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Small RNAs and extracellular vesicles in filarial nematodes: from nematode development to diagnostics

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Abstract

Parasitic nematodes have evolved sophisticated mechanisms to communicate with their hosts in order to survive and successfully establish an infection. The transfer of RNA within extracellular vesicles (EVs) has recently been described as a mechanism that could contribute to this communication in filarial nematodes. It has been shown that these EVs are loaded with several types of RNAs, including microRNAs, leading to the hypothesis that parasites could actively use these molecules to manipulate host gene expression and to the exciting prospect that these pathways could result in new diagnostic and therapeutic strategies. Here we review the literature on the diverse RNAi pathways that operate in nematodes and more specifically our current knowledge of extracellular RNA (exRNA) and EVs derived from filarial nematodes *in vitro* and within their hosts. We further detail some of the issues and questions related to the capacity of RNA-mediated communication to function in parasite-host interactions and the ability of exRNA to enable us to distinguish and detect different nematode parasites in their hosts.

Introduction

Filarial nematodes, the causative agents of some of the most prevalent poverty related diseases, are tissue-dwelling nematodes that are transmitted by blood-feeding arthropods to terrestrial vertebrate hosts, from amphibians to mammals. For those nematodes infecting humans, their distribution is confined to tropical and subtropical regions, therefore representing a matter of public health in developing countries [1]. Latest estimations of the World Health Organization suggest that over 120 million people are infected by filarial parasites, causing considerable morbidity despite long-term chemotherapy-based control programmes [1,2]. The clinical manifestations (for example, lymphedema, hypertrophy of the skin and blindness) are rarely associated with high mortality rates but their chronicity and morbidity impose a tremendous socio-economic burden on these countries.

From a host-pathogen standpoint, filarial nematodes are fascinating organisms for their ability to persist in their hosts for long periods, surviving and reproducing for over a decade in some cases [3]. This can be attributed to a repertoire of adaptations and strategies that the parasites employ, including secretion of factors with immunomodulatory properties [4,5]. Furthermore, the

complex life cycles and ecological interactions of parasitic nematodes make them an interesting object of study with regard to moulting, growth, and survival in challenging environments, such as those encountered upon infection of the definitive host. However, many mechanistic and molecular aspects associated with the biology of these parasitic nematodes have not been fully elucidated. RNA interference (RNAi) pathways have been shown to play important roles in the free-living nematode *Caenorhabditis elegans*, including regulation of developmental timing, genome defense and adaptation to the environment [6–8]. Here we describe the current understanding of how different RNAi pathways operate in filarial nematodes, making use of comparisons with studies in *C. elegans* in which many mechanistic aspects of RNAi were discovered [9,10].

In the last 8 years it has been shown that the small RNAs involved in RNAi within cells are also found extracellularly. Their association with extracellular vesicles (EVs) in parasite infections may implicate them as novel players in the transmission of information between the parasites and their hosts [11]. We will describe recent evidence of extracellular RNAs derived from filarial nematodes, their potential use for diagnostics, as well as current challenges and outstanding questions in the field.

RNAi pathways in filarial nematodes

Three primary RNAi pathways have been characterized in animals: the microRNA (miRNA) pathway, the endo/exo-small interfering RNA (endo/exo-siRNA) pathway and the P-element Induced Wimpy testis (PIWI)-interacting RNA (piRNA) pathway [9,12]. These pathways are distinguished by the origin and identity of the small RNA guide and target, as well as the properties of the Argonaute (AGO) protein to which they bind. In general AGOs have two main functions: 1) recognizing and binding small RNA and 2) mediating the interaction with other proteins required for small RNA loading, association with targeted RNAs, gene silencing activity, and/or subcellular localization[8,13]. From a structural standpoint, they are generally ~90-100 kDa monomeric proteins containing at least two domains: a PAZ domain (involved in 3'-end recognition and binding of the small RNA) and a PIWI domain, which binds to the 5' end of the small RNA and in some cases includes an RNaseH-like activity that can carry out

endonucleolytic cleavage ("slicing") of the targets [13,14]. The ancestral AGOs that bind to miRNAs are called ALG1 and ALG2 (Ago-like gene). The piRNA pathway is thought primarily to operate in genome defense through targeting transposable elements, mediated by the PIWI-clade of AGOs. Homologs to these proteins are not present in clade III nematodes; the phylogenetic classification proposed by Blaxter et al is used throughout this manuscript [15]. Rather, it is thought instead that other AGOs and small RNA classes could be involved in genome defense in this clade [16]. Indeed a remarkable feature of nematodes is their extended AGOs (27 identified in *C. elegans* [8,13,17]), reflecting the diversity of RNAi pathways that can operate in these animals. The majority of the AGOs in *C. elegans* belong to the WAGO (worm-specific-Ago) clade and many members of this clade are expected to be found in filarial nematodes [8,13]. From studies in *C. elegans* the WAGOs are thought to bind to a class of secondary siRNAs that can act through a range of mechanisms including chromosome segregation and epigenetic modifications [18–20] and can mediate transgenerational inheritance [21]. A more extensive description of different structural, functional and mechanistic aspects of AGO proteins is provided in recent reviews [8,13,22].

Biogenesis of miRNAs

The microRNA pathway is one of the best characterized RNAi pathways in nematodes [23]. These molecules, first described in *C. elegans* over two decades ago, are encoded within the genome as stem-loop structures that undergo a series of maturation events to produce the short RNA guide. In nematodes, as in other animals, miRNAs can either derive from within intragenic sequences (generally within the introns) or from independent, intergenic transcriptional units [24]. These transcripts, termed the primary miRNAs (pri-miRNAs), are mostly derived from the activity of RNA polymerase II **(Figure 1)**. Some miRNAs are clustered together in discrete genomic regions suggesting coordinated expression [10].

Once transcribed, miRNA biogenesis involves a series of maturation events starting with cleavage by the microprocessor complex in the nucleus [10,25]. The microprocessor is composed of the RNase III endonuclease DROSHA and DCRG8, among other scaffold proteins, and cleaves the pri-miRNA to produce a shorter hairpin (pre-miRNA) with a 5' monophosphate and

a ~2 nt overhang at the 3' end **(Figure 1)**. The pre-miRNA is then actively transported to the cytoplasm by Ran-GTP protein and members of the Exportin family (predominantly EXP-5). Once in the cytoplasm, the pre-miRNA is recognized by a second RNase III endonuclease called DICER that catalyses cleavage of the hairpin to produce a double stranded duplex approximately 22 nt in length, where both 3'ends display a ~2 nt overhang [10,25]. One strand of this miRNA duplex is then incorporated into the RNA-Induced Silencing Complex (RISC) through association with the AGO protein **(Figure 1)**. The miRNA then guides RISC to target messenger RNAs to elicit inhibition of translation, accelerated mRNA de-adenylation and/or endonucleolytic cleavage of the mRNA, depending on the degree of complementarity between the miRNA and its target [10,25]. In animals, miRNAs generally are not perfectly complementary to their targets and recognition is dominated by the "seed" site defined as nucleotides 2-7 in the 5' end of the miRNA.

miRNA discovery and evolution in filarial nematodes

A number of studies have now documented miRNAs in filarial nematodes as well as the related clade III nematodes *Ascaris suum* and *Ascaris lumbricoides* [26–28] **(Table 1)**. Poole et al. first reported miRNAs in the filarial nematode *Brugia malayi* **(Table 1)** [29] using bioinformatic predications as well as classical cloning from mixed life stages: adult males, gravid adult females and microfilariae (Mf). The authors identified 32 miRNAs including families well conserved in nematodes. A subsequent report by Winter et. al identified miRNAs in the genome of *B. pahangi*, reporting a total of 132 miRNA loci that encode 104 unique mature sequences, including 29 of the 32 miRNAs previously discovered in *B. malayi* [30]. Winter et al. carried out a side-by-side comparison of miRNAs sequenced from the clade V gastrointestinal parasite *Haemonchus contortus* and the clade III filarial parasite *B. pahangi* and were able to show that most of the miRNAs in each organism were not conserved in the other. Some of the newly evolved miRNAs were highly abundant and/or showed stage specific expression.

Beyond the studies with *Brugia spp*., miRNAs have also been identified in the dog heartworm parasite *Dirofilaria immitis* [31]. Here a total of 1063 miRNA candidates were identified by sequence alignment of mixed-adult stage libraries against the miRBase repository [32],

corresponding to 808 miRNA families [31]. While the large number of miRNAs reported here could reflect an expanded miRNA repertoire in this parasite, it also highlights the fact that different studies use different criteria for assigning a small RNA sequence as a miRNA. In the study by Winter et al., for example, the authors used both miReap and miRDeep prediction programs, but then further filtered the results manually with the requirement that both arms of the hairpin must be present in their datasets [30]. All of these factors, along with the depth of sequencing that is carried out, will affect the number and identity of miRNAs identified in different nematode species, in addition to the quality of the genomes available. This becomes an issue when trying to examine acquisitions and losses, as well as species-specificity of miRNAs for use in diagnostic applications (detailed further below).

While it is tempting to speculate that the evolution of miRNAs in filarial nematodes relates to parasitism, it should also be noted that a study comparing miRNAs in the free-living nematode *Pristionchus pacificus* to the *Caenorhabditis* spp. (clade V nematodes) also showed that the majority of miRNAs were not conserved [33]. Likewise, another study examining miRNAs in nematodes spanning clades I-V showed that at least 20% of *C. elegans* miRNAs were conserved. This work also demonstrated that homology inversely correlated with phylogenetic distance for both free-living and parasitic nematodes [16]. Consequently, it seems likely that different miRNAs follow diverse evolutionary trajectories linked to various aspects of nematode biology in both free-living and parasitic organisms. It is still challenging to pinpoint correlations between specific behavioral and physiological adaptations and the fluidity at which miRNA families are lost or gained. Gene duplication and "arm switching" (a process that leads to a switch in the arm from which the functional mature miRNA is derived), have been proposed as common mechanisms for the evolution of miRNAs and expansion of some miRNA family members [30,34].

Functional implications of stage-specific expression of miRNAs

A number of observations suggest that discrete miRNA subsets might be important regulators of processes in a particular life stage of filarial nematodes. For example, many of the miRNAs identified in *B. malayi* showed stage specific expression, including miR-2b and let-7 which are

abundant in adult stages [30]. Interestingly in a further study, Winter et al., demonstrated that one particular member of the let-7 family, miR-5364 is the most upregulated miRNA in the infective L3s (iL3s) during the vector-to-host transition (~12X compared to vector-derived L3s), as soon as 24h post-infection [35]. Analysis of the pre-miRNA sequence suggests that this let-7 family member is present in all clade III nematodes for which there is sequence information but is absent in other nematodes, including the Clade V nematodes *C. elegans, Heligmosomoides polygyrus* and *H. contortus.*

It has also been reported that miR-71 was one of the most abundant miRNAs in small RNA libraries prepared from total RNA from mixed adult worms in *D. immitis* [31]. A later study in *B. malayi* showed that miR-71 represents ~27% of the total miRNA reads identified in Mf datasets, and is 3-5X more enriched in Mf than adult worms [36]. It is possible that some of miR-71 detected in the study with *D. immitis* could potentially originate from Mf found in gravid female worms and not from adult worms *per se,* although this is still unclear. A recent report demonstrated the functional activity of miR-71 in developmentally competent *B. malayi* embryos using a luciferase reporter assay, concluding that miR-71 can act as a post-transcriptional repressor of mRNA targets in this life stage [37]. In *C. elegans*, miR-71 regulates longevity and life span [38], where it is upregulated in L1 diapause and dauer larvae but not particularly in other life stages [39].

It has been shown that filarial nematodes adjust their developmental schedule and fecundity in response to host-derived immunological factors [40]. This is indicative of different developmental trajectories depending upon environmental signals, a phenomenon referred to as phenotypic plasticity [41–44]. miRNAs, as well as other RNAi pathways, have been shown to control developmental choices and life history traits in post-dauer *C. elegans*, which shares behavioral and physiological traits with infective L3 larvae in parasitic nematodes [41,43,45–47]. Therefore, it is likely that the same mechanisms operate in filarial nematodes to control development and fertility in response to immunological cues from the host. A comparative analysis evaluating the RNAi landscape throughout filarial development in different environmental contexts will help to clarify if such molecular "switches" (discrete small RNA populations) could be the drivers or modulators of such morphological and developmental choices.

Endogenous small-interfering RNA (endo-siRNA) pathways in filarial nematodes

Most commonly, RNAi pathways in parasitic nematodes are discussed in relation to the ability to trigger an RNAi-mediated gene silencing response upon stimulation with exogenous (or environmental) double-stranded RNAs (exo-dsRNAs). This requires uptake of double-stranded RNA (dsRNA), processing this into primary siRNAs, amplification involving an RNA-dependent RNA polymerase to produce the secondary siRNAs, and ability to spread the signal, (reviewed in [48–51]). In many nematodes the absence of the dsRNA import protein SID-1 is thought to explain the lack of efficient RNAi carried out experimentally [52]. However endogenous pathways are expected to exist in these organisms where siRNAs are generated by a variety of mechanisms and these can have a variety of functions [50,53]. In *C. elegans*, two major categories of endo-siRNAs have been identified experimentally and bioinformatically: 22G-RNAs and 26G-RNAs, both displaying a strong bias for guanine at the 5' end. The endogenous 26G-RNAs are normally produced from mature mRNA transcripts by the action of the RNA-dependent RNA Polymerase (RdRP) RRF-3 [54]. These 26G-RNA precursors act as triggers for the production of a second class of 22G-RNAs that are synthesized *de novo* by RdRPs [54]. Other triggers such as piRNAs can also induce the *de novo* synthesis of 22G-siRNAs [53,54]. The function of the secondary siRNAs is dictated by the association with different types of AGO proteins. These have been shown to have multiple roles in *C.elegans* including chromosome segregation [18], genome defense, surveillance and integrity [13], as well as transgenerational epigenetic inheritance [55].

In *Ascaris*, it was shown that 26G-RNAs as well as 22G-RNAs were predominantly detected in the germline through to 128-cell embryos [28]. The majority of these endo-siRNAs mapped to a broad spectrum of coding genes in an antisense fashion [28]. On the other hand, a total of 40 repeat-associated siRNAs were identified in adult stages of *B. malayi* [29]. Similarly, several sense and antisense siRNAs were detected in the small RNA data from iL3s and mixed adult stages in *B. pahangi,* with at least eight sequences derived from repetitive element*s* [30]. A closer examination revealed that these sequences were mainly associated with retrotransposons and mapped to non-annotated repeats. Interestingly, a phylum-wide survey suggested that in clade III nematodes, the 22G-RNAs preferentially target antisense to predicted repetitive elements, and have been proposed as a mechanism to control transposon activity in the absence

of piRNAs [16]. Beyond genome defense, it is possible that endo-siRNAs might be involved in a wide range of biological processes in nematodes, including sophisticated (and potentially novel) gene silencing mechanisms as well as epigenetic regulation. Our understanding of these phenomena will be greatly enhanced with further studies of the post-transcriptional regulatory networks of different life stages across this clade.

Extracellular vesicles and extracellular RNA in filarial nematodes

It is now recognized that RNA molecules can also operate beyond the limits of the cell. One key feature of extracellular RNA (exRNA) is its remarkable stability in hostile environments such as human biofluids. Several studies have demonstrated that the stabilization of exRNA can occur through direct association with protein and lipid partners such as AGO complexes or LDH particles or encapsulation within extracellular vesicles (EVs), reviewed in [56–58]. EVs and exRNAs have been found in excretion/secretion (ES) products from a diverse range of parasites, from microbes to nematodes (reviewed in [11]). This has been suggested as an active exchange of genetic material that can mediate communication between organisms of the same species, or even between evolutionarily distant organisms [59,60].

Most of the literature detailing exRNA in helminths focuses on their encapsulation within EVs although the origins of these are not all well documented **(Figure 1)**. EVs that pellet upon ultracentrifugation can derive from the endocytic pathway (termed exosomes) or from budding off the plasma membrane (often termed microvesicles) and these can be difficult to distinguish by their sizes: exosomes are generally 40-100 nm and microvesicles can range from 100-1000nm. These can also be difficult to distinguish based on their protein content, for example recent research with mammalian EVs has demonstrated that proteins previously referred to as "canonical exosomal markers" (MHC I and II, flotilins, actin or Heat-shock proteins 70, among others [57,58]) can be detected in other classes of EV. The authors further showed that even within small EVs of the same density and size there were multiple categories that could be distinguished by displaying different combinations of protein markers [61]. It seems likely that such heterogeneity exists in parasite EVs, an area that remains largely unexplored, which could be key to understanding the diversity of their functions [11].

Initial reports in the trematodes *Echinostoma caproni* and *Fasciola hepatica* suggested that EVs (30-100 nm) could derive from tegumental structures and could be a mechanism for transferring material to host cells [62,63]. EVs with similar sizes have also been characterized in the human pathogenic trematodes *Schistosoma mansoni* [64] and *Schistosoma japonicum* [65], the carcinogenic liver fluke *Opisthorchis viverrini* [66], the clade V gastrointestinal nematode *Teladorsagia circumcincta* [67], and the clade I whipworm *Trichuris suis* [68]. In our own work we showed that the clade V gastrointestinal parasitic nematode *H. polygyrus* secretes EVs that are enriched in proteins known to be abundant in the intestinal tissue of the parasite as well proteins associated with exosome biogenesis (e.g. Alix) [69]. In the context of filarial infections, a recent report focusing on *B. malayi* showed that both iL3s and gravid adult females secreted EVs *in vitro* [70]. The EVs detected in excretion/secretion (ES) products from iL3s were described as homogeneous, based on size, ranging between 50 -120 nm. Proteomic analysis of the iL3s revealed an enrichment for several proteins previously termed exosome markers, including HSP70 and Rab-1 [70].

Nematode-derived miRNAs were identified in both *H. polygyrus* and *B. malayi* EVs with some overlap in those that were found including miR-71 and members of the let-7 and miR-100 families. Both studies in *H. polygyrus* [69] and *B. malayi* [70] showed that the secreted RNA population is distinct from the RNA isolated from the total worm. Similar observations were made when comparing the RNA from EVs and worms in *F. hepatica* [71]. While this suggests distinct miRNAs are secreted, it does not inform on whether this subset is selectively exported from the cell from which it derives. Some mechanisms for selective sorting of miRNAs into EVs have been described in mammalian systems, involving RNA-binding proteins [72,73]. The presence of ribosomal proteins in the EV's secreted by *B. malayi* iL3s was also noted [70], though it is not known if these were associated with rRNA fragments that were also found. It is unclear whether or how different RNA processing pathways converge with EV biogenesis and secretion. In some systems, components of the RISC complex have been detected in EVs or shown to co-migrate with endosomal MVB fractions in density gradients [74]. Interestingly, one AGO protein was also identified in both vesicle and vesicle-depleted fractions from *H. polygyrus in vitro* [69], although the mechanistic aspects associated with secretion of AGO proteins in nematodes or others parasites are unknown.

Regulation and plasticity of EV secretion?

The population of EVs detected in ES products seems to be variable between life stages, with reduced content observed in *B. malayi* gravid adult females compared to iL3s [70]. Interestingly, the EV release rate from iL3s was reduced by ~2 fold between 24h and 72h (estimated as the amount of particles released by parasite over time), and this is thought to be associated with worm viability in the culture conditions tested. Indeed, one outstanding question in the field is whether exRNA and EVs could derive from dead or moribund worms. It is intriguing to think of vesicle secretion as a regulated mechanism involving specialized tissues and/or organs in the nematode, whereby release could occur in response to environmental conditions, vector-to-host transition, activation of specific receptors stimulated by host hormones, etc. However, there is very little evidence at present to support this, in part because of the youth of this field. It was proposed by Zamanian et al. that the release of EVs could be a phenotype restricted to larval stages and might be involved in invasion during the onset of infection by modulating immune responses in the host [70]. Given the precedent for immune-modulation by parasite EVs [11,69,75], it does seem likely that their release would be subject to control, possibly by both parasite and host. It is also possible that properties of the EVs and their cargo can change throughout filarial development and in response to particular environmental challenges and/or cues. In the context of filarial parasites for example, iL3s could release exRNA-loaded EVs aiming to ensure successful migration through an active interaction with cells at the site of the infection and/or evasion of early innate immune cells. Similarly, gravid adult females could release exRNAs involved in down-regulation of immune response against Mf, thus ensuring their survival. Although exciting, the hypothesis of "plasticity" in the exRNA signals and EVs secreted by different life stages in filarial nematodes remains unstudied.

Extracellular small RNAs as biomarkers for filariasis – towards diagnostic applications

One potential application of these parasite-derived exRNAs is in the area of biomarkers for helminthiases. This is based on a key observation that parasite-derived exRNAs can be detected in biofluids from their hosts as demonstrated by small RNA sequencing and qRT-PCR. This was first documented in schistosomiasis [76–78], but has also been examined by multiple groups in

the context of filarial infections [79–81]. In an initial report, Tritten et al. documented a total of 245 miRNA candidates of potential nematode origin in the plasma of dogs infected with the heartworm *D. immitis* based on sequencing [79]. In a subsequent report they documented a total of 22 unique sequences derived from *L. loa* in human serum and 10 sequences derived from *O. ochengi* in infected cattle serum [80]. We have also identified 62 *O. ochengi*-derived miRNAs in onchocercomata fluid [81] and found a total of 16 *L. sigmodontis*-derived miRNAs in the serum of mice during the patent stage of the infection [69]. Common to all of these studies was the presence of extracellular miR-71 and miR-100 family members. Further comparisons are challenging due to differences in the technical methods and analysis reported from the studies, which use different cut-offs for defining a candidate miRNA and are carried out at different depths of coverage. Identification of nematode-derived small RNAs in host fluids is also challenging given the dominance of host-derived sequences in these samples, and it may be appropriate to remove all sequences that could derive from the host prior to assignment of these as parasitic in origin.

Whilst it might be expected that all nematode parasites can or do release exRNA and EVs, there are a number of factors that will influence the ability to detect these molecules in different host fluids. It is logical that the localization of the parasite within the host dictates the presence of parasite-derived exRNAs in different biofluids. In line with this, nematode miRNAs could be identified in the serum of mice infected with *L. sigmodontis*, but not in serum from mice infected with *H. polygyrus* (which resides in the small intestine) in a side-by-side comparison [69]. The close contact between some filarial nematodes and the lymphatic system (*Wuchereria bancrofti*, *L. loa*, *Brugia spp.*) could account for a widespread distribution of secreted parasite products into the bloodstream, such that they are readily detectable in serum and plasma (and perhaps urine) (**Figure 2**). On the other hand, the presence of a nodular structure in onchocerciasis may impose a physical barrier for the trafficking of locally secreted parasite products to the bloodstream. However, the presence of a vascularized system surrounding the onchocercomata nodule may be seen as a "window" for dissemination of such products [82] (**Figure 3**). The mechanisms by which parasite EVs and exRNAs can bypass physical barriers (for example, those imposed by the nodular structure in *Onchocerca spp.*) and reach the bloodstream are not well understood yet but could help inform to what extent these molecules can be effectively used as biomarkers for different filarial infections.

The potential species-specificity of some miRNAs makes them attractive candidates as diagnostics where co-infection is an issue, for example in distinguishing *O. volvulus* and *L. loa* in co-endemic communities. A pan-filarial small RNA-based biomarker could also be useful as a point-of-care diagnostic test aiming to monitor populations subjected to mass drug administration (MDA) or for elimination programs. Studies conducted in biofluids from several filarial infections suggest that, for instance, miR-71 can be used as a biomarker for filarial infection [79–81]. However, it is expected that several technological approaches will be considered in order to improve not only the platforms currently available for exRNA detection (reviewed in [83,84]) but also the way in which these technologies can be transferred in a field-friendly manner. Advancing inexpensive technologies and streamlined purification protocols will certainly increase the likelihood of adopting small-RNA based biomarkers in the field.

Final considerations & outstanding questions

The field of EVs and small RNAs in parasitic nematodes is in its infancy and rapidly growing alongside efforts to exploit these in therapeutic and diagnostic applications. From a biological perspective, several outstanding questions should be addressed in order to drive this field forward. It is still unknown if the secretion of exRNA-loaded EVs is developmentally regulated in parasitic nematodes, whether there are mechanisms to sort and package different small RNAs into EVs and if all the different types of exRNAs reported so far in ES products play a role in parasite-to-host communication. If there are mechanisms in place in the parasitic nematodes to control EV secretion and dictate the cargo that is exported, then it is plausible that these mechanisms might have evolved as an additional axis of adaptation of the parasites to regulate their hosts' immune system [85].

If this is the case, several aspects need to be considered. First, if the parasites effectively use EVs and small RNAs as a mechanism for invasion, colonization and immune evasion, then one possibility is that these functions are specifically compartmentalized within the parasites. We therefore should expect that certain tissues or organs be directly involved in their production and secretion/excretion; for example, those with glandular functions such as the pharynx or cells producing ES products. Building from this idea, we could also propose that the profile and

exRNA content of EVs, as well as the diversity of exRNAs, will be different between life stages, as a result of their development. For example, gravid adult females could actively secrete a plethora of exRNAs and EVs, which, together with other soluble proteins in the ES products, aim to maintain a downregulated immune status in the host in order to aid in survival not only of the adults but of the Mf as well, a life stage that is particularly less complex. This interplay with the host could begin early in the infection, and would be maintained throughout. The idea of "maternally-mediated" Mf survival is exciting as it offers a new possibility for treatment and intervention. However, the ideas proposed so far remain speculative and will require further analysis.

Although less explored in helminth infection, exRNAs and EVs could also have functions as mediators of parasite-to-parasite cross talk. Studies in the protozoan *P. falciparum* showed that EVs can be involved in communication between parasite populations as well as with the host [86]. Other organisms such as fungi and bacteria, which are typically found in the normal microbiota in the host, also secrete EVs. EVs may not only be involved in communication with the vertebrate host but also could be relevant for the establishment of relevant ecological interactions between pathogens that might co-exist in natural conditions; e.g. in situations where multiple infections occur at the same time [87]. For example, it remains possible that exRNAs and EVs could be involved in: *a*) intraspecies communication, e.g. chemoattractant derived from female worms to increase adult male motility or fertility, maternally derived pro-survival signals to increase Mf survival or *b*) interspecies communication, e.g. *Wolbachia*-derived EVs that confer nutritional advantages to the filarial host [88–90], modulation of the host's immune response by filarial-derived EVs [70].

Although still far off, further work in this area could contribute to the development of novel technological applications to diagnose and control filarial infections (reviewed in [91]). Basic research in this area also offers new scenarios to understand the real complexity that exists in the interaction between organisms at a molecular level. This is useful for understanding how parasitic nematodes can manipulate the gene expression machinery in their host for their own benefit, or the molecular basis of the mutualistic interactions between endosymbionts, e.g. *Wolbachia* and their nematode hosts. An exciting and intriguing avenue is the possibility of merging genome editing and functional genomic tools (reviewed in [92,93]) to engineer specific

EVs cargos. These "tailored" EVs could be used as vehicles to further our understanding on how multiple organisms use these extracellular systems to transfer information and to maintain a dialogue with their surroundings. Towards this goal, further work will be required to improve the genetic manipulation toolkit currently available for filarial nematodes and to advance the basic research on EV and exRNA secretion and function in these parasites.

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Figures & Table legends

Figure 1: Simplified schematic of biogenesis and potential export pathways of microRNAs. MiRNAs are produced from primary miRNA transcripts that are processed by the microprocessor in the nucleus and exported to the cytoplasm where they are further processed by Dicer to produce a 22 nt duplex RNA. One strand of the duplex (the mature miRNA) is loaded onto an AGO protein and guides the RISC complex to mediate control of gene expression by translational repression or accelerated miRNA decay (**A**). The microRNA can also be exported out of the cell, either in association with AGO or in another form (the uncertainty is depicted

with a question mark), through directly fusing with components of the plasma membrane into EVs termed microvesicles (**B**), or through incorporation into the exosomal biogenesis pathway into multivesicular bodies (MVBs) (**C**).

Figure 2: Proposed routes of EV secretion *in vivo* in lymphatic filariasis. Depiction of filarial nematodes (for example *Brugia* spp.) residing within a lymph node. A hypothesis is that the lack of a nodular structure (as observed in infection from *Onchocerca spp*) might facilitate the accessibility of EVs into the circulation**.** It is also unclear whether the detection of EVs and small RNAs is exclusively associated with viable worms or can be also derived from moribund or dead worms

Figure 3: Proposed routes of EV secretion *in vivo* in Onchocerciasis. Depiction of a nodule-forming species member of the *Onchocerca* genus (for example, *O. volvulus* or *O. ochengi*) residing within a nodular structure termed an onchocercoma. It is not yet clear whether or how the nodular structure imposes a physical barrier for dissemination of small RNA-loaded EVs into the bloodstream. As in Figure 2, it is unclear whether the detection of EVs and small RNAs is exclusively associated with viable worms or can be also derived from moribund or dead worms

Table 1: miRNA identification in clade III nematodes

Clade III nematode	Life stage(s)	Method	Depth of coverage	miRNA diversity	Reference
Filarial nematodes					
B. malayi	Mixed AM, gAF & Mf	RNA 5' ligation independent protocol + RT-PCR/ Capillary Sequencing	503 inserts cloned	32 miRNAs	[29]
B. pahangi	iL3s & mixed AM & gAF	Small RNA library prep kit / Illumina Sequencing platform	~13 million reads for *B. pahangi* iL3s / ~13 million reads for *B. pahangi* mixed adults	125 precursor sequences that produce 99 mature miRNAs and 81 unique star sequences	[30]
B. malayi	AM, gAF & Mf	RNA 5' ligation-dependent protocol + RT-PCR / Illumina Sequencing platform	~3.5 to 3.7 million reads in adult stages and Mf (8 to 10 million reads in Mf libraries with alternative treatments)	129 precursor sequences that produce 145 mature miRNAs	[36]
D. immitis	Mixed AM & gAF	RNA di-tagging + by RT-PCR / Solexa Sequencing platform	9.8 million reads	1063 miRNA candidates	[31]
Ascaris genus					
A. suum	Embryonic stages & early development	RNA di-tagging approach+ RT-PCR / Illumina Sequencing platform	~12 to 18 million reads for early stages / ~70 to 350 million reads in larval stages	97 miRNAs grouped into 59 *Ascaris* seed families.	[28]
A. suum	AM & gAF	RNA di-tagged approach / Solexa sequencing platform	11.7 million reads for each life stage	494 and 505 miRNA candidates in gAF and AM, respectively	[26]
A. suum, A. lumbricoides	gAF	Illumina small RNA library prep kit / Solexa Sequencing platform	14.69 and 9.76 million reads in *A. lumbricoides* and *A. suum* libraries, respectively	494 miRNA candidates in *A. suum*; 171 miRNA candidates in *A. lumbricoides*	[27]

iL3s = infective L3s, AM = adult male, gAF = gravid adult female, Mf = microfilariae

Table 2: Extracellular filarial-derived miRNAs reported *in vitro* and *in vivo*

Clade III nematode	Host	Sample type	Depth of coverage (Total parasite-specific RNA reads)	miRNA diversity	Reference
In vitro					
***B. malayi* (iL3s + adult males and females)**	iL3s derived from *A. aegypti* / Adult worms obtained from NIAID-NIH/FR3	Excretion/Secretion (ES) products	11,139 *B. malayi* reads in EV's (~2% of total reads) / 1,519,403 *B. malayi* reads in iL3s (~50% of total reads)	52 miRNAs detected in iL3s-derived EVs	[70]
In vivo					
L. sigmodontis	*BALB/c* mice	Serum (d60p.i. – Patent infection)	1,188 *L. sigmodontis* reads (~1.5% of total reads)	16 *L. sigmodontis*-miRNAs in mouse serum	[69]
D. immitis	Dog	Plasma	418,415 *D. immitis* reads (~2.7% of total reads)	245 *D. immitis* miRNAs in dog plasma	[79]
O. volvulus	Human	Serum	~2,180 *O. volvulus* reads (0.1% of total reads)	21 *O. volvulus* miRNAs in Human serum	
L. loa	Baboon	Plasma	Unknown	22 *L. loa* miRNAs in baboon plasma	[80]
O. ochengi	Cattle	Plasma	Unknown	10 *O. ochengi* miRNAs in bovine serum	
O. ochengi	Cattle	Nodular fluid	157,633 *O. ochengi* reads (~1.1% of total reads)	62 *Onchocerca* miRNAs in onchocercoma fluids	[81]
O. volvulus	Humans	Serum/plasma	~108,323 and 355,397 *O. volvulus* reads in two separate libraries (~1.1 and 1.5% of total reads)	6 *Onchocerca* miRNAs in human serum/plasma