UTILIZING MOLECULAR MARKERS TO CHARACTERIZE FILARIAL INFECTIONS

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ABSTRACT

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Filarial nematode infections represent a tremendous burden for both animal and human medicine. Millions are infected and billions live in endemic regions. Molecular techniques offer a novel approach to understanding these infections and the agents of infection. In the first experiment the generation of biomarkers is addressed by characterizing the secretome of *Dirofilaria immitis*, the dog heartworm. This experiment revealed 110 proteins secreted by the organism. Of those 110, 52 were unique to *D. immitis* while the remainder was shared among the other nematode species for which secretomes have been compiled. In the second experiment molecular markers were applied to *Onchocerca volvulus* nodules to describe the health of the worm specifically. Those data showed a statistically significant difference in the staining intensity between Ivermectin treated and untreated worms.

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KEY TO SYMBOLS or ABBREVIATIONS

NRas: Neuroblastoma RAS viral oncogene homolog

ML: macrocyclic lactone

HARD: Heartworm Associated Respiratory Distress

GO: gene ontology

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

NCBI: National Center for Biotechnology Information

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

LC-MS/MS: liquid chromatography tandem mass spectroscopy

AOI: Area of Interest

CHAPTER 1

Literature Review

Filarial nematode infections are a tremendous burden to both human and animal medicine. In humans, millions of people are infected with filarial nematodes. Most commonly these infections are *Onchocerca volvulus*, *Brugia malayi* and *Wuchereria bancrofti*. Most of these infections occur in tropical regions of the world and in those who live in poverty [1, 2]. In animals the infections are most common in livestock and companion animals and represent many different species *Dirofilaria immitis* included. Taken together these infections represent a significant drain on the capabilities and productivity of those infected both human and animal.

In the pharmaceutical industry filarial nematodes remain a constant target. The infections in livestock and companion animals offer a more profitable market than do the human infections. It is because of that profitability that most new drugs to treat filarial nematodes come from the animal medicine market. In particular, *D. immitis*, the dog heartworm, is a major concern [3]. There are numerous drug combinations aimed at controlling *D. immitis* by halting transmission of the parasite and killing the adult worms in the mammalian host. The most common drugs used are avermectins, milbemycin oxime, tetracycline antibiotics and arsenicals. There are, however, questions about recent loss of efficacy (LoE) in the most common drugs used to treat *D. immitis* infections [4, 5]. However resistance remains a controversial topic in parasitological circles.

In conjunction with new drug developments the ability to gauge the effects of the new drugs must also be refined; utilizing biomarkers developed with that analysis in mind should greatly supplement the ability to measure drug effects, whether that be the effects of Ivermectin-

induced muscular paralysis or benzimidazole-induced destruction of beta tubulin. Conventionally the analysis of drug efficacy on a microscopic examination has been performed by experts in the field using subjective measurements and scales. Molecular diagnosis kits use proprietary antigens believed to be derived from female worms alone [6-8]. This leaves a field of parasitologists relying on the findings of a minority or, at best, an unknown quantity. Developing new markers to characterize both filarial infections and the effects of drugs on the nematode itself must be a high priority. It is honoring this priority that part of this report addresses the development of an antigen-based analysis of worm viability comparing Ivermectin treated and untreated *O. volvulus*.

The biology of filarial nematodes covers much evolutionary adaptation to the environment in which each organism finds itself. However, there are many features that are conserved. Nematodes are pseudocoelomates, meaning they have a body cavity that is partially lined with tissue derived from the mesoderm. This pseudocoelom is filled with fluid, the pseudocoel. The pseudocoel transports nutrients through the process of diffusion to the organs surrounded by it. The pseudocoel also transports waste away from the organs. The pseudocoel serves another and very important function; it is the mechanism by which the hydrostatic skeleton operates. A hydrostatic skeleton acts under an internal pressure that gives a nematode its shape and the ability to move. By longitudinal muscle contraction localized pressure of the pseudocoel increases and acts against the internal organs and the lining of the body cavity to allow movement. The nematode moves in a sinusoidal pattern by these pressure changes [9].

All nematodes are protected by a cuticle that covers the hypodermis and longitudinal muscles. The cuticle is a weave of cross-linked collagen impregnated by lipids and glycoproteins. The cuticle allows some chemicals to diffuse across it. Glucose, among others is

capable of bypassing the cuticle so that even when the pharyngeal pumping is paralyzed by the administration of certain drugs the nematode is able to acquire the nutrients needed to maintain basic life functions. Oxygen and other gasses can also diffuse across the cuticle. The body openings are also covered by the cuticle though this is of a different construction [9]. The outermost layer of the cuticle is a negatively charged glycoprotein rich covering. This layer is synthesized and secreted from the secretory system and gland cells. In parasitic nematodes it has been implicated in immune system evasion [10]. In *C. elegans* it is hypothesized this surface coat may help in locomotion and the prevention of the adherence of pathogenic bacteria [11].

The hypodermis lies just beneath the cuticle and may be cellular or a cellular syncytium, meaning a multinucleated cell. The hypodermis thus lines the inner surface of the cuticle and is divided into four zones: a dorsal, ventral and two lateral hypodermal cords. The hypodermis also forms the attachment point for the longitudinal muscles which line the hypodermis in distinct locations [9].

Most species of filarial nematodes harbor intracellular organisms or endosymbionts. These organisms have been identified to be *Wolbachia*, the same genus of organisms that are symbionts in many insect species. In most filariae, *Wolbachia* are found in both sexes, but unlike in the male, they are found in the reproductive cells of the female. In both sexes *Wolbachia* are also found in the lateral hypodermal chords and throughout the hypodermis. They are not present in the longitudinal muscle of either sex. The immunolocalization of *Wolbachia* in *B. malayi* is shown in figure 1. When a species has the endosymbiont, every member of that species contains *Wolbachia* suggesting they are integral to the survival of the worm. This suggestion leads to the inevitable conclusion that *Wolbachia* may afford a new pathway to controlling filarial nematode infections. Clinical trials testing the efficacy of the

tetracycline antibiotics against filarial nematode infections have yielded positive results [3, 12, 13].

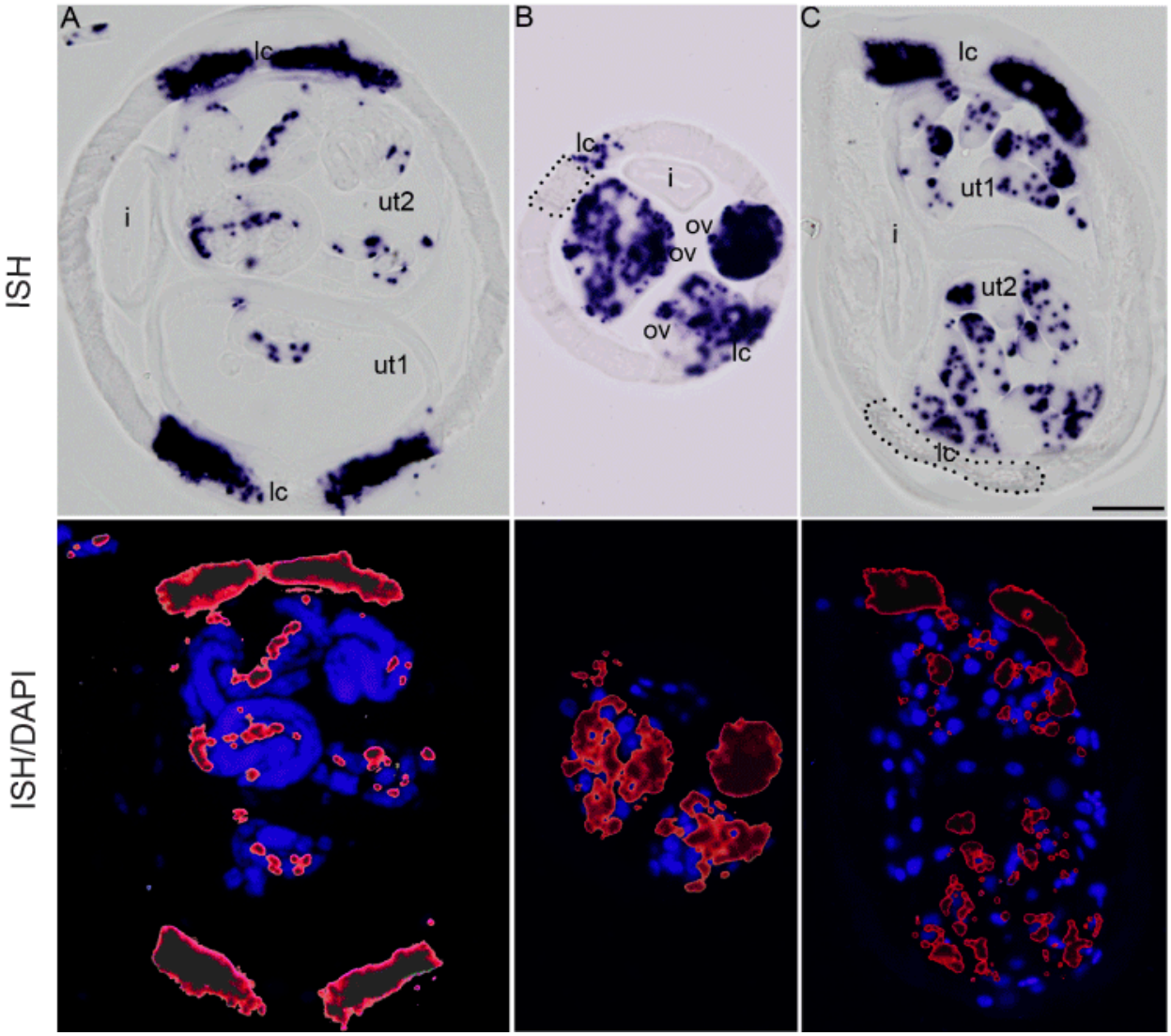

Figure 1: The localization of *Wolbachia* [14].

However, a deeper understanding of the *Wolbachia* organism and its interaction with its host might reveal more promising chemotherapeutic targets. To do this, the genome of the *Wolbachia* endosymbiont of the filarial nematode *Brugia malayi* was sequenced and annotated [15, 16]. Once completed, the annotated genome was compared to those of other organisms to

gain a wider understanding of features of *Wolbachia*, such as metabolism, to other organisms. This analysis demonstrated that *Wolbachia* had a higher conservation of metabolic genes than other closely related *Rickettsia* spp. Notwithstanding this conclusion, *Wolbachia* did not appear to have a robust biosynthetic capability. This capacity for biosynthesis is heavily involved in the relationship between symbionts. The relationship is defined by what each organism provides for the other. Understanding this limited biosynthetic capability must call into question what substances are provided for the worm host and whether it was more similar to insect *Wolbachia* that behave more like parasites than endosymbionts.

Analysis of the annotated genome revealed that the *B. malayi* species of *Wolbachia* possesses the complete molecular machinery to produce both purines and pyrimidines. Purines and pyrimidines are the base components of the nucleotides that comprise DNA and RNA. It has been suggested that *Wolbachia* may manufacture an excess of purines and pyrimidines, exporting some to the worm host during times of high consumption, offering a possible explanation as to their locations in the worm host. The *Wolbachia* are found in the female reproductive cells and both the male and female hypodermis. These locations are both areas of growth and cell division where the need for purines and pyrimidines is high. Another significant complement of biosynthetic capabilities was discovered for riboflavin and flavin adenine dinucleotide. No evidence for the worm's ability to manufacture these reducing agents has been found, suggesting another very important contribution *Wolbachia* makes to its worm host. One of the more important discoveries made by searching the annotated genome was that of *Wolbachia*'s almost complete ability to synthesize heme. A final gene encoding protoporphyrinogen oxidase is missing from the sequence, though it is possible the biosynthetic pathway is supplemented by another enzyme that has yet to be identified. Heme is an iron-

containing compound that acts as a reducing agent and most notably, in blood cells as the carrier of oxygen in hemoglobin. Heme is a critical component of the worm's living systems and as yet there is no evidence of *B. malayi*'s ability to synthesize heme. Indeed, the Phylum Nematoda is the only metazoan grouping in which heme synthesis pathways are not encoded in the genome; nematodes must acquire heme from the environment, this includes the host in which they live by consumption as heme cannot diffuse across the cuticle [17].

Cell-surface proteins of *Wolbachia* have been implicated in the development of the various manifestations of pathology observed in filarial nematode infections. Experiments using the mouse model for *O. volvulus* infections demonstrated that worm segments containing *Wolbachia* induced the pathology commonly associated with River Blindness while those segments that had been depleted of *Wolbachia* developed no pathology. This pathology is thought to heavily involve neutrophil performance as they surround invading microfilariae in the ocular fluid and thereby induce the inflammation that leads to the onset of pathology upon death of microfilariae and release of these bacterial antigens [18, 19]. There is also evidence from *D. immitis* that removal of *Wolbachia* by doxycycline treatment prior to melarsomine therapy reduces pathology due to dying worms in the lungs, but this is not settled as too few studies have been reported to confirm the initial observations [20].

Nematodes have a lifecycle that is composed of distinct parts, the egg or embryo, four juvenile stages and the adult. The parasitic members of the nematode family often have a complex lifecycle consisting of two hosts, the intermediate host, which incubates and transmits the infection and the definitive host, where the parasites sexually mature and reproduce. This arrangement breaks the lifecycle into two parts. In filarial nematodes the microfilariae (L_1) are taken up by the invertebrate intermediate host where they undergo two molts to become the

infective L_3 larval stage. At a subsequent blood meal of the mosquito the infective larvae pass into the definitive host where they undergo two more molts to become the sexually mature adults (L_5). These adults then mate and begin the cycle anew. The lifecycle of *Dirofilaria immitis* is shown in Figure 2.

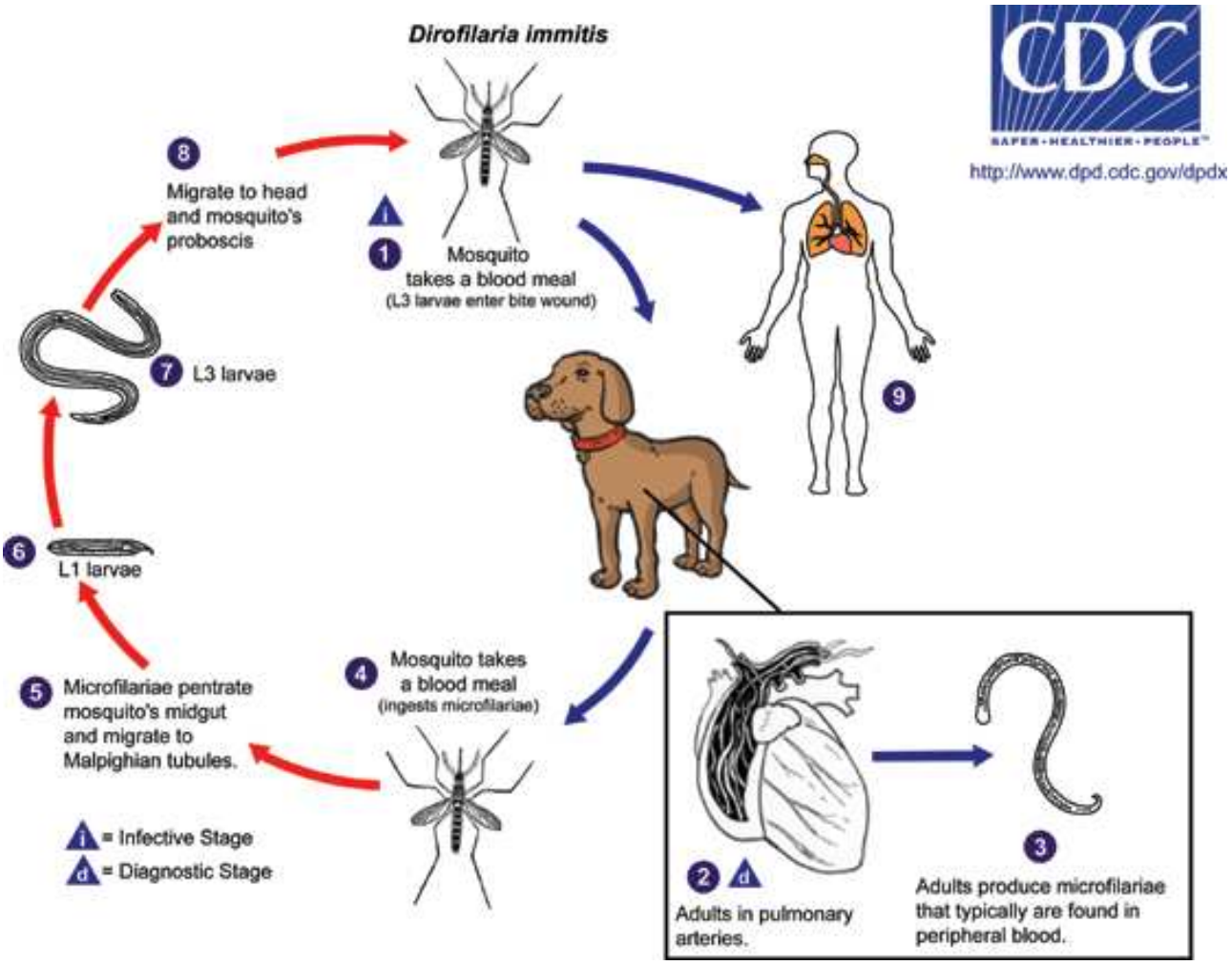

Figure 2: The lifecycle of *D. immitis*.

In the case of *D. immitis* the microfilariae are taken up by a mosquito of the genus of *Aedes, Anapheles* and *Culex* upon feeding on a microfilaremic host. Within the mosquito vector the microfilariae undergo two molts to reach the infective L_3 larval stage. These larvae then

penetrate to the mosquito's labium where they await the next blood meal. It is this infective stage that reenters the mammalian host upon a mosquito feeding. The L_3 larvae undergo two subsequent molts to reach the sexually mature adult, L_5. The sexually mature adults mate and the female releases sheathed microfilariae that begin the lifecycle [3, 21]. A collection of adult *D. immitis* worms is shown in figure 3.

Figure 3: An image of the typical residence of *D. immitis* in the heart of a dog [22].

Diagnostic tests aim to accomplish the identification of infectious agents with two key factors: sensitivity (the ability to detect even small numbers of the infectious agents) and specificity (the ability to distinguish between infectious agents). There are a number of different methods to accomplish this and each has specific strengths and weaknesses. In the filarial diagnosis there are generally 3 tests that can be performed: 1) a microscopic analysis of the filariid, the L_1 stage, 2) polymerase chain reaction (PCR) to detect specific DNA sequences and 3) enzyme-linked immunosorbent assay (ELISA) to detect either specific antigens or specific

antibodies. The commercial kits available today rely on proprietary female worm antigens to achieve detection [3, 6, 23, 24]. Figs 4 and 5 show these common diagnostic tests.

The development of a safe and effective macrofilaricide, a drug that eliminates the adult worms, has been challenging. Larvacidal activity is highlighted by most of the antifilarials and in this it is an attempt to control transmission. Detailed below are the most common chemotherapeutic agents used in filarial infections.

Avermectins are widely used in both human and animal medicine. In livestock these drugs are used to control gastrointestinal worms, lungworms, lice, mites and flies. In the companion animals they are of course used to control filariae, GI worms, bot flies, lice, fleas, mites and ticks. The avermectins are robust and highly efficacious drugs that are widely used and have improved the quality of life for countless humans and animals [1, 2, 27, 28].

The mechanism of action of the avermectins has recently received much attention. In illuminating this mechanism it became clearer how the avermectins functioned and how the worm was affected by the presence of the compound. The chemical structure of Ivermectin and a highly related compound, milbemycin oxime are shown in figs 6 and 7, respectively.

Glutamate-gated chloride channels (GluCl) are a subfamily of the larger group of ligand-gated ion channels that are common features of all nervous systems. GluCls are the targets of the macrocyclic lactone anthelmintic class (avermectins and milbemycins). These compounds act as reversible but long term agonists of the channel, locking it open and preventing transmission of subsequent currents, thus paralyzing the neuron. The interaction between an avermectin and this channel is of such high affinity that once opened; the drug-bound channel does not close again until the drug is cleared from the system [29].

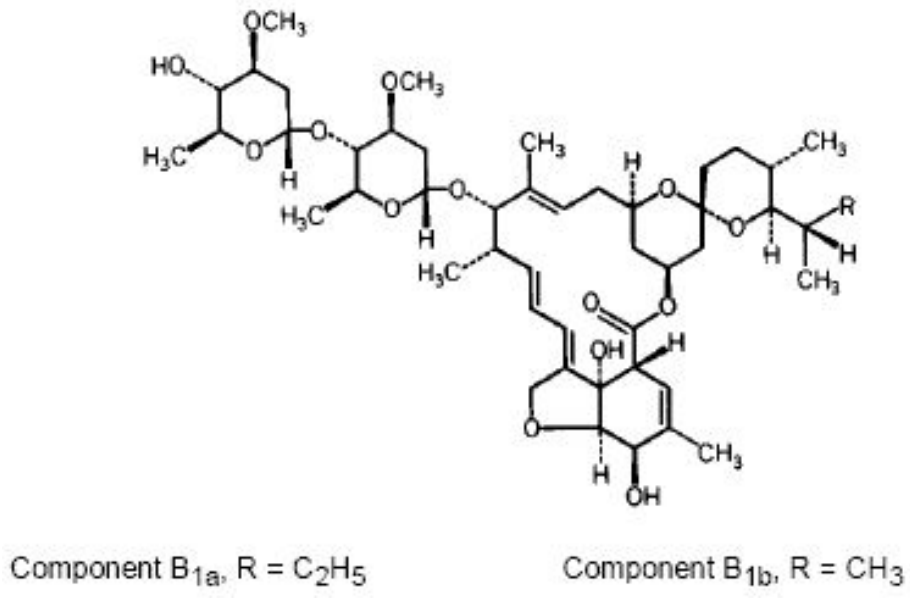

Figure 4: The chemical structure of Ivermectin [30].

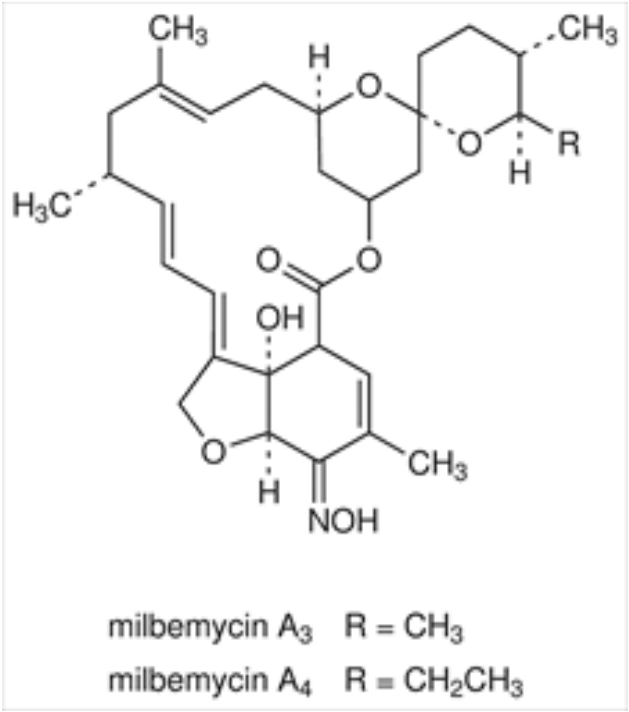

Figure 5: The chemical structure of milbemycin oxime [31].

In nematodes, GluCls are encoded by a small family of genes. A widely conserved gene, *avr-14*, found in both parasitic and free- living species, is of particular relevance [32]. *In vivo* this gene is alternatively spliced to produce two subunits, one of which, GluClα3B, is capable of forming channels that bind Ivermectin. This is the only type of IVM-binding GluCl identified in the filarial nematode *B. malayi* [33]. When expressed in *Xenopus laevis* oocytes, the channel was sensitive to L-glutamate and Ivermectin, in a concentration-dependent manner. Ivermectin resistance in *C. elegans* requires loss-of-function mutations in at least 3 GluCl genes; whether this situation pertains to parasitic species is unknown. Work in *O. volvulus* and *D. immitis* suggests that changes in function of multi-drug resistance pumps may be involved, but proof of this association has yet to be provided. The demonstration of macrocyclic lactone resistance in filarial nematodes presents a great challenge for the effective treatment of those infections [4, 34-36].

In microfilaria it appears that GluCl expression is limited to the musculature surrounding the excretory-secretory vesicle. In *C. elegans* and gastrointestinal parasitic species, GluCl expression extends to the motor-nervous system, especially noted in the pharyngeal neurons and both hypodermal nerve cords, suggesting a wide-spread distribution of these channels and implicating them in many different functions, including locomotion, ingestion and possible sensation. These diverse locations also provide insight into the activity of the avermectins in the parasite itself as a paralytic agent that interrupts pharyngeal pumping, locomotion, the release of mature microfilariae from the ovijector and possibly interfering with the parasite's ability to sense the local environment [29, 32, 33, 37].

A second class of drugs, the benzimidazoles, is widely used in human and animal medicine as well. Benzimidazoles bind selectively to beta-tubulin in parasitic nematodes. The

chemical structure of albendazole and flubendazole are shown in figs 6 and 7, respectively. This binding inhibits microtubule formation. Microtubules are hollow organelles and are a fundamental component of several cell processes including cell division, cell motility, shape and intracellular transport. Microtubules are heterodimeric constructs of alpha- and beta-tubulin [38-40]. An image of a microtubule is shown in figure 8. The binding of benzimidazole halts the assembly of the microtubule and leads to its disappearance. In *Ascaris suum*, this was found to affect the intestinal cells of the parasite. By inhibiting microtubule formation, the cells could no longer transport secretory vesicles, leading to failure to take up glucose and presumably other nutrients, subsequently starving the parasite. An effect was also noted on egg production [39, 40].

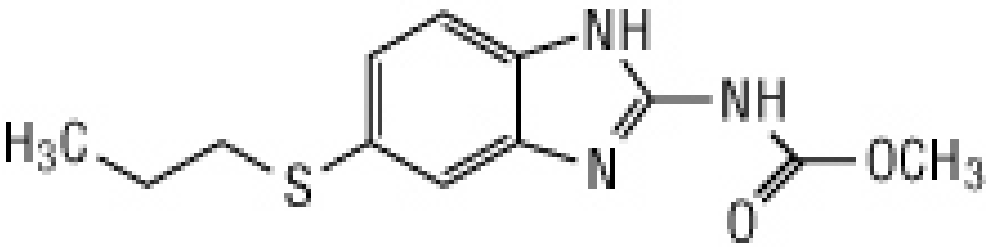

Figure 6: The chemical structure of albendazole [41].

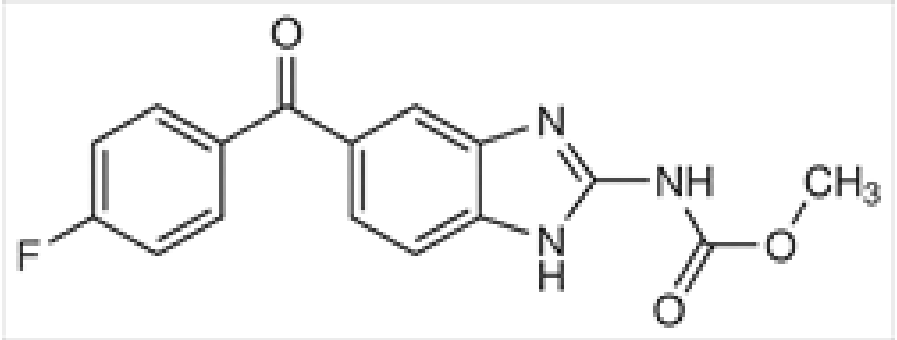

Figure 7: The chemical structure of flubendazole [42].

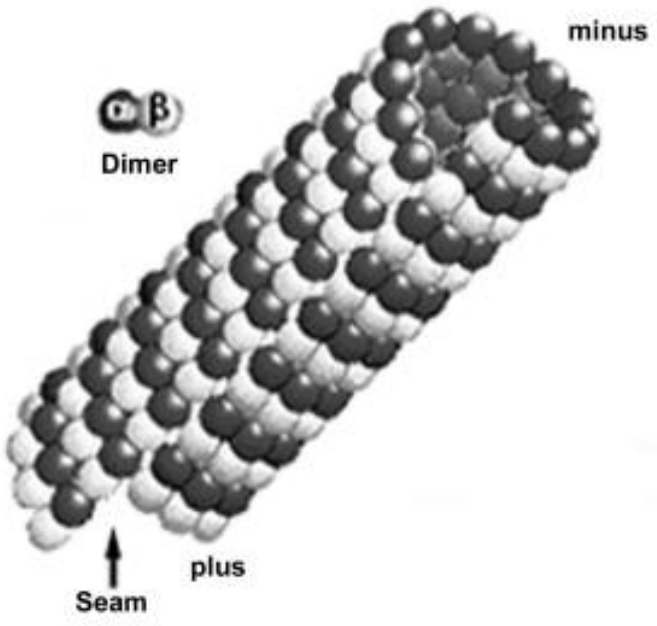

Figure 8: An image of a microtubule formed from both alpha and beta tubulin [43].

Benzimidazole resistance has become a significant cause for concern, especially in intestinal parasites of great importance in veterinary medicine. This resistance was traced to a single amino acid substitution linked to a single nucleotide polymorphism in the gene encoding beta-tubulin. This substitute removes a phenylalanine and replaces it with a tyrosine at position 200. This has the effect of markedly reducing the affinity of benzimidazoles for beta-tubulin. Resistance may be difficult to overcome because the mammalian host beta-tubulin already has a tyrosine group at position 200 [44, 45]. Resistance has spread through the selection pressure of the drug exerted upon individuals promoting the appearance and stabilization of resistance in the population. This has the coincidental effect of selecting against non-resistant phenotypes in the presence of drug pressure, thereby removing those individuals from the population. Finally, the selection pressure of the drug causes the resistant phenotype to become fixed in the population and removes any fitness deficit originally associated with the onset of resistance [38, 40, 44].

Mutations at position 167 and 198 have also been associated with resistance in veterinary parasites. The danger for human use is that the single-day regimen used in human MDA campaigns is suboptimal in terms of efficacy against hookworms and whipworms, leading to a real threat of resistance development as mass distribution of albendazole and mebendazole take off.

Albendazole is routinely used for lymphatic filariasis elimination in humans; however the evidence for its efficacy is sparse [27, 46]. It is known that benzimidazoles are poorly absorbed from the gastrointestinal tract subsequent to oral dosing and thus achieve a low blood level leading to questions of efficacy against blood dwelling filariae such as *D. immitis*. Benzimidazoles, especially albendazole have been used to treat neurocysticercosis [47].

While oral doses of albendazole may have questionable efficacy for filarial elimination injections of the drug flubendazole have shown promise in that field. Injections achieve a higher blood level as the GI tract is bypassed by delivery straight into the body [48]. Flubendazole may demonstrate a higher efficacy against the adult worms, the macrofilariae, something no commercially available drug has yet to do well.

A third class of drugs, tetracycline antibiotics, was developed as targeting the bacterial endosymbiont *Wolbachia*. The chemical structure of tetracycline is shown in figure 9. Doxycycline is a member of the tetracycline antibiotic family. The chemical structure of doxycyline is shown in figure 10. These antibiotics work by interfering with protein synthesis at the molecular level [49]. It is through this mechanism that the *Wolbachia* endobacteria are targeted by doxycyline treatment. This treatment serves to sterilize adult worms, though through a different mechanism than Ivermectin. Doxycyline treatment also has an adulticidal effect by

depleting the worms of the endosymbiont *Wolbachia*, thereby removing the contributions of the bacteria to the parasite [12, 50-52].

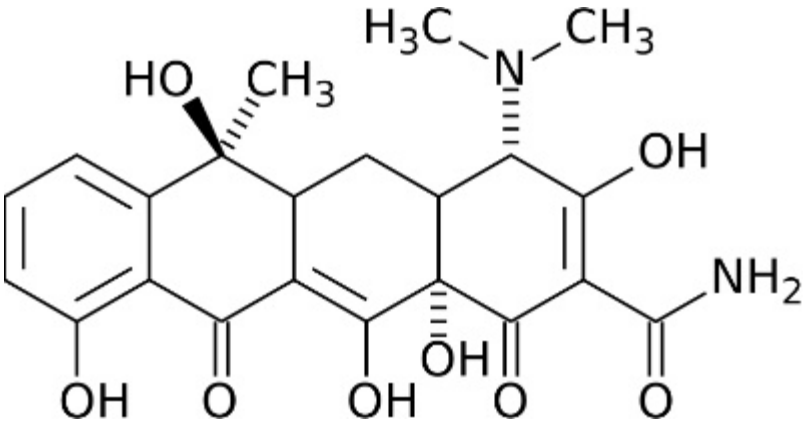

Figure 9: The chemical structure of tetracycline [53].

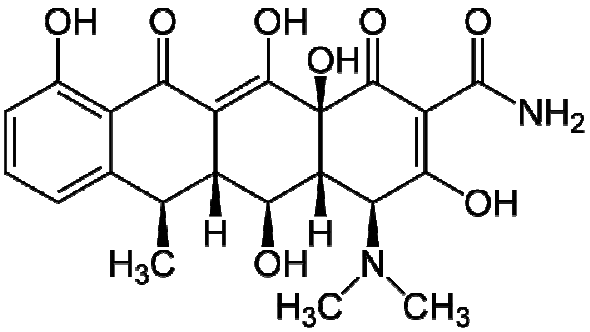

Figure 10: The chemical structure of doxycycline [54].

The tetracycline class of antibiotics binds reversibly to the 30S subunit of the bacterial ribosome. This has the effect of blocking aminoacylated – tRNA from accessing the ribosomal A site and preventing the peptidyl – transfer reaction. Because it binds reversibly to the 30S subunit it requires a higher concentration of the drug to be internalized into the bacterium. This need renders the pathway vulnerable to efflux pumps. These pumps are implicated in tetracycline resistance by externalizing the drug [49].

If the mode of action is similar in *Wolbachia* it does not necessarily explain the apparent disappearance of the *Wolbachia* from the worm as suggested by immunohistological studies. Tetracycline acts as a bacteriostatic, inhibiting the growth of the bacteria in most bacteria. It is possible that doxycyline is a bacteriacidal in *Wolbachia* [51, 55].

Melarsomine dihydrochloride is a melaminyl thioarsenite that is available to treat *Dirofilaria immitis* infections in dogs. The chemical structure of melarsomine dihydrochloride is shown in figure 11. Melarsomine is a trivalent arsenical that comes as a lyophilized powder soluble in water or physiological saline. It is administered through a deep intramuscular injection in the lumbar muscles. Its therapeutic concentration is 2.2mg/kg administered twice, 3 hours apart [56, 57]. The mode of action of this drug is unknown but presumably related to the effect arsenic has on glycolysis. Arsenic itself is an extremely potent poison. It disrupts ATP production. In the citric acid cycle arsenic inhibits lipoic acid, which is a cofactor for pyruvate dehydrogenase and by competing with phosphate it uncouples oxidative phosphorylation, inhibiting the energy reduction of NAD+. Hydrogen peroxide production is also increased which may lead to oxidative stress and cell death [58].

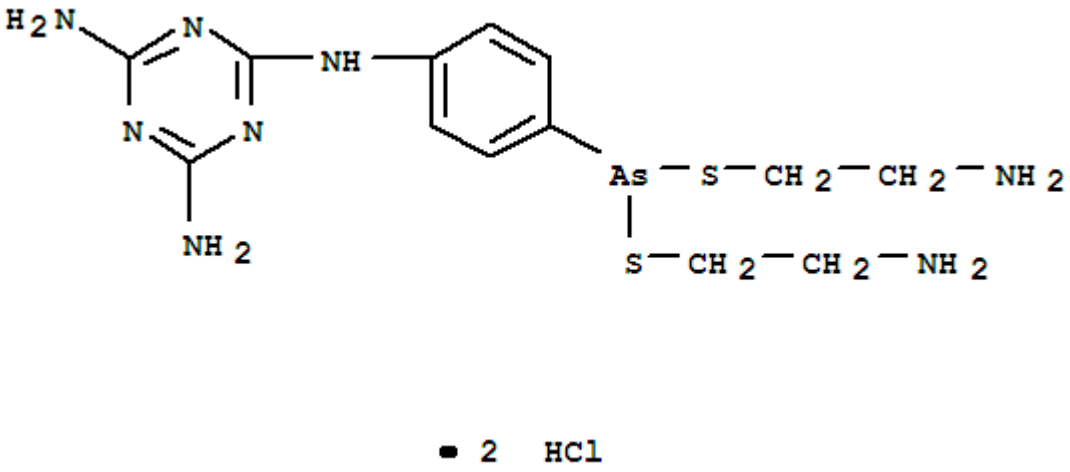

Figure 11: The chemical structure of melarsomine dihydrochloride [59].

This treatment however is not without risk to the animal. Both pulmonary thromboembolism and pulmonary hypertension have been noted with pulmonary hypertension largely a result of the presence of the macrofilariae [3, 20].

The secretome is the collection of proteins secreted and excreted by an organism. Once collected and analyzed, these proteins provide an interesting and illuminating view of how the organism survives in its environment [60-67]. These proteins are collected by incubating the organism in selected culture media. For example, *Dirofilaria immitis* can be cultured in RPMI 1640 with the addition of antibiotics, antifungals, glucose, sodium bicarbonate and HEPES. Incubated at 39°C, the worms survive and continue to metabolize. It is these metabolites that alter the pH of the media requiring the addition of the buffers mentioned above. The media, once collected is passed through a barrier to filter out molecules and detritus larger than a specified molecular weight. The remaining media is now concentrated with the proteins in a higher concentration relative to the liquid media. The proteins then must be brought out of solution, precipitated using trichloroacetic acid (TCA). The residual TCA is washed away using

acetone. The proteins are then re-pelleted using a microcentrifuge. Once pelleted, the proteins are resuspended in a sample buffer suitable for use in SDS-PAGE (sodium dodecyl sulfate poly-acrylamide gel electrophoresis). This procedure separates the proteins into bands based on molecular weight and charge. The bands are then excised from the gel and digested using an enzyme, typically trypsin, and the resulting liquid is pumped through a tandem mass spectrometer which analyzes the amino acids based on mass and charge [68, 69]. A computer program then digitally reforms the amino acids into the proteins from which they originated using a data file containing the amino acid sequences of known proteins from a database that the user selects. Once reformed the proteins are now ready for analysis using a number of computer programs. Blast2GO is one of the more popular programs to use[70]. Blast2GO scans a number of databases to describe the proteins in more detail [71, 72]. Blast2GO assigns each protein a number of gene ontology (GO) terms that illuminate biological process, molecular function and cellular localization. Additional programs can analyze the proteins and predict signal sequences that indicate the protein is likely secreted [73]. In this manner the secretome can then be compiled and analyzed regarding the biological functional role of individual proteins in aiding the organism to survive in its environment.

The importance of the secreted proteins is worth exploring further. Analyzing the secretome provides clues as to the importance of certain proteins in parasite survival based upon their overall abundance in the secretome or their functional motifs; for example proteases and protease inhibitors. In the filariids, proteins such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) were found as well (Figure 14). In particular superoxide dismutase is of great importance in surviving the host immune response as oxygen radicals are a feature of immune cell destruction of foreign bodies [74, 75]. In the case of *B. malayi* superoxide dismutase was

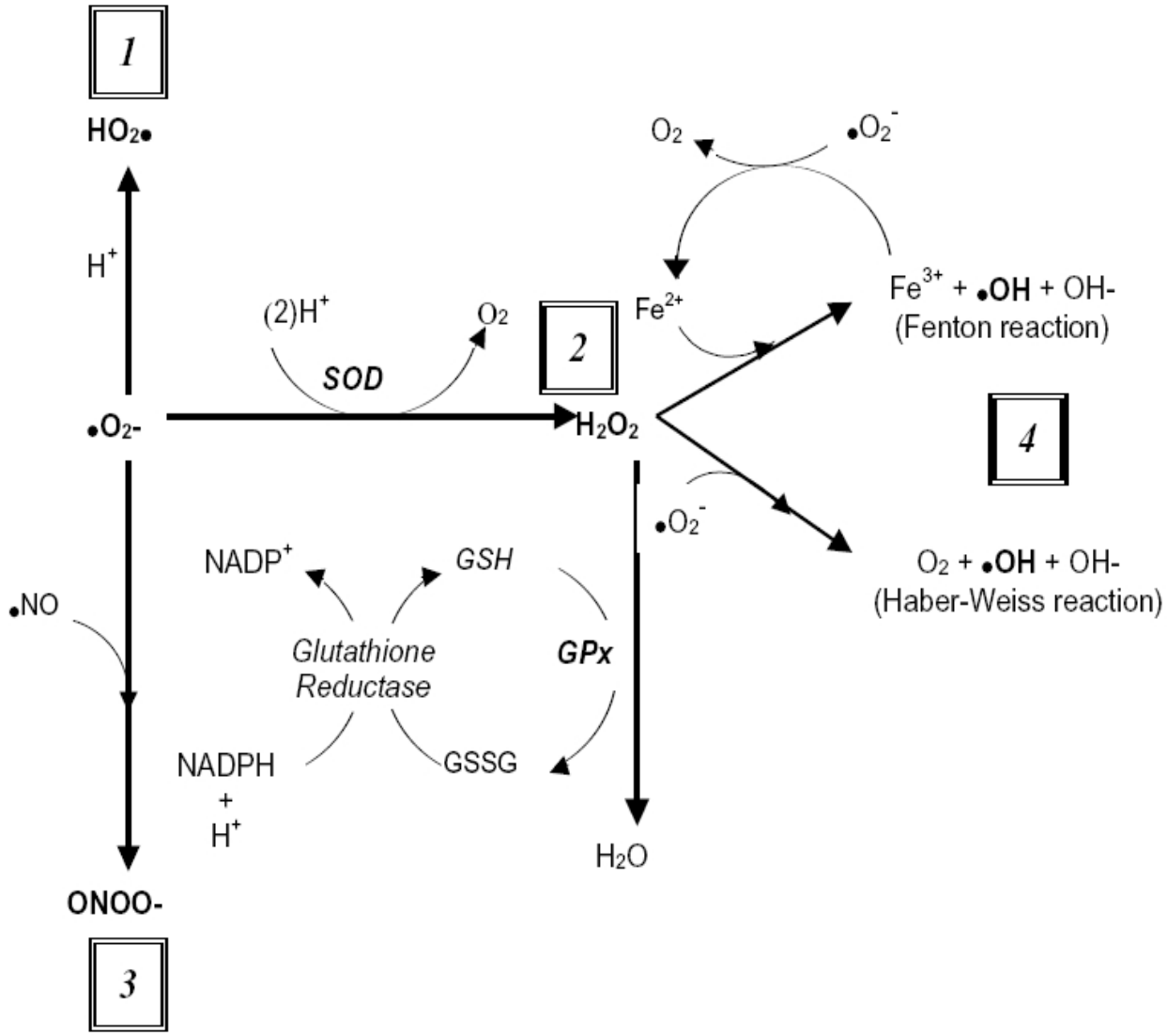

Figure 12: The pathways of neutralizing reactive oxygen species, including GPx and SOD [76].

found in lifecycle stages L_1 and L_5 of the parasite suggesting its critical importance for survival in the mammalian host environment [61-63]. Several pathways for neutralizing reactive oxygen species are shown in figure 12.

The anatomy of the secretory machinery in the adult nematodes is relatively poorly described. Overall it consists of either a glandular or tubular structure that links to the external surface by a pore that may be muscularly controlled [9]. However the secretory apparatus has been well documented in microfilariae [33]. It was found that the secretory pore in the microfilariae was surrounded internally by muscle. This suggests muscular control of the release of secreted products. When treated with Ivermectin the release of proteins from the microfilariae was lessened though not eliminated. If the same were true in adults it could help explain Ivermectin activity against the adult worms.

In attempting to characterize filarial infections molecular methods have become the dominant mode of investigation. This is a testament to their robustness and usefulness. Molecular markers are used in a variety of approaches from drug design and analysis to diagnosis of cancer in both humans and animals [77-80]. The two main reports featured in this thesis demonstrated the exceptional usefulness of biomarkers, both their generation through characterizing a secretome and their application in differentiating treated and untreated worms and assessing their viability.

It is a challenge for the modern pharmaceutical industry to come up with new drugs to treat filarial infections, drugs that are needed to address these infections in the face of resistance [4, 35, 36]. Though resistance remains a controversial topic it is understandable given the profitability of the current avermectin-based chemotherapies, not only in companion animal medicine but also in livestock care. Avermectin-based drugs are used to treat a wide variety of infections and infestations from dog heartworm to fleas and biting flies. If resistance is confirmed, and many reports have tended to reinforce this [5-7], the pharmaceutical companies

will be under immediate pressure to develop a new line of drugs to combat resistant phenotypes. Given the market for the current drugs this is readily understandable as the worldwide need for these drugs is high. Achieving success with this process will be greatly aided by the use of biomarkers, both generated by secretome compilations and also by monitoring the status of the worms themselves in response to the new treatments and comparing them to the old treatments where resistant alleles have become fixed in the population.

In compiling the secretome of adult *D. immitis* there are many new diagnostic markers waiting to be tested. When screened against the existing secretomes novel biomarkers can be developed into quantifiable diagnostic tests. It may also be possible to investigate these biomarkers as potential chemotherapeutic targets. The use of immunotherapy in cancer biology has become a standard practice in human medicine [81]. Utilizing monoclonal antibodies targeted to specific cell receptors, cancer cells can be destroyed with precision as opposed to therapies that result in collateral damage to the human body beyond the destruction of cancer cells [82]. Immunomodulatory therapy is also common in the treatment of rheumatoid arthritis where antibodies are injected to halt and subsequently suppress inflammation and stop the progression of joint destruction [83]. These examples suggest that immunotherapies for parasitic infections may not be based on speculation alone. It might be possible to utilize the biomarkers identified in the *D. immitis* secretome as immunotherapy targets. Using these biomarkers it may be possible to develop an antigen vaccine that would inhibit the colonization of the host. A better understanding of the adult secretory process is required, perhaps simultaneously with development of unique proteins as immunotherapy targets. Though a comprehensive analysis of the secretory process in microfilariae has been completed [33] for *B. malayi*, the adult secretory system has received little attention. Though Ivermectin was found to decrease the secretion

levels of proteins in microfilariae and presumably leave them vulnerable to immune system killing, this explanation is harder to apply to the action in adult worms given the remarkable longevity of adult filariae even when exposed to constant drug pressure in the form of avermectins. Analysis of the secreted proteins over time in adult filariae exposed to Ivermectin may lead to new conclusions about the adult secretory system. For example, if the levels did not decrease in a statistically significant manner it might be that the neuromuscular control of the adult secretory system relies on a different modality of signaling that is unaffected by the presence of Ivermectin. Further studies, for example confocal microscopic examination of the adult secretory system using a filariid-specific antibody to the avermectin receptor could illuminate the possible effects of Ivermectin on the adult secretory system. Such studies should be considered a priority in order to not only compare the adult and microfilaria secretory systems but also to investigate the mode of action of Ivermectin on the adults beyond the paralysis of pharyngeal pumping and ovijector resulting in the cessation of release of microfilariae, embryostasis. The understanding of the neuromuscular control of the ovijector can be accomplished and would also serve to illuminate the location of the avermectin receptor and its implications for embryostasis.

In respect to using biomarkers generated in the secretome as diagnostic markers the approach could be fairly simple. By screening the secretome against the *C. lupus familiaris* proteome a subset of the secreted proteins would be identified that had limited or no homology to dog proteins. Once identified these proteins could be used to generate polyclonal antibodies which could then be used to detect those proteins in blood samples using a simple ELISA. In this manner a universally secreted, non-proprietary protein could be used as a rapid diagnostic marker capable of detecting both male and female worms. It might also be possible to use the

relative levels of the protein to determine the worm burden in the infected animal. To accomplish this end a set of adult worms should be cultured in the same procedure from which the initial secretome was derived. By measuring the amount of protein secreted into the culture medium through time a rate of secretion could be determined. Using this rate of secretion relative to the number of worms cultured a relatively exact determination could be made as to the worm burden. There is no data available as to the clearance rate of filarial secreted proteins from the blood of infected animals though it should be expected that there is some clearance in the normal biological processes of the infected animal. A relatively simple experiment could be used to determine the rate of clearance from the blood of an infected animal. By experimentally infecting dogs with *D. immitis* and then measuring the blood levels of the targeted secreted proteins and comparing that with the rate of secretion established in culture medium a corrective variable could be introduced to the equation to compensate for clearance from the blood. Upon death of the animal the adult *D. immitis* should be recovered to confirm the rate of secretion and corrective variable by comparing the number of worms and the levels of secreted proteins in the blood versus those secreted in culture medium. By using the experimentally established corrective variable based on the clearance of secreted proteins from the blood of infected animals a reliable diagnostic test that accounts for worm burden could be developed. Along the same reasoning this diagnostic test could be used in experiments to investigate the rapidity of clearance of the infection using adulticidal therapies. By monitoring the levels of secreted proteins in the blood of infected animals and its decrease under drug pressure a timeline could be established of worm death.

In my reports the importance of biomarkers cannot be overstated in understanding not only the biology of the worm in response to drug pressure, the onset of resistance and its

implications on worm biology, but also the diagnosis, monitoring of chemotherapy and resistance. Ultimately a greater understanding of worm biology, resistance and chemotherapy may lead to innovative and novel drug targets, strategies to counteract the onset of resistant phenotypes and illuminating the basic biology of the worm itself, a subject that is relatively poorly explored beyond the world of *C. elegans*.

CHAPTER 2

The Secretome of *Dirofilaria immitis*

There have been a number of secretomes already reported for nematodes [60-65, 67]. In this report we add to that list the secretome of adult *D. immitis*. Our hypothesis driving this research was the addition of another secretome to the available literature. In this manner it was undertaken to not only characterize a secretome for an organism that resides in a different location in the host as opposed to many of the compiled secretomes, blood vs. lymph, but also to illuminate the differences between the filarial nematodes and what those differences might suggest in terms of adaptation to survive in a different environment. These data demonstrate that there is a significant difference between *D. immitis* and both *Brugia malayi* and *Heligmosomoides polygyrus*. This was not totally unexpected. Although initially it was thought that the difference in location within the host would contribute little to the difference in secreted proteins that might not be the case. In this report the 10 most abundant proteins were shared among the filariae but the remaining highly abundant proteins included those unique to *D. immitis*. The functional implications of this are discussed further in the report.

The characterization of proteins released from filariae is an important step in addressing many of the needs in the diagnosis and treatment of these clinically important parasites, as well as contributing to a clearer understanding of their biology. This report describes findings on the proteins released during *in vitro* cultivation of adult *Dirofilaria immitis*, the causative agent of canine and feline heartworm disease. Differences in protein secretion among nematodes *in vivo* may relate to the ecological niche of each parasite and the pathological changes that they induce.

This study identified 110 proteins. Of these proteins, 52 were unique to *D. immitis*. A

total of 23 (44%) were recognized as proteins likely to be secreted. Although these proteins were unique, the motifs were conserved compared with proteins secreted by other nematodes.

The present data indicate that *D. immitis* secretes proteins that are unique to this species, when compared with *Brugia malayi*. The two major functional groups of molecules represented were those representing cellular and of metabolic processes. Unique proteins might be important for maintaining an infection in the host environment, intimately involved in the pathogenesis of disease and may also provide new tools for the diagnosis of heartworm infection.

The filarial nematode *Dirofilaria immitis*, the aetiologic agent of heartworm infection in dogs and cats, is widely distributed in the United States, South America and parts of Europe and Asia [3]. The adult worms can be found mainly in the pulmonary arteries, and sometimes the right heart, atrium and vena cava in heavy infections; this differs from many other filariae that tend to favour lymphatic vessels. Infections with small numbers of adult *D. immitis* may be asymptomatic and have limited pathological effects; however, high adult worms loads usually cause exercise intolerance, a wet cough and lethargy in dogs [84]. Cats are inherently resistant to Dirofilarial infections and thus usually have much lower adult worm burdens than do dogs. However, as cats have a much smaller pulmonary arterial tree they are more sucsceptible to embolism. In addition, dirofilariasis in cats is often more difficult to diagnose due to lower loads and the differing clinical signs from those in dogs [84].

Although *D. immitis* has been controlled through several different strategies, the most successful has been the prophylactic administration of a range of drug combinations and administration schedules, most usually involving tablets or topical preparations containing a macrocyclic lactone (ML) anthelmintic to uninfected dogs and cats to protect them by killing infective L_3 larvae and developing L_4 larvae [85]; drugs in this class of agents are also

microfilaricidal. MLs also affect adult worms, thus inducing long-term suppression in the production of microfilariae (mff) [86]. There are, however, concerns relative to the development of ML resistance [4, 5]. A course of arsenical drugs, such as the currently preferred malarsomine, is adulticidal, although this regimen is not without risk to the animal due to the hepato- and nephron-toxicity of these compounds [56, 57]; 'slow-kill' strategies for use of MLs in infected dogs have also been developed [87], and the potential for anti-*Wolbachia* treatment options to reduce transmission and pathological effects following adulticidal therapy is promising [12, 20].

It has long been recognized that parasitic nematodes release factors, primarily proteins, which alter the immune responses of their hosts [88-90]. Recently, the use of sophisticated mass spectroscopy-based approaches, coupled to genome and transcriptome sequencing, has enabled the identification of proteins released by *Brugia malayi* [61-63] and *Heligmosomoides polygyrus polygyrus* (now considered to represent *H. bakeri*- [*18*]) into culture medium [60, 64]. Secreted proteins have also been characterized from the canine hookworm, *Ancylostoma caninum* [21], the plant parasitic nematode *Meloidogyne incognita* [22] and from *Strongyloides stercoralis* [23]. Not all of these nematode datasets were analyzed against complete genomes (or transcriptomes), and, therefore, some of the compilations may be less completely assigned than others. Nonetheless, it can be concluded that a large number of proteins have been detected in the secretome from these parasites, with marked differences observed among them. The complexity of the nematode secretome compromises the ability to define the most biologically important proteins through a systematic analysis. One approach to provide some focus to this question is to define the secreted proteins that are conserved among parasites that share a niche (e.g., tissue *versus* gastrointestinal tract), and to consider those shared between phylogenetically related

organisms (for instance those in Clade III vs. Clade V; [91]). Thus far, the data sets for nematodes are limited to parasites from different clades and different habitats. The present study describes the secretome of *D. immitis*, as distinct from that of *B. malayi* which resides in a different niche in the mammalian host.

A more pragmatic reason to study the composition of parasite secretomes is to identify the most abundant proteins released into host fluids and tissues which could be candidates for the development of new diagnostic tests, and possibly new treatments. Current diagnostic procedures for nematodes typically rely on poorly characterized or proprietary antigens or antibodies, or the counting of eggs in faecal specimens: the identification of abundantly secreted proteins may allow the development of tests which can assess worm burdens, a goal not readily attainable using current diagnostic tools [92].

Eighty mixed sex, adult *D. immitis* worms were collected from the pulmonary vessels and right heart chamber from mf test-positive dogs immediately after euthanasia, and the healthy worms placed in the culture fluid, as described below. These procedures were approved by the Animal Use Committee of St. Matthews University School of Veterinary Medicine (Grand Cayman, British West Indies). At the end of each 24 h period, immotile worms were removed from the culture system; thus, 56 worms were cultured on day 2 and 51 on day 3, the two days on which culture medium was collected for analysis.

Worms were cultured in large Petri dishes (1 worm/4 mL medium, 5 worms per dish) at 39 °C in RPMI 1640 medium, supplemented with 200 mM L-glutamine, 20 mM HEPES, 200 IU/mL penicillin, 200 IU/mL streptomycin, 25 µg/mL amphotericin B (Gibco Invitrogen, Grand Island, NY), 1% w/v D-glucose and 1% w/v sodium bicarbonate, pH 7.2. Medium was collected

and changed every 24 h. To limit potential contamination of the samples with host proteins, first-day medium was discarded. Medium from the subsequent 2 days was collected for molecular analysis. To determine the vitality of the worms, the change in colour (pH) of the medium was monitored to verify that all worms present were actively metabolizing. Petri dishes that exhibited a colour change were used for analysis, whereas those that remained unchanged or contained immotile worms were discarded. Based on this protocol, medium from 107 worm-days of cultures was collected. On the third day, the concentration was decreased to 1 worm/6 mL. Protease inhibitors (Complete EASYpack Roche, Indianapolis, IN) were added to batches of 50 mL of collected medium.

Immediately after the removal of adult worms, the medium samples were centrifuged at 1000 x *g* for 5 min to pellet mff released during the incubation. The supernatant was removed, passed through a 0.22 µm filter and frozen at -20 ℃ for shipment to Michigan State University. There, the combined volume (775 mL) was concentrated to 40 mL using an Amicon Ultra 3000 MWCO (Millipore, Billerica, MA). Proteins were then precipitated using trichloroacetic acid (final concentration of 20%). Pelleted proteins were washed with cold (-20 °C) acetone 3 times and allowed to air dry [61-63].

Protein pellets were dissolved in 100 µL sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol,1% β-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue, pH 6.8) and re-precipitated with chloroform:methanol (1:4). Pellets were re-solubilized in SDS-PAGE sample buffer and run on a BioRad Criterion precast 12.5% Tris-Glycine gel at 50 V for 15 min, followed by 120 V until the dye front reached the bottom of the gel (~ 90 min). The gel was fixed overnight in 40% methanol/20% acetic acid, followed by staining with colloidal Coomassie Blue. The entire gel

lane was sectioned into 10 equal slices, and each slice was digested in-gel, essentially as described previously [68]. Briefly, gel bands were dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate, pH~8, at 56 °C for 45 min, dehydrated again and incubated in the dark with 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 min. Gel bands were then washed with ammonium bicarbonate and dehydrated again. Sequencing grade, modified trypsin was prepared to 0.01 µg/µL in 50 mM ammonium bicarbonate and ~50 µL were added to each gel band, so that the gel was submerged. Bands were incubated at 37 °C overnight. Extracted peptides were re-suspended in 20 µL 2% acetonitrile/0.1% trifluoroacetic acid.

A 10 µL aliquot of each sample was automatically injected by a Waters nanoAcquity Sample Manager (www.waters.com) and loaded for 5 min on to a Waters Symmetry C18 peptide trap (5 µm, 180 um x 20 mm) at 4 µL/min in 2% acetonitrile /0.1% formic acid. Bound peptides were eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% acetonitrile/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3 µm, 200 A, 100 U x 150 mm, www.michrom.com) and eluted over 90 min with a gradient of 5% B to 35% B in 78 min at a flow rate of 1 µL/min. Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (www.thermo.com) using a Michrom ADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top ten ions in each survey scan subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resultant MS/MS spectra were converted to peak lists using BioWorks Browser v3.3.1 (ThermoFisher) using the default parameters and the peptide masses were compared with a FastA dataset of predicted tryptic peptides derived from a genome sequence of *D. immitis* (P. Maser, personal communication; see

http://nematodes.org/downloads/959nematodegenomes/blast/db/Dirofilaria_immitis_v1.3_20110901.fna) and the NCBI database, including the *Canis familiaris* genome, using the Mascot algorithm v2.3 (www.matrixscience.com). Search parameters were restricted to allow up to two missed tryptic sites, fixed modification of carbamidomethyl cysteine, variable modification of oxidation of methionine, peptide tolerance of +/- 10 ppm and MS/MS tolerance of 0.6 Da. The Mascot output was analyzed using Scaffold, v3.2, (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet algorithm [69]. Assignments validated above the Scaffold 95% confidence filter were considered true. The data have been submitted to Tranche.

Blast2Go [70] was used to analyze the returned proteins as described elsewhere [61-63]. Briefly, an initial BLASTP search was performed against the non-redundant NCBI protein database. Subsequently, the annotation was completed using default parameters [71, 72]. SecretomeP [93] and SignalP [73] were used to assess secretory motifs in the proteins.

Approximately 60 µg of protein was collected from the *D. immitis* cultures. An initial SDS-PAGE run revealed a complex pattern of proteins (Figure 1). The three lanes were combined for tryptic digestion and MS/MS analysis. A second gel consisted of a single lane that contained the same amount of protein analyzed in the first PAGE run.

The initial analysis revealed a total of 110 proteins. Results from the second run confirmed the first, with an additional 17 low abundance proteins appearing in this run. Following manual curation, redundant proteins (attributed to the same *D. immitis* locus) were removed, leaving a total of 110 proteins in this analysis of the heartworm secretome

(Supplemental Table 1). Previous reports identified 193 proteins in the adult *B. malayi* secretome using similar methods [16].

Of these 110 proteins identified in the heartworm secretome, 52 were unique to *D. immitis*, not being described as being present in the *B. malayi* secretome [15-17]. Two of these proteins returned no BLAST hits, leaving 50 defined proteins unique in this context. The proteins found in common in the filariae were concentrated among the more abundant hits, but were distributed through the set, with the 15 most abundant proteins generally shared between the two filariae. Table 1 shows the 15 most abundant proteins detected in the heartworm secretome. The 15 most abundant proteins in the heartworm-unique sample are shown in Table 2. Of these unique defined proteins, 47 (90%) had Gene Ontology (GO) terms assigned in Blast2Go.

Catalytic activity (GO:0003824) and binding (GO:0005488) were the two major molecular function categories, those using GO terms (Figure 2) and those according to biological process (Figure 3). This distribution was highly conserved with that reported for *B. malayi* [16]. The distribution of level 4 biological process GO terms was fairly flat (Figure 4), with no marked bias for particular functions. The category of 'cellular macromolecule metabolic process' was the most frequent term in this category, while 'ribonucleotide binding' was the most frequent term in the set of returned level 4 molecular function GO terms (Figure 5). For both, the distribution of term frequency was generally similar to those reported for *B. malayi* [16].

Analysis for secretion signals by SecretomeP and SignalP showed 10 (19%) of the *D. immitis* unique proteins include a canonical signal sequence for secretion. An additional 13 (25%) include peptide sequences associated with non-classical secretion pathways, for a total of

23 (44%) that can be recognized as proteins likely to be secreted in some manner. This figure is somewhat less than the corresponding percentages previously reported for *B. malayi* [16].

Several mammalian proteins, clearly not of nematode origin, were also detected in the protein samples (data not shown). These proteins included keratin, titin and serum albumin, among others, but did not interfere with the characterization of the heartworm secretome. No bacterial proteins were present.

The initial analysis revealed a secretome consisting of 110 proteins, identified through the analysis of ~30 µg protein collected during cultivation of adult heartworms. MS/MS analysis of the same amount of protein was repeated independently on the second gel; these results confirmed the first, with 17 additional low-abundance proteins being displayed in this run; manual curation revealed that 17 of the identified proteins were duplicates. The high agreement between independent replicates of the MS/MS analysis suggests that a reliable and reasonably complete accounting of the proteins present in this sample was obtained, in consideration of the amount of protein available and the intrinsic sensitivity of the methods. The 110 proteins identified in these two experiments (Supplemental Table 1) were combined for further analysis. Using similar methods, 193 proteins were reported in the adult *B. malayi* secretome from ~ 100 µg of protein, suggesting that the procedures generated similar efficiencies of protein recovery and identification [63].

Of the 110 proteins identified in the heartworm secretome, 52 were not present in the published secretome of *B. malayi* [15-17]. The degree of relatedness of the secretome composition of these two species was higher than that of either with the secretomes of the other nematode species for which comprehensive datasets are available (not shown) [18-22]; since

these data were generated using different methods and produced quite different numbers of identified proteins from multiple developmental stages, a detailed species-species comparison is unwarranted at this time. As an example, however, the comparative data reveal that the secretomes of the filarial species are much more closely related (53% identical) than either is to *H. polygyrus*, a gastrointestinal nematode in a different clade (V *versus* III; <20% identical). It is possible to discern a set of 17 secretome proteins which are common to species that parasitize mammals, including *D. immitis* (Table 3), and so constitute a minimal consensus secretome of species from distinct clades [23] which inhabit different niches as adults. All of these proteins, except cystatin, macrophage migration inhibition factor, triosephosphate isomerase and phosphatidylethanolamine-binding protein, have also been detected in the *M. incognita* secretome [21]. The functions embodied in this list can, in general, be associated with roles in modifying host responses or in protein-release pathways. The inclusion of additional nematode species in secretome analyses will enable this list to be refined, but the available data suggest that secretome composition may be highly adapted to the site of residence of these parasites. It is interesting, in this regard, that we did not find in the *D. immitis* secretome some classes of proteins which have been reported in the secretomes of both other nematodes, including *B. malayi*, such as a variety of proteases and globins. Whether their absence from the current secretome is due to the lower amount of heartworm protein available for this analysis or to a fundamental difference in the menu of secreted proteins among these species requires additional research.

The proteins identified in common in the filariae were distributed throughout the data set. However, the shared proteins were much more likely to be among the most, as opposed to the least abundant molecules, indicating a high correlation between proteins secreted in abundance

by the two filariae. Two of the *D. immitis*-unique proteins returned no BLAST hits, leaving 50 defined proteins unique, in this context, in the *D. immitis* secretome. Of these proteins, 45 (87%) could be assigned GO terms in Blast2Go. Catalytic activity (GO:0003824) and binding (GO:0005488) were the two major molecular function categories (Figures 2 & 3), while cellular (GO:0009987) and metabolic (GO:0008152) processes were the two major biological process categories (Figures 2 & 3) for the heartworm-unique proteins. This distribution was, in general, quite conserved with that reported for proteins in the *B. malayi* secretome using similar methods [16]. Consideration of the functions of the heartworm-unique secretome did not identify any molecules with special relevance to the niche inhibited by this species, compared with that of *B. malayi*.

About 40% of the heartworm-unique proteins contained amino acid sequences that are associated with classical or non-classical secretion pathways. This figure is somewhat lower than the corresponding figure (~ 65%) reported for *B. malayi* using similar methods [16]. An explanation for this discrepancy is not readily apparent; additional data on the identification of proteins released in the host (as opposed to in culture) could resolve the biological relevance of their detection in these experiments. It should also be noted that many secreted proteins are now recognized as being released in exosomes, which represent a significant pathway in eukaryotic and prokaryotic organisms [33, 34]. Indeed, exosomes-mediated secretion events have been detected in the *C. elegans* excretory canal [35]. Evidence is not available on the anatomical localization of exosomes in parasitic nematode secretory systems, but many proteins detected in nematode secretomes, including actin, elongation factors, aldolase, enolase, HSP70 and cyclophilin, are common components of mammalian exosomes (http://www.exocarta.org/exosome_markers). A recent report identified 27 *Onchocerca ochengi*

proteins recovered from nodules [36], including many homologs of secretome proteins in other filariae. The majority of the *O. ochengi* proteins lacked secretion signals and are associated with exosomes in other organisms; these data support the relevance of the antigens detected *in vitro* and suggest that the role of exosomes, as a source of secreted proteins, warrants further investigation.

The impact of *D. immitis* infections on companion animal health and veterinary practice in endemic areas cannot be overstated. In endemic areas, the ?prevalence?incidence of infection can be as high as 20% in areas in which prophylaxis treatment is irregular [1]. The development and introduction of the highly efficacious and relatively inexpensive ML-based regimens for prophylaxis have produced one of the most successful mass drug administration programs in history. However, there are emerging concerns of resistance to the MLs most commonly used for heartworm prevention [6, 7]. Data obtained from this experiment may assist in addressing this situation in several ways. First, current methods of testing for prophylactic activity against *D. immitis* are exceptionally time-constrained, as they monitor the onset of microfilaremia in treated dogs, which occurs ~ 8 months after infection [1]. A biomarker based on abundantly secreted proteins might allow detection of worms that survive the prophylactic regimen shortly after infection, and the proteins reported here are candidates for the development of such a test. Similarly, a number of current diagnostic tests based on antigen detection have been advanced for the diagnosis of *D. immitis* infection, but all of them have some problems with sensitivity and none is associated with a reported parasite protein [37]. A legitimate goal is the development of a test that can accurately predict adult worm burdens [1, 38], which can be an important factor in deciding on a course of treatment for infected animals. A test based on the most abundantly secreted parasite proteins may be better able to fulfil that role. Antigen-based diagnostic tests for

human filarial infections have similar limitations, including the lack of well-described antigens in some tests, which have not been selected based on abundance in serum, concerns about sensitivity and an uncertain correlation with adult worm burden [39–43].

From a therapeutic standpoint, efforts to limit survival or development of heartworms with immunological interventions, such as vaccination, could be enhanced if proteins essential for the success of an infection were targeted as vaccine antigens. Previous work in this area seems to have been typically focused on parasite proteins that generated significant immune responses in dogs [45], which is not necessarily a predictor of value as a protective antigen. Instead, down-stream experimental work on the functional role of secreted proteins could identify candidates for which a strong antibody response would prevent establishment of an infection. A menu of secreted proteins, provided here, is essential for that work to proceed. This same line of reasoning suggests that the proteins identified may yield novel therapeutic targets. At least some of these secreted proteins may be critical for the survival of the parasite within the host. Any interference with their function *via* the administration of a therapeutic antibody may have a detrimental effect on the parasite's ability to remain viable, offering a possible alternative to the current arsenical-based strategy to cure established infections [1]. As the composition of the secretome varies according to life-cycle stage and sex in *B. malayi* [16, 17], it will be important to determine the contribution to the current *D. immitis* secretome from male, female and mff before advancing into new research in this area.

The data obtained from this experiment yielded some information on putative functions of these proteins, which may help to illuminate the difference between the niches exploited by the various worms whose secretomes are compiled. As *D. immitis* resides in the bloodstream of the host, it is reasonable to expect some level of difference between both the gut-dwelling *H.*

polygyrus and the lymph-residing *B. malayi*, which can be seen in these data. Of the 110 identified proteins, 52 (47%) were unique to *D. immitis* compared to the nematode secretomes compiled previously.

It is known that a number of proteins are commonly conserved across nematodes species and are from this current study are also found in *D.immitis* (Table 3). A few of the proteins characterized as unique share a common family with proteins secreted by *B. malayi*. For instance, galectin was highly abundant in the *B. malayi* secretome, but the closest homolog in the *D. immitis* secretome was a galactoside-binding lectin family protein, which presumably has a similar or related functional role. However, a BLASTp analysis revealed that the *D. immitis* genome encodes a predicted protein that is almost identical to the *B. malayi* galectin (not shown), but which was not secreted proteins. Similarly, the glutathione s-transferase 1 found in the heartworm secretome was related to a homolog identified in the *B. malayi* secretome [16-18], but the closest homolog of the *B. malayi* protein in the *D. immitis* genome (data not shown) was not found in the current study. The implications of these findings are not clear; the functional conservation of these protein families in the secretomes of the two filarial species does not account for the discrepancy in secretion of the most closely related proteins between the two species.

A hypothesis driving investigations into the composition of the *D. immitis* secretome is that at least some of them should be adapted for the task of living in blood. Protease inhibitors and proteins that detoxify oxygen radicals are likely important for any parasite in a host; candidates specifically pertinent for life the bloodstream are not readily apparent. The family of transthyretin-like proteins is highly represented in the heartworm secretome. This family is represented by a large number of genes in *C. elegans* [46], the functions of which are largely

unknown. However, transport functions have been associated with this family [44], and it would be advantageous to study their biological function in this regard in nematodes in general and tissue-dwelling species in particular.

The anatomy of secretory apparatuses in adult *D. immitis* is unknown. In general, the adult filariid secretory system is either glandular or tubular. In each kind of system, a duct links the secretory cells and opens to the exterior through a secretory pore that may be muscularly controlled [47]. In addition to a discrete secretory compartment analogous to that found in mf [6], proteins may be discharged into the medium from uterine fluid during the release of mf by females, from the release of cuticle-associated materials or from defecation of incompletely digested parasite or host proteins. Several canine proteins were detected in these samples; whether they arose from incomplete washing of the worms or from excretion *via* the faecal route cannot be concluded. The contribution of proteins secreted *versus* those excreted or discharged into the medium (e.g., intestinal waste and/or uterine fluid) could be resolved by further experiments.

This is the first report of the secretory proteome of *D. immitis*, which lives in the circulatory system rather than the lymphatic vessels (*B. malayi*). Adult *D. immitis* were collected from dogs immediately after euthanasia and cultured for 3 days in RPMI 1640 media. This media was processed and yielded 110 proteins, 52 of which have not been reported in the secretomes of any other nematodes studied to date. Although these proteins were unique, their functional categories and motifs are generally similar to those of proteins released by other nematode species.

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SEQUENCE DESCRIPTION
Phosphatidylethanolamine-binding protein
Ladder protein
Ml domain containing protein
Transthyretin-like protein 5
Glycosyl hydrolases family 31 protein
Transthyretin-like protein 5
LL20 15kda ladder antigen
Transthyretin-like protein partial
Plasma glutamate carboxypeptidase-like
NADH-dependent fumarate reductase
Cysteine protease inhibitor
Abhydrolase domain containing isoform cra_a
Transthyretin-like protein partial
Immunogenic protein 3
Exocyst complex component 2

Table 1: The 15 most abundant proteins detected in the *Dirofilaria immitis* secretome.

SEQUENCE DESCRIPTION	ABUNDANCE RANK
Abhydrolase domain containing isoform cra_a	12
Glutathione s-transferase 1	18
Elegans protein partially confirmed by transcript evidence	22
cre-pqn-85 protein	22
Nipped-b-like protein	22
Pdz domain containing protein	26
Jheh1	27
Alpha-actinin	30
Epoxide hydrolase 1	32
Protein dek isoform 1	35
Kh domain containing protein	37
Protein szt2	38
Hypothetical protein LOAG_04081 [Loa loa]	39
Elongation factor tu homologue precursor	40
Pan domain containing protein	44

Table 2: The 15 most abundant *Dirofilaria immitis* unique proteins.

Sequence Description
LL20 15 kDa ladder antigen
Enolase
Fatty acid-binding protein
Glutathione-S-transferase
Cysteine protease inhibitor/cystatin
Transthyretin family proteins
Actin
Triosephosphate isomerase
Phosphatidylethanolamine-binding protein
Protein disulfide isomerase
Heat shock protein 70
Immunogenic protein 3
Fumarase
Macrophage migration inhibition factor
Cyclophilin
Lectins (galectin/galactoside-binding protein)
Aldolase

Table 3: Proteins/protein functions generally conserved in nematode secretomes.

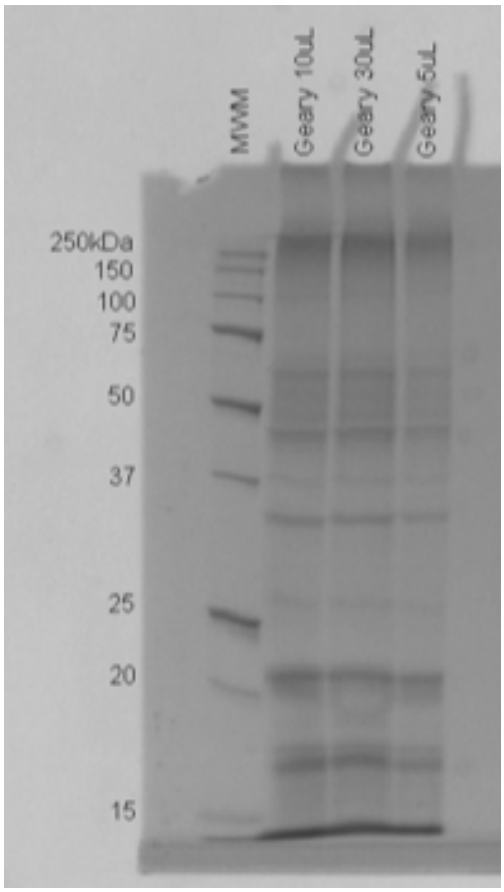


Figure 13: SDS-PAGE gel of the culture fluid collected from adult mixed sex *Dirofilaria immitis* adult worms.

A

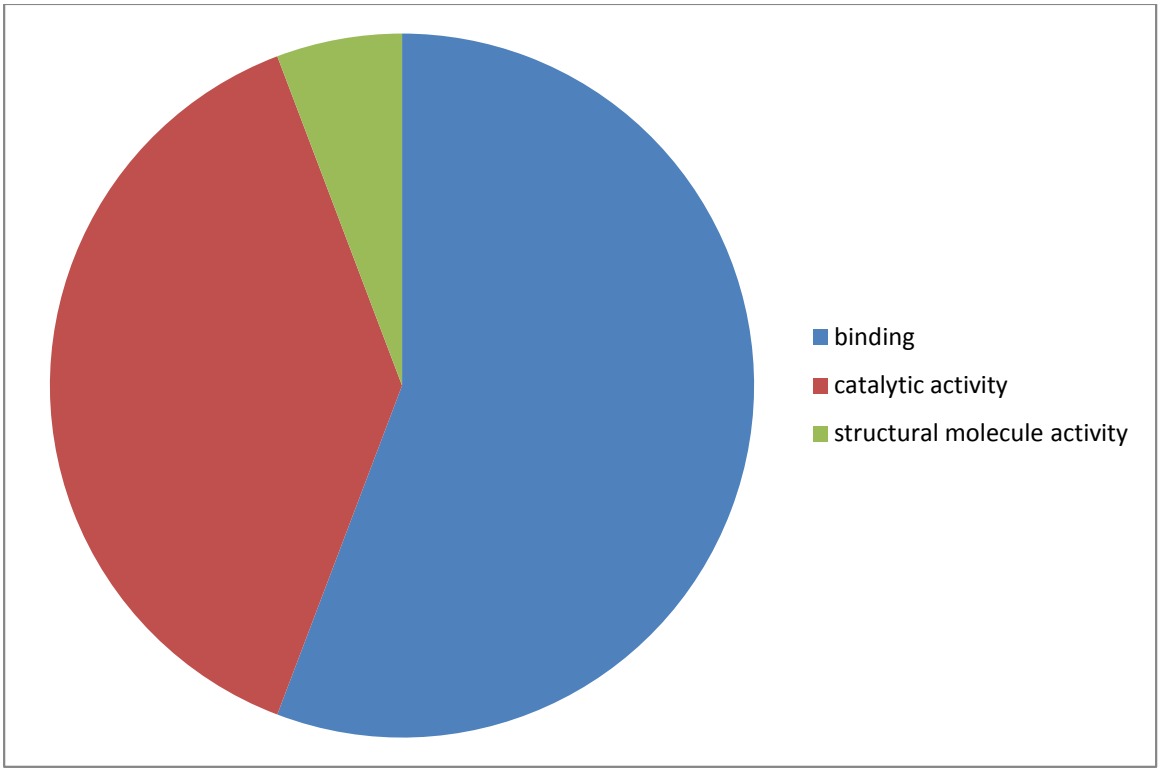
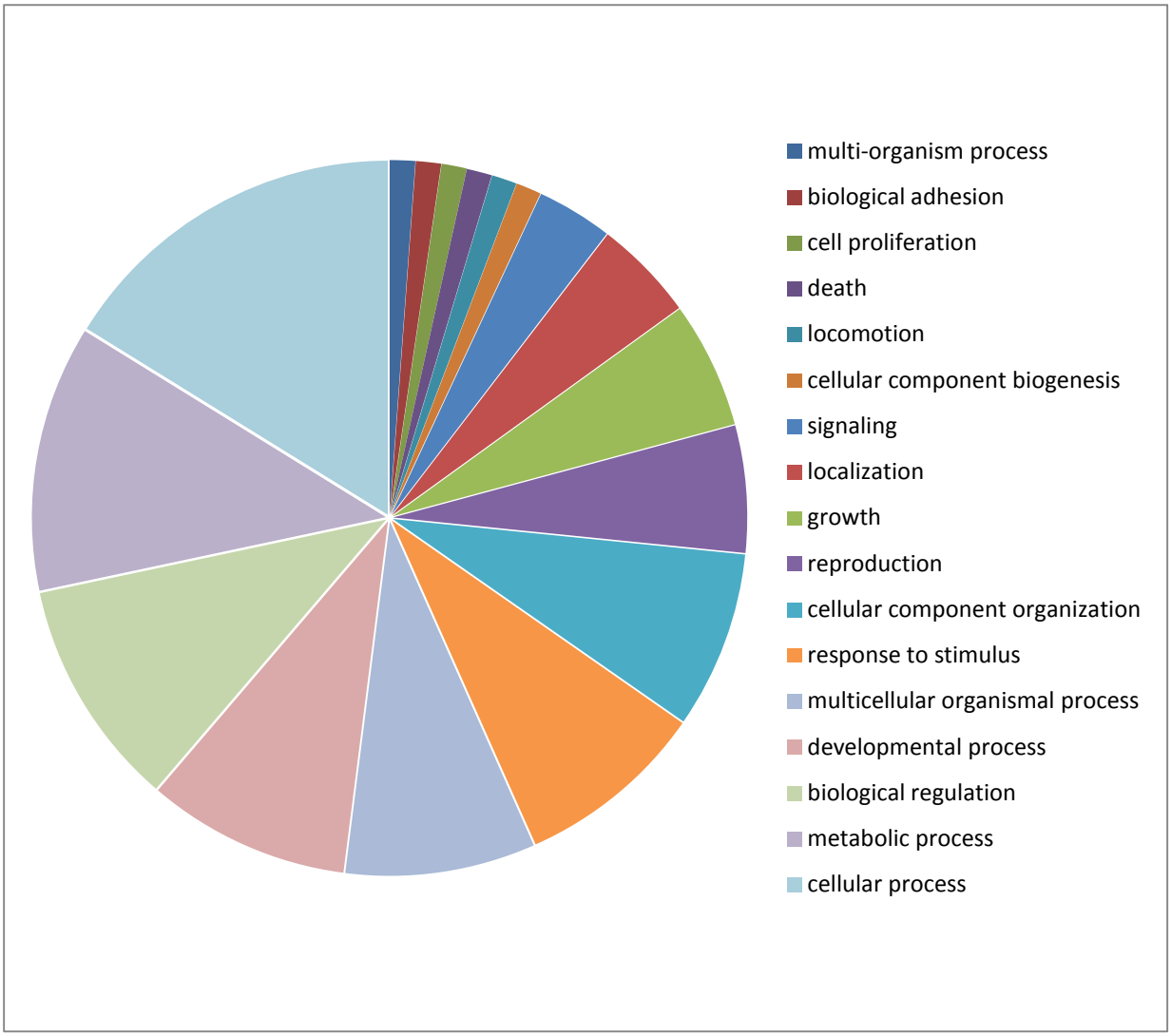


Figure 14: *Dirofilaria immitis* protein analysis: A. Distribution of the most abundant (level 2) molecular functions using GO terms. B. Distribution of the most abundant (level 2) biological process GO terms.

Figure 14 (con't)

B



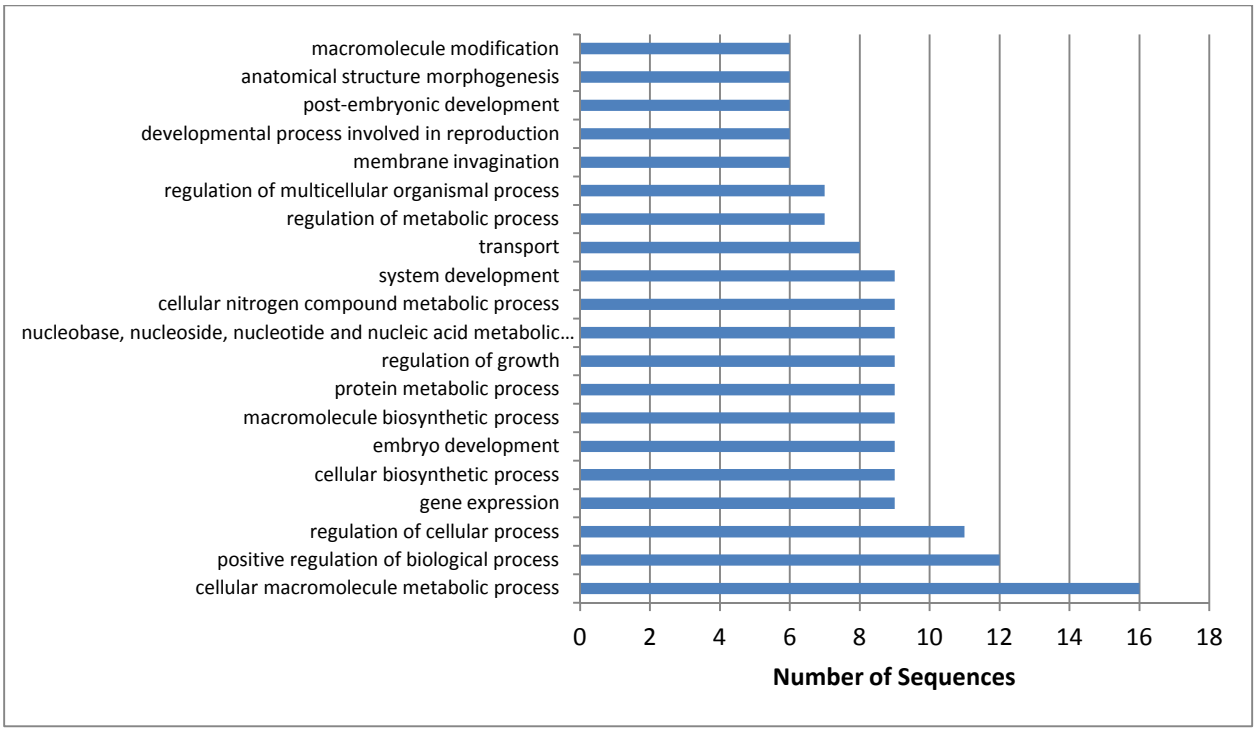


Figure 15: *Dirofilaria immitis* protein profile: Distribution of the top 20 most abundant (level 4) biological processes GO terms.

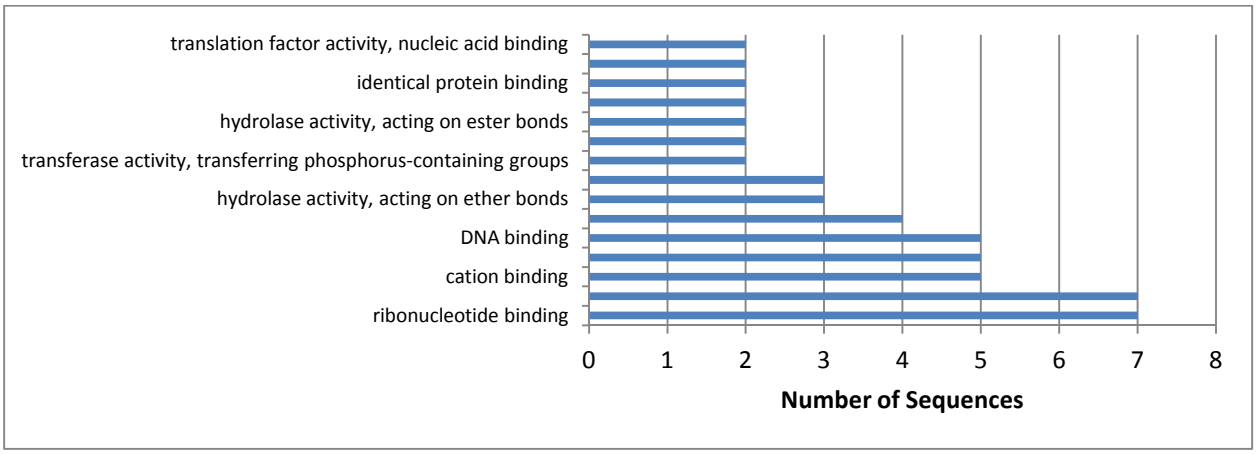


Figure 16: *Dirofilaria immitis* protein profile: Distribution of the most abundant (level 4) molecular functions GO terms.

CHAPTER 3

Characterizing *Onchocerca volvulus* Nodules

Developing accurate, quantifiable tests of parasite viability, both under drug pressure and free from drug pressure must be a priority for assessing the efficacy of new drugs and evaluating the pathways they act upon. The same is true for understanding the current drugs used to treat filarial nematode infections.

Many major investigative activities, such as assessing the effects of macrofilaricides and the immune system on the complicated parasites, depend most commonly on assessment of their morphological status. The morphological assessment of the viability and the stages of degeneration of mature filarial worms however is a difficult and often very subjective process. The use of in situ molecular markers by immunocytochemistry has been used by a number of investigators often with reagents that are directed against mammalian antigens rather than using those that have been clearly defined as nematode constituents. We have identified markers that appear to have homologous presence and function in both mammals and nematodes. These immunocytochemical reagents directed against putative nematode components of cellular metabolism and replication have been used to reflect the effects of Ivermectin on adult *Onchocerca volvulus*. The presence of these markers can be quantitated and provide more objective data as to the status of adult worms than has been previously used. The results from the use of these markers suggest that the long-term use of Ivermectin has a general depressive effect on the health and in all likelihood the longevity of the worm. This approach to assessing worm viability and degenerative status is believed to be suitable for general use and allows this

important assessment activity to be carried out by a wider range of scientists than only those with extensive parasistological knowledge.

O. volvulus is a parasitic filarial nematode that infects humans and is the etiologic agent of river blindness. There are an estimated 37 million currently infected with at least 300 thousand permanently blinded. It has an indirect lifecycle and is transmitted through the bite by a black fly of the genus *Simulium*. Current control programs aim to interrupt transmission by mass administration of the macrocyclic lactone Ivermectin. Ivermectin is primarily a larvacidal. However, it induces in the adult females embryostasis, the cessation of the release of microfilariae. The nodules used in this experiment have been previously described and characterized [2]. Many procedures have been developed to characterize and describe nodules in specific relevance to treatment status and worm health [28, 94]. Our attempt utilizes a computer program to analyze untreated and treated worm sections to determine if there is a statistically significant difference between the two in the expression of NRas.

A BLAST search of the *Brugia malayi* proteome [95] was undertaken to establish the presence of a homologous protein to human NRas. This search identified Ras protein Let-60. A global sequence alignment using lalign [96], was performed to investigate the homology between the human and worm proteins to determine how likely a polyclonal anti-NRas antibody raised to the whole protein would cross-react with the worm Let-60. Western blots were performed to demonstrate the cross-reactivity of the human antibody to worm NRas. That blot is shown in figure 17. The global sequence alignments are shown in figs 18 and 19.

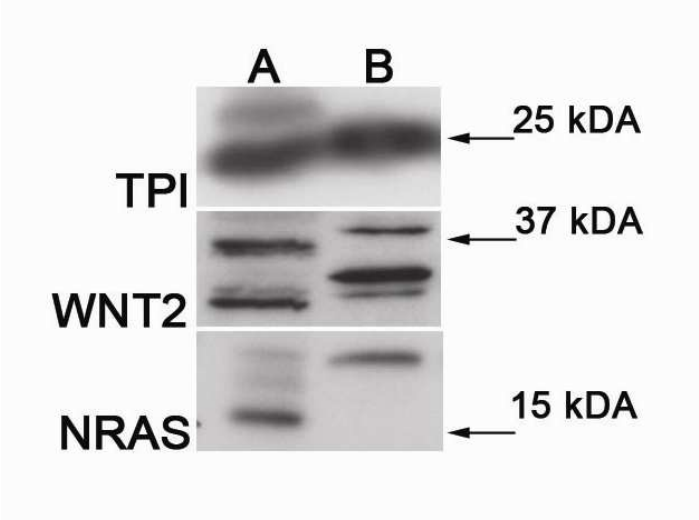


Figure 17: Western blot of 3 proteins, including NRas. Lane A is *C. elegans* protein extracts, lane B is a commercially purchased ladder.

```
 10        20        30        40        50
Brugia ---MTEYKLVVVGDGGVGKSALTIQLIQNHFVEEYDPTIEDSYRKQVVIDGETCLLDILD
          :::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C_     KRVMTEYKLVVVGDGGVGKSALTIQLIQNHFVEEYDPTIEDSYRKQVVIDGETCLLDILD
               10        20        30        40        50        60

         60        70        80        90       100       110
Brugia TAGQEEYSAMRDQYMRTGEGFLLVFAVNEAKSFENVTQYREQIRRVKDSDEVPMVLVGNK
       :::::::::::::::::::::::::::::::::::::::::..::.:::::::::.:::::::::
C_     TAGQEEYSAMRDQYMRTGEGFLLVFAVNEAKSFENVANYREQIRRVKDSDDVPMVLVGNK
               70        80        90       100       110       120

       120       130       140       150       160       170
Brugia CDLAQRTVESRAILDASRSIGMPAVETSAKTRMGVDDAFYTLVREIRKHKEKQ-CIKPRK
       ::::.:.:. :.. ..... :.: :.:::::::::.:::::::::::::.:..    ::.:
C_     CDLSSRSVDTRTVSETAKGYGIPNVDTSAKTRMGVDEAFYTLVREIRKHRERHDNNKPQK
              130       140       150       160       170       180

         180
Brugia KRKCVII
       :.:: :.
C_     KKKCQIM
```

Figure 18: Global sequence alignment of *B. malayi* Let-60 EDP31853.1 to *Caenorhabditis elegans* Ras protein AAA28103.1.

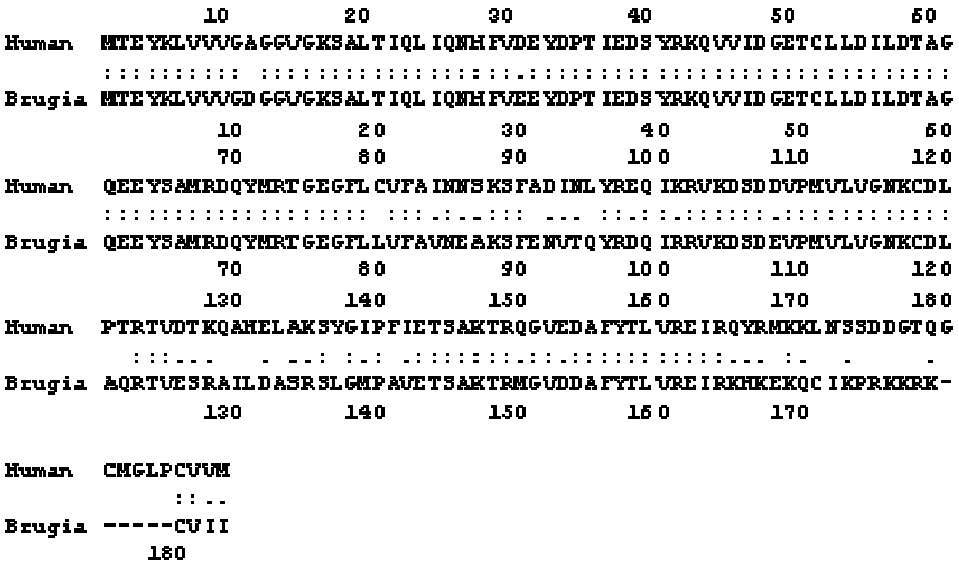


Figure 19: Global sequence alignment of *B. malayi* Let-60 EDP31853.1 to human NRas NP_002515.1.

Fixed, paraffin-embedded nodules were sectioned and stained with a polyclonal goat anti-human NRas antibody. The sectioned nodules were analyzed by JG to quantify NRas staining using ImagePro (Media Cybernetics, Bethesda, MD). A photograph taken of each section was first converted to an 8-bit grayscale image. To control for the fixation process and any differences inherent thereof, 5 host plasma cells from each tissue section were selected and quantified. Five worms were selected around the geometric center of each nodule. For each worm custom Area of Interest (AOI) were drawn to include only the hypodermis of each worm. These AOIs were then analyzed using a bitmap. A selection of the images used in this experiment is shown in figs 21 through 28. The bitmap analysis was exported to Microsoft Excel where a mean was calculated. This mean for the worm tissue was compared against the mean of the 5 host plasma cells to generate a ratio. This ratio was compared against a ratio developed in the identical way for Ivermectin treated worms using an unpaired t-test.

The difference between treated and untreated worms was found to be statistically significant with a p value of 0.01. Figure 20 shows the difference between the two groups. This difference was manifested with treated worms staining less darkly than untreated worms. A total of 15 worms were examined from each group.

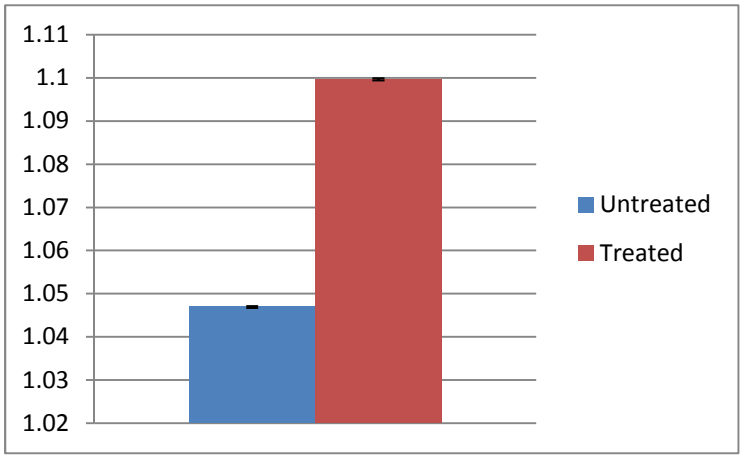


Figure 20: Bar graph showing a statistically significant difference between the staining of Ivermectin treated and untreated worms.

Detecting a statistically significant difference between the untreated and Ivermectin treated worms suggests this NRas stain may be a useful marker in assessing the viability of filarial parasites. As a visual comparison it is interesting to note a high degree of difference between the *Wolbachia* staining in untreated and Ivermectin treated worms suggesting Ivermectin may induce a decrease in *Wolbachia* metabolic activity, either through starving the bacteria of essential nutrients or affecting the worm in such a way as to signal a decrease in *Wolbachia* metabolic activity.

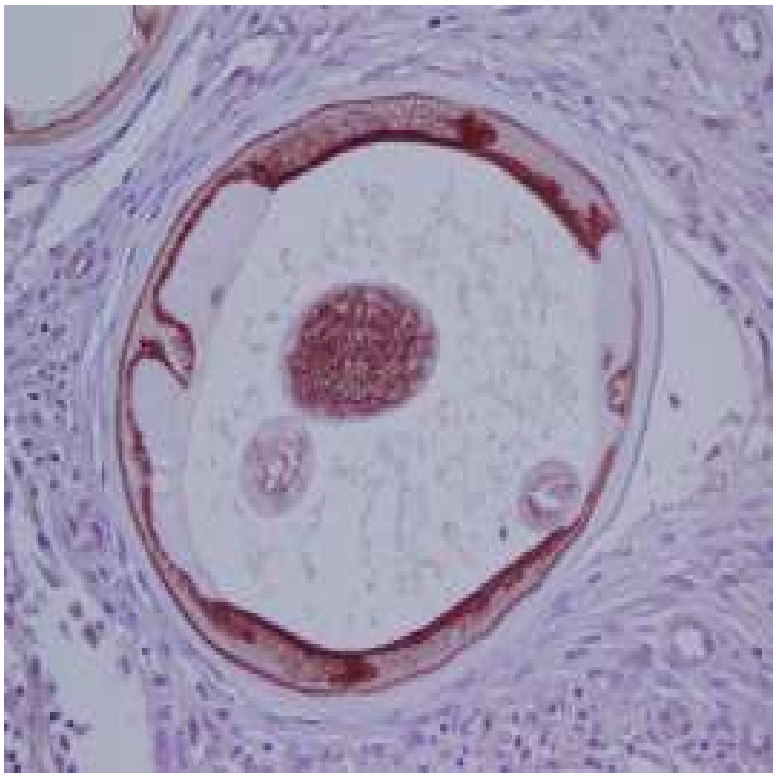


Figure 21: Image of an untreated adult female *O. volvulus*.

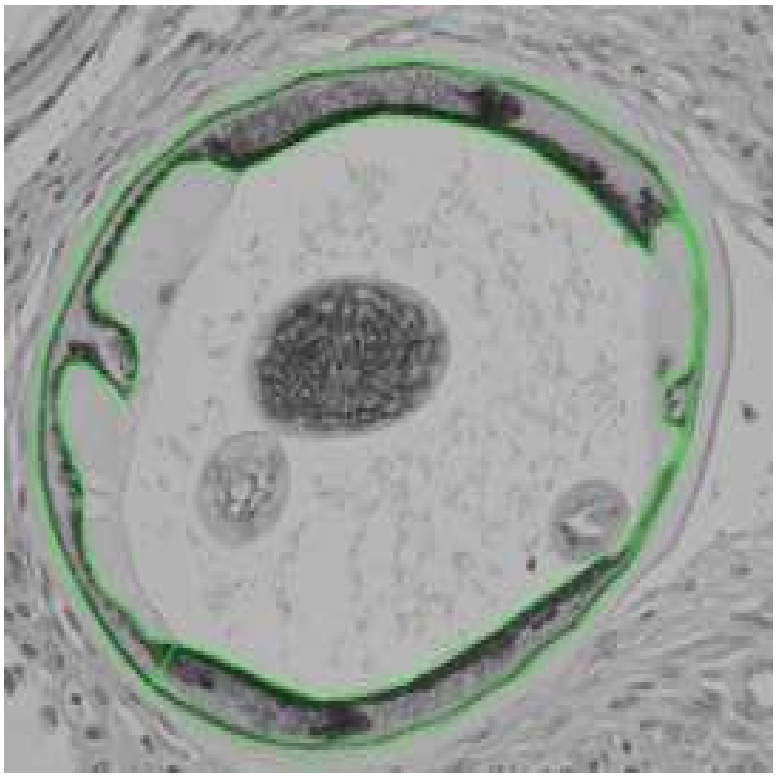


Figure 22: Grayscale image of the above figure showing custom AOI drawn to include only the hypodermis.

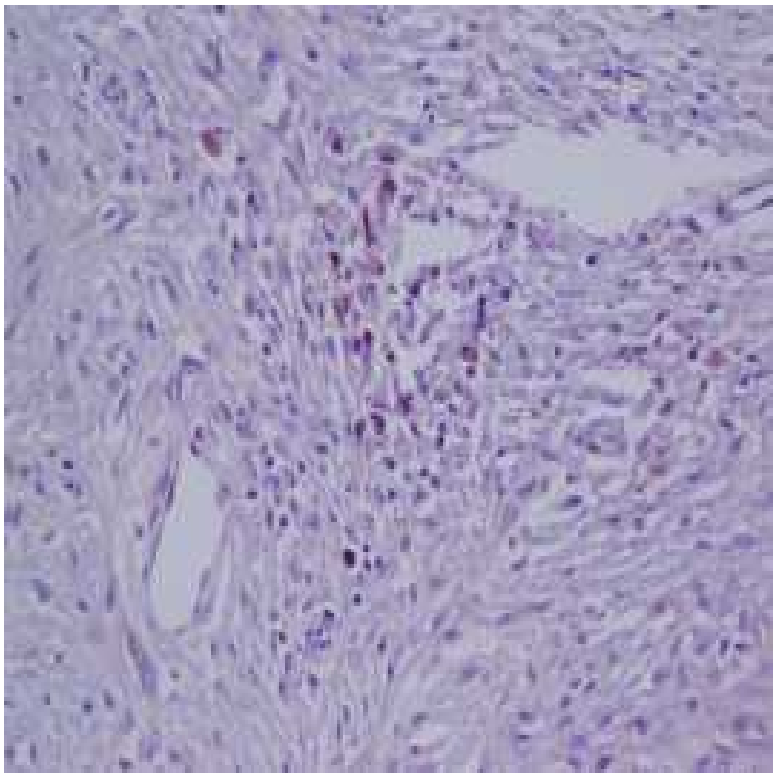


Figure 23: Image of host plasma cells from an untreated nodule.

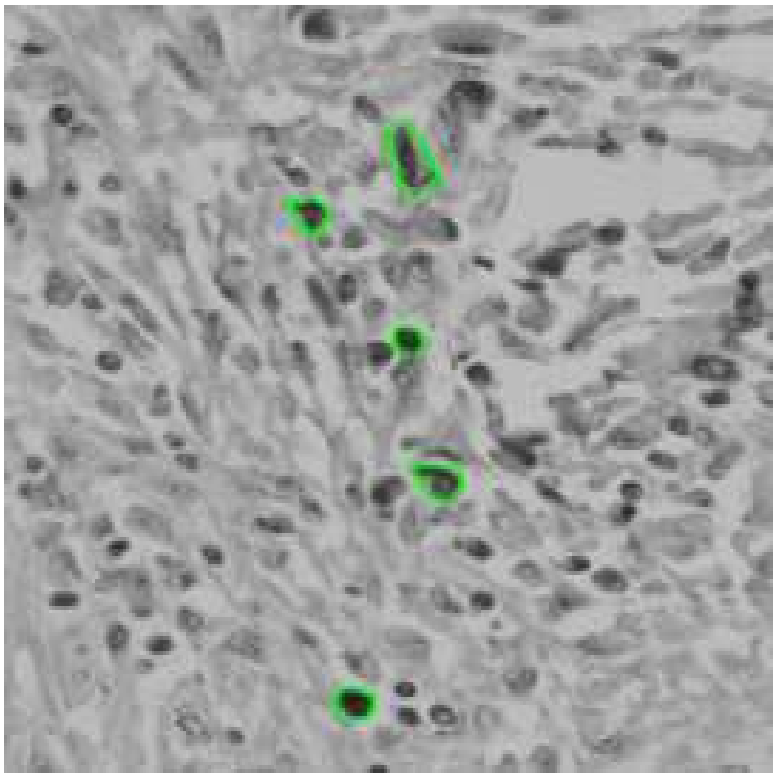


Figure 24: Grayscale image of above figure showing custom AOIs drawn around 5 host plasma cells.

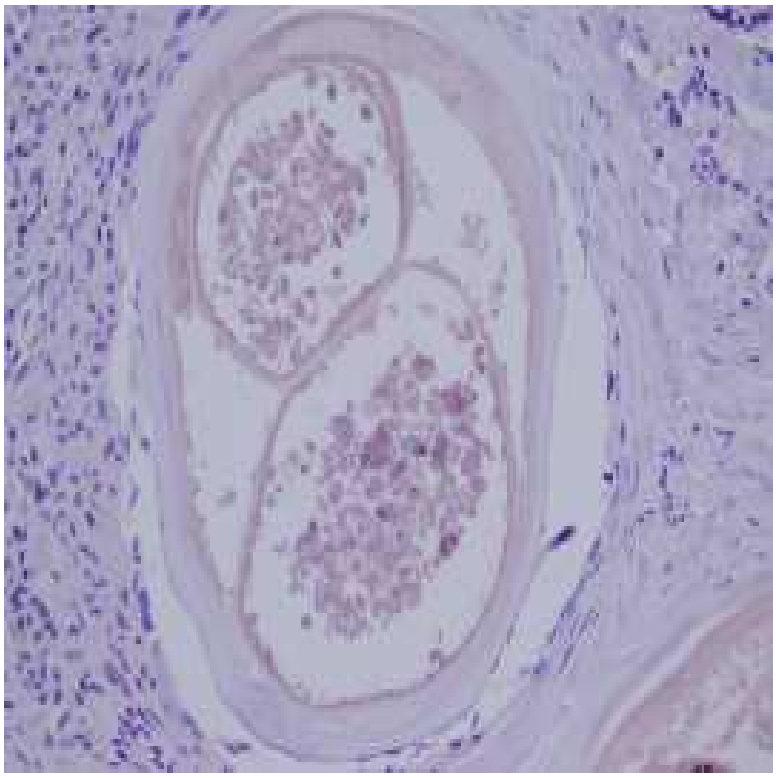


Figure 25: Image of an Ivermectin treated adult female *O. volvulus*.

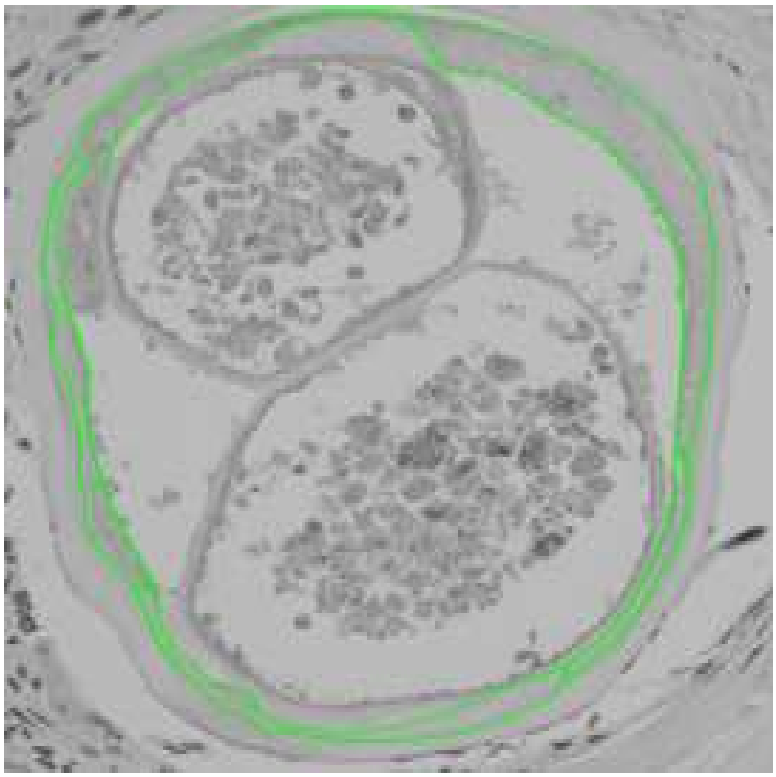


Figure 26: Grayscale image of the above figure showing the custom AOI drawn to only include the hypodermis.

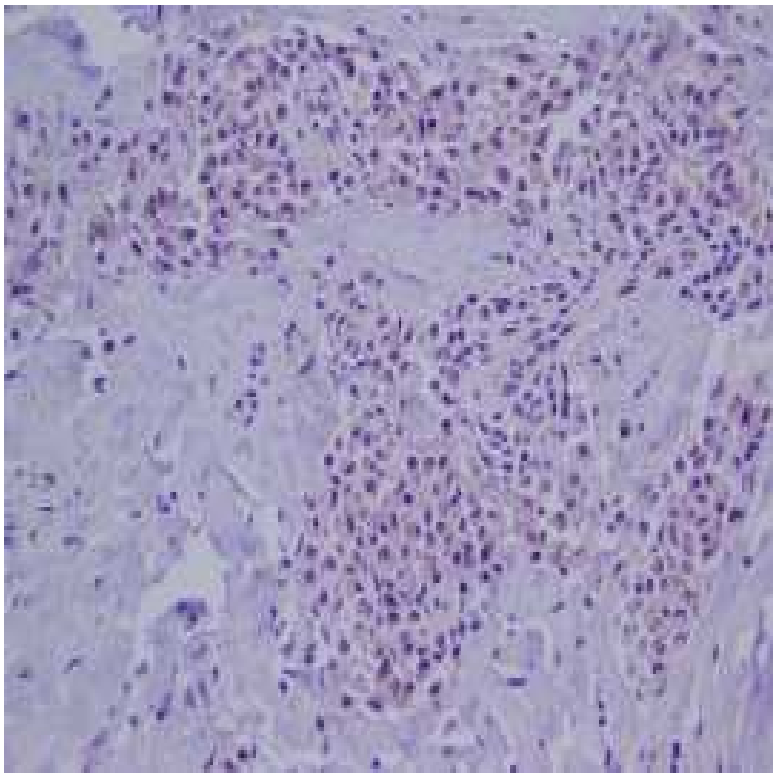


Figure 27: Image of host plasma cells from an Ivermectin treated nodule.

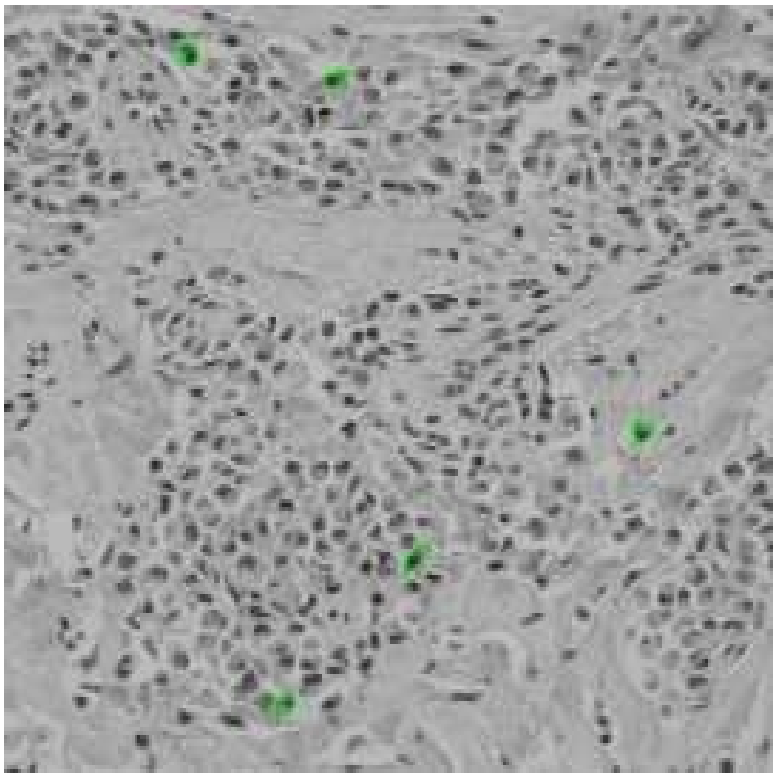


Figure 28: Grayscale image of the above figure showing custom AOIs drawn around 5 host plasma cells.

CHAPTER 4

Discussion

In the first report of the *D. immitis* secretome a total of 110 proteins were detected from both male and female worms cultured together. This is somewhat less than the reported secretome for *B. malayi* that most closely matches the procedure [63]. However, the sample was almost half the size of that reported in [62]. Given the current understanding of LC/MS MS increasing the sample size should increase the detection of proteins of low abundance. When analyzing the secretome it became apparent that the filarial nematode secretomes are quite similar in their most abundantly secreted proteins. The 10 most abundantly secreted proteins were conserved among the filarial nematodes with abundance position 11 the first noted departure from that trend. In addition, several proteins or protein functions were conserved through the several different secretomes [60-67]. This information offers a suggestion as to the functional implications of these proteins and protein functions. It may be that they are required for successful survival within a host, given that of an animal or a plant. The differences noted in the secretome of *D. immitis* when compared to that of *B. malayi* may also offer suggestions as to the different proteins required to survive in blood as opposed to lymph. The successful completion of characterizing the secretome, as presented in chapter 2, has enabled a wider understanding of *D. immitis*.

Understanding the functional implications of secreted proteins should offer clues as to the ability of the parasite to evade host immune responses [90]. In analyzing the secretome attention should also be paid to potential immunotherapeutic targets, not just in curing the infection but also which proteins may influence the immune system and what uses that may have in treating

chronic inflammatory diseases. This area is not new but it offers an interesting perspective on the progression of filarial infections and what is required by the parasites to successfully establish an infection and survive within the host.

In chapter 3, a process to utilize molecular markers was detailed. While not found in any of the secretomes, from which molecular markers were generated, the NRas homolog was found in the *B. malayi* genome and proteome. When the sequences were compared they were found to be remarkably well conserved. This level of conservation not only suggested the likelihood of cross-reactivity but also perhaps a functional conservation as well. This would implicate Let-60 to act in a manner similar to NRas. The work detailed in chapter 3 demonstrates the robustness of molecular markers and their diverse uses, in this case, using NRas to mark the effects of Ivermectin treatment on *O. volvulus* in nodules. Using NRas to characterize the health of the worm brought to light some possible metabolic effects of exposure to Ivermectin. Though Ivermectin is known to paralyze neuromusculature of the nematode the nematode still acquires enough nutrients to persist for over a decade in the host. However, it does not appear that the nematode acquires enough nutrients to continue normal life processes.

A different take on the NRas staining is a negative feedback loop within the biology of the nematode. Perhaps, when exposed to Ivermectin the effects signal the worm to slow down metabolism, growth and development. This should manifest as a decrease in staining intensity.

Understanding that decrease in staining intensity may help to illuminate the role that NRas/Let-60 plays in the nematode and may lead to more information about the effects of Ivermectin outside of the accepted neuromuscular paralysis.

REFERENCES

REFERENCES

1. Osei-Atweneboana, M.Y., et al., *Prevalence and intensity of Onchocerca volvulus infection and efficacy of Ivermectin in endemic communities in Ghana: a two-phase epidemiological study.* Lancet, 2007. **369**(9578): p. 2021-9.

2. Cupp, E.W., et al., *The effects of long-term community level treatment with Ivermectin (Mectizan) on adult Onchocerca volvulus in Latin America.* Am J Trop Med Hyg, 2004. **71**(5): p. 602-7.

3. McCall, J.W., et al., *Heartworm disease in animals and humans.* Adv Parasitol, 2008. **66**: p. 193-285.

4. Bourguinat, C., et al., *Macrocyclic lactone resistance in Dirofilaria immitis.* Vet Parasitol. **181**(2-4): p. 388-92.

5. Geary, T.G., C. Bourguinat, and R.K. Prichard, *Evidence for macrocyclic lactone anthelmintic resistance in Dirofilaria immitis.* Top Companion Anim Med. **26**(4): p. 186-92.

6. Foreyt, W.J., *Diagnostic parasitology.* Vet Clin North Am Small Anim Pract, 1989. **19**(5): p. 979-1000.

7. Tanaka, K.I. and R.B. Atwell, *Immunoperoxidase staining characteristics of Dirofilaria immitis in the dog.* Res Vet Sci, 1991. **50**(1): p. 33-7.

8. Weil, G.J. and R.M. Ramzy, *Diagnostic tools for filariasis elimination programs.* Trends Parasitol, 2007. **23**(2): p. 78-82.

9. Wharton, D., *A Functional Biology of Nematodes*. 1986, Baltimore, Maryland: The Johns Hopkins University Press.

10. Blaxter, M.L., et al., *Nematode surface coats: actively evading immunity.* Parasitol Today, 1992. **8**(7): p. 243-7.

11. Girard, L.R., et al., *WormBook: the online review of Caenorhabditis elegans biology.* Nucleic Acids Res, 2007. **35**(Database issue): p. D472-5.

12. McCall, J.W., et al., *Heartworm and Wolbachia: therapeutic implications.* Vet Parasitol, 2008. **158**(3): p. 204-14.

13. Rossi, M.I., et al., *Effects of doxycycline on the endosymbiont Wolbachia in Dirofilaria immitis (Leidy, 1856)--naturally infected dogs.* Vet Parasitol. **174**(1-2): p. 119-23.

14. Jiang, D., P.U. Fischer, and G. J.Weil, *Use of in situ Hybridization to Localize Wolbachia during Embryogenesis in Brugia malayi.* Journal of Bacteriology and Parasitology, 2011. **2**(112).

15. Pfarr, K. and A. Hoerauf, *The annotated genome of Wolbachia from the filarial nematode Brugia malayi: what it means for progress in antifilarial medicine.* PLoS Med, 2005. **2**(4): p. e110.

16. Foster, J., et al., *The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode.* PLoS Biol, 2005. **3**(4): p. e121.

17. Wu, B., et al., *The heme biosynthetic pathway of the obligate Wolbachia endosymbiont of Brugia malayi as a potential anti-filarial drug target.* PLoS Negl Trop Dis, 2009. **3**(7): p. e475.

18. Pearlman, E. and I. Gillette-Ferguson, *Onchocerca volvulus, Wolbachia and river blindness.* Chem Immunol Allergy, 2007. **92**: p. 254-65.

19. Brattig, N.W., *Pathogenesis and host responses in human onchocerciasis: impact of Onchocerca filariae and Wolbachia endobacteria.* Microbes Infect, 2004. **6**(1): p. 113-28.

20. Kramer, L., et al., *Evaluation of lung pathology in Dirofilaria immitis-experimentally infected dogs treated with doxycycline or a combination of doxycycline and Ivermectin before administration of melarsomine dihydrochloride.* Vet Parasitol. **176**(4): p. 357-60.

21. Cheville, N., *Introduction to Veterinary Pathology*. 3 ed. 2006, Ames: Blackwell Publishing.

22. Lobeck, C. and K.S. Latimer. [cited 2012; Available from: http://www.vet.uga.edu/vpp/clerk/Lobeck/index.php.

23. Njenga, S.M. and C.N. Wamae, *Evaluation of ICT filariasis card test using whole capillary blood: comparison with Knott's concentration and counting chamber methods.* J Parasitol, 2001. **87**(5): p. 1140-3.

24. Atkins, C.E., *Comparison of results of three commercial heartworm antigen test kits in dogs with low heartworm burdens.* J Am Vet Med Assoc, 2003. **222**(9): p. 1221-3.

27. Geary, T.G., et al., *Unresolved issues in anthelmintic pharmacology for helminthiases of humans.* Int J Parasitol. **40**(1): p. 1-13.

28. Duke, B.O., et al., *Effects of three-month doses of Ivermectin on adult Onchocerca volvulus.* Am J Trop Med Hyg, 1992. **46**(2): p. 189-94.

29. Wolstenholme, A.J. and A.T. Rogers, *Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics.* Parasitology, 2005. **131 Suppl**: p. S85-95.

30. 2006 [cited; Available from: http://www.stanford.edu/class/humbio103/ParaSites2006/Scabies/Management&Treatment.html.

31. 2009 [cited; Available from: http://en.wikipedia.org/wiki/Milbemycin_oxime.

32. McCavera, S., et al., *An Ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode Haemonchus contortus.* Mol Pharmacol, 2009. **75**(6): p. 1347-55.

33. Moreno, Y., et al., *Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of Brugia malayi.* Proc Natl Acad Sci U S A. **107**(46): p. 20120-5.

34. Bourguinat, C., et al., *Analysis of the mdr-1 gene in patients co-infected with Onchocerca volvulus and Loa loa who experienced a post-Ivermectin serious adverse event.* Am J Trop Med Hyg. **83**(1): p. 28-32.

35. Bourguinat, C., et al., *Correlation between loss of efficacy of macrocyclic lactone heartworm anthelmintics and P-glycoprotein genotype.* Vet Parasitol. **176**(4): p. 374-81.

36. Bourguinat, C., et al., *P-glycoprotein-like protein, a possible genetic marker for Ivermectin resistance selection in Onchocerca volvulus.* Mol Biochem Parasitol, 2008. **158**(2): p. 101-11.

37. Portillo, V., S. Jagannathan, and A.J. Wolstenholme, *Distribution of glutamate-gated chloride channel subunits in the parasitic nematode Haemonchus contortus.* J Comp Neurol, 2003. **462**(2): p. 213-22.

38. Lacey, E., *The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles.* Int J Parasitol, 1988. **18**(7): p. 885-936.

39. Borgers, M., et al., *Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells.* Am J Vet Res, 1975. **36**(08): p. 1153-66.

40. Martin, R.J., A.P. Robertson, and H. Bjorn, *Target sites of anthelmintics.* Parasitology, 1997. **114 Suppl**: p. S111-24.

41. GlaxoSmithKline. 2009 [cited 2012; Available from: http://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=13022.

42. Edgar181. 2009 [cited; Available from: http://en.wikipedia.org/wiki/File:Flubendazole.png.

43. Böhm, K.J. 2007 [cited; Available from: http://www.fli-leibniz.de/~kboehm/Tubulin.html.

44. von Samson-Himmelstjerna, G., *Molecular diagnosis of anthelmintic resistance.* Vet Parasitol, 2006. **136**(2): p. 99-107.

45. Diawara, A., et al., *Assays to detect beta-tubulin codon 200 polymorphism in Trichuris trichiura and Ascaris lumbricoides.* PLoS Negl Trop Dis, 2009. **3**(3): p. e397.

46. Geary, T.G. and C.D. Mackenzie, *Progress and challenges in the discovery of macrofilaricidal drugs.* Expert Rev Anti Infect Ther. **9**(8): p. 681-95.

47. Abba, K., S. Ramaratnam, and L.N. Ranganathan, *Anthelmintics for people with neurocysticercosis.* Cochrane Database Syst Rev, (1): p. CD000215.

48. Mackenzie, C.D. and T.G. Geary, *Flubendazole: a candidate macrofilaricide for lymphatic filariasis and onchocerciasis field programs.* Expert Rev Anti Infect Ther. **9**(5): p. 497-501.

49. Seputiene, V., et al., *Tigecycline - how powerful is it in the fight against antibiotic-resistant bacteria?* Medicina (Kaunas). **46**(4): p. 240-8.

50. Hoerauf, A., et al., *Depletion of wolbachia endobacteria in Onchocerca volvulus by doxycycline and microfilaridermia after Ivermectin treatment.* Lancet, 2001. **357**(9266): p. 1415-6.

51. Hoerauf, A., et al., *Wolbachia endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study.* Med Microbiol Immunol, 2008. **197**(3): p. 295-311.

52. Turner, J.D., et al., *Macrofilaricidal activity after doxycycline only treatment of Onchocerca volvulus in an area of Loa loa co-endemicity: a randomized controlled trial.* PLoS Negl Trop Dis. **4**(4): p. e660.

53. Dimech, A. 2012 [cited; Available from: http://www.adonline.id.au/terminatorseeds/genetic-use-restriction-technology/.

54. Ju. 2012 [cited; Available from: http://en.wikipedia.org/wiki/Doxycycline.

55. Slatko, B.E., M.J. Taylor, and J.M. Foster, *The Wolbachia endosymbiont as an anti-filarial nematode target.* Symbiosis. **51**(1): p. 55-65.

56. McCall, J.W., et al., *Clinical prophylactic activity of melarsomine dihydrochloride (RM 340) against Dirofilaria immitis in heartworm-naive beagles exposed to natural infection in three southeastern states.* Vet Parasitol, 1994. **55**(3): p. 205-19.

57. McTier, T.L., et al., *Use of melarsomine dihydrochloride (RM 340) for adulticidal treatment of dogs with naturally acquired infections of Dirofilaria immitis and for clinical prophylaxis during reexposure for 1 year.* Vet Parasitol, 1994. **55**(3): p. 221-33.

58. Casarett Louis J, D.J., Klaassen Curtis D ,Watkins John Barr *Casarett and Doull's Essentials of Toxicology*. 2003, New York: McGraw Hill.

59. 2008 [cited; Available from: http://www.lookchem.com/cas-891/89141-50-4.html.

60. Moreno, Y., et al., *Proteomic analysis of excretory-secretory products of Heligmosomoides polygyrus assessed with next-generation sequencing transcriptomic information.* PLoS Negl Trop Dis. **5**(10): p. e1370.

61. Bennuru, S., et al., *Stage-specific proteomic expression patterns of the human filarial parasite Brugia malayi and its endosymbiont Wolbachia.* Proc Natl Acad Sci U S A. **108**(23): p. 9649-54.

62. Hewitson, J.P., et al., *The secretome of the filarial parasite, Brugia malayi: proteomic profile of adult excretory-secretory products.* Mol Biochem Parasitol, 2008. **160**(1): p. 8-21.

63. Moreno, Y. and T.G. Geary, *Stage- and gender-specific proteomic analysis of Brugia malayi excretory-secretory products.* PLoS Negl Trop Dis, 2008. **2**(10): p. e326.

64. Hewitson, J.P., et al., *Proteomic analysis of secretory products from the model gastrointestinal nematode Heligmosomoides polygyrus reveals dominance of venom allergen-like (VAL) proteins.* J Proteomics. **74**(9): p. 1573-94.

65. Mulvenna, J., et al., *Proteomics analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, Ancylostoma caninum.* Mol Cell Proteomics, 2009. **8**(1): p. 109-21.

66. Bellafiore, S., et al., *Direct identification of the Meloidogyne incognita secretome reveals proteins with host cell reprogramming potential.* PLoS Pathog, 2008. **4**(10): p. e1000192.

67. Soblik, H., et al., *Life cycle stage-resolved proteomic analysis of the excretome/secretome from Strongyloides ratti--identification of stage-specific proteases.* Mol Cell Proteomics. **10**(12): p. M111 010157.

68. Shevchenko, A., et al., *Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels.* Anal Chem, 1996. **68**(5): p. 850-8.

69. Nesvizhskii, A.I., et al., *A statistical model for identifying proteins by tandem mass spectrometry.* Anal Chem, 2003. **75**(17): p. 4646-58.

70. Conesa, A., et al., *Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research.* Bioinformatics, 2005. **21**(18): p. 3674-6.

71. Mulder, N.J., et al., *New developments in the InterPro database.* Nucleic Acids Res, 2007. **35**(Database issue): p. D224-8.

72. Myhre, S., et al., *Additional gene ontology structure for improved biological reasoning.* Bioinformatics, 2006. **22**(16): p. 2020-7.

73. Petersen, T.N., et al., *SignalP 4.0: discriminating signal peptides from transmembrane regions.* Nat Methods. **8**(10): p. 785-6.

74. Rada, B. and T.L. Leto, *Oxidative innate immune defenses by Nox/Duox family NADPH oxidases.* Contrib Microbiol, 2008. **15**: p. 164-87.

75. Halliwell, B., *Phagocyte-derived reactive species: salvation or suicide?* Trends Biochem Sci, 2006. **31**(9): p. 509-15.

76. 2001 [cited; Available from: http://www.noiseandhealth.org/showbackissue.asp?issn=1463-1741;year=2001;volume=3;issue=11;month=April-June.

77. Bolanowski, M.A., L.A. Jacobson, and R.L. Russell, *Quantitative measures of aging in the nematode Caenorhabditis elegans: II. Lysosomal hydrolases as markers of senescence.* Mech Ageing Dev, 1983. **21**(3-4): p. 295-319.

78. Artal-Sanz, M. and N. Tavernarakis, *Mechanisms of aging and energy metabolism in Caenorhabditis elegans.* IUBMB Life, 2008. **60**(5): p. 315-22.

79. Antebi, A., *Genetics of aging in Caenorhabditis elegans.* PLoS Genet, 2007. **3**(9): p. 1565-71.

80. Russell, R.L. and R.I. Seppa, *Genetic and environmental manipulation of aging in Caenorhabditis elegans.* Basic Life Sci, 1987. **42**: p. 35-48.

81. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome.* Nature, 2009. **458**(7239): p. 719-24.

82. Scott, A.M., J.D. Wolchok, and L.J. Old, *Antibody therapy of cancer.* Nat Rev Cancer. **12**(4): p. 278-87.

83. Ajeganova, S., et al., *Effect of biological therapy on levels of atheroprotective antibodies against phosphorylcholine and apolipoproteins in rheumatoid arthritis - a one year study.* Clin Exp Rheumatol. **29**(6): p. 942-50.

84. Hoch, H. and K. Strickland, *Canine and feline dirofilariasis: life cycle, pathophysiology, and diagnosis.* Compend Contin Educ Vet, 2008. **30**(3): p. 133-40; quiz 141.

85. Bowman, D.D. and C. Mannella, *Macrocyclic Lactones and Dirofilaria immitis Microfilariae.* Top Companion Anim Med. **26**(4): p. 160-72.

86. Lok, J.B., et al., *Studies of reproductive competence in male Dirofilaria immitis treated with milbemycin oxime.* Trop Med Parasitol, 1995. **46**(4): p. 235-40.

87. McCall, J.W., *The safety-net story about macrocyclic lactone heartworm preventives: a review, an update, and recommendations.* Vet Parasitol, 2005. **133**(2-3): p. 197-206.

88. Maizels, R.M., et al., *Helminth parasites--masters of regulation.* Immunol Rev, 2004. **201**: p. 89-116.

89. Harnett, W. and M.M. Harnett, *Therapeutic immunomodulators from nematode parasites.* Expert Rev Mol Med, 2008. **10**: p. e18.

90. Hewitson, J.P., J.R. Grainger, and R.M. Maizels, *Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity.* Mol Biochem Parasitol, 2009. **167**(1): p. 1-11.

91. Blaxter, M.L., *Nematoda: genes, genomes and the evolution of parasitism.* Adv Parasitol, 2003. **54**: p. 101-95.

92. Gioia, G., et al., *Highly sensitive multiplex PCR for simultaneous detection and discrimination of Dirofilaria immitis and Dirofilaria repens in canine peripheral blood.* Vet Parasitol. **172**(1-2): p. 160-3.

93. Bendtsen, J.D., et al., *Feature-based prediction of non-classical and leaderless protein secretion.* Protein Eng Des Sel, 2004. **17**(4): p. 349-56.

94. Specht, S., et al., *Criteria for the differentiation between young and old Onchocerca volvulus filariae.* Parasitol Res, 2009. **105**(6): p. 1531-8.

95. Ghedin, E., et al., *Draft genome of the filarial nematode parasite Brugia malayi.* Science, 2007. **317**(5845): p. 1756-60.

96. Myers, E.W. and W. Miller, *Optimal alignments in linear space.* Comput Appl Biosci, 1988. **4**(1): p. 11-7.