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Mining filarial genomes for diagnostic and therapeutic targets

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Abstract

Filarial infections of humans cause some of the most important neglected tropical diseases. The global efforts for eliminating filarial infections by mass drug administration programs may require additional tools (safe macrofilaricidal drugs, vaccines and diagnostic biomarkers). The accurate and sensitive detection of viable parasites is essential for diagnosis and for surveillance programs. Current community-wide treatment modalities do not kill the adult filarial worms effectively; hence, there is a need to identify and develop safe macrofilaricidal drugs. High-throughput sequencing, mass spectroscopy methods and advances in computational biology have greatly accelerated the discovery process. Here, we describe post-genomic developments toward the identification of diagnostic biomarkers and drug targets for the filarial infection of humans.

Recent advances in filarial infections

Parasitic nematodes have a significant impact on human and animal health caused primarily by filarial worms, the common roundworm, hookworms, whipworms and others [1]. Among the eight filarial infections of humans, those caused by *Brugia malayi*, *Wuchereria bancrofti*, *Loa loa* and *Onchocerca volvulus* are responsible for most of the filarial disease burden. The life cycles of all of the filarial parasites are similar, and each involves an intermediate vector host and long-lived adult parasites, that, depending on the species involved, reside in connective tissues (*O. volvulus*), the lymphatics (*Brugia* spp, *W. bancrofti*) or subcutaneous tissues (*L. loa*). These adults release microfilariae that circulate in the peripheral circulation (most species) or in the dermal or ocular tissues (*O. volvulus*) (Figure 1). The human filarial worms (except *L. loa* and some species of *Mansonella*) harbor an endosymbiotic bacterium *Wolbachia* that can affect the viability and sterility of the worms [2].

During the past decade, advances in sequencing technology, mass spectroscopy as well bioinformatic capabilities have led to a major increase in the number of helminth genomes available. High quality reference genomes provide a platform to investigate the underlying biological makeup and processes by enabling transcriptomics, proteomics, glycomics, metabolomics and other specialized ‘omic’ technologies (Table 1).

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While the current anti-filarial drugs have variable efficacy against the microfilariae (ivermectin (IVM) and diethylcarbamazine (DEC)) or against the adults (albendazole (ALB), DEC, and doxycycline), depending on their mode of action and stage-specificity, they must often be administered once or twice annually for up to 20 years for ivermectin-based MDA for onchocerciasis and for 5-8 years for the standard two-drug regimen of ALB/DEC or IVM/ALB used for lymphatic filariasis elimination programs [3, 4]. Moreover, the development of drug resistance [5, 6] or the induction of severe adverse events following treatment [7-9], or contraindications for DEC in areas endemic for onchocerciasis drives the need for the identification and development of alternative therapeutics, along with sensitive diagnostic tools to assess for efficacy of these alternative treatment strategies [10].

Early genomic studies of the filarial parasites used expressed sequence tags (ESTs) derived from stage-specific cDNA libraries to drive the filarial genome project [11]. Following the elucidation of the first filarial genome (*B. malayi*) [12], the draft genomes of the human filarial parasites *L. loa* [13, 14], *W. bancrofti* [13], *O. volvulus* [15] and the non-human filarial parasites *Brugia pahangi* [16], *Dirofilaria immitis* [17] and *Onchocerca ochengi* [15] became available. Recent reviews highlight the advances and insights gained from the comparative analyses of the genomes, transcriptomes and proteomes of these filarial nematodes [18, 19].

Although the genomic revolution has been touted as a means of driving vaccine target discovery for filarial parasites, to date, there is currently no vaccine against any of the filarial infections [20], although a number of potential vaccine candidates have been identified for *O. volvulus* using an “immunonomic” approach [21] or immunoinformatics [22]. Here, we review the implications of the post-genomic developments towards the identification of novel diagnostics and additional drug targets in human filarial infections.

Diagnostic toolbox

The diagnostic toolbox for filarial infections is fairly limited and, until nucleic acid detection and recombinant antigen production became possible, largely relied on classic parasitological methods [23] or serology based on the use of crude parasite extracts [24]. With the advent of recombinant antigen production, polymerase chain reaction (and other methods of nucleic acid detection), based mainly on pre-genomic information [25, 26], sensitive and species-specific diagnostics became available [27-34].

Post-Genomic Protein Biomarker Discovery

When the draft genomes of *B. malayi, W. bancrofti, O. volvulus* and *L. loa* became available, they were used quite successfully to identify species-specific antigens [12-15]. An example of this was the identification of Wb123 as a marker of early *W. bancrofti* infection [35] that was subsequently used for surveillance efforts following mass drug administration (MDA) [36-43]. Nevertheless, there remained a need for new tools with even greater specificity and sensitivity, especially in areas where multiple filarial species were co-endemic.

Having the genome of *L. loa* allowed for comparative proteomic analysis of both urine and sera from *Loa*-infected individuals that resulted in the identification of novel proteins (antigens) that could be detected in infected individuals. Among several *Loa*-derived proteins detected using mass spectrometry and genome-based identification in body fluids of infected patients, we were able to identify LOAG_16297 as a potential biomarker for *L. loa* microfilarial loads in infected individuals [44]. Likewise, the use of microfilarial RNA-seq data and comparative bioinformatics led to the identification of LOAG_14221 as another quantitative biomarker for *Loa*-microfilarial density [45].

The genomes of filarial parasites exhibit a range (12,000 – 15,000) of protein-coding genes that can also be found in multiple proteoforms as a result of polymorphisms, post-translational modifications and alternative splicing events [13, 15, 46, 47]. For most genomes (including the filarial genomes), there is a large proportion of uncharacterized or hypothetical genes that have orthologues in other related species. Clearly, most of these hypothetical proteins, whether conserved or unique, are detectable as proteins [21, 48-52] and could be useful biomarkers of infection [21]. The proteins (in the thousands) can be gridded on to high-density arrays. High-throughput protein microarrays, though currently quite expensive, have become a valuable tool in translational research towards discovering and validating potential biomarkers and identifying vaccine candidates. The availability of different protein array formats (protein fragment, peptide, full-length) provides platforms for different queries [53]. For example, the sero-reactivity to a peptide (on a peptide-array) can be MHC- restricted and thus not be recognized by antibody, whereas the full-length protein containing multiple B cell epitopes would be more suitable for protein arrays.

Post-Genomic Nucleotide-based Biomarkers

Nucleic acid-based tools often offer better sensitivity and specificity than parasitological or serological methods. In the peri-genomic era, these nucleic acid-based tools were identified empirically [54-56], and were primarily based on the use of interspersed repeats and internal transcribed spacer sequences [57-62] as genetic markers. Strategies have been designed to utilize qPCR and loop-mediated isothermal amplification (LAMP) methods for the detection of parasite DNA [25]. However, they are not yet applicable in the field and often not deployable in low-resource settings. Isothermal amplification methods circumvent the need for expensive and bulky instrumentation, requiring a simple heat block or water bath. Among the various isothermal-based technologies, loop-mediated isothermal amplification (LAMP) methods have been reported for a variety of filariae in humans [63-67] or in the vectors [66, 68, 69].

The nucleic-acid based tools in the post-genomic era have been largely focused on small RNAs [70, 71]. Among the various small RNAs, microRNAs (miRNA) have been garnering increasing interest not only as important regulators of nematode development but also as diagnostic markers of infection [72-77]. The detection of miRNAs and their potential for use as biomarkers is influenced by: i) sensitivities in depth of coverage, ii) miRNA identification methodology employed, iii) niche of the parasite (blood *versus* other tissues), and iv) the nature of the miRNA (conserved or unique) itself. For example, while the detection of heartworm specific miRNAs in *Dirofilaria*-infected (but not from uninfected) dogs

demonstrated the utility and specificity of miRNAs [74], the detection of conserved miRNAs in the serum of *O. volvulus*-infected individuals [73] was not uniform across populations from differing geographic locations [75, 76].

A recent study [78] points out the feasibility and diagnostic power of using plasma-derived miRNA as diagnostic biomarkers. Among the suggested ways to optimize the detection of miRNAs were to use: a) a higher volume of plasma; b) a pre-amplification step to boost sensitivity; c) miRNAs from fractions enriched in exosomes. While each of these options are likely to enhance detection, the practical feasibility of handling larger volumes of plasma and/or purification of exosomes in the field or even in a centralized regional laboratory need to be considered. This is especially important because small amounts of parasite-derived miRNAs were detected in plasma in individuals infected with the skin-tissue dwelling *O. volvulus,* when compared to individuals infected with blood-tissue dwelling *L. loa, W. bancrofti* or *B. malayi* [74]. Nevertheless, adopting small RNA-based biomarkers for inexpensive assays for applications in the field or at point-of-care still requires technological advancements.

Post-Genomic Metabolite-based Biomarkers

Prior to the genomic era, metabolite mapping was used to understand the composition of the filarial worms [79]. Metabolic reconstructions at the genome-level utilizing flux balance analysis (FBA) of the genomes of *B. malayi*, *L. loa*, *O. volvulus* and the relative contribution of *Wolbachia* highlighted potential metabolic chokepoints [15, 80]. Metabolomic approaches identified urine-derived N-acetyltyramine-O-b-glucuronide (NATOG) as a potential biomarker for *O. volvulus* [81], though the utility of NATOG measurements is somewhat controversial [82, 83].

Using a slightly different approach, metabolite profiling of *O. volvulus*-infected individuals resulted in the identification of many unknown metabolites as potential biomarkers of infection [84]. Because metabolite profiling of the parasites is largely limited by availability of live worms, it would be important to leverage the metabolic composition identified empirically to fill the holes in the metabolic maps based on genomic information. Furthermore, because filarial worms source their nutrients from their host(s) or from the *Wolbachia* endosymbiont, the significantly depleted levels of metabolites in the infected individuals suggests the likely dependence of the filarial worms on specific metabolites [84].

Therapeutic targets

Current control measures, as part of the MDA campaigns, are largely based on annual or bi-annual distribution of ivermectin (IVM) (onchocerciasis endemic areas) or diethylcarbamazine citrate (DEC; areas non-endemic of onchocerciasis) either alone or in combination with albendazole. At the individual patient level, a number of drugs, including DEC, albendazole, ivermectin, and doxycycline, are in use and have demonstrated efficacy against a number of the filarial infections [85, 86]. However, there have been substantial efforts in the past 10 years to identify more effective macrofilaricides [87]. Although biochemical extracts from medicinal plants have been explored for bioactive compounds

with filaricidal activity [88], current efforts are largely focused on repurposing approved drugs that could accelerate more effective therapies.

Post-Genomic Drug Targets

Comparative genomics of the parasite(s) and human host provide a dataset of potential targets. The process of comparing the genomes and their inferred biochemical pathways yields features that are evolutionarily conserved and features that are divergent between organisms. Currently, considering the logistics and length of time between drug discovery and market introduction, the use of existing natural, synthetic or semi-synthetic compounds with known target and safety profiles are being identified and explored as potential alternatives. When targeting metabolic networks present in both the parasite and the host, selectivity for the parasite is the most important property.

Wolbachia appear to be essential for the development and survival of the filarial parasites that harbor them. Depletion of *Wolbachia* by antibiotics (e.g., the tetracyclines) disrupts embryogenesis, microfilariae (mf) development and worm survival [2], but, despite the loss of *Wolbachia*, filarial worms can survive for long periods of time [89]. This longevity, the length of time needed for antibiotic treatment (4-6 weeks) and contraindications in children under 8 years of age and in pregnant women, has led to the search for novel anti-*Wolbachia* agents [90]. Repurposing drugs such as minocycline [91] and rifampicin [92] as anti-*Wolbachia* therapy could potentially reduce the treatment duration from 6 weeks to 1-2 weeks, allowing for more ease in delivering these regimens on a community-wide basis.

Post-Genomic Drug Targets: High-Throughput Screens

Using a yeast-based model, screening of 400 drugs available from the Malaria Box project [93] yielded filarial-specific active compounds that affected adult females of *B. pahangi* (used as surrogate for human infections) *in vitro* and did not affect the corresponding human homologues [94]. Repurposing of approved drugs also led to the identification of auranofin, approved for the treatment of rheumatoid arthritis, as a lead candidate for treating lymphatic filariasis and onchocerciasis, whose likely target is thioredoxin reductase (TrxR) [95]. It is likely that auranofin treatment renders the parasite susceptible to oxidative damage allowing for subsequent clearance of the parasite by the host immune system. Likewise, screening for developmental inhibitors of filarial parasites identified closantel, a known anthelmintic drug that acts like a proton ionophore, and targets L3-expressed chitinase [96, 97]. Further, proteomic studies resulted in the identification of 62 gender-associated proteins expressed during embryogenesis or spermatogenesis [98]. Most of these gender-associated proteins have homologues in *C. elegans* with severe RNAi phenotypes and, hence, might be targets for new drugs or vaccines. High-throughput drug screening for repurposing approved drugs targeting *Wolbachia* yielded effective compounds from the tetracycline, fluoroquinolone and rifamycin classes [99].

Post-Genomic Drug Targets: Genome-wide Screens

Predictive genome-wide screening of drugs approved for human use with the potential use as anthelmintics led to the identification of 16 *O. volvulus* proteins (predominantly enzymes and proteins involved in ion transport and neurotransmission) as likely targets [15].

Information gained from the filarial genome projects identified other protein kinases in the filarial genomes —EGFR, Src, Raf/Raf, FRAP and AGC/DMPK/ROCK —for which there are orally available small molecule inhibitors on the commercial market [13]. Aside from kinases, other targets found based on genome mining of the *O. volvulus* genome, that link to FDA-approved drugs with likely activity include well-known and commonly used drugs such as metformin, baclofen, acetaminophen, and sertraline [15].

Proteins that are unique to nematodes and sufficiently distinct from homologous human proteins are particularly attractive as drug targets. Comparison of filarial and non-filarial nematodes revealed the presence of filarial-nematode specific kinases which could be targeted using existing approved drugs for human use. Of the 205 conventional and 10 atypical protein kinases encoded in the *B. malayi* genome, 142 are deemed essential based on the RNAi phenotype in *C. elegans* [12]. Similarly, 168 kinases of *O. volvulus* have no significant human matches [15]. One of the post-genomic insights from *L. loa* genome was the existence of a tyrosine kinase c-Abl like protein in filarial worms. Moving from a theoretical basis, the applicability of repurposing drugs based on *L. loa* genome, was tested with the tyrosine kinase inhibitors (TKI) imatinib, nilotinib and dasatinib [100]. The TKIs were able to effectively affect all life-stages of *B. malayi* at concentrations that are physiologically achievable. Given the structural similarity of these c-Abl like proteins in the filarial nematodes, and the conserved binding site of the TKI, it is likely to be successful in targeting the parasites that cause lymphatic filariasis, loiasis and onchocerciasis [100]. Because the expression of c-abl-like protein localized predominantly to the reproductive organs, muscle and intestine of the adult *B. malayi* worms, an effort to perform clinical trials in humans (e.g., NCT02644525) with drugs such as these TKIs that are effective as both micro- and macrofilaricides might greatly shorten the length of MDA treatment and significantly boost elimination efforts [101].

The availability of stage-specific transcriptional and proteomic data, coupled to comparative genomics identified potential genes and pathways of *Wolbachia* that can be targeted. This focus has largely arisen from work on the metabolic pathways of the parasites [102] that are perceived to be complemented by the *Wolbachia*-containing filarial parasite and its endosymbiont [15]. However, given that the genetic makeup of *L. loa* (filarial parasite devoid of *Wolbachia*) is the same as other filarial worms, the nature of the metabolic provisioning in the symbiotic relationship remains enigmatic [13]. Further, the observed heterogeneity and extreme variations in *Wolbachia* copy numbers within and between populations in *O. volvulus* raises the issue of how metabolic provisioning by *Wolbachia* is balanced, and a complicating factor in finding an efficacious antibiotic for MDA programs [103]. Another genome-based approach relies on the prediction of essential *Wolbachia* genes that have experimental evidence or verified genes in other bacterial taxa and phylogenetically conserved in Rickettsiales [104]. Incidentally, many of the potential inhibitors identified by the A-WOL screens target these essential genes/gene products [105].

From the endosymbiont perspective, Filobase [106] — a database derived from post-genomic insights from *B. malayi* and its *Wolbachia* endosymbiont (*w*Bm) using a combination of bioinformatic tools, pathway analyses and data mining -- provides a list of essential proteins that could be potential drug-targets for the parasites that cause lymphatic

filariasis. This approach, however, requires *de novo* drug development, a time consuming and costly endeavor with uncertainty about safety.

Based on a curated set of 'essential genes' across bacteria, archaea and eukaryotes [107] and sequence homology to humans, a subset of *B. malayi* predicted genes have been postulated as potential drug targets [108]. This strategy is, however, limited to genes that exhibit sequence homology with genes of known functional characteristics; they are not applicable to the gene families restricted to filarial parasites that are probably essential [21, 46, 48].

Concluding Remarks

Comparative genomic and post-genomic investigations have provided a vast amount of molecular information. Through these studies, sets of genes, proteins or critical pathways have been identified and can be further exploited to develop improved diagnostic and therapeutic tools. Moreover, the availability of stage-specific expression data allows for the identification of parasite-derived biomarkers that can reflect different periods of parasite development. These in turn can be exploited for various point-of-care tools that may be needed for support of intervention strategies aimed at control and elimination of filarial infections. For example, we now have the ability to identify markers of: 1) early infection or of recrudescence (L3-L4 based); 2) active patent infection (adults and microfilariae based); or 3) ongoing transmission (vector stages). Whatever the approach, good point-of-care tools are needed for aiding and evaluating the priorities towards intervention strategies aimed at control and elimination of filarial infections [109, 110].

Rational design-based approaches toward the identification of biomarkers and toward the development of rapid format assays is now of primary importance if the goals of the MDA programs are to be achieved. Moreover, it is important to have the political will to provide to the global health community the resources needed for testing and validating these biomarkers and therapeutic targets along with models that can be used to predict drug efficacy in human infection (see Outstanding Questions).

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Trends

- Current diagnostic methods are not sufficient to reliably detect active filarial infection once transmission is controlled.
- Accurate detection of viable filarial parasites requires diagnostic tools with better specificity and sensitivity to detect low to very low-level infections.
- Diagnostic tools should preferentially be point-of-care, be able to distinguish among related filarial species or detect the presence of multiple infections.
- Current treatment strategies are not adequately effective against the adult filarial worms. Triple drug therapy looks promising.
- Identification and development of novel and safe macrofilaricides is largely focused on repurposing existing approved drugs and those that target metabolic chokepoint reactions.

Outstanding Questions

- What is the in vivo efficacy of the FDA-approved drugs identified through post-genomic approaches in human filarial infection?
- Can POC tests for filarial infections be made more sensitive and specific?
- Can we develop an animal model to test drug efficacy reflective of human responses?
- Can there be community wide resources such as:
    1. banked material that includes sera/plasma/urine from filarial-infected individuals pre- and post-treatment
    2. parasite material for genomics/transcriptomics for population biology and drug resistance studies
    3. arrayed proteins or glycans or metabolites for screening for discovery and validation of promising biomarkers?

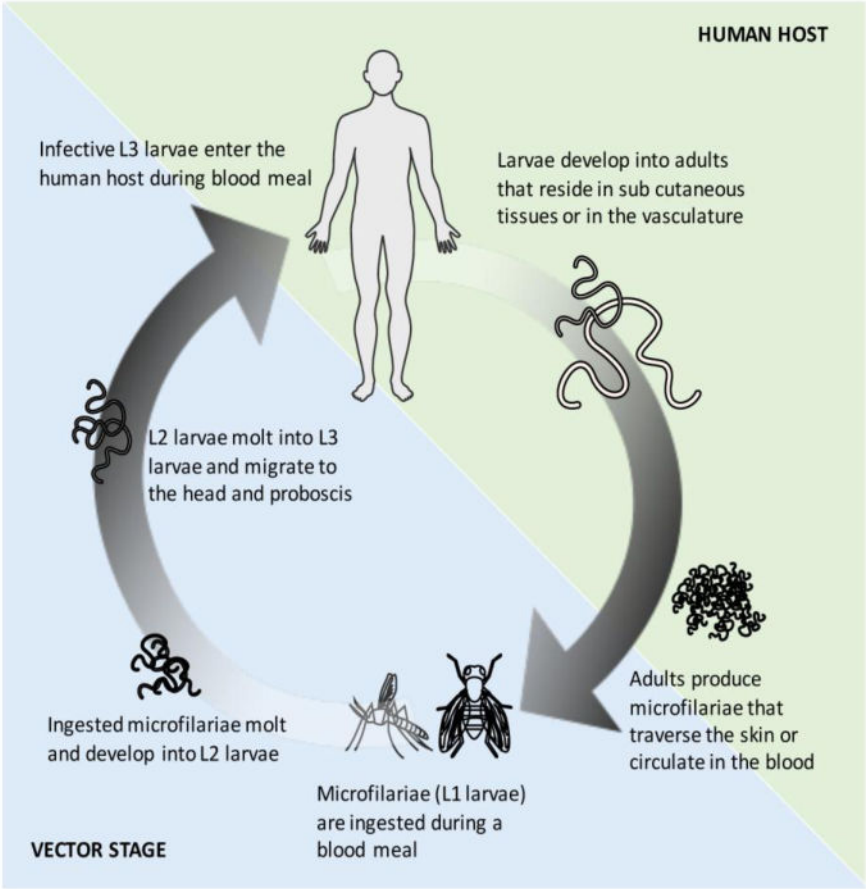

Figure 1. Life cycle of filarial parasites

The human filarial parasites are digenetic with a primary human host and an intermediate vector stage. Infection occurs during the blood meal when the infective L3 larvae enter the human host. The larvae develop into adults that reside in subcutaneous tissue or vasculature (blood or lymph). The microfilariae produced by the adults are found in the skin or in the peripheral circulation from which they can be picked up by the vector during a blood meal. In the vector, the ingested microfilariae develop into infective L3 larvae that migrate to the head and proboscis.

Table 1

Post-genomic human filarial datasets

	Molecule	Life-stages / Samples	Wolbachia	Platform	Accession	Ref
Brugia malayi	mRNA	Egg; MF; L3; L4; AF; AM		Illumina Genome Analyzer IIx	E-MTAB-811	[46]
		Intra-mosquito host		Illumina Genome Analyzer IIx	GSE53664	[111]
		20-Hydroxyecdysone treated AF		Illumina MiSeq	SRP064921	[112]
		16-days(L4), 30-,42-, and 120-days post infection	Yes	Illumina HiSeq	SRP090644	[113]
		MF and MF post-ingestion		Microarray	GSE15017	[114]
		L3 and L3 post-infection		Microarray	www.nematode.net	[115]
		AM; AF		Microarray	www.nematode.net	[116, 117]
		Doxycycline treated AF	Yes	Microarray	GSE34976	[118]
		Tetracycline treated adults		Microarray	E-MEXP-2185	[119]
		AM; MF	Yes	Illumina NextSeq	GSE93139	[120]
		AF and MF exposed to Heme		Illumina Genome Analyzer IIx		[121]
		AF exposed to bacteria		Illumina MiSeq		[122]
		Exosomes		Illumina MiSeq		[123]
		AF exposed to Flubendazole		Illumina MiSeq		[124]
		AF exposed to Ivermectin		Illumina MiSeq	GSE75341	[125]
	Small RNA	Adults		Illumina MiSeq	GSE56651	[126]
		Exosomes		Illumina MiSeq		[123]
		AM; AF; MF		Illumina Genome Analyzer IIx		[76]
		AM; AF; MF			Multiple sequences	[77]
	Protein	ES proteins from Adults		2-DE/MALDI-TOF/LC-MS/MS		[50]
		ES proteins from AM; AF; MF		SDS-PAGE/LC-MS/MS		[49]
		ES proteins from AM; AF; MF; L3; molting L3	Yes	LC-MS/MS		[51]
		Somatic proteins of L3; MF; UTMF; AF; AM	Yes	LC-MS/MS		[48]
		Body-wall, gut and uterine tissue of AF	Yes	LC-MS/MS		[52]
		20-Hydroxyecdysone treated AF		LC-MS/MS		[112]
		AM; AF		2DE-MALDI-TOF		[98]
		Exosomes		LC-MS/MS		[123]

	Molecule	Life-stages / Samples	Wolbachia	Platform	Accession	Ref
		Tetracycline treated AF	Yes	2-DE/LC-MS/MS		[127]
	Meta-genomics	Metabolic potential of nematodes				[80]
Loa Loa	mRNA	MF			PRJNA60555	[13]
	Small RNA	Circulating miRNA				[75]
	Protein	Urine of infected individuals				[44]
Onchocerca volvulus	mRNA	Nodular MF; Skin MF; AF; AM; L3; *in vitro* derived L3 Day1; L3 Day3			PRJEB2965	[15, 21]
	Small RNA	Circulating miRNA				[75]
		Circulating miRNA				[73]
		Circulating miRNA				[78]
	Protein	EMB; MF; AF; AM; L3; *in vitro* derived L3 Day1; L3 Day2; L3 Day3; L4	Yes		PXD003585	[21]
		Plasma of infected individuals				[128]
		Proteome-wide peptide array				[53]

AF: Adult Female; AM: Adult Male; MF: Microfilariae; L3 and L4: larval stages; EMB: Embryos; UTMF: Uterine Microfilaria; ES: Excretory-secretory proteins. “Yes” in *Wolbachia* column denotes transcriptome or proteome of *Wolbachia* was analyzed. Empty fields denote either not applicable or data not available.