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CICLO XXXII

In vitro study of the molecular mechanisms responsible for the filaricide effect of macrocyclic lactones combined with doxycycline against _Dirofilaria immitis_.

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To my father,

thank you for always beliving in me

and for showing me that hard work always pays off.

Abstract

Macrocyclic lactones (MLs) are antihelminthic drugs that can prevent heartworm disease in dogs, caused by *Dirofilaria immitis* infections, during transmission season. They have been also shown to eliminate adult parasites after long-term administration, with a so called "slow-kill" effect. In addition, recent studies have established that antibiotic treatment of *Dirofilaria immitis*-infected dogs inhibit parasite embryogenesis, larval development, microfilarial production, and long-term survival of adults. This is due to the presence of an bacterial endosymbiont, *Wolbachia*, which is required for normal parasite development, fertility and survival. These bacteria, whose presence have been reported in most human filarial nematode parasites, due to their obligate nature has been a target for several drug discovery initiatives. Thus, several protocols combining of the antibiotic doxycycline with a ML have been tested. Previous studies have shown them to provide a more rapid adulticidal and microfilaricidal effect than either one of the two drugs alone. Unfortunately, female worms have been observed to have a higher tolerance to treatments compared to male worms.

The actual reason behind the increase in efficacy of the doxycycline/ML combination therapy on *D. immitis* is still unknown. Therefore, to understand better the mechanism of action laying behind the observed synergistic effect of the two drugs when administered in combination, an *in vitro* study on adult worms of *D. immitis*, focused on the response to different treatments has been carried out.

In particular, since ivermectin, one of the most common ML used for prevention HWD, has been reported to interact with membrane transporters, primarily ABC transporter, the present study was aimed at evaluating whether transporters may be involved in the synergic effect of the combination treatment.

For this purpose, adult worms of *D. immitis* were collected and treated *in vitro* for either 24 or 48 hours with either doxycycline alone, ivermectin alone or the two drugs combination. Changes in modulation of seven transporter genes have been analyzed by relative quantitative real-time PCR and doxycycline residual concentration in the treatments media were measured by HPLC analysis.

Results of the gene expression study following 24 hours of treatments reported a sex-dependent gene expression. In female worms, this was characterized by a down-regulation following single ivermectin administration, a dramatic up-regulation of all transporter genes following the single doxycycline treatment, with balanced effect when the two drugs were given together. In male, the expressions were

different. In fact, while ivermectin alone induced a general up-regulation, the single doxycycline treatment induced a slight down-regulation in all genes. Similarly to what reported in female, when administered in combination a balanced effect was reported also in male worms.

Although the sex-dependence was conserved also in the results obtained following the 48 hours of treatment, the actual gene expression trends within each sex change entirely. Indeed, while females' gene expression of all transporters per all tested treatments returned to the control level, males' genes expression profile resemble the trend observed in female after 24 hours of treatments. Furthermore, no definitive proof of the actual change in excretion of either doxycycline have been collected with HPLC analysis.

These results correlate the synergic adulticide effect observed in previous *in vivo* studies with the actual change in modulation of the transporter genes, and they highlighted a sex-dependent transporter genes modulation, which could be connected to the different *Wolbachia* load found in the two sexes.

In conclusion, despite these results, further studies are required to further elucidate whether the modulation of cellular efflux may be connected, also partially, to the adulticide effect of DOXY/ML combinations in heartworm-infected dogs.

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1. Introduction

1.1 *Dirofilaria*

The nematode genus *Dirofilaria* belongs to the Onchocercidae family of the order Spirurida (Anderson 1992). Among all *Dirofilaria* species, the most relevant in Europe are *D. immitis* and *D. repens,* which are responsible for vector-borne parasitic infections primarily of dogs, cats, and other species of wild mammals (Fig.1) (Genchi et al. 2009; Simón et al. 2017). These parasitic nematodes are transmitted by hematophagous arthropods.

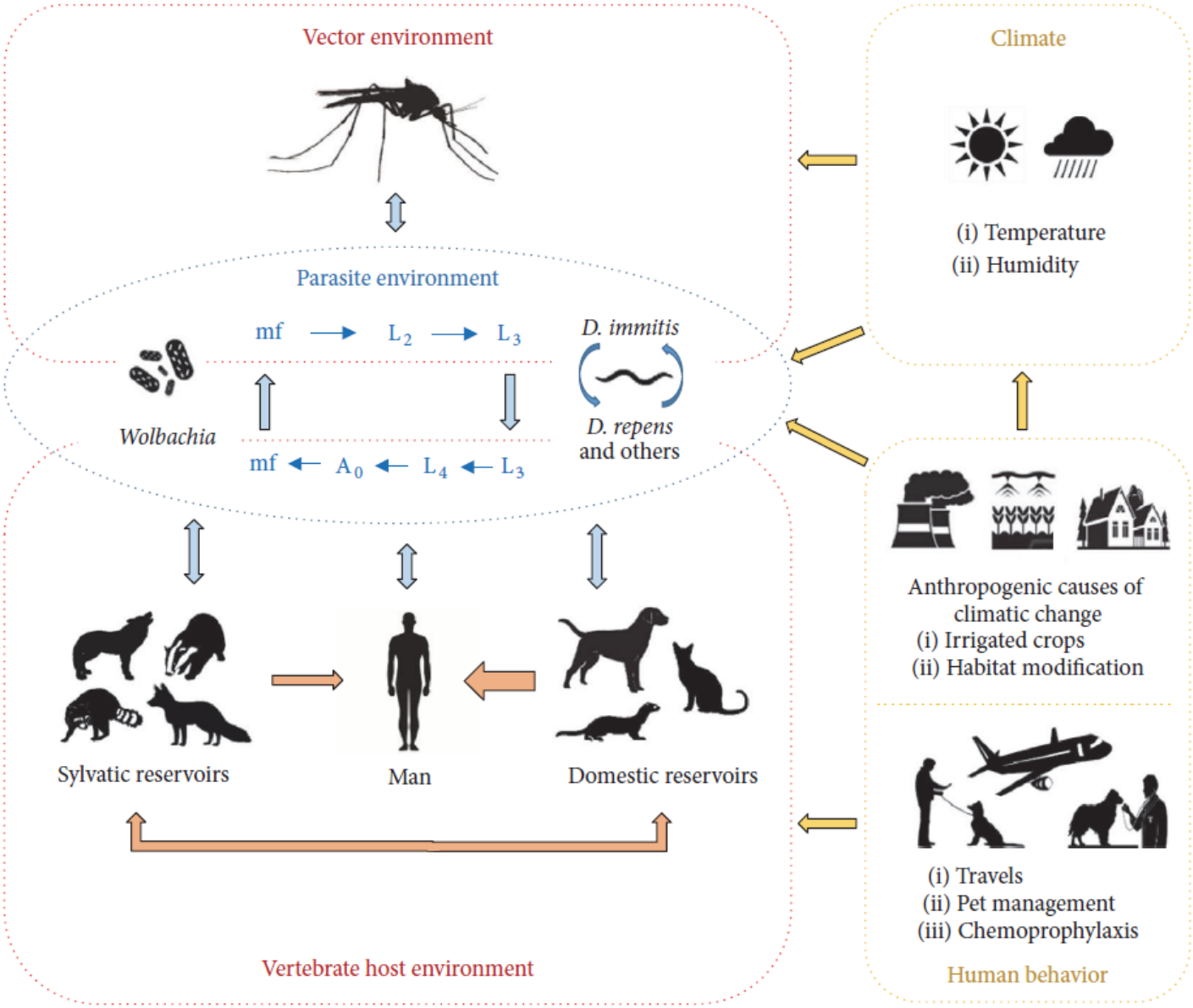


Fig. 1: The episystem of *Dirofilariosis*. (Simón et al. 2017)

Due to the low specificity of feeding by their vectors, both *Dirofilaria* species are zoonotic, thus representing a risk for both human and animal health (Simón et al. 2017; Genchi et al. 2009). In particular, in animals *D. immitis* is the causative agent of heartworm disease, while *D. repens* causes subcutaneous dirofilariosis (*D. repens*); in humans, they are responsible for pulmonary and subcutaneous/ocular dirofilariosis (Pampiglione et al. 1995; Simón et al. 2005).

In recent years, Europe has been experiencing an increase in the spreading of vector-borne infections. This phenomenon has multiple drivers, such as globalization, with the increase in tourism as well as international trades, and the development of insecticide and drug resistance. However, one of the key factor responsible for this spread is climate change (Harrus and Baneth 2005). Indeed, due to the increase in temperature observed, new arthropod vectors have become available, as well as new pathogens (Hendrickx et al. 2004; Roger and Randolph 2006). More importantly, pathogens such as *Dirofilaria*, which used to be restricted to the Mediterranean countries, have reported important variations in both transmission and distribution (Simón et al. 2012). These changes in parasites' epidemiology are becoming more evident; *Dirofilaria* infections are now an issue concerning also the northern and eastern areas of Europe (Fig. 2) (Genchi et al. 2009).

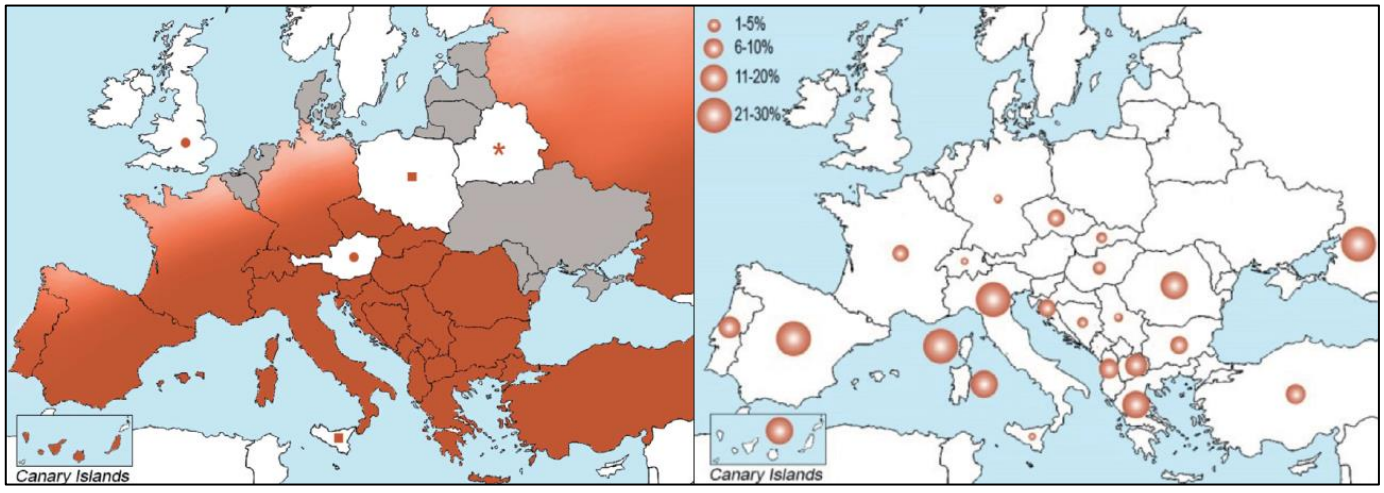


Fig. 2: *Dirofilaria immitis* distribution (left) and mean prevalence rates (right) in Europe.

(ESDA guidelines 2017)

A recent study conducted by Genchi et al. (2019a) have reported both *Dirofilaria* spp. to be widely spread throughout Italy. Although the presence of both parasites has been reported from a large number of areas, monoinfections by *D. immitis* have been reported prevalently in the northern regions (Fig.3).

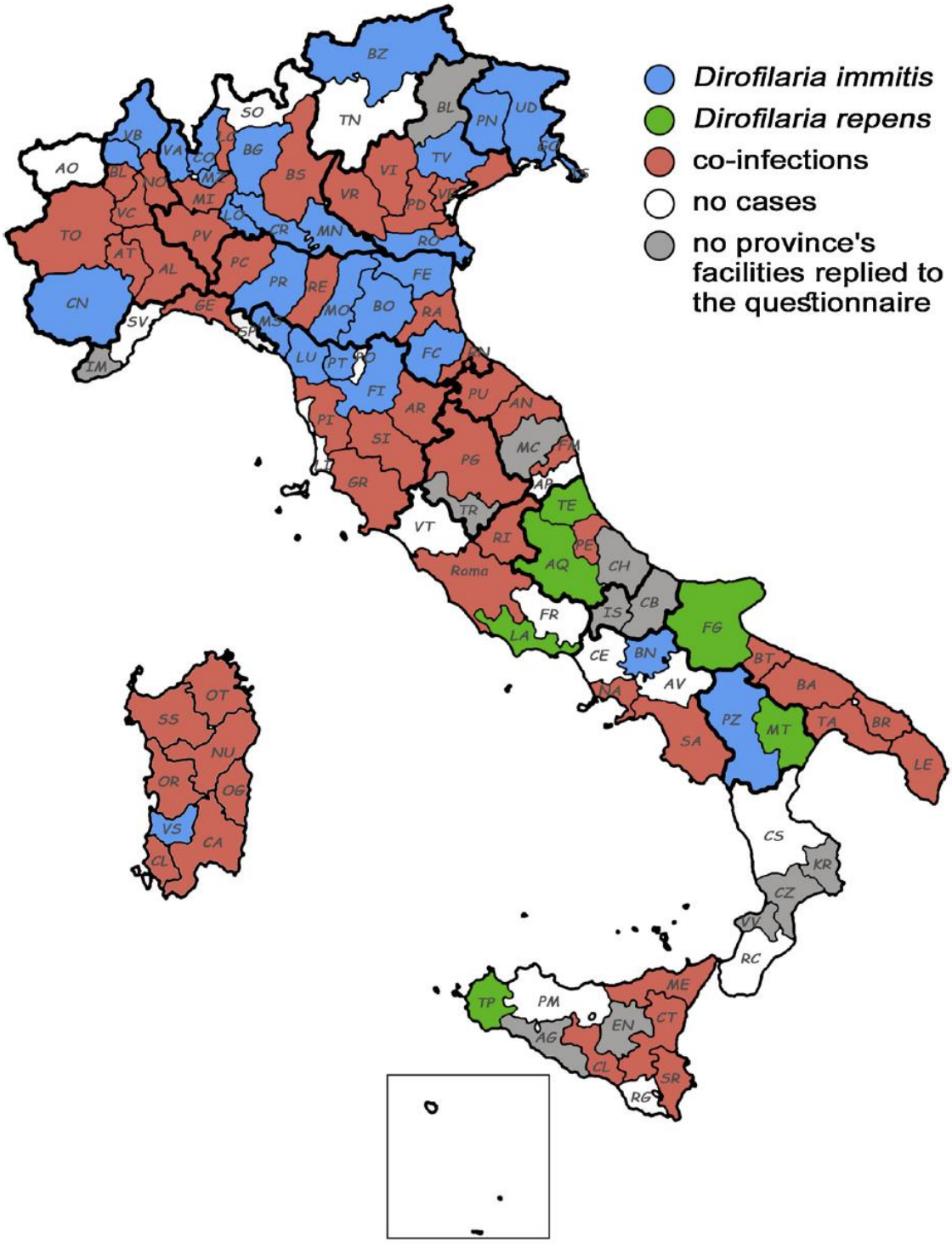


Fig. 3: Distribution of *D. immitis* (blue) and *D. repens* (green) and co-infections (brown) in Italy.

(Genchi et al. 2019a)

1.1.1 *Dirofilaria immitis*

Dirofilaria immitis is commonly known as heartworm, since it was first described as residing in dogs' right ventricle. Despite its name, *D. immitis* adult worms reside in the pulmonary artery of their definitive host.

The first known reference to a *D. immitis* infection in dogs has occurred at the beginning of the 17th century, by the Italian scientist Birago, who detected a worm in his hunting dog (Birago 1626). Unfortunately, Birago confused it with the larvae of another parasite. Thus, it was not until much later that it was identified as *D. immitis*. As mentioned above, *D. immitis* infects primarily dogs and cats. However, *D. immitis* infection has also been reported in unusual hosts, including pinnipeds (Alho et al. 2017).

Its vectors are females of various mosquitoes belonging to the *Culicidae* family, such as *Anopheles* spp., *Aedes* spp., and *Culex.* Spp. (Cancrini et al. 2001;2007).

During their blood meal, infected mosquitoes release hemolymph containing L3 stage larvae onto the wound, allowing larvae to actively penetrate the skin of the host (McGreevy et al. 1974). Between 3 to 12 days postinfection (d.p.i.) the L3 molts to L4, which then at 50/70 d.p.i. molts into a pre-adult stage called L5 (Kume and Itagaki 1955; Kotani and Powers 1982; Lichtenfels et al. 1985). This pre-adult stage of the parasite migrates to the pulmonary artery. Once there, at about 120 d.p.i. it reaches sexual maturity (Orihel 1961; Kotani and Powers 1982). Adults can leave over 7 years (Venco et al. 2011). After mating, adult females, which are ovoviparous, lay microfilariae directly into the blood stream (between 6 to 9 months post infection), allowing their access to the vector (Bowman and Mannella 2011). These highly motile unsheathed pre-larvae, can live in the final host for up to two years (Bowman and Mannella 2011; Venco et al. 2011). Naïve mosquitoes during their blood meal ingest these circulating microfilariae (mf). From the digestive tract, mf reach mosquitoes' Malpighian Tubules and there, in approximately 8 to 10 days, they molt to L2 (also called sausage stage). Then about three days after they molt again into L3 stage, which finally migrate to the mosquito mouthpart of the vector, starting again the parasite cycle (Fig.4) (Taylor et al. 1960).

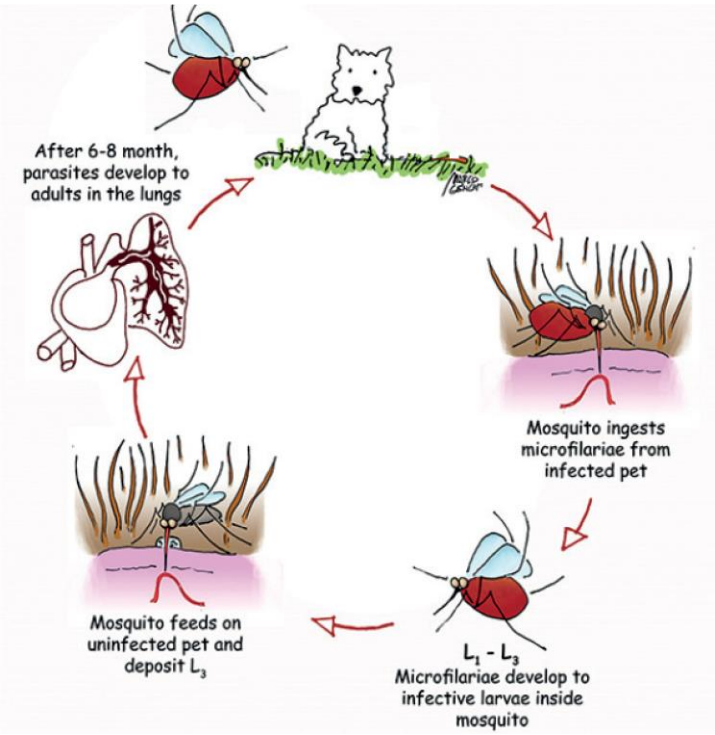


Fig. 4: *Dirofilaria immitis* life cycle. (ESDA guidelines 2017)

Between all stages of the *Dirofilaria* development, only adult worms have reported to have a fully functional digestive tract where the nutrient uptake is carried out; whereas for microfilariae, the nutrient uptake is exclusively transcuticular (Jaffe and Doremus 1970; Ando et al. 1980; Barret 1993).

Sexual dimorphism is characteristic of adult nematodes and *D. immitis* is no exception, particularly regarding dimensions. In fact, while adult female worms range from 250-310mm long and 1-1.3mm width, male worms are considerably smaller, ranging from 120-200mm in length and 0.7-0.9mm in width (Manfredi et al. 2007). Although the same dichotomy has also been observed for adults of *D. repens*, their final size are much smaller compared to those of *D. immitis*, ranging between 100-170mm in length the females and 50-70mm the males (Manfredi et al. 2007). On the contrary, *D. repens* microfilariae are larger compared to those of *D. immitis*, in fact, while the first measure between 350-385µm in length and 7-8 µm in diameter, the latter space between 290 to 330µm in length and 5 to7µm in diameter. These differences in size, together with other difference visible at the microscope, at the cephalic and caudal level, make the discrimination between the two species possible, without necessarily requiring molecular analysis.

1.2 *Wolbachia*

Wolbachia is an α-proteobacterium and is part of the order *Rickettsiales*. This genera represents one of the most widespread and well-studied intracellular bacteria (Bandi et al. 1998; Werren et al. 2008). It has been estimated to infect two-thirds of all arthropods species. Whereas in nematodes, it is restricted to two families: the plant parasitic Pratylenchidae (Haegeman et al. 2009) and the animal-parasitic Onchocercidae (Ferri et al. 2011).

Previous studies based on multi-locus phylogeny have divided this genus into 16 subgroups, labelled in alphabetical order from A to G (Werren et al. 1995; Lo et al. 2007; Bordenstein et al. 2009; Augustinos et al. 2011; Glowska et al. 2015). Subgroup C and D, which contain *Wolbachia* associated with filarial nematodes, are characterized by features associated with mutualistic behaviours toward their hosts, such as vertical inheritance and metabolic integration, as well as 100% prevalence (Bandi et al. 1998; Casiraghi et al. 2001; Taylor et al. 2005a; Hosokawa et al. 2010; Darby et al. 2012; Gill et al. 2014).

Sironi and colligues (1995) discovered the first *Wolbachia* observed in filariae in *D. immitis*. Although it was first described just as a bacterium-like body, later studies carried out in other filarial parasites, such as *Onchocerca volvulus*, recognized its bacterial nature (Kozek and Marroquin 1977). Previous studies have reported this intracellular bacterium symbiotic nature toward several organism, including filariae of the Onchocercidae family, such as those responsible for lymphatic filariasis (*Wuchereira bancrofti* and *Brugia malayi*), onchocerciasis (*Onchocerca volvulus*), as well as heartworm (*Dirofilaria immitis*) (Fenn and Blaxter 2006; Taylor et al. 2013).

In filarial nematodes, *Wolbachia* is vertically transmitted from mother to offspring and it is located in host-derived vacuoles (Dumler et al. 2001). Due to their symbiotic nature, *Wolbachia* has lost most of the cell wall structure typical of other Gram-negative bacteria. In male filarial nematodes these bacteria is located only in the somatic hypodermal cords, which run along the length of the parasite, while in female worms it is harboured also in germinal zones (Fig. 5) (Taylor et al. 2005a). In particular, *Wolbachia* is absent from the reproductive tissues of both male and female worms until at least the fourth stage larvae (L4) and starts invading the female germinative zones from the hypodermal cords just after the parasite moults to the L5 stage (Fischer et al. 2011; Landmann et al. 2012). In arthropods, *Wolbachia* can affect several biological aspects, including reproduction, i.e. cytoplasmic incompatibility (CI), viral immunity and iron metabolism (Teixeira et al. 2008; Werren et al. 2008; Kramer et al. 2009);

in filarial nematodes, previous studies have shown that *Wolbachia* is involved in both moulting and embryogenesis of these organisms (Hoerauf et al. 2003; Rossi et al. 2010).

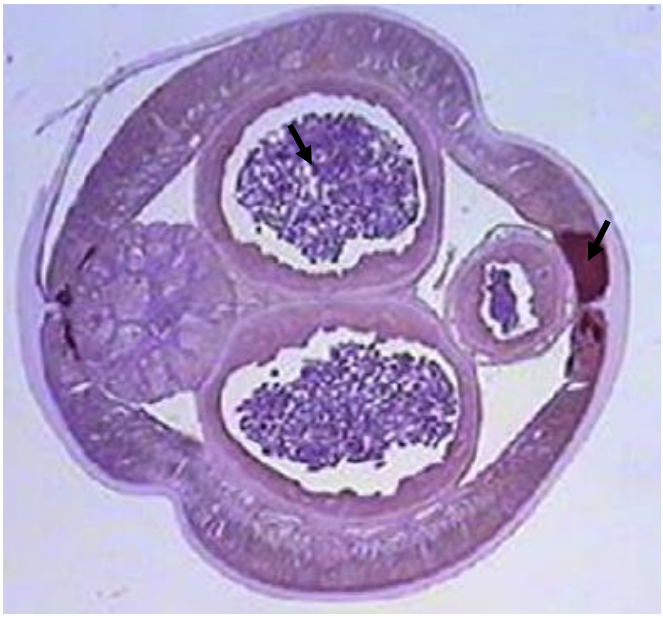


Fig. 5: Anti-*Wolbachia* surface protein (WSP) immunohistochemistry and histology of a female worm of *D. immitis*. Arrows indicate the presence of *Wolbachia* in the ovaries and in the lateral cords of the worm. (Bazzocchi et al. 2008)

Moreover, despite its constant presence in all stages of the filarial nematode life cycle, previous studies have reported a change in the size of *Wolbachia* populations according to the stage of development taken into consideration (Fenn and Blaxter 2004; McGarry et al.2004; Fischer et al. 2011). The highest increase has been observed when the parasite reaches the mammalian host, inducing larval development (McGarry et al. 2004). To regulate bacterial population and in particular to limit its overgrowth, nematodes activate autophagy, a conserved intracellular defence mechanism as well as regulator of cell homeostasis (Voronin et al. 2012).

The observed dynamic changes in *Wolbachia* loads inside filarial parasite have led scientists to suggest its involvement in provision of essential nutrients and metabolites required for their developmental processes (Taylor et al. 2013). Thanks to the new full genome sequences techniques, i*n silico* studies aimed at the comparison of the molecular pathways present in *Wolbachia* and in its host, revealed the interdependent nature of the relationship between these two organisms (Stalko et al. 2010). In fact, while these nematodes have been shown to rely on *Wolbachia* for the synthesis of several metabolites

including haem, riboflavin and nucleotides (Foster et al. 2005; Ghedin et al. 2007; Darby et al. 2012; Scott et al. 2012), the endosymbiont has been shown to be metabolically dependent on some of the host products such as several vitamins and cofactor, as Coenzyme A (Foster et al. 2005). In particular, when considering haem metabolism, previous studies have reported a lack of haem biosynthesis genes in filarial nematode host (Foster et al. 2005). Furthermore, evidence indicates that *Wolbachia'*s role in haem metabolism is not limited to its provision, but it might be also involved maintenance of iron homeostasis (Darby et al. 2012). In addition to the provision of metabolites, *Wolbachia* has been shown in *O. ochengi* to have a mitochondrial-like function, by generating ATP, as well as an immune-modulating activity (Darby et al. 2012). Taken together, all studies would suggest that *Wolbachia* symbiont has become an essential partner for key biological processes in these nematodes.

Wolbachia can be considered as a defensive mutualist. By releasing Toll-like receptors (TLR) ligands such as WSP, it activates neutrophils, thus preventing worm degradation by eosinophils (Bandi et al. 2001; Brattig et al. 2001; Taylor et al. 2001; Hansen et al. 2011). This interaction with the innate immune system of the mammalian host has been reported to contribute to the pathogenesis as well as immunology of the filarial diseases (Bazzocchi et al. 2003). In fact, when the adult worm dies, release of *Wolbachia* can shift the host immune response from a Th2-response toward a Th1-type response, causing inflammation and degeneration of the vascular environment, as well as suppression of the microfilariemia (Kramer et al. 2005a; Morchón et al. 2007).

Previous studies have also shown that the administration of antibiotic treatment induces a delay in larval growth as well as apoptosis in the female reproductive tract (Taylor and Hoerauf 2001; Taylor 2012). For this reason, this symbiotic relationship has been widely exploited to treat filarial infections, in particular using doxycycline (Taylor et al. 2005b, Debrah et al. 2006; 2007; Hoerauf et al. 2008; 2009; Supali et al. 2008; Mand et al. 2009; Turner et al. 2010). However, due to the possible side effects associated with this antibiotic, new research has been focussing on the discovery of novel anti-*Wolbachia* compounds. This has been the goal of the Anti-*Wolbachia* (A-WOLL) consortium (http://a-woll.com/). This group composed both academic as well as industrial partners, funded by The Bill & Melinda Gates Foundation, has as its ultimate goal the development of novel drugs against human filariasis such as onchocerciasis and lymphatic filariasi.

1.3 Heartworm disease

Canine cardiopulmonary dirofilariosis, also known as heartworm disease (HWD), is a serious and potentially fatal disease. It is a chronic disease caused primarily by the presence of adult *D. immitis* in the pulmonary artery, and by the release of their antigenic products, including *Wolbachia* (Furlanello et al. 1998; McCall et al. 2008b; Venco and Vezzoni 2001; Vezzoni and Venco 1998; Kramer et al. 2005a).

The most common symptom is a persistent, chronic cough, which increases with exercise. This can be followed by moderate or severe dyspnea. Lesions of the pulmonary artery are associated with the subsequent pathology. Endothelial cells enlarge leading to a narrowing of the vessels causing endarteritis (Simón et al. 2012). The damaged arterial surface leads to proliferation of endothelial cells, resulting in proliferative pulmonary endarteritis (Calvert et al. 1985; Rawlings 1986). The severity of villus proliferation is strictly connected to the length of time of the infection, parasite load and the intensity of the host immune system response (Simón et al. 2012). In addition to the chronic disorder, an acute pathology can occur, due to either spontaneous or treatment induce adult worms' death (Simón et al. 2012). This acute phase of the disease is characterized by thromboembolism events, which can be life threatening for the animal (Fig 6).

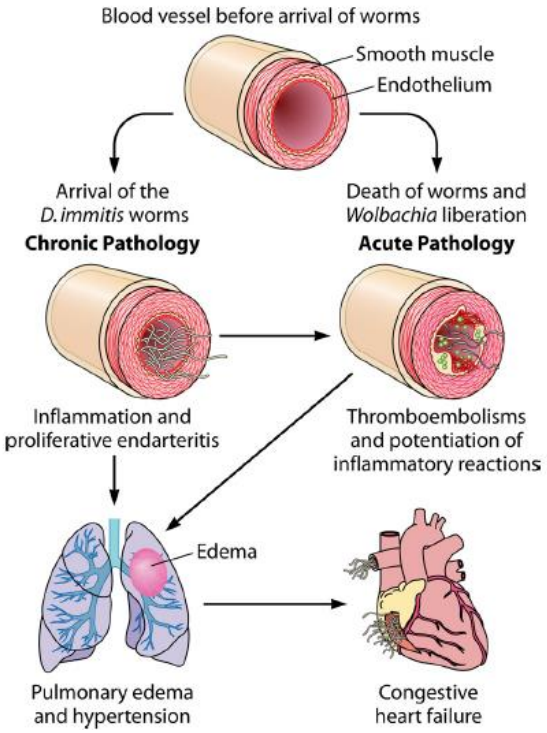


Fig. 6: Progression of the Heartworm disease in dogs. (Simón et al. 2012)

Conversely, cats are generally asymptomatic carriers, and some of them die suddenly without any premonitory sign (Atkins and Lister 2006; Lister and Atwell 2008). In few cases, an acute vascular and parenchymal inflammatory response can be activated in cats causing evident, but nonspecific, signs of the infection, which are caused by the arrival of immature worms in the pulmonary artery (Simón et al. 2012). Misdiagnosis of these clinical signs is quite common, in fact are often confused with asthma or allergic bronchitis (Lee and Atkins 2010).

Although most commonly observed in smaller size dogs, the caval syndrome is a condition caused by the sudden rise pf the pulmonary pressure, which then leads to the displacement of the worms' mass from the pulmonary artery into the right ventricle (Furlanello et al. 1998). The resultant tricuspid insufficiency is followed by an increase in the venous pressure and difficulty in return circulation, which eventually leads to death of the patient for hemoglobinuria, hemolysis and disseminated intravascular coagulations in about two days (Simón et al. 2012).

1.3.1 Diagnosis of HWD

Heartworm disease can be diagnosed by either the identification of circulating microfilariae, by using the modified Knott test or filtration test, or by the detection of adult female worm antigen by using specific commercially available antigen tests, such as enzyme-linked immunosorbent assays (ELISAs) (Courtney and Cornell 1990; Georgi and Georgi 1992; Knott 1939). While the first allow discrimination between the microfilariae of *D. immitis* from those of *D. repens*, some infections could also be amicrofilaremic, therefore the use of the second diagnostic technique in these case can unravel the presence of the parasite in the host (Simón et al. 2001). The use of these two techniques in combination has been advised by the American Heartworm Society (AHS) and the European Society of Dirofilariosis and Angiostrogylosis (ESDA) to allow an accurate detection of dirofilariasis (ESDA guidelines 2017; AHS guidelines 2018). To confirm diagnosis other diagnostic methods can be applied such as radiography or echocardiography (ESDA guidelines 2017; AHS guidelines 2018).

1.4 Prevention and Treatment of HWD

The American Heartworm Society (AHS) and the European Society of Dirofilariosis and Angiostrogylosis (ESDA) are the two principal, international associations dedicated to promoting knowledge and research in canine and feline HWD. Both societies have published specific guidelines describing prevention and treatment practices to be carried out against this disease.

For prevention of HWD, both societies recommend the administration of monthly dosage of macrocyclic lactones (MLs), a wide sread anthelmintic class of drugs, all year round (Table 1). These molecules are active against larvae that have been inoculated by mosquitoes in the previous thirty days.

Table 1: Macrocyclic lactone dosages used for heartworm prevention. (ESDA guidelines 2017)

Drug	Administration	Dose	Interval
Ivermectin	Oral	> 6 mcg/kg	Monthly
Milbemycin	Oral	> 0.5-0.75 mg/kg	Monthly
Moxidectin	Injection SR	0.17 mg/kg	every 6-12 months
Selamectin	Spot on	6 mg/kg	Monthly
Moxidectin	Spot on	>2.5 mg/kg	Monthly

In addition to MLs, repellents and ectoparasites, such as pyrethroids, are also available. Although they are quite helpful in inhibiting blood-feeding of mosquitoes and/or killing mosquitoes, they cannot be used as substitute for MLs. In fact, according to both societies, they need to be applied in combination with MLs (ESDA guidelines 2017; AHS guidelines 2018).

The use of preventive treatment needs to be preceded by a proper diagnosis. In fact, even though the long-term administration of a preventive is known to induce a “slow kill” effect, in alternative to a proper adulticidal therapy, this ML stand-alone therapy may increase the risk of selecting for MLs-resistant strains of the parasite (McCall 2005; Geary et al.2011**;** Bowman 2012).

For adulticide treatment, the only molecule currently approved by Food and Drug Administration (FDA) is melarsomine dihydrochloride. This molecule has been shown to act on immature worms of two and four months old (Dzimianski et al. 1989, 1990; McCall et al. 2010). The official therapy reported by the AHS is based on a three-dose protocol, where melarsomine dihydroclhloride is administered at a dose of 2,5 mg/Kg via intramuscular injection in lumbar muscles. The first injection is followed at least thirty days by other two injections of the same dose after 24 hours apart. The two injection of melarsomine 24 hours apart have been shown to kill 90% of the adult worms, while the three-dose protocol kills 98%

of the worms (Keister et al. 1992; Vezzoni et al. 1992). Thus, the three doses protocol has the advantage of decreasing the chance of complications by inducing a two-step killing.

Both societies suggest combining the official therapy with a pretreatment, consisting of a combination of thirty days antibiotics (doxycycline at 10mg/Kg bid (Bandi et al. 1999; Kramer et al. 2011; Nelson et al. 2017) and two monthly doses of macrocyclic lactone (ML). This pretreatment will eliminate the migrating larvae, the circulating microfilariae and weaken the adult worms. In particular, early administration of doxycycline is based on the observation that the major *Wolbachia* surface protein (WSP) induces in the infected host a specific IgG response, contributing to pulmonary and renal pathology (Kramer et al. 2005b). Thus, the administration of doxycycline prior melarsomine treatment reduces *Wolbachia* population, minimizing the occurrence of side effects as well as the severity of pulmonary thromboembolism. In addition to the pre-treatment protocol, several other recommendations and supportive treatment options are recommended in order to minimize cardiopulmonary complications. One of the most important factors in avoiding post-treaemtn complications is to adopt exercise restriction. Finally, after 9 months from the start of adulticidal treatment, an antigen test needs to be conducted to confirm the success of the therapy.

More recently, due to the presence of multiple issues related both to costs as well as side effects, scientist are putting their effort into developing alternative protocols to the adulticidal therapy with melarsomine (Kramer et al. 2018). These treatments include the use of a ML in combination with doxycycline. Both AHS and the ESDA in their guidelines advise to use combination treatments for all those cases where either melarsomine is not possible or is contraindicated. A recent survey carried out on Italian veterinary facilities, has reported an increase in the use of the alternative protocol for the treatment of *D. immitis*. In fact, while melarsomine treatment is used in 35% of the cases, the ML/doxy combination is used 29% (Genchi et al. 2019a).

An alternative to the chemotherapeutic treatments described above is the minimally invasive surgical heartworm removal. This procedure represents the only option of treatment for dogs with caval syndrome or for dogs with heavy worm burden, where no pharmacological treatment can be performed due to the high rick of post-adulticidal complications (ESDA guidelines 2017).

1.4.1 Macrocyclic lactones

Macrocyclic lactones are still the class of drugs most widely used to treat nematode infections of both veterinary and medical significance. In human health, these molecules, in particular ivermectin, have been widely used against *Onchocerca volvulus*, the causative agent of river blindness (Brown and Neu 1990). This group of molecules have been used for more than 30 years for the control of HWD. MLs are divided in two classes: the avermectins, such as ivermectin, and the milbemycins (which include moxidectin). These molecules are natural fermentation products of soil dwelling streptomycete micro-organisms (Danaher et al. 2006). MLs are large complex ringed structures. Avermectins have a 16-membered macrocyclic ring, containing a spiroketal group, a benzofuran ring and a disaccharide functionality (Figure 7), while milbemycins lack the disaccharide group (Fisher and Mrozik 1989).

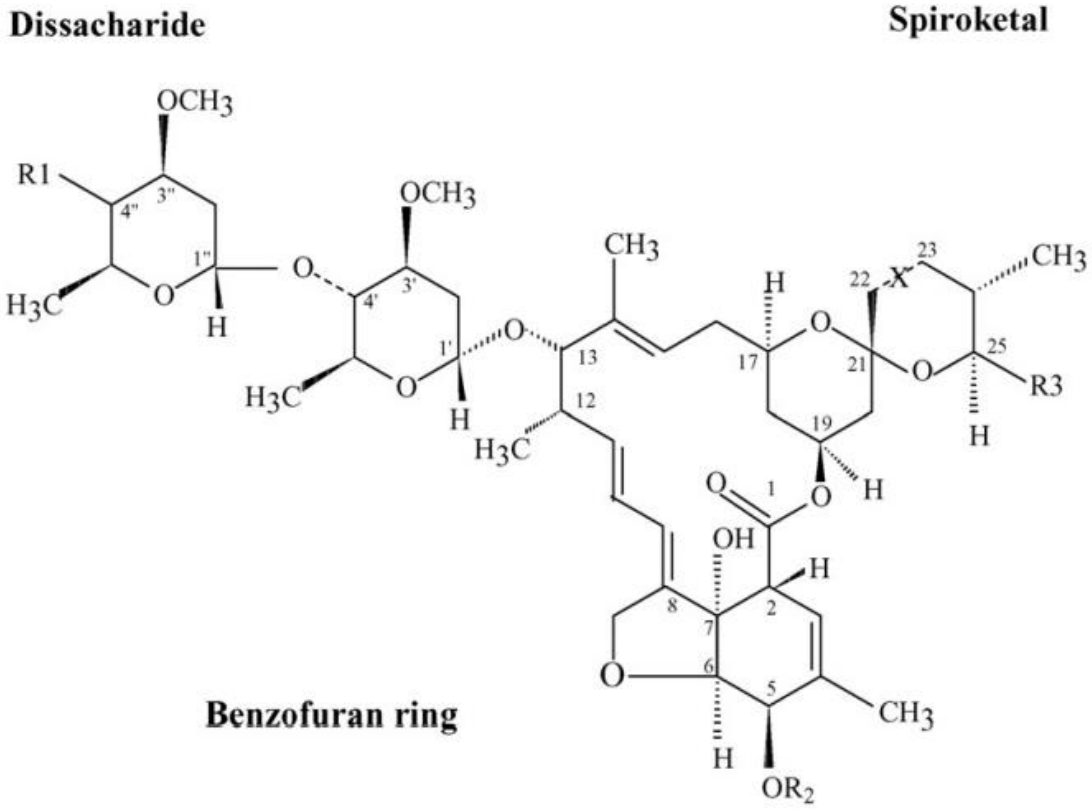


Fig. 7: Chemical structure of avermectins. (Danaher et al. 2006)

Use of MLs for HWD prevention was first developed to act on L3/L4 stage larvae of *D. immitis*, in order to prevent the establishment of the adult parasite. More recently, the monthly administration of MLs

has been shown to suppress reproduction in adult worms and to act against microfilarial stage, thus leading to a reduction in the transmission incidence and a gradual elimination the adult parasites (Bowman et al. 1992; Courtney et al. 1998; McCall et al. 1998, 2005; Tompkins et al. 2010; Stitt et al. 2011).

The exact mode of action of MLs on different stages of *D. immitis* is still unknown. They are known to act as modulators of a plethora of ion channels both in vertebrate as well as in invertebrates, the most important group being the glutamate-chloride channels (GluCl) (Wolsteinholme and Rogers 2005; Wolsteinholme 2012). To investigate the molecular mechanism of action of MLs in nematodes, a number of studies have been carried out: some have demonstrated direct activation of recombinant GluCls channels expressed in *Xenopus* oocytes (Cully et al. 1994; Dent et al. 1997; Vassilatis et al. 1997; Horozok et al. 2001; McCavera et al. 2009; Yates and Wolstenholme 2004); others have shown a high-affinity binding in receptors expressed in cell lines (Cheeseman et al. 2001); while several others have reported the association of drug resistance phenotype of *C. elegans* strains with the presence of mutations in genes encoding for those channels (Dent et al. 2000; Ghosh et al. 2012). Although, based on the evidence collected so far, MLs should act on glutamate-gated chloride channels leading to the inhibition of pharyngeal pumps and paralysis of the worm, in filarial nematodes this hypothesis has been disproved (Avery and Horviz 1990; Geary et al. 1993; Brownlee et al. 1997; Dent et al. 1997, 2000). In fact, when administered at recommended dosage to adults of *D. immitis*, this acute effect does not occur (Bennet et al. 1988).

In 2005, Hampshire published the first report on loss of efficacy of MLs in *D. immitis*. Since than many more reports focusing on MLs resistance have been produced, hence inducing scientist to focus on the search for genetic markers to monitor these emerging resistant phenotypes (Bourguinat et al. 2011a,b; 2015; Pulaski et al. 2014). Bourguinat et al. in 2011 showed evidence of ML selection on P-glycoproteins (PgP) in *D. immitis*. They reported a strong correlation between the occurrence of a specific PGP genotype (GG-GG, homozygosity) and the increased resistance to ivermectin. These results suggest the PGP genotype a plausible candidate for use as a genetic marker of ML resistance.

MLs remain so far, the only class of heartworm prevention available, with moxidectin and ivermectin being the two molecules most used against *D. immitis*. Therefore, preserving their effectiveness is of critical importance (Bowman et al. 2011).

1.4.1.1 Ivermectin

Ivermectin is a derivative of avermectin, and it was first created and commercialized by Merck in 1981(Figure 8) (Van Voorhis et al. 2015). This molecule is active against a broad spectrum of organisms, which not only include nematodic parasites, but also arthropods, such as lice and mites (Victoria and Trujillo 2001). In humans, ivermectin is still considered the drug of choice against both lymphatic filariasis and river blindness (Taylor et al. 2010). Due to its wide spread use, resistant parasite strains have emerged in almost all target species causing a real threat for the both human and animal health (Tompkins et al. 2011).

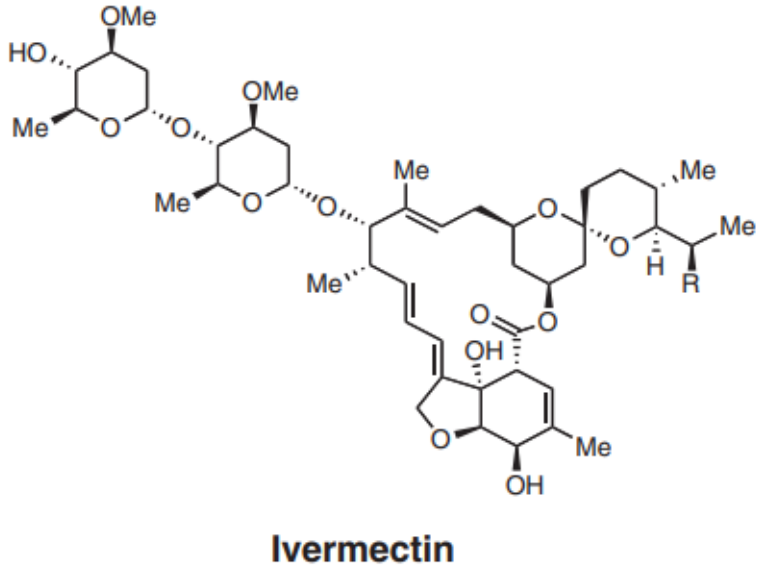


Fig. 8: Ivermectin molecular diagram (adapted from Crump and Omura 2011).

Previous studies carried out in *C. elegans* have confirmed irreversible binding of IVM to the glutamate-gated chloride ion. This phenomenon leads to an increase in permeability of the membrane, which induces a paralysis of the worms and eventual death (Dent et al. 1997, 2000). In several nematodes, IVM administration has been shown to cause a block of the pharyngeal pumping, which results into worm starvation (Geary et al 1993; Martin 1996; Kotze 1998; Ardelli et al. 2009). This does not occur in *B. malayi*. In this organism no pharyngeal pumping is involved in feeding, since its gut is atrophied, and thus food is most likely obtained through the cuticle (Chen and Howells 1981; Howells and Chen 1981).

Although the feeding pathway has not yet been fully described in *D. immitis*, due to its close genetic similarities to *B. malayi*, the use of the same mechanisms can be plausible. In addition, IVM is very effective against the reproductive system of filarioid nematodes, which have been reported to be more sensitive to this molecule than other nematodes (Gardon et al. 2002; Ardelli et al. 2009; Tompikins et al. 2011).

1.4.1.2 Moxidectin

Moxidectin is a member the milbemycin subfamily of MLs and it is a derivative of nemadectin (Figure 9). It is used for control and prevention of heartworm disease (*D. immitis*), as well as for treatment of trichostrongylid gastrointestinal nematodes. This molecule has a longer half-life compared to other milbemycins; and compared to ivermectin, moxidectin has both a larger volume of distribution and a slower clearance rate (Prichard et al. 2012).

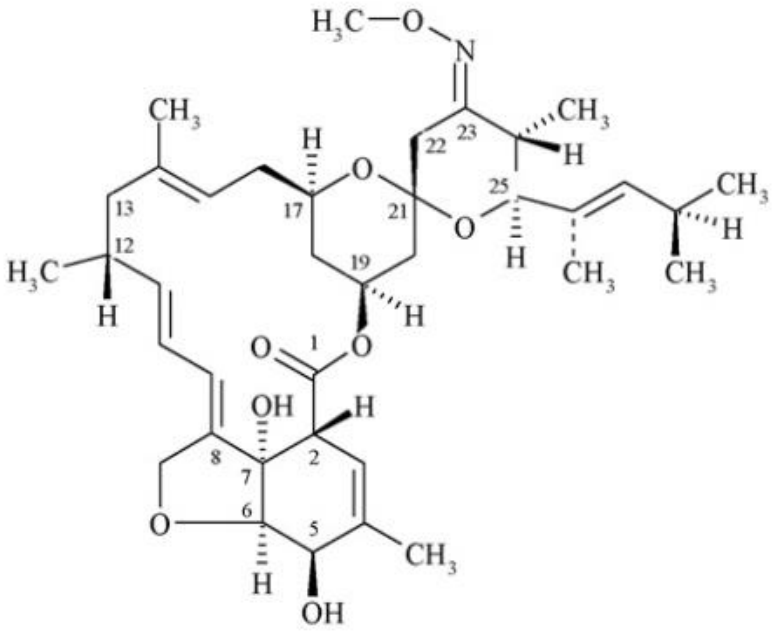


Fig. 9: Moxidectin molecular structure (adapted from Danaher et al. 2006).

Previous studies have reported how moxidectin kinetics is not dependent on PgPs or other ABC transporters, whereas avermectins, such as ivermectin, are. This clear discrepancy in the molecule's interaction with these pumps is due to the presence of structural differences between groups. Indeed, the integrity of the disaccharide moiety of the ivermectin, which is not found in moxidectin, plays a

central role in affinity for PgPs (Figure 10) (Lespine et al. 2007; Kiki-Mvouaka et al. 2010). Thus, inducing a weaker interaction between moxidectin with mammalian P-gp compared with ivermectin (Lespine et al. 2006). Moreover, recent studies, focused on ML resistance in nematodic parasites, have reported moxidectin having a reduced effect on causing overexpression of both P-gps and MRPs in nematodes (Prichard and Roulet, 2007; Ardelli and Prichard, 2008).

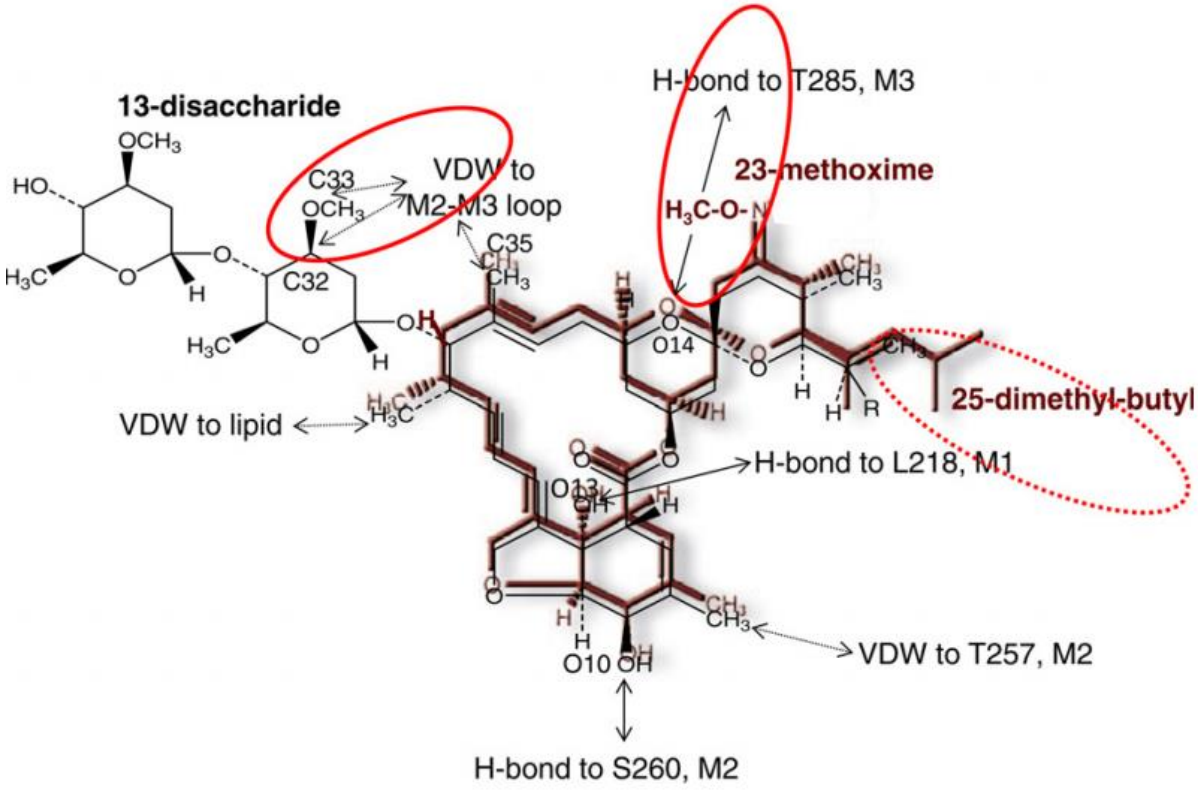


Fig. 10: Interaction of ivermectin (IVM) with a glutamate-gated chloride channel (GluCl), showing moxidectin (maroon) superimposed over ivermectin (black). While some of the interaction sites are in common between the two molecules (O10, O13, C18, C35 and C48), other interaction sites of ivermectin with the GluCl are either absent (C32, C33;) due to the absence of any saccharide group in moxidectin, or are blocked/altered (O14) by the C23 methoxime group of moxidectin. The sites where interactions will be different are highlighted by a solid red circle. (Prichard et al. 2012)

1.4.2 Doxycycline

Doxycycline is an antibiotic derived from first generation tetracyclines (Piacentini et al. 2019). It has broad-spectrum bacteriostatic action. It has been shown to inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit of both gram-positive and gram-negative bacteria (Nelson and Levy 2011).

Since the bacterial endosymbiont *Wolbachia* has been shown to be present in all developmental stages of those filarial parasites that harbor the bacteria, the inclusion of antibiotics to the adulticide protocol is beneficial (ESDA guidelines 2017). In *Wuchereira bancrofti*, another filarial nematode, the administration of doxycycline has been shown to eliminate 98% of its *Wolbachia* population (Hoerauf et al. 2003). Similar results have been also reported for *D. immitis*, including inhibition of parasite embryogenesis, larval development, microfilarial production, as well as long-term sur-vival of adults (Bandi et al. 1999; McCall et al. 1999; Kramer et al. 2007; Bazzocchi et al. 2008; Grandi et al. 2010). Moreover, doxycycline therapy has been shown to be macrofilaricidal against both human (*Wuchereria bancrofti*, Debrah et al. 2007; *Onchocerca volvulus*, Hoerauf et al. 2008) and animal (*Onchocerca ochengi*; Gilbert et al. 2005) filarial worms. In *D. immitis*-infected dogs it has been reported having a slow-kill effect on adult worms (24-36 months) (Grandi et al. 2010). During the first two month of treatment, it has been shown to also act on third and fourth stage larvae (McCall et al. 2011; Kramer et al. 2018).

Furthermore, similarly to what observed by Albers et al. (2012) in human hosts with *O. volvulus* infections, this treatment has been showed to induce sterility of the next generation larvae which, although are still capable of infecting mosquitoes, are no longer able to develop into adult worms (McCall et al. 2008a, 2014).

In addition to its antimicrobial effect, in 1983, Golub et al. demonstrated that tetracyclines possess potent anti-inflammatory activity, by inhibiting collagenase activity in rat gingival tissue. This finding might contribute to the observed reduction in filarial-induced pathology when patients are treated with doxycycline (Debrah et al. 2006). Moreover, when administrated to *D. immitis*-infected dogs, doxycycline has been reported to eliminate *Wolbachia* population and to induce a marked decrease in pro-inflammatory responses to either dead or dying worms (Kramer et al. 2008, 2011; Mavropoulou et al. 2014).

Doxycycline administration has been demonstrated to reduce respiratory complications as well as mortality rates (Nelson et al. 2017). Alternative antibiotic therapies are now been investigated. Although there are no available data for *D. immitis*, previous studies in *Onchocerca gutturosa* have suggested minocycline as a plausible substitute to doxycycline (Townson et al. 2006).

1.4.3 Combination Treatment

Although MLs are very effective at killing larval stages of *D. immitis*, they are much less active against adults (Rawling et al. 2002). For this reason, researchers have started investigating the effects of antibiotic/MLs combination treatments. Several studies combining MLs with doxycycline have demonstrated that these protocols are both safe and effective (Bazzocchi et al. 2008; Grandi et al. 2010; Bendas et al. 2017; Mavropoulou et al. 2014; Savadelis et al. 2017). Furthermore, these new combinations have been reported to provide a more rapid adulticidal effect rather than either of the two drugs administered alone (Bazzocchi et al. 2008; Savadelis et al. 2017).

So far, the two most widely studied combination protocols have been the ivermectin/doxycycline and, more recently, the moxidectin/doxycycline.

Both combination treatments have been shown to be highly effective against adult worms in both experimentally-infected and naturally-infected dogs (Bazzocchi et al. 2008; Grandi et al. 2010; Savadelis et al. 2017; Genchi et al. 2019b)

Interestingly, previous results suggest that the combination moxidectin/doxycycline has a greater adulticidal effect compared to ivermectin/doxycycline (Savadelis et al. 2017; Genchi et al. 2019). In fact, while with the ivermectin/doxycycline combination *D. immitis*-infected dogs after 10 months of treatment just a 78,3% of the mature adults were eliminated, with moxidectin/doxycycline combination, the value raised to 95,9%, getting closer to the adulticidal efficacy reported for the melarsomine 3-doses therapy (99%) (Bazzocchi et al. 2008; McCall et al. 2008a; Savadelis et al. 2017; Genchi et al. 2019b; Keister et al. 1992).

The moxidectin/doxycycline combination treatment has been reported by Genchi et al. (2019b) to also have a superior microfilaricidal effect compared to that of the ivermectin/doxycycline treatment. Indeed, for moxidectin/doxycycline treatment, a dramatic reduction in circulating microfilariae was observed starting after thirty days of treatment (Genchi et al. 2019b). This does not occur either for the

ivermectin/doxycycline treatment nor for the melarsomine one, where complete clearance of microfilaremia is reached for the first treatment at day 90, while for the second one it takes several months longer (Grandi et al. 2010; Genchi et al. 2019b). This quick reduction in microfilaremia observed after moxidectin/doxycycline treatment is of fundamental importance, since it would lead to a much faster interruption of the parasite transmission cycle.

The differences illustrated above, for adulticidal and microfilaricidal efficacy, between the two combination treatments, as suggested by Savadelis and colleagues (2017), could be due to the much larger concentration of moxidectin required for the treatment compared to ivermectin. In fact, while the minimum dosage for moxidectin is 2.5 mg/Kg, for ivermectin this is 6 µg/Kg.

Overall, both treatments have been shown to reduce the incidence of pulmonary pathology associated with the death of the parasite (Kramer et al. 2011; McCall et al. 2008a). Although well tolerated by dogs, the length of the ivermectin/doxycycline therapy, together with the cost of this treatment have discouraged practitioners from considering it as a valid alternative to melarsomine (Bazzocchi et al. 2008).

1.5 ABC transporters

ABC (ATP-binding cassette) transporters are one of the most abundant protein families. They are membrane transporters, which are spread throughout a large variety of kingdoms, including archea, bacteria and eukarya (Stitt et al. 2011; Ford and Beis 2019). They belong to the ABC system superfamily, which consist of three classes. Class 1 systems function as importers and have been described exclusively in prokaryotes (Boos and Lucht 1996). Class 2 systems work exclusively as exporters. This second class is found in both prokaryotes and eukaryotes (Fath and Kolter 1993). These first two classes are known as ABC transporters. The last class, class 3, is not involved in membrane transport, as it acts on cellular process, such as DNA repair, translation and regulation of gene expression (Dassa 2003). Thus, while in the first two classes the energy derived from the ATP hydrolysis is used to transport target molecules across the membrane, in class 3 the ABC ATPases involved in DNA repair use the energy derived from ATP to recognize and bind miss paired DNA bases or DNA insertion loops (Hopfener and Tainer 2003).

Members of the ATP-binding cassette (ABC) protein superfamily have two characteristic regions: a highly conserved ATP-binding cassette (ABC_2 domain) and a less conserved transmembrane domain (ABC_TM1F domain) (Walker et al. 1982), which is usually found in those that function as transmembrane transporters (Ardelli et al. 2010). The ABC_2 domain contains several conserved regions: the Walker A, which contains a phosphate-binding loop; the Walker B, which contains the magnesium-binding site; the switch region, characterised by a histidine loop; a signature sequence, which differentiate ABC systems from other ATP dependent proteins; and a Q-motif that is localized between the Walker A and the signature sequence. All together, the Walker A, Walker B, Q-motif and the switch region constitute the nucleotide binding site (Schneider and Henke 1998; Higgins 1992, 2001). The difference between the first two classes and the last class is the localization of the signature sequence. While in the first two classes, it is positioned between the Wolker A and Wolker B sequences; in class 3, it is distant from those two reference sequences (Hopfner and Tainer 2003).

While in vertebrates ABC protein superfamily has been extensively studied, it is less so for helminths. Among ABC transporter proteins, the two nematodes in which this protein superfamily has been best characterized are *Cenorabditis* species and the filarioid *Brugia malayi* (Sheps et al. 2004; Zhao et al. 2004, 2007; Ardelli et al. 2010).

Thanks to recent structural studies, the number of hypothesis proposed regarding their substrate translocation mechanisms have increased. In fact, in addition to the original alternation access model, in which the transporter switched from an inward- to an outward-facing stage (Beis et al. 2015) Fig. 11, new models have now been proposed, such as: the occluded-mechanism with transient opening (Bountra et al. 2017), the outward-only mechanism (Perez et al. 2015) or the substrate-capture from periplasm mechanism (Crow et al. 2017; Okada et al. 2017) Fig. 11**.**

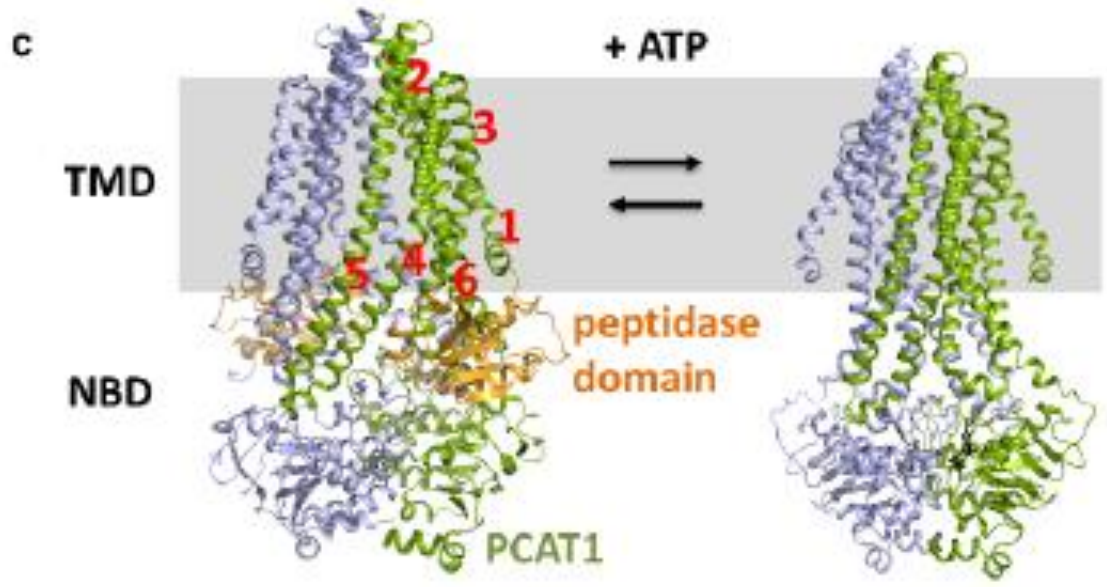


Fig. 11: Schematic representation of the alternating access and outward-open only mechanism. The crystal structure of the PCAT1 bacterial transporter is presented; The protein is showed: on the left with an inward-open position, while on the right with a nucleotide-bounded outward occluded position. The membrane is depicted in grey, while the peptide domain are shown in orange. Transmembrane domain (TMD) are numbered in red. (Ford and Beis 2019)

ABC transporter proteins are responsible for a great number of functions (Holand 2003)**.** The most common task of these proteins is to transport a great variety of substrate, such as metabolites, metal ion, amino acids or also drugs, across membranes (Higgins 2001). In eukaryotes, some ABC transporters function as a defence mechanism. In fact, they have been showed to prevent drugs accumulation in tissues by actively up regulating efflux pumps, such as PGPs. Nonetheless, ABC transporters are also required for normal physiological functions (Ardelli 2013). Several studies in nematodes have demonstrated that, similarly to what happens in other organisms, there is an association between ABC

transporters and selection for drug resistance (Blackhall et al. 1998; Ardelli and Prichard 2004, 2007, 2008; Ardelli et al. 2005, 2006a, b; Bourguinat et al. 2008; Bartley et al. 2009; Eng and Prichard 2005; Tompkins et al. 2011). In fact, ABC transporters have also been reported to be correlated with ivermectin resistance in different parasites (Ardelli and Prichard 2004, 2007; Ardelli et al. 2005, 2006a, b; Bourguinat et al.2008).

1.5.1 ABC-B subfamily and drug resistance in filarial nematodes

The core structure of members of the ABC system class 2 is composed of a transmembrane domain (TMD) of twelve transmembrane (TM) helices and a nucleotide-binding domain (NBD). While the first domain, which contains the substrate-binding site, is responsible for the translocation pathway, the second one is responsible for the ATP binding and hydrolysis (Beis 2015). Members of this class are divided into several subfamilies based on their domain organization. In *C. elegans*, eight subfamilies were identified and named with letters, A to H (Sheps et al. 2004).

Of these eight, subfamilies A, B, C and G have been shown to confer multidrug resistance in humans. In nematodes only subfamily B and C have been related to ivermectin resistance (Stitt et al. 2011).

ABCB transporters can include either half-transporters, containing a single ABC_TM1F domain followed by a single ABC_2 domain, or full-transporters, which instead are characterized by two ABC_TM1F domain alternated to two ABC_2 domain (Sheps et al. 2004). In most cases, the distribution of half versus full-transporters is quite even (Ardelli 2013).

Half-transporters have been showed to carry out important function in the cell, as for example in *C. elegans haf1* is required for mitochondrial signalling, while *haf-4* and *haf-9* are involved in intestinal granular formation (Haynes et al. 2010; Kawai et al.2009).

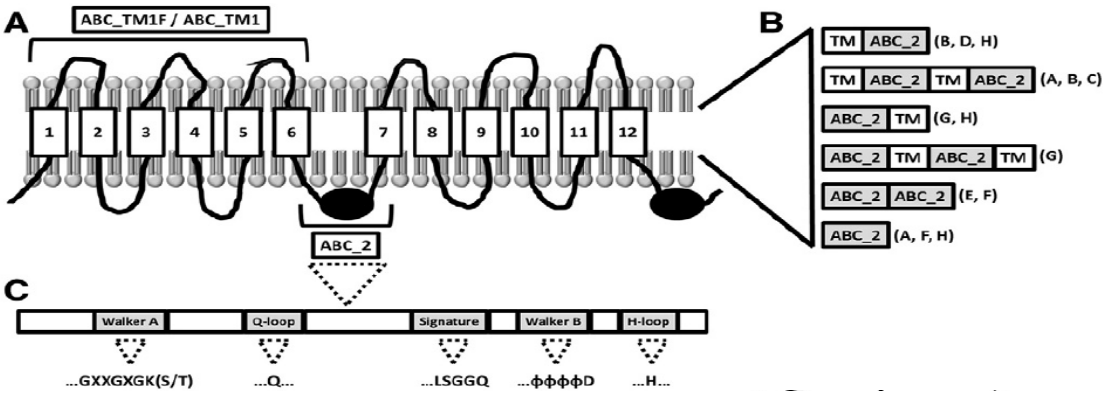


Fig 12: A) Schematic representation of a full transporter containing two transmembrane domains (ABC_TM1F or ABC_TM) that loop through the plasma membrane 12 times and two ATP-binding domains(ABC_2). B) The arrangement of the transmembrane domains and the ABC_2 domains in the subfamilies of ABC transporters. C) Detailed structure of the highly conserved ABC_2 domain showing the Walker A, Q-loop, signature sequence, Walker B and H-loop, together with each respective sequence. (Ardelli 2013)

Bourguinat et al. in 2016 have identified in *D. immitis* seven completed gDNA sequence of ABC-B transporter. Following bioinformatic characterization, three of these were recognized PGP (Dim-*PgP-3,* Dim-*PgP-10*, Dim-*PgP-11*), two were identified as ABC-B half transporters genes (Dim-*haf-1* and Dim-*haf-4*), one ABC half transporter gene contained an ABC-C motif (Dim-*haf-5*). Several other genes still need further investigation in order to be fully characterized (Bourguinat et al. 2016).

Unfortunately, despite the advances in whole genome sequencing only a few nematode genomes have been annotated and even fewer have been completed (Ardelli 2013). Therefore, due to the lack of a finished genome sequence of *D. immitis*, the number of ABC transporters reported by Bourguinat et al. (2016) cannot yet be considered definitive (Godel et al. 2012).

When the phylogenetic tree of full and half ABC-B transporters is considered the sequences from *D. immitis* consistently clustered together with *B. malayi*, *L. Loa* and *O. volvulus*, while it clearly separates from *C. elegans* (Bourguinat et al.2016). This is consistent with the much lower concentration of ABC transporters carried by these organisms, only 20% of the number found in *C. elegans* (Sheps et al. 2004). This might be connected their different style of life. In fact, whereas free-living organisms compose the first, the other two are parasitic species, leading to a reduction in the normal functions of the parasites, since the host already performs them (Ardelli 2013). Despite this difference in the genetic abundance

of the ABC transporter genes, the distribution across subfamilies is relatively constant, being the subfamily B the most abundant (Ardelli 2013).

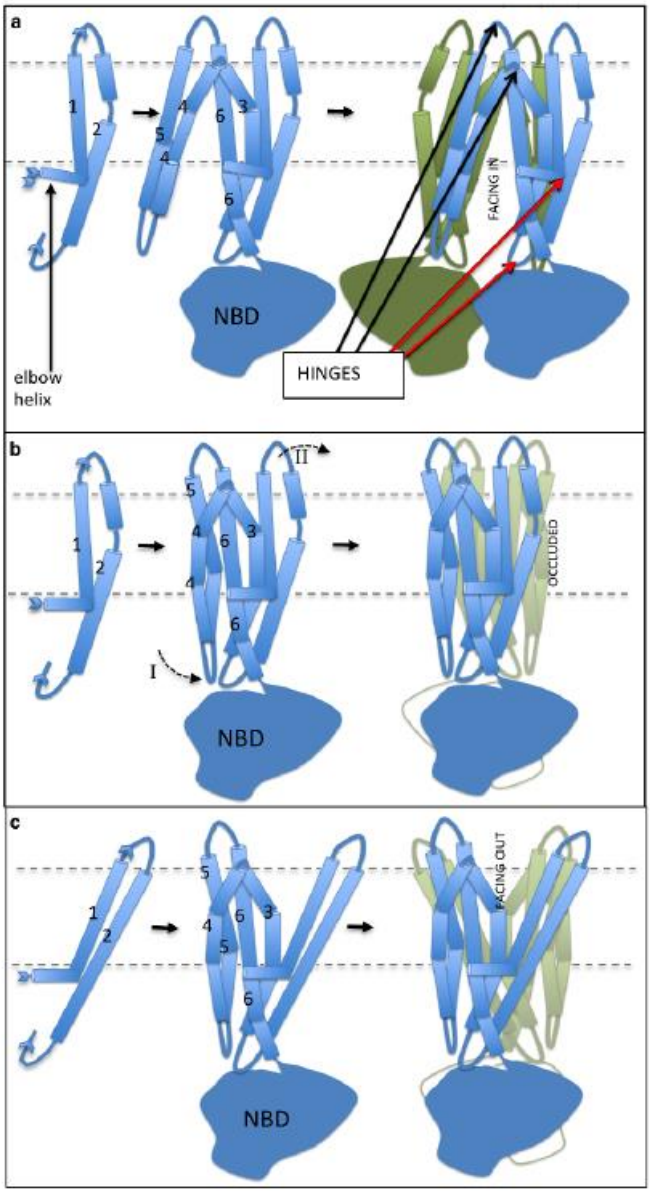


Fig. 13: Topology of the transmembrane helices (numbered in black) of euckaryotic ABCB transporter. The figure shows how the different parts of the protein move during the conformational transition: from and inward-facing state (**a**), to outward-occluded state (**b**), to the final outward-facing state(**c**). The nucleotide-binding domain (NBD) are also indicated in the figure. The hinges formed by the second and third extracellular loop which connecte transmembrane helices 3 with 4 and 5 with 6 are indicated with black arrows. The hinges formed by the N-terminal elbow helix and the first cytoplasmic loop are indicated with red arrows. Movements of the two "swinging arms" are indicated both with courved arrows and by Roman numerals. (Ford and Beis 2019)

1.5.1.1 P-glycoproteins and multidrug resistance

One of the most intensively studied ABC-B transporters is the multidrug transporter P-glycoprotein. This protein, which was first identified by Juliano and Ling in 1976 in clinical oncology, it was shown to confer multidrug resistance to a great variety of structurally unrelated molecules by overexpression in target tissues (Juliano and Ling 1976; Ambudkar et al. 1999).
PGP are responsible for the membrane transport of neutral and cationic hydrophobic compounds, and they have a pivotal role in bioavailability and tissue distribution of drugs. Thus, they were showed to affect IVM distribution inside the target nematode (Tompkins et al. 2011).

Riodan and Ling discovered the first mechanism of MDR in 1979 in tumour cells. This mechanism involved an overexpression of PGP. Since then, several other ABC transporters have been shown to confer resistance in a similar manner as PGP (Cole et al. 1994; Allikments et al.1998; Litman et al. 2000; Bates et al. 2003; Borst et al. 2003). Due to the conserved structure and functionality of the ABC systems throughout different domains, it is not surprising the occurrence of similarities in their roles in drug action and resistance between mammals and nematodes (Higgins et al. 1992; Ardelli 2013). Although several mechanisms for cellular resistance to single class of cytotoxic drugs have been suggested, such as down-regulation of target genes, overexpression of transporters or changes in mRNA stability; the principal MDR system described so far in literature has been the active efflux of drugs by cellular membrane transporters with a broad spectrum of substrate specificity (Higgins 2007; Ménez 2011). In mammals as well as in several nematodes, such as *C. elegans*, *H. contortus*, *T. circumcinata*, *B. malayi* and *O. volvulus*, the PGP associated resistance mechanism is often observed as change in gene expression (Sangster et al. 1999; Huang and Prichard 1999; Higgins 2007; Ardelli and Prichard 2008; James and Davey 2009; Dicker et al. 2011; Williamson et al. 2011). In fact, previous studies have reported an overexpression of PGP in ivermectin resistant strains of *Caenorhabditis elegans* after exposure to ivermectin (Ardelli and Prichard, 2008).

Further studies have reported the occurrence of specific genetic changes in PGPs' sequences in drug resistant strains of nematodes. This has also been shown in *D. immitis* by Bourguinat and colligues (2011b), which reported a GG-GG genotype in ivermectin resistant *D. immitis*. These mutations, which occur as single nucleotide polymorphisms (SNPs), were identified in the alleles of the ABC transporter genes and are a result of the exerted selection pressure of the long-term drug regimen. Although most

of these SNPs cause silent mutations, some are correlated with a reduced sensitivity to specific drugs (Ardelli 2013).

It has also been suggested that the increase in expression of PGP may facilitate the activation of other resistance mechanisms, as the stimulation of coexisting drug transporters (e.g. MRP1, ABC2) (Stitt et al. 2011).

2. Aim of the study

As already mentioned in the introduction, several *in vivo* studies have been focusing on the analysis of the effects on both dogs and parasites of the alternative therapies available for heartworm disease, either moxidectin/doxycycline or ivermectin/doxycycline. All of them have reported for the combination treatments (DOXY+IVM/MOXI) a superior and more significant adulticidal effect against *D. immitis* compared to when either one of the drugs was administered alone (Bazzocchi et al 2008; Savadelis et al. 2018; Genchi et al. 2019). In addition, previous studies have reported a higher tolerance to adulticide treatments of female worms compared to male worms of *D. immitis*, both when exposed to melarsomine dichlorohydrate or to the ivermectin/doxycycline combination (Keister et al. 1992**;** Bazzocchi et al. 2008). Hence, a better understanding of how MLs and doxycycline work together is very important improving treatment of filarial parasites, not only in animals, but also for all human filariasis, which are still causing disease in millions of people worldwide.

Therefore, in light of these findings, the main goal of this study was to elucidate the molecular mechanisms laying behind the increase in efficacy of the MLs and antibiotic combination against *D. immitis.* To do so, the role played by membrane transporters of *D. immitis*' adults, and in particular of the ABC transporters, in the response to the two drugs of interest was investigated, not only when the two drugs were administered in combination but also when they were given separately. Moreover, in an attempt to to shed light on the reasons connected to the observed female higher tolerance to treatments, it seemed appropriate to consider the results according to worms' gender.

Previous studies carried out on several eukaryotic organisms including *D. immitis*, have reported that ATP-transporters (ABCTs), are involved in the active carriage of drugs across cell membranes (Wilkens et al. 2015). Doxycycline has also been described to inhibit the expression of the membrane ABC transporters in mammals (Agbedanu et al 2015). Therefore, based on this knowledge, the starting hypothesis of this PhD project was that the increased adulticide effect of the doxycycline/ML combination might be due to the interaction of either one of the two drugs with cellular efflux mechanisms. This could lead to a change of the membrane transporter genes' modulation, causing an increase in the intracellular concentration of the other drug.

3. Materials and Methods

3.1 Membrane transporter sequence selection and analysis

Genome analysis and ABCB identification in *D. immitis* was previously described by Bourguinat et al. (2008). The study reported the presence of seven genes annotated as having secretory functions. Of these seven genes, one, which was a pseudogene, has been excluded from the present study. The expected protein sequence of each of the six genes was imported into Geneious 10.0.9 and BLASTp analysis was performed. The protein sequence of each transporter was blasted against the non-redundant protein sequences (nr) database of NCBI. Ten sequences from each Blast were selected between the hits with identity greater than 55%. A seventh gene, previously reported by Yates and Wolstonholme (2004), encoding a glutamate-gated chloride channel alpha3B subunit, has been also selected for the gene expression study. Since this channel, as well as the ABCB transporter genes, have been previously extensively described, no additional sequence analysis were carried out.

3.2 Reagents

All reagents were purchased from Sigma-Aldrich® (Missouri, USA), except where indicated.

3.3 Collection and maintenance of *D. immitis* adults

Seven privately-owned dogs that had never received macrocyclic lactones or doxycycline were diagnosed with patent *D. immitis* infection. Following owners' consent, dogs underwent minimally-invasive surgical heartworm removal (AHS guidelines 2018). In addition to those dogs, two control dogs from a concluded study at TRS Labs (Athens, GA, USA), were used as worm donors.

A total of 36 female and 36 male adult worms were collected. Extracted worms were washed in Hank's Balanced Salt Solution (HBSS) medium at room temperature, checked for viability, counted, sexed, and then placed individually into 50mL of Roswell Park Memorial Institute (RPMI) Medium and stored overnight at 37°C 5% CO2.

3.4 Optimization of DNA and RNA co-extraction

The following procedure (Fig. 14) is part of a publication in press (Lucchetti et al. 2019b*)*.

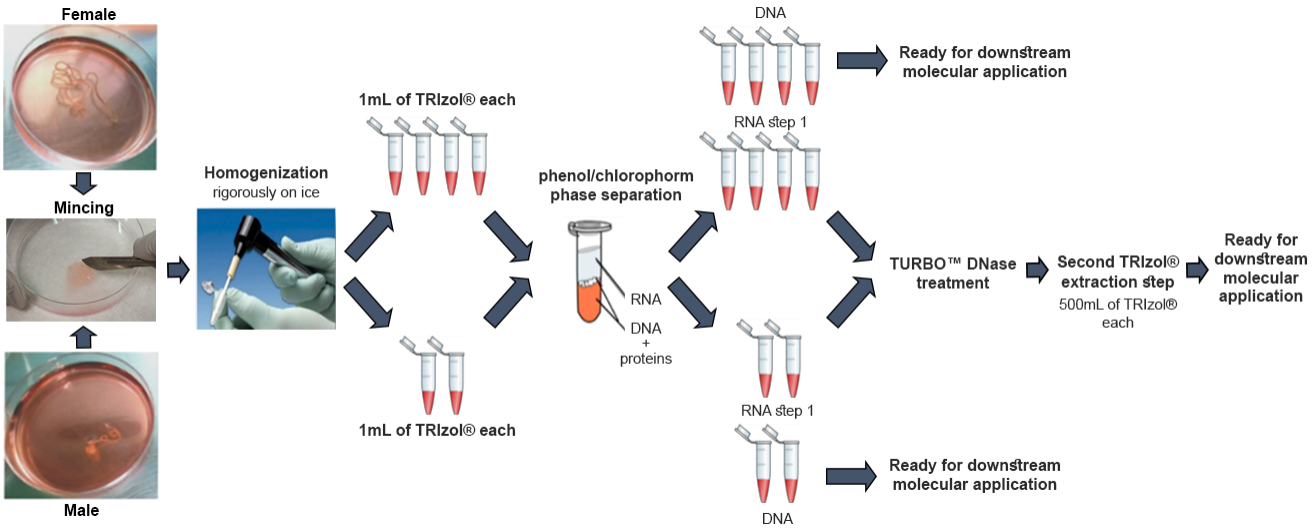


Fig. 14: Schematic representation of the optimized process of RNA/DNA co-extraction described in detail below (Lucchetti et al. 2019b).

3.4.1 Preparation of adult worms

12 adult worms of *D. immitis*, respectively 6 females and 6 males, were used for the optimization step. Extracted worms were washed in HBSS medium (Sigma-Aldrich®, Missouri, USA) at room temperature, checked for viability, counted and sexed. Three female and three male worms were individually placed into 15mL Falcon tubes and stored at -80°C. Single worms were used as starting material for the RNA/DNA co-extraction.

3.4.2 Sample homogenization

Since female *D. immitis* are longer than males (30 cm vs. 20cm, approximately), the following steps of extraction were performed slightly differently between the two sexes.

Each frozen worm was homogenized individually. The worm was placed into a precooled Petri dish with 500µL of cold TRIzol® Reagent (Ambion®, Foster City, USA). With the help of a scalpel, the worm was chopped into tiny pieces, thin enough so that they could be picked easily up by trimmed 1000µL tips, and then placed it into a sterile RNase and DNase free 1.5mL Eppendorf tube. Each worm was homogenized using an electric pellet pestle (Sigma-Aldrich®, Missouri, USA) for 3 minutes. Then, an

additional 500µL of TRIzol® Reagent were added to partially homogenized sample, and after mixing it thoroughly, 500µL of it were transferred into a new sterile tube. Homogenization with the electric pestle was repeated once more per each of the two tubes, for 2 minutes. For females, since their initial weight was twice that of males (~220 mg and ~115 mg respectively), the homogenization step was repeated one more time per each of the two samples just prepared. A further 500µL of TRIzol® Reagent was added to each of the two tubes and, after mixing the sample thoroughly, 500µL of sample were transferred into a new tube. Each sample was homogenized again for a 1 minute. All steps were performed on ice, to avoid RNA degradation.

3.4.3. RNA extraction through Phasemaker™ Tubes

Three adult worms of *D. immitis* per each sex were analyzed with a product specific for the optimization and simplification of RNA isolation: Phasemaker™ Tubes (Thermo Fisher Scientific, Waltham, USA). Thus, two samples per each female and one per each male were used for the extraction procedure with Phasemaker™ Tubes according to the manufacturer's protocol. All remaining samples, respectively two samples per each female and one per each male, were processed using the proposed co-extraction protocol.

3.4.4. DNA and RNA co-extraction

To each tube containing 500µL of homogenized sample a further 500µL of TRIzol® reagent were added. They were treated according the TRIzol® Reagent manufacturer protocols (Chomczynski 1993). Once the three phases were separated and the aqueous phase set apart from the other two, the two isolation steps were performed once again according to the manufacturer protocol, although a few changes were made at the elution step for both RNA and DNA procedures.

The final elution of RNA samples was performed in 100µL of DEPC water, and the rehydration of the sample was performed at 56°C per 13 minutes. Some samples, due to the presence of a greater pellet, were placed in a water bath at 56°C for a longer time, until the pellet was fully rehydrated.

The final elution of DNA samples was performed in 300µL of DEPC water, and they were then placed in a water bath at 46°C for 15 minutes.

After resuspension, all samples were briefly vortexed and analyzed at the spectrophotometer (BioSpectrometer® fluorescence with µCuvetta® G 1.0, Eppendorf, Hamburg, Germany). For each

sample, data concerning concentration (ng/ µL) and quality, described as 260/280 and 260/230 values, were collected.

In order to guarantee RNA quality which would allow down-stream analysis, such as quantitative/relative RT-PCR, each sample required the elimination of any possible residue of genomic DNA. A solution of 150 ng/µL of RNA in a final volume of 50µL was prepared per each sample. To each samples were added 5µL of TURBO™ DNase buffer and 2µL of TURBO™ DNase (Thermo Fisher Scientific, Waltham, USA) and then they were incubated at 37°C for 30 minutes. After the first incubation, to each samples were added other 2µL of TURBO™ DNase enzyme and incubated again at 37°C for other 30 minutes.

A 500µL of TRIzol® Reagent was added to each tube and a second RNA extraction was performed following the manufacturer protocol. The final elution was performed in 35µL of DEPC water and as performed for the first RNA extraction, pellets were rehydrated at 56°C for 13 minutes. All samples were quantified at the spectrophotometer.

3.4.5. Preparation of cDNA

To obtain a fully pure RNA sample to be used for gene expression analysis, an additional purification step was performed. RNA samples were treated with AccuRT Genomic DNA Removal Kit (Abm, Richmond, Canada) according to the manufacturer protocol. Starting with 300ng of final RNA, and followed by an incubation at room temperature of 8 min. For cDNA synthesis the OneScript® cDNA Synthesis Kit (Abm, Richmond, Canada) was used. The presence of any genomic contamination was checked by PCR reaction using GOTAQ Flexi DNA Polymerase Green G2 (Fisher MB, Rome, Italy) and 0.35 µM of specific primers designed on Dim-*pgp-10* for cDNA (see Table 2) (Sigma-Aldrich®, Missouri, USA) (Bourguinat et al. 2016). The amplification protocol was characterized by a denaturation step at 95°C for 1,30 min, followed by 40 repeated cycles: 95°C for 15 sec; 59.4°C for 1min with a final elongation of 72°C for 5 min. Amplicons were run on a 2% agarose gel.

3.5 In-vitro treatment of adults

The remaining extracted adult parasites, respectively 30 females and 30 males, were placed individually in tubes with 50 mL of NI media (Evans et al. 2013). They were then treated as follows: 1% DMSO alone (DMSO), ivermectin (3.54 nM) with 1% DMSO (DMSO+ivermectin), 1% DMSO in combination with doxycycline (56.5 µM) (DMSO+doxycycline) and, finally, the combination of doxycycline (56.5 µM) and ivermectin (3.54 nM) together with 1% DMSO (DMSO+doxycycline+ivermectin). Untreated samples, plain NI media, served as controls. Concentrations of the two drugs were chosen according to their highest plasma concentrations measured after *in vivo* treatments of dogs (Daurio et al. 1992; Gutiérrez et al. 2012). Given that the present *in vitro* study attempts to mirrored what occurs *in vivo*, drug concentrations were not calibrated based on the parasite's gender and study media volumes were standardized to 50 mL for both sexes. Each treatment was performed in triplicates, for both sex. Half of the worms were maintained for 24h at 37°C, 5% CO_2, and then washed in HBSS and then stored at -80°C. The other half of the collected worms, after the first 24h of treatment, was placed into a fresh treatment media tube to continue treatment exposure for 24h more, for a total of 48h. These exposure times were chosen based on a previous study on IVM treatments both in vivo as well as *in vitro* of other nematodes (Maté et al. 2018; Reaves et al. 2018). After treatment all samples were checked for vitality and, after being washed in HBSS, they were promptly frozen at -80°C. After the treatment, all media were stored at -20°C.

3.6 RNA extraction and cDNA preparation

After treatment, each of the 60 worm was used for RNA extraction following the procedure just described above in chapter 3.4.4. The Resulting RNA was then used for cDNA preparation according to the procedure described above (see chapter 3.4.5).

3.7 Relative quantitative reverse-transcription PCR (qRT-PCR)

The sequences of three ABC-B transporters genes (Dim-*pgp*-3, Dim-*pgp-10*, Dim-*pgp-11*) and of three ABC-B half transporter gene (Dim-*haf*-1*,* Dim-*haf*-4 and Dim-*haf*-5) and that of a glutamate-gated chloride channel (*Di-avr-14*) were previously described (Bourguinat et al. 2016; Yates and Wolstonholme 2004). Primers sets were tested in order to optimize the efficiency and the dynamic range of the reaction. As endogenous control, the 18S ribosomal subunit was selected (Bazzocchi et al. 2008). All primers used for this study are listed in Table 2. Three biological replicates of both sex were prepared per each treatment of interest. Three technical replicates were analyzed per each of the biological replicate. Gene expression study was carried out by mean of a relative qRT-PCR, where results were normalized, first to the internal control gene (18S rDNA) and then to the treatment control (NI alone). The BrightGreen 2X qPCR Mastermix (Abm, Richmond, Canada) was used according to the manufacturer's instructions. The amplification protocol was characterized by a denaturation step at 95°C for 1 min and 30 sec, followed by 40 repeated cycles (95°C for 15 sec; 59.4°C per 1min for all genes except for Dim-*haf-4* gene which instead required an annealing time of 57.7°C per 1 min for; 72°C for 20 sec). Final concentration for each primer in all reactions was 0.35 µM. Fluorescence signals were collected at every cycle and to avoid the presence of unspecific products, a melting curve analysis was performed.

Table 2: Primers used in the present study to evaluate the expression of the two ABC-B transporter genes, of the ABC-B half transporter gene and of the glutamate-gated chloride channel.

Gene	Primer's Name			Primer Sequences	
Dim-*pgp*-3	DimmScaf46-cDNA-F6	F:	5' -	TGCGTTGAGCGTTAGTTTCATGCCA	- 3'
	DimmScaf46-cDNA-R7	R:	5' -	TTCGTACCAACCAAAGGCCA	- 3'
Dim-*pgp*-10	DimmScaf48-cDNA-F8	F:	5' -	GCCATCGTAGGTCCATCAGGTTCTGGT	- 3'
	DimmScaf48-cDNA-R12	R:	5' -	TGTTCAACTGAAACGACCACACGTC	- 3'
Dim-*pgp*-11	DimmScaf04-cDNA-F6	F:	5' -	TTAACAGTGTTGATGAAGGATCAAATCC	- 3'
	DimmScaf04-cDNA-R6	R:	5' -	ATATTTCGCTGCGGTCTTGTTGG	- 3'
Dim-*haf-1*	DimmScaf23-cDNA-F4	F:	5' -	AGCACAGGAACCCATTCTAT	- 3'
	DimmScaf23-cDNA-R5	R:	5' -	AGTTCCGTGTTCACCAACAA	- 3'
Dim-*haf*-4	DimmScaf101-cDNA-F5	F:	5' -	GTGCAAACTCGAGGTTTTGCTGT	- 3'
	DimmScaf101-cDNA-R2	R:	5' -	TCCACCTCGAAGACCTCCAGCA	- 3'
Dim-*haf*-5	DimmScaf496-cDNA-F4	F:	5' -	TCCAACTGCATCCAAGGAAGAGG	- 3'
	DimmScaf496-cDNA-R3	R:	5' -	TCGAATCACCGAATCGTGCAA	- 3'
Di-*avr-14*	Di-avr-14_Fw	F:	5' -	GCAAGAAGACATCTTATTGACAAG	- 3'
	Di-avr-14_Rev	R:	5' -	CGGGCAAGATAATACCAATGAAAG	- 3'
18S rDNA	18SQ-F	F:	5' -	GGGACAAGCGGTGTTTAGC	- 3'
	18SQ-R	R:	5' -	GCACGCTGATTCCTCCAGT	- 3'

3.8 Data analysis

Results were presented as the relative change in gene expression (2^ -ΔΔCt) ± standard deviation (SD) of the three biological replicates. All Ct values were managed by CFX Manager Software (Bio-Rad, Hercules, California, USA) and the 2^ -ΔΔCt was calculated according to the Livak method (Livak and Schmittgen 2001). Results were normalized, first to the internal control gene (18S rDNA) and then to the treatment control (NI alone). Three biological samples were analyzed per each sex. Standard deviation between each technical replicate was calculated. All standard deviations were lower than 0.3, which is the threshold values for a Ct standard deviation to be considered accurate, as described in the "Guide to performing relative quantitation of gene expression using real-time quantitative PCR"

published by Thermofisher Scientific (https://tools.thermofisher.com/content/sfs/manuals/cms_042380.pdf.; Anonymous, 2008).

Standard deviation per each 2^ -ΔΔCt was calculated according to the same manual. Data normality and distribution were tested respectively with Pearson test and one-way ANOVA using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego California USA, www.graphpad.com), and P-values < 0.05 were considered significant.

3.9 HPLC analysis for doxycycline quantification in culture media

The concentrations of doxycycline in different treatment media (DMSO+DOXY and DMSO+DOXY+IVM) post-treatment were measured by means of HPLC method as described by Menozzi et al. 2015. A standard curve was prepared with doxycycline in NI media. The internal control selected for the study was the DMSO+DOXY.

As previously described by Lucchetti et al. (2019a), for this analysis a Prostar LC Workstation (Varian Co., Walnut Creek, CA, USA), with a Prostar325 UV-Vis detector and a 10µL loop were used. While, for the chromatographic separation Syncronis C18 ana-lytical column (Thermo, Milan, Italy) (5 µm particle size, 150 mm × 4.6 mm) was selected. Acetonitrile and 0.01 mol/L trifluoroacetic acid (30:70, v/v) were used as mobile phase, with a flow rate of 1.0 mL/min and an analytical wavelength of 350 nm. The software Star Chromatography Workstation System control version 0.41 by Varian was used.

4. Results

4.1 Characterization of membrane transporter genes

To investigate whether any membrane transporter genes have already been described in *D. immitis*, a bibliographic research has been carried out. Seven genes were found relevant for this study, all of which have already been extensively described by previous literature (Table 3). Six ABC transporter genes were included in the study: three full ABCB transporter (Dim-*pgp-3*, Dim-*pgpg-10*, Dim-*pgp-11*) and three half transporters two belonging at the ABCB subfamily (Dim-*haf-1* and Dim-*haf-4*), while the third to the ABCC subfamily (Dim-*haf-5*). The last selected transporter is Di-avr-14 and is a glutamate-gated chloride channel, highly conserved in nematodes (Yates and Wolstonholme 2004).

Table 3: The table lists the name of the reference sequences of all genes taken into account, the protein encoded and other information such as the transporter localization and/or the proposed function.

SELECTED GENES	REFERENCE SEQUENCES	STRUCTURE	ADDITIONAL DETAILES
Dim-*pgp-3*	nDi.2.2.scaf00046	PGP	Localized on the apical membrane of excretory an intestinal cells;
Dim-*pgp-10*	nDi.2.2.scaf00048	PGP	Expressed in the intestine of the nematode;
Dim-*pgp-11*	nDi.2.2.scaf00004	PGP	Involved in the detoxification of the worm from ivermectin;
Dim-*haf-1*	nDi.2.2.scaf00023	ABC-B region	Required for the transport of peptides and for the activation of the unfolded protein response (UPR), located in the internal mitochondrial membrane;
Dim-*haf-4*	nDi.2.2.scaf00101	ABC-B region	Required for excretion, growth and normal conformation of the worm;
Dim-*haf-5 (5.1-5.2)*	nDi.2.2.scaf00496	ABC-C region	Transport of heme group form the mitochondria to the cytoplasm;
Di-*avr-14*	AJ581673.1	GluCl	glutamate-gated chloride channel alpha3B subunit

4.2 Optimization of DNA and RNA co-extraction procedure

Since adult worms of *D. immitis* can only be obtained either through necropsy of experimentally infected dogs or by minimally-invasive surgical heartworm removal of naturally infected dogs, each individual worm sample is extremely important. For this reason, being able to exploit fully each sample it is essential. Therefore, to have the possibility of performing multiple analysis on a single adult worm, an already existing method for simultaneous DNA/RNA isolation from a single adult of *D. immitis* was adapted and optimized. This new protocol allows co-extraction of DNA and RNA from the same sample, thus providing the opportunity to perform multiple molecular analysis which otherwise would be impossible to perform on the same sample. In particular, this new protocol allows to correlate genes expression by the parasite with bacterial load; hence it may offer new insights into the effect of *Wolbachia* reduction on the parasite host's biology and function, and thus into the adulticide effects of specific treatments.

Results obtained from the co-extraction procedure applied on three female worms and on three male worms show that the DNA extraction works better for female worms than for male, for which mean concentrations are respectively 183.88 ng/µL and 31.8 ng/µL (Table 4).

Female concentrations measured after the first round of extraction (RNAstep1) were also much higher compared to those obtained for male (408.47 and 261.8 respectively) although both purity (260/280) are close to the 1.8 value. The two sex concentrations got closer after the second round of RNA extraction, in fact the mean concentrations for both sex were about ~160ng/µL, with much higher purity (260/280= ~1.9).

Table 4: Data collection reporting concentrations (ng/µL) as well as the 260/280 and 260/230 parameters measure by spectrophotometer analysis of three biological replicates per each sex. All samples were analyzed individually and per each of the six worms the mean values of the technical replicates were calculated and listed in the table. In the table are reported values for the overall mean and standard error of mean (SEM). (Lucchetti et al. 2019b).

SEX	DNA			RNA STEP 1			RNA FINAL		
Female	ng/µL	260/280	260/230	ng/µL	260/280	260/230	ng/µL	260/280	260/230
1	137.65	1.31	0.88	358.63	1.81	0.82	152.95	1.84	0.70
2	212.83	1.32	0.91	354.00	1.82	0.79	181.55	1.95	1.19
3	201.18	1.31	0.74	512.78	1.72	0.57	161.38	1.83	1.15
MEAN	183.88	1.31	0.84	408.47	1.78	0.73	165.29	1.88	1.01
SEM	±35.10	±0.00	±0.09	±41.48	±0.02	±0.06	±18.65	±0.05	±0.15
Male									
1	55.45	1.37	0.61	261.80	1.75	0.37	160.45	1.86	1.03
2	29.60	1.36	0.39	323.25	1.83	1.07	168.10	1.86	1.46
3	10.30	1.75	0.17	304.10	1.87	1.25	166.60	1.80	1.68
MEAN	31.8	1.5	0.4	261.8	1.8	0.4	160.5	1.9	1.0
SEM	±14.59	±0.11	±0.14	±14.07	±0.02	±0.17	±2.24	±0.03	±0.14

4.2.1 Validation of efficiency of the proposed protocol

To confirm that the co-extraction protocol developed was optimized, its results were compared with those obtained using Phasemaker™ Tubes (Thermo Fisher Scientific, Waltham, USA). Since these tubes cannot be used for co-extraction, as they are specific for RNA isolation, only results for RNA could be compared (RNA step1 and RNA final). The concentrations obtained of the RNA step 1 are generally higher when using the Phasemaker Tubes although the purity of the extraction results higher when the optimized protocol is applied

Results collected following the two protocol of RNA extraction, reported in Table 5, suggest no particular difference between both quality and quantity of the RNA extracted. In fact, no statistical difference has been observed between the two different applied procedures when the single worms were considered separately. On the other hand, when the overall results from each sex were compared,

the statistical analysis showed a marked significant difference (p-value=0.005) in female RNA step 1 and a less significant difference for RNA final (p-value=0.03). No difference was observed for any RNA extracted from males.

Table 5: Comparison between concentration and quality of samples from untreated worms (Control) extracted following either the newly developed protocol or using the Phasemaker™ Tubes manufacturer protocol. Mean ± SEM values calculated between the three biological samples are reported. (Lucchetti et al. 2019b)

Samples		RNA step 1			RNA final		
		ng/µL	260/280	260/230	ng/µL	260/280	260/230
Female	Novel protocol	616.00 ± 44.67	1.90 ± 0.01	1.50 ± 0.10	141.52 ± 14.00	2.11 ±0.13	1.57 ± 0.22
	Phasemaker™ Tubes protocol	859.52 ± 46.13	1.81± 0	1.31 ± 0.10	100.75 ± 10.77	2.16 ±0.15	1.44 ± 0.44
Male	Novel protocol	320.87 ± 16.62	1.76 ± 0.06	0.98 ± 0.15	205.33 ± 12.42	1.75 ± 0.06	1.16 ± 0.36
	Phasemaker™ Tubes protocol	346.23 ± 43.81	1.66 ± 0.06	0.81 ± 0.10	167.50 ± 0.02	1.70 ± 0.02	0.81 ± 0.27

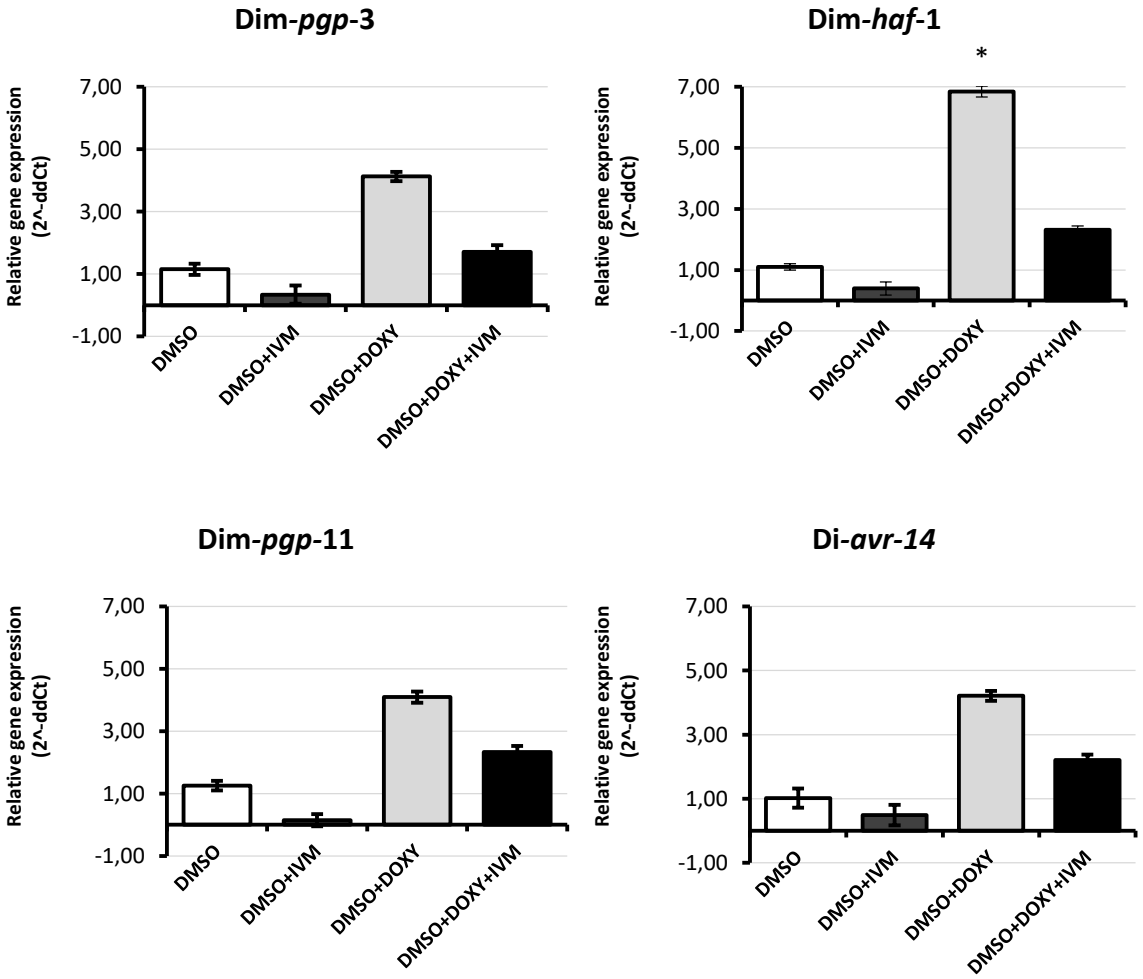
4.3 Determination of gene expression profile following *in vitro* treatments

To investigate the role played by membrane transporters of the parasite in response to the different drugs combination treatment performed, a gene expression study was carried out. Seven membrane transporter genes were analyzed through Relative Quantitative RT-PCR analysis. For all the analysis very little variation between NI controls (whose values were set to 1) and solvent control composed of medium with DMSO alone (whose values ranged between 0.9 and 1.4-folds) was observed. Thus, results for the remaining treatments were compared to the values obtained for the DMSO control.
All profiles are presented with the same scale for the relative ratio.

4.3.1 Membrane transporters' gene expression following 24 hours treatment

Results collected for female worms after 24 hours of treatment are reported in Fig. 15 after normalization. All genes expression, made exception for the Dim-*haf-4* gene, followed a similar trend.

All transporter genes underwent a down-regulation when ivermectin alone was administered. The greatest reduction was observed for Dim-*pgp-10* with 0.11 fold. On the contrary, doxycycline alone induced a marked increase in all gene expression, reaching a fold change greater than 4 for Dim-*pgp-3*, Dim-*pgp-10*, Dim-*pgp-11*, Di-*avr-14*, with a peak of 6.85 fold change for Dim-*haf-1*. With the combination treatment (DMSO+DOXY+IVM) there was a general up-regulation of all genes compared to the DMSO control. Made exception of results obtained for Dim-*haf-4* gene, where the expression reaches 14 fold, for all other genes, a balanced effect of the two drugs is observed. Exposure to the combination induced an up regulation that is not as high as that from the DMSO+DOXY treatment (reaches ~2 fold).



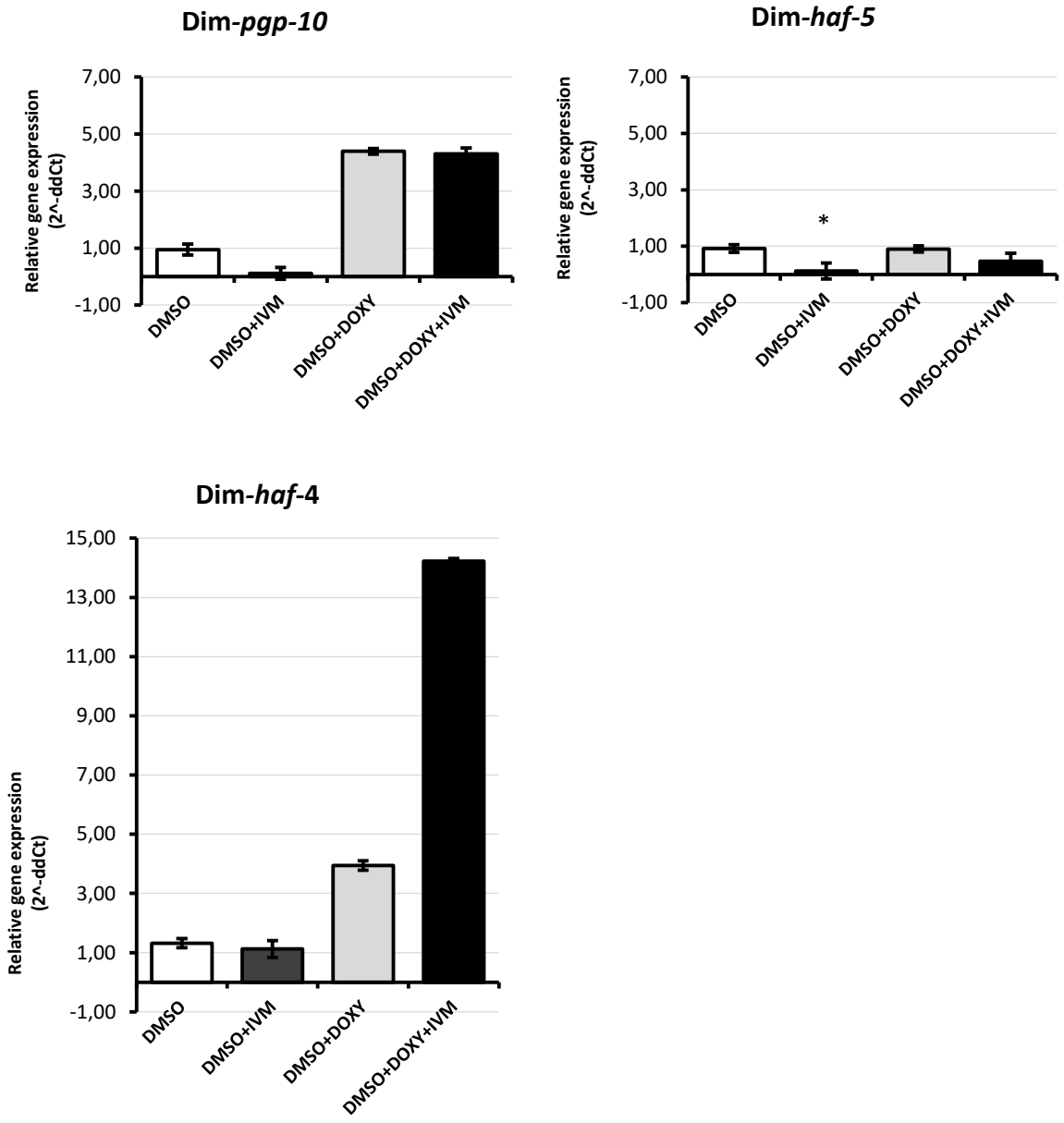
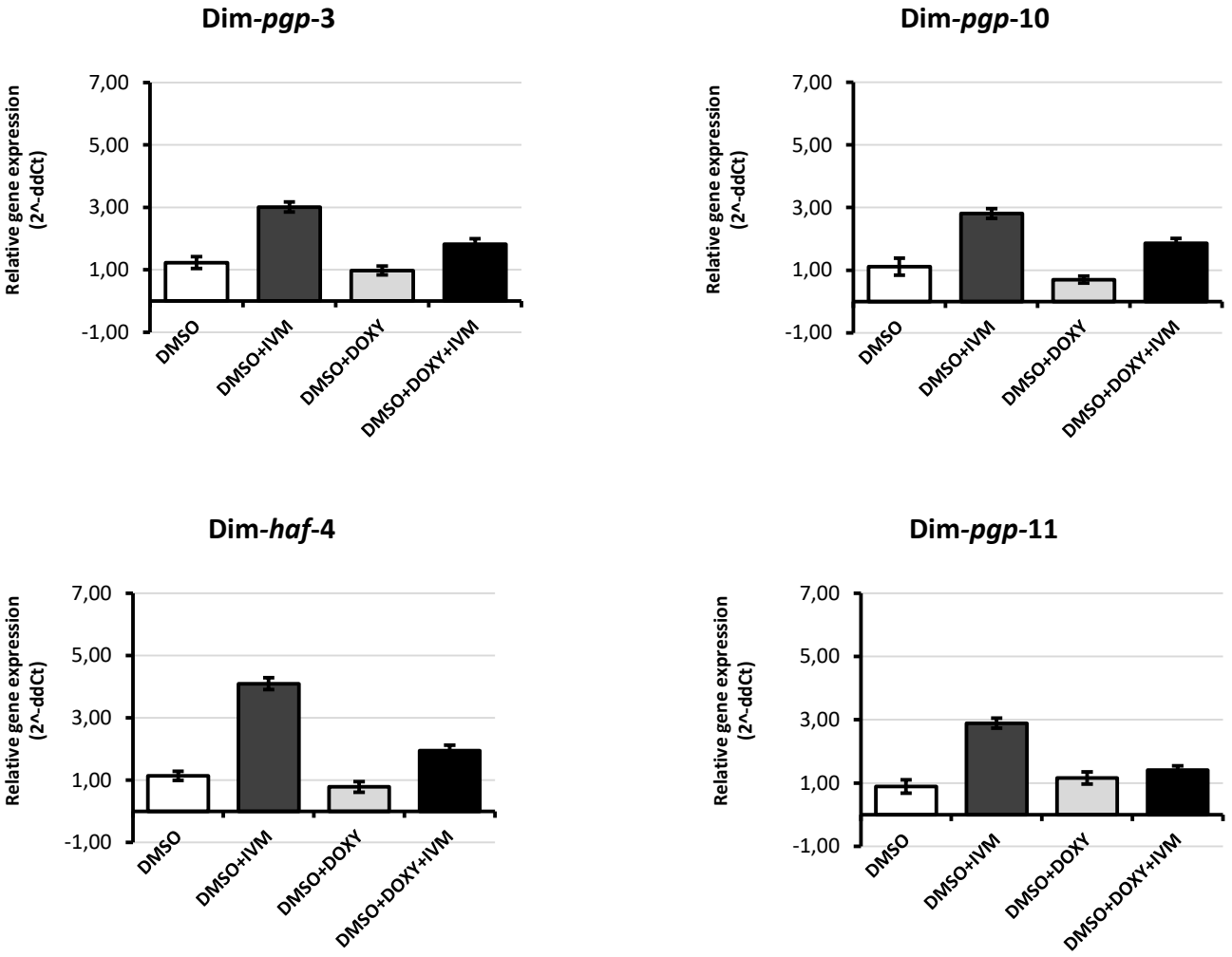


Fig. 15: Gene expression analysis of female worms treated for 24h with the following treatments: DMSO(control treatment with NI media + 1% DMSO); DMSO+IVM (ivermectin + 1%DMSO); DMSO+DOXY (doxycycline+1%DMSO); DMSO+DOXY+IVM (doxycycline + ivermectin + 1% DMSO). All results were expressed as fold change of the gene of interest (2^-ΔΔCt), normalized first to the internal control (housekeeping gene: 18S) and then to the control treatment (plain NI media). Three worms were considered per each treatment and each of them was analyzed in triplicate. Data resulting from these analyses are as the mean ± S.D. of each treatment. Statistical significance (p value < 0.05) are reported in the graph as (*).

On the contrary, results obtained for male worms underline a very different general trend (Fig. 16). In fact, when ivermectin was administered alone there was a general up-regulation, of ~3 folds, for all transporter genes analyzed, with the highest increase observed for Dim-*haf-4* reaching 4.10 fold.

In contrast to what observed for females, the administration of doxycycline alone in male did not induce any change in expression compared to control values. In this case, the combination treatment also showed an up-regulation compared to the control level (up to1.86 fold for Dim-*pgp-10*), but as reported also in female, the up-regulation did not reach the values obtained by the single drug administration (DMSO+IVM).



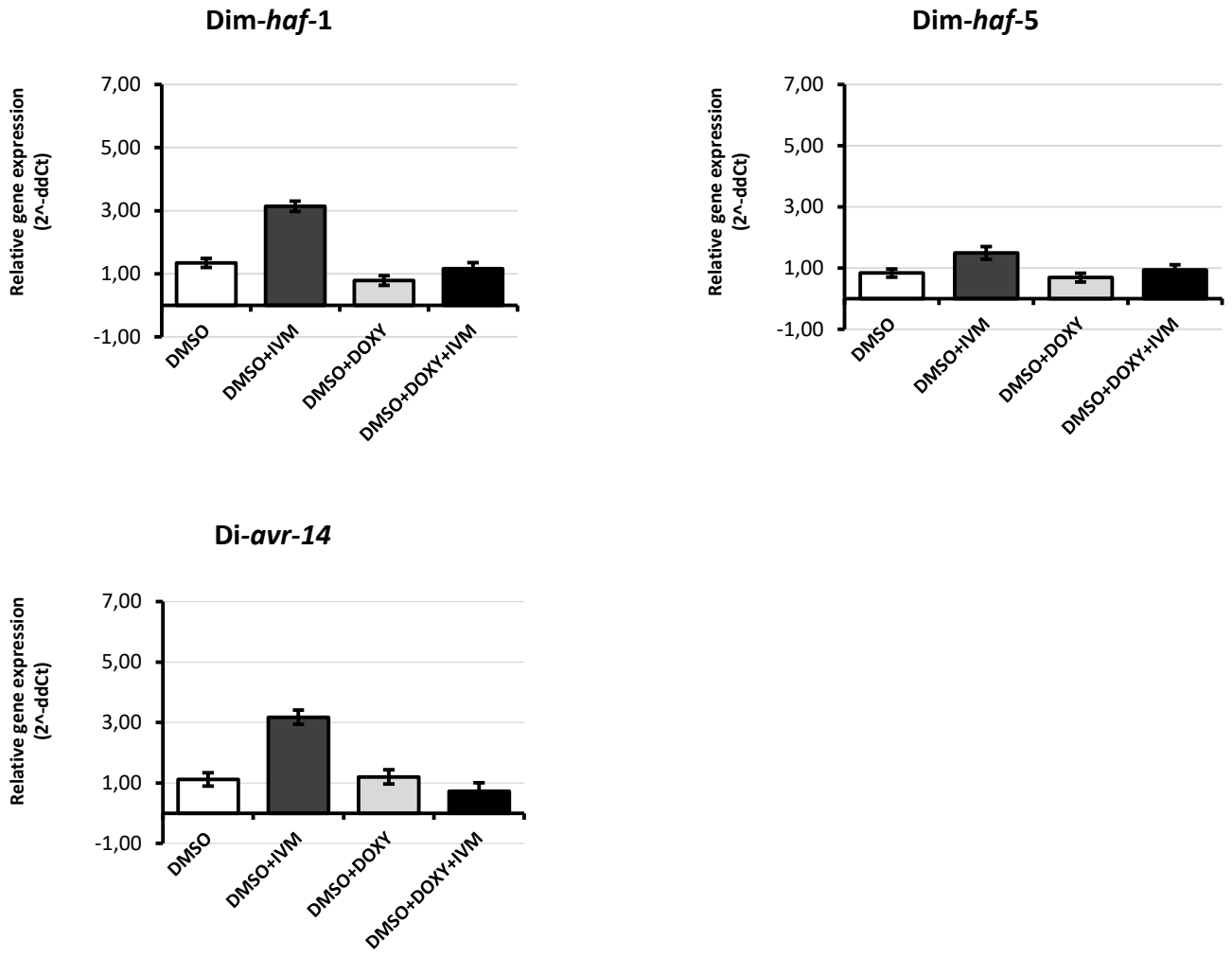
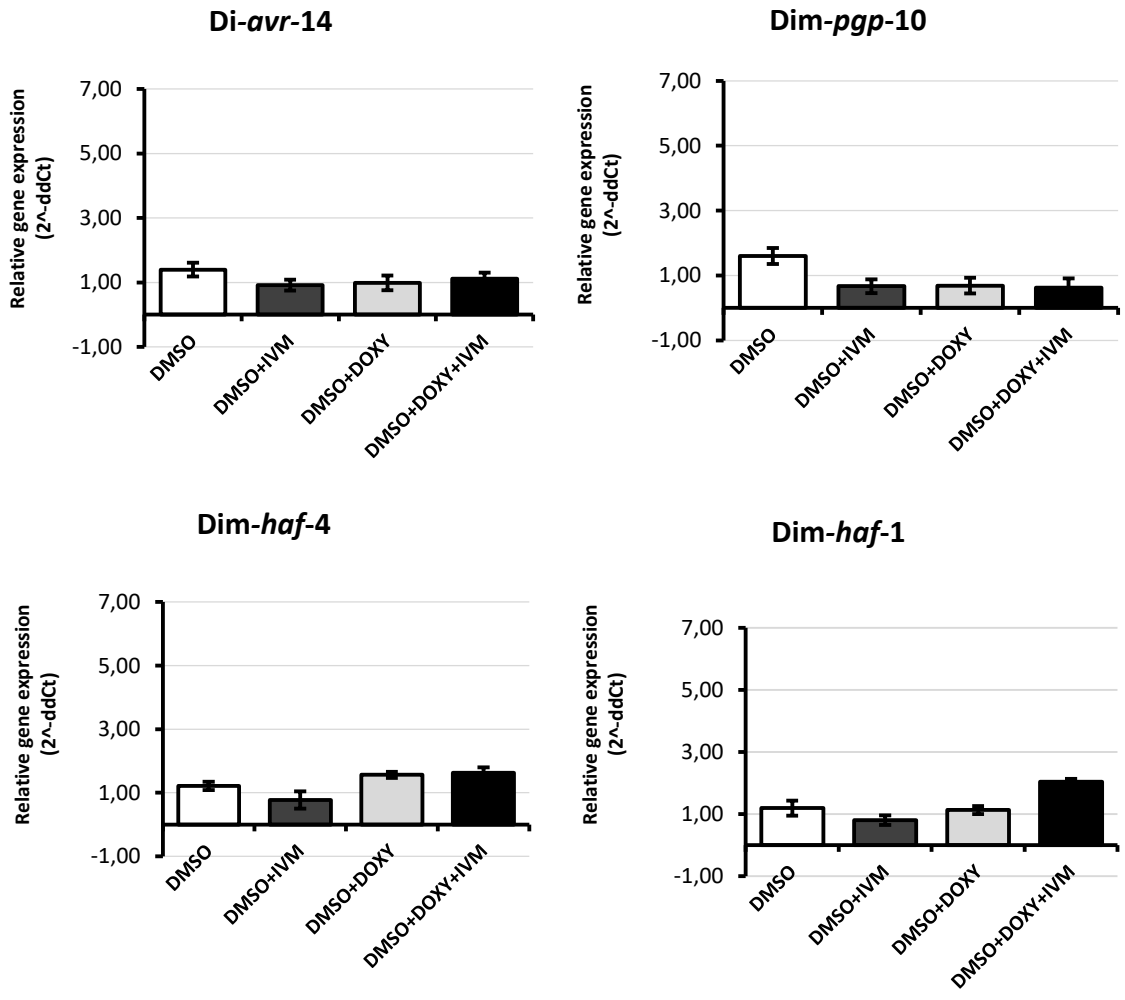


Fig. 16: Gene expression analysis of male worms treated for 24h with the following treatments: DMSO (control treatment with NI media + 1% DMSO); DMSO+IVM (ivermectin + 1%DMSO); DMSO+DOXY (doxycycline+1%DMSO); DMSO+DOXY+IVM (doxycycline + ivermectin + 1% DMSO). All results were expressed as fold change of the gene of interest (2^-ΔΔCt), normalized first to the internal control (housekeeping gene: 18S) and then to the control treatment (plain NI media). Three worms were considered per each treatment and each of them was analyzed in triplicate. Data resulting from these analyses are as the mean ± S.D. of each treatment.

Among all gene expression taken into account following the 24 hours treatment study, the only two differential gene expression that resulted in a statistical significance difference are reported for Dim-*haf-1* (p value of 0.0281) and Dim-*haf-5* (p value of 0.0147) in female following treatment with respectively single doxycycline and single ivermectin.

4.3.2 Membrane transporters' gene expression following 48h treatment

Results for collected for female worms reported in Fig. 17, showed that none of the treatment performed had any effect on transporter gene expression after 48 hours of treatment. With the exception of a two specific cases, where the combination treatment induced a slight increase in pump expression (up to 2.04 fold in Dim-*haf-1*) and an increase of 3.01 fold observed in Dim-pgp-3 after single ivermectin administration, all gene expression levels were comparable to that of the control. As observed for Di-*avr-14* whose level range between 0.9 fold to 1.11 fold.



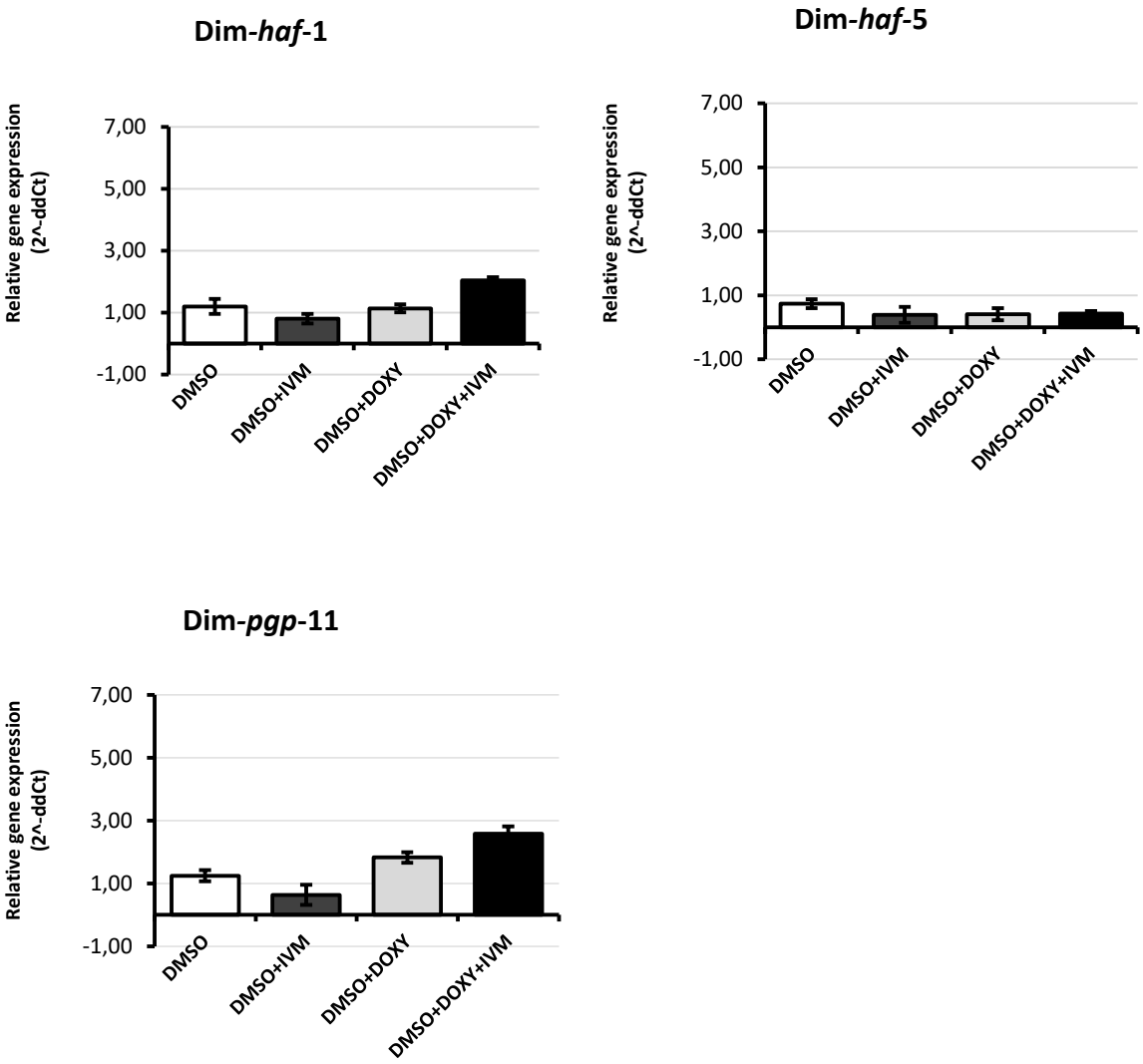
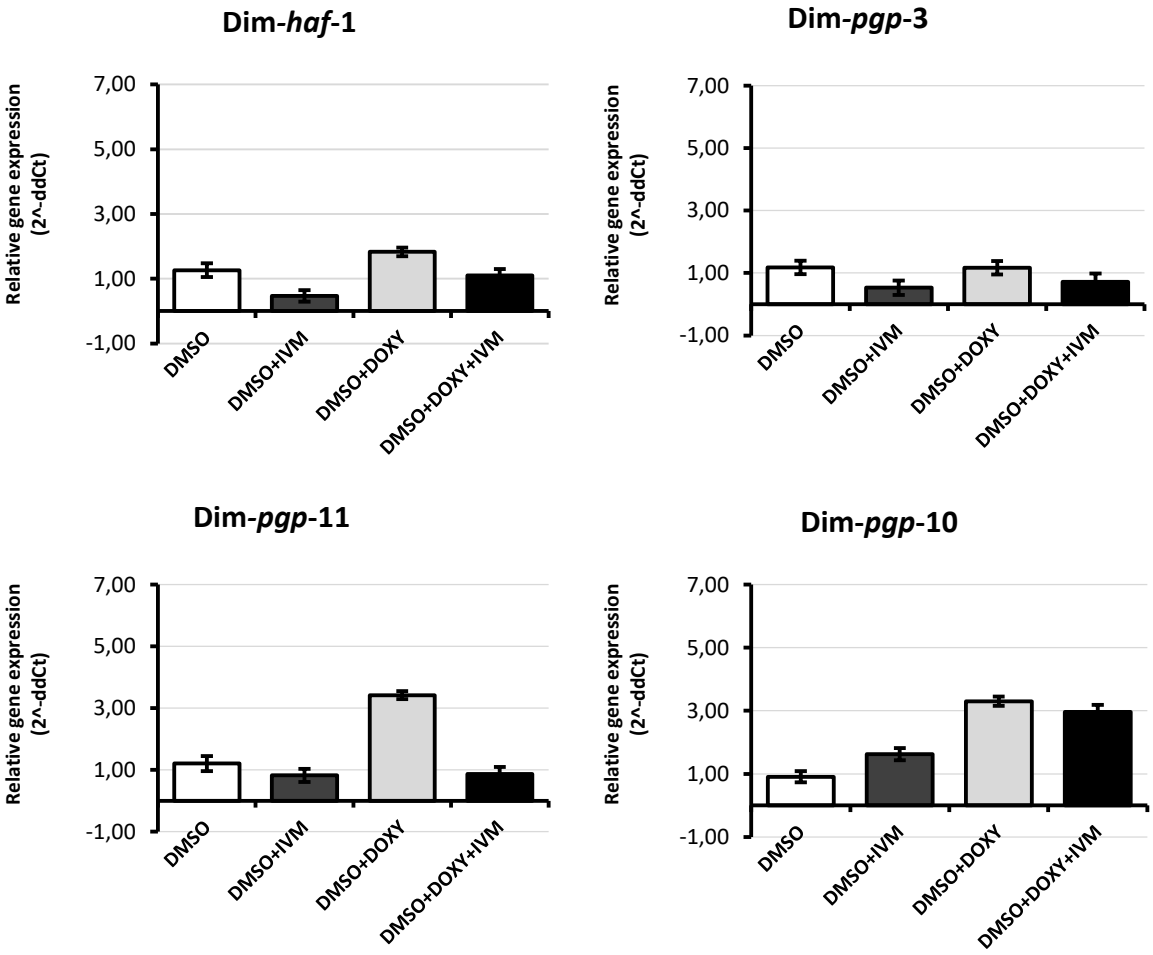


Fig. 17: Gene expression analysis of female worms treated for 48h with the following treatments: DMSO (control treatment with NI media + 1% DMSO); DMSO+IVM (ivermectin + 1%DMSO); DMSO+DOXY (doxycycline+1%DMSO); DMSO+DOXY+IVM (doxycycline + ivermectin + 1% DMSO). All results were expressed as fold change of the gene of interest (2^-ΔΔCt), normalized first to the internal control (housekeeping gene: 18S) and then to the control treatment (plain NI media). Three worms were considered per each treatment and each of them was analyzed in triplicate. Data resulting from these analyses are as the mean ± S.D. of each treatment.

Quite interestingly the male gene expression results obtained following 48h of treatment showed a general trend which resembled that observed for females after 24h of treatment (Fig. 18). In fact, also

in this case ivermectin when administered alone induces down-regulation of the transmembrane genes, reaching in Dim-half-1 0.47 fold. Gene expression raises when males are treated with doxycycline alone, reaching a maximum increase of ~3.4 fold for Dim-*haf-4* and Dim-*pgp-11*. As previously observed in female treated with the combination treatment for 24 hours, the two molecule administered in combination induce a balanced effect also in male worms when treated for 48 hours. As in the case of the Dim-haf-1 gene its expression following the two single drug administration is 0.47 fold for ivermectin alone while 1.83 fold for doxycycline alone, whereas following the combination treatment the level of expression was 1.10 fold.



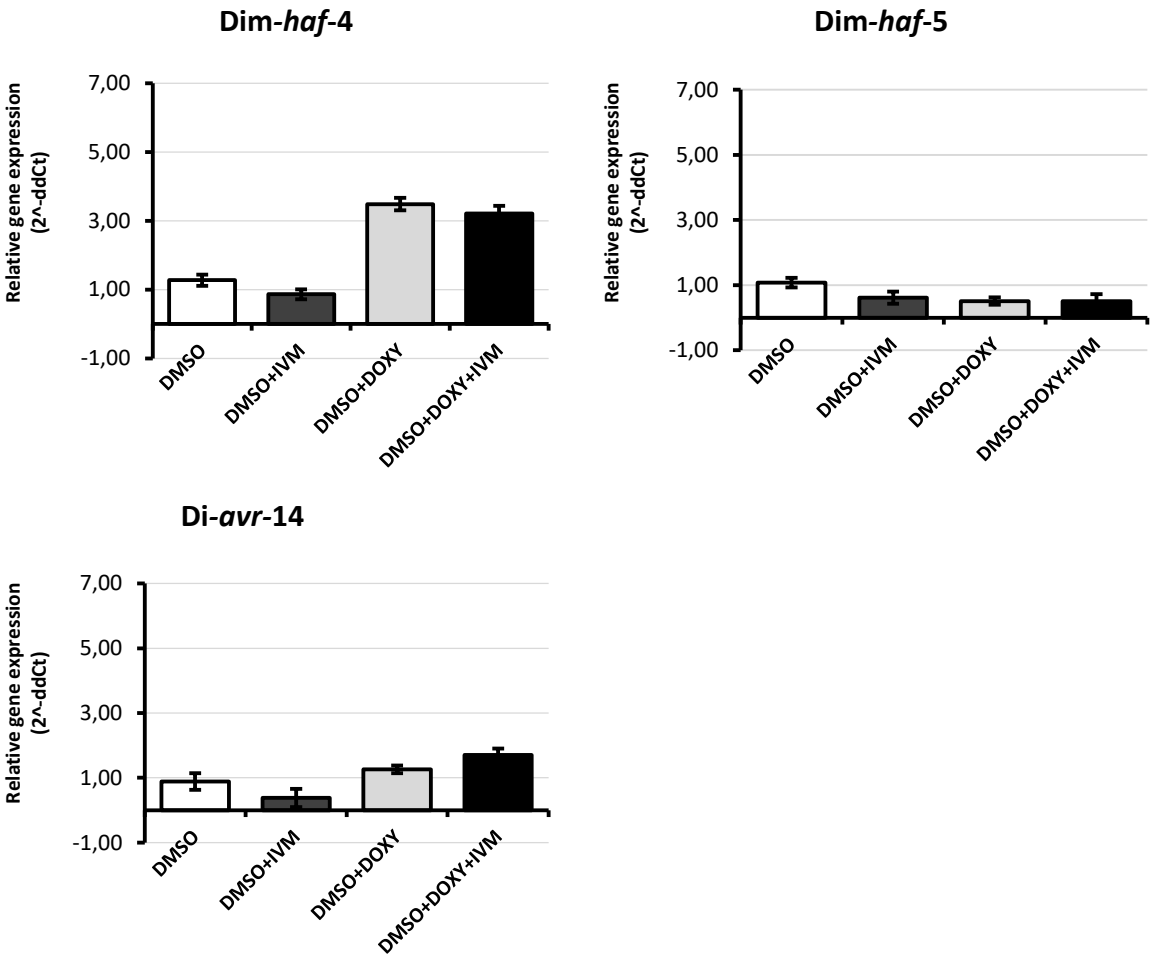


Fig. 18: Gene expression analysis of male worms treated for 48h with the following treatments: DMSO (control treatment with NI media + 1% DMSO); DMSO+IVM (ivermectin + 1%DMSO); DMSO+DOXY (doxycycline+1%DMSO); DMSO+DOXY+IVM (doxycycline + ivermectin + 1% DMSO). All results were expressed as fold change of the gene of interest (2^-ΔΔCt), normalized first to the internal control (housekeeping gene: 18S) and then to the control treatment (plain NI media). Three worms were considered per each treatment and each of them was analyzed in triplicate. Data resulting from these analyses are as the mean ± S.D. of each treatment.

None of the differences in gene expression following the various treatment protocols performed for 48 hours were statistically significant when compared to the negative controls. The lowest p value (p value of 0.108), was observed between the DMSO control and the single ivermectin treatment for Di-*avr-14* gene expression in males. As reported by Amrhein V and colleagues (2019), statistically non-significant

results cannot be taken as proof of the null-hypothesis. Therefore, these results can still be considered as reliable despite their statistical “non-significance”.

4.4 Determination of doxycycline concentrations in treatment media

To investigate the possibility of a correlation between the differential gene expression observed above and the actual pumps secretion activity, the overall doxycycline concentrations of the media containing either doxycycline alone or ivermectin + doxycycline were analyzed at the HPLC after the *in vitro* treatments of worms. Results showed a slight difference between the two sexes (Fig. 19). As already described in Lucchetti et al. 2019a, the media from female worms contained a lower concentration of doxycycline in the media compared to male media for both treatment protocols. When comparing the two treatment media concentrations, the doxycycline samples had lower concentrations of doxycycline than media from all worms treated with the combination doxycycline/ivermectin. None of the observed differences were statistically significant (p value > 0.9).

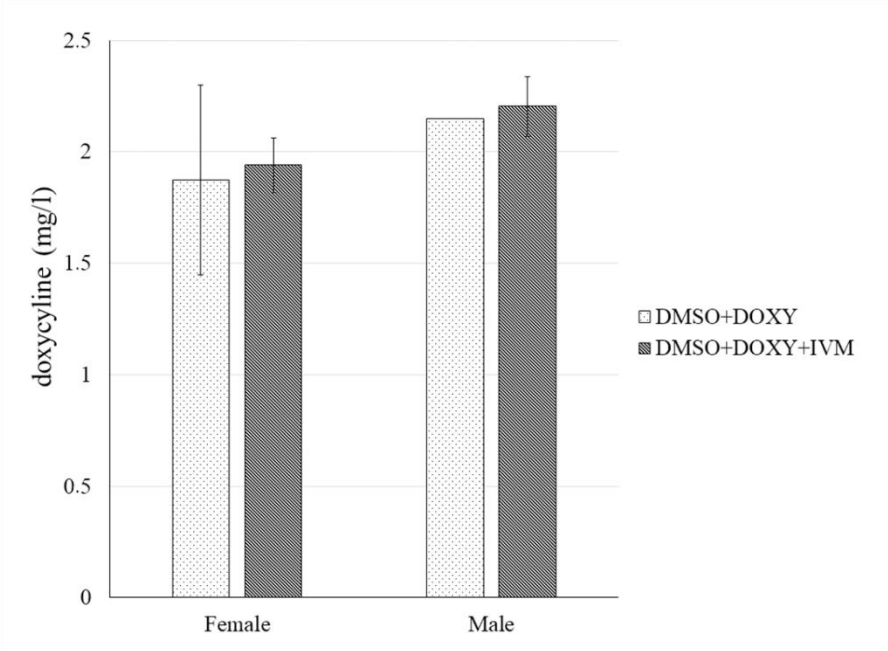


Fig. 19: HPLC analysis of doxycycline levels found in different treatment media after adult *D. immitis* treatment. Media analyzed were as follows: 1% DMSO in combination with 56.5 µM DOXY (DMSO + DOXY), 1% DMSO in combination with 3.54 nM IVM and 56.5 µM DOXY (DMSO + DOXY+ IVM). This histogram shows the average doxycycline concentrations, expressed in mg/l and standard errors calculated from all data collected for three biological replicates. (Lucchetti et al. 2019a)

5. Discussion

D. immitis is a filarial nematode transmitted by several mosquito species, which makes it a very cosmopolitan parasite. Although dogs represent its final host, where it causes heartworm disease, this parasite can accidentally infect humans, and thus is a potential zoonosis. Since the majority of the methods used to control parasitic nematodes rely on drugs and in particular macrocyclic lactones, the their misuse, under-dosing, and overuse has led to the development of drug resistant phenotypes in many target species, including *D. immitis* (Ardelli 2013). Thus, due both to its wide distribution and to the increase in the occurrence of MLs-resistance strains, research is now focusing on finding alternatives to currently available ttreatment and in particular to the use of MLs.

To better understand the efficacy of MLs/doxycycline combinations, and to possibly improve it by reducing the frequency and concentrations of the administrations, an *in vitro* study focusing on the molecular mechanisms of action of the two drugs on the parasite has been carried out. In this regard, particular attention has been given to the role played by membrane transporters in response to the synergic effect of the ivermectin/doxycycline combination at two different time points (24 and 48 hours of treatment).

5.1 Optimization of DNA/RNA co-extraction

So far, most of the existing methods for molecular analysis of filarial nematodes have focused on single RNA or DNA extraction. In fact, as described in previous studies, DNA is generally isolated by means of commercial kits such as DNeasy Blood and Tissue Kit (QIAGEN, Germany) and QIAamp® DNA Mini Kit (Qiagen, Venlo, The Netherlands). A few alternative protocols involving a combination lysis buffers, composed primarily of Tris-HCl and K proteinase enzyme, are also available (Masny et al. 2009; Varela-M et al. 2018; Verocai et al. 2018; Torres-Chable et al. 2018). The use of commercial kits has been reported for RNA extraction too. A few examples of these methods are the Micro Fast Track™ purification kit (Thermo Fisher Scientific, Waltham, USA) and the RNeasy kit (Qiagen, Valencia, CA, USA). Previous studies have also extensively described the use of TRIzol® reagent for single RNA extraction, which is usually proceeded by sample digestion in Tris-EDTA (Kron et al. 2003; Desjardins et al. 2013; Ballesteros et al. 2016). The possibility of a simultaneous extraction of both molecules from a same

sample has not yet been taken into consideration in this organism, although it could allow maximum exploitation of a single sample, increasing in this way the amount of information obtained at once.

Until now, co-extraction has never been described for nematodes; in fact, there are no reports of this procedure being applied on single adult nematode samples, in particular with such a large size as that of *D. immitis*. Thus, to the authors' knowledge, this represents the first attempt of developing an optimized DNA/RNA co-extraction in *D. immitis*. In this context, results obtained following the proposed co-extraction procedure validate the possibility of using this protocol as an efficient option for simultaneous extraction of good quality of both DNA and RNA from a unique sample of adult *D. immitis*. In fact, good concentrations of both DNA and RNA were extracted from each sex, although there was a significant difference between results of male and female worms. In fact, both DNA and RNA step 1 concentrations of males were lower compared to those obtained for females. Since a similar variation in RNA step 1's concentrations was observed also when the Phasemaker™ Tube protocol was applied, thus this could be associated with the size difference of the two genders, which results in a difference in starting material. In addition, the RNA results collected following the proposed protocol are similar to those obtained using the Phasemaker™ Tube protocol, therefore further validating the optimized procedure.

The described extraction procedure grants maximum exploitation of a single sample of adult *D. immitis* worm, allowing the possibility of focusing the molecular analysis on different levels, such as either on the parasite itself or on its symbiont (*Wolbachia*), and thus allowing the investigation of different aspects of their metabolisms.

5.2 Gene expression analysis and doxycycline quantification

Several *in vivo* studies, carried out both in experimentally- as well as naturally- infected dogs, have demonstrated a remarkable adulticidal effect against *D. immitis* of treatments combining doxycyline with MLs, much greater also compared to the effect of the single drugs administrations (Bazzocchi et al. 2008; Savadelis et al. 2017; Genchi et al. 2019b). In particular, the combination of ivermectin with doxycycline has been reported to have a greater effect on the parasite's endosymbiont *Wolbachia* population (Bazzocchi et al. 2008).

The actual mechanism laying behind the increase in efficacy of the combination therapy is still unknown. In fact, although Bourguinat and collogues (2011b) have correlated the resistant phenotype of *D. immitis* against IVM with the presence of mutations on the genetic sequences encoding for ABC transporters, their role on the antibiotic resistance is still unknown.

In the present study, the author evaluated the differential expression of seven transmembrane transporter's genes following either 24 hours or 48 hours *in vitro* treatment of the *D. immitis*' adult worms. In particular, the genes encoding six ABC transporter were taken into account, three of which were predicted to encode for PgPs, and a seventh gene encoding for a GluCl channel.

Previous studies have suggested that, as already observed in *C. elegans*, also in *B. malayi* some of the PgP are expressed in areas involved in the worm reproduction, such as uterus or vulva (Stitt et al. 2011; Zhao et al. 2004). Similarly, the GluCl channels were reported to be present both in the excretory/secretory pore of microfilariae and in the reproductive tissue of adult filaria (Moreno 2010; Li 2014). Despite these information, their actual tissue expression in *D. immitis*, as in many similar filarial nematodes, is still unknown. Therefore, in this study an initial homogenization of the sample has been preferred to a tissue specific RNA isolation.

Results for the 24h study, were partially already published in Lucchetti et al. (2019a). They highlighted a sex-dependent differential gene expression of the all transporters genes taken into account following treatments. As also reported by Mani and collogues for Dim-*pgp-11* when expressed in mammalian cells, the administration of ivermectin in female worms has led to a general reduction in the expression of all transporter genes analyzed (Mani et al. 2016). This is also consistent with what observed in previous studies, where ABC transporters, which are part of drug resistance mechanisms in many organisms, were inhibited by ivermectin (Ballent et al. 2012). As already proposed in Lucchetti et al. (2019a), these down-regulations, reaching its lowest value with 0.11-folds for the Dim-*pgp-10*, suggest an inhibitory activity of ivermectin on the transports. On the contrary, in male worms, ivermectin administration resulted in the up-regulation of all seven genes, with the highest increase of 4.1-fold in Dim-*haf-4*, suggesting a stimulatory effect of the molecule on the pumps. This up regulation of the transporters has already been described in other nematodes such as *H. contortus* and in *C. elegans*, and it could represent a drug resistance mechanism activated against MLs by the parasite (Yan et al. 2012; Raza et al. 2016). Since for this study all worms, which were collected from naturally infected dogs,

come from areas of Europe where the first autochthonous infection was presented in 2007 and where regular prevention is not yet practiced, the connection of these results to the presence of resistant strains is highly unlikely (Tolnai et al. 2014; Bacsadi et al. 2016).

Differential expression is evident for all genes also following the single doxycycline treatment. In this case, the reaction was the opposite of what just described for ivermectin. In fact, while in male worms doxycycline alone induced just a slight down-regulation, since all gene expression levels are close the control one, reaching the lowest value of 0.69-fold in Dim-*haf-5*; in female worms, doxycycline alone induced a significant up-regulation, of all transporter genes (up to 6.8-fold increase in Dim-*haf-1*). As already proposed by Lucchetti et al. (2019a), and as results from the present study suggest, *Wolbachia* might be playing a role in the detoxification of the intracellular environment of female worms from the antibiotic. Keeping in mind that transporters and in particular ABC transporters are known to be ATP-dependent, and that according to previous studies carried out in *O. ochengi*, one of the primary contributions of *Wolbachia* to the host is the ATP provision, the proposed hypothesis becomes more consistent (Darby et al. 2012). Moreover, considering the different distribution of the endosymbiont in two sexes, not only due to difference in size, but also to the presence of the bacteria in the reproductive tissues of the female, which implies a much greater *Wolbachia* population in female worms; this could be the cause of the diverse response to the treatments observed between the two sexes.

The results obtained for the combination treatment suggest a balanced effect of the two drugs. In fact, for both sexes this treatment induces an up-regulation of all genes. Although, when taking into consideration each single gene, the levels of expression following the ivermectin/doxycycline treatment did not reach the levels of up-regulation obtained following single drug administration (for female the doxycycline treatment, while for male the ivermectin treatment).

All together, these results could explain the observed increase in efficacy of the combination treatment on male worms, compared to female. In addition, a more intense intracellular detoxification from the drugs in females, may explain why they have been reported to be more resistant to the combination treatment compared to males (92% male mortality against the 69% of female mortality) (Bazzocchie et al. 2008).

The dichotomy of transporters' genes expression in response to the performed treatments between the two sexes of the parasite is also evident after the 48 hours of treatment. While in females none of the treatments had any effect on the transporters gene expression, in males the modulation of transporters' genes expression resembled the trend observed in female worms treated for 24 hours, although with a much lower fold change magnitude. In fact, the greatest up-regulation was observed for Dim-*haf-4* following single doxycycline treatment (3.48-fold). Moreover, while the doxycycline induced a general up-regulation of all transporter genes analyzed, the ivermectin alone induced a general down-regulation. In addition, also in this case, when the two drugs were administered together, they had a balancing effect on the genes expression.

These results would suggest that, in females, transporters' genes expression are modulated following initial contact with the molecules (both singularly and in combination), and then after 48 hour it normalizes, restoring the control expression levels. A similar trend has been described by Epis et al. (2014) for the ABC transporter genes expression in insects following insecticidal treatments. On the contrary, in male worms the response to treatments seems to start with a delay of 24 hours compared to what observed in females. This lag in the response to treatments between female and male worms is likely due to the *Wolbachia* load present in each sex. Since males have a lower *Wolbachia* population size, their transporter genes up-regulation was delayed of 24h compared to what observed in females. Furthermore, as reported by Ardelli et al. (2010), which have quantified the relative abundance of ABC transporters in *B. malayi*, the difference in response could be also connected to the significantly higher amount ABC transporters present in female worms, particularly of subfamily B and C, compared to what observed in males and microfilariae.

The presence of a link between the change in modulation of the transporters genes expression and the actual efflux of the antibiotic was investigated by means of HPLC analysis. As expected from the results of the gene expression study, the concentration of doxycycline were similar between the two media taken into consideration (doxycycline alone and doxycycline/ivermectin) for both sexes. On the other hand, despite what observed by the RT-PCR, when compared, the concentrations measured inside the media were higher in male rather than female, which might indicating a higher efflux in male. This is most probably not the case, and the results could be in fact due to the volume used for the in vitro treatment, which was kept standard (50mL) per both sexes. Moreover, on the contrary to what

described by Menozzi et al. (2015), no clear potential summation effect induced by a higher uptake of doxycycline when administered in combination with ivermectin was observed in this study.

Finally, since many other factors could be responsible for this synergic effect, as for example alterations of the immune response, further studies are required to better elucidate whether the modulation of cellular efflux may be connected, also partially, to the adulticide effect of DOXY/ML combinations in heartworm-infected dogs (Passeri et al. 2014).

6. Conclusions

Better understanding the mechanisms of action of the combination of MLs and doxycycline is important not only to improve the treatment of *Filaria* in animals, but also to ameliorate the treatment of all human filariasis that are still causing disease in millions of people worldwide. In light of this, the present study represents, to the authors' knowledge, the first evaluation attempt of the expression of transporter genes in adult worms of *D. immitis* following treatment with MLs/doxycycline.

This study demonstrates a sex-dependent transporter genes modulation, which might be connected to the different *Wolbachia* load found in the two sexes. Moreover, this study correlates the synergic adulticide effect observed in previous *in vivo* studies, with the actual change in modulation of the transporter genes. The combination of the two drugs has been reported to induce a balanced effect of the activity of the two molecules when given separately. Since no definitive proof of the actual change in excretion of either doxycycline or ivermectin has been collected in this study, further investigation is necessary to determine if the differential modulation of cellular efflux is in part responsible for the adulticide effect of doxycycline/macrocyclic lactone combinations in heartworm-infected dogs.

The greatest limitation of this study is represented by the small number of samples analyzed per each treatment, which is due to working mainly with naturally infected dogs. The main problems associated with working with naturally infected dogs are: the need of surgical removal of the parasite, which is a relatively uncommon practice; the very low worm burden found in natural infections; the difficulty in identifying dogs which have not undergone previous MLs/doxycycline treatment. All these limitations could be overcome by working with experimentally infected dogs.

Several future studies would be interesting to carry out in order to get a better picture of the molecular mechanisms involved in this synergism. As reported by the Epis et al. (2014), in insects ABC transporters reach their highest level of expression already after the first 6 hours of treatment. Therefore, it might be interesting to evaluate the pumps' genes expression at two extra time point (6 hours and 12 hours). Furthermore, in *B. malayi*, the inhibition of ABC transporters has been shown to induce an increment in sensitivity of the parasite to moxidectin (Stitt et al. 2011). This has not been demonstrated yet for *D. immitis.* Therefore, it would be interesting to carry out a second *in vitro* study to evaluate moxidectin and modidectin/doxycycline combination effects on the parasite. Moreover, with latter would be

interesting to investigate whether the resulting differential modulation would follow the same trends as the one observed in this study for ivermectin/doxycycline combination. Finally, by performing an *in vitro* study on *Wolbachia* cultures without the parasite host, its response to doxycycline could be tested, and a transcriptomic analysis aimed at evaluating bacterial response to the drug could be carried out. This study could be central to shed light on whether *Wolbachia* plays a direct role in transporter genes modulation, hypothesis proposed by the results obtained in this study, and in that case, what is the cascade of events that the endosymbiont activates in order to do so.

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