conserved exosomal proteins, and specific proteins delivered to host cells to modulate host immune response, and increase *T. vaginalis* adherence to vaginal and prostate epithelial cells, respectively[112]. To quantify *T. vaginalis* EVs internalization by host cells, an American study established biochemical assays to monitor their uptake by host cells. By such assays, the investigators succeeded to identify their surface components and mechanism of uptake. The study demonstrated interaction of EVs with host glycosaminoglycans, specifically with heparan sulfate (HS) on host cell surface proteoglycans. The result was confirmed when the investigators used a competition assay to remove HS, and they observed complete inhibition of EV uptake. Moreover, the study identified a protein on EVs surface (4-α-glucanotransferase, *Tv*4AGT) with high binding ability to HS. Similarly, using competition assay for *Tv*4AGT inhibited EV uptake. Besides, the study demonstrated that EV internalization was dependent on host cell cholesterol and caveolin-1[113].

Host immune modulation: It was demonstrated that *T. vaginalis* surface lipophosphoglycan released small exosome-like vesicles capable to induce marked macrophage IL-10 production with modulation of IL-8 secretion by epithelial cells. Treatment of experimentally infected mice with *T. vaginalis* exosomes exhibited significant decrease in the inflammatory process due to decrease of ILs 6, 13, and 17 production[114].

Virus endosymbiosis: Apparently, EVs released from *T. vaginalis* carrying an endosymbiotic *Trichomonas* virus (TVV) play crucial roles in disease pathogenesis and drug resistance. Based on phylogenetic analyses and genomic sequences, four different TVV subspecies (1-4) were identified. The first study to demonstrate the release of TVV proteins within small EVs (sEVs) was published in the year 2022. The investigators recorded a significant impact of TVV positive isolates on sEVs proteome and RNA cargo than that observed for TVV negative isolates. Moreover, they showed that TVV loaded in sEVs stimulated a high pro-inflammatory cytokine response of human cell lines. There was significant increase in the production of IL-8, IL-6, and IL-1β expression. Proteomic analysis of sEVs revealed significant enrichment of 12 molecules including eight unique proteins, and four of viral origin. Among the enriched proteins were those implicated in parasite adhesion such as *Tv*BspA, and adhesine, localized on *Trichomonas* surface, and ~2.5-fold increase in regulatory RNA of viral origin (tsRNAs)[115]. In the same year, another study compared EVs cargo between *T. vaginalis* isolates that were either TVV free, with single, two, or three TVV subspecies. Presence or absence of TVV subspecies was confirmed by reverse transcription (RT)-PCR. Identified EVs (30-150 nm) showed *Tv*TSP1 as classical exosome marker in all EVs isolates. All TVV positive isolates showed the viral capsid proteins in a sEV that belonged to TVV subspecies, as proved by RT-PCR. Using new generation sequencing RNAseq datasets, the study investigated the distribution of TVV subspecies in another twenty-four isolates. Results revealed that the majority of isolates were infected with TVV1, followed by TVV3 and TVV2, while few were TVV free, suggesting the possible transmission of virus subspecies among different isolates. The investigators hypothesized that EVs released from TVV positive isolates deliver their soluble content and transmit TVV subspecies to host cells via its sEV[116].

Virulence factors: Cargo of all studied *T. vaginalis* exosomes contained TSPs among several molecules participating in adherence and colonization[117]. Another virulence factor identified in *T. vaginalis* exosomes was ESCRT-III subunit vacuolar protein sorting-associated protein 32 (VPS32). It was demonstrated that *Tv*VPS32 over-expression was significantly linked with increased adherence. Accordingly, *Tv*VPS32 was proved to have essential role in EVs biogenesis, and their cargo sorting that modulated *T. vaginalis* adherence[118].

Entamoeba histolytica

The first comprehensive proteomic study conducted in *E. histolytica* EVs was published in the year 2020 when American investigators showed EVs enrichment of cytoskeletal proteins, and several molecules involved in EVs formation, cell signaling, and metabolism. Small RNAs (sRNAs), previously characterized in *Entamoeba* RNA interference pathway proteins, e.g.,

argonaute were also identified. Sequencing analysis of sRNAs showed that the majority were 27 nucleotides representing a subset of the cellular antisense sRNA population. It is worth mentioning that highly potential virulent *E. histolytica* isolates possess a sRNA population that controls gene expression with strong capability of transcriptional gene silencing in target genes. Since EVs isolated from encysting trophozoites promoted encystation in other trophozoites, the investigators concluded EVs impact on encystation, and intercellular communication among trophozoites to influence their growth kinetics[119].

It was described that *E. histolytica* trophozoites activated the release of neutrophil extracellular traps (NETs) with subsequent neutrophil lysis. In an attempt to evaluate the effects of *E. histolytica* EVs on NETs, a Mexican study isolated and characterized EVs co-cultured with human neutrophils. The study demonstrated EVs uptake by neutrophils with subsequent significant reduction in ROS production. Comparative proteomic analysis followed by gene ontology revealed abundance of the virulence factors namely N-acetyl-D-galactosamine (GalNAc), and calreticulin, and various antimicrobial molecules, e.g., lactoferrin and myeloperoxidase in EVs of *E. histolytica* and neutrophils, respectively. The investigators hypothesized that the uptake of EVs released by *E. histolytica* trophozoites by human neutrophils led to transfer of EVs cargo to produce immunomodulatory effects[120].

Free-living amoeba

***Acanthamoeba* spp.:** Since *Acanthamoeba* spp. are professional phagocytes, i.e., are able to secrete several phagocytosing enzymes expressed in released EVs that efficiently access host cytoplasm. Therefore, Brazilian reviewers claimed that EVs are efficient carriers of several glycosidase, lipases and proteases, and act as drug delivery system. Besides, their immunogenic cargo was able to activate the host immune system and induce a partial protective response. It was observed that glycosidases (glycosyl hydrolases, alpha amylases, beta-glucosidases, galactosidases, and mannosidases) were enriched in *A. castellanii* EVs. Notably, glycosydases are involved in several cellular biological processes such as cell growth, and recognition. Proteases, which are major virulence factors in *Acanthamoeba* spp. responsible for cyst/trophozoite differentiation and disease pathogenicity, were also enriched in EVs cargo. Among EVs cargo also were kinases involved in cellular signaling, and *Acanthamoeba* plasma membrane lipids such as ergosterol, free sterol, and free fatty acids[121].

Although *Acanthamoeba* type 5 is commonly detected in environmental samples, few cases were recorded in human cases. A collaborative work from Costa Rica, and Spain confirmed EVs release from T5 isolate collected from a water source. The study investigated trophozoites effects on cultured cell monolayers at two different temperatures and demonstrated abundance of virulence factors (proteases) at 37^0C. To determine EVs nano-mechanical properties, the investigators used atomic force microscopy, and the results revealed higher adhesion activity. Therefore, it was concluded that EVs have crucial role in *Acanthamoeba* survival and its damaging potential[122].

***Naegleria fowleri*:** The same group of investigators from Costa Rica, and Spain characterized EVs released from *N. fowleri* trophozoites isolated from a clinical case. Large EVs (>200 nm) were identified, and SDS-PAGE revealed protein bands that ranged from 25 to 260 kDa. Western blotting revealed significant reaction with rat polyclonal antibodies (pAbs) raised against *N. fowleri* antigens at three intense bands (70-80 kDa), and 3 weak bands (~23 kDa), i.e., identification of immunogenic cargo used either in serological diagnosis or as protective candidates. In order to identify the predominant proteins recognized by pAbs intense bands, the 3 more intense bands were submitted to proteomic analysis. Results revealed actin, HSP70, serine protease (leucine aminopeptidase), cysteine protease (fowlerpain), EF1-α, glyceraldehyde-3-phosphate dehydrogenase, and adenosyl homocysteinase. The latter participates in methylation reactions required for growth and gene regulation. Additionally, protease activity assays demonstrated serine protease abundance. The investigators recommended further researches on *N. fowleri* EVs to reveal new rapid and accurate tools for diagnosis of the lethal primary amoebic meningoencephalitis[123].

***Blastocystis* spp.**

In the year 2022, the first two proteomic studies of *Blastocystis* EVs were conducted in Iran and Singapore. Iranian investigators exposed a human leukemia monocytic cell line to *Blastocystis* EVs that were extracted from subtypes (STs 1-3). Pro-inflammatory (TNF-α, and IL 6) and anti-inflammatory cytokines (ILs 4 and 10) were assessed using quantitative PCR. While all extracted exosomes (30-100 nm) downregulated IL-10 expression, ST1- and ST3-derived exosomes upregulated IL-6. Interestingly, only ST1-derived exosomes also recorded the significant increased expression of TNF-α, and IL-6, and decreased expression of ILs 4 and 6. The study confirmed EVs role on pro-inflammatory and anti-inflammatory cytokines in ST1 that modulated host immune response[124]. On the other hand, *Blastocystis* ST7, in comparison to other subtypes, exhibited higher virulent activities such as induction of intestinal epithelial cell damage, gut microbiota dysbiosis, and increased resistance to commonly used drugs. The second study was conducted to investigate EVs role in ST7. The investigators isolated EVs from *Blastocystis* ST7 (7B, and 7H) and co-cultured them with three relevant human gut microbiota (*Lactobacillus brevis, Bifidobacterium longum,* and *Escherichia coli*) to record their effect on prokaryote growth. Results revealed that EVs released from ST7 isolates possessed specific protein cargo with significant variability between isolates. Fluorescence

microscopy demonstrated human cells viability loss as observed by increase in propidium iodide uptake. Moreover, on increasing the concentration of 7B EVs, the study recorded significant increase of IL-1β expression, a pro-inflammatory cytokine, but not IL-6 and TNF-α[125].

CONCLUDING REMARKS

1. Three EVs categories are associated with parasitic diseases. First is the EVs directly released from helminthes: *Schistosoma*, *Fasciola*, *Echinococcus*, and filarial nematodes. Second are those released by intracellular protozoa: *Plasmodium* spp., *Leishmania* spp., *T. gondii*, *T. cruzi*, and *T. brucei*. The last category of EVs are released by host immune cells that are stimulated by parasite-derived antigens either intra- or extracellular parasites.
2. In spite of EVs discovery ~40 years ago, there is still much to be explored regarding the clinical applications of parasite derived EVs. Since exosomes are rich in lipids, proteins, and miRNAs, they have several advantages including stable structure, high content in plasma, and alternative abundance and constitution under stress conditions. Therefore, the majority of studies investigating their clinical applications focused on their use as prognostic biomarkers in cancer. In parasitic diseases, studies are encouraged to investigate use of miRNAs, HSPs, TSPs and virulence factors as potential molecules with diagnostic, therapeutic, and protective efficacy.
3. Host vascular endothelium contributes with parasite-derived EVs to facilitate cell-to-cell communication in intracellular protozoa. While erythrocyte membrane protein-1 (EMP1) and intercellular adhesion molecule-1 (ICAM-1), mediate the adhesion of infected RBCs to the vascular epithelial cells in malaria, GP63 contributes with ICAM-1, and vascular cell adhesion molecule-1 (VCAM-1) to facilitate migration of mononuclear cells, and lymphocytes to the endothelial cells initiating the inflammatory process in leishmaniasis. In American trypanosomiasis, released cruzipain invades the vascular epithelial cells through a calcium-dependent mechanism. In African trypanosomiasis, exosomes fuse to RBCs, and the released virulence factors causes RBC membrane alteration and anemia.
4. **Virulence factors:** Several studies documented expression of virulence factors in EVs released from *Leishmania* spp., *Trypanosoma* spp., *G. lamblia*, *E. histolytica*, *T. vaginalis* and free-living amoeba. Host immune response modulation favoring for progressive inflammatory process was hypothesized for their functional mechanisms. Chagas' chronic cardiomyopathy is also attributed to *T. cruzi* EVs enriched with virulence factors. Virulence factors induce mitochondrial dysfunction in cardiomyocytes with increase of mitochondrial ROS to activate NF-κB transcription factor and gene expression of pro-inflammatory cytokines.
5. **Diagnostic and prognostic biomarkers:** Several miRNs were suggested to diagnose schistosomiasis with low infection intensity and proved valuable in grading hepatic fibrosis. They were also proposed potential biomarkers for hydatid cyst fertility, and differentiation between *Plasmodium* spp. A putative filamin domain-containing protein was identified as diagnostic marker for diagnosis of asymptomatic hypnozoite *P. vivax* infection. Determination of pyruvate phosphate dikinase (PPDK) in circulating EVs was suggested as a prognostic assessment in Chagas' disease.
6. **Therapeutic efficiency:** Since EVs are implicated in improving parasite attachment and virulence, blocking EVs synthesis and release, or altering their cargo to prevent parasite survival, represents novel potential therapeutic approaches. Treatment of *G. lamblia* with nystatin and oseltamivir, two inhibitors of lipids rafts, induced alteration of proteome profile of the secreted *G. lamblia* EVs. Use of *L. tarentolae* and *A. castellanii* EVs proved suitable as drug delivery systems in treating leishmaniasis, and *Acanthamoeba* keratitis, respectively.
7. **Immunization:** Beside their effects in host immune evasion, EVs carry TSPs among several molecules participate in adherence and colonization. Therefore, immunization using EVs enriched with parasite TSPs were suggested in schistosomiasis, hydatid cyst, and trichomoniasis. In toxoplasmosis, exosomal immunization reduced parasitemia and increased survival time with significant production of IFN-γ, IL-10 and TNF-α in brain, and spleen. A novel strategy for immunotherapy against Chagas' disease was suggested. Mice treated with co-cultured dendritic cells with *T. cruzi* trypomastigotes showed partial protection after challenge infection.
8. **Drug resistance:** Several studies documented involvement of EVs in spread of drug-resistant strains of *P. falciparum* and *L. infantum*. It was hypothesized that EVs released by drug resistant isolates carry enriched proteins involved in the parasite survival and drug-resistance genes according to its drug resistance mechanism.
9. **Viral endosymbiosis:** EVs released from *T. vaginalis* with endosymbiotic *Trichomonas* virus play crucial roles in disease pathogenesis, and drug resistance with high possibility of virus transmission to *T. vaginalis* with negative virus endosymbiosis. It was hypothesized that EVs released from virus positive isolates deliver their cargo to host cells.
10. **Host behavioral changes:** In chronic toxoplasmosis, EVs are linked with host behavior. It was hypothesized that *T. gondii* exosomes influenced host cells proliferation through involvement of miRNAs in regulating host target genes related to cell division.
11. Clinical applications of *Schistosoma* and *Giardia* EVs proved to be a novel therapeutic approach to ameliorate the severity of inflammatory bowel diseases.

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