Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

THE ROLE OF NEMATODE P-GLYCOPROTEINS IN THE MECHANISM OF MACROCYCLIC LACTONE RESISTANCE

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
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Tierarzt
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Berlin 2021

Journal-Nr.: 4288

Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus): Equidae, *Parascaris univalens*, P-glycoprotein, *Caenorhabditis elegans,* ivermectin, moxidectin, drug resistance, anthelmintics, nematoda, parasitology

Tag der Promotion: 11.08.2021

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LIST OF ABBREVIATIONS

µL	Microliters
µm	micrometers
^{3}H	Tritium (Hydrogen-3)
A	Alanine
aa	Amino Acid
ABC	ATP binding cassette
ABCB	ABC-transporter subfamily B
ATP	Adenosintriphosphate
ben-1	Beta-tubulin gene 1
BZ	Benzimidazole
Cas9	CRISPR-associated Protein
cDNA	Complementary DNA
Ceg	*Cylicocyclus elongatus*
Cel	*Caenorhabditis elegans*
CeNDR	*Caenorhabditis elegans* natural diversity resource platform
CNV	Copy number variation
col-19	*collagen-19*
Con	*Cooperia oncophora*
CRISPR	Clustered regularly interspaced short palindromic repeats
Cyt-P450	Cytochromes P450
Dim	*Dirofilaria immitis*
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
Dyf	Dye filling
E	Glutamic acid
EC_{50}	Half maximal effective concentration
ESCCAP	European Scientific Counsel Companion Animal Parasites
F	Phenylalanine
FACS	Fluorescence-activated cell sorting
FEC	Faecal egg count
FECRT	Faecal egg count test
FPKM	Fragments per kilobase of transcript per million mapped reads
F_{st}	Fixation index

GABA	γ-Aminobuttersäure
ges-1	gut esterase-1
GFP	Green fluorescence protein (*Aequorea victoria*)
GluCls (protein) or *glc* (gene)	Glutamate-gated chloride channel
GO	Gene ontology
GWAS	Genome-wide association study
Hco	*Haemonchus contortus*
IVM	Ivermectin
KCON	Ketoconazole
kDa	kilo Dalton
kbp	kilo Base Pairs
lacZ	β-galactosidase gene
L1	First stage larva
L2	Second stage larva
L3	Third stage larva
L4	Fourth stage larva
LDA	Larval development assay
log P	Logarithm base 10 of the partition coefficient (P)
MDR1	Multidrug-resistance-protein 1
MDR-1 or *mdr-1*	Multi drug resistance protein/*gene*
ML	Macrocyclic lactone
mm	millimetres
MOX	Moxidectin
mRNA	*messenger* RNA
nAChR	nicotinic acetylcholine receptor
NBD	Nucleotide binding domain
NCBI	National Center for Biotechnology Information
ng	nanogram
NGS	Next-generation sequencing
p.i.	post infectionem
PCR	Polymerase chain reaction
Peq	*Parascaris equorum*
Pgp	P-glycoprotein
Pgp-i	P-glycoprotein inhibitor
PP	Pharyngeal pumping

Pun	*Parascaris univalens*
RNA	Ribonucleic Acid
RNAi	RNA-interference
RNAi	RNA interference
SNP	Single nucleotide polymorphism
spp.	species pluralis
TBZ	Thiabendazole
TMD	Transmembrane domain
TPM	Transcripts per kilobase million
UK	United Kingdom
US	United states
UTR	Untranslated region
VPM	Verapamil
WAAVP	World Association for the Advancement of Veterinary Parasitology
WT	Wildtype
Y	Tyrosine

1 INTRODUCTION

Drug resistance has become a major obstacle to treating infectious pathogens of humans, animals and crops. The collateral damage from indiscriminate chemical intervention is a major driver of species extinction, but the concurrent development of drug resistance in species across all phyla reflects the extraordinary potential to adapt which is universal to life. Nematodes are no exception to this global phenomenon. In fact, resistances to all available classes of anthelmintics have quickly arisen in a large number of different parasitic species of animals, humans and plants (Wolstenholme et al. 2004, Von Samson-Himmelstjerna 2012, Prichard and Geary 2019, Tinkler 2020). In addition to the importance of finding a sustainable solution, drug resistance is an opportunity to study ongoing evolutionary mechanisms.

Parasitic nematodes represent a major burden to animal and human health globally and currently chemotherapeutic intervention is currently the most effective control strategy (Nielsen 2016, Hodgkinson et al. 2019). In veterinary medicine, the rapid advance of macrocyclic lactone (ML) and multi-drug class resistances spell the pressing need to find new strategies to reduce or reverse their spread. For example, the important pathogen of foals, *Parascaris univalens*, has developed widespread resistance to MLs as well as multi-resistance against multiple anthelmintic classes (Von Samson-Himmelstjerna 2012, Nielsen et al. 2014b, Nielsen 2015). Concurrently, first signs of reduced susceptibility to MLs (Eng et al. 2006, Doyle et al. 2017) and other classes of anthelmintics, e.g. the benzimidazoles (BZs) (Krücken et al. 2017), have also emerged in human parasitic nematodes. Essentially, with no novel chemotherapeutic treatment alternatives or vaccination in sight, optimizing drug treatment strategies is pivotal to prolong the effectiveness of MLs and other anthelmintics. To this end, reliable, quick and cheap resistance diagnosis is essential (Kaplan 2020) which in turn requires a deep understanding of the mechanisms of drug resistance. However, the underlying mechanism of ML resistance are unknown. With the increasing availability of next-generation sequencing (NGS) data for parasitic nematodes, utilization of genomic tools in combination with functional validation is the most promising strategy to elucidate the ML resistance mechanisms (Doyle et al. 2019a, Doyle and Cotton 2019). While state-of-the-art gene editing is widely used in the model nematode *Caenorhabditis elegans*, these genetic tools still need to be established for most parasitic nematodes (Lok 2019). Until then, model systems represent the best alternative approach to validate candidate genes identified comprehensively using genetic and genomic appraoches.

ATP-binding-cassette (ABC)-transporters and in particular P-glycoproteins (Pgps) were proposed as candidates for a contribution to ML resistance. Pgps are transmembrane transporters which facilitate increased drug efflux and thereby can reduce the concentration at the drug target site. While mammals only have a few Pgp genes, most nematodes possess a much larger repertoire but which of these genes are relevant for drug resistance is unclear. Despite advances in the last decade, their general functional role in nematodes and specifically in ML resistance has remained a conundrum. Merely, their tissue expression patterns and their role in mammal blood-brain barrier and intestinal excretion offer some indication of their putative function.

The general objective in this PhD project was to better understand the role of *P. univalens* Pgps in modulating ML susceptibility in nematodes.
To this end, the complete Pgp repertoire in *Parascaris univalens* was first deciphered using novel and available NGS resources and their expression patterns were characterized.
Consecutively, the functional mechanism of Pgp-mediated resistance was investigated using selected model systems. In a yeast growth assay, their interaction with different anthelmintics was analyzed. Utilizing the model nematode *C. elegans*, the role of Pgp expression in specific tissues was examined bv overexpressing a parasite-derived Pgp exclusively in the intestine or the epidermis.

As the uptake and dispersion routes of MLs are unknown, the role of active drug ingestion for susceptibility against two important MLs, ivermectin and moxidectin, was also investigated within the context of tissue-specific Pgp expression.

Finally, with the aim of finding novel inhibitors of nematode Pgps, several compounds were examined for their effect on *C. elegans*.

2 LITERATURE

2.1 Nematodes

Nematodes have conquered most ecosystems, occupy a broad range of different ecological niches, and have engaged in parasitic and symbiotic relationships. Reflecting their extraordinary adaptability, nematodes can be found in polar regions (Caruso et al. 2019) and over 1 kilometer underground (Borgonie et al. 2011). The phylum Nematoda consists of over 25.000 described species (Poinar 2016) and some estimates claim a species diversity of 100,000 to 10 million (Poinar 2011). As the most abundant metazoan in many ecosystems (Platonova et al. 1985, Danovaro et al. 2008, Van Den Hoogen et al. 2019), nematodes play a central role in food webs (Sochová et al. 2006). Concurrently, interest in nematodes has been sparked by a conspicuously large number of parasitic species (Dobson et al. 2008). The most commonly used system for phylogeny distinguishes five clades (I-V) (Blaxter et al. 1998) and parasitic species occur within each clade, parasitizing a broad spectrum of host including plants, vertebrates and invertebrates in marine, fresh-waterr or terrestrial ecosystems. Importantly, many parasitic species have a strong impact on human and livestock health and agriculture. Corroborating their potential to adapt, the development of drug resistance in many relevant parasitic nematode species has further raised concerns.

2.1.1 Anatomy and Biology

Equivalent to the diversity of occupied ecological niches, adult nematodes range in size from as large as several metres (*Placentonema gigantissima* parasitizing the placenta of sperm whales) (Gubanov 1951) to as small as 0.2 mm (*Protorhabditis hortulana*) (Abolafia and Peña-Santiago 2016). Despite their morphological differences, most nematodes share a common body plan (*Bauplan*).

Adult stage nematodes and larvae are bilaterally symmetrical and unsegmented. Their most outer layer is the non-cellular cuticle which is produced by the epidermis (also referred to as the hypodermis in particular in the older literature and still in a large proportion of the *Caenohrbaditis elegans* research community) (Poinar 2015). The most outer layer of the cuticle is made mostly from cuticulin, i.e. lipids and glycoproteins, and is important in signaling, sensing and other physiological processes (Page and Johnstone 2007). This layer is followed by a collagen-rich layer. Together, over 170 collagen genes (in *C. elegans*) encode up 80% of the soluble protein in cuticle extractions (Page and Johnstone 2007). These collagen proteins undergo complex post-translational modifications and processing before being excreted from the apical epidermis membrane. The cuticle represents the worm's exoskeleton and plays a crucial role in maintaining body morphology. It is also the first barrier against toxins (Xiong et al. 2017) and additionally plays a role in environmental stress sensing and the innate immune response (Taffoni and Pujol 2015) in interplay with the epidermis. During development, the cuticle is synthesized five times: during in the late embryogenesis and prior to each molt (Singh and Sulston 1978). The epidermis is formed by a syncytium of cells or by a single cell layer (Altun 2020b). For example, in *C. elegans* the main part of the epidermis is formed by the so called hyp7 syncytium and it is complemented by additional cells in the head and tail region. Between each of the epidermis cells, zonula adherens link the cells tightly and allow a distinction between the apical and the inner membrane (Michaux et al. 2001). Along with the intestine, the cuticle-epidermis complex has also been shown to be a major tissue for fat storage and metabolism in the model nematode *Caenorhabditis elegans* (Mullaney and Ashrafi 2009). In development, the epidermis plays a crucial role in establishing the body plan and regulating cell fate (Johnstone and Barry 1996, Greenwald 1997). Based on genetic screens in *C. elegans*, many genes were identified that play a role in cuticle structure and function. For example, common phenotypes which have been implicated with cuticle defects are the roller (rol, worms turn around their own axis) and the dumpy (dpy, uneven surface and jolted appearance) phenotype. These phenotypes were traced back to deletions in genes encoding a broad range of cuticular components (Page and Johnstone 2007).

Locomotion is facilitated by a layer of longitudinal muscle cells (body wall muscles) and the high hydrostatic pressure in the fluid-filled pseudocoel (body cavity). It appears as side-by-side movements resulting from repeated relaxation and tension of opposite sided muscles regulated by neuronal loops (Cohen and Sanders 2014). A unique feature of the nematode neuromuscular junctions is that the muscles grow protrusions towards the motor neurons (White et al. 1986). In other organisms, the nerves innervate the muscles via dendrites or axons. The muscular protrusions (also called arms) form the neuromuscular junctions *en passant* and the longitudinal muscular nerves are almost entirely covered with these muscular branches (White et al. 1986). The protrusions can originate from myoblast moving away from the nerve during embryogenesis but with their membranes staying in touch with the motor neuron. During larval development muscular protrusions are actively formed in an actin mediated process (Dixon et al. 2006). In addition to controlling locomotion, the nematode nervous system coordinates several important processes such as the regulation of food uptake by pharyngeal pumping through serotonin and other neuronal transmitters (Song and Avery 2012) but it is also capable of performing other more complex functions. Studies in model nematodes such as *C. elegans* and *Pristionchus pacificus* have revealed the intricate behaviors that nematodes can exhibit such as learning (Hart (Ed.) 2006), dietary choices (Avery and You 2012, Wilecki et al. 2015, Worthy et al. 2018) and other behaviors in response to chemo-sensing which require sophisticated feed-back loops. In *C. elegans*, sensory structures are well described anatomically and are supported by sheath cells, socket cells and specific glia ("GLR") cells. These support cells enclose the sensory neurons. The central components of the sensory neuronal system are the amphids, the cephalic sensilla, the inner and outer labial sensilla, the deirid sensilla and the phasmid sensilla (Altun 2020a). Specifically, the amphids have been implicated in chemotaxis, mechanosensation, osmotaxis and dauer pheromone sensation (Bargmann 1997, Geffeney 2017). Notably, the dyf (increased fluorescent DYe Filling) phenotype results from the knockout of a diverse set of genes which are expressed predominantly in the support cells which among other phenotypes result in amphidal defects (Inglis et al.).

In the German literature, an important component of the nematode anatomy is the "Hautmuskelschlauch", which consists of the cuticle, epidermis, nerves and muscles. This distinction is made because these layers are tightly linked and cannot be separated by dissection. However, this term is only found in the German literature (Kühnel 2001, Bang-Berthelsen et al. 2012).

The digestive system opens with an oral cavity (stoma) lined by the cuticle and the underlying epidermis (Poinar 2015), leading into an ectodermal pharynx which in most nematodes sucks in food by pumping. The comparatively large intestine, anchored in the pharynx, is usually formed by a single layer of cells and is responsible for nutrient digestion and absorption. The intestine also allows excretion of waste products through the rectum. However, the excretory system also includes the epidermis-cuticle where metabolic waste products and a large number of small molecules are excreted. The majority of the excretory system is formed by the large H-shaped excretory cell (canal cell) which is supplemented by a pore cell, a duct cell and a fused pair of gland cells. The opening of this system is the excretory pore which is tightly fused to the epidermis. The secreted molecules referred to as the excretome/secretome perform a number of important functions such as signaling and defense (e.g. anti-microbial peptides) (Kong et al. 2016).

Many nematode females (or hermaphrodites) possess large reproductive organs allowing a rapid population expansion under favorable conditions. However, reproductive processes are not conserved between nematode species and nematodes reproduce by a number of different reproductive modes (Trivers 1983) including dioicous (e.g. Strongylida and Ascarida) or androdioecious (e.g. *Caenorhabditis* spp.) reproduction. Despite these differences, after hatching all nematodes undergo a life cycle with four molts (shedding of the cuticle) before reaching the reproductive adult stage (Poinar 2015). These life cycles can become exquisitely complex in parasitic nematodes.

2.1.2 Parasitism

Parasitism is very common in animals and besides arthropods and a few other phyla, it is particularly common in nematodes (Weinstein and Kuris 2016). Parasitism can appear in many forms but generally the parasites are much smaller than ther host. Parasites can live and feed on (ectoparasite) or in (endooparasite) the host, thereby causing it harm and reduce its fitnes. Nematodes parasitize most species of plants and animals and it has been suggested that over the course of evolutionary history nematodes have adapted parasitism on least 15 independent occasions (Blaxter and Koutsovoulos 2015). Only very rarely, a reversion from parasitism might occur (Dorris et al. 2002).

Life cycles of parasitic nematodes are often highly specialized to a specific eco-system. Transition between life cycle stages can be necessary to reach specific check-points (Albarqi et al. 2016) or to go through multiple intermediate hosts. Such life cycles with one or more intermediate hosts are referred to as heteroxenous while the host in which sexual reproduction takes place is referred to as the definitive host. In contrast, other parasitic nematodes might only have one host (monoxenous), e.g. in case of Ascarididae. As a common feature, most parasitic nematodes transition from a free-living to a parasitic life-style during the third larval stage (L3) (Sudhaus 2010).

Parasitism has also driven host-parasite co-evolution giving rise to highly specialized immune-evasion strategies by modulating the host's immune system, e.g. through the molecules from the excretome/secretome (Soblik et al. 2011, Borloo et al. 2013, Lok 2016). All hosts including mammals have continuously evolved under the selection pressure of parasites (Bañuls et al. 2013), and still today parasitic nematodes impose a major burden on livestock and human health (Eziefula and Brown 2008). It has been estimated that up to a quarter of the human population is infected with parasitic nematodes (Hotez et al. 2008) and they are ubiquitous pests in livestock (Kaplan 2020) and agriculture (Nicol et al. 2011). Co-infections are common in many hosts including all domesticated animals e.g. ascarids and cyathostomins (a diverse species complex by itself) co-infecting equines (Saeed et al. 2019) and can also occur in humans (Lepper et al. 2018). Noteworthy, humans and their activities, such as translocation of animal hosts, are a major force in spreading parasitic disease geographically thereby further contributing to their genetic diversification (Sallé et al. 2019).

2.1.3 *Parascaris univalens*

2.1.3.1 *Taxonomy and Biological Peculiarities*

Parascaris spp. parasitize equines and they are important pathogens of foals (Deplazes et al. 2012). The genus *Parascaris* belongs to the Ascarididae family within the order of Ascaridida. This order includes many important pathogens of marine mammals (Anisakidae), amphibians (Cosmocercidae), Carnivora including dogs and cats (Toxocaridae) and birds (Ascaridiidae), of which many are also important zoonoses, such as *Ascaris suum*, *Anisakis* spp. and *Toxocara* spp. The horse roundworm *Parascaris* spp. strictly infects equines.

Within the genus *Parascaris*, there are two species which are morphologically identical and can only be differentiated through karyotyping, *P. univalens* (one germline chromosome pair) and *P. equorum* (two germline chromosome pairs). Due to their large size with females reaching up to 50 cm, they are easily visible when expelled with the feces or when opening an infected equine's intestine and early historic descriptions date back more 130 years in France (Van Beneden 1883) and almost 240 years in Germany (Goeze 1782). For a long time, *P. equorum* was considered the predominantly prevalent species and for decades no-one investigated the prevalence of the two species. However, several recent studies reported that all investigated samples were identified as *P. univalens* in Sweden, Norway, Germany, Iceland, Brazil and USA (Tydén et al. 2013, Nielsen et al. 2014c, Martin et al. 2018), suggesting that at least today, *P. univalens* is the globally more prevalent species.

Ascarids have unusually large genomes, e.g. 253 Mbp in *P. univalens* and 298 Mbp in A. suum. Peculiarly, ascarids have evolved a genetic mechanism of programmed DNA elimination which reduces their genome size by a variable amount, e.g. 13-15 % in A. suum (Müller et al. 1982,

Wang et al. 2017) and up to 88 % in *Parascaris* sp. (Niedermaier and Moritz 2000). Generally, programmed DNA elimination is the removal of a proportion of the genetic material during the germline-soma differentiation and this has arisen multiple times in different phylogenetic lineages while the eliminated genetic content can vary considerably. For example, this process can take place either as part of a chromatin reorganization (diminution) which has been reported in ciliates (Chen et al. 2014), lampreys (Smith et al. 2018), hagfish and ascarids (Müller and Tobler 2000) or by the complete loss of a chromosome which has been reported in insects and several vertebrates including fish (Kohno et al. 1998), mammals (Close 1984) and birds (Kinsella et al. 2019). While eliminated DNA sequences are mostly repetitive, they also include many protein-coding regions and it is speculated that this process is involved in gene regulation and silencing and might serve to resolve or reduce conflict between germline and somatic tissues (Wang and Davis 2014). Comparing *P. univalens* and *A. suum*, it was shown that the mechanism for the identification of chromosome break sites is sequence independent and only 35% of the eliminated genes (mostly involved in spermatogenesis) were conserved between these two ascarid species (Wang et al. 2017).

Remarkably, a recent study on horse genomics found that the part of a testis expressed gene ETSTY7 is shared between *Parascaris* spp. and horses and provides evidence for eukaryotic horizontal transfer of genetic material and inter-chromosomal mobility (Janečka et al. 2018).

2.1.3.2 *Life Cycle and Clinic*

The *Parascaris* spp. life cycle is divided into a free and a parasitic stage. Unfortunately, the older literature, which has dissected the life cycle and has laid the biological and parasitological groundwork does not discriminate between *P. univalens* and *P. equorum*. Likewise, the following description is inconsiderate of potential differences between the two species.

The free-living part of the life cycle starts with the expulsion of eggs with the feces. The infectious larvae develop within the eggs which takes between 10-21 days depending on the temperature (Clayton 1986). In most nematode parasites, the third stage larva (L3) is the infectious stage, but in some of the older literature the possibility of only one molt in the egg and three molts in the host was discussed, which would mean that the egg containing the second stage larva (L2) is the infectious stage (Clayton and Duncan 1979). However, the current prevailing notion is that indeed the L3 covered by a lose L2 cuticle is the infective stage in ascarids as well (Fagerholm et al. 2000). Eggs measure ca. 100 µm in diameter and have a particularly high tenacity and a sticky shell which leads to the characteristic thick brown uneven eggshell surface (**Fig. 1**) caused by adhesion of dirt and fecal particles and allows survival of the infectious stage on both pasture and in stables for many years. When ingested by susceptible hosts, which are usually foals under 18 months (Poynter 1970), larvae will hatch in the small intestine and migrate via the liver to the heart and then to the lungs which takes about 14-17 days (Nicholls et al. 1978) (**Fig. 1** red arrow). This migration can cause pathology from tissue damage and inflammation in the liver (Brown and Clayton 1979) and the lungs leading to coughing, nasal discharge and nervousness (Clayton and Duncan 1978). The coughing facilitates migration to the pharynx where they are swallowed. After reaching the small intestine they develop to adults and pre-patency is concluded after about 90-110 days p.i. (Lyons et al. 1976) and two molts in the host. During patency, which usually can last 1-2 years, *Parascaris* spp. females shed large amount of eggs and estimates range from >150,000 to 60,000,000 eggs per day (Lyons et al. 1976, Clayton 1986). The main pathology is caused by accumulation of adults in the small intestine which may lead to obstipation, even rupture and consequently severe colic (Clayton and Duncan 1978). This occurs mostly in foals and yearlings as immunity develops with age and continuously increases, thereby resulting in a continuous reduction of worm and egg counts (Fabiani et al. 2016). In a few cases, older horses fail to eliminate the infection and these horses can be a major source of infection for foals (Hinney et al. 2011).

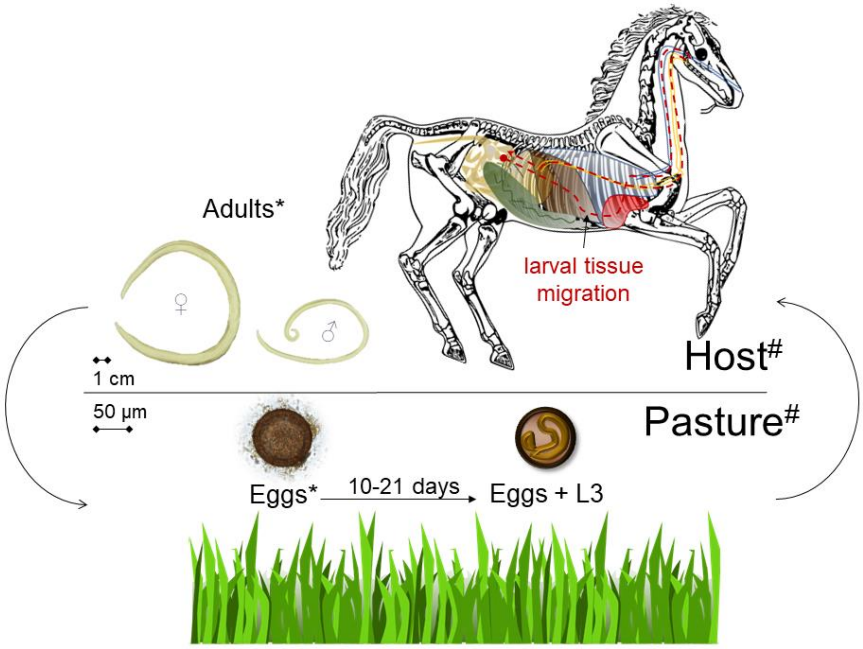

Figure 1 *Parascaris* spp. life cycle

**pictures of adult Parascaris univalens, modified from pictures by K. Seidl ©Institute for Parasitology and Tropical Veterinary Medicine*

#https://pixabay.com/vectors/animal-bone-horse-mammal-1298826/ modified manually to illustrate the anatomy of the inner organs and https://pixabay.com/vectors/grass-lawn-nature-green-summer-303857/ visited 25.03.2020

The life cycle of *Parascaris* spp. is similar to that of other ascarids and although they are monoxenous their extensive tissue migration has been suggested as an evolutionary remnant of an abandoned intermediate host (Anderson 1988). An alternative explanation is that tissue migration is a way of evading the host's immune system because despite its tremendous energy expenditure, tissue migration appears to result in a survival advantage for parasitic helminths (Mulcahy et al. 2005).

2.1.3.3 *Epidemiology*

Due to the high tenacity of the eggshell, *Parascaris* spp. are environmentally resilient. Among other factors this has contributed to their global spread, as *Parascaris* spp. is ubiquitous in equid populations worldwide (Nielsen et al. 2014b) infecting domesticated and wild equines. For example, they can be found in donkeys (Mohammed Jajere et al. 2016) and zebras (Wambwa et al. 2004).

In domesticated horses the reported prevalences vary geographically and also depending on the counting method, i.e. by farm or by animal and separated by age. For example, 53% (179/341) of foals in Saudi-Arabia (Alanazi et al. 2017) were positive for *Parascaris* spp. In northern Germany, *Parascaris* spp. was found on 16.7% of investigated farms (Hinney et al. 2011), with 1% (13/1294) of adult horses, 6% (5/83) of yearlings and 33% (10/30) of foals <1 year shedding *Parascaris* spp. eggs. In a study from the USA, 34% (yearlings) or 20% (adults) were tested *Parascaris* spp. positive (Hautala et al. 2019). As outlined in the previous section (section 2.1.3.2), the most important influence on *Parascaris* spp. prevalence is age, however, other important factors can also have a major impact on the prevalence and egg shedding e.g. seasonal variation (dependent on the geographical and climatic conditions), immunological status of the host, farm management and treatment strategies (Lyons and Tolliver 2004, Hinney et al. 2011, Rehbein et al. 2013, Fabiani et al. 2016, Hautala et al. 2019). A study examining a population of feral horses on an isolated island (Sable Island, Canada) which has been protected from human interference since 1961 reported an unusually high amount of

adult patent infections but confirmed the higher prevalence of *Parascaris* spp. in younger animals (Jenkins et al. 2020).

Interestingly, a recent study reported an increase in *Parascaris* spp. infections diagnosed in post-mortem examinations from the year 2000 onwards and a significant 1.97-fold higher prevalence post-2007 compared to pre-2007 (Sallé et al. 2020), which is the time when reports of ML resistance started to accumulate (see section 2.2.7).

2.1.3.4 *Diagnosis and Therapy*

Commonly, diagnosis is performed by coproscopic examination (fecal flotation with a saturated saline solution) to identify the characteristic eggs (Esccap 2019) which can be quantified with various techniques such as the McMaster or Mini-Flotac (Barda et al. 2013, Cringoli et al. 2013) method. However, egg numbers in the feces do not correlate well with worm counts as many factors can influence egg shedding in *Parascaris* spp. females and thus is an improper predictor to determine the number of adults in the intestine (Nielsen et al. 2010). For example, high population densities reduce the number of eggs shed per female suggesting quorum sensing mechanisms (Lyons et al. 1976, Clayton and Duncan 1979). Alternatively, adult worms expelled with the feces or post-mortem examinations (naturally the gold standard) allow the diagnosis of parascariosis. As an additional diagnostic tool to monitor the burden of ascarids in the intestine an ultrasonographic scoring method was developed (Nielsen et al. 2016).

The prevalence and spread of anthelmintic resistance in *Parascaris* spp. are outlined in detail in section 2.2.7 but in general, resistance is widespread against MLs, spreading against pyrimidines (pyrantel) and emerging against BZs. At the same time, resistance to BZs (and multiple resistance) is widespread in other horse parasites, in particular the cyathostomins (Matthews 2014, Nielsen et al. 2014b, Nielsen 2015). This development is the result of high frequency preventive (= without prior diagnosis) or metaphylactic (= treatment for the complete herd if one animal has been tested positive) anthelmintic applications at herd level which have been the recommendation for many decades. As this strategy has exhausted its lifetime, diagnosis and treatment have become closely intertwined as this represents a path towards more sustainable strategies which are currently discussed and refined in the veterinary parasitological community. Such strategies which aim at preventing or at least postponing the development of anthelmintic resistance include selective or strategic treatment and management. Currently, pyrantel and BZs (fenbendazole) are still effective on most farms to target *Parascaris* spp. in young horses and it is recommended to reduce the use of one drug class to twice a year. In line with recommendations from computer models (Leathwick et al. 2017), current guidelines suggest strategic treatments to target ascarids in young animals (Esccap 2019) and selective treatments (only high egg shedders) in adult horses. Strategic treatment is based on seasonal and age-specific treatments at herd level. Importantly to this strategy, regular fecal egg count monitoring including fecal egg count reductions tests (FECRT) should be performed, which are the gold standard (without sacrificing the animal) for determining the presence of anthelmintic resistance. For this, a fecal egg count should be performed before and 14 days after treatment. In addition, improved hygiene in stables and pastures are critical to reduce the infection pressure and thereby, worm burdens and symptoms. In this regard, to avoid the introduction of resistance from other farms, newly acquired horses should be quarantined until the success of treatment has been ensured by a FECRT. Recommended disinfectants should contain cresol or peracetic acid which are active against the highly resilient *Parascaris* spp. eggs. It remains unclear, whether these approaches can impact resistance development at this stage.

2.1.4 *Caenorhabditis elegans*

2.1.4.1 *Biology*

Caenorhabditis elegans, first described at the beginning of the 20th century (Maupas 1900), is a free-living clade V (Rhabditida) nematode (Blaxter et al. 1998).

Visually, *C. elegans* appear as transparent worms. While usually they are hermaphrodites, a low proportion (roughly 0.1%) of the population will develop as males (Emmons 2005, Ellis and

Schedl 2007). Adult hermaphrodites are about 1110 µm to 1150 µm in length, 65 µm wide and generally larger than males. Interestingly, *C. elegans* and several other nematode species exhibit eutely. This means that the number of somatic cells in every adult animal is fixed (1090 cells in the adult hermaphrodites and 1178 cells in the adult males). The embryonic and larval developmental process has been elucidated in great detail and includes a programmed cell death of 131 or 147 somatic cells and hence resulting in a total of 959 somatic cells in the adult hermaphrodites and 1031 in the adult males, respectively (Sulston and Horvitz 1977, Nigon and Félix 2017). For each cell, a transcriptomic profile as well as a detailed description is available (Wormatlas 2020). For example, the hermaphrodite epidermis is made from a few syncytial cells, hyp1-13, of which some fuse during development, and seam cells. Interestingly, the largest proportion of the epidermis, is constituted by hyp7 which contains 139 nuclei. The intestine is made of rather large cuboidal cells which are laterally sealed by adherens and adherens junctions (Labouesse 2006) forming a lumen. At their apical pole microvilli are covered in a proteoglycan matrix containing highly modified glycoproteins and chitin microfibrils, possibly serving as a protection from mechanical and toxic injuries or as a filter (Lehane 1997).

At 20 °C, the complete life cycle from a newly fertilized and laid egg to reproductive maturity takes about 65 hours and in the presence of food, a hermaphrodite will lay about 280 eggs (Byerly et al. 1976). After hatching, each first stage larva (L1) will undergo four molts, each marking the end of a larval stage (L2, L3, L4, adult). Crowding, lack of food and other limiting factors will induce production of a pheromone which disrupts physiological development and triggers the formation of a so called dauer stage following the L2 stage rather than development to L3 (Golden and Riddle 1982, Ludewig et al. 2017). Like development, reproduction in *C. elegans* is regulated by many different external factors, most importantly temperature (Byerly et al. 1976) but also other factors such as food availability (Trent et al. 1983), chemical stimuli such as salts (Horvitz et al. 1982) or mechanical stimulation, i.e. the vibration of the culture medium inhibits egg laying (Sawin 1996).

2.1.4.2 *Relevance for Life Sciences*

Caenorhabditis elegans is one of the most widely studied species since Sydney Brenner established it as a model organism for multicellular eukaryotes (Brenner 1974). One of the reasons for its success is that they can be cultured with low maintenance costs and efforts on agar plates and can rapidly expand their population based on an *Escherichia coli* diet (usually using the OP50 strain, a mutant requiring uracil for growth). Their cost-effective culturing as well as their transparency has allowed detailed analysis of many aspects of biology. In fact, scientific discoveries in *C. elegans* were rewarded in 2002 with a Nobel Prize in Physiology or Medicine to Sydney Brenner, H. Robert Horvitz, and John Sulston (organ development and programmed cell death), in 2006 with a Nobel Prize in Physiology or Medicine to Andrew Fire and Craig C. Mello (RNAi discovery) and 2008 with a shared Nobel Prize in Chemistry (green fluorescent protein) to Martin Chalfie.

While *C. elegans* has been of considerable importance to understand nematode biology, it has become quite clear that many findings in *C. elegans* cannot be transferred one-to-one to other nematodes in the same genus, neither to nematode parasites with an entirely different lifestyle nor generally to nematodes from other clades (Viney 2017, Blanchard et al. 2018). Along the same lines, most research has been conducted on the N2 Bristol strain isolated from mushroom compost near Bristol, England (Riddle et al. 1997) and its mutants. However, global sampling has revealed the high genotypic and phenotypic diversity (Crombie et al. 2019). As the *C. elegans* community has become aware that this is a problem in terms of the particularities of this strain and the lack of representation of genetic diversity which easily leads to false conclusion, these global strains have been made available from the *Caenorhabditis elegans* natural diversity resource platform (CeNDR) (Cook et al. 2016). Only rather recently, *C. elegans* has become increasingly popular as a model organism for nematode parasites. Its relevance as a model organism for anthelmintic resistance and nematode parasite research is further outlined in section 2.3.2.3.3.

2.2 Anthelmintic Resistance

2.2.1 Introduction to Anthelmintic Resistance

The emergence of drug resistance to all available classes of anthelmintics in a vast number of parasite species reflects the extraordinary potential to adapt in the nematode phylum. This development has quickly turned into a global challenge to maintaining animal health in particular in sheep (Rose Vineer et al. 2020a) and to an increasing extent also in horses, cattle (Rose Vineer et al. 2020a) and very recently in dogs (Kitchen et al. 2019).

Strikingly, in contrast to vaccination no pathogen has ever been fully eradicated through chemotherapy, however, chemotherapy has proven extremely successful in the control and treatment of many diseases of bacterial, fungal and parasitic origin. In veterinary medicine, the regional almost eradication of the very pathogenic horse parasitic nematodes commonly known as large strongyles through high frequency herd anthelmintic treatment gives an example for such successful control (Reinemeyer 2009). Unfortunately, this success has come at a grave cost as resistance to multiple anthelmintics has evolved in other horse parasitic nematodes in parallel (Von Samson-Himmelstjerna 2012).

Before further outlining the current knowledge on anthelmintic resistance, it is necessary to define what makes a nematode population resistant. The World Association for the Advancement of Parasitology (WAAVP) guideline 2012 defines anthelmintic resistance “as a heritable change in susceptibility to an anthelmintic in a population of parasitic nematodes such that a dose which normally provides ≥95% clearance of adult worms provides ≤80% clearance” (Geary et al. 2012). Effectively, this increased survival rate results from an increased frequency of a single or multiple resistant alleles in a worm population which in turn results in a shift in the dose-response curve.

In line with Theophrastus von Hohenheim’s general adage “*Sola dosis facit venenum”*, only the dose determines whether an individual parasite is killed by a drug or not, however, this dose varies considerably between different genotypes. Over time, those genotypes within a population will be selected that harbor protection against the drug but do not impose a too heavy burden on worm fitness. Considering the biodiversity in the nematode phylum and the anatomical transformations during each specific life cycle, it is not surprising that this dose varies also quite considerably between nematode species. For example, for drugs interfering with neuronal receptors the subunit repertoire of the target receptors within a species is critical for the drug efficacy (Blanchard et al. 2018). At the same time, host pharmacokinetics are a major influencing factor for the actual dose reaching the parasite (Lifschitz et al. 2017). Among other factors, these differences make it challenging to evaluate whether anthelmintic resistance is present in a parasite population and to compare between different species. In addition, determining the dose (rather than exposure to a certain concentration) is extremely difficult to achieve, even *in vitro*.

For several reasons, parasitic nematodes have been quite successful in evolving mechanisms to withstand anthelmintic exposure. In veterinary medicine, livestock is usually kept in herds which is associated with relatively large parasite populations (in particular for those animals kept on pasture) and for decades regular and frequent anthelmintic application of the whole herd has been common practice. Combined with the high genetic diversity of several nematode species (Gilleard and Redman 2016) and their reproductive potential, this treatment practice bears a high risk for the selection and spread of resistant alleles. Similarly, humans living in densely populated areas with insufficient sanitary infrastructure and here in particular places with regular crowding such as schools result in a similar situation (Webster et al. 2008, Krücken et al. 2017). Despite thriving under the right conditions, as the offspring of many (gastro-intestinal) soil-transmitted parasitic nematodes are expelled with the feces, parasitic nematodes can be effectively driven back by drinking water treatment, hygiene and the establishment of a sanitary system (Vaz Nery et al. 2019). For livestock such measurements are difficult to implement and are mostly limited to indoor animal farming. Therefore, preventive and metaphylactic treatment strategies to control nematode diseases have been common

practice for decades in veterinary medicine which has driven the selection of resistance alleles. Noteworthy, whether or not resistance develops in a parasitic nematode population depends on a large number of variables, including but not limited to the drugs pharmacokinetics and -dynamics (Yu et al. 2018a), commonly used treatment strategies, the genetic diversity in a parasite population, the number of resistance alleles necessary to confer a sufficient resistance level, the mechanism of resistance, the life cycle stage and the resistance and immunological status of the host (e.g. domesticated animals bred for productivity versus natural systems).

In general, drug resistance mechanisms can be categorized as specific or unspecific. Specific mechanisms are alterations of the drug target site, e.g. single nucleotide polymorphism (SNPs) F167Y (Silvestre and Cabaret 2002), E198A (Ghisi et al. 2007), F200Y (Kwa et al. 1995) and other mutations in the β-tubulin (*ben-1*) gene conferring resistance against the anthelmintic class of BZs (Driscoll et al. 1989). Also, expression changes such as down or upregulation or gene deletions can lead to resistance (Dent et al. 2000). In addition, alternative splicing can lead to anthelmintic resistance, e.g. in case of levamisole or monepantel resistance (Fauvin et al. 2010).
In contrast, unspecific resistance mechanisms are usually the result of changes in drug metabolism or drug efflux. In turn, these changes can result from overexpression or SNPs leading to increased drug affinity in the relevant metabolic genes. These unspecific mechanisms can include metabolizing enzymes such as members of Cyt-P450 superfamily (Matoušková et al. 2016) or efflux pumps such as transporters of the ATP-binding cassette (ABC) gene superfamily (Lespine et al. 2012). Metabolizing enzymes can alter the active site or the chemical properties allowing more efficient efflux. Transporters can reduce the effective drug concentration.

It should be noted that most resistance-associated alleles are already present in a susceptible population of nematodes but at very low frequencies. In addition, in particular in those parasite with a high mutation rate (and reproductive rate) such as *H. contortus*, mutations which can confer resistance are constantly appearing (Gilleard and Redman 2016). The metabolic and regulatory pathways which are rapidly selected by treatment had initially evolved over millions of years as nematodes have been in a constant evolutionary battle with other species in their ecological environment such as fungi and bacteria which in turn have been developed a wide range of toxins to target their competitors and predators. Noteworthy, many drugs including some of the most influential drugs are products of fungi e.g. penicillin (Gaynes 2017), plants e.g. artemisinin or bacteria e.g. the MLs originating from the soil-dwelling *Streptomyces avermitilis* (Burg et al. 1979, Chabala et al. 1980).

2.2.2 History of Drug Discovery and Resistance

Anthelmintic drugs can act by a number of ways to directly kill nematodes (nematocidal) or to facilitate their elimination (e.g. by temporary paralysis or by incapacitating their ability to evade the immune system) from the host. Early anthelmintics such as piperazin, phenothiazine and organophosphates were effective only against a few selected nematode species and exhibited high toxicity in the host and were replaced between the 1960s and 1980s (**Fig. 2**) by the three main groups of broad spectrum anthelmintics still used today. In the 1960s the BZs (**Table 1**) as the first broad-spectrum anthelmintic revolutionized treatment against parasitic helminths including most parasitic nematodes in animals and humans. Shortly after the BZs in the early 1970s the introduction of the imidazothiazoles/tetrahydropyrimidines (Table 1) was the second impactful broad-spectrum anthelmintic class, which however exhibited a narrower safety margin. At this time, first resistances against BZs had already been reported in some parasitic nematode species (**Fig. 2**). Another decade later, the discovery of the MLs improved treatment options considerably due to a very high efficacy, broad spectrum against helminth and arthropods and a large therapeutic index (Burg et al. 1979, Chabala et al. 1980). The extraordinary success of MLs diminished efforts to discover and market new anthelmintics temporarily (Geary et al. 1999) but with increasing levels of ML resistance in several economically important parasites in the early 2000s, these efforts were reinforced leading to the market introduction of emodepside (Bayer Health Care group now part of Elanco Animal

Health) and monepantel (Novartis and Elanco Animal Health) (Kaminsky et al. 2008b) in the early 2010s (**Fig. 2**). Many derivatives of the major classes of anthelmintics (BZs, MLs, imidazothiazoles/tetrahydropyrimidines) are available as oral formulations by a number of producers. Alternative formulations include topical, injectable or slow release preparations (injectable or bolus) (Vetpharrm accessed 27.03.2020). The formulation depends on the chemical properties of the anthelmintic and on the biology of the target parasite.

Due to the lack of efficacious and cost-effective vaccines (see section 2.2.8) chemotherapy remains the main option for parasitic nematode control in veterinary medicine in general and also specifically in horse farming. However, as visible in **Figure 2** resistances have emerged against each class of anthelmintic for all anthelmintics which are still in use, except for the newly emerged drugs. Nonetheless, even for monepantel, drug resistance has already been reported in *Teladorsagia circumcincta, H. contortus* and *T. colubriformis* (Ploeger and Everts 2018). For controlling *Parascaris* spp., BZs and imidazothiazole/tetrahydropyrimidine derivatives have sustained efficacy for a longer time than MLs. However, in recent years reports of resistance against fenbendazole and pyrantel are increasing also in *Parascaris* spp. (**Table 1**).

In addition to this development in farm animals, ML and multiple resistance is has been reported in parasitic nematodes of dogs: the heartworm *D. immitis* and the hookworm *Ancylostoma caninum* (Jimenez Castro et al. 2019). In the latter, the development and spread had gone unnoticed until the description of a multi-resistant isolate (Kitchen et al. 2019) which was found not to be a unique case but rather an extreme case within a general trend (Jimenez Castro et al. 2019). In contrast, the situation of ML resistance in the dog heartworm has been monitored very closely for a decade and is considered the result of large-scale preventive and treatment programs in the US (Bourguinat et al. 2011b, Geary et al. 2011, Bowman 2012, Pulaski et al. 2014, Vatta et al. 2014, Bourguinat et al. 2015, Wolstenholme et al. 2015, Bourguinat et al. 2016, Mani et al. 2016a, Mani et al. 2016b, Mani et al. 2017, Willi et al. 2018).

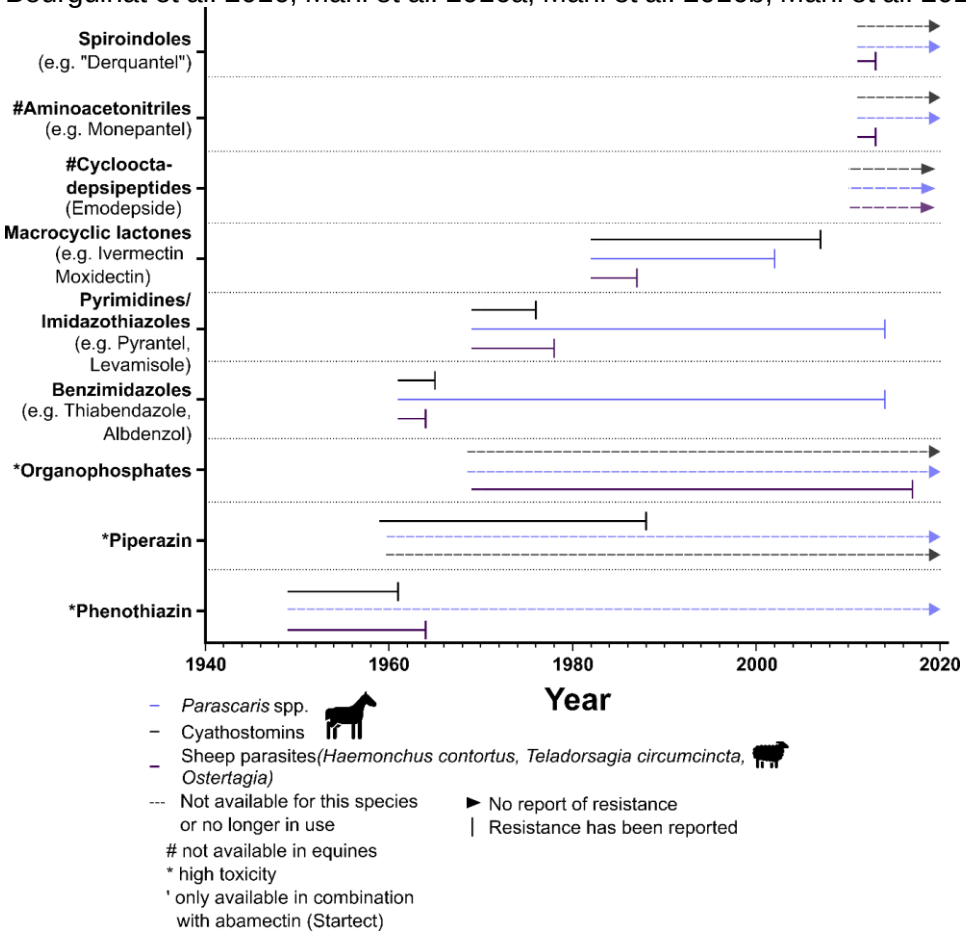

Figure 2 Duration between market introduction and first report of anthelmintic resistance

Tabelle 1 References corresponding to Figure 2 - anthelmintic class market introduction and first report of resistance in important parasites of horses and sheep

Anthelmintic class	Anthelmintic	Market introduction	First resis-tance	Publication	Nematode species	Host species
Phenothiazine		1940	1961 1964	(Drudge and Elam 1961) (Bennett and Todd 1964)	Cyathostomins *H. contortus*	Horse Sheep
Piperazine		1950s (Mckellar and Jackson 2004)	1988	(Drudge et al. 1988)	Cyathostomins	Horse
Organophosphates	e.g. coumaphos, naphthalophos	1960s (Krücken et al. 2012)	2017	(Lamb et al. 2017)	*H. contortus*	Sheep
Benzimidazole	Thiabendazole (1961), Fenbendazole (1974), Albendazole (1976)	1960s–1980s (Mckellar and Jackson 2004)	1964 1965 2014	(Drudge et al. 1964) (Drudge and Lyons 1965) (Relf et al. 2014a)	*H. contortus* Cyathostomins *Parascaris* spp.	Sheep Horse Horse
Imidazothiazole/ Tetrahydropyrimidine	Pyrantel[1], Levamisole[2], Morantel[2], Oxantel	1960s–1980s (Mckellar and Jackson 2004)	1976[1] 1978[2] 2014[1]	(Chapman et al. 1996) (Le Jambre et al. 1978) (Relf et al. 2014b)	Cyathostomins *Ostertagia circumcincta* *Parascaris* spp.	Horse Sheep Horse
Macrocyclic lactones	Ivermectin[1,] Moxidectin, Abamectin and more	1980s (1981[1]) (Mckellar and Jackson 2004)	1987[1] 2002[1] 2007[1]	(Carmichael et al. 1987) (Boersema et al. 2002) (Von Samson-Himmelstjerna et al. 2007)	*H. contortus* *Parascaris* spp. Cyathostomins	Sheep Horse Horse
Cycloocta-depsipeptides	Emodepside	2005 (Krücken et al. 2012)	-	Not available in equines/ruminants	-	-
Aminoacetonitrile	Monepantel	2010 (Kaminsky et al. 2008a) Not available for use in equines	2013	(Scott et al. 2013) (Ploeger and Everts 2018)	*Teladorsagia circumcincta*, H. contortus	Sheep
Spiroindoles	Derquantel (only available in combination with abamectin)	2010 (Little et al. 2011) Only available for sheep	2011	(Kaminsky et al. 2011)		

*Multiple years in "*First report of resistance" *correspond to the "*Nematode species" in the same *row.*
[1, 2] Indicate the anthelmintic against which resistance was reported or the corresponding market introduction year

2.2.3 The Macrocyclic Lactones

Ivermectin as the first ML was discovered by William C. Campbell who worked at Merck Institute for Therapeutic Research and Satoshi Ōmura working at the Kitasato Institute (Burg et al. 1979, Chabala et al. 1980). Due to the extraordinary success of the MLs to drive back human parasitic disease, their discovery was rewarded with a Nobel Prize in Physiology or Medicine in 2015. In addition to their safety and efficacy, a major advantage is that the broad spectrum includes many important parasitic species of humans, domesticated animals and agricultural plants such as arthropods and nematodes (Shoop et al. 1995, Molyneux et al. 2003). Specifically, MLs allowed substantial improvements in the control of onchocercosis and lymphatic filariosis and at the same time, these drugs were very successful in veterinary medicine. In fact, they are the most commonly used anthelmintic for the control of parasitic nematodes in sheep, cattle and horses as well as domestic pets (Burgess et al. 2012, Relf et al. 2012, Mcarthur and Reinemeyer 2014). Since the discovery of IVM, many different ML derivatives with different pharmacodynamics and pharmacokinetics were discovered and marketed.

2.2.3.1 *Pharmacodynamics*

The main effect of MLs on nematodes results from the binding and opening of glutamate-gated chloride channels (GluCls) which results in an uncontrolled influx of chloride ions and a hyperpolarization of the post synaptic neurons (Arena et al. 1991, Martin 1997) as depicted for IVM in **Fig. 3**. This binding is considerably slower than that of the natural ligand glutamate but is irreversible thus permanently increasing chloride ion permeability.

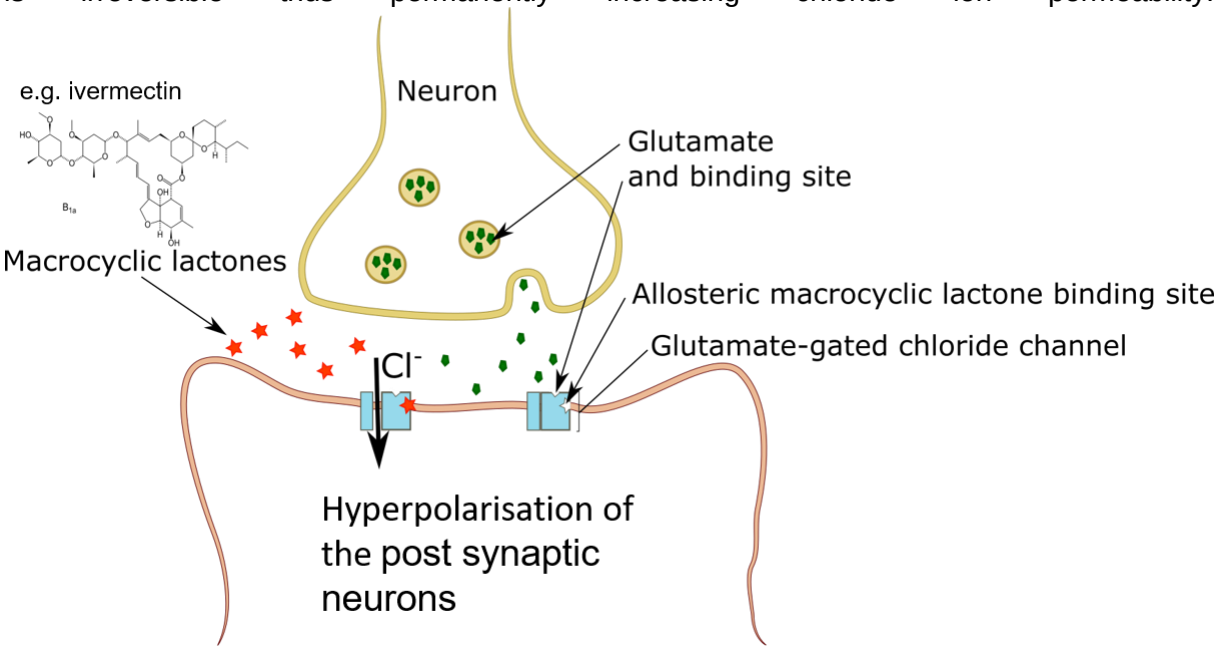

Figure 3 Schematic illustraton of the mode of action of macrocyclic lactones

In nematodes, the hyperpolarisation of the post synaptic neurons leads to a flaccid paralysis of the body wall muscle and the pharynx (Wolstenholme and Rogers 2005a). Consequentially, pharyngeal paralysis leads to starvation of the worm and the immobility facilitates expulsion with the feces and makes the worm susceptible to the immune system. Noteworthy, GluCls are not expressed in muscles cells and therefore, an indirect effect of IVM on the neuronal regulation of locomotion and pharynx is assumed. Locomotion and pharyngeal pumping are controlled by command interneurons in the head of the worm (Zheng et al. 1999) as well as motor neurons which are the sites where GluCls are expressed primarily (Dent et al. 1997, Dent et al. 2000, Portillo et al. 2003). The hyperpolarisation of these neurons inhibits the formation and transmission of action potentials.

The expression of *C. elegans* GluCls in *Xenopus laevis* oocytes allowed the identification of two GluCl subunits which together formed a heteromeric channel (Cully et al. 1994) susceptible to IVM, *glc-1* (GluCl α subunit) and *glc-2* (GluCl β subunit). In addition, two more GluCl subunits were discovered, GluClα2A and GluClα2 which were alternatively spliced versions of the *avr-15* (Dent et al. 1997) gene. Expression pattern analyses from the same study showed that the subunits exhibited different tissue localization i.e. GluClα2 and β in the pharynx and GluClα2 in the motor neuronal system. Furthermore, another ML susceptible GluCl gene was identified, *avr-14*, also exhibiting alternative splice forms, GluClα3A and GluClα3B. A strain lacking all three genes (*avr-14*, *glc-1* and *glc-2* deletions) exhibited very high resistance to IVM (Dent et al. 2000). Corroborating the complexity of the mode of action, more GluCl genes were identified shortly after in *C. elegans* including *glc-3* and *glc-5* of which some subunits were also susceptible to IVM (Horoszok et al. 2001). In addition to this complex interaction in *C. elegans*, the parasite GluCl repertoires are similarly diverse and vary considerably between species and clades. This complexity is considered to be one of the main reasons for differences in susceptibility of different nematode species (Wolstenholme and Rogers 2005b, Blanchard et al. 2018), e.g. a 40-50 times higher susceptibility of *Ancylostoma ceylanicum* compared to *Necator americanus* (Richards et al. 1995). In *Parascaris* spp., major differences exist compared to *C. elegans*. For instance, the *avr-14b* subunit does not form a homomeric IVM susceptibility receptor as it does in *C. elegans*, however, when co-expressed with *glc-2*, the resulting novel sub-type heteromeric receptor is indeed susceptible to IVM (Lamassiaude et al. 2018, Lamassiaude et al. 2020). At the same time, expression patterns of receptors vary between developmental stages in nematodes and the parasitic stage specific requirements might differ substantially (e.g. mechanisms for tissue migration and host evasion). For example, in *D. immitis* and *Brugya malayi* microfilariae, the *in vivo* effect requires lower doses than *in vitro* (Vatta et al. 2014) which is probably the result of IVM interfering with the secretory system hence leading to reduced capability for immune modulation and host invasion (Moreno et al. 2010, Harischandra et al. 2018). In addition, due to the pharmacokinetics of MLs the tissue localization of a parasitic stage determines the dose reaching the worm (discussed in section 2.2.3.2).

Notably, the number of neuronal targets of MLs in nematodes is not limited to GluCls and is known to also include ionotropic γ-aminobutyric acid ($GABA_A$) receptors which were actually the first known targets of these drugs (Holden-Dye et al. 1988). Overall, MLs can interfere with GABA, GluCl, glycine, histamine and nicotinic acetylcholine receptors in vertebrates and invertebrates but probably with a lower affinity thus offering an explanation for the differing toxicity between species (Wolstenholme and Rogers 2005a). Accordingly, this might explain why MLs do not have sufficient activity against trematodes and cestodes (Laing et al. 2017). Between the different derivatives of MLs (discussed in section 2.2.3.2) it is assumed that there are a number of differences in the target receptors and receptor subunits (Prichard et al. 2012) and likewise, there are differences in the effect of these derivatives on nematodes (Ardelli et al. 2009).

2.2.3.2 *Chemical Properties and Pharmacokinetics*

To date, many ML derivatives have been marketed. Generally, MLs can be divided into two groups, the avermectins and the milbemycines. The avermectins are fermentation products of *S. avermitilis* and the milbemycins are fermentation products of *Streptomyces hygroscopicus*. Both contain the 16-membered macrocycle fused to a benzofurane (C2, 7, 8) and spiroketal group (C17-19) (**Fig. 4**) and exhibit a similar range of drugs with only subtle differences. Thus, this core structure is considered responsible for their anthelmintic activity (Mckellar and Benchaoui 1996). Explicitly, the milbemycines lack the C13 saccharide side chain (**Fig. 4** examplarily shown for MOX) while IVM and other avermectins exhibit a dissacharide (or monosaccharide in case of selamectin) at this position. In addition, there is considerable variation at the C25 and C23 position of the spiroketal between the derivatives. These chemical disparities lead to differences in their physico-chemical properties, for example their size, molecular weight and lipophilicity. While IVM exhibits a molecular weight of 875.1 g/mol, MOX

exhibits a molecular weight of only 639.8 g/mol. It should be noted that typically, IVM is present as two homologues, B1a (over 80%) and B1b (<20 %, slightly s molecular weight, 861.1 g/mol) (Sutherland and Campbell 1990). Generally, MLs exhibit poor or no solubility in water and high lipophilicity. MOX has been proposed to have a higher lipophilicity than IVM, possibly as a result of the lack of the C13 sugar moiety (Prichard et al. 2012). In turn, these chemical differences also lead to differing pharmacodynamics and -kinetics.

Due to their lipophilicity MLs distribute well throughout the body and accumulate in fatty tissues including the liver independent of the dose (Andrews et al. 1993). This accumulation and subsequent slow release leads to a long-lasting persistence and activity (Entrocasso et al. 1996). In this regard, MOX has been shown to have a longer half-time in different species including humans (Cotreau et al. 2003), dogs (Al-Azzam et al. 2007), cattle (Bousquet-Mélou et al. 2004), sheep (Lloberas et al. 2013a) and horses (Perez et al. 1999) and this effect has been suggested to be the result of the higher lipophilicity of MOX (Lespine 2013). Generally, plasma concentrations and mean residence time vary between species and study. A study in horses comparing different ML derivatives reported peak plasma concentrations after oral administration of 200 mg/kg bodyweight of 21.4 ng/ml IVM, 21.3 ng/ml doramectin, and 30.1 ng/ml MOX after around the 8 hours following application. The area under the curve for MOX was higher than for both avermectins in this study (Gokbulut et al. 2001). This might indicate a persistence or it also indicates a higher bioavailability but it should be noted that the actual drug effect is determined by the dose available in target tissues. For example, adult stages of gastro-intestinal nematodes dwell in the intestine or stomach but larval stages of some parasite species migrate through tissues. In some filarial nematodes both the larval and adult stages can reside in the blood stream. While a higher mean plasma residence time and area under the curve for MOX compared to IVM is consistent between studies, the actual peak plasma concentrations might vary, i.e. 70.3 ng/ml for MOX and 44.0 ng/ml for IVM. It should be noted,

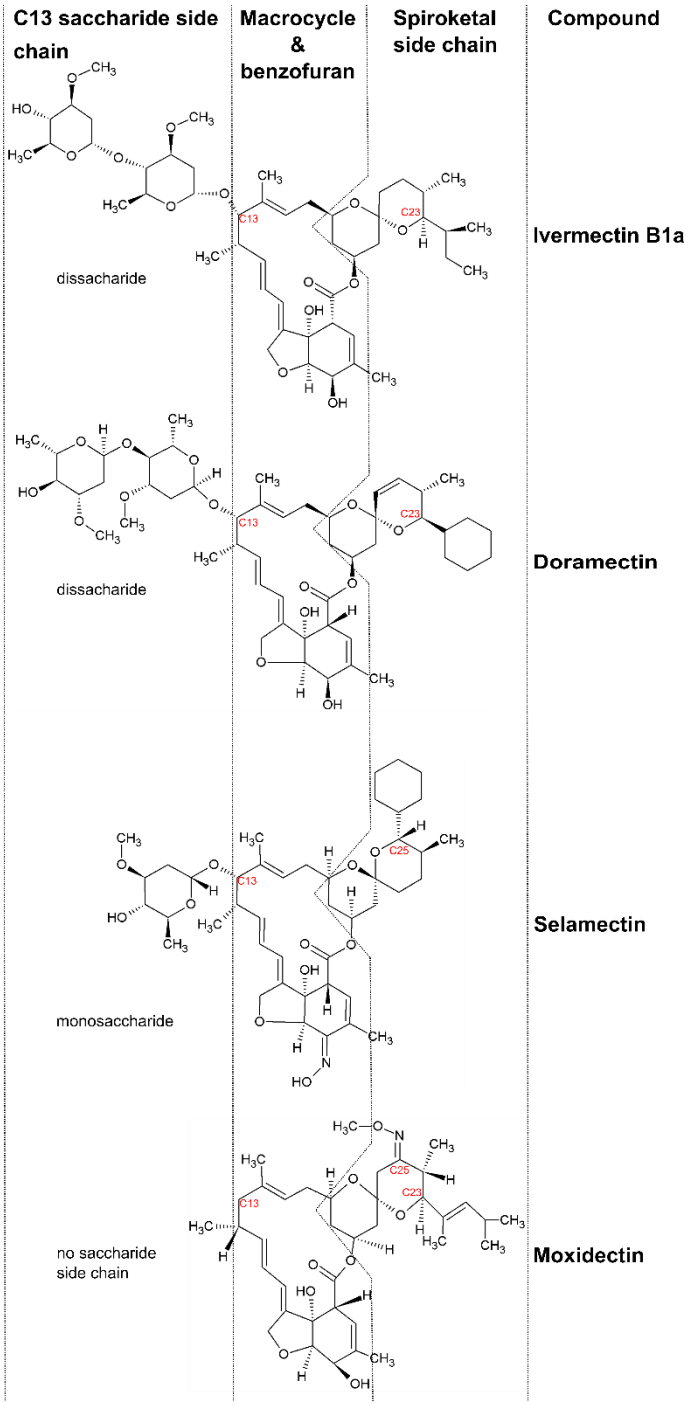

Figure 4 Chemical structure of macrocyclic lactone derivatives

Chemical structures visualized using ChemSketch v12.01 (ACD/Labs)

that in this study MOX was administered at a higher dose of 400 mg/kg bodyweight (Pérez et al. 1999).

MLs are available as oral, pour-on or subcuticular and in rare cases intravenous injectable formulations. The injectable formulation has been shown to result in longer persistence in the body than oral administration (Hennessy and Alvinerie 2002). Pour-on formulations are common in livestock due to their easy and stress-free applicability but were shown to only achieve roughly 15% of bioavailability in cattle compared to subcuticular injection possibly as a result of wasting and skin trapping (Gayrard et al. 1999). In mammals, MLs are almost exclusively excreted with the feces and with only 1-3% renal elimination and most of the parental compound is excreted without metabolization (<10% metabolites) (Gokbulut et al. 2001, Laffont et al. 2002, Ballent et al. 2006a, Ballent et al. 2006b, Mckellar and Gokbulut 2012). For excretion, mammalian Pgps play an important role (Ballent et al. 2006a, Ballent et al. 2006b). This excretion route also has consequences for ecotoxicity and for the availability at parasite location tissues, i.e. a high exposure of intestinal dwelling stages.

2.2.3.3 *Toxicology and Environmental Toxicity*

As mentioned in section 2.2.3.1, MLs interfere with a broad range of vertebrate and invertebrate receptors. While this has contributed to their extraordinary success and is the reason for their broad applicability, it is also the reason for toxicity in the host and in ecosystems. The main target of MLs in targeted pathogens, the GluCls, are only found in protostome invertebrate phyla, but IVM also interacts with the most closely related vertebrate glycine and GABA receptors which can be found in the spinal cord and the brainstem (Lynch 2004) at micromolar concentrations (Vassilatis et al. 1997). However, mammals are better protected from ML induced neuronal toxicity due to the impermeability of the blood brain barrier (Schinkel et al. 1994b) and hence the tolerated dose is much higher leading to a broad therapeutic index. The main contributor to this barrier function is in fact the mammalian Ppg (*mdr-1* or *Abcb1*) and in individuals with a gene defect, application of MLs leads to severe neurotoxicity. This has been shown for several species including mice (Schinkel et al. 1994b), dogs and cats (Merola and Eubig 2012). This gene defect is very common in several dog breeds, in particular in Collies (Geyer and Janko 2012).

Unfortunately, unwanted effects are not limited to side effects in animals and humans with a gene defect. Rather, as a flipside of their broad target spectrum, MLs exhibit toxicity for many ecosystems and the frequent usage can lead to environmental contamination. This contamination can cause eco-toxicity in aquatic and terrestrial eco-systems (Jacobs and Scholtz 2015).

2.2.4 Benzimidazoles

Similar to the MLs, many different BZ derivatives exist today such as thiabendazole, fenbendazole, albendazole and flubendazole. Thiabendazole (TBZ) was the first BZ marketed for veterinary medicine but has mostly been replaced by more effective compounds with a broader range. In equines, the most commonly used BZ derivative is fenbendazole. Most BZs have a broad spectrum but exhibit some variation. TBZ for example targets many different nematodes as well as many fungi including pathogenic species (Zhou et al. 2016). Regarding their pharmacodynamics, BZs bind at β-tubulins and inhibit their polymerization with other α- or β-tubulins (Lacey 1990). Several BZ derivatives also exhibit activity against trematodes and cestodes (Mehlhorn 2008). Depending on the targeted helminth it is therefore necessary to choose the right BZ derivative. Between nematodes and even between stages there are considerable differences in the activity spectrum of the individual derivatives (Kuhlmann and Fleckenstein 2017). However, binding of mammalian β-tubulin is almost completely absent for all licensed BZ derivatives leading to a low toxicity for treated animals and humans. Nevertheless, their broad spectrum and wide application results in moderate environmental toxicity in particular to some aquatic organisms (Oh et al. 2006, Wagil et al. 2015).

Benzimidazole pharmacokinetics also differ between derivatives, application and host species and hence, every derivative has a recommended dose for target nematode species and its

host. Benzimidazoles are metabolized in the liver (first-pass) and this has a significant effect on the availability of active compound. This first-pass effect is exploited in pro-benzimdazoles (e.g. febantel, thiophanate and netobimin), which require host metabolization to the active derivative. Depending on the animal species, the availability of metabolites depends on the species-specific metabolizing enzymes. In addition, there appear to be differences between the sexes, age as well as immunological and hormonal status of an individual (Křížová-Forstová et al. 2011). Strikingly, plasma concentrations following oral administration are higher for most BZs e.g. fenbendazole (Mckellar et al. 2002) and albendazole (Lifschitz et al. 1997) in unfed than in fed animals, hence feeding is also an important factor. Also, overall bioavailability is rather low for most BZ derivatives and with several exceptions (e.g. albendazole, oxfendazole, and triclabendazole) many derivatives exhibit a moderate absorption from the intestine, possibly as a result of their low water solubility. In fact, it is assumed that the reason for the low absorption during starvation is the decelerated gastro-intestinal passage which allows more time for solubilization (Křížová-Forstová et al. 2011).

2.2.5 Other Veterinary Relevant Anthelmintics

Another relevant anthelmintic drug class for veterinary medicine are the tetrahydropyrimidines e.g. pyrantel and imidazothiazoles e.g levamisole which are both nicotinic acetylcholine receptor (nAChR) agonists (Aubry et al. 1970, Robertson and Martin 1993, Qian et al. 2006, Martin et al. 2012) and are often grouped together. Like GluCls, these receptors are very diverse in nematodes leading to differences in the efficacy for different species. The more recently developed aminoacetonitriles (e.g. monepantel) target only clade V nematodes, which however include most relevant parasitic nematodes in small ruminants. They also target several nAChRs which are ligand-gated ion-channels. In particular, *mptl-1* appears to be a major target (Baur et al. 2015) whichis also a nAChR named after monepantel. With monepantel there is currently one aminoacetonitrile on the market and it is only available in ruminants. Derquantel belongs to the spiroindoles. As an acetylcholine antagonist it induces a flaccid paralysis. Initial studies have shown a high toxicity in horses (Nielsen and Reinemeyer 2018). Likewise fairly new to the market, cyclooctadepsipeptides target a broad spectrum of nematodes of veterinary importance (Harder and Von Samson-Himmelstjerna 2001, Harder et al. 2005) and have rather low toxicity (Von Samson-Himmelstjerna et al. 2000). In nematodes, they target a calcium-gated and voltage-dependent potassium channel, slo-1 (Kulke et al. 2014). Emodepside is currently only available for use in dogs and cats. To target cestodes and trematodes, praziquantel is the most commonly used drug (Chai 2013).

Furthermore, many plants and plant extracts exhibit anthelmintic activity. This is usually a synergistic effect of several compounds which might diminish when the individual compounds are separated (Githiori et al. 2006, Hoste et al. 2015). Probably the most famous and best-studied plant with anthelmintic activity is chicory. In several studies it was demonstrated that extracts *in vitro* and *in vivo* as well as the addition of whole plants to the animal feed or promoting chicory growth on pasture has a significant effect on nematodes, i.e. paralysis *in vitro* and reduced worm burdens and fecal egg count (FEC) *in vivo* (Peña-Espinoza et al. 2017, Peña-Espinoza et al. 2018). Furthermore, many different compounds in chicory which by themselves exhibit anthelmintic activity *in vitro* were described and characterized. In general, it is assumed that resistance development is slower against plant-based approaches as plants contain many different active compounds (see section 2.2.8).

2.2.6 Epidemiology of Anthelmintic Resistance in *Parascaris* spp.

For unknown reasons anthelmintic resistance in *Parascaris* spp. has emerged in particular against the class of MLs while in most other nematodes, development of BZ resistance has seen a much faster development and spread (see section 2.2.2). In this context it should be noted that *P. equorum* is the dose-limiting parasite in equines for MLs (Reinemeyer 2012), meaning MLs have had the lowest efficacy against *Parascaris* sp. initially. A potential reason for the slow development of resistance against BZs might be a higher number of susceptible beta-tubulin genes (Kotze et al. 2014). Nonetheless, indications for emerging resistance

against BZ derivatives has been incidentally reported in ascarids e.g. in *Ascaris lumbricoides* (Krücken et al. 2017) and in *P. univalens* (Armstrong et al. 2014, Alanazi et al. 2017). At the same time, report of resistance against pyrantel are accumulating (Armstrong et al. 2014, Lassen and Peltola 2015, Alanazi et al. 2017, Martin et al. 2018). Moreover, multiple resistance (resistance against >1 anthelmintic class) have also been reported (Armstrong et al. 2014, N. Kettner 2017). Regarding the potential of *Parascaris* spp. to adapt spp., a study reported that globally *Parascaris* spp. populations appear to be genetically homogenous and to exhibit a rather low mutation rate (Tyden et al. 2013). In light of the high resistance levels, the high reproduction rate, the phenotypically diverse degree of susceptibility to different anthelmintics, this finding was rather surprising. A potential but unlikely explanation would be the extremely rapid spread of a resistant isolate filling the ecological niches made available world-wide from regular anthelmintic treatments.

In the past two decades reports of resistance against the MLs have accumulated from all over the world (**Fig. 2**) and in most of the investigated farms resistance was detected(Jenkins et al. 2020, Tang et al. 2020). Compared to a map reviewing the spread of ML resistance in the preceding PhD thesis on *Parascaris* spp. at the Institute of Parasitology and Tropical Veterinary Medicine at the Freie Universität Berlin (Janssen 2014), an increasing number of studies from a larger number of countries have reported ML resistance demonstrating the continuous spread (**Fig. 5**). In fact, in contrast to the previously mentioned study, the global pattern of resistance against MLs suggests that resistance has most likely risen several times independently. From the geographical distribution of resistance reports (**Table 2**, **Fig. 5**), it appears that countries with a stronger economy (i.e. Europe, US, Australia, Canada, UK) are affected more. It can be speculated that the reason is that on the one hand horse farming in these countries is more intense and high frequency deworming with MLs has been practiced out of economic and veterinary feasibility for decades. On the other hand, these countries maintain more extensive veterinary parasitological surveillance, hence detection and publication of resistance is more likely.

Country (in order of first report of resistance)	**Publication**
Netherlands	(Boersema et al. 2002, Van Doorn et al. 2007, Peaty 2008)
Canada	(Hearn and Peregrine 2003, Slocombe et al. 2007)
USA	(Slocombe 2003, Craig et al. 2007, Lyons et al. 2008)
United Kingdom	(Stoneham and Coles 2006, Relf et al. 2014b)
Denmark	(Schougaard and Nielsen 2007)
Germany	(Von Samson-Himmelstjerna et al. 2007)
Brazil	(Molento et al. 2008)
Italy	(Veronesi et al. 2009, Veronesi et al. 2010)
Finland	(Näreaho et al. 2011, Hautala et al. 2019)
Poland	(Basiaga and Kornas 2011, Studzińska et al. 2020)
Turkey	(Cirak et al. 2011)
France	(Laugier et al. 2012, Sallé et al. 2020)
Czech Republic	(Janssen et al. 2013a)
Australia	(Armstrong et al. 2014, Beasley et al. 2015, Wilkes et al. 2017)

New Zealand	(Bishop et al. 2014, Morris et al. 2019)
Estonia	(Lassen and Peltola 2015)
Saudi-Arabia	(Alanazi et al. 2017)
Switzerland	(N. Kettner 2017)
Ethiopia	(Seyoum et al. 2017)
Sweden	(Martin et al. 2018)
Argentina	(Cooper et al. 2020)

Table 1 Publications of macrocyclic lactone resistance in *Parascaris* spp.

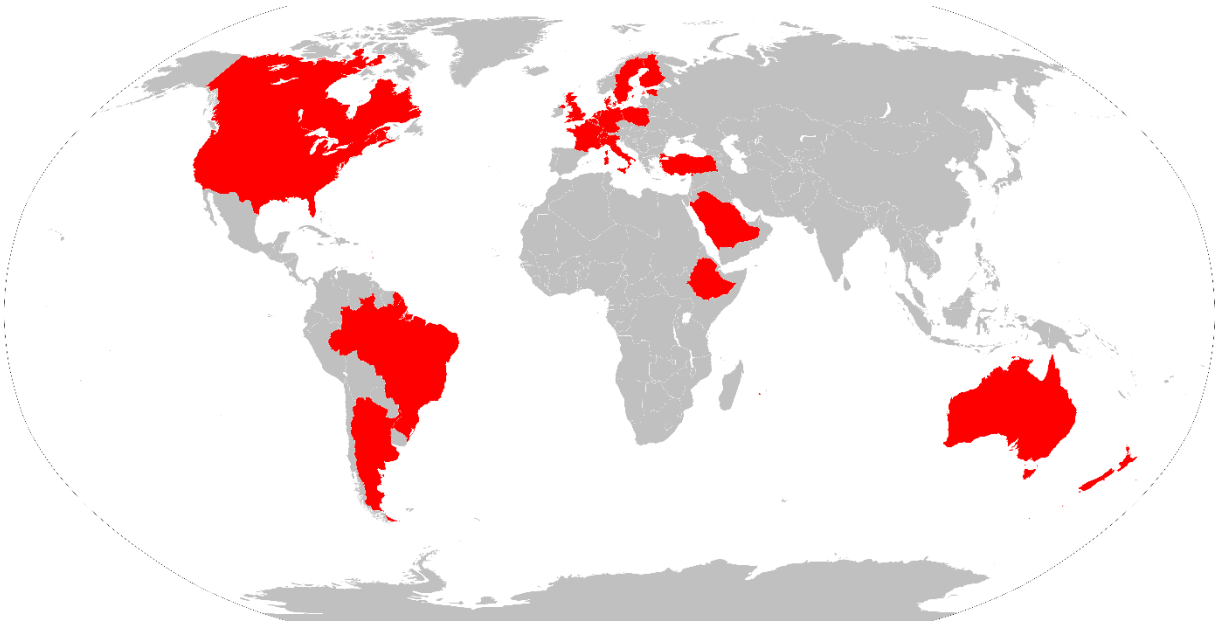

Figure 5 World map showing the geographical distribution of macrocyclic lactone resistance in *Parascaris* spp.

World map https://en.wikipedia.org/wiki/Wikipedia:Blank_maps#/media/File:BlankMap-World.svg accessed 02.04.2020 and modified with Inkscape v.1.0; corresponds to Table 2

2.2.7 Detection of Anthelmintic Resistance

Detection of anthelmintic resistance in a population is of critical importance in managing the spread of anthelmintic resistance and determining which drugs to use (Kotze and Prichard 2016, Esccap 2019).

The detection and diagnosis of anthelmintic resistance can be done either phenotypically or genotypically. Currently, the gold standard is the diagnosis using the FECRT because it can reliably detect the ineffectiveness of a drug inconsiderate of the resistance mechanism. For this reason, it is recommended by academic and clinical experts worldwide for diagnosing anthelmintic resistance in veterinary medicine (Geary et al. 2012, Ema 2016) including equines (Esccap 2019). Quite importantly, the test can be conducted by any veterinarian or other instructed personnel without special training as it simply requires a FEC prior and 14 days after anthelmintic treatment.

Another phenotypic test for resistance is the egg reappearance period test, which is more sensitive than the FECRT to detect emerging drug resistance in some helminth species (Nielsen et al. 2013, Nielsen et al. 2014a). However, it is very time consuming and not suited

for routine diagnostics in veterinary medicine as it requires regular testing of egg counts over a period of time after treatment. In addition, it is only suited for detecting ML resistance.

With growing knowledge of the anthelmintic modes of action and the resistance mechanisms, more specific molecular approaches have been designed which might allow resistance profiling of a specific population in the future. This has the potential to reduce and avoid unnecessary application of anthelmintics and reduce the time until results on the resistance status are available. Also, molecular test can improve sensitivity and eliminate errors from anthelmintic application and the natural variation of egg shedding (Fabiani et al. 2016). The design of such tests is intricately connected to the resistance mechanisms; hence a good understanding of these mechanisms is necessary. Currently, such tests are only available for the detection of BZ resistance as the main resistance mechanism against these drugs is comparatively well understood. Specifically, resistance against BZs results from mutations (SNPs) F167Y (Silvestre and Cabaret 2002), E198A (Ghisi et al. 2007), E198L (Dilks et al. 2020), F200Y (Kwa et al. 1995) and other mutations in beta-tubulins (*ben-1* in *C. elegans*). Hence, PCR or more advanced sequencing approaches such as pyrosequencing (Von Samson-Himmelstjerna et al. 2009) and droplet digital PCR (Baltrušis et al. 2019) can be applied to detect the presence of resistant alleles in a population of parasitic nematodes. Recently, advances in sequencing technology have led to the development of even more sensitive and precise methods to determine species specific allele frequencies (Avramenko et al. 2019, Sargison et al. 2019). Currently the applicability of such approaches remains limited to academia. Noteworthy, while beta-tubulin gene mutations are the main source of resistance, other mechanism are also considered capable of conferring resistance to BZs (Hahnel et al. 2018). Hence, reliance on SNP quantification, like any other molecular test assuming the presence of a specific resistance mechanism, might lead to the false diagnosis of BZ susceptibility.

Furthermore, several *in vitro* assays have been developed to test the susceptibility of adults or larval parasite stages. These tests are limited to academic research as they are comparatively laborious and time consuming. In principle, *in vitro* assays tests whether a shift in the concentration-response curve is present in comparison to a susceptible reference population. The larval development assay tests the ability to develop from L1 to L3 or L4 (only in *C. elegans*) and susceptibility in larval stages (Gill et al. 1995, Demeler 2005). The larval migration inhibition assay examines for the motility phenotype in larval stages (Boisvenue et al. 1983). The egg hatch assay examines embryo susceptibility in the egg and the ability to hatch (Le Jambre 1976). The larval feeding inhibition assay investigates feeding L1 taking up fluorescencely labelled *E. coli* (Álvarez-Sánchez et al. 2005). In general, to conclude with certainty that anthelmintic resistance is present in a population can be challenging as fold changes can vary between the investigated phenotype, species and life cycle stage (Demeler 2005, Matthews et al. 2012).

2.2.8 Overcoming Anthelmintic Resistance

2.2.8.1 *Risk Factors and their Consequences for Management*

As explained in section 2.2.2 drug discovery and marketing of new classes of anthelmintics has halted after the discovery of MLs. Despite the recent marketing of Aminoacetonitrile as Zolvix™ and emodepside e.g. in Profender®, treatment options are extremely limited, especially in horses as for those no new drug class has been marketed during the past decades (Nielsen and Reinemeyer 2018). Particularly problematical, foals are often co-infected by BZ and/or pyrantel resistant strongyles and ML and/or pyrantel resistant *P. univalens* which leaves no anthelmintic to treat both infections (Nielsen 2016).

Therefore, to prolong the lifetime of existing drugs, several approaches have been discussed by the veterinary parasitological community to overcome or at least slow down the development of anthelmintic resistance. There is a consensus that the optimal path includes a combination of several approaches. Furthermore, management and good veterinary practice are critical prerequisites for success. To help decision making for farm management, several studies analyzing risk factors have been published in the last decade, mostly focusing on

resistance in *H. contortus*, but also on equine parasites. In a meta-analysis of such studies focusing on sheep parasites, the most important identified risk factor was high frequency treatment. Other risk factors were also mixed-species grazing, herd size, drench-and-shift pasture management, the use of long-acting drug formulations, a lack of testing for resistance and under dosing (no weighing) (Falzon et al. 2014). Most of these risk factors can be broken down to a few concepts which should be applied in management. These are refugia management (Hodgkinson et al. 2019) by selective and strategic treatment in combination with surveillance of nematode infection loads and regular efficacy testing on farms (Geary et al. 2012, Esccap 2019). The overall aim of these measures is reduction of (unnecessary) treatment which is the main driver for selection of resistance-associated alleles (Rose et al. 2015a) and this concept is described in more detail in section 2.2.8.3. In horses, a risk factor analysis advocated FEC-based treatment regimens for individual determination of anthelmintic dose, and targeted treatment of those animals with high worm burdens and high egg shedding. Furthermore, the study reported that extensive outdoor keeping of horses without or with little rotation were high risk factors promoting the development of resistance significantly (Sallé et al. 2017).

2.2.8.2 *Computer Modelling*

In addition to risk factor analyses, computer modelling allows to examine the impact of treatment practice and other factors such as climate and drug pharmacology on resistance development (Rose Vineer 2020). Pioneers Smith and Grenfell have laid the basis for most models developed in the last decade (Smith and Grenfell 1994). In particular, the GLOWORM project has allowed generation of modified and advanced models based on the Smith and Grenfell models to investigate the impact on the free-living larval stages (e.g the impact of climate) (Rose et al. 2015b) and the parasitic stages (e.g. the impact of pharmacokinetics) (Rose Vineer et al. 2020b) of gastrointestinal nematodes with a particular focus on sheep parasites such as *H. contortus*.

In addition, a series of models have been developed to simulate the development of resistance in *P. univalens* as well as in cyathostomins (Leathwick 2013, Leathwick et al. 2015a, Leathwick et al. 2016, Leathwick et al. 2017, Wang et al. 2018).

Overall, these simulations and models reinforce refugia management strategies as a central outcome (Leathwick et al. 2019), but also warn that the success of refugia management strategies might differ between climatic zones, herd structures and other factors. A few of the available models and simulations allow exploring the effect of different treatment regimens including high or low frequency, rotation and combinational treatments within a single or poly-genic context (Sauermann et al. 2019). Based on such models, it has been suggested to use combination treatment (Scare et al. 2019). However, the potential outcomes and risks of combination treatment are still discussed in the parasitological community (Ema 2016).

A more generally developed computer model indicates that irrespective of the biology of the pathogen, the concentration range between killing and no killing as well as the killing time of a drug are both critically influencing the rate of resistance development (Yu et al. 2018a).

Finally, first attempts are made to establish databases at EU level which would provide a necessary basis of real-world data to utilize the the emerging availability of big data approaches in veterinary parasitology (Rose Vineer et al. 2020a).

2.2.8.3 *Refugia*

The concept of refugia was already introduced in section 2.2.8.1 and 2.2.8.2. In the context of anthelmintic resistance, the refugia refers to those nematodes in a population which are not affected by treatment, hence are not under selection pressure thereby assuring that susceptible alleles are maintained in the population. The refugia includes free-living stages (on the pasture, i.e. eggs and developing larvae), stages not affected by the treatment (e.g. somatic stages when drugs act only intra-intestinal) and worms in non-treated animals, i.e. those identified as individuals with low worm burdens. To maintain refugia, treatment should not be done when the refugia is small, e.g. during draught or winter (Falzon et al. 2014). Hence,

pursuing a refugia based strategy does not aim at pathogen eradication but rather at slowing down resistance development and sustainable parasite management. In general, the refugia concept was already proposed two decades ago (Van Wyk 2001) but has recently seen a steep increase in popularity in the parasitological community as a relevant contributor to combat anthelmintic resistance (Shalaby 2013, Hodgkinson et al. 2019, Leathwick et al. 2019). The concept was originally developed for pest control in agriculture where it is still applied (Jin et al. 2015).

As part of the concept of refugia, selection pressure on parasites stems not only from human intervention but from many sources most importantly the parasites ecological environment, i.e. the host immune system, climate (Sallé et al. 2019) and competitors for the same ecological niche both inside and outside of the host as well as the availability of food. In addition, each individual organism must allocate its resources to different processes e.g. in case of parasites to reproduction, environmental resilience and host immune evasion and modulation. Evolutionary theory would predict that drug resistance bears fitness costs either from altered resource allocation (e.g. overexpression of resistance associated genes) or by reduced or diminished gene function (e.g. mutants). For this reason, given enough time with substantially reduced selection pressure, it might be possible to re-establish susceptibility by restoring the original genetic composition in a population. In fact, one study reported the reestablishment of susceptibility against levamisole and IVM in *T. circumcincta* as a result of strict resistance management programs (Leathwick et al. 2015b). However, the F200Y allele associated with resistance against BZs does not seem to have a fitness cost in *C. elegans* (Hahnel et al. 2018). If this is true for parasites, to restore the initial genetic state will take much longer and would not be sustainable as resistance alleles would be easily re-selected. It can be speculated that in *P. univalens*, SNPs conferring resistance to BZs bear a higher fitness cost which could explain the slow-paced development of resistance. In contrast, compared to BZ resistance, ML resistance development in most nematodes happened at a much slower pace and *a priori,* it can be speculated that in those species ML resistance bears a higher fitness cost. Alternatively, the multi-genic nature of ML resistance might explain this difference in development pace.

In order to manage this evolutionary process successfully, many factors need to be taken into account of which several are closely related to the parasite's biology (section 2.2.8.1 and 2.2.8.2).

2.2.8.4 *Other Strategies to Overcome Anthelmintic Resistance*

If a specific resistance mechanism is known, it is also possible to develop targeted countermeasures. For example, if resistance would be conferred by a transporter protein (e.g. Pgp), inhibition of Pgps might allow reversal of the resistance and extension of the lifetime of a drug. In antibiotic resistance, such approaches were temporarily successful against resistant bacteria e.g. inhibitors against beta-lactamase (a bacterial enzyme deactivating beta-lactam antibiotics such as penicillin). However, even then resistance mechanisms evolved against the inhibitory drug (inhibitor-resistant β-lactamases) (Chaïbi et al. 1999). For pesticide resistance, piperonyl butoxide can be used to increase the efficacy of carbamates, pyrethrins and pyrethroids (Tozzi 1999) and it has been experimentally used as a synergist to anthelmintics as well (Kotze et al. 2006). However, piperonyl butoxide is not used *in vivo*, but only as a pestizide, possibly due to the safety concerns which would also be relevant for inhibitors of Pgp.

A more sustainable approach to parasitic nematode control would be vaccination. However, currently vaccination development appears to be a major challenge in eukaryote pathogens with only a few successful examples e.g. a tick vaccine (Willadsen et al. 1995) which in many cases elicits only limited protection and can require several boosts. Despite immense investments, an impactful *Plasmodium* spp. vaccine has not yet been developed (Draper et al. 2018). Against nematodes, only few vaccines were so far developed and marketed: Barbervax® (Wormvax Australia Pty Ltd.) to prevent haemonchosis, a natural gut-antigen and Bovilis Huskvac® (MSD Animal Health), a live attenuated vaccine for bovine lungworm control.

Both vaccines are relatively expensive, require multiple applications and isolation of adult or larval worms to prepare the vaccine, hence limiting their applicability. Since decades, extensive efforts to develop recombinant vaccines have not yielded any well-performing candidates (Matthews et al. 2016, González-Sánchez et al. 2018), although for the tapeworm *Echinococcus granulosus* such a vaccine has even recently been marketed: Providean Hidatil EG95® (Tecnovax). The main reason for these obstacles lies within the natural immune response. For example, there is high variability in the immune response between individuals and a generally less efficient induction of an immune memory compared to other diseases of bacterial or viral origin. For many nematodes, with age a protective immunity develops which protects in particular from severe infections, e.g. in *Parascaris* spp. In addition, infection with nematodes can lead to immunity upon re-infection and indeed, the potential of immunity has fueled vaccine research (Yasuda et al. 2018). However, currently a breakthrough in vaccine research appears rather unlikely due to the intricacy of the host-parasite relationship and the ability of many nematodes to modulate the host immune system (Matthews et al. 2016).

Likewise, plant extracts containing many different active compounds are discussed as a more sustainable intervention to nematode control (see section 2.2.5). However, it is possible that such approaches drive selection pressure to unspecific general resistance mechanisms which provide protection against a broad range of substrates.

Several biological control agents are also studied as potential alternative treatment approaches. Most famously the nematophagous fungi (Braga and De Araújo 2014, Luns et al. 2018). Recently, the nematode pathogenic bacterium *Chryseobacterium nematophagum* has also been discussed as a potential agent for biological control (Page et al. 2019). However, such strategies have been the subject of controversy and have had tremendous impact on local ecosystems. For example, the introduction of the cane toad to control the cane beetle led to the endemic spread of the cane toad and extinction of other species (Burnett 1997). Likewise, the rosy wolf-snail was introduced to control another invasive snail species, the giant African land snail, but instead drove many other snail species in Hawaii to extinction (Curry and Yeung 2013).

Even more controversial than biological control, environmental engineering through gene drives have been proposed for pest control which originally focussed on the control of *Anopheles gambiae*, the malaria vector (Kyrou et al. 2018). These genetic drives spread infertility genes through a population and thereby reduce the spread and reproductive rate (Champer et al. 2016). In principal, these drives should be selected against over time but there is a concern that once unleashed, these engineered systems might lead to the extinction of the target species, or worse, of other non-target species as well. Arguably, they are considerably more save and more specifically targeted at the pest species than the current approaches using indiscriminate chemical intervention (Oye et al. 2014, Esvelt and Gemmell 2017). Because of the population size and structure of parasitic nematodes in farm animals, which is usually rather large and mostly restricted to a farm, parasitic nematodes might be a good target for gene drives. However, the active trade of livestock is a driver of parasite spread, e.g. of resistant populations (Sallé et al. 2019).

Finally, parasite resilient breeds of domesticated animals are an intuitively sustainable appraoche for farming but many other factors influence breeder choices. Notably, parasite resilient and resistant breeds elective breeding has indeed been performed with success (Mcmanus et al. 2014). In addition, current novel NGS might allow insights into the genetics of resistance to nematode parasites and could help guide breeders make better choices (Sweeney et al. 2016, Al Kalaldeh et al. 2019). For many food-producing animals, breeding has focused on extreme productivity i.e. high milk production or increased reproduction at the cost of disease susceptibility. In the equine industry, focus has been put on other factors such as speed or endurance. In the future, less intensive farming, smart breeding of more animals more resistant to disease might resolve current issues in the combination with a sophisticated vaccine. Notably, as more resilient breeds are less efficient for food production, this will need to be accompanied by a reduced intake of animal products.

For now, strategies to improve the combat against anthelmintic resistance will need to aim at prolonging the life of available drugs. For this, it is essential to know the resistance mechanism. As also discussed in section 2.2.7, this will facilitate the development of more sensitive and less time intensive (molecular) diagnostic approaches as well as of targeted counter measures.

2.3 Mechanisms of Macrocyclic Lactone Resistance

2.3.1 Insights from Next-Generation Sequencing

Despite considerable efforts to elucidate the ML resistance mechanism our current understanding remains superficial. Recently, NGS approaches have allowed substantial advancement in the understanding of the evolutionary mechanisms leading to anthelmintic resistance and have led to the identification of novel candidate genes and novel potential pathways to resistance. At the current state of technology, genome-wide and transcriptome analyses represent the most promising comprehensive starting point to identify lead candidate genes (Doyle and Cotton 2019). The main strategy is to compare susceptible and resistant populations. Despite the technological advancements, studies applying NGS to elucidate resistance mechanisms in nematodes are rare. Currently, initiatives for nematode parasite research such as the “Building Upon the Genome” consortium (Bug Consortium 2013), the “50 Helminth Genomes Initiative” (Wellcome Trust Sanger Institute 2014) and platforms such as “WormBase Parasite” (Bolt et al. 2018) are built and expanded to make these resources more easily available and accessible to researchers without a bio-informatic background.

In addition to genomics and transcriptomics, specialized NGS approaches also allow insights into other biological processes, such as from single-cell sequencing, methylome sequencing (not relevant for nematodes) or small RNA sequencing but these technologies have so far not been applied to anthelmintic resistance except for one exception which have connected microRNA expression and anthelmintic resistance (Gillan et al. 2017). However, some of these technologies have already been applied to other questions in nematology (Cao et al. 2017, Marks et al. 2019).

2.3.1.1 *Genomics*

The genome is the complete set of DNA of an organism and NGS technology has allowed high-resolution insight into the genetic make-up of many different species. Some currently relevant sequencing technologies are outlined in section 2.3.1.2.

The identification of relevant genes for ML resistance in parasitic nematodes can be done by identifying genomic loci with signatures of selection. As a first step a reliable reference genome must be assembled which requires among other factors deep sequencing depth and high coverage but this process will not be discussed in detail here. The bioinformatic pipelines for whole genome *de novo* assembly and annotation are subject of constant reconstruction and refinement (Khan et al. 2018). Currently, the development of third generation sequencing methods such as Oxford Nanopore Technologies and “Single Molecule, Real-Time Sequencing” (PacBio®) which allow much longer reads than sequencing by synthesis (see also section 2.3.1.2) have resulted in a paradigm shift (Sohn and Nam 2018). Following reference genome assembly, population genomics and evolutionary modelling can give insight whether a locus is under selection in a resistant population.

Problematically, as a result of the genetic diversity in different populations correlating phenotypes with signatures of selection is considered error prone (Doyle et al. 2019b). As a solution to this problem, studies in *H. contortus* and *T. circumcincta* utilized back-crossing of resistant isolates under constant anthelmintic selection pressure into a susceptible phenotype to reduce the background and purify resistance loci. This process is the repeated cross over many generations of worms experimentally selected for resistance with worms from the same susceptible population which has not been kept under selection with an anthelmintic. The expected result is that the resistance alleles are introgressed into the susceptible genomic background which can be monitored after every cross. The aim is to purify the resistance alleles and the prevention of false positive association of genotypes with resistance. Currently,

the main statistic to identify a locus under selection is the fixation index (F_{st}) which is a measure of genetic differentiation. It can be calculated by mapping SNPs from two populations onto a reference genome and for each SNP position, a value of 0 indicates no differences between two population (unimpeded interbreeding) while a value of 1 indicates complete differentiation. Usually, F_{st} is calculated for a certain region of several kilo base pairs (kbp) windows for pairwise comparisons of isolates to improve the confidence that selection is actually present at a locus (Nagamine et al. 2012). Another measure to identify candidate genes is copy number variation (CNV), however, it is often difficult to be determined reliably from NGS data and depends on the quality of the genome assembly. Assigning a read (e.g. for Illumina sequencing 50, 150 or 250 bp) to a specific genomic locus can be near to impossible if two or more loci share an identical genetic sequence but there are more specialized tools to assess CNV, e.g. microarray-based comparative genomic hybridization or nanopore sequencing (producing long reads).

2.3.1.2 *Transcriptomics*

The transcriptome represents the total of all messenger RNA molecules expressed by an organism. Transcriptome assemblies and differential gene expression analyses from RNA-sequencing (RNA-Seq) data offer insight into the transcript sequences and abundance as well as their regulation (Wang et al. 2009). Comparisons between resistant and susceptible isolates can then reveal differences with regard to splicing, constitutive expression levels or upregulation upon drug exposure of individual genes. RNA-sequencing can answer many questions on the transcriptome and there are many specialized applications. For example, RNA-Seq can be performed on tissues obtained from manual dissection of worms and can be even done even single-cell specific (Cao et al. 2017).

In principle, the workflow can be divided in several steps and is exemplarily outlined for a research question on anthelmintic resistance in a parasitic nematode. The workflow is similar for DNA and RNA-Seq but there are some differences. For sample worms need to be collected either from slaughtered of sacrificed infected animals (adults) or from larval culture from feces of infected animals, which allows larvae to hatch and develop to third or fourth stage (larval stages). If worms are obtained from slaughtered animals, their viability needs to be supported by suited conditions (Scare et al. 2020) during which worms can be exposed to drug. Library preparation depends on the RNA-Seq method which in turn should be chosen specifically to answer the question. In this step, RNA is extracted, abundant transcripts are removed (e.g. ribosomal RNA which represent the most abundant RNA molecules and they are usually not of interest), fragmented, transcribed to cDNA and finally repaired at the end, dA-tailled and ligated to adaptors (Illumina Inc. 2020, New England Biolabs Inc. 2020). For DNA sequencing, library preparation contains different steps.

There are many different methods for sequencing of DNA (or cDNA) or directly of RNA (e.g. Oxford Nanopore Technologies and PacBio® Single Molecule, Real-Time Sequencing). The most commonly used sequencing instruments are produced by Illumina Inc. and perform sequencing by synthesis using reversible dye terminators (Reuter et al. 2015). These are referred to as NGS or second generation sequencing. More recently, technologies such as nanopore sequencing sometimes referred to as third generation sequencing are in development. These technologies overcome problems such as the rather short read length and a bias by PCR preparation to amplify a library (Ambardar et al. 2016). After DNA sequencing, data analysis represents the most time-consuming and arguably most difficult task. For data analysis, many commercial (e.g. from Geneious) and open-source algorithms and software tools are available to answer a broad range of question. For RNA-Seq, a common first step is the assembly of a transcriptome, which includes all sequence variants for each transcript. This process can be rather difficult, however available algorithms and data analysis software have improved considerably in the last decade. These algorithms map and cluster the raw-read data and assemble contigs as the largest assembled unit. A high-quality assembly will have a low number of contigs. Transcript sequences can then be detected from the assembled contig sequences. If a high-quality reference genome exists, the assembly process is considerably less complicated and less reliant on the quality of sequences and

sequencing depth. However, if instead a reference genome of a closely related species or geographically distant population is used, assembly is biased towards already existing contigs (Huang et al. 2016) but it can be beneficial to combine it with a *de novo* assembly. In general, assembly of transcriptomes and genomes is naturally error prone as it is based on high-throughput sequencing data which in turn stems from a method with an inherent error rate of 0.1 (Fox et al. 2014) to 0.24% (Pfeiffer et al. 2018) for sequencing by synthesis. Based on data from Illumina RNA-Seq, it was shown that overall error rate in assemblies is between 1-3% and increases from the 5'-end towards the 3'-end (Liu et al. 2012). Most of these errors are substitutions or deletions (Indels) (Yang et al. 2013). Third generation sequencing such as nanopore sequencing, which can generate much longer reads but have a higher error rate, might be a good solution in the future to even further improve *de novo* assembly by providing a mapping template for low-error rate short reads from sequencing by synthesis.

Probably the most common analysis based on RNA-Seq raw data is the estimation of gene expression of individual transcripts to compare expression between different samples. Two very common statistics are fragments per kilobase of transcript per million mapped reads (FPKM) or transcripts per million (TPM) and many tools (e.g. for use in R or python) are available and are continuously improved to calculate these statistics and visualize them. FPKM is calculated as

$FPKM_g = \frac{r_g * 10^9}{gene\ length\ in\ bp * total\ number\ of\ reads\ in\ a\ sequncing\ run\ (per\ sample)}$ where *g* defines a specific gene region and r_g is the number of reads (fragments in case of paired-end data) mapped to a particular gene region. Hence FPKM normalizes for the two main technical biases, sequencing depth and transcript length. In contrast TPM is calculated as

$TPM = \frac{r_g * rl * 10^6}{gene\ length\ in\ bp * T}$ where $T = \sum_{g \in G} \frac{r_g * rl}{gene\ length\ in\ bp}$, *g* defines a specific gene region, r_g is the number of fragments/reads mapped to a particular gene region and *rl* is the fragment/read length. Simplified, instead of normalizing for the total number of reads in a sample, TPM normalizes for the total number of transcripts in a sample and it was shown that thereby TPM eliminates a statistical bias in FPKM which on average inflates statistical significance on transcript abundance differences between samples (Wagner et al. 2012).

Another common RNA-Seq analysis step is the combination with genomic resources to allow annotation of coding regions on the genome. In addition, the combination with genomic resources can help elucidate alternative splicing of genes, fusions and other post-transcriptional modifications.

2.3.1.3 *Candidate Genes and Multigenicity*

Prior to NGS, research was focused mainly on a few candidate gene groups, meaning genes which were identified by forward genetic screens, chosen for analysis out of rational reasons or based on a hypothesis. These were i) the target of MLs, the GluCls, ii) Cyt-P450 metabolizing enzymes, iii) ABC transporters, particularly the Pgps (*ABCB* genes) and iv) genes causing the dyf phenotype which were associated with amphidal defects. The early forward genetic screens using the vast *C. elegans* mutant libraries identified primarily GluCl deletions (*avr-14*, *glc-1* and *glc-2* deletions) and secondarily dyf-phenotype genes deletions (Dent et al. 2000). Although experimental deletion of GluCls leads to strong reduction in susceptibility in the model nematode *C. elegans*, GluCl or dyf-associated gene deletions in wild parasite populations could so far not be detected and linked to resistance. This could be due to the broad target range of MLs and the genetic diversity of target receptors (see section 2.2.3.1) or as particularly GluCl deletions have a high fitness cost. One of the reasons why the P-glycoproteins have emerged as one of the major candidate genes is that they play a central role in blood-brain barrier function against MLs (see section 2.2.3.3), hence it was known that ML derivatives were substrates of these transporters. In addition, evidence is accumulating for a contribution to the resistance mechanism in nematodes (see section 2.3.3.2).

In the last decade, the increasing availability of NGS approaches has impacted the understanding of ML resistance considerably. Although it had already been assumed because

previous experimental findings could not fully explain or be linked to the high level of ML resistance seen in field isolates, comprehensive evidence for multigenicity was provided in *T. circumcincta* (Choi et al. 2017) and *H. contortus* (Luo et al. 2017, Khan et al. 2019, Khan et al. 2020). In these studies, neuronal genes including voltage gated, ligand-gated, acetylcholine-gated (*acc-2*), serotonin-gated, GluCl (*glc-3, α*-subunit), GABA-like and G-protein-coupled receptors as well as vesicle-mediated (e.g. *cni-1*) and ion-transmembrane transport genes exhibited elevated F_{st} values in resistant (in case of Choi et al. introgressed into a susceptible background) compared to susceptible isolates. Also, loci containing genes involved with lipid metabolism and transcriptional regulators (in particular nuclear hormone receptor (NHR) genes showed signs of selection. Finally, loci with elevated F_{st} values also included classical candidate genes from the xenobiotic metabolism including several ABC-transporters (i.e. *pgp-10* in *H. contortus* and *pgp-9* in *T. circumcinta* study) and Cyt-p450 genes (*cyp-33C1* in *H. contortus*). In the *T. circumcinta* study, *pgp-9* exhibited higher CNV and based on RNA-Seq data also a higher expression level.

Results from two different independent studies on *H. contortus* conflicted with these findings and in contrast rather pointed towards one major IVM resistance quantitative trait locus (QTL) (Rezansoff et al. 2016). This locus contained 360 genes on 5 Mb but none of the classical candidate genes. Based on this, a more detailed study in collaboration with the Sanger Institute confirmed the same QTL for the two genetically and geographically distinct populations back-crossed into the same susceptible back-ground under selection pressure of IVM (Doyle et al. 2019b). Here, candidate genes including *glc-3* and *avr-15* as well as *pgp-9* and *pgp-3* and other candidate genes were located on the very edge of the genomic region showing only weak signatures of selection and were excluded as the contributors to resistance in these isolates. The causal genetic element within the QTL, to this date, in these isolates is unknown. In an extension of this study presented at the WAAVP 2019 conference, several minor QTL were also identified in these isolates, hence the result indicated one major resistance allele but could not exclude multigenicity (Doyle et al. 2019a). A mapping of differentially expressed genes from transcriptome analyses in this back-cross study revealed enriched gene ontology (GO) terms for regulation of neuronal differentiation in males and contractile fiber in females (Laing et al. 2019). The authors also proposed a few potential candidate genes, among them kinases and genes involved with neuronal regulation and behavioral plasticity.

This conflicting evidence suggests that different nematode populations and species can evolve unique ML resistance mechanisms.

2.3.2 Forward and Reverse Genetics: The Importance of Genetic-Functional Validation

While NGS approaches can provide evidence whether a gene is under selection and thereby navigate candidate gene choice, functional genotype-phenotype analyses are essential to understand how a gene contributes to resistance and to experimentally verify the indications provided by NGS. Despite sophisticated statistical methods, genome-wide association studies (GWAS) can lead to false positive results (Shen and Carlborg 2013, Biedrzycki 2018, Rohde et al. 2018). For instance, in the case of anthelmintic resistance, an identified locus might actually contain a gene selected from another causal variant which has changed in the last decades due to selection by e.g. changes in the climate, animal farming systems or changes in the breed and hence the changed immunity of the available host animals in a particular region. Hence, in addition to deciphering the details of gene function, forward and reverse genetics allow to identify false positives from GWAS. While back-crossing (see section 2.3.1.1) appears to be a reliable strategy to reduce error rates compared to conventional GWAS, functional validation and characterization of candidate genes remains indispensable to understand the resistance mechanism.

Exemplarily, a transcription factor was found at a locus exhibiting strong evidence for selection (Khan et al. 2020). While this might represent a promising lead, it remains unclear which genes are regulated by this transcription factor and how the regulated genes contribute to resistance. Complementary transcriptome analyses could give a first lead to answer the first question, but

leave uncertainty with regard to which differentially expressed genes contribute to resistance and which are a biproduct. Without forward or reverse genetics, it is difficult to link the transcription factor to the regulated genes. In the case of the identified putative ML resistance locus in *H. contortus* on chromosome V (Doyle et al. 2019b), the relevant genes still need to be identified and validated but even transcriptome analyses could not identify a clear drug specific response (Laing et al. 2019). A broad range of components which contribute to a general stress response which help survival nonetheless might conceal essential drug specific response genes. In the end, all novel identified candidates will need to be rigorously characterized functionally to validate an interaction with the ML and to elucidate the mechanism by which they contribute to resistance. This functional characterization is usually conducted by examining phenotype-genotype relationship, e.g. by examining the phenotype of a gene-deletion strain obtained from mutagenesis (forward genetics), targeted gene silencing or phenotype analyses of transgenic strains (reverse genetics). If the candidate gene was found to be a major contributor to resistance in a species at the functional and genetic level, a specific counter strategy e.g. co-administration of an inhibitor could be devised.

For effective forward and reverse genetics, it is necessary to alter the genomic or transcriptomic information specific to a gene and to have a reliable read-out phenotype. The read-out phenotype is intricately linked to gene function and drug mode of action, hence choosing the right assay is essential. In order to modify gene expression, the biotechnological revolution in the last decades has led to the availability of a broad range of methods for genetic manipulation, including RNAi and genetic engineering. Some of these methods are available in parasitic nematodes which are outlined below. In addition, diverse genetic modification methods as well as diverse range of assays are well established in the model nematode *C. elegans* and a large community of researchers contribute to the *C. elegans* toolbox (Wormbook 2005-2020, Nance and Frøkjær-Jensen 2019, Harris et al. 2020, Wormatlas 2020). These have allowed genome-wide forward genetic screens to elucidate the gene function for a large number of phenotypes in many aspects of biology.

2.3.2.1 *Gene silencing*

Gene silencing is the interference with transcription or translation and usually results in an incomplete reduction of the overall gene expression. The most common tool for gene silencing is RNA interference (RNAi). It is well established in *C. elegans* using feeding of bacteria expressing double-stranded RNA for candidate gene studies and high-throughput approaches which have been continuously refined (Fraser et al. 2000, Kamath and Ahringer 2003, Jagadeesan and Hakkim 2018). These high-throughput approaches have played a major role in annotating genes and deciphering gene function. In fact, the first discovery of the mechanism of RNAi by Mello and Fire, which was rewarded with a Nobel Prize in physiology and medicine in 2006, was made in *C. elegans* (Fire et al. 1998). Since the discovery of this physiological gene regulatory pathway in which, extremely simplified, a small RNA molecule (~20-25 bp) binds a mRNA and neutralizes it, gene silencing has been developed as a tool for reverse genetics. Currently, it has also been developed as a treatment against infectious diseases, cancer and genetic diseases (Berkhout 2004, Weng et al. 2019) of which some are in clinical phase I and II or already on the market (Onpattro, Alnylam Pharceuticals). To date, many different small RNA molecules have been discovered and the general RNAi pathway was found to be conserved in all eukaryotes including mammals (Cerutti and Casas-Mollano 2006).

In addition to *C. elegans*, the method has been described in the parasitic nematode *A. suum* (Mccoy et al. 2015) and *H. contortus* (He et al. 2020, Khan et al. 2020, Naqvi et al. 2020).

2.3.2.2 *CRISPR/Cas9 and transgenesis*

Many different approaches for transgenesis have been developed. As a common feature, a foreign transgene is delivered usually as a plasmid into the cell to be expressed under the regulation of a promotor and untranslated region (UTR) of the receiving host organism. For transgenesis of multicellular organisms, the germline optimally at the unicellular stage should be targeted to assure that all cells and offspring carry the same modification. A genetic modification can either result in a gene deletion or transgene expression while transgenes can

be either permanent (through genomic integration) or transient (no genomic integration or RNA based). For gene delivery, many different options have been developed depending on the model organism. Gene delivery in unicellular organism is referred to as transformation and can be achieved for example by heat shocking (common in *E. coli*), electroporation (an electric impulse resulting in micropores in the membrane) or chemical permeabilization such as the lithium acetate method in yeast (Gietz and Woods 2002). In *C. elegans*, gene delivery can be achieved by feeding worms on bacteria transformed with the plasmid of choice or, more commonly by microinjection (Evans (Ed.) 2006). Alternatively, microparticle bombardement can be used but this method is used less commonly than microinjection (Evans (Ed.) 2006). The latter method can be used to deliver plasmids, double tranded RNA (for RNAi) or proteins such as Cas9. In principle, a small volume of the injection mixture is microinjected into the gonads of one day adult hermaphrodite. Then, the offspring can be examined by PCR or by the presence of a co-expression marker. Commonly such markers are pharyngeal GFP expression or a gene resulting in a visible movement change, e.g. the roller phenotype (Cox et al. 1980).

Following RNAi, CRISPR/Cas9 represents the next major breakthrough in biotechnology. Initially discovered as an adaptive immune response in prokaryotes (Barrangou et al. 2007), it was later shown that the mechanism could be used to design a powerful gene editing method (Deltcheva et al. 2011, Gasiunas et al. 2012, Jinek et al. 2012). In prokaryotes, the interplay between CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) sequences and the enzymes from the Cas (CRISPR-associated) family result in immune memory. In brief, the detection of viral DNA is followed by their integration into the bacterial genome at a locus marked by the CRISPR sequences (Terns and Terns 2011). Upon re-encounter of the viral DNA, the expression of the integrated DNA as crRNAs (Deltcheva et al. 2011) facilitates the binding and ultimately cleavage of the viral DNA through the formation of a crRNA-Cas9 complex. Currently the endonuclease enzyme which is most famous and most widely used in gene-editing is *Streptococcus pyogenes* Cas9 but the *cas* gene repertoire in prokaryotes is immense (Makarova et al. 2011). This cleavage process which involves many genes in prokaryotes has been adapted and simplified to be used with incredible flexibility in many different organisms to target any genome sequence (Jinek et al. 2012, Cong et al. 2013, Mali et al. 2013). In principle, the CRISPR/Cas9 system uses either a single-guide RNA which contains both a Cas9 binding site as well as the complementary genomic target sequence or a hybrid between a target guiding crRNA and a trans-activated crRNA for Cas9 binding and cleavage. These guide RNAs can be specifically synthesized to target a specific locus, which is only limited by the availability of a protospacer adjacent motif (PAM) (in case of *Spy*Cas9 5'-NGG-3'). If administered with synthetical Cas9 or with a plasmid encoding Cas9, the targeted genomic locus will be cut with high efficiency and precisely at the target sequence site.

In *C. elegans*, this system is highly reliable to insert mutations either by error prone end joining (faulted repair at the cleavage site) or by homologous recombination using a short DNA fragment complementary to the target site containing the desired change in DNA as repair templates. The insertion of transgenes is also possible with CRISPR/Cas9. Introduction of short fragments into the *C. elegans* genome is also possible with a relatively high efficiency (Paix et al. 2015), however introduction of fragments longer >2kbp is much more difficult and requires considerable screening (Dickinson et al. 2015). Both small and large insertions rely on homologous recombination with a large template.

As RNAi only reduces gene expression to an amount which can vary and is therefore unreliable, the *Caenorhabditis elegans* Gene Knockout Consortium aims to improve the accuracy of actual genotype-phenotype relationships by using CRISPR/Cas9 to comprehensively re-analyze all genes on the *C. elegans* genome (Au et al. 2019).

2.3.2.3 *Model organisms*

As mentioned previously, the approach of reverse genetics is to express or knockout a gene of choice to analyze the phenotype. For this, a wide range of different model organisms are established and choosing the best model system depends on the research question, the

experimental set-up and the expected phenotype. To express nematode genes transgenically, e.g. nematode Pgps, it is necessary that the transgene is correctly transcribed to an mRNA which is then transported out of the nucleus and that the translated protein undergoes the correct post-translational modifications. For example, to my best knowledge there are no reports of mammalian Pgps expressed in prokaryotes but in several other eukaryote expression systems which might be attributed to the inability to express these genes in a prokaryote system. Below, a few of the common model systems used to decipher anthelmintic resistance mechanisms and specifically the role of Pgps is introduced.

2.3.2.3.1 Yeast

Many different yeast species are utilized for numerous biotechnological applications and research questions. The budding yeast *Saccharomyces cerevisiae*, one of the most commonly used yeast species, has a wide application range in fermentation in alcohol production and bakery and has been widely used in research to study metabolism, biochemistry, genetics and other research questions (Mattanovich et al. 2014). For this reason, the *S. cerevisiase* genome was the first eukaryote genome completely sequenced and the genome sequence has since been refined and annotated comprehensively (Engel et al. 2014). As it is a single-cell organism with a short generation time (Herskowitz 1988), low maintenance costs and easy culturing at 30°C (Salari and Salari 2017), it is an excellent model organism. Furthermore, a large toolbox for genetic manipulation including cutting edge technology such as CRISPR/Cas9 is available as well as many specialized protocols for handling and processing. Pgps as candidate genes have been characterized in this model organism (see section 2.3.3.3).

2.3.2.3.2 Cell culture

Cell culture allows the growth of eukaryote (and in particular mammalian) cells under controlled conditions and most cell lines are immortalized, monoclonal cell lines. They are used in research to answer a wide range of questions. In the last two decades, primary cell culture, organoid and tissue cell culture have been developed and refined which are considered superior as they are closer to a physiological situation. Nonetheless, their rather easy and cost-effective culture retains the usefulness of cell lines as well as the rather large toolbox for genetic manipulation. In nematodes, primary cell cultures of *C. elegans* cells from different stages have been established for example for single-cell profiling and electrophysiology (Zhang and Kuhn 2013).

Both, nematode and mammalian Pgps as candidate genes have been characterized using the cell line LLC-PK1 (a pig kidney epithelial cell line with low endogenous Pgp expression) (see section 2.3.3.3).

2.3.2.3.3 Caenorhabditis elegans

As outlined in section 2.1.4, *C. elegans* is an important model organism and has played a central role in understanding anthelmintic drug mode of action, novel drug discovery (Burns et al. 2015) and anthelmintic resistance. As mentioned previously in section 2.3.2, a large community of researchers contribute to the *C. elegans* resources and toolbox (Wormbook 2005-2020, Nance and Frøkjær-Jensen 2019, Harris et al. 2020, Wormatlas 2020). In addition to the previously mentioned resource for globally collected strains (CeNDR) (Cook et al. 2016), an immense repertoire of strains of single or multiple gene deletions for most genes by the National BioResource Project (Japan) (Yamazaki et al. 2010) and the Caenorhabditis Genetics Center (CGC, University of Minnesota, USA) is available. Furthermore, specifically tailored protocols for cutting-edge technology such as genetic manipulation (e.g. CRISPR/Cas9) and single-cell sequencing as well as a large number of established assays and corresponding protocols are freely accessible (Wormbook 2005-2020). More recently, automated systems enable large-scale drug discovery, high-throughput genotype-phenotype analyses (Andersen et al. 2015, Partridge et al. 2018, Rodríguez-Palero et al. 2018, Atakan et al. 2019, Au et al. 2019, Daniels et al. 2019), automated genetic manipulation (Song et al. 2016) as well as a more detailed analysis of specific behavior and phenotypes.

In addition to CRISPR/Cas9 gene editing, transgene overexpression from extra-chromosomal arrays of transgenes can be easily induced in *C. elegans* by gonadal injection of a plasmids containing the transgene under regulation of a *C. elegans* or parasite promotor. This technique represents another effective and time-efficient method to allow the characterization of the interrelation of genotypes and phenotypes of parasite-derived transgenes. Extrachromosomal arrays are repetitive sequences formed from the injected plasmids which have undergone homologous recombination (Mello et al. 1991) and are usually high molecular weight DNA arrays (Stinchcomb et al. 1985). This process occurs with surprisingly high efficiency regardless of plasmid linearization so that transgenic worms can be obtained rather easily. In particular, sharing a common sequence element, such as the backbone of plasmids makes this process even more efficient (Mello and Fire 1995). Within a transgenic line, the transgene will be passed on to a proportion of the offspring with varying efficiency of up to 80 % but usually around half of the offspring. The transmission rate is influenced positively by the array size (Mello et al. 1991). As many promotors are well described, expression can be directed to a specific stage or tissue (Riddle DI 1997).

For anthelmintic resistance research, wild-type (James and Davey 2009, Laing et al. 2012, Ardelli and Prichard 2013), transgenic (extrachromosomal array) (Janssen et al. 2015) and knock-out *C. elegans* strains (Bourguinat et al. 2011a, Janssen et al. 2013d) have been used to examine ML resistance candidate genes (see section 2.3.3.3). Likewise, expression of parasite genes such as neuronal receptors can be directly used to rescue gene function such as an increased susceptibility phenotype (Glendinning et al. 2011, Blanchard et al. 2018, Charvet et al. 2018). To analyze resistance phenotypes in *C. elegans*, many different methods are available. The proper read-out phenotype depends on the effect of the drug. For MLs, the inhibition of pharyngeal and body wall muscle are considered the main effect. Thus, motility scores offer a robust phenotype. Common motility assays are the thrashing assay, which measures the number of body bends in liquid media, or the body bend assay on agar plates (Nawa and Matsuoka 2012). Using the latter, it is more difficult to detect a statistically significant marginal reduction of susceptibility as body bends are several folds lower on agar plates and less reliable. In general, availability of food should be avoided during motility scoring as worm will seize to move in the presence of food. More recently, products by InVivo Biosystems (formerly NemaMetrix Inc.) such as the ScreenChip™ system for electrophysiological recordings of pharyngeal pumping, the NemaImager and the wMicroTracker for locomotor activity allow a considerably more delicate and reliable phenotype analysis. Like motility, inhibition of pharyngeal pumping can also be used as a read-out of ML effect (Ardelli et al. 2009, Weeks et al. 2018b). For these phenotypes, state-of-the-art automated approaches should be favored as they are considerably less time-consuming and independent of the experimenter's experience. In addition, larval development assays are a common tool to detect resistance phenotypes in *C. elegans* (Ardelli et al. 2009, Ardelli and Prichard 2013, Janssen et al. 2013d) as well as in parasitic nematodes (Gill et al. 1995). As a disadvantage, larval development assay is not suited to analyze the role of genes in adult nematodes as they require the gene of interest to be expressed in the larval stages, transgenically or in the WT (for gene knockout). For this assay, synchronized L1 are incubated over a certain period and then development is scored compared to a no drug control. Usually, development is only scored as the expected development within a time frame, for example commonly L4 and adult are counted as "fully developed" while L1 to L3 are counted as not fully developed. Unfortunately, the generated data from this assay does not allow a discrimination between completely inhibited development and different nuances of delayed development unless counting each stage separately. Furthermore, several additional assays have been developed, such as the larval motility inhibition assay/test and the larval feeding inhibition assay.

2.3.2.3.4 Other model organisms

Several other model organisms exist which are used for anthelmintic resistance research, most notably the *Xenopus* system for electrophysiological investigation of neuronal receptors (see section 2.2.3.1). While in biology most research focusses on forward and reverse genetics,

these can be combined with other approaches such as metabolomics to identify candidate genes.

2.3.3 P-glycoproteins

2.3.3.1 *Evolution, Function and Structure*

P-glycoproteins belong to the ABC transporter superfamily defined by a highly conserved ATP-binding-cassette (ABC) motif. This ancient gene superfamily contains genes involved in the translocation of a large range of different substrates across membranes, including essential regulatory genes which tend to be conserved across different phyla as well as many efflux transporters which are more diverse both in terms of genetic variation and domain shuffling (Sheps et al. 2004b). According to their transmembrane domain (TMD) and ABC motif organization ABC transporters are classified into six (Rogers et al. 2001) or seven (Dean and Allikmets 1995) subfamilies and Pgps belong to the B subfamily (ABCB). Interestingly, a driver for the diversity of ABC transporters is their dynamic coherent evolution, meaning similar rates of gene duplication and gene loss and resulting in a high diversification despite their universal occurrence (Sheps et al. 2004b). Most commonly, the mammal gene is referred to as *ABCB1* or *mdr* and the protein as Pgp or ABCB1, however, in nematodes (and other phyla) the nomenclature is less organized, possibly as a result of a much larger repertoire of genes, and the genes are usually named *pgp* (based on *C. elegans* according to WormBase, version WS276) despite their classification as *ABCB* genes.

P-glycoproteins have first been described in cholchicin resistant Chinese hamster ovary (CHO) cells (Juliano and Ling 1976). The authors concluded that a 170 kDa protein with carbohydrate components on the surface of the cell was responsible for reduced permeability and hence named it P glycoprotein. With the emergence of improved sequencing technology in the late 70s (Sanger et al. 1977) which then continuously improved leading up to the biotechnological (and specifically NGS) revolution, it became clear that this gene was conserved in eukaryotes (Bosch and Croop 1998, Sheps et al. 2004b). Orthologs of Pgps have been reported in a large number of vertebrate species (Annilo et al. 2006). For example, two Pgps have been reported in humans, one more commonly referred to as MDR-1 (*mdr-1* or *ABCB1* gene) (Isacke et al. 1986) is associated with xenobiotic metabolism while the other termed MDR-2 (*mdr-2* or *ABCB4*) is associated with phospholipid transport but not MDR and is usually not called Pgp (Poupon et al. 2013). In rodents, three Pgp genes have been described in hamsters (Ng et al. 1989) as well as three in mice (*mdr1a/ABCB1a, mdr1b/ABCB1b* und *mdr2/ABCB4*). Likewise, Pgps can be found in invertebrates for example in arthropods (Vaché et al. 2006) and mollusks (Minier et al. 1993). In nematodes, Pgp evolution has resulted in a particularly large repertoire such as 10, e.g. in *H. contortus* (Laing et al. 2013), to 15 Pgps, e.g. in *C. elegans* (Sheps et al. 2004b) and gene duplications have been the major driving force for their diversification (Zhao et al. 2004b).

Structurally, Pgps consist of two homologous parts each formed by a TMD followed by a nucleotide binding domain (NBD). This structure is considered to have originated from a gene duplication of a gene containing a single TMD and a NBD (Croop 1993). Such single TMD genes are also referred to as half-transporters which are also very diverse and present across all kingdoms including bacteria (Chen et al. 1986) but to form a functional ABC transporter, half-transporter proteins always form homo- or hetero dimers (Biemans-Oldehinkel et al. 2006). Furthermore, it has also been demonstrated that some half-transporters can form homo- and heterotetramers and even larger molecules (Geillon et al. 2017). In Pgps, each TMD is formed by 6 transmembrane helices (Aller et al. 2009) which form an internal cavity open to the cytoplasm and the inner leaflet of the membrane. Within this internal cavity, several bindings sites have been detected and similar suggestions have been proposed for the *Cel*Pgp-1 crystal structure (Jin et al. 2012). *In silico* modelling for IVM and MOX of *C. elegans* (Jin et al. 2012, David et al. 2016) has helped to elucidate the various binding pockets in several Pgps (Aller et al. 2009, Bikadi et al. 2011). Particularly, binding of substrates is considered to take place in the membranes inner leaflet. Upon substrate binding, the two NBDs in the cytoplasm catalyze ATP hydrolysis which drives a conformational change leading to the

extracellular expulsion of the substrate. Their function as efflux transporters has been extensively studied (Theodoulou and Kerr 2015) and they have been described as “a vacuum cleaner for hydrophobic molecules embedded in the membrane” (Raviv et al. 1990). Despite a wealth of data, the exact mechanism how ABC transporters use ATP to translocate their substrate has remained controversial. Several models have been proposed: most popularly the “switch model” suggests binding of two ATP which leads to dimerization of the two ABC motifs followed by a dissociation upon hydrolysis which in turn results in a conformational change of the inner channel from inward (cytoplasm) to outward (extracellular) facing, hence principally a flippase function; the constant model proposes independent ATP hydrolysis at each ATP binding site resulting in a see-sawing motion (George and Jones 2012); a more recent model is the reciprocating twin-channel model which is supposedly most coherent with the available data and proposes that a “sequence of ATP binding, hydrolysis, and product release in each active site is directly coupled to the analogous sequence of substrate binding, translocation and release in one of two functionally separate substrate translocation pathways”. However, with more crystal structures and Cryo-electron microscopy becoming available, it has also become clear that this mechanism differs between different ABC transporters (Ford and Beis 2019). This increasing availability of high resolution data is leading towards a consensus “twist-and-squeeze” model for how Pgps translocate their substrate which is a distinct mechanism from that of other transporters (Kodan et al.). Likewise, a consensus model for the polyspecific substrate recognition is being reached (Xia et al. 2019).

With regard to the relatively large repertoire of nematode Pgps, the differences between the different analogous Pgp genes in nematodes remain unknown. Why exactly nematodes maintain this large repertoire as well as their specific functions can only be speculated. While Pgps mostly are considered components of the xenobioteic metabolic machinery, they have been implicated with other processes, such as transport of cholesterol and other lipids both in humans (Poupon et al. 2013) as well as in *C. elegans* (Nunes et al. 2005, Sharom 2014, Riou et al. 2020). Furthermore, differences in the substrate repertoire between different nematode Pgps remain to be elucidated.

2.3.3.2 *Genetic and transcriptional evidence for a contribution to macrocyclic lactone resistance*

Initially, Pgps were implicated with IVM toxicity in mammals by serendipity when *mdr1a* deficient mice were treated routinely against a mite infestation with IVM. Following the administration, most of the homozygous *mdr1a* deletion mice died or showed severe neurological symptoms and a more systematic analysis revealed that the Pgp deficiency lead to a significantly increased permeability of the blood-brain barrier (Schinkel et al. 1994a). Since then, it has become clear that Pgps play a vital role in blood-brain barrier in many different mammals including humans (Kiki-Mvouaka et al. 2010, Menez et al. 2012, Merola and Eubig 2012). In some dog breeds such as Collies, the *mdr1a* defect is very common (Mealey et al. 2001), hence a genetic screening is recommended.

Shortly after this discovery in mammals, the first study reported that a Pgp homolog might be responsible for reduced IVM susceptibility in a parasitic nematode (Xu et al. 1998). Since then, many studies have investigated the involvement of different Pgp orthologues and have produced conflicting evidence based on the population, species and Pgp while at the same time many studies have also confirmed evidence from prior studies. In general, evidence for an involvement of Pgps has been provided by i) candidate gene studies comparing resistant and susceptible isolates either at the transcriptional (e.g. qPCR or transcriptomics) or genomic level, ii) genome-wide studies both with backcrossing or simple GWAS and iii) at the functional level using various model systems (outlined in section 2.3.3.3). In addition, the use of Pgp-is has been a vital tool both within parasite populations and model organsisms.

The most abundant publications reported evidence provided by candidate gene studies. In different species, comparisons between susceptible isolates have produced evidence for selection and differential expression. For instance, in a resistant *T. circumcinta* population, the *Tcipgp-9* gene was found to be under purifying selection (Turnbull et al. 2018). Likewise, in *P.*

univalens several SNPs were identified in the *pgp-11.1* gene which were enriched in a resistant population. In *D. immitis*, identified SNPs which were more abundant in resistant populations (Mani et al. 2017) were even proposed as markers, but in a field study these were found to be unsuited as reliable resistance markers (Willi et al. 2018).

At the transcript level, many studies have investigated upregulation upon exposure or differential constitutive expression levels in *C. elegans* and parasitic nematodes. In *C. elegans*, step-wise increase of the IVM concentration led to a decreased susceptibility (max. tolerated concentration change from 1 ng/µL to 10 ng/µL), and higher Pgp expression correlated with a low susceptibility phenotype at low to intermediate (<6ng/µL) concentrations (James and Davey 2009). A different study reported increased expression of *Cel*Pgp-12 following several generations of selection (Figueiredo et al. 2018).

Several studies investigated changes or differences in the expression of Pgps with regard to ML resistance in different parasitic species.
In *P. univalens*, increased constitutive expression of *Pun*Pgp-11.1 (5.9-fold in male adults and 1.5-fold in female adults) but not of *Pun*Pgp-16.1 was reported in a resistant isolate compared to randomly chosen isolate (the study named *P. equorum*, but in fact all samples originating from the same study which were later karyotyped and shown to be *P. univalens*) (Janssen et al. 2013a).
In cyathostomins, increased constitutive expression compared between L3 larvae from resistant and susceptible isolates was described and following chemical Pgp inhibition the susceptibility of larvae *in vitro* was increased (Peachey et al. 2017).
In *H. contortus*, numerous studies reported both upregulation or increased constitutive expression of Pgp in resistant *H. contortus* (Riou et al. 2005a, Roulet and Prichard 2006, Mate et al. 2018, Reyes-Guerrero et al. 2020). Using both RNA-Seq and qPCR on adults of an H. contortus isolate, Maté et al. observed inconclusive evidence for a contribution of Pgps. Depending on the Pgp lineage small increases, decreases or no change at all was observed (Mate et al. 2018). A recent study reported inducible Pgp expression up to an astonishing 127-fold upregulation under *in vitro* conditions in a resistant isolate compared to a susceptible isolate (Reyes-Guerrero et al. 2020). The upregulated Pgp lineages varied between developmental stages, but notably, upregulation of Pgps was observed in all tested developmental stages, in adults *Hco*Pgp-1, -9, -12, -14 and -16, in L4 *Hco*Pgp-2 and -10 in L4, *Hco*Pgp-16 in eggs and generally to a lesser degree (<29.72 fold) in adults, L3 and L4 *Hco*Pgp-1, -4, -11, -12 and -16 (Reyes-Guerrero et al. 2020). Based on genome-wide studies, some of the few GWAS have reported significant selection at *Hco*Pgp-10 and other ABC-transporters (Khan et al. 2019, Khan et al. 2020).
In *T. circumcincta*, increased constitutive expression between resistant and susceptible isoaltes of *Tci*Pgp-9 was reported in a candidate gene approach (strongest change in eggs). The most comprehensive and arguably reliable evidence for a contribution of a Pgp thus far has been provided by a genome-wide backcross study in adult *T. circumcinta* which reported increased constitutive expression and an increase in CNV of *Tci*Pgp-9 following several rounds of selection and backcrossing (Dicker et al. 2011b, Choi et al. 2017).
In the cattle parasite *Cooperia oncophora*, increased expression of *Con*Pgp-9 following selection with IVM (although no phylogenetic analysis was conducted and the sequence has not been published but showed according to the authors 83% identify with *Cel*Pgp-9, possibly not actually *Con*Pgp-9) (Areskog et al. 2013). Following *in vivo* treatment with MOX or IVM lead to a 3 to 5 fold increase in *Con*Pgp-11 expression in adults and L3 in the resistant isolate compared to before treatment (De Graef et al. 2013). A similar result was reported by another study using two different isolates with the strongest upregulation in *Con*Pgp-16 (Tydén et al. 2014). Likewise, co-administration of a chemical Pgp-i, verapamil (VPM) diminished differences in susceptibility between a resistant and a susceptible isolate *in vitro* (Demeler et al. 2013b, Algusbi et al. 2014a).
In contrast, some studies have also demonstrated that Pgps are not involved with ML resistance in the studied populations for example in a different *H. contortus* isolate (ISE-IVM^R) (Williamson and Wolstenholme 2012), but the lack of such reported results in candidate genes

studies might also be the unfortunate result of publication bias (Ioannidis 2005). More comprehensively, a lack of selection or differential expression has been described by the vast majority of genome-wide backcross, GWAS and transcriptome studies in *H. contortus* (Redman et al. 2012, Rezansoff et al. 2016, Luo et al. 2017, Doyle et al. 2018, Doyle et al. 2019a, Doyle et al. 2019b, Laing et al. 2019) and *Onchocerca volvulus* (Doyle et al. 2017).
Furthermore, it was suggested that Pgps and other xenobiotic detoxification components are regulated by the transcription factor *nhr-8* in *C. elegans* and *H. contortus* (Ménez et al. 2019) and in *C. elegans nhr-8* deletion strain or following RNAi inhibition of *nhr-8* significantly increased IVM susceptibility (Ménez et al. 2019). Hence, selection for changes in Pgp expression pattern could actually be located at another locus encoding transcription factors or microRNAs.
In summary, there appears to be compelling evidence for a contribution of Pgps to ML resistance at a first glance, which has been provided by a large number of different techniques, both candidate and comprehensive genome-wide approaches and for all larval stages, eggs and adults in a large number of species. While Pgps have have been very popular popularity as possibly the most promising candidates for ML resistance for more than a decade, their alleged inability to explain the resistance of field isolates by themselves but also the conflicting evidence and new candidates genes produced by NGS studies might be the reason for the subtle trend towards a decline in publications focusing on nematode Pgp after 2016 (**Fig. 6**).

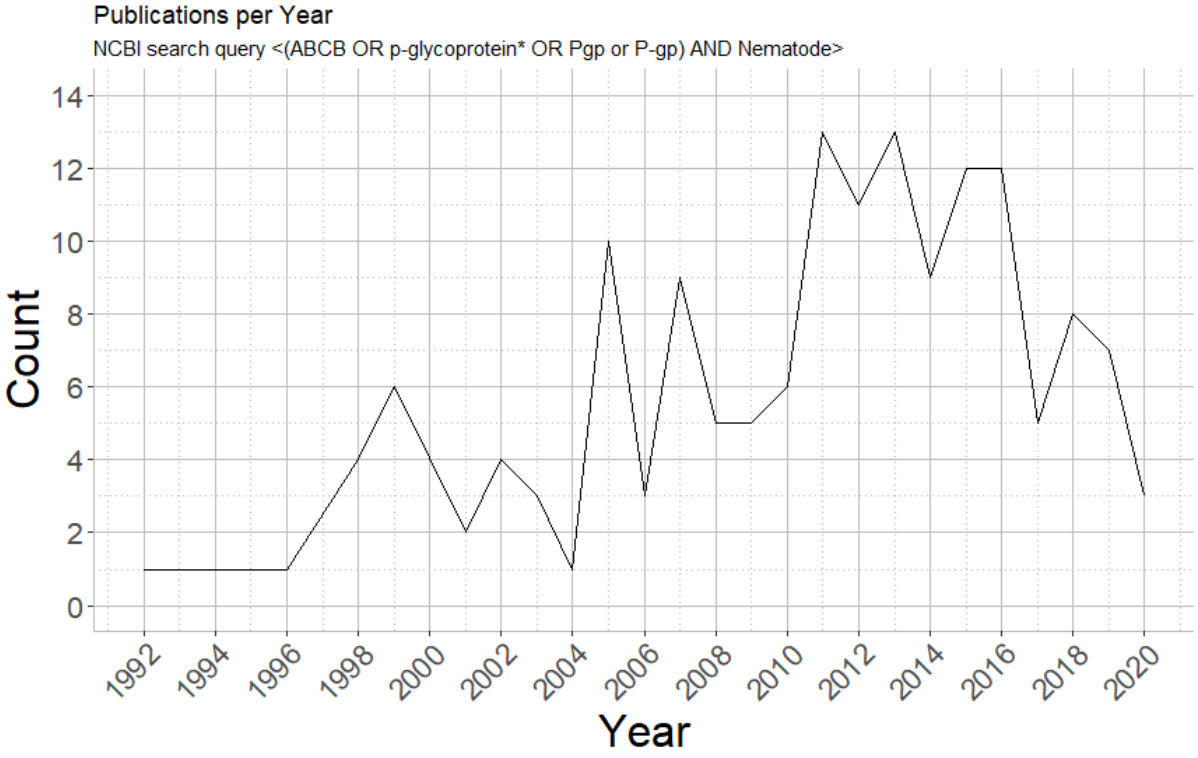

Figure 6 Publications on nematode P-glycoproteins per year

Based of NCBI PubMed https://pubmed.ncbi.nlm.nih.go *search result accessed 21.05.20, visualized with ggplot (Wickham 2016) in R version 4.0.0*

In light of this conflicting evidence, Pgps can be considered as potential contributors to ML resistance in some populations within a multigenic context while they might not play an important role in other populations.

2.3.3.3 *Interaction of P-glycoproteins with macrocyclic lactones*

With regard to the functional role of Pgps in ML resistance, it remains unclear whether SNPs can induce functional changes permitting a more efficient ML transport, whether increased constitutive Pgp expression is selected, whether Pgp inducibility is selected over generations and whether overexpression is actually a pathway to resistance (see section 2.3.3.2).

Rationally, any of these reported changes could lead to ML resistance and although Pgp and ML interaction has been shown in several studies, comprehensive functional evidence demonstrating how Pgps might contribute to ML resistance is lacking.

Increased Pgp expression has been correlated with resistance in many parasitic nematodes (section 2.3.3.2), but a causal relationship for an interaction with ML has usually been inferred from application of chemical inhibitors, for example to reverse a resistant phenotype. While the effect on Pgps can only be described as highly speculative in these type of studies, Pgp interaction with MLs has also been characterized by using model systems at the molecular level. As a common restriction to most model organisms except *C. elegans*, characterization of ML transport is only possible in relation to a second known Pgp substrate due to the lack of a measurable direct effects on the model system. Direct molecular evidence of binding of MLs by nematode Pgp proteins is not yet available and only an *in silico* study based on the crystal structure of *Cel*Pgp-1 identified one single unique site for all MLs within the inner chamber (David et al. 2016).

As a cost-effective model system, a *S. cerevisiae* strain lacking 7 endogenous ABC-transporter genes (AD1-7, genotype *Δyor1 Δsnq2 Δpdr5 Δpdr10 Δpdr11 Δycf1 Δpdr3*) (Rogers et al. 2001) has been used to examine the interaction of a resistance candidate nematode ABC transporter, *Cylicocyclus elongatus* Pgp-9 with different macrocyclic lactone derivatives in a yeast growth assay (Kaschny et al. 2015). As macrocyclic lactones are not directly affecting yeast growth, MLs were co-incubated with a secondary known Pgp substrate and antimycotic KCON. In general, nematode *Con*Pgp-9 was characterized with KCON, actinomycin, valinomycin, daunorubicin and TBZ (no interaction) as direct Pgp substrates. Then, using the co-incubation *Con*Pgp-9 and ML interaction was shown for ivermectin, eprinomectin, moxidectin but not for selamectin.

The cell line LLC-PK1 has been used to study gene function of transgenic nematode Pgps (as well as human MDR-1), *H. contortus* Pgp-2, -9.1 and 16, *D. immitis* Pgp-11 and Pgp-16 (Godoy et al. 2015b, Godoy et al. 2016, Mani et al. 2016a). Similar to the yeast model, ML interaction with Pgps is inferred from the ML concentration dependent inhibition of transgenic Pgp-mediated efflux of a secondary Pgp substrate, a fluorescent dye (e.g. Rhodamine 123 or *Hoechst* 33342). The results from these studies suggest a trend towards a slightly better Pgp-mediated fluorophore efflux inhibition by ivermectin, abamectin (both in *Hco*Pgps) and selamectin (only *Dim*Pgp-11) in contrast to only weak or no inhibition by moxidectin (all tested Pgp) and milbemycine oxime (*Dim*Pgp-11). Noteworthy, the use of different fluorophores revealed differences in the inhibitory potential of each ML which demonstrates the issue with using a secondary Pgp substrate. Despite these limitations, the authors speculate that avermectins are better substrates than milbemycines, also potentially as a result of different binding sites which is also supported by the *in silico* analysis (David et al. 2016). In addition, in the yeast growth assay MOX was also a less potent inhibitor than IVM, however Selamectin did not elicit any affect (Kaschny et al. 2015). Interestingly, Selamectin does only exhibit a monosaccharide at the C13 side chain while other avermectins have a disaccharide (**Fig. 4**). Thus, the sugar side chain might influence the Pgp substrate specificity but the general conclusion that the avermectins are better substrates than milbemycines is not supported by these findings.

Pharmacogenetic analyses suggest that non-synonymous mutations might result in changes of substrate specificity or altered phenotypic resistance in human *mdr-1* (Kioka et al. 1989, Gow et al. 2008, Fung and Gottesman 2009) as well as in a related bovine ABC transporter, *ABCG2* (Real et al. 2011). In humans, polymorphisms have also been correlated with diseases such as ulcerative colitis (Li et al. 2006). However, to date conclusive functional and pharmacogenomic evidence in humans or nematodes for a causal relationship for altered substrate specificity is lacking. With regards to mutations in non-coding (promotor) regions, many studies have proposed altered expression in mammals, but not in nematodes (Li et al. 2006). In nematodes, SNPs in Pgps have been proposed to be selected in resistant isolates but could so far not be functionally implicated with resistance nor confirmed as markers of resistance (Bourguinat et al. 2011b, Janssen et al. 2013a, Bourguinat et al. 2015).

In contrast to yeast and cell culture, *C. elegans* is directly susceptible to MLs and the effect of candidate genes from the xenobiotic metabolic machinery on ML susceptibility can be directly examined. As a disadvantage, the highly complex mode of ML action and the multi-cellular nature limit to what extent interpretation for an interaction between ML and Pgp can be inferred at the molecular level. However, transgenic expression of *Pun*Pgp-11 (*Peq* in the publication) has been shown to reduce the IVM susceptibility in a thrashing assay (Janssen et al. 2015). Prior Pgp studies in *C. elegans* have focused on examining the phenotype of Pgp deletion strains. Here, all tested Pgp loss of function *C. elegans* strains showed a moderate increase in susceptibility to IVM in a larval development assay in two independent studies (Ardelli and Prichard 2013, Janssen et al. 2013d).

Generally, MLs and particularly IVM are well-established as Pgp substrates, but whether all or just some nematode Pgps are ML transporters is unknown, in fact, even the Pgp repertoire in most ML resistant nematode species has not been described. In this regard, any differences between different populations and species is similarly unexplored. Even more interestingly, how Pgps might contribute to ML resistance remains to be elucidated.

2.4 Objectives

The overall objective of this dissertation was to gain a better understanding of the mechanism of Pgp-mediated ML resistance. To this end, the state-of-the-art molecular approaches and -omics resources were utilized. Briefly, four main objectives were driving this research.

1) The decipherment and characterization of the complete Pgp repertoire in *P. univalens* in order to provide a comprehensive annotation from reliable sequencing data of all coding sequences
2) Examination of the inducibility of individual Pgps by ivermectin and the tissue specific Pgp expression using -omics technology
3) Characterization of the interaction of specific *P. univalens* Pgps with MLs, specifically with IVM and MOX
4) Elucidation of the functional role of tissue-specific Pgp expression within the context of active drug ingestion
5) Screening of compounds to identifiy inhibitors of nematode Pgps

3 PUBLICATIONS

3.1 The P-glycoprotein repertoire of the equine parasitic nematode *Parascaris univalens*

Publication Date: 12th August 2020

Available at: https://rdcu.be/b646E DOI: https://doi.org/10.1038/s41598-020-70529-6

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The P-glycoprotein repertoire of the equine parasitic nematode *Parascaris univalens*

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P-glycoproteins (Pgp) have been proposed as contributors to the widespread macrocyclic lactone (ML) resistance in several nematode species including a major pathogen of foals, *Parascaris univalens*. Using new and available RNA-seq data, ten different genomic loci encoding Pgps were identified and characterized by transcriptome-guided Rt-pcRs and Sanger sequencing. phylogenetic analysis revealed an ascarid-specific Pgp lineage, Pgp-18, as well as two paralogues of Pgp-11 and Pgp-16. comparative gene expression analyses in P. univalens and Caenorhabditis elegans show that the intestine is the major site of expression but individual gene expression patterns were not conserved between the two nematodes. In P. univalens, *Pun*Pgp-9, *Pun*Pgp-11.1 and PunPgp-16.2 consistently exhibited the highest expression level in two independent transcriptome data sets. Using RNA-Seq, no significant

upregulation of any Pgp was detected following in vitro incubation of adult *P. univalens* with ivermectin suggesting that drug-induced upregulation is not the mechanism of Pgp-mediated ML resistance. expression and functional analyses of *Pun*Pgp-2 and *Pun*Pgp-9 in Saccharomyces cerevisiae provide evidence for an interaction with ketoconazole and ivermectin, but not thiabendazole. Overall, this study established reliable reference gene models with significantly improved annotation for the P. univalens pgp repertoire and provides a foundation for a better understanding of pgp-mediated anthelmintic resistance.

Parasitic nematodes are important pathogens of livestock, companion animals and humans and the emergence and spread of anthelmintic resistance has compromised veterinary helminth control. In equines, *Parascaris univalens* poses a major threat particularly to juvenile h orses[1,2]. At present, chemotherapeutic metaphylaxis and therapy remain the most effective and commonly used strategies for veterinary helminth control although widespread anthelmintic resistance to one or multiple drug classes compromise their success[3–5]. The frequent and unrestricted use of macrocyclic lactones (MLs) in domestic horses over decades has driven selection of resistant parasite populations[6]. In *Parascaris* sp., ML resistance was first reported in the early 2 000s[7] and since then has developed into a global challenge. Its presence has been demonstrated in several European countries[8–13], North America[14–16], as well as New Z ealand[17], Australia[18] and more recently Ethiopia[19] and Saudi-Arabia[20]. Similarly, the development and spread of ML resistance has been reported in many other parasitic nematodes, including *Teladorsagia circumcincta*[21] and *Haemonchus contortus* in sheep[22], *Dirofilaria immitis*[23] in dogs and even in human filarial nematodes such as *Onchocerca volvulus*[24].

Prior to genome-wide approaches, P-glycoproteins (Pgp) were already presumed to be important contributors to ML resistance in a number of parasitic nematodes[25]. Nonetheless, deciphering the mechanisms of ML resistance is challenging, partly due to the multi-genic nature of the resistance traits[26,27]. Furthermore, inconsistency between different species or populations in different studies reveals the high complexity of this problem. For instance, multigenicity has become apparent in light of recent genome-wide studies[28–30], which also substantiate evidence for Pgp as candidate genes. Choi et al. showed in a backcross experiment conducted in *T. circumcincta* that ABC-transporters were among the genes that had undergone selection in response to treatments with ivermectin (IVM), a commonly used ML. In this study, *Tci*-Pgp-9 exhibited higher copy numbers in the genome, four strongly selected single nucleotide polymorphisms (SNPs) and a higher number of transcripts in RNA-seq in the resistant backcrossed isolate[29]. In contrast, genome-wide association studies in *H. contortus* are conflicting and either point towards a single major resistance locus, which does not directly correspond to any previous candidate resistance gene[31,32] or multiple selected loci including Pgp[30].

P-glycoproteins belong to the superfamily of ATP binding cassette (ABC) transporters and form the ABCB subfamily[33]. The substrates of these xenobiotic transporters are usually neutral or lipophilic[34,35] and they exhibit a broad substrate range[36]. Their versatility as transmembrane transporters is most likely the reason for their evolutionary success — Pgps can be found in almost all eukaryotes and seem to have experienced an expansion through duplication events in several species giving rise to different Pgp lineages[37]. This evolutionary diversification has been particularly strong in nematodes[38]: while mammals only possess one or two different Pgp genes[37], nematodes typically have a much larger and more diverse repertoire, for example 15 Pgps in *Caenorhabditis elegans*[39], including a pseudogene[40]. Pgp open reading frames (ORFs) are comparatively large (usually 3,800–4,000 bp) and translate into proteins of about 170 kDa with a central pore formed by two transmembrane domains each containing six transmembrane helices followed by an ATP binding s ite[41].

Although genome and transcriptomes of parasitic nematodes provide a crucial resource for anthelmintic resistance research, to date in silico prediction of transcripts from draft genome and limited transcriptomes is error prone and often leads to incomplete gene models for many, in particular larger genes. In the case of the diverse Pgp gene family, this has been a particular challenge as many studies (e.g. Janssen et al., 2013 and Jesudoss et al., 2019 on *Parascaris* sp.) have focused only on the few validated parasite Pgp genes, resulting in a research bias towards these genes.

The mechanism of anthelmintic drug resistance due to Pgps, has been linked to overexpression of individual Pgps in several parasitic nematode species, i.e. of Pgp-2 and Pgp-9 in *H. contortus*[42], Pgp-

9 in *T. circumcincta*[43] as well as of Pgp-11 in *P. univalens*[44]. In line with findings for other nematode Pgps such as for *Tci*Pgp-9 from candidate gene and whole-genome s tudies[29,45] as well as early studies on a *H. contortus* Pgp[46], three SNPs identified in a *Parascaris* sp. Pgp-11 orthologue were strongly increased in field isolates of a resistant population compared to susceptible populations[44]. This suggests that both overexpression and drug target site mutations of Pgps may lead to a reduced IVM susceptibility. At a functional level, MLs have been shown to be substrates of Pgps. In this regard, avermectins such as IVM appear to be better substrates than milbemycins such as moxidectin[47–49], most likely due to the lack of a sugar moiety of the latter[50]. Furthermore, the deletion of individual Pgps in *C. elegans* resulted in a modest increase of IVM susceptibility[51], which appeared to vary slightly between individual Pgps. From the Pgp orthologues previously linked to ML and specifically IVM resistance through expression changes or signatures of selection, functional evidence for an interaction with MLs of *P. univalens* Pgp has only been provided for a Pgp-11 orthologue with IVM[52] but not for orthologues of Pgp-2 and Pgp-9. In other nematodes, the Pgp-11 orthologue of *D. immitis*[53] and the Pgp-2 and Pgp-9 orthologues of *H. contortus*[54,55] have been shown to interact with MLs using the cell line LLC-PK1. Likewise, the Pgp-9 orthologue which has been linked most often to ML resistance in different parasitic nematodes at the epidemiological level, has also been shown to interact with MLs from *Cylicocyclus elongatus* using the *Saccharomyces cerevisiae* AD1-7 yeast strain[47]. Transgenic Pgp expression in this yeast strain lacking 7 endogenous ABC-transporters (AD1-7) has been established as an easy and cost-effective experimental approach to examine interaction directly with the antimycotic and anthelmintic thiabendazole (TBZ) and indirectly with MLs through co-incubation with the antimycotic and known Pgp-substrate ketoconazole (KCON).

In light of the aforementioned candidate gene and genome-wide studies and with respect to the high number of Pgp genes in nematodes, evidence is accumulating that only some Pgps are directly involved in ML resistance. To gain a better understanding of the anthelmintic resistance mechanisms, it is essential to know the whole inventory of Pgps in resistant nematode species. On these grounds we have identified and characterized the whole Pgp gene family of the frequently ML resistant ascarid species *P. univalens* at the genomic and transcriptomic level. Following this comprehensive approach, the putative candidate Pgp orthologues *Pun*Pgp-9 and *Pun*Pgp-2 were characterized regarding their interaction with two important anthelmintic drug classes, macrocyclic lactones (IVM) and benzimidazoles (TBZ).

Results

Comprehensive annotation of full length P-glycoproteins. As Pgp annotation based on the *P. univalens* transcriptome assembly WormBase ParaSite version WPBS14 was incomplete for most Pgps and resulted in discontinuous ORFs, a transcriptome-guided RT-PCR approach was used allowing amplification and sequencing of all identified Pgps, revealing a total repertoire of ten Pgps in *P. univalens*.

Overall, annotation was considerably improved compared to the automatic annotation by Wang et al.[56] automatic annotations WormBase ParaSite version WPBS14 for all Pgps with several novel exons added to the gene models (Supplementary Fig. S1). Specifically, 5′ and 3′ coding exons were missing for several Pgps (Supplementary Fig. S1), a large number of insertions, mismatches and deletions was detected disrupting the originally predicted ORFs (Supplementary Table S1) and in case of *Pun*Pgp-3 and *Pun*Pgp-12, annotation was severely fragmented and incomplete (Fig. S1b,g). For several splice junctions of previously annotated (in the WormBase

ParaSite version WPBS14) and correctly identified exons, accuracy was improved through elimination of a deviation of a few base pairs. The updated Pgp annotation was integrated into the whole genome annotation in gff3 format and uploaded on WormBase ParaSite (parasite.wormbase.org).

Heat maps of splice events of Splign-analysed cDNA sequences were mostly consistent between replicates and reflected by another approach calculated transcripts per kilobase million (TPM) expression levels (in heatmaps as fragments per kilobase of exon per million [FPKM]) with highest expression in the intestine for most transcripts. However, in several cases varying exon support depending on tissues was detected, e.g. E28 (3′ exon) in *Pun*Pgp-2 is found only in the testis (Supplementary Fig. S1a) and E1 (5′ exon) in *Pun*Pgp-9 is not detected in testis or carcass tissue samples (Supplementary Fig. S1c).

The only experimentally identified alternative splice variant was found in *Pun*Pgp-18 for E13/14 and named *Pun*Pgp-18A and *Pun*Pgp-18B. Both variants were also found in both transcriptome data sets (Supplementary Fig. S1i) including the RNA-Seq experiment where worms where incubated with IVM, henceforth addressed as the IVM transcriptome ("Biological material, genome and transcriptome resources"), which had high junction support for variant A in all samples as well as weak support for variant B in the control samples NTC3 and NTC7 (data not shown).

Phylogenetic relationship of *Parascaris univalens* and other nematode p-glycoproteins. Phylogenetic analysis using complete protein sequences (Fig. 1) revealed that all outgroup sequences formed a single cluster, while nematode Pgps were separated into several strongly supported clusters containing *P. univalens* Pgps as well as their *C. elegans* orthologues. If applicable due to a very limited number of published full-length nematode Pgps, subclusters within a group of Pgp resembled general nematode phylogeny[57] with separate ascarid, rhabditid and trichocephalid Pgp subclusters. Pgps of *P. univalens* and *A. suum* were named in accordance with CGC standards as orthologues of *C. elegans* as *Pun*Pgp-2, -3, -9, -10, -12, -11.1, -11.2, 16.1, and -16.2 and for hypothetical (manually corrected using the *P. univalens* orthologue full length ORF sequence) *A. suum* sequences *Asu*Pgp-X_hp. One Pgp lineage without a currently known orthologue in *C. elegans* or any parasitic nematode was identified in both *A. suum* and *P. univalens* and named Pgp-18. Several published reference Pgps were also renamed according to the result of the phylogenetic analysis. Remarkably, *P. univalens* appears to have two paralogues of Pgp-11 and Pgp-16, which were named Pgp-X.1 (previously published[44]) or -X.2, accordingly. Both *Pun*Pgp-11 paralogues possess an orthologue in *Toxocara canis* and *A. suum*. Here, *Pun*Pgp-11.2 shows stronger relatedness with filarial nematode Pgp-11 than *Pun*Pgp-11.1 for which no orthologue was identified in filariae. In contrast, *Pun*Pgp-16 paralogues bear stronger resemblance to each other than to the few other published Pgp-16 sequences of *Caenorhabditis briggsae* and *Brugia malayi*, but both paralogues have orthologues in *A. suum* as well. *Asu*Pgp-16.1 could not be assembled from the available contig sequences and was hence omitted from the phylogenetic analysis. With regard to their genomic positions, the Pgp-11 paralogues are separated and appear on different genomic scaffolds, NINM01000018.1 and NINM01000098.1 (Supplementary Fig. S1e,f), while *Pun*Pgp-16.1 and -16.2 are localized in close proximity (6,651 bp intergenic region) in a head to tail (Supplementary Fig. S1H) orientation on the genomic scaffold NINM01000014.1 with genomic coordinates 2,435,368:2,453,320 and 2,459,971:2,480,921, respectively.

All *P. univalens* Pgps have orthologues in *A. suum* and vice versa. Inspection of the alignment of complete translated cDNA sequences revealed large gaps and incongruity in regions localising in both TMDs of the Pgp. Despite that, a maximum likelihood tree (data not shown) calculated from a GBLOCKS edited alignment excluding sequence blocks with high divergence and ambiguous alignments, did not improve branch support values and was almost identical to the full length tree except for a shift in the position of the Pgp-10 cluster, which appeared in a sister position to the Pgp-2 cluster in the GBLOCKS edited tree in contrast to the tree calculated from the complete alignment (Fig. 1).

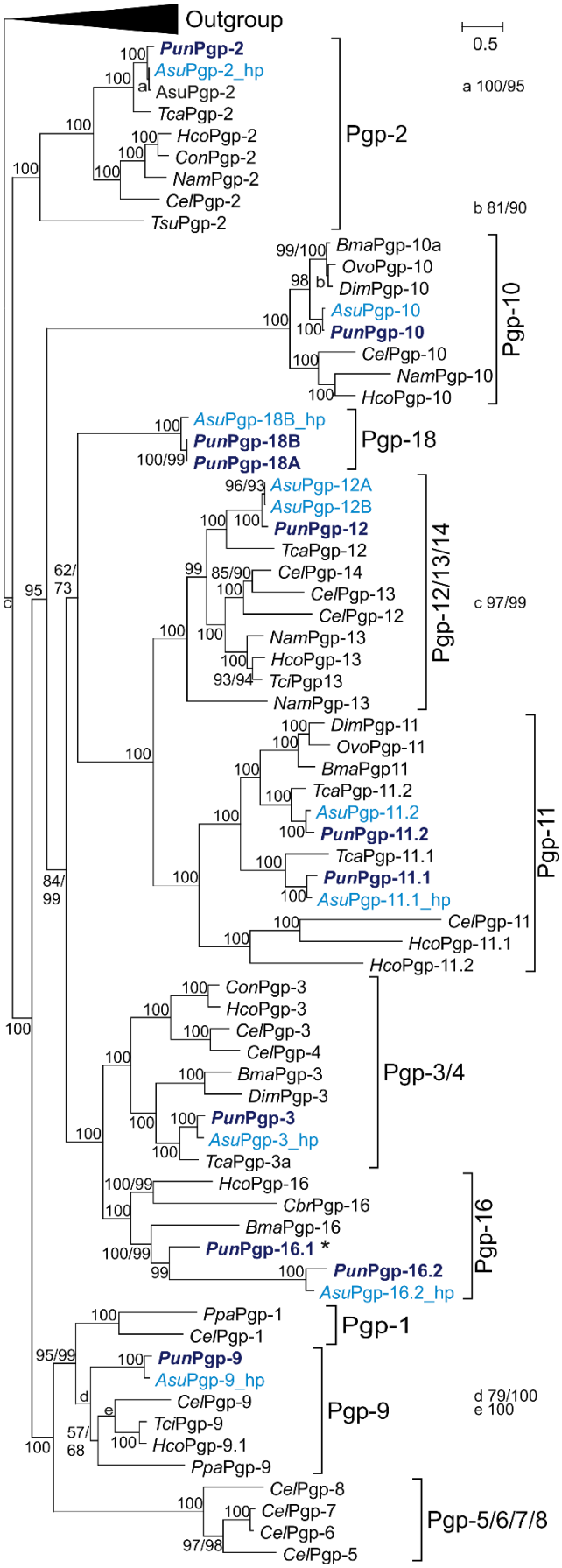

Figure 1. Phylogenetic analysis of *Parascaris univalens* and nematode Pgp. The consensus tree was calculated ▸ from Pgp protein sequences using RaxML and LG + F + G model allowing nearest neighbour interchange (NNI) and subtree pruning and regraftment (SPR) with 1,000 bootstrap replicates (first value). Thereafter, this tree was used to restrict tree topology in RaxML additional branch support values were calculated using the ShimodairaHasegawa (SH) approximate likelihood ratio test (second value). Nodes with only one value had the same support value calculated with both methods. A collapsed representative outgroup of *Drosophila melanogaster*, *Mytilus* spp., *Mus musculus*, *Pediculus humanus corporis* and *Homo sapiens* Pgps was used for rooting. *Parascaris univalens* Pgps are indicated in dark blue. *Ascaris suum* Pgps (light blue) from the sequence data of the most recent transcriptome assembly (GCA_000187025.3) are shown here as *Asu*Pgp-X_hp (hypothetical). *Asu*Pgp-16.1 was identified in the transcriptome but omitted in the analysis because of fragmented and incomplete sequence data and to indicate its presence *Pun*Pgp-16.1 is highlighted with an *. The scale bar represents the indicated number of substitutions per site. Accession numbers are given in Supplementary Table S1 as well as annotated protein names (changed in this figure according to the result of the phylogenetic analysis). Pgp: P-glycoprotein.

Expression profiles of *Parascaris univalens* and *Caenorhabditis elegans* p-glycoproteins. IVM incubation of *P. univalens* did not result in a significantly changed expression level in terms of TPM of any of the Pgps compared to the DMSO incubated control group (Kruskal–Wallis, Dunn's post-hoc test, $p > 0.05$) (Fig. 2). However, base expression levels within both the control group and the IVM incubated group were significantly higher for *Pun*Pgp-11.1, -16.2 and -9 compared to *Pun*Pgp-3, -12 and -18 (Kruskal–Wallis test, Dunn's post-hoc test, $p < 0.05$) (Fig. 2). In between the high (TPM > 40) and low expression Pgps (TPM < 5), *Pun*Pgp-2, -10, -11.2 and -16.1 formed a group with intermediate TPM expression (Fig. 2).

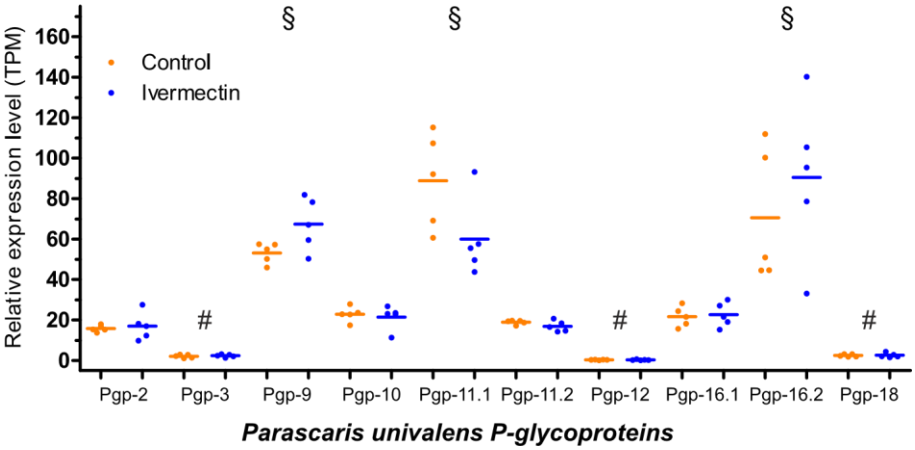

Figure 2. Relative expression levels of ivermectin and control incubated adult *Parascaris univalens*. Relative expression levels of *Parascaris univalens* adult females incubated with 1 0^{-9} M ivermectin (blue) or a DMSO control (orange) for twelve hours with 5 worms for each condition. Relative expression levels were derived from RNA-Seq raw read libraries. Reads were mapped onto the augmented (with *P. univalens* Pgp cDNA sequences) *P. univalens* genome (genome assembly ASM225920v1, version WBPS14) using STAR and featureCounts and normalised as transcripts per million (TPM), then each replicate was visualized per Pgp using GraphPad Prism v. 8.3.3 (GraphPad Software, San Diego, California USA, https ://www.graph pad.com). No significant upregulation of any Pgps was found following the incubation of worms with ivermectin compared to the DMSO control groups (Kruskal–Wallis test, Dunn's post-hoc test, $p > 0.05$) but constitutive expression levels of *Pun*Pgp-11.1, 16.2 and -9 (§) were significantly higher compared to *Pun*Pgp-3, -12 and -18 (#) (Kruskal–Wallis test, Dunn's post-hoc test, $p < 0.05$) in both the control and the ivermectin incubated group. Pgp: P-glycoprotein.

In a *P. univalens* transcriptome data set with tissue specific samples from a population of worms naïve to anthelmintics, henceforth addressed as the tissue specific transcriptome ("Biological Material, genome and transcriptome resources"), both replicates exhibited almost identical TPM expression levels for all Pgps (Supplementary Fig. S2). As sample size (n = 2) was too small, no statistical analysis was conducted. However, expression levels and patterns varied considerably between different tissues. To improve information depth in the graphs, expression levels were visualised on a l og_{10} scale for *P. univalens* (Fig. 3a) and as a comparison for *C. elegans* (Fig. 3b) as well as on a linear scale for both nematodes (*P. univalens* Fig. 3c and *C. elegans* Fig. 3d). Similar to the IVM-transcriptome, *Pun*Pgp-9, -11.1 and the two *Pun*Pgp-16 paralogues were the most strongly expressed Pgps in the tissue specific transcriptome (Fig. 3c). The intestine exhibited by far the strongest overall expression and in particular of those same four aforementioned Pgps (Fig. 3c). This strong intestinal Pgp expression was also found in *C. elegans* where expression analysis was based on a single cell transcriptome data set[58], although here the extraordinarily strong pharyngeal expression of *Cel*Pgp-14 (missing in *P. univalens*) was most striking (Fig. 3d). Second highest Pgp expression in *P. univalens* was found in the carcass tissue (hypodermis, muscle, neuron and pharynx) (Fig. 3a). In contrast, in *C. elegans* neuronal, body wall muscle and hypodermis expression was observed for almost all Pgps although at very low levels (Fig. 3b). In the gonadal tissues only moderate to low Pgp expression was found in *P. univalens* (Fig. 3a) with differences between the sexes, i.e. strong expression of *Pun*Pgp-10 and -11.2 in the ovaries which

was low in the testis and in any of the other tissues (Fig. 3c). In contrast in the testis, expression of *Pun*Pgp-9 and -11.1 was strongest, which in turn was very low in the ovary (Supplementary Fig. S2). Pgp expression in *C. elegans* was almost completely absent in the gonads, except for very low expression of *Cel*Pgp-2, -8 and -14 (Fig. 3b) and low in glia and neurons (Fig. 3b). Overall, *Pun*Pgp-9 and -11.1 and the Pgp-16 paralogues were the most strongly expressed in almost all tissues, except for the ovary (Supplementary Fig. S2a).

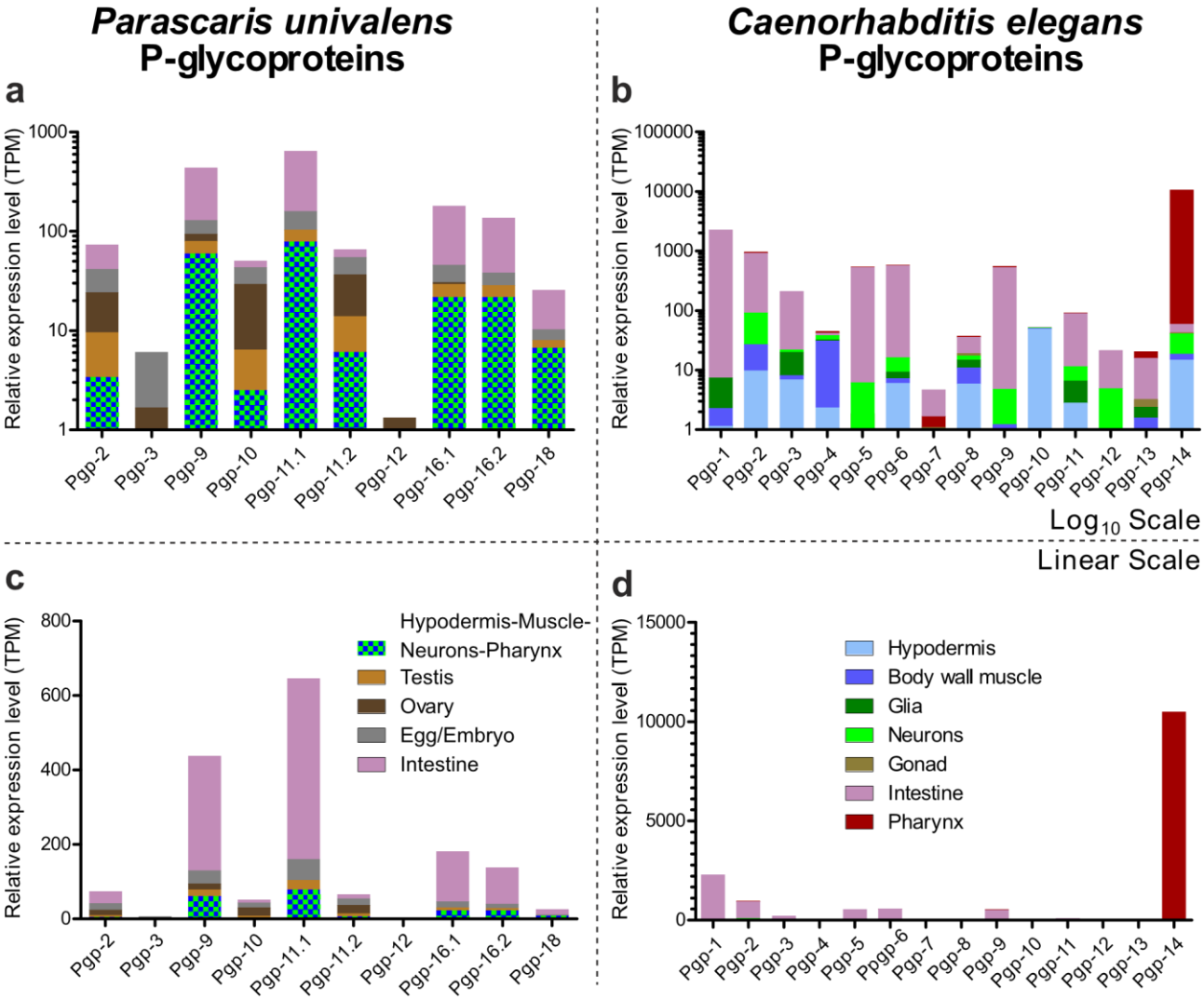

Figure 3. Relative tissue expression levels of *Parascaris univalens* and *Caenorhabditis elegans* P-glycoproteins. Tissue expression levels of *Parascaris univalens* worms were calculated based on transcriptome raw reads data sets (Geo Accession GSE99524, samples GSM2645460 to GSM2645464) mapped onto the augmented (with *P. univalens* Pgp cDNA sequences) *P. univalens* genome (WormBase ParaSite genome assembly ASM225920v1, version WBPS14) using STAR. Expression levels were normalised as transcripts per million (TPM) from raw read counts obtained with featureCounts. *Caenorhabditis elegans* tissue expression levels (TPM) were obtained from a single cell transcriptome (Geo accession number: GSE98561) and calculated with R [87] package monocle3[88–90]. Then expression levels of *P. univalens* (**a**) and (**b**) and *C. elegans* (**b**) and (**d**) were visualized on a $\log_{10}$ scale (**a**) and (**b**) and a linear scale (**c**) and (**d**). The different tissues were color-coded as followed. Digestive tract: *P. univalens* and *C. elegans* intestine (purple), *C. elegans* pharynx (red); Hypodermis/Cuticle/Neuron: *C. elegans* hypodermis (light blue), body wall muscle (dark blue), glia (dark green) and neurons (light green), *P. univalens* hypodermis-neuronal-muscle tissue (blue-green); Reproductive tissue *C. elegans* gonads (brown); *P. univalens* ovary (dark brown), testis (light

brown). Visualisation was done using GraphPad Prism v. 8.3.3 (GraphPad Software, San Diego, California USA, https ://www.graph pad.com). *Pgp* P-glycoprotein.

Functional analysis of *Pun*Pgp-2 and *Pun*Pgp-9 in a yeast growth assay. The *Saccharomyces cerevisiae* AD1234567 strain was successfully transformed with the genes *Pun*Pgp-2, *Pun*Pgp-9 and lacZ in the pYes2 vector and expressed under the control of the *gal-1* promoter. For each gene, transcription was validated in at least one clone by RT-PCR (Supplementary Fig. S3a for lacZ and b for *Pun*Pgp-2/-9). For each clone, clone 1 was used for all analyses. The generated strains are henceforth referred to as AD1-7Pgp-9, AD1-7Pgp-2 and AD1-7lacZ. The AD1-7 strain lacks seven endogenous xenobiotic ABC-transporters and hence exhibits an increased susceptibility to antimycotic ABC-transporter substrates such as KCON. Therefore, transgenic Pgp overexpression is expected to lead to measurable shifts in susceptibility.

To confirm that the identified Pgps have a function in xenobiotic transport two Pgp orthologues implicated with ML resistance in other nematodes but which have not been studied in *P. univalens* were chosen, i.e. *Pun*Pgp-2 and *Pun*Pgp-9. Ketoconazole inhibited yeast growth in a concentration dependant manner with an E C_{50} of 0.28 µM KCON in the AD1-7lacZ strain (Table 1). Expression of both *Pun*Pgp-2 and *Pun*Pgp-9 resulted in a reduction of KCON susceptibility visible in a marked curve shift to the right (Fig. 4 a and b) with significantly increased EC_{50} values ($p = 0.0002$) of 0.58 µM KCON (2.46 fold increase) and 0.44 µM KCON (1.86 fold increase) for the two strains, respectively (Table 1). Thiabendazole also inhibited the growth of yeasts with an E C_{50} of 0.16 mM TBZ in the AD1-7lacZ strain (Table 1). In contrast to KCON, expression of both Pgp resulted in only minor changes in the concentration–response-curves (Fig. 1c) and not in a significant change in TBZ susceptibility with EC_{50} values of 0.15 mM TBZ and 0.24 mM TBZ for AD1-7Pgp-2 and AD1-7Pgp-9, respectively (Table 1).

Strain	EC_{50} ketoconazole (µM)	EC_{50} 95% CI (µM)	Fold change[a]	R^2	p-value[b]
AD1-7lacZ	0.28	0.25–0.31	–	0.89	
AD1-7Pgp-2	0.69	0.58–0.82	2.46	0.57	0.0002
AD1-7Pgp-9	0.52	0.44–0.62	1.86	0.69	0.0002
Strain	**EC_{50} thiabendazole (mM)**	**EC_{50} 95% CI (mM)**	**Fold change[a]**	**R^2**	**p-value[b]**
AD1-7lacZ	0.16	0.13–0.48	–	0.47	
AD1-7Pgp-2	0.15	0.101–0.39	0.94	0.65	0.79
AD1-7Pgp-9	0.24	0.04–0.94	1.47	0.54	0.498

Table 1. EC_{50}s of transgenic AD1-7 *Saccharomyces cerevisiae* relative growth in the presence of ketoconazole or thiabendazole. *Pgp* P-glycoprotein; *EC_{50}* half maximal effective concentration calculated from four parameter non-linear regression model; *CI* confidence interval. [a] EC_{50} fold change compared to the lacZ expressing control strain. [b] Extra sum-of-squares F test with Holm's correction for multiple testing.

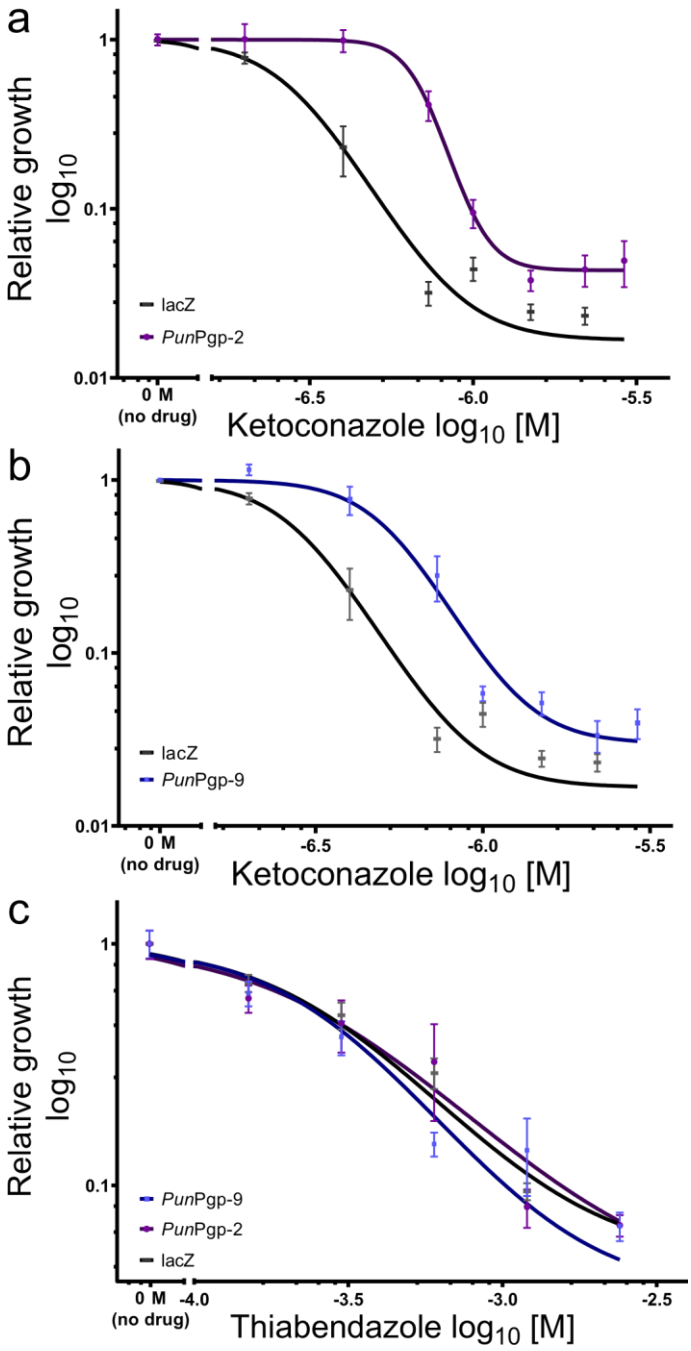

Figure 4. Susceptibility of AD1-7 expressing *Pun*Pgp-2 and *Pun*Pgp-9 to ketoconazole and thiabendazole. Susceptibility to ketoconazole was determined as relative growth of transgenic *Saccharomyces cerevisiae* AD1-7 strain in the presence of a dilution series of ketoconazole (final concentration of 1% DMSO) or thiabendazole (dissolved in water) with $\log_{10}$ transformation of concentrations, setting the negative no drug control to $1\ 0^{-7}$ M (ketoconazole) and $1\ 0^{-4.15}$ M (thiabendazole) but visualising it as "0 M (no drug)" separated by a break in the x-axis. Yeast were grown in 48-well plates with 12 replicates, four each on three separate days, per strain and concentration for 48 h and measuring OD_{600} every 10 min. From blanked reads (to wells containing only medium but no yeast), the area under the curve was calculated and then normalised to the no-drug control for the same strain on the same day. Then, four-parameter non-linear regression models were calculated and compared using the extrasum-of-squares F test with Holm's adjustment. Relative growth curves for AD1-7Pgp-2 (**a**) (purple circles) and AD1-7Pgp-9 (**b**) (blue squares) exhibited significantly increased E C_{50}s for ketoconazole ($p = 0.0002$) but not to

thiabendazole (**c**) (p = 0.7897 and AD1-7Pgp-9 p = 0.498) compared to AD1-7lacZ strain (black rectangles). Calculation and visualisation was performed using GraphPad Prism v. 8.3.3 (GraphPad Software, San Diego, California USA, https ://www.graph pad.com). Pgp: P-glycoprotein.

Ivermectin did not inhibit yeast growth in the AD1-7Pgp-2 and AD1-7lacZ strain (p = 0.796 and 0.168, oneway ANOVA). In the AD1-7Pgp-9 strain relative growth varied significantly between concentrations ($p < 0.0001$, one-way ANOVA) but was not significantly inhibited at any concentration (adjusted $p > 0.05$, Tukey's honestly significant difference (HSD) post-hoc test). However, relative growth was significantly elevated at 0.4 µM IVM compared to the control strain (adjusted $p < 0.0001$, $p > 0.05$ Tukey's HSD post-hoc test with Holm's correction for multiple testing). Since IVM did not directly inhibit yeast growth (Fig. 5 black curves), an indirect assay was used to detect potential interaction with Pgps. A low KCON concentration, that only marginally reduced growth of the particular yeast strains, was used and combined with increasing IVM concentrations. A concentration of 0.2 µM KCON was used for AD1-7lacZ strain, while 0.73 µM KCON was chosen for both Pgp expressing strains (Fig. 4 a and b). However, in the presence of KCON, increasing IVM concentrations resulted in the inhibition of yeast growth in the AD1-7Pgp-2 and AD1-7Pgp-9 strains (Fig. 5b,c) but not in the AD1-7lacZ strain (Fig. 5a). For both AD1-7Pgp-2 and AD1-7Pgp-9, a significant growth inhibition was measured at concentrations higher than 0.73 µM IVM in the presence of KCON (all adjusted $p < 0.05$ two-way ANOVA and a Dunn's post hoc test with Holm's p-value adjustment). In contrast, no growth inhibition was observed in AD1-7lacZ in the presence of IVM (Fig. 5a) (all adjusted $p > 0.05$ two-way ANOVA and a Dunn's post hoc test with Holm's p-value adjustment).

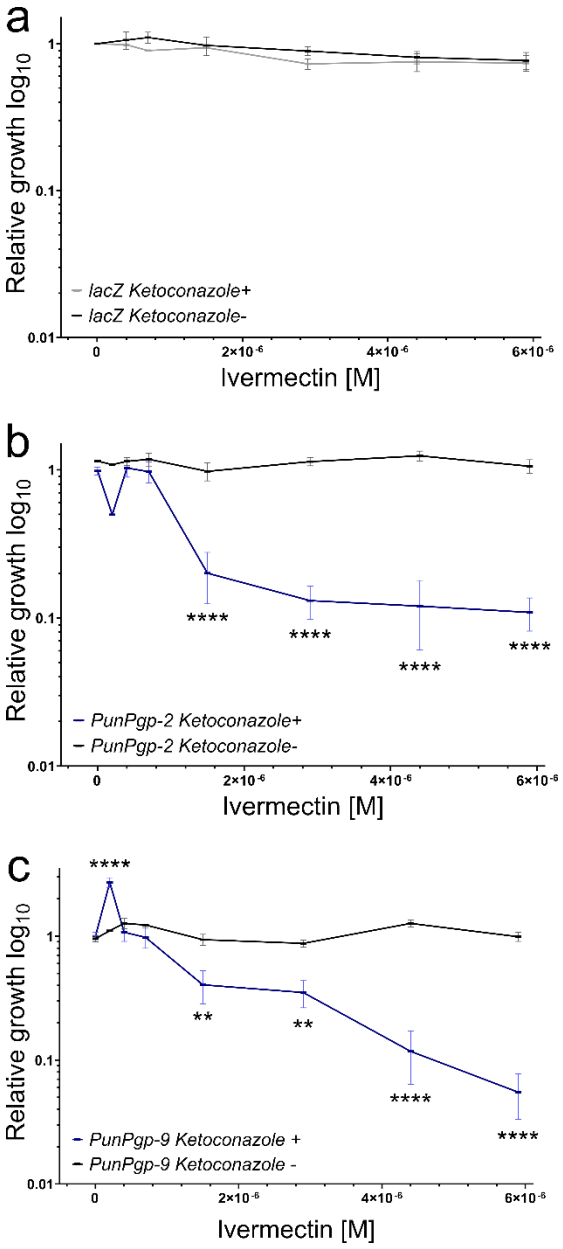

Figure 5. Relative transgenic yeast growth inhibition by ivermectin in presence or absence of ketoconazole.

Relative growth was determined by growing transgenic *Saccharomyces cerevisiae* AD1-7 strain expressing lacZ (**a**) (grey/black) *Pun*Pgp-2 (**b**) (purple/black) or *Pun*Pgp-9 (**c**) (blue/black) in the presence of a dilution series of ivermectin either with or without (black) a maximum tolerated ketoconazole concentration (0.73 µM in AD1-7Pgp-2 and Pgp-9 and 0.2 µM in AD1-7lacZ) on a 48 well plate for 48 h, measuring O D_{600} every 10 min for 12 replicates on three separate days. Then, following blanking to empty wells, the area under the curve was calculated which was normalised to the growth of the negative no drug control of the same day. For each strain, relative growth was compared at each concentration in the presence or absence of ketoconazole using a two-way ANOVA followed by a Dunn's post-hoc test. Calculations were done using R version 3.6.2[87] and package dunn. test[93]. **p < 0.01, ****p < 0.0001. *Pgp* P-glycoprotein.

Discussion

Pgps are central components of the xenobiotic detoxification machinery in nematodes and the evolutionary success of this gene family in nematodes is reflected by its high diversity. For the first time the present study has identified the complete, manually curated Pgp repertoire of a clade III nematode with reliable gene models. The *P. univalens* repertoire comprising 10 Pgps resembles the *A. suum* repertoire and is similar in size to that of other parasitic nematodes such as 10 in *H. contortus*[59] and 8 in *B. malayi*[60]. In addition, for the first time evidence for an interaction between *Pun*Pgp-2 and *Pun*Pgp-9 with IVM was provided, whereas TBZ did not appear to be a substrate of either of the two Pgps.

In ascarids, the evolution of this gene family remains a dynamic process in recent evolutionary history with gene duplications and subsequent divergence in Pgp-11 and Pgp-16 and a novel ascarid-exclusive lineage, Pgp18. The closer relatedness between nematode Pgp lineages compared to the outgroup containing mammalian, mollusc and arthropod Pgps confirms the expansion of Pgps in an early nematode ancestor, but several nematode groups have lost some ancient or evolved new Pgp lineages. Regarding the novel Pgp-18 lineage, the lineage branches deep in the Pgp family but support for the particular position of this cluster appears to be comparatively weak in our phylogenetic analysis (62/73). No matter if it should be considered as a sister group to Pgp11/12/13/14 or alternatively to Pgp-3/4/16, the position suggests that this family has split from the other clusters early in the evolution of ascarids. Comparably, the duplications in *Pun*Pgp-11 and *Pun*Pgp-16 have probably occurred after ascarid secession from filarial worms but before the differentiation into extant ascarid species, as orthologues for both *Pun*Pgp-11 paralogues can be found not only in *P. univalens* and *A. suum* but also the more distantly related *T. canis*. Both *Pun*Pgp-16 paralogues have at least orthologues in *A. suum*. Currently, genomic resources for *P. univalens* are extremely limited (only one genome representing a small number of individuals) but it is likely that Pgp evolution is continuously driven through treatment practices. Furthermore, our results indicate that there is a likelihood of alternative splicing in Pgps of nematodes, e.g. as demonstrated for *Pun*Pgp-18. The calculated support (FPKM) for individual exons suggests several alternative splicing events based on present or absent exon support in the different tissues. However, alternate endings of Pgps found in the different contigs and in the annotation as well as alternating support of individual exons in different tissues (Supplementary Fig. S1) could not be confirmed by RT-PCR. Missing exons in the Wang et al., 2017 annotation concentrated at 5′- and 3′ ends (Supplementary Fig. S1) and led to truncated gene models. While we cannot exclude isoforms for Pgps that were not identified with our approach, we were able to significantly improve the annotation of all *P. univalens*. Future improved genome assembly may allow further refinement of the annotation of these genes.

In GenBank, the inventory of nematode Pgps is immense and chaotic, i.e. many published and annotated Pgps including those published for *A. suum* do not adhere to wormbase.org nomenclature guidelines and some genes annotated as hypothetical Pgp are likely to be artefacts of automated assembly and annotation pipelines. Consequently, many of the deposited sequences and gene-models are unreliable and incorrectly named, hindering comparative analysis. Automated annotation and transcriptome resources have immense value. However, as has been demonstrated in model organisms, genome data from next-generation sequencing, automatic assembly and annotation should be validated by independent experimental methods for studies on particular genes and transcripts. Furthermore, transgenic expression and functional analysis as conducted in this study demonstrate that identified protein sequences lead to functional genes.

Considering the efflux mechanism of Pgps, it can be assumed that in order to contribute to resistance a high expression level is necessary. However, from the data provided by the IVM transcriptome dataset, no significant upregulation of any Pgp was detected following IVM treatment. Nonetheless, in a previous study, worms from a resistant *P. univalens* population showed a small but significant higher expression of *Pun*Pgp-11.1 but not of *Pun*Pgp-16.1[44] but other Pgps were not examined due to the lack of validated Pgp sequences in gene databases. In the present study, the resistance status of IVM incubated worms was unknown and it may be that the worms had a susceptible phenotype, which could be a potential reason for the observed absence of IVM treatment associated inducibility of Pgp

genes. From a methodical point of view, it is possible that the IVM concentration and incubation duration are responsible for the encountered lack of significant transcriptomic response or that

the artificial culture conditions were unsuited to induce an upregulation of xenobiotic transporters upon IVM encounter in vitro. The experimental set-up has several limitations, for example it does not include a positive control, which is difficult to design since nothing is known about gene regulation in *P. univalens*. Furthermore, some other limitations set by culture conditions to transcriptomes of ascarids derived from in vitro culture were recently revealed[61]. However, with regard to studies of other nematodes e.g. *C. elegans*[62] or *T. circumcincta*[29] it appears most likely that constitutively high expression levels of Pgps that can contribute to resistance may be selected over many generations as a heritable trait.

Interestingly, the Pgp intestinal expression by far exceeded that of the other tissues, substantiating evidence for a Pgp-mediated barrier function against uptake of xenobiotics including anthelmintics at the intestine, but this mechanism is not yet understood. As the population of worms used for the tissue specific transcriptome study has never been treated with MLs, it appears that the strong intestinal expression level is innate to *P. univalens.*

Likewise, the similarly strong intestinal expression of *C. elegans* Pgps allows the assumption that strong intestinal Pgp expression might be conserved in nematodes, but not at the individual gene level. In *P. univalens*, *Pun*Pgp-9, -11.1 and -16.2 consistently exhibited the strongest expression throughout both independent transcriptome data sets, but in *C. elegans* a different repertoire of high expression Pgp was identified, including Pgp-1 and Pgp-14 which might also be attributed to the different developmental stages. Pgp expression in the carcass tissue (hypodermis, muscle, neuron and pharynx) was second strongest, with the aforementioned set of identical dominant Pgps. It remains unclear whether their protective role to MLs is of any importance in the hypodermis below the cuticle or even directly at the target sites in neurons. To this end, *Pun*Pgp-11.1 and *Pun*Pgp-16.1 mRNA expression was recently detected at the H-shaped excretory system, which forms a continuous canal from the nerve ring to the middle of the body, and the nerve cords. The expression pattern of mRNA expression levels from the same study are in line with our findings[63]. Strikingly, in a previous study, a *C. elegans* strain with a loss-of-function allele of the predominantly pharyngeally expressed *Cel*Pgp-14 showed the strongest IVM susceptibility increase of all *C. elegans* Pgp mutant lines in a development a ssay[51]. Moreover, this Pgp was upregulated in an IVM resistant (avr-14/avr-15/glc-1 deficient) *C. elegans* strain[64], suggesting that Pgp expression at the pharynx could protect relevant regulatory neurons and alter IVM susceptibility. Further investigations on the mechanism and role of tissue specific ABC-transporter expression in nematodes are needed, e.g. tissue specific transcriptomic analysis upon ML or other anthelmintic exposure as small to moderate gene expression fold changes of individual genes can be missed when analysing whole worm samples.

To compare the Pgp transport potential of important anthelmintics, two previously resistance associated Pgp orthologues were chosen for functional characterization, with *Pun*Pgp-2 being a moderate expression Pgp and *Pun*Pgp-9 a high expression Pgp. Transgenic expression of *Pun*Pgp-11.1 was previously shown to decrease IVM susceptibility of *C. elegans*[52]. Here, we show that both *Pun*Pgp-2 and *Pun*Pgp-9 can also interact with IVM. This confirms previous reports[47–49,51,53,55] that different Pgp orthologues can transport MLs (in this case IVM) which also explains why following selection pressure different Pgp and other ABC-transporters underwent expression changes depending on the study and the nematode species[56,62,65]. Our results support that both *Pun*Pgp-2 and *Pun*Pgp-9 are transmembrane transporters and excrete KCON and IVM out of the yeast cells. Notably, for Pgp to effectively reduce the effective concentration of a toxin or drug in nematodes, for example by efflux in the intestine, their localisation and predominant efflux activity at the apical intestinal membrane is a prerequisite. Conversely, localisation at the baso-lateral surface would result in influx and thus increased susceptibility. For example, *C. elegans* Pgp-1 was shown to localize exclusively to the apical membrane[66]. In addition to their role in ML detoxification, Pgps have been linked to resistance against other anthelmintics such as benzimidazoles[67] and more recently monepantel[68]. However, the results of the present study do not indicate an interaction between either *Pun*Pgp-2 or *Pun*Pgp-9 and the benzimidazole derivative TBZ. Nonetheless, the demonstrated interaction between both Pgps and the structurally unrelated KCON and IVM show that these Pgps are indeed multi-drug transporters with a

wide substrate range and their interaction with monepantel remains to be elucidated. With respect to the conserved large repertoire of Pgps in nematodes, it also appears likely that several Pgps are also involved in endogenous physiological processes. For instance, *Cel*Pgp-2 has been linked to lipid storage[69] and in *P. univalens*, *Pun*Pgp-10 and -11.2 expression is focused to the ovary (Fig. 3a), suggesting a role in reproductive processes. Noteworthy, while Pgp-2 orthologues have an important physiological function in *C. elegans*, the results of this study show the broad substrate range of a Pgp-2 orthologue which results in functional flexibility. Therefore, it can be speculated that evolutionary changes in the life cycle and behaviour accompanied by changes in tissue expression patterns alter the actual function and role of a Pgp orthologue. In the context of resistance and with respect to the previously mentioned differences in the Pgp repertoire implicated with ML resistance in different nematode species, it is likely that the Pgp expression pattern in target and barrier tissues of individual Pgp in a specific nematode population at the time of treatment is a major confounding factor influencing which Pgp contribute to ML resistance.

In conclusion, this study provides a valuable resource for Pgp-mediated anthelmintic resistance research through improved and reliable gene models from experimentally verified Pgp sequences in *P. univalens.* Furthermore, the analysis of tissues specific expression level and characterization of the interaction of two *P. univalens* Pgps with two important anthelmintic classes represented by IVM and TBZ, improve the foundation for understanding Pgp-mediated anthelmintic resistance. To this end, there is an urgent need to improve the understanding of how individual Pgp orthologues interact with different ML derivatives and contribute to ML resistance as well as to elucidate the functional role of tissue specific expression of individual Pgps.

Materials and methods

Biological material, genome and transcriptome resources. Two independent transcriptome data sets of *P. univalens* were used in this study.

A previously published total RNA transcriptome data set (GeoDataset accession: GSE99524; GenBank transcriptome assembly accession: GCA_002259205.1 WormBase ParaSite version WPBS14) was used which included samples with two replicates each of male and female gonads, embryo, intestine, carcass (remainder after dissection of intestine and germline tissue including pharynx, hypodermis, muscle and neuronal tissue) as well as a male and female mixed sample, the latter consisting of a 5′-untranslated region (UTR) spliced leader PCR library and a 5′-UTR TeloPrime PCR l ibrary[56], referred to as tissue specific transcriptome. Originating from the same study, a reference genome assembly (GenBank assembly accession: genome assembly ASM225920v1, version WBPS14) and an *Ascaris suum* transcriptome assembly (GenBank transcriptome assembly: GCA_000187025.3) were used[56]. The corresponding annotation of the *P. univalens* reference genome assembly was obtained from WormBase ParaSite, version WBPS14. The worms for this study are from a research herd maintained at the University of Kentucky with no anthelmintic usage since 1979, which means no prior exposure to M Ls[70].

A second total RNA transcriptome data set was generated from a total of 10 individual *P. univalens* adult worms, referred to as IVM-transcriptome. All *P. univalens* were collected from an abattoir in Kraków and their resistance status and treatment history was unknown. For in vitro culture, only female worms were selected and handled as described previously[44]. In brief, worms were incubated at 37 °C in artificial perienteric fluid (APF, 5 mM $MgCl_2$, 6 mM C aCl_2, 24 mM KCl, 23 mM NaCl, 110 mM N aCH_3COO, 11 mM dextrose, 10 mM Tris and was adjusted to a final pH of 7.5 at 37 °C) in ventilated tissue culture flasks, 1–2 worms per flask, for 18 h. Then, 10 worms were further incubated in pairs of two in fresh APF containing 10^{-9} M IVM (Sigma-Aldrich) for twelve hours while 10 control worms were incubated only in the presence of the vehicle (1% DMSO). After incubation, individual worms were rapidly frozen on dry ice and stored at − 80 °C until use. RNA was isolated from all worms using the whole worm and TriFast reagent (Peqlab). Before further use, quality of all RNAs was controlled on the Bioanalyzer 2,100 (Agilent) with the RNA 6,000 Nano Kit. All RNAs used in the experiments had RNA integrity numbers (RIN) ≥ 9.1. In order to confirm the sex of worms as obtained using morphological criteria, an RT-PCR targeting the vitellogenin-6 RNA was conducted and only vit-6 positive samples were included[44]. For deep-sequencing, five control and five samples

from IVM incubated worms were chosen. After construction of 10 individual (barcoded), random-primed libraries for paired-end sequencing, all libraries were sequenced together on a single lane of an Illumina HiSeq 2000 to obtain 100 bp paired end reads. CASAVA software version 1.8.2 (Illumina) was used to demultiplex all samples and to clip adapters from all reads. Ribosomal RNA sequences were identified and filtered out using RiboPicker 0.4.3[71]. Data were quality filtered by (i) removing all reads containing more than one N, (ii) removal of bases or complete reads containing sequencing errors, (iii) trimming of reads at the 3′-end to obtain a minimum average Phred quality ≥ 10 over a window of 10 bases and (iv) discarding all reads with less than 20 bases left after the above quality adjustments. Worms from the same population as the IVM-transcriptome were used for RNA extraction for RT-PCR[44].

Saccharomyces cerevisiae strain AD1234567 (AD1-7) deficient in seven endogenous ABC-transporters[72] was kindly provided by André Goffeau (Université Catholique de Louvain, Belgium). The strain was maintained and grown at 30 °C in Yeast Extract-Peptone-Dextrose (YPD) medium plates (10 g yeast extract, 20 g peptone, 20 g dextrose, 15–20 g Agar in 1 L H_2O bidest, (Sigma-Aldrich)) at 250 rpm or on YPD agar plates with 15–25 g/L additional agar.

All reagents were purchased from Thermo Fisher Scientific unless stated otherwise.

Amplification and cloning of full-length P-glycoproteins. To identify contigs encoding ABCB transporters, both transcriptomes were analysed with NCBI software Basic Local Alignment Search Tool (BLAST)[73] TBLASTN using *P. univalens* Pgp-11.1 and Pgp-16.1 protein sequences (GenBank accession numbers in Supplementary Table S3) as query. To examine full gene coverage, the identified contigs were then compared to the other nematode Pgps in the GenBank database using BLASTX. For each putative Pgp, the contig or a combination of contigs with the highest coverage of a complete Pgp was used for the following steps, while putative halftransporters were excluded. Gene specific primers for RT-PCR were designed manually from 3′- and 5′- UTR of all potential alternative endings for amplification of full length Pgps. For contigs spanning merely a fraction of a Pgp gene, primer pairs were designed at the 5′- and 3′-ends of each sequence (all primers in Supplementary Table S2).

First strand cDNA was synthesized from 1–2 µg of total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions. The PCR reaction mixture contained ca. 1–2 ng/µL cDNA, 0.02 U/µl Phusion Hot Start II polymerase, 1 M betaine (Sigma-Aldrich), each primer at 0.5 µM and each dNTP at 200 µM in 25 µL 1 × HF buffer. An initial denaturation at 98 °C for 30 s was followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at a primer pair specific Tm (Supplementary Table S2) for 30 s and elongation at 72 °C for fragment specific elongation time (Supplementary Table S2). A final elongation was performed at 72 °C for 300 s. Purified PCR products were directly cloned using the StrataClone Blunt PCR Cloning Kit (Agilent) according to the manufacturer's instructions. Extracted plasmids (ZymoPURE II Plasmid Midiprep Zymo Research) were verified by restriction analysis and sequenced by primer walking technique (LGC Genomics).

In case of missing transcriptome sequence data at the 5′- or 3′-end, contig sequences were first verified by RTPCR as described earlier followed by the 5′-end amplification by nested RT-PCR using a primer targeting the *A. suum* spliced leader s equence[74] as a forward primer and two gene specific reverse primers. 3′-end amplification was performed using the 5′/3′ RACE Kit, 2nd Generation (Roche) according to the manufacturer's instructions (all primers Supplementary Table S2).

Phylogenetic analysis. Pgp protein sequences of nematodes and a representative outgroup were obtained from GenBank and WormBase (GenBank accession numbers or WormBase IDs in Supplementary Table S3). Additionally, *A. suum* transcripts were obtained through BLASTX analysis of the latest transcriptome assembly using *P. univalens* cDNA sequences as query. Open reading frames were then manually corrected using the *P. univalens* orthologue as a template. Alignment was carried out using M-Coffee[75–78] with default parameters. For determination of an optimal amino acid substitution model, the alignment was analysed with ProtTest 3.0[79]. Thereafter, a consensus tree was calculated using RAxML 8.2.9[80] and the LG + F + G model[81]. with 1,000 bootstrap replicates allowing both nearest neighbour interchange (NNI) and subtree pruning and regraftment (SPR) moves. Using

the tree obtained by bootstrapping to restrict the tree topology, the Shimodaira-Hasegawa (SH) approximate likelihood ratio test was used to calculate additional branch support values in RAxML. To prevent potential analysis errors due to the high variability in certain sequence regions, a second tree was computed following analysis and editing of the original alignment with GBLOCKS 0.91b[82] with default parameters to exclude highly variable positions. Finally, the best tree was visualized using MEGA 7[83] rooting the tree using the outgroup.

Re-annotation of the *Parascaris univalens* genome and expression analyses. Genomic coordinates of intron and exon borders were predicted in the *P. univalens* genome (assembly ASM225920v1, WormBase ParaSite version WPBS14) based on mapping of the cDNA sequences using the NCBI tool Splign (version 2.1.0; based on compart version 1.35)[84]. Coordinates were converted to gff3 format and used to "patch" the genome annotation (removing the original annotation in the overlapping areas). Mapping of raw reads of individual sample data sets against the genome was conducted using STAR (version 2.5.4b)[85] and expression levels of Pgps were estimated using featureCounts[86] against the patched annotation. Resulting raw counts were normalised for the overall size of the sequencing library as Transcripts per Kilobase Million (TPM).

TPM expression levels were visualized with GraphPad Prism version 8.3.3 (GraphPad Software, San Diego, California USA, https: //www.graphpad.com). For the IVM incubated transcriptome, each data point was visualized and differences in expression levels of individual Pgp were analysed with the Kruskal–Wallis test followed by a Dunn's Post-Hoc ($\alpha = 0.05$) test with corrections for multiple testing. For the tissue specific transcriptome data set, the mean of two replicates was visualized and no statistical analysis was conducted. Finally, single cell transcriptome was used to compare tissue expression levels, calculated with R [87] package monocle3[88–90] of individuals Pgps in *C. elegans* L2[58] (Geo Accession number Accession: GSE98561). For visualization of low expression tissues, expression was visualized on a $\log_{10}$ scale and for better comparison of overall expression levels, on a linear scale. Mappings (bam files from STAR) were also analysed using the R package S GSeq[91] to visualize coverage of individual exons (as FPKM) in a heatmap. For comparison of the new annotation to the original Pgp annotations from Wang et al., 2018 (WormBase ParaSite version WPBS14), both annotations were visualized with Geneious Prime 2019.2.3 (https ://www.genei ous.com) and then combined with exon coverage heatmaps of the experimental cDNA exons using CoralDRAW X7 (https ://www.coral draw.com).

Plasmid assembly. cDNA sequences of *Pun*Pgp-2 and *Pun*Pgp-9 were codon optimized for expression in *S. cerevisiae* and synthesized excluding the stop codon (BioCat GmbH) (sequences in Supplementary Table S2) in two overlapping parts and then assembled by PCR. For each gene, a reaction mixture was prepared containing the two fragments at equimolar concentrations of 5 fmol each along with 0.02 U/µL Phusion Hot Start II polymerase (Thermo Fisher Scientific), each dNTP at 200 µM in 25 µL 1 × HF buffer. Denaturation at 98 °C for 30 s was followed by 12 cycles each of denaturation at 98 °C for 10 s, annealing at 68 °C for 30 s and elongation at 72 °C for 200 s. Then forward and reverse primers (Supplementary Table S2) were added at 0.5 µM to 28 µL total volume in 1 × HF buffer matching the 5′ and 3′ end of the sequence and cycled 35 times under the same conditions and ending with a 5 min elongation. Purified PCR products were incubated with Taq DNA Polymerase (Thermo Fisher Scientific) in 20 µL of 1 × Taq DNA Polymerase Buffer, 2 µL $MgCl_2$ for 72 °C at 20 min to add 3′ desoxyadenosine overhangs. Then, products were purified and ligated into the pYes2.1 vector followed by transformation using the pYES2.1 TOPO TA Yeast Expression Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequences of plasmids extracted from *Escherichia coli* Top10 cultures were validated by Sanger sequencing (LGC Genomics).

Transformation into *Saccharomyces cerevisiae*. pYES2.1-his plasmids containing *Pun*Pgp-2 and *Pun*Pgp-9 or the β-galactosidase gene (lacZ) (supplied control vector) were transfected by the lithium acetate method into AD1-7 yeast using the pYES2.1 TOPO TA Yeast Expression Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Successfully transformed yeast were grown for 48 h on synthetic minimal medium plates lacking uracil (SC-U) (6.7 g/L yeast nitrogen base

with amino acids, 1.92 g/L yeast drop-out medium supplement without uracil, 2% glucose, 1% raffinose and 2% agar, omitting agar when making synthetic minimal medium, see recipes in user manual pYES2.1 TOPO TA Yeast Expression Kit, Sigma-Aldrich).

Verification of expression by RT-PCR. Transcription of *Pun*Pgp-2 and *Pun*Pgp-9 was analysed by RTPCR. Yeast were grown in induction media containing galactoses (SC-U medium but substituting the carbon source to 2% galactose and 1% raffinose) which induces activation of the GAL1 promotor and results in expression of the inserted gene. After 24 h of incubation at 30 °C and 250 rpm, total RNA was extracted using the Maxwell Simply RNA kit (Promega). cDNA was synthesized using the Maxima H- First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) from ~ 1 µg of RNA using Oligo$(dT)_{18}$Primers according to the manufacturer's instructions. RT-PCR was performed using 0.02 U/µL Phusion Hot Start II polymerase (Thermo Fisher Scientific), 0.5 µM each primer, each dNTP at 200 µM, 1–2 ng/µL cDNA in a total volume of 25 µL 1 × HF buffer for two different primer sets (Primers in Supplementary Table S2). After a denaturation at 98 °C, 35 cycles of 98 °C for 10 s, a primer pair-specific annealing temperature for 15 s and 72 °C for 30 s were performed. Amplified PCR product were visualised after gel electrophoresis with GrGreen.

Yeast growth inhibition assay. Yeast growth inhibition assays were performed in principle as explained elsewhere [47,92]. Briefly, prior to the actual assay transgene expression of *Pun*Pgp-2, *Pun*Pgp-9 or the lacZ gene was induced in AD1-7 *S. cerevisiae* strain by growing yeast in 5 mL induction media at 30 °C and 250 rpm for 24 h. Then, after harvesting the yeast by centrifugation, yeast cells were seeded at 4×10^4 cells/well into a 96 well plate in a final volume of 100 µL induction medium containing drugs as described below dissolved in DMSO at a final concentration of 1% (IVM and KCON) or directly in water (TBZ). The plates were sealed with parafilm to avoid concentrations shifts due to evaporation and incubated in the Synergy 4 plate reader (Biotech) for 48 h at 30 °C and 250 rpm shaking. Every 10 min, shaking was interrupted and absorption at 600 nm (OD_{600}) was measured.

To determine the susceptibility of yeast strains to KCON and TBZ, yeast were incubated with a dilution series of KCON (2.94 µM, 2.2 µM, 1.46 µM, 1.09 µM, 0.37 µM and 0.18 µM, 0.00 µM KCON) at a final total 1% DMSO concentration or TBZ (2.4 mM, 1.2 mM, 0.6 mM, 0.3 mM, 0.15 mM, 0 mM TBZ) without DMSO generating a minimum of 12 replicates split equally on three separate days. For *Pun*Pgp-2 and *Pun*Pgp-9 expressing strains the maximum tolerated KCON concentration of 0.4 µM was chosen and co-incubated with an IVM dilution series (0.0 µM, 0.1 µM, 0.2 µM, 0.4 µM, 0.73 µM, 1.5 µM, 2.9 µM, 4.4 µM, 5.9 µM IVM) at a final total 1% DMSO concentration and also generating a minimum of 12 replicates split equally on three separate days.

Analysis was done similarly as described elsewhere[47] and all calculations and visualizations were conducted using GraphPad Prism 8.3.0 unless specified otherwise. In brief, raw absorption data which correlates with yeast growth was exported from Gen5 Data Analysis Software (BioTek), blanked to wells containing no yeast and only medium and excluding the first 50 measurements. Then, the area under the curve (AUC) was calculated for each well. To determine relative growth, the AUC was then normalised to the mean AUC of the negative controls (containing no TBZ, no KCON but 1% DMSO or IVM or for IVM + KCON co-incubation a fixed KCON concentration) of the same yeast strain and on the same day. For non-linear regression analysis concentrations were $\log_{10}$ transformed. For this, the 0 M concentration (no drug negative controls) were set to 0.1 µM KCON and 0.7 µM TBZ. To determine susceptibility to directly antimycotic substances KCON and TBZ, four parameter non-linear regression models (model: Y = Bottom + (Top − Bottom)/(1 + 10^((Log EC_{50} − X)*Hillslope)) were calculated from a minimum of 12 replicates per concentration and due to the normalisation to relative growth top values were constrained to a maximum of 1 and bottom values to a minimum of 0. From the non-linear regression models, half maximal effective concentrations (EC_{50}) and corresponding 95% confidence intervals as well as R^2 values as a determinant for goodness of fit were calculated. To compare susceptibility of AD1-7Pgp-2 and AD17Pgp-9 to AD1-7lacZ, the extra sum-of-squares F test for the L ogEC_{50} was used and p-values were corrected for multiple testing with the Holm's method in R version 3.6.2[87], considering corrected p-values > 0.05 as significant.

The direct inhibitory effect of IVM in the presence or absence of yeast was analysed in R. First, direct effect of IVM on yeast growth was analysed for using a one-way analysis of variance (ANOVA) followed by a Tukey's HSD post-hoc test with Holm's p-value adjustment. Then, using a two-way ANOVA the effect on yeast growth of IVM concentration (factor 1) in the absence or presence of a fixed KCON concentration (factor 2) was analysed. To analyse differences between the absence and presence of KCON at each IVM concentration within each strain, a Dunn's post-hoc test[93] was conducted with Holm's p-value adjustment. For all analyses, an adjusted p-value less than 0.05 was considered significant.

Data availability

BankIt2307849 PunPgp-2: MT001899, BankIt2307849 PunPgp-3: MT001900, BankIt2307849 PunPgp-9: MT001901, BankIt2307849 PunPgp-10: MT001902, BankIt2307849 PunPgp-11.2: MT001904, BankIt2307849 PunPgp-12: MT001905, BankIt2307849 PunPgp-16.2: MT001907, BankIt2307849 PunPgp-18A: MT001908, BankIt2307849 PunPgp-18B: MT001909. The updated annotation (gff3) of P-glycoproteins has been shared with WormBase ParaSite and will be available on the next WormBase Parasite release.

Received: 3 April 2020; Accepted: 21 July 2020

Published online: 12 August 2020

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Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (grant number 111144555). Additional funding was provided by the DFG (grant number 251133687/GRK 2046) and the Karl-Enigk Foundation (grant number S0229/1100191/19). Open access funding provided by Projekt DEAL. Deutsche Forschungsgemeinschaft Grant Number: 111144555. Deutsche Forschungsgemeinschaft Grant Number: 251133687/GRK 2046.

Author contributions

A.G.: Investigation, Conceptualization, Formal analysis, Data Curation and Visualization, Writing—original draft preparation, review and editing; J.K.: Conceptualization, Formal analysis, Writing—review and editing, Supervision, Funding acquisition; E.H.: Conceptualization, Data Curation, Formal analysis, Writing—review and editing, Funding acquisition; J.I.J.: Investigation, Resources, Writing—review and editing; M.B.: Resources, Writing—review and editing; SK: Resources, Writing—review and editing; C.B.: Investigation; R.D.: Resources, Writing—review and editing; J.W.: Resources, Writing—review and editing; M.K.N.: Resources, Writing—review and editing; G.V.S.: Conceptualization, Supervision, Writing—review and editing, Funding acquisition.

3.1.1 Supplementary Material

Supplementary information is also available for this paper at https://doi.org/10.1038/s41598-020-70529-6.

Supplementary Material

Supplementary Figure S1 Comparison of annotation of Parascaris univalens P-glycoproteins

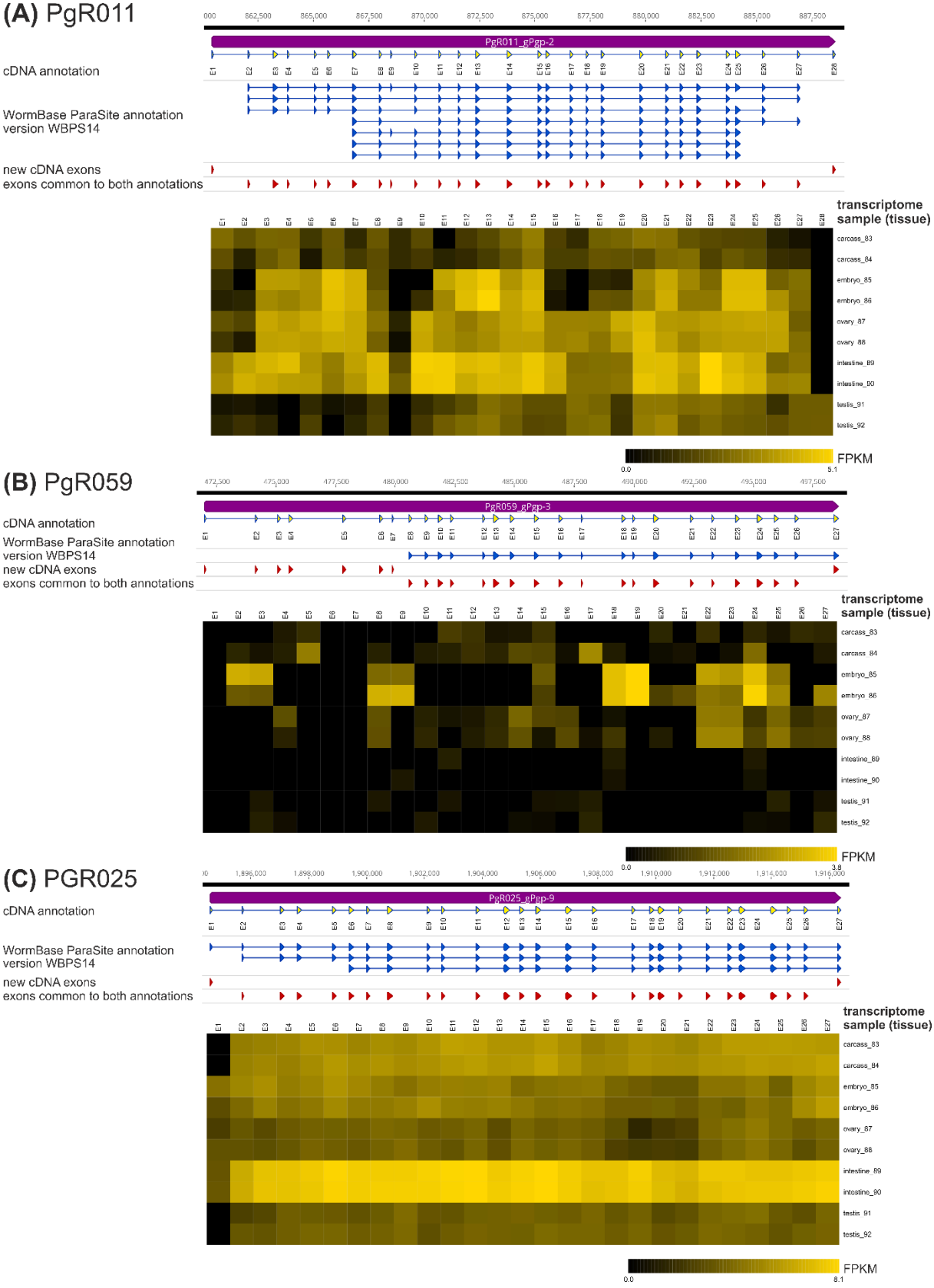
Supplementary Figure S2 Tissue expression levels

Supplementary Figure S3 RT-PCR *Pun*Pgp-2 and *Pun*Pgp-9

Supplementary Table S1 Contig Coverage

Supplementary Table S2 Primers and sequences

Supplementary Table S3 Accession numbers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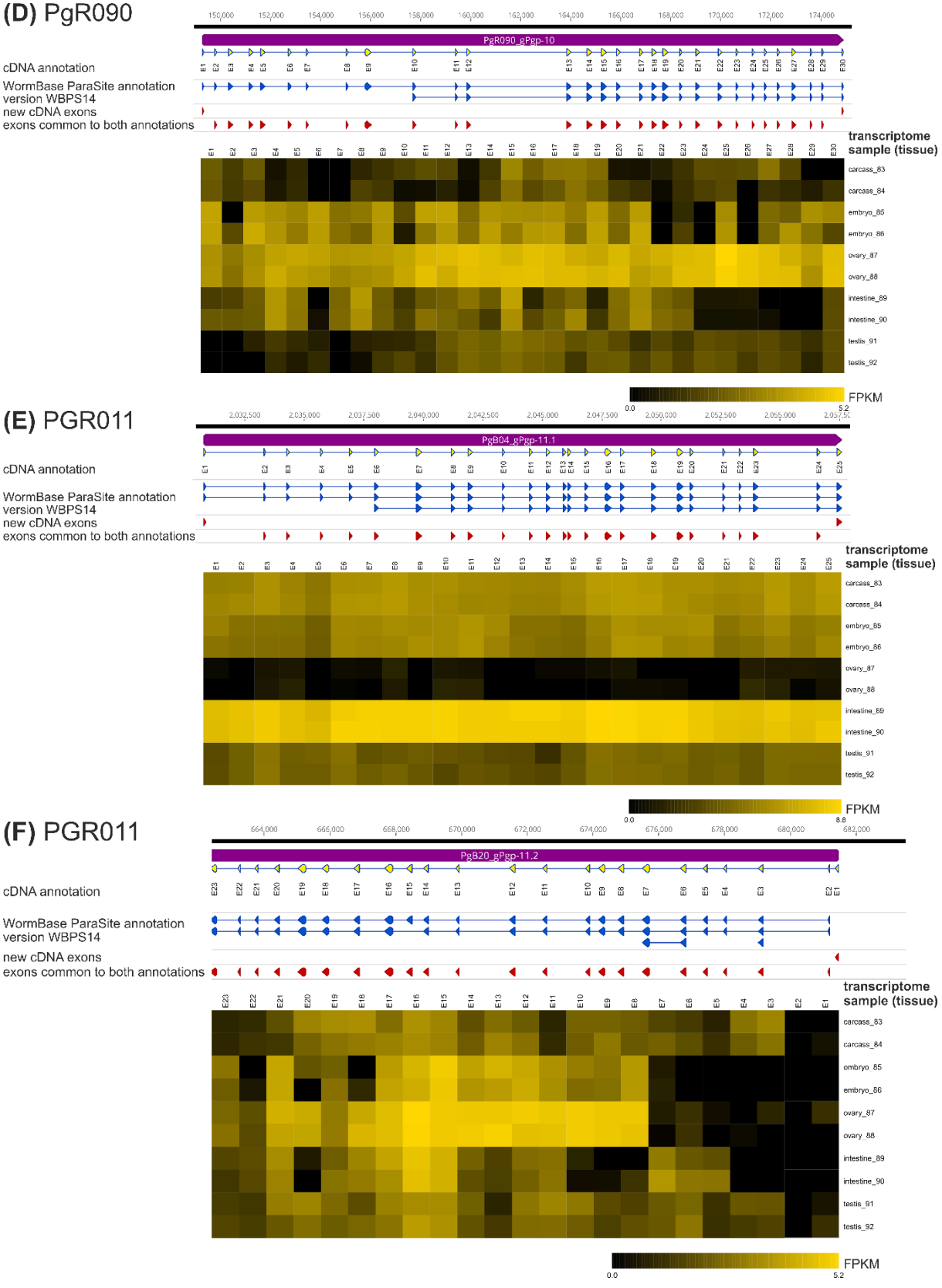
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intestine_89
intestine_90
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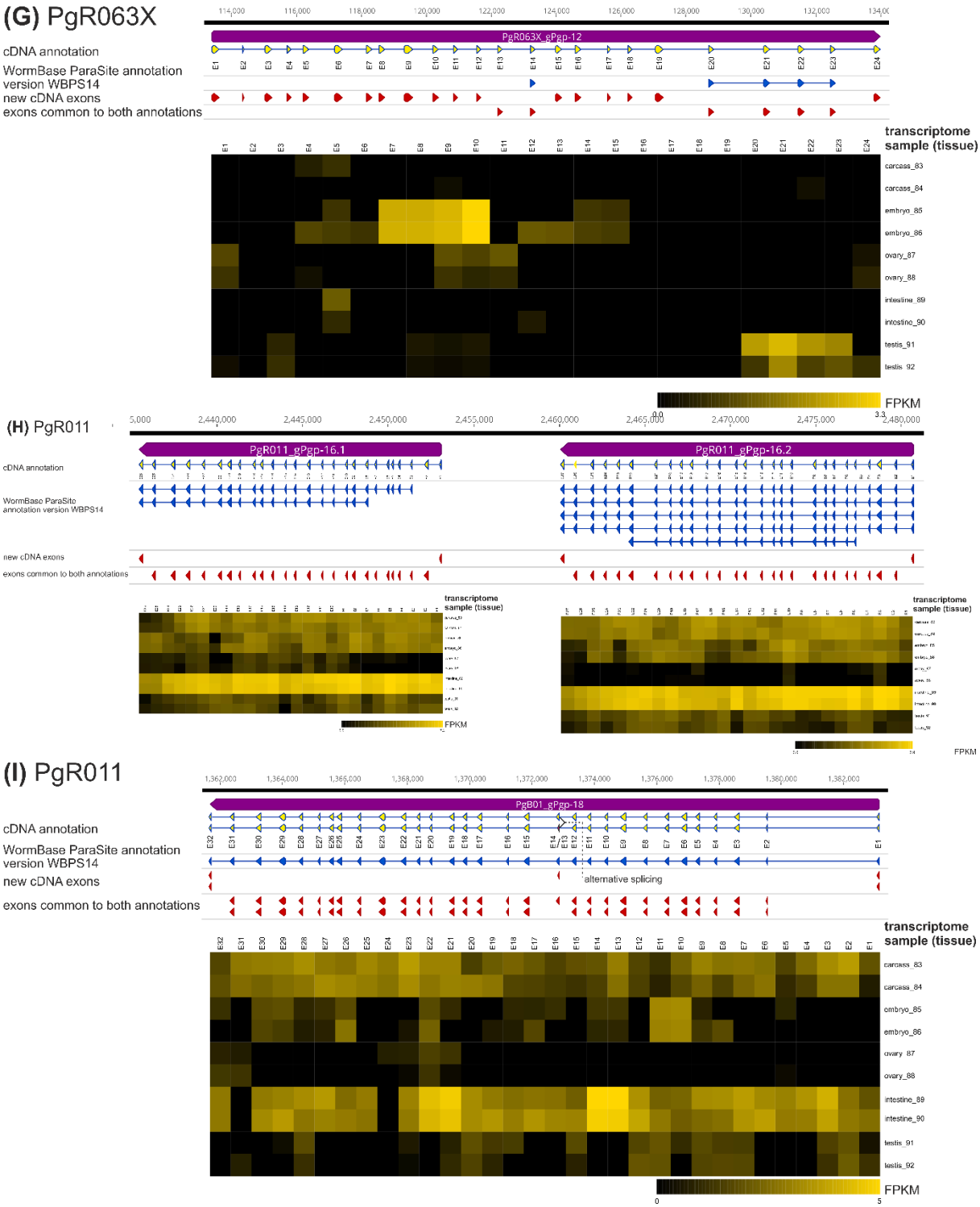
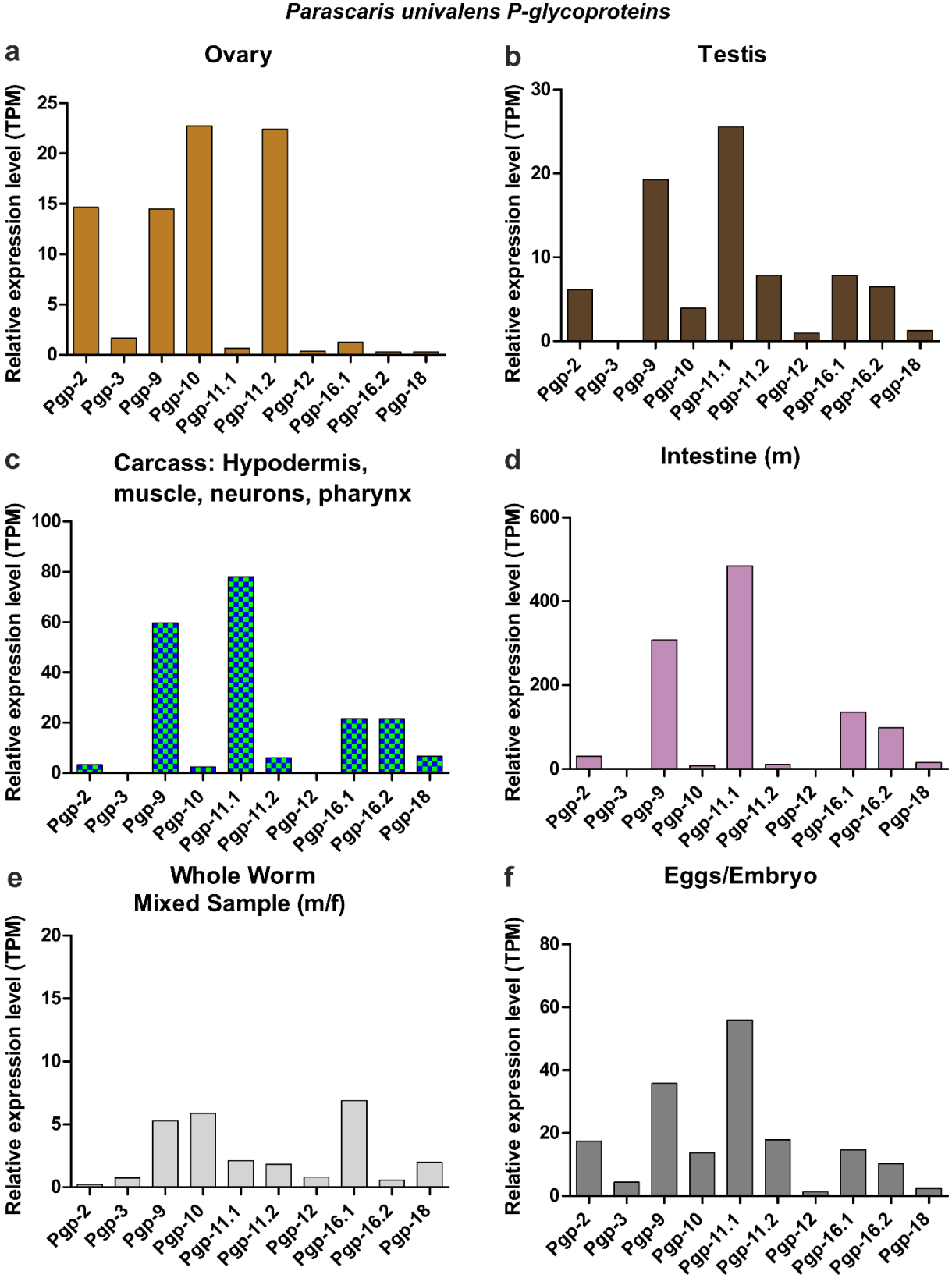


Supplemental Figure S1 Annotation of *Parascaris univalens* Pgp

Improved annotation of experimentally verified *Parascaris univalens* Pgp exons (yellow) numbered 5' to 3' end were compared to original automatic annotation (WormBase ParaSite annotation version WBPS14, blue) and differences of new exons and exons common to both annotation highlighted (red). cDNA sequences of Parascaris Pgps were mapped onto the *P. univalens* genome with Splign version 2.1.0 to identify genomic coordinates of exons. Visualisation of original annotation and the newly generated annotation was made using Geneious Prime 2019.2.3. Coverage of individual exons of the new experimental cDNA exons was calculated with R package SGSeq and visualized as a heatmap (FPKM, yellow) for each tissue sample of the tissue specific transcriptome (GeoDataset accession: GSE99524). For

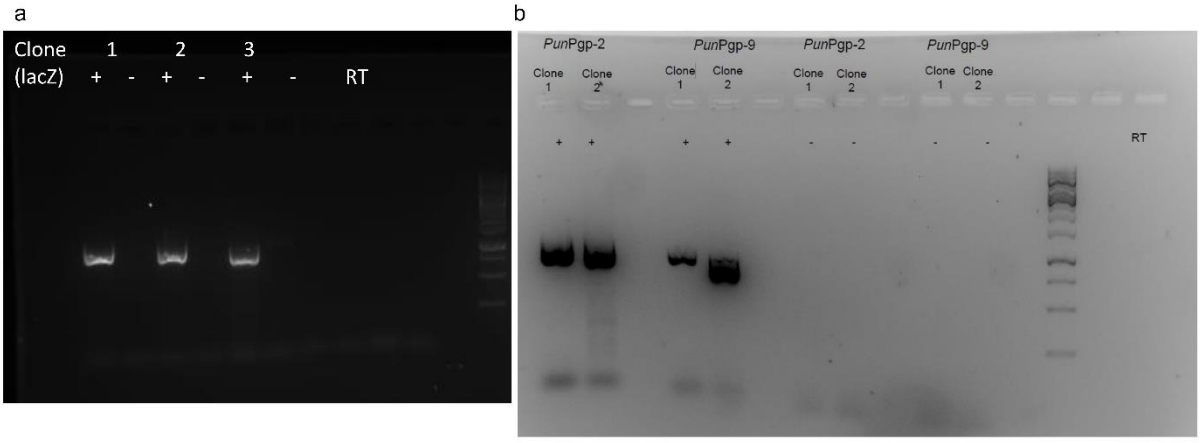
each Pgp (A) Pgp-2, (B) Pgp-3, (C) Pgp-9, (D) Pgp-10, (E) Pgp-11.1, (F) Pgp-11.2, (G) Pgp-12, (H) Pgp-16.1 and Pgp-16.2, (I) Pgp-18 were visualized on the respective genomic locus of the respective genomic scaffold (genome assembly ASM225920v1, version WBPS14). Pgp: P-glycoprotein



Supplementary Figure S2 Tissue expression levels of *Parascaris univalens* P-glycoproteins

Expression levels of *Parascaris univalens* Pgp were calculated based on transcriptome raw reads data sets (Geo Accession GSE99524, samples GSM2645460 to GSM2645464) mapped onto the the augmented (with *P. univalens* Pgp cDNA sequences) *P. univalens* genome (WormBase Parasite genome assembly ASM225920v1, version WBPS14) using STAR. Expression levels were normalised as transcripts per million (TPM) from raw read counts obtained with featureCounts. Then expression levels were visuliased by tissue of (a) the ovary (light brown), (b) the testis (brown), (c) carcass tissue including the epidermis, neurons, muscles and the pharynx (blue and green), (d) intestine (purple), (e) a whole worm sample (5'-TeloPrime library), (f) eggs. Pgp: P-glycoprotein; m: Male; f: female; TPM: transcripts per million

Supplementary Figure S3 RT-PCR of Saccharomyces cerevisiae AD1-7 expressing *Pun*Pgp-2, *Pun*Pgp-9 or lacZ



Supplementary Figure S3 RT-PCR of *Saccharomyces cerevisiae* AD1-7 expressing *Pun*Pgp-2, *Pun*Pgp-9 or lacZ
Saccharomyces cerevisiae AD1-7 expressing lacZ, *Pun*Pgp-2 or *Pun*Pgp-9 were generated through transformation. Following total RNA extraction and cDNA synthesis including a no-reverse transcriptase (RT) control, RT-PCRs were performed. PCR products were visualised with Gel-electrophoresis. Several clones were screened but only clone 1 for all genes was used for analyses. Pgp: P-glycoprotein. RT: Reverse transcriptase

Scientific Reports | (2020) 10:13586 | https://doi.org/10.1038/s41598-020-70529-6

Supplementary Table S1
Contig Coverage of ORFs in the tissue specific transcriptome assembly

***Pun*Pgp-2**	cDNA ORF length:	3864	Number of contigs:	7	Coverage Start:	1	Coverage End:	3864	Full ORF Coverage:	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t01	3653	1	3658	21	3653	25	3622	99.0%	94.7%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t02	4339	1	3864	21	3857	27	3825	99.0%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t03	3833	1	3841	21	3833	28	3803	99.7%	99.4%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t04	3638	407	3583	114	3269	21	3148	99.1%	82.2%
			3376	3511	3271	3406	0	136	100.0%	3.5%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t05	3552	407	3583	114	3269	21	3148	99.1%	82.2%
			3376	3658	3271	3552	1	281	99.3%	7.3%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t06	4009	407	3864	114	3527	44	3404	99.7%	89.5%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t07	3525	407	3583	1	3156	21	3148	99.1%	82.2%
			3376	3511	3158	3293	0	136	100.0%	3.5%
***Pun*Pgp-3**	cDNA ORF length:	3723	Number of contigs:	5	Coverage Start:	591	Coverage End:	3971	Overall ORF Coverage (%)	90.8%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**

	hub_106215_PG_hub_106215_all_transcripts_PgR059_g034_t01	3248	2113	3723	1604	3205	9	1602	100.0%	43.3%
			591	2112	1	1512	10	1509	99.8%	40.9%
***Pun*Pgp-9**	cDNA ORF length:	3948	Number of contigs:	3	Coverage Start:	1	Coverage End:	3948	Full ORF Coverage:	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR025_g103_t03	5506	1	3948	793	4714	26	3922	99.3%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR025_g103_t02	5620	76	3948	981	4828	25	3848	99.4%	98.1%
	hub_106215_PG_hub_106215_all_transcripts_PgR025_g103_t01	6823	276	3948	2382	6031	23	3650	100.0%	93.0%
			76	278	1749	1950	1	202	100.0%	5.1%
***Pun*Pgp-10**	cDNA ORF length:	3924	Number of contigs:	6	Coverage Start:	1	Coverage End:	3924	Overall ORF Coverage (%)	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR090_g012_t01	4387	1	3924	266	4160	29	3888	99.8%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR090_g012_t02	2987	1144	3924	1	2760	21	2757	99.9%	70.9%
***Pun*Pgp-11.1**	cDNA ORF length:	3858	Number of contigs:	3	Coverage Start:	1	Coverage End:	3858	Full ORF Coverage:	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**

	hub_106215_PG_hub_106215_all_transcripts_PgB04_g139_t01	5657	1	3858	1574	5430	24	3825	99.1%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgB04_g139_t02	4968	1	3858	885	4718	24	3825	99.8%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgB04_g139_t03	4359	388	3858	659	4109	20	3442	99.7%	90.0%
***Pun*Pgp-11.2**	cDNA ORF length:	3909	Number of contigs:	4	Coverage Start:	1	Coverage End:	3909	Overall ORF Coverage (%)	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t01	4103	1	3909	5	3889	24	3876	99.8%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t02	4097	1	3909	5	3883	30	3870	99.8%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t03	620	448	1070	1	620	3	618	99.7%	15.9%
	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t04	338	166	386	119	338	1	219	99.5%	5.7%
***Pun*Pgp-12**	cDNA ORF length:	4056	Number of contigs:	3	Coverage Start:	1464	Coverage End:	3971	Overall ORF Coverage (%)	61.83%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	1038	2929	3971	1	1038	5	1037	99.9%	28.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g007_t01	650	1917	2418	152	650	3	499	100.0%	13.5%
	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g006_t01	264	1464	1728	1	264	1	264	100.0%	7.1%

***Pun*Pgp-16.1**	cDNA ORF length:	3855	Number of contigs:	2	Coverage Start:	84	Coverage End:	3855	Overall ORF Coverage (%)	97.85%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g140_t01	5610	84	833	1165	1909	5	744	99.2%	19.5%
			829	3855	2490	5497	19	3004	99.2%	78.5%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g140_t02	5024	84	3855	1165	4911	25	3742	99.9%	97.8%
***Pun*Pgp-16.2**	cDNA ORF length:	3864	Number of contigs:	5	Coverage Start:	1	Coverage End:	3864	Overall ORF Coverage (%)	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t01	5174	1	3864	625	4462	26	3835	99.2%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t02	5170	1	3864	621	4458	26	3835	99.2%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t03	6530	1	3864	1981	5818	26	3835	99.9%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t04	7109	1	3864	2560	6397	26	3835	99.9%	100.0%
	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t05	3529	411	3124	810	3505	20	2692	99.9%	70.2%
***Pun*Pgp-18**	cDNA ORF length:	3888	Number of contigs:	1	Coverage Start:	1	Coverage End:	3888	Overall ORF Coverage (%)	100.00%
	Contig	**Length (bp)**	**From (cDNA)**	**To (cDNA)**	**From (contig)**	**To (contig)**	**Gaps**	**Identities**	**Identities (%)**	**ORF Coverage (%)**

hub_106215_PG_hub_106215_all_transcripts_PgB01_g089_t01	4070	1	3888	26	3892	37	3858	99.8%	100.0%

Supplementary Table S1. Contigs encoding P-glycoproteins of the tissue specific transcriptome (GEO accession number: GSE99524). For each P-glycoprotein ORF and for each contig length, ORF coordinates (from/to cDNA) as well as the corresponding contig coordinates (from/to contig), gaps, identities, identity percentage and ORF coverage (ignoring gaps) is listed. Number of contigs vary strongly between Pgp and do not correspond to their expression level. Complete ORF coverage was not available for several Pgp. Pgp: P-glycoprotein

Supplementary Table S2

a) Full Length P-glycoprotein Open Reading Frame Primers

Gene	Direction	contig of origin	Sequence (5'-3')	Primer length	Tm (°C)	Fragment Size (bp)
*Pun*Pgp-2	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t02	ACTGGTGGTTATCAACGTG	19	71.4	4065
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgR011_g057_t02	ATCCACACTACAAAACGTGA	20		
*Pun*Pgp-10	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR090_g012_t01	CAACTTGTTCGGCAGCTTCAGTTTACCGACAA	32	55.0	4547
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgR090_g012_t01	GAGAAGTGGTAGAACAAAGGATGAGGTGATAA	32		
*Pun*Pgp-3	Forward	comp75122_c0_seq1	TGAGAGATGTCTTCACGAAGAAAGG	25	55.0	4091
	Reverse	comp75122_c0_seq1	CAACTTAAAAGATCTTGTTTCCTGA	25		

*Pun*Pgp-18	Forward	hub_106215_PG_hub_106215_all_transcripts_PgB01_g089_t01	CTATCAGGTAAAGTGAATCCAAGAAATGGGT	31	65.0	4120
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgB01_g089_t01	ATTGATTAAGAGATGAAATGAATGATAGCGAA	32		
*Pun*Pgp-11.2	Forward	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t01	AAAATGACCGACAACGACAAGTCAAAATGCAG	32	60.0	4105
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgB20_g052_t01	TTTCCTTGTTAGATATAACGAAACGCTCATGG	32		
*Pun*Pgp-3	Forward	comp75122_c0_seq1	ATGGCACGATTCATGTCAAGGTTCTATC	28	55.0	3765
	Reverse	comp75122_c0_seq1	GATGGTTTCTGTGATATATAGCGTTTCTCTCT	32		
*Pun*Pgp-16.2	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t01	GCTACTAATCGTCTAGCTCTATTGGTCATCAA	32	60.0	4332
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgR011_g141_t01	TTTCTAGGCCTGCTGCTATTCCTTTATTGCAA	32		
*Pun*Pgp-9	Forward	hub_106215_PG_hub_106215_all_transcripts_PgB04_g139_t01	TCTCTCGTCGATGTTCACACGTT	23	59.0	4110
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgB04_g139_t01	TGGTGATTATCGCAGCAACAGG	22		

b) Additional Primers

Gene	Direction	contig of origin	Sequence (5'-3')	Primer length	Tm (°C)	Fragment Size (bp)	Purpose

*Pun*Pgp-12	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	TCAACTTCTAAGCAGAGAGGCACG	24	65	634	Verification of fragment
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	ATCGTGGTTCTTGTCCCACCAATG	24			
	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	AGGTCTCGACACAGAAGTTGGAGA	24	62	226	
	Reverse	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	TGCTGAATTG ACGATAGTCG ATGTGC	28			
	Forward	comp29735_c0_seq1	TCTTcACGAAGAAAGGTTCAAGATGAGCA	29	65	421	
	Reverse	comp29735_c0_seq1	GATAGGCGGCAAGGAGAAATTCG	23			
	Forward	comp29735_c0_seq1	CGGCAGGTTTCATAACAATTTATCG	25	65	270	
	Reverse	comp29735_c0_seq1	TAACATACGAGCGTCGATTACCGC	24			
	Forward	hub_106215_PG_hub_106215_all_transcripts_PgR063X_g008_t01	TCAACTTCTAAGCAGAGAGGCACG	24	65		Verification of g008/29735 as one Pgp
	Reverse	comp29735_c0_seq1	TAACATACGAGCGTCGATTACCGC	24			
	Forward	Splice Leader (SL) *Ascaris suum*	GGTTTAATTACCCAAGTTTGAG	22			Amplification of 5' end
	Reverse	Sequencing product of PgR063X_g008_t01	AGTGATACTGGTTTTGCCTCG	21	60	unspecific	Reverse for SL
	Reverse2	Sequencing product of PgR063X_g008_t01	TTGTTTCGCCAAATCAAGCC	20	60	~220bp	Nested SL reverse

	Forward	Sequencing product of comp29735_c0_seq1	GTGCGTTTCGATGGACGT GAC	22	70		3'RACE
	Forward	Sequencing product of comp29735_c0_seq1	AGAAATCAGATGGCATTG GCGG	23		700bp	nested_1 (3'RACE)
	Forward	Sequencing product of3' RACE SP6	GGGTCTCGACACAGAAGT TGGAGA	24		600bp	nested_2 (3'RACE)

c) Full Length P-glycoprotein Sequencing Primers

Gene	Forward Primers	Sequence (5'-3')	Reverse Primers	Sequence (5'-3')
*Pun*Pgp-2	M13-24F-BLUE	GTAAAACGACGGCCAGTGAGCGCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	CGTCAGAAATATCTGAAAGCAATA	R1	GTGTATGTTGGCTAATTTGGCG
	F2	GCTGGATCGAGAAGTTTCACAC	R2	CATCAGTGAGACGAGCGGTAAG
	F3	TATTCGATGGAACCATCGAGAGTA	R3	TCCCGGTGGAATGTCTTTGATCAT
	F4	TTGTGCACTCGATTTGCGACCGAT	R4	CGGTTCTTGCGATACGATTCCGAT
	F5	GTGCAGCAATCGTCAAAGAACT		
*Pun*Pgp-10	M13-24F-BLUE	GTAAAACGACGGCCAGTGAGCGCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	TCTCGTTGGCGGTTTGTTTC	R1	AGGTAAAATCGGCCTTGAAGG
	F2	TCCAATGGGCGTTTAATGCT G	R2	ACATCACCCAGTGTCACATC

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	F3	TCTGGTGGTCAAAAGCAACG	R3	ACCGGAAGAGCTATTCCAAG
	F4	TCGTTACCCGGTAAGGATAC	R4	ACGTATAACAGCTCGGGCGA
*Pun*Pgp-18	M13-24F-BLUE	ACGATGATCGATCGATACTCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	TCGATTCTCACAGGTGCAAC	R1	TGCGAAGTCCATTGCTACTG
	F2	ATGGGCGAGTGGTAGAGACG	R2	AGTGACATCTGCCAACCGAAG
	F3	ATGTCACTCGTTACTCTTGTAA	R3	AGATCGACTGTCGTCCATGT
	F4	GATGACGATCCAGATGACGGG	R4	CAGGTTCTATCTCACCTGAGC
*Pun*Pgp-12	M13-24F-BLUE	ACGATGATCGATCGATACTCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	AGGTGCTATTGCCGAGGAATC	R1	TAGCCCAGCAGCAGTTCTTG
	F2	TCCTGAGGGATATCAGACTC	R2	ATATGCACCCGCAAGTGCTC
	F3	ATGTCTTTCGAGCACTTGCG	R3	GTCTGATATCCCTCAGGAAG
	F4	CAAGAACTGCTGCTGGGCTA	R4	ATTCCTCGGCAATAGCACCTG
*Pun*Pgp-11.2	M13-24F-BLUE	ACGATGATCGATCGATACTCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	TGCTACAGGC GATATTGACG	R1	ATCGCCTGTAGCACTCTCTG
	F2	TGCTACAGGC GATATTGACG	R2	TCGAGTATCGATCGATCATCG

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	F3	TACTGGATGAAGCGACTAGC	R3	TGCTCTGGGTTCTCTTGTTC
	F4	TTCACTTTCGGCATCGAGC	R4	AGAGAACCCAGAGCATGGAA C
*Pun*Pgp-3	M13-24F-BLUE	ACGATGATCGATCGATACTCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	TGAAGAAGGCTCGTAAGATGGG	R1	AGCACCGGTAAATATTCCCGTC
	F2	TTCAAAAGGCTCTTGACCAGGC	R2	TCGCTTTCTGTGTCCAACGCAC
	F3	TGATAGCAGGAGTTGGAATCGC	R3	GATAGCAGGAGTTGGAATCGCA
	F4	TATCTGAAGCGGGTCTACGTCAG	R4	TTGTCGTCGATTGGGATAAGCG
*Pun*Pgp-16.2	M13-24F-BLUE	ACGATGATCGATCGATACTCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	ACATCTCGTGAAGCGACGTTG	R1	GTACAAGGTCAGCTCGATTG
	F2	CAATCGAGCTGACCTTGTAC	R2	TCTTCCAACGTCGCTTCACG A
	F3	GATTGCAAGACGTCCATCAG	R2-1	TTGGTGACAAACTGGGCAAG
	F4	TAGTTCCTCTGATGCAGGAC	R3	CCACTTATAGTCGTCGCAAC
	F5	GATGCGGCTATTTTGTCGAG	R4	GTGTCTGTCG CATTCTTCTT C
	F6	AAGTGGCCAATAAACCGGCAC	R5	TCATCGAGCGTTCCGAATTG
	F7	TCGCATTCGCCTATTGTGCAG	R6	TGGTGCACAATTTCCGCAAC

*Pun*Pgp-9	M13-24F-BLUE	GTAAAACGACGGCCAGTGAGCGCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	F1	GAGTCAGTTCTTTGCCGGATTT	R1	GTGAGAGTGACATCATAATCAA
	F2	TTGGACCAAGTGGATGTGGTAA	R2	ACAGTTCCATCAATTGGGTCAT
	F3	GGAAACGAGGATGTCACTGAG	R3	CGCTCCTACGACAAGAAACATG
	F4	GATCAGATGC TCAGCAATGG TC	R4	CTCAGTGACATCCTCGTTTC C
	F5	TTCGCATATCCAGAACGACCAG		

d) Codon optimized constructs for expression in *Saccharomyces cerevisiae* of *Pun*Pgp-2 and *Pun*Pgp-9

>*Pun*Pgp-2_codon_optimized

ATGATGCGTTCAAGTCAGGATAATGAAAAAGTTCCGCTGATTTCAGCCAGTATTGCGTCATACGAGTCTTTTAACTCAAGCAATTGCA
AGCGTCCTTCATCCCTTAAGGTCAAGGAGAAAGGGCTTTTCTCATACACGACGTGTCTAGACTATTGTCTAATCCTTATGGGCACCT
TCGCAGGTATAGCACACGGAACCGGCTTTCCTCTTTTAAGTATAGTGCTAGGGGGGATGACAACGATATTCCTGAGAGCCGAGAAC
TCCGACTTTGTTCGTGGTTATTCTGTTATAGTCAACAATTCAGCCCTTAGCCAGATTACGAAGGAAGAGTTCGACGCTTCTGTTACCA
CCTATTGCCTTTACTACCTATTGATAGGCGTATTTATGTTTATATCCAGCTATATACAGATCGCTTGCTGGGAGTCTTTTAGTGAGAGA
ACCACTCATAGGATAAGACAAAAATATTTAAAAGCGATACTGAGGCAGCAAATTGCCTGGTTCGATACCCAACAGAGTGGCAATCTT
ACGGCCCGTTTGACTGACGACCTGGAACGTGTTAGAGAGGGGTTAGGCGATAAGCTAAGCATGATGATTCAACTGATGGCCGCCTT
CATCGCGGGATTTATAGTCGGCTTTATATATAATTGGCGTATGACTCTAGTGATGATGGCTTTCGCCCCATTGAACGCCCTGACAGG
CGCATGGATGTCTCGTATGGCGGCGACGAGAACACAGGTCGAGCAAGAGAAGTATGCTGTGGCCGGGGCTATAGCAGAAGAAACC
TTTAGTAGCATAAGAACCGTTCATAGTCTGAACGGAGCAACCAGAGAAATCGCAAGATATGAGAAGGCCTTAGAGGATGGGAGGCG
TACTGGGCGTTTGAAATACTTATATCTTGGGACCGGAATGGCGTTGAATTACCTTATTGTGTATGCCAGCTACGCAGTGGCCTTTTG
GTACGGCAGTCTGATCATAATCGGTGATCCGACTTTCGACAGAGGCTCCGTGTTCACGGTATTCTTTAGTGTGATGAGTGGAAGCAT
GGCGCTAGGTGGTGCCCTTCCAAACATGGCTACTTTTGCTATGGCGAGAGGTGCAGCAAGAAAAGTACTGAGTGTCATCAACAGTG
TGCCTATTATTGACCCTTACTCCAGCAGTGGTACATTTCCCTCAAAGCTAAAGGGGGCGATCAGTTTTCAAAATGTGTCCTTTTCCTA
TCCGATCCGTAAAGACATTCAAATTTTGGACCGTATTACTTTTGATATTAGTCCCGGTAGAAAAATCGCGTTGGTGGGAAGCAGTGG
TTGTGGGAAATCTACCGTGATGCACCTGCTTTTGAGGTTTTATGATCCGGACCTTGGTATGGTGACTCTTGATGGTTATGACATTCGT
AGTCTTAATGTCCGTCGTCTGAGGGATGCAATAGGGATAGTGAGCCAAGAGCCGATACTGTTTGATGGCACGATAGAATCAAATATC

CGTCTTGGGTGGGAGAAAGCTACGAGAGAGGACATTGTCAGGGCATGTAAACAAGCTAACGCTTGGGAATTTATTCAGCTTCTGCC
CGACGGGCTTAGTACCAGAGTAGGCGAGCGTGGCGTGCAACTATCAGGGGGCCAGAAACAAAGAATTGCCATTGCACGTGCACTA
ATAAAGGATCCCCAAATTTTGCTTCTGGACGAGGCAACCAGCGCACTTGACACGGAGAGCGAATCCGTAGTACAAAAGGCTCTTGA
ACAGGCTCAGATCGGTAGAACTACAATCACGATTGCACACCGTCTGTCAACTATCAGGGACGTGGATGAGATCCTAGTCTTTAAAAA
CGGATCCATCGTCGAAAGGGGAACGCACATAGAGTTAATTGCCGCGCGTGGACTATACTACGGAATGGTGCTTGCGCAAGATATAA
ACCAACAGACTGAAATGATCGATGATGAAATGGACGAGGTCGACGATGTAGACGACCGTAGTTCTAATTTGGATGTCGTTCGTAAGA
AAAGGTCAATTGCGAATTCTTACCACAGATCCGTGTCCGACCCTTCAGAATTATCTCTTAGGTCTTCAGCCGCAATCGTAAAAGAGC
TTCAGGACGCCGCGGAGGAGAGCTCAGTAAGACCTACACCGATGTCACGTATTTTTCTTATGAATAGAGAAACTTGGCCATATCTTT
TCGTGGGACTGGTCGGTTGCTGCTTAAGTGGTATCGTCCCGCCCTTCTTTGCATTGGTTTATAGTCAGATATTCAGCGTCTTCTCCG
AACCGGTAGACAGACTTGGGAGCGACGCTCGTTTCTGGTCCCTTATGTTCTTAGCATGTGGAGTGATCGATGCTATAGGTTTTTTCA
TTTCCGCCAACATGCTTGGGCTATGTGGAGAAACGCTGACGAAAAAAATTAGGTTGATGGCTTTCACCAACTTATTGCGTCAAGATA
TAGCCTTCTATGACGATCAGAGACACTCAACGGGGAAGCTTTGCACACGTTTTGCGACAGACGCGCCGAACGTCCGTTACGTCTTT
ACCCGTTTACCACTGGTAGTTGCGAGCGTTGTAACTCTAGTCGGCGCTATTGCGATTGGGTTCCTGTTTGGGTGGCAACTAGCCTTA
ATCTTGCTAGCAATCGTACCGCTAATTCTTGGCTCTGGTTATGTAGAAATGAGACTTCAGTTCGGGAAGCAGTTGAGGGAGACCGAG
CTACTAGAAGAAGCCGGGAGAACAGCGACCGAGGCCGTAGAGAATATACGTACAGTACAGTCACTTAATAAGCAGTCCGCTTTTAT
CAGAGAATACTCACAACACTTGGAAACCCCTTTTAGGGAAAATATGCAGAGAGCACACATCTACGGAGCTGTTTTCGCTTTCTCACA
ATCCTTAATCTTCTTCATGTATGCGTTAGCATTTTGGTTAGGTTCTTTATTTGTCGACAATGCGGTGATGCAACCCATCAACGTTTACA
GGGTGTTTTTCGCGATTGCTTTCTGCGGCCAAAGCGTGGGGCACATCAGTGCCTTTATTCCGGACGTGGTTAAAGCACGTTTAGCC
GCAAGTTTGGTCTTTCACCTAAGCGAGTATCCGACGGCCATAGACTCATTGAGCGATCAAGGTAGTAGAATAACTATAAAGGGTGCT
ATCCAGTTAAAGAATGTTTTCTTTTCCTACCCGACAAGACGTAATACAAGAATATTAAGAGGCTTGACACTTAATGTCAAGGAGGGCG
AAACGGTTGCACTTGTTGGGCACTCTGGCTGTGGGAAATCCACAGTTATGGGTCTGCTTGAGCGTTTTTACGACCCTAACAGAGGC
AACATATACGTTGACGGAGAGAACATCAGAGACGTAAACATTAAGTGCTTGAGGTCCCAAATGTGTATTGTAAGCCAGGAACCCATA
CTATTTGATTGCACGATTGAGGAGAACATAATGTACGGACTGGACAGGGAAGTGAGCCATGAGGAGGTGGTCAACGCTGCCAAACT
GGCTAATATCCATAAATTTATCTTAAGCTTGCCCCTGGGGTATGAAACTAGGGTCGGTGAAAAAGGGACCCAGTTGAGCGGCGGAC
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AATCTGAACAGGTCGTTCAAGAAGCTTTAGAAAACGCGCGTAAAGGTAGGACTTGTCTTGTGATAGCCCATAGACTGAGCACAATTC
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>*Pun*Pgp-9_codon_optimized
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e) Primers for validation of constructs by sequencing

Gene	Forward Primers	Sequence (5'-3')	Reverse Primers	Sequence (5'-3')
*Pun*Pgp-2 codon optimized	M13-24F-BLUE	GTAAAACGACGGCCAGTGAGCGCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	Pgp-2_fw_1	TGATGCGTTCAAGTCAGGATAATG		
	Pgp-2_fw_2	AACATGCTTGGGCTATGTGGAG		
Gene	**Forward Primers**	**Sequence (5'-3')**	**Reverse Primers**	**Sequence (5'-3')**
*Pun*Pgp-9 codon optimized	M13-24F-BLUE	GTAAAACGACGGCCAGTGAGCGCG	M13-24R-BLUE	AACAGCTATGACCATGATTACGCC
	Pgp-9_fw_1	ATGTCCATTCGTAGCTCTCATAAG		
	Pgp-9_fw_2	AACGAGATTCTGGCGATCTTCG		

f) Primers for RT-PCR for validation of transgene expression in yeast

Gene	Direction	Sequence (5'-3')	Tm (°C)	Fragment Size (bp)
*Pun*Pgp-2 Codon optimized	Forward	GCATAATCAGGGACATC GTATGG	63.9	962
	Reverse	GCGGTGATGCAACCCAT CAAC		
*Pun*Pgp-9 Codon optimized	Forward	TGGCCACGGACACACCA AATGTG or TAGCCCAGGGCATGGCT TACGGTTT (used for RT-PCR in Supplemental Fig. 3)		1441 or 1085
	Reverse	GCATAGTCCGGAACGTC GTATGG		
lacZ	Forward	CTTTTGACACCAGACCAA CTGG	63.1	1141
	Reverse	TGTTCCGTCATAGCGATA ACG		

Supplementary Table S2. Primers and sequences
Sequences of a) full length open P-glycoprotein reading frame amplification (RT-PCR) primers for all *Parascaris univalens* Pgp, b) additional primers used to obtain sequences missing in both transcriptomes, verification primers, RACE PCR and spliced leader PCR) c) sequencing primers for all *Parascaris* Pgp, made by primer walking, d) Codon optimized constructs of *Pun*Pgp-2 and *Pun*Pgp-9 for expression in *Saccharomyces cerevisiae*, e) primers for validating correctness of codon optimized yeast expression constructs e) primers for RT-PCR for validating transgene expression at the transcription level. *Pun*: *Parascaris univalens*; Pgp: P-glycoprotein

Supplementary Table S3

Gene Name in Maximum Likely Hood Tree	Annotated Name (if applicable)	Organism	Accession Number Genbank	WormBase ID	Contig
***Pun*Pgp-11,1**	***Peq*Pgp-11**	*Parascaris univalens*	AGL08022.1		

***Pun*Pgp-16.1**	***Peq*Pgp16**	*Parascaris univalens*	AGL08023.1		
***Pun*Pgp-2**		*Parascaris univalens*	MT001899		
***Pun*Pgp-3**		*Parascaris univalens*	MT001900		
***Pun*Pgp-9**		*Parascaris univalens*	MT001901		
***Pun*Pgp-10**		*Parascaris univalens*	MT001902		
***Pun*Pgp-11.2**		*Parascaris univalens*	MT001904		
***Pun*Pgp-12**		*Parascaris univalens*	MT001905		
***Pun*Ppg-16.2**		*Parascaris univalens*	MT001907		
***Pun*Pgp-18A**		*Parascaris univalens*	MT001908		
***Pun*Pgp-18B**		*Parascaris univalens*	MT001909		
***Cbr*Pgp-16**	Hypothetical protein CBG12969	*Caenorhabditis briggsae*	CAP31851.1	BP:CBP17602	
***Cel*Pgp-1**		*Caenorhabditis elegans*	NP_502413	WP:CE11932	
***Cel*Pgp-2**		*Caenorhabditis elegans*	NP_491707	WP:CE41207	
***Cel*Pgp-3**		*Caenorhabditis elegans*	NP_509901	WP:CE03818	
***Cel*Pgp-4**		*Caenorhabditis elegans*	NP_001257143	WP:CE03308	
***Cel*Pgp-5**		*Caenorhabditis elegans*	NP_001257116	WP:CE43003	
***Cel*Pgp-6**		*Caenorhabditis elegans*	NP_001041287	WP:CE40818	
***Cel*Pgp-7**		*Caenorhabditis elegans*	NP_509812	WP:CE36668	
***Cel*Pgp-8**		*Caenorhabditis elegans*	NP_509811	WP:CE31624	
***Cel*Pgp-9**		*Caenorhabditis elegans*	NP_507487	WP:CE15714	

Scientific Reports | (2020) 10:13586 | https://doi.org/10.1038/s41598-020-70529-6

***Cel*Pgp-10**		*Caenorhabditis elegans*	NP_509205	WP:CE40807	
***Cel*Pgp-11**		*Caenorhabditis elegans*	NP_495674	WP:CE34788	
***Cel*Pgp-12**		*Caenorhabditis elegans*	NP_510126	WP:CE03260	
***Cel*Pgp-13**		*Caenorhabditis elegans*	NP_510127	WP:CE40253	
***Cel*Pgp-14**		*Caenorhabditis elegans*	NP_510128	WP:CE0262	
***Ppa*Pgp-1**		*Pristionchus pacificus*		PP:PP30697	
***Ppa*Pgp-9**		*Pristionchus pacificus*		PP:PP38391	
***Asu*Pgp-2**	Multidrug resistance protein 1	*Ascaris suum*	ADY40620		
***Asu*Pgp-11.2**	Multidrug resistance protein 3	*Ascaris suum*	ADY40644		
***AsuPgp*-16.2**	Multidrug resistance protein 3	*Ascaris suum*	ADY40573		
***Asu*Pgp-2_hp**		*Ascaris suum*			AgR011_g279_t03
***Asu*Pgp-3_hp**		*Ascaris suum*			AgR049_g026_t04
***Asu*Pgp-9_hp**		*Ascaris suum*			AgB03_g189_t01
***Asu*Pgp-10_hp**		*Ascaris suum*			AgR054_g025_t01
***Asu*Pgp-11.1_hp**		*Ascaris suum*			AgB01_g256_t02
***Asu*Pgp-12_hp**		*Ascaris suum*			AgR009X_g036_t04
***Asu*Pgp-16.1_hp**		*Ascaris suum*			AgR016_g207_t01
***Asu*Pgp-18A_hp**		*Ascaris suum*			AgR067_g008_t02
***Asu*Pgp-18B_hp**		*Ascaris suum*			AgR067_g008_t02
***Hco*Pgp-2**		*Haemonchus contortus*	AAC38987		
***Hco*Pgp-3**		*Haemonchus contortus*	AFX93749.1		

***Hco*Pgp-13**		*Haemonchus contortus*	ARE67135.1		
***Hco*Pgp-16**		*Haemonchus contortus*	AFX93754.1		
HcoPgp-11.2		*Haemonchus contortus*	CDJ81808.1		
***Hco*Pgp-11.1**		*Haemonchus contortus*	CDJ84338.1		
***Hco*Pgp-10**	unnamed protein product	*Haemonchus contortus*	CDJ83820.1		
***Hco*Pgp-9.1**		*Haemonchus contortus*	JX430397		
***Con*Pgp-2**		*Cooperia oncophora*	AGJ71178		
***Con*Pgp-3**		*Cooperia oncophora*	AGJ71177		
***OvoPgp-3 (*10280)**		*Onchocerca volvulus*		OV:OVP11414	
***Ovo*Pgp-10**		*Onchocerca volvulus*		OV:OVP13603	
***Ovo*Pgp-11**		*Onchocerca volvulus*		OV:OVP14503	
***Dim*Pgp-3**		*Dirofilaria immitis*	ALI16766.1		
***Dim*Pgp-11**		*Dirofilaria immitis*	ALI16773.1		
***Dim*Pgp-10**		*Dirofilaria immitis*	ALI16764.1		
***Bma*PGP-10**		*Brugia malayi*		BM:BM29899	
***Bma*Pgp-21**		*Brugia malayi*	XP_001900095		
***Bma*Pgp-10a**		*Brugia malayi*	XP_001897744		
***Bma*Pgp11**		*Brugia malayi*	CRZ23051.1		
***Bma*Pgp-3**	Bm11309	*Brugia malayi*		BM:BM42091	
***Bma*Pgp-16**	Bm2594, isoform a	*Brugia malayi*	CDP97625.1		
***Nam*Pgp-2**	ABC transporter, ATP-binding protein	*Necator americanus*	XP_013305476.1		
***Nam*Pgp-13**	ABC transporter, ATP-binding protein	*Necator americanus*	XP_013298646.1		

***Nam*Pgp-10**	ABC transporter, ATP-binding protein	*Necator americanus*	XP_013299120.1		
***Nam*Pgp-14**	hypothetical protein NECAME_00050	*Necator americanus*	XP_013309418.1		
***Celo*Pgp-9**		*Cylicocyclus elongatus*	AJM87336.1		
***Tci*Pgp-13**		*Teladorsagia circumcincta*	PIO77339.1		
***Tci*Pgp-9**		*Teladorsagia circumcincta*	PIO69295.1		
***Sma*MRP1_1 (1,2,3)**		*Schistosoma mansoni*	XP_018648325.1		
***Sma*MRP1_2 (1,2,3)**		*Schistosoma mansoni*	XP_018651783.1		
TcaPgp-2	Multidrug resistance protein 1A	*Toxocara canis*	KHN80157.1		
***Tca*Pgp-3a**		*Toxocara canis*	KHN78383.1		
***Tca*Pgp-12**	Multidrug resistance protein pgp-3	*Toxocara canis*	KHN87068.1		
***Tca*Pgp-11.1**	Multidrug resistance protein pgp-1	*Toxocara canis*	KHN73709.1		
***Tca*Pgp-11.2**	Multidrug resistance protein pgp-3	*Toxocara canis*	KHN89031.1		
TsuPgp-2	hypothetical protein M514_09538	*Trichuris suis*	KFD70158.1		
Outgroup (collapsed)					
***Dme*PgpA**		*Drosophila melanogaster*	AAA16186		
***Dme*PgpB**		*Drosophila melanogaster*	AAA28679		
***Dme*PgpC**		*Drosophila melanogaster*	AAA28680		
***Mca*Pgp-L**		*Mytilus californianus*	ABS83556		

***Mga*Pgp-L**		*Mytilus galloprovincialis*	CAX46411		
***Mmu*ABCB-1b**		*Mus musculus*	NP_035205		
***Mmu*Pgp**		*Mus musculus*	AAA39514		
***Phu*MRP-1**		*Pediculus humanus corporis*	XP_002425149		
***Phu*MRP-2**		*Pediculus humanus corporis*	XP_002432260		
***Phu*MRP-3**		*Pediculus humanus corporis*	XP_002426586		
***Phu*MRP-4**		*Pediculus humanus corporis*	XP_002425021		
***Hsa*ABCB1**		*Homo sapiens*	NP_000918.2		

Supplementary Table 3. NCBI Accession numbers, WormBase IDs and contigs (*Ascaris suum* transcriptome, GEO: GSE38470) of protein sequences used in the maximum likelihood tree. Annotated gene names were changed according to the result of the phylogenetic analysis in the tree and original gene names are listed.

3.2 Pharyngeal Pumping and Tissue-Specific Transgenic P-Glycoprotein Expression Influence Macrocyclic Lactone Susceptibility in *Caenorhabditis elegans*

pharmaceuticals

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Article

Pharyngeal Pumping and Tissue-Specific Transgenic P-Glycoprotein Expression Influence Macrocyclic Lactone Susceptibility in *Caenorhabditis elegans*

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Citation: Gerhard, A.P.; Krücken, J.; Neveu, C.; Charvet, C.L.; Harmache, A.; von Samson-Himmelstjerna, G. Pharyngeal Pumping and Tissue-Specific Transgenic P-Glycoprotein Expression Influence Macrocyclic Lactone Susceptibility in *Caenorhabditis elegans*. *Pharmaceuticals* **2021**, *14*, https://doi.org/10.3390/ph14020153

Academic Editor: Marcelo J. Nieto
Received: 12 January 2021
Accepted: 9 February 2021
Published: 13 February 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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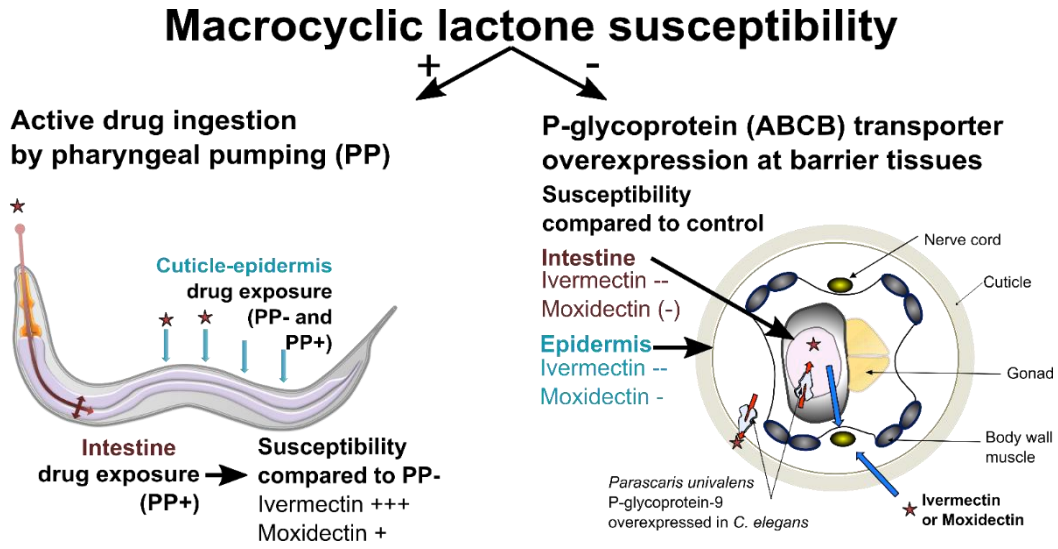
Abstract: Macrocyclic lactones (MLs) are widely used drugs to treat and prevent parasitic nematode infections. In many nematode species including a major pathogen of foals, *Parascaris univalens*, resistance against MLs is widespread, but the underlying resistance mechanisms and ML penetration routes into nematodes remain unknown. Here, we examined how the *P*-glycoprotein efflux pumps, candidate genes for ML resistance, can modulate drug susceptibility and investigated the role of active drug ingestion for ML susceptibility in the model nematode *Caenorhabditis elegans*. Wildtype or transgenic worms, modified to overexpress *P. univalens* PGP-9 (*Pun*-PGP-9) at the intestine or epidermis, were incubated with ivermectin or moxidectin in the presence (bacteria or serotonin) or absence (no specific stimulus) of pharyngeal pumping (PP). Active drug ingestion by PP was identified as an important factor for ivermectin susceptibility, while moxidectin susceptibility was only moderately affected. Intestinal *Pun*-PGP-9 expression elicited a protective effect against ivermectin and moxidectin only in the presence of PP stimulation. Conversely, epidermal *Pun*-PGP-9 expression protected against moxidectin regardless of PP and against ivermectin only in the absence of active drug ingestion. Our results demonstrate the role of active drug ingestion by nematodes for susceptibility and provide functional evidence for the contribution of *P*-glycoproteins to ML resistance in a tissue-specific manner.

Keywords: macrocyclic lactones; nematode; parasitology; P-glycoprotein; drug resistance; macrocyclic lactones; *Caenorhabditis elegans*



Graphical Abstract

1. Introduction

The treatment of parasitic nematode infections in animals and humans relies on chemotherapy, and macrocyclic lactones (MLs) represent the most widely used drug class in veterinary medicine due to their high efficacy, low toxicity, and broad spectrum of target parasites [1]. However, drug resistance to MLs is now widespread in parasitic nematodes of ruminants [2], horses [3], companion animals [4,5], and humans [6]. With over 1.5 billion infected humans [7] and essentially all domestic animals exposed to nematode infections, the ongoing development and spread of ML drug resistance is an obstacle to maintaining health standards. In the pathogenic equine parasite *Parascaris univalens*, resistance against MLs is widespread, which poses a health threat to young animals worldwide [8].

In *Caenorhabditis elegans* and in parasitic nematodes, the primary pharmacological targets of MLs are the glutamate-gated chloride channels (GluCls) [9,10]. The irreversible binding of MLs to GluCls leads to a hyperpolarisation of the respective neurons that control locomotion, pharyngeal pumping, and egg laying, resulting in flaccid paralysis of the muscles and pharynx [9,10]. However, the underlying resistance mechanisms are insufficiently understood.

The ATP-binding-cassette subfamily B member 1 (ABCB1) transporter genes, more commonly referred to as P-glycoproteins (Pgps), were among the first candidate genes for ML resistance in parasitic nematodes [11,12] and in *C. elegans* [13,14]. These xenobiotic efflux pumps are conserved in eukaryotes and have a broad lipophilic substrate range [15]. Their role in ML resistance has been inferred from observed differences of individual Pgp expression in association with drug exposure [16] and findings of constitutively high expression levels in resistant compared to susceptible isolates [11]. Recently, next-generation sequencing (NGS) approaches allowed the identification of genetic signatures of selection in close proximity to Pgp loci in resistant and introgressed populations [17–19]. In addition, several functional studies in *C. elegans* have provided evidence for an involvement in the ML resistance phenotype. For example, individual Pgp deletion strains, and silencing or inhibition of Pgps increased ivermectin (IVM) susceptibility [13,14]. However, our understanding of how Pgps contribute to ML resistance functionally remains superficial and fragmented. In contrast to mammals, which only possess one (human) to three (rodents) Pgps, the nematode Pgp gene family comprises a diverse repertoire that differs between nematodes species [20], e.g., 15 Pgps in *C. elegans* [21] and 10 Pgps in *Haemonchus contortus* [22]. More recently, the Pgp repertoire

of *P. univalens* was completely deciphered, revealing 10 different Pgp genes with particularly strong expression levels for *Pun-pgp-11.1, Pun-pgp-16.2*, and *Pun-pgp-9* [23]. Across different species, the *pgp-9* gene lineage has been repeatedly associated with ML resistance in several nematode species such as cyathostomins [19,24], *Teladorsagia circumcincta* [18,25], and *H. contortus* [26] and is regarded as one of the most relevant candidates for a contribution to ML resistance. While Pgps are expressed in most tissues, expression is most prominent in the intestine in different nematode species, including *P. univalens* and *C. elegans* [23,27–29]. In addition, the epidermis (= hypodermis) also exhibits moderate Pgp expression levels in *P. univalens* [27] and *C. elegans* [23], but the tissue-specific function of these genes is unclear.

Many marketed ML derivatives have been shown to exhibit differing pharmacokinetics, efficacies, and chemical properties [1]. Generally, MLs are classified into two groups, the avermectines (including IVM) and the milbemycins (including moxidectin, MOX), the latter lacking a polysaccharide side chain at C13 of the lactone ring. This chemical disparity has been suggested to lead to the differences in the affinity of nematode Pgps for different ML derivatives [24,30]. In mammals, the main route of ML elimination is the intestine, and here, Pgps have been shown to play an important role [31]. In contrast, the uptake and elimination routes of MLs in nematodes and the role of Pgps in these processes remain to be elucidated. Mammalian Pgps also reduce the permeation of MLs through the blood–brain barrier and Pgp (i.e., *ABCB1* or *mdr-1*) deficiency causes acute neurotoxicity upon IVM treatment [32]. In this regard, the capacity of MDR-1 to restrict effective crossing of MOX and IVM over the barrier varies between IVM and MOX [33].

In the present study, we investigated whether the tissue-specific Pgp expression patterns in nematodes are relevant for modulation of ML susceptibility. Based on their role in mammals, we hypothesised that intestinal and epidermal Pgp expression in nematodes can reduce ML permeability of these tissues. Our objective was to examine the effect of tissue-specific Pgp overexpression at the intestine and at the epidermis on IVM and MOX susceptibility using transgenic overexpression of the ML resistance candidate gene *Pun*-PGP-9 in *C. elegans*. Additionally, we took advantage of *C. elegans* behaviour to induce active drug ingestion by pharyngeal pumping (PP) in the presence of bacteria [34] or through chemical stimulation of PP by 5-hydroxytryptamine (5-HT = serotonin) [35], while in turn mostly limiting exposure to the cuticle–epidermis barrier in the absence of an appropriate PP stimulus [36,37]. Our results show that active drug ingestion increases susceptibility to IVM considerably and to MOX only moderately and indicate that both derivatives are taken up by *C. elegans* via the intestine and cuticle–epidermis. In both tissues, Pgps have a protective function by reducing the worms' susceptibility to IVM and MOX.

2. Results

2.1. Tissue-Specific Expression Patterns of Pun-PGP-9 *in a* Cel-pgp-9 *Mutant Strain*

To address the function of *Pun-pgp-9* using *C. elegans*, the *Cel-pgp-9 (tm830)* null allele was used to generate transgenic strains carrying extrachromosomal arrays driving tissue-specific overexpression of *Pun-PGP-9*. Two lines, henceforth referred to as intestine-Pgp-9 line 1 (*IntPgp-9Ex1*) and line 2 (*IntPgp-9Ex2*), were generated with *Pun*-PGP-9 expression driven by the intestine-specific *gut esterase 1* promotor (*ges-1p*) [38] and pharyngeal green fluorescence protein (GFP) expression. Another line, henceforth addressed as epidermis-Pgp-9, was generated with *Pun*-PGP-9 expression driven by an epidermis-specific *collagen-19* promotor (*col-19p*) [39] and pharyngeal GFP expression (*EpiPgp-9Ex1).* Finally, a line serving as the control strain expressing only GFP in the pharynx (*myo-2p*::*gfp*) (*CtrlEx1)* was generated.

Transcription of *Pun-pgp-9* was confirmed by RT-PCR for all *Pun*-PGP-9 transgenic strains by amplifying a 1043 bp PCR product, while no PCR product was amplified in the control

strain (Figure 1a). Protein localisation in the epidermis and the intestine were confirmed by the immunofluorescence detection of a FLAG-tag fused to the NH_2-end of *Pun*-PGP-9 and visualised using a secondary DyLight405 antibody and confocal microscopy (Figure 1b–e). *Pun*-PGP-9 protein expression in intestine-Pgp-9 line 1 appeared intestinal specific (Figure 1b). At a higher magnification, a strong apical expression was apparent (Figure 1e). Expression in the epidermis-Pgp-9 strain was detectable in the epidermis (Figure 1c). Specific blue fluorescence was not visible in the control strain following staining, while pharyngeal GFP expression was visible, indicating that the antibody staining was specific to the FLAG-tagged *Pun*-PGP-9 (Figure 1d).

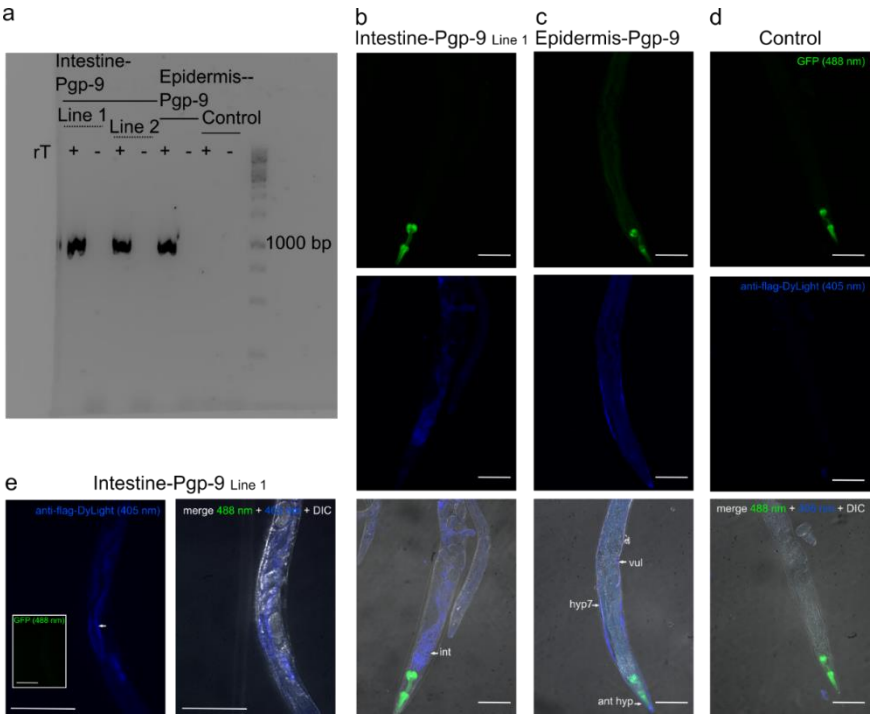


Figure 1. Tissue-specific expression of *Pun*-PGP-9 in *Cel-pgp-9* loss-of-function strain, strain tm830. Verification of transcription and tissue-specific expression. (**a**) Visible bands for all reverse-transcriptase (RT) PCRs on cDNA templates made from whole-worm total RNA, and no bands for no RT controls and the control strain. (**b**–**e**) Confocal microscope images of tissue-specific expression patterns of fixed, freeze cracked, and immunofluorescence-stained transgenic adult *Caenorhabditis elegans* (blue lookup table (LUT), 405 nM excitation), pharyngeal GFP expression (green LUT, 488 nm excitation) and merged images (DIC +405 nM + 488 nM). Primary antibodies target the FLAG-tag fused to *Pun*-PGP-9 and the secondary antibody is conjugated with DyLight405. Images were acquired with a confocal Eclipse Ti-U inverted research microscope and processed and merged with ImageJ [40]. All scalebars are 100 µm. (**b**) Immunostaining in the intestine-Pgp-9 line 1 (white arrows indicate the intestine) and GFP expression at the pharynx. (**c**) Immunostaining of the epidermis-Pgp-9 strain (white arrows indicate the epidermal syncytium) and pharyngeal GFP expression. (**d**) Immunostaining in the control strain and pharyngeal GFP expression. (**e**) Higher magnification of the intestine-Pgp-9 strain (white arrow indicates the apical membrane), *Pun*-PGP-9: *Parascaris univalens* P-glycoprotein-9, GFP: green fluorescence protein, DIC: differential interference contrast, int: intestine, vul: vulva, hyp7: hyp7 syncytium, N2Δ*Cel*Pgp-*9* is tm830 (NBRP) [*Cel-pgp-9(-)*]; Transgenic strains genotypes: Epidermis-Pgp-9 *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*]; Intestine-Pgp-9 Line 1 *IntPgp-9Ex1* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*]; Control strain *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp:: Cel-unc-54_3'-UTR*].

2.2. *Motility Assays in Transgenic and Wildtype* Caenorhabditis elegans

All mentioned half-maximal effective concentration (EC_{50}) values, fold changes, and other parameters calculated from the non-linear regression models as well as p-values from

statistical significance tests are given in Supplementary Table S1 for IVM and Supplementary Table S2 for MOX and are not specifically referenced in the text.

Motility assays were conducted as described elsewhere [41] with modifications for the stimulation of PP as schematically summarised in Figure 2a. Prior to drug incubations, induction of PP by feeding OP50 or 5-HT treatment or the lack of PP in the absence of a stimulus was confirmed visually under a stereo microscope. By PP stimulation, MLs dissolved in DMSO within the incubation medium were actively ingested. After an 18–24 h incubation, the motility of worms incubated without drugs in 1% DMSO (negative, vehicle control) was tested; neither transgene expression nor induction of pharyngeal pumping by 5-HT or OP50 significantly influenced motility (Kruskal–Wallis test with Dunn's post hoc test) compared to the wildtype (WT, untransformed N2 Bristol strain worms)/OP50$^-$ condition (no PP stimulation) (Figure 2b).

2.2.1. The Effect of Active Drug Ingestion on Ivermectin and Moxidectin Susceptibility

Strikingly, the EC_{50} for the WT in the OP50$^+$ condition was 11.1-fold lower than in the absence of OP50 bacteria (Figure 2c). In order to evaluate whether this effect is the result of potentially confounding factors such as IVM metabolisation by the OP50 bacteria or starvation, OP50 were substituted by 5-HT in the incubation medium, thereby eliminating the bacteria and food source but maintaining a stimulus for pharyngeal pumping. This resulted in an 8.0-fold decrease in the EC_{50} compared to OP50$^-$ to a level just above that achieved by incubation with OP50 bacteria (Figure 2c).

For MOX, stimulation of PP by OP50 also resulted in overall lower EC_{50} values than in the absence of PP (OP50$^-$), but the effect of PP was considerably smaller than that observed for IVM (Figure 2c,d).

Comparing PP induction by 5-HT$^+$ and OP50$^+$, IVM EC_{50} values were higher when stimulated by 5-HT across all strains except intestine-Pgp-9 (all $p < 0.05$, extra-sum-of-squares F test, Bonferroni corrected) while MOX EC_{50} values varied slightly in the control and WT and were not significantly different in both *Pun*-PGP-9 expressing strains (both $p > 0.05$, extra-sum-of-squares F test, Bonferroni corrected) (Figure 2e).

2.2.2. *Cel-pgp-9* Loss-of-Function Strain Susceptibility Phenotype in Adult Stage

The control strain, which is the *Cel-pgp-9* null mutant (*CtrlEx1 [Cel-pgp-9(-); Cel-myo-2p::gfp:: Cel-unc-54_3'-UTR*]), did not exhibit increased susceptibility to IVM or MOX (Supplementary Figure 1a,b). Serving as a control for extrachromosomal array transgene expression (pharyngeal GFP expression), the calculated EC_{50} values in the control strain matched those of the WT for IVM. Likewise, the control strain's response to MOX was similar to that of the WT strain despite minor variations (Supplementary Figure 1c,d. The control strain was used as a reference for both the *Pun*-PGP-9 overexpression strains, epidermis-Pgp-9 and intestine-Pgp-9, as it exhibits the same genetic background and transgenic extra-chromosomal expression.

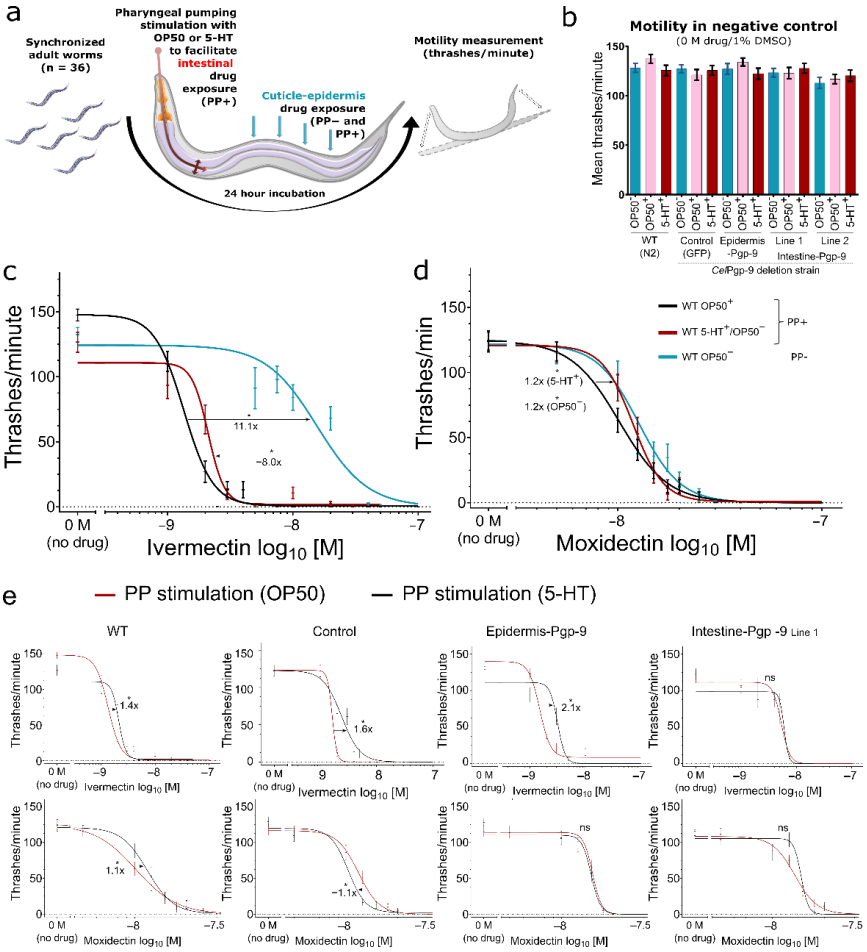


Figure 2. Pharyngeal pumping increases ivermectin and moxidectin susceptibility. Effect of pharyngeal pumping (PP) stimulation by OP50 food bacteria or serotonin in *Caenorhabditis elegans* on ivermectin and moxidectin susceptibility. (**a**) Schematic illustration of the experimental setup with active ingestion and intestine drug exposure by PP stimulation. (**b**) Mean thrashes/minute ± standard error of the mean (SEM) in the negative control (no drug, 1% DMSO) between strains and conditions. Each strain/condition combination was compared to WT OP50^{-} with a Kruskal–Wallis test with Dunn's post hoc, and $p > 0.05$ was considered not significant. (**c**,**d**) Ivermectin and moxidectin concentration–response curves calculated with GraphPad v8.3.0 (GraphPad v8.3.0, www.graphpad.com) based on thrashes/minute in the WT strain with n = 36 per concentration spread equally on three separate days. PP stimulation by OP50 bacteria (OP50^{+}) (black), 4 mM 5-HT (red), or in the absence of a PP stimulus (OP50^{-}) (blue). Significant differences between half-maximal effective concentration (EC_{50}) were calculated using the extra-sum-of-squares-F test and Bonferroni correction. (**e**) Comparison of the effect of PP stimulation by OP50 *Escherichia coli* food bacteria (red) or 5-HT (black) in different transgenic and wildtype strains. (**c-e**) All calculated four parameter non-linear regression models and SEM at each concentration correspond to Supplementary Tables S1 and S2. Prior to the calculation, the no-drug negative control was set to 0.1 nM and all concentrations were $\log_{10}$ transformed. On the x-axis, the negative control was visualised as "0 M (no drug)" and separated by a break in the axis. P-values <0.05 were considered significant and are indicated with an asterisk, while corresponding fold-changes are indicated with arrows. 5-HT: serotonin/5-hydroxytryptamine; WT: *C. elegans* N2 Bristol; Control strain genotype *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp::Cel-unc-54_3'-UTR*].

2.2.3. The Effect of Epidermal Pun-PGP-9 Expression on Ivermectin and Moxidectin Susceptibility

Epidermal *Pun*-PGP-9 expression reduced susceptibility to IVM only in the absence of PP stimulation but to MOX regardless of PP.

Concerning IVM susceptibility, the epidermis-Pgp-9 line resembled the control strain in the presence of an OP50 PP stimulus (Figure 3a), but in the absence of a PP stimulus, the EC_{50} increased significantly by 2.9-fold compared to the control strain. Similarly, the MOX EC_{50} in the absence of PP in the epidermis-Pgp-9 strain was also significantly increased (Figure 3d). However, significantly increased MOX EC_{50} values were observed regardless of PP in this strain (Figure 3b). Notably, the differences to the control strain were only moderate, i.e., a 1.3-fold in the absence of PP (OP50^{-}) and a 1.3-fold (OP50^{+}) or a 1.5-fold (5-HT^{+}) increase in the presence of PP stimulation.

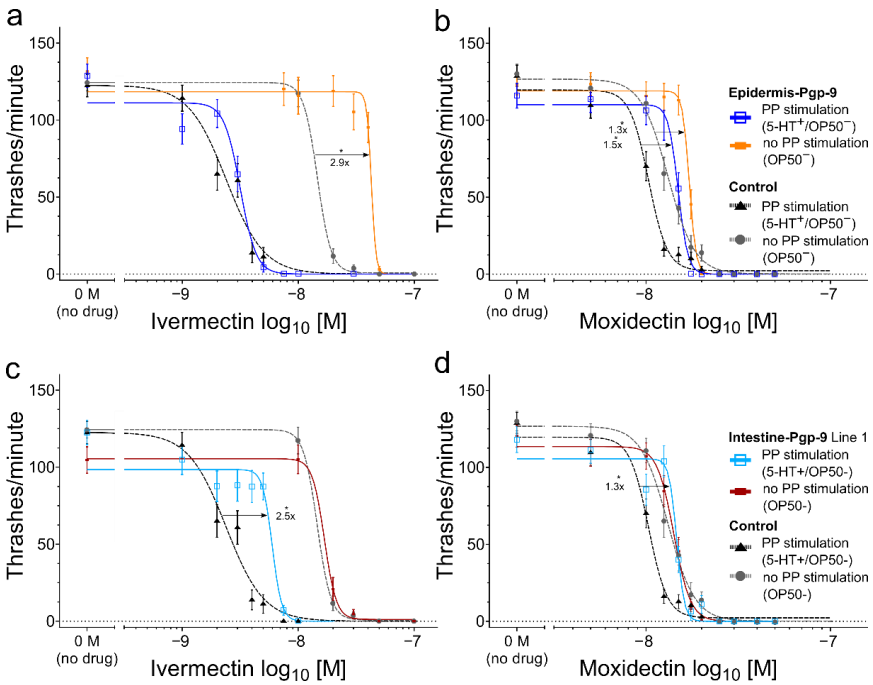


Figure 3. Modulation of ivermectin and moxidectin susceptibility in *C. elegans* by transgenic tissue-specific *Pun*-PGP-9 expression. (**a**–**d**) Concentration–response curves corresponding to Tables 1 and 2 for ivermectin (**a**/**c**) and moxidectin (**b**/**d**) in the absence of a PP stimulus ($OP50^{-}$) or PP stimulation by serotonin ($5\text{-}HT^{+}/OP50^{-}$) in liquid S-medium. (**a**–**d**) show the control strain's response for $5\text{-}HT^{+}$ (black dashed line with triangles) or $OP50^{-}$ (grey dashed line with circles), (**a**,**b**) show the epidermis-Pgp-9 strain for $5\text{-}HT^{+}$ (dark blue with open squares) or $OP50^{-}$ (orange with rectangles) and (**c**,**d**) show the intestine-Pgp-9 line 1 for $5\text{-}HT^{+}$ (light blue with open squares) or $OP50^{-}$ (red with rectangles). All concentration–response curves are based on four-parameter non-linear regression models (GraphPad v8.3.0, www.graphpad.com) calculated from the motility response (thrashes/minute) of 36 synchronised 1-day adults per concentration, and error bars represent standard error of the mean. Prior to calculation, concentrations were log_{10} transformed, and the no-drug negative control was set to 0.1 nM. On the x-axis, the negative control was visualised as "0 M (no drug)" and separated by a break in the axis. Significant differences in EC_{50} values were compared using the extra-sum-of-squares-F test and Bonferroni correction; p-values <0.05 were considered significant and indicated with an asterisk (*), while corresponding fold-changes are indicated with arrows. *Pun*-PGP-9: *Parascaris univalens* P-glycoprotein-9; N2Δ*Cel*Pgp-*9* is tm830 (NBRP) [*Cel-pgp-9(-)*]; Transgenic strains genotypes: Epidermis-Pgp-9 *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*]; Intestine-Pgp-9 Line 1 *IntPgp-9Ex1* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*]; Control strain *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp:: Cel-unc-54_3'-UTR*].

2.2.4. The Effect of Intestinal *Pun*-PGP-9 Expression on Ivermectin and Moxidectin Susceptibility

Intestinal *Pun*-PGP-9 expression reduced susceptibility to both tested MLs, but this effect was always dependent on active drug ingestion.

Concerning IVM, intestinal *Pun*-PGP-9 expression reduced the susceptibility to IVM in the presence of a PP stimulus compared to the control strain (Figure 3c) in two independent lines (Supplementary Figure 1c,d). In contrast, IVM EC_{50} values for both lines were not significantly different from the control strain when PP was not stimulated (Figure 3c).

MOX EC_{50} values in the two intestine-Pgp-9 lines were also significantly elevated when PP was stimulated by OP50 or 5-HT compared to the control strain (Figure 3d). However, in contrast to IVM, the corresponding fold-changes were considerably smaller (<1.3-fold). In addition, all MOX EC_{50} values for the two lines were overall lower than the MOX EC_{50} values calculated for the epidermis-Pgp-9 line. In the absence of PP stimulation, EC_{50} values did not increase compared to the control strain, which is in line with the observations for IVM.

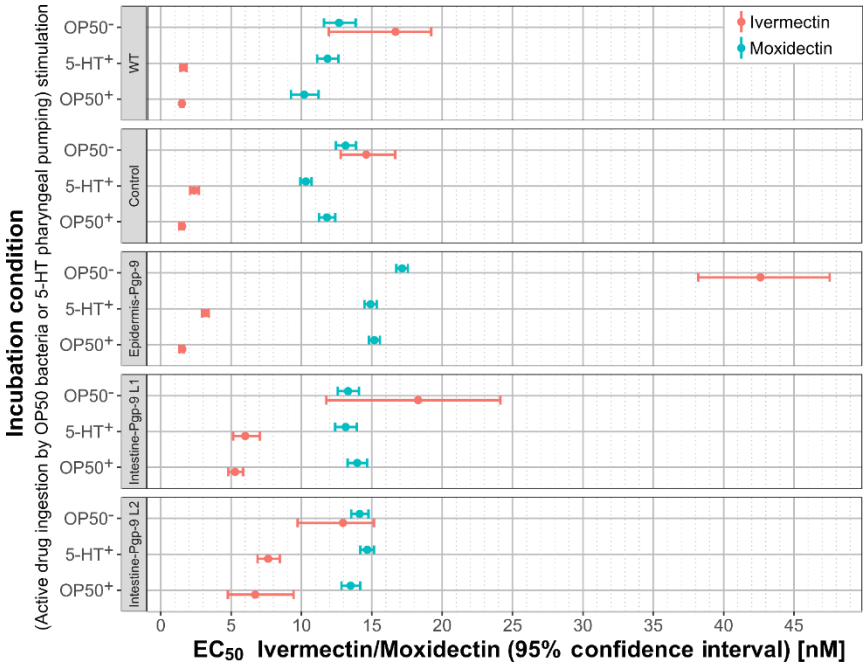


Figure 4. Comparison of moxidectin and ivermectin in wildtype and transgenic *Caenorhabditis elegans* strains. Forrest plot visualising the EC_{50} and corresponding 95% confidence intervals for ivermectin (red) and moxidectin (turquoise) in transgenic and wildtype *Caenorhabditis elegans* strains in the presence of pharyngeal pumping (PP) stimulation by OP50 food bacteria ($OP50^{+}$) or 5-hydroxytryptamine ($5\text{-}HT^{+}$) or no PP stimulation ($OP50^{-}$) were visualised using ggplot2 [42] in R v4.0.3 [43]. EC_{50} values were inferred from four-parameter linear regression models calculated from 36 synchronised 1-day adult worms per concentration, strain, and condition using GraphPad v8.3.0 (GraphPad v8.3.0, www.graphpad.com). Worms were incubated for 24 hours in S-medium containing a concentration series of ivermectin or moxidectin in a final DMSO concentration of 1%. Epidermis-Pgp-9 genotype is *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*];. Intestine Pgp-9 Line 1 (L1) and Line 2 (L2) genotype is *IntPgp-9Ex1 or 2* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*];. Control strain genotype is *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp::Cel-unc-54_3'-UTR*]; WT is N2 Bristol. Pgp: P-glycoprotein; *Pun*-PGP-9: *Parascaris univalens* P-glycoprotein-9; IVM: ivermectin; MOX: moxidectin; PP: pharyngeal pumping; WT: wildtype.

2.2.5. A Comparison of Moxidectin and Ivermectin Susceptibility in Transgenic and Wildtype Strains

Compared to IVM, EC_{50} values for MOX were generally higher across strains, when PP was stimulated but lower in the absence of a PP stimulus (Figure 4) as detailed below. For example, MOX EC_{50} values in the WT in the presence of PP stimulation were significantly higher, by 6.7- and 7.6-fold, compared to IVM (Figure 4) for co-incubation with OP50 or 5-HT, respectively. In contrast, when a PP stimulus was not provided, the MOX EC_{50} was not significantly different compared to that of IVM (Figure 4). Across strains, only small fold-changes below 1.2-fold for the MOX EC_{50} values were detected in the presence or absence of PP stimulation, which is in a sharp contrast to those for IVM. As for IVM, a marked EC_{50} increase (up to 11-fold in WT) from PP stimulation to no PP was observed in all strains (Figure 4). Likewise, *Pun*-PGP-9 expression only resulted in low fold changes for MOX EC_{50} values (<1.5-fold) in comparison to considerably higher fold changes (e.g., >3.5-fold in intestine-Pgp-9) for IVM (Figure 4). Despite the marked effect on IVM susceptibility from intestinal *Pun*-PGP-9 overexpression, all EC_{50} values for both intestine-Pgp-9 lines in the presence of PP stimulation remained lower compared to EC_{50} values of any other strain in the absence of a PP stimulus (Figure 4). In contrast, MOX EC_{50} values in the intestine-Pgp-9 strains in the presence and absence of PP were similar (Figure 4).

3. Discussion

3.1. The Role of Pun-PGP-9 in Ivermectin and Moxidectin Susceptibility

The objective in this study was to elucidate the general functional role of Pgp expression in specific tissues in the context of ML susceptibility. To this end, we chose to exemplarily

characterise *Pun*-PGP-9, as it has already been shown to interact with IVM in a yeast model [23]. An important question addressed in this work was whether there are differences in the protective effect of Pgp overexpression between different ML derivatives, by testing IVM as an avermectin and MOX as a member of the milbemycins, which are summarised in Figure 4.

Currently, functional evidence regarding the interaction between specific parasite Pgps and different MLs has mostly been inferred from indirect assays using the above-mentioned yeast model [23,24] or the cell line LLC-PK1 [24,29,30,44,45]. In these model systems, MLs were used as inhibitors for a secondary Pgp substrate with a discernible effect in the model systems to characterise the interaction between a transgenic parasite Pgp and different MLs. These studies have consistently suggested that MOX has a lower affinity to the examined nematode Pgps than IVM. This conclusion is further supported by our findings in *C. elegans* that *Pun*-PGP-9 overexpression induces larger susceptibility shifts for IVM than for MOX, which were observed without the use of a secondary substrate. Whether the low fold-changes for MOX have any biological significance remains to be elucidated [46]. However, *Pun*-PGP-9 overexpression reduced both MOX and IVM susceptibility, which indicates that both MLs are probably *Pun*-PGP-9 substrates. This substrate range could facilitate cross-resistance, which indeed has been reported in *Parascaris* sp. [47].

In *C. elegans*, only one study had previously demonstrated the impact of a transgenic Pgp on IVM susceptibility, by overexpressing *Pun*-PGP-11.1 under the control of the native *Cel-pgp-11* promotor [41], which has been shown to primarily drive intestinal expression [48]. The concordantly observed moderate fold changes in this and Janssen et al., despite the extrachromosomal array overexpression, might indicate that Pgps cannot cause resistance by themselves in parasitic nematodes but rather that Pgps are contributors within a multigenic context as proposed by NGS studies in other nematode species [17,18].

3.2. The Intestinal and Transcuticlular-Epidermal Uptake of Ivermectin and Moxidectin

Interestingly, the extent of the protective effect against IVM resulting from the *Pun*-PGP-9 expression at the intestine or the epidermis was markedly influenced by the selective intestinal exposure from active drug ingestion by PP. By itself, this newly described effect of active drug ingestion on ML susceptibility was considerable and, for IVM, even surpassed the extent of the effect of Pgp overexpression in either tissue.

The differences between the effect on MOX and IVM susceptibility of active drug ingestion and intestinal or epidermal *Pun*-PGP-9 overexpression might be interpreted as differing uptake capacities at specific *C. elegans* tissues. As a general mechanism, PP stimulation is required for food uptake, and the increased uptake of incubation fluid also facilitates the accumulation of its contents [36].

For IVM, the strong effect of active drug ingestion and the PP dependency of the protective effect of intestinal *Pun*-PGP-9 expression indicate that higher intestinal exposure increases the overall concentration and effect of IVM. Nonetheless, the measurable impact of epidermal *Pun*-PGP-9 expression on susceptibility also indicates that IVM is taken up, although less efficiently, via the cuticle–epidermis.

In contrast, the effect of epidermal *Pun*-PGP-9 expression on MOX susceptibility regardless of PP could suggest that the epidermis–cuticle is the predominant MOX uptake route. For IVM and a few other anthelmintics, transcuticular-epidermal permeability had already been described by biophysical studies on cuticle–epidermis preparations of *Ascaris suum* and experiments using live *A. suum* with a surgically ligated pharynx [49,50]. Likewise, experiments in *C. elegans* have demonstrated a considerably quicker onset of pharyngeal paralysis in a cuticle defective *C. elegans bus-8* loss-of-function strain [51]. A study by O'Lone and Campbell indicated that PP inhibition by refrigeration, and thus inhibition of oral

ingestion, reduced the susceptibility to IVM in *C. elegans* [52]. The authors suggested that oral ingestion increases IVM susceptibility but that transcuticular-epidermal uptake is sufficient to induce paralysis at comparatively low concentrations [52], which is in high concordance with our observations and conclusions.

In contrast to IVM, the uptake of MOX in nematodes has not been studied at all. The absence of a similarly strong effect of PP on MOX susceptibility as observed for IVM could indicate that the cuticle–epidermis represents the main MOX penetration route. This conclusion is also supported by the lowest MOX susceptibility regardless of PP stimulation in the epidermis-Pgp-9 strain. The main factor determining permeability through the nematode cuticle is compound size [49,50,53], and MOX is considerably smaller than IVM due to the lack of the C13 glycosyl side chain. Additionally, lipophilicity is an ancillary but important factor for transcuticular permeability [49,50,53], and indeed IVM has a lower lipophilicity than MOX [54]. These differences in size and lipophilicity seem to offer a reasonable explanation for a less efficient transcuticular IVM uptake but cannot explain why intestinal drug exposure from active drug ingestion increased only IVM but not MOX susceptibility. Therefore, more detailed studies are needed to understand this difference. Both ML derivatives are very lipophilic and accumulate well in high-fat-content tissues in the host [55]. In this regard, the mainly intestinal and epidermal fat storage in *C. elegans* [56] should facilitate passive diffusion and accumulation in these tissues.

3.3. Experimental Limitations and Relevance for Parasitic Nematodes

The novel insights from the present study are, to some extent, limited to the model nematode *C. elegans* that was utilised, since the required forward genetic tools are not yet available for *Parascaris* sp. or any related parasitic nematode species [57]. However, it may be anticipated that the general conclusions of the tissue-specific protective function in *C. elegans* may be extrapolated as a general functional mechanism of Pgps in nematode barrier tissues, such as the epidermis and the intestine, in light of a common nematode body structure. Nevertheless, it should be noted that parasitic nematodes exhibit differences in size, life cycle, stage-specific feeding behaviour, genetics, e.g., in their receptor repertoire [58] and, hence, in their drug susceptibility [59]. For example, IVM and MOX EC_{50} values for adult *C. elegans* were several fold higher than those measured in the adult stage of examined parasitic nematodes in vitro, e.g., *T. circumcincta* [60]. The reasons for these differences remain elusive and have been a matter of speculation. In non-feeding third larval stages, exposure to MLs (or any other anthelmintic) is restricted to the cuticle and, indeed, non-feeding larval stages of parasitic nematodes exhibit considerably lower susceptibilities [60,61]. These differences might be explained by an increased susceptibility from active IVM ingestion. Furthermore, the sheath (i.e., the residual cuticle of the second larval stage) of the third-stage strongylid larva might also reduce susceptibility by limiting drug penetration into the worm. Regarding the uptake of MLs in adult stages of parasitic worms, an in vivo study using an ML-resistant population of the sheep parasite *H. contortus* found no difference of initial drug accumulation in parasites between MOX, IVM, and abamectin 0.5 days after treatment [62]. However, MOX concentrations in the host dropped significantly compared to IVM and abamectin despite similar plasma levels within 2 days post treatment, suggesting that drug elimination may vary between different ML derivatives [62]. In *Ostertagia ostertagi*, a cattle parasite dwelling on the surface of the abomasal mucosa and feeding by sucking on the mucosa, IVM levels in the worms were shown to correlate with IVM concentration in the abomasal mucosa and were maximised by subcutaneous drug application [63]. These findings would support that parasite nematode IVM susceptibility is also increased by feeding. In the same study, IVM levels in the cattle parasite, *Cooperia oncophora*, which dwells in the small intestine, correlated well with

intestinal content, and parasite exposure was maximised by oral drug application [63]. Overall, the study by Leathwick et al. highlights how parasite exposure considerably differs between parasitic species with regard to their biology [63], a concept that can also be extended to the different developmental stages, e.g., migrating/histotropic or hypobiotic stages. Based on the feeding behaviour, the blood-sucking *H. contortus* would be expected to experience high intestinal ML exposure during the early peak in plasma concentrations and, thereafter, prolonged low intestinal exposure, while the parasite cuticle would be exposed from the drug in the abomasal fluid [62,64,65]. Measuring IVM concentrations in adult *H. contortus* 3 days after subcutaneous injection, Lloberas et al. reported only a weak correlation with plasma levels, possibly because of the timing of the measurement [64]. In contrast, intraruminal IVM application resulted in very high abomasal drug concentration, which also resulted in high concentration in the parasite tissues 3 days after IVM application, suggesting an efficient transcuticular uptake [64].

Regarding the observed tissue-specific expression patterns, some reservations remain whether expression was exclusive to the targeted tissues, the intestine and the epidermis. The representative images in Figure 1 show the principal expression pattern differences observed in the stained worms between the lines with transgenic expression induced by two well-characterised promotors, ges-1p and col-19p. However, extrachromosomal array overexpression might result in background expression in other tissues and in particular epidermal specificity was difficult to verify as fluorescence was visible throughout the worm and staining appeared variable along the length of all stained worms. The fluorescence visible in the entire worm might be explained by autofluorescence as it appeared in both the green and blue channels (Figure 1c). The variability in the strength of the staining was also observed in other worms and could be the result of a non-uniform antibody exposure and deformation resulting from the patchy freeze-cracking. 3D-images would be better suited to verify tissue specificity and to rule out background expression in unwanted tissues [66].

4. Conclusions

Our results demonstrate that Pgps can contribute to reducing ML susceptibility in a tissue-specific manner in nematodes and that active drug ingestion increases ML susceptibility. The observed dependency of the protective effect of intestinal or epidermal Pgp overexpression on active drug ingestion would suggest a role of Pgps in barrier fortification by reducing tissue drug permeability as a possible mechanism of a Pgp-mediated ML susceptibility as illustrated in Figure 5. As the tissue-specific expression patterns vary considerably between the diverse Pgp lineages in different nematode species [23,27], the protective capacity of a specific Pgp lineage will vary between species and developmental stages.

Furthermore, our findings emphasise that more attention should be placed on how target parasite species take up ML drugs, since this might lead to differences in susceptibility to individual ML derivatives. Whether changes in barrier permeability represent a relevant mechanism of anthelmintic resistance remains to be elucidated in parasitic nematodes.

In conclusion, this study significantly improves the understanding of a Pgp-mediated ML resistance mechanism by demonstrating how transgenic Pgp expression at specific barriers can impact the susceptibility to different ML derivatives. Furthermore, the differing relevance of active drug ingestion for IVM and MOX susceptibility suggests thus far unknown pharmacological differences and demonstrates the importance of drug barriers and uptake routes for susceptibility.

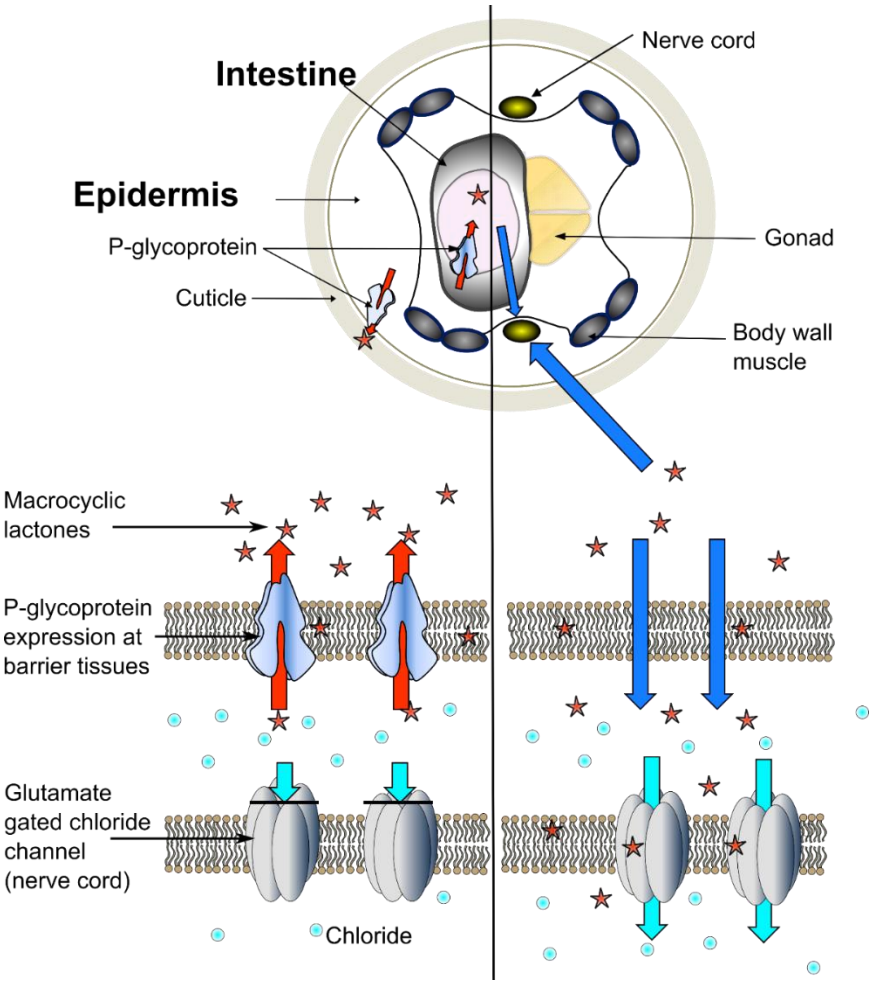


Figure 5. Schematic illustration of P-glycoprotein-mediated barrier function. Hypothetical schematic illustration of Pgp-mediated barrier function in a *Caenorhabditis elegans* adult. Expression of P-glycoproteins in specific barrier tissues, i.e., the epidermis and the intestine prohibit MLs from reaching target tissues, thereby preventing an ML-induced hyperpolarisation of the neurons and muscle paralysis.

5. Materials and Methods

5.1. Plasmids and Plasmid Construction

Plasmids for transgenesis were assembled using the NEB HIFI DNA Assembly Kit (New England Biolabs Inc.) according to the manufacturer's instructions into the pUC19 vector linearised with *Sma*I (ThermoFisher). Plasmid constructs were *Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR* utilising the *col-19* promotor [39] to drive epidermal *Pun*-PGP-9 expression (Supplementary Figure 3a) and *Cel-ges-1p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR* (Supplementary Figure 3b) utilising the *ges-1* promotor [38] to drive intestine-specific *Pun*-PGP-9 expression. The *C. elegans unc-54* 3'-UTR [41] and the *Pun*-pgp-9 cDNA [23] were amplified from verified plasmids, while the 3' end primer for the *Pun-pgp-9* amplification introduced an in-frame FLAG-tag (DYKDDDDK) before the stop codon (all primers in Supplementary Table S3). The *C. elegans* promotors *col-19p* and *ges-1p* [38] were amplified from genomic DNA extracted from the Bristol N2 strain [39]. A co-injection marker plasmid (pPD118.33) driving pharyngeal GFP expression was used (Addgene L3790 plasmid #1596 was a gift from A. Fire). Sequences of all constructs were confirmed by Sanger-sequencing (LGC Genomics).

5.2. Generation of Caenorhabditis elegans Strains and Maintenance

Caenorhabditis elegans WT strain Bristol N2 was obtained from the *Caenorhabditis* Genetics Centre (CGC; University of Minnesota, Minneapolis, MN, USA) and the *Cel-pgp-9* deletion strain tm830 [*Cel-pgp-9(-)*] was obtained from the National BioResource Project (NBRP; Tokyo, Japan). Strains were maintained at 20 °C and standard conditions on NGM plates [67].

Plasmid constructs for intestinal and epidermal *Pun*-PGP-9 expression were loaded onto Eppendorf FemtoTips II needles at 25.0 ng/µL along with pPD118.33 as a co-injection marker at 12.5 ng/µL. Injection into the gonads of 1-day adults of the *Cel-pgp-9* deletion strain was performed using an Eppendorf Femtojet 4 connected to an Eppendorf micromanipulator and mounted onto a Leica inverse microscope. Preparation of worms for injection was carried out as described elsewhere [68]. Additionally, a strain controlling for pharyngeal GFP expression and presence of extrachromosomal arrays in general was generated by injecting only the co-injection marker. Transgenic strains carrying extrachromosomal arrays *IntPgp-9Ex1* and *IntPgp-9Ex2* with genotype [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR], EpiPgp-9Ex1* with genotype [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR]*) and *CtrlEx1* with genotype [*Cel-pgp-9(-); Cel-myo-2p::gfp::Cel-unc-54_3'-UTR*) were maintained by regular transfer of GFP-positive individuals to a new plate.

5.3. Verification of Pun-PGP-9 Expression by RT-PCR and Immunofluorescence

Transcription of *Pun-pgp-9* in GFP-positive offspring of injected worms was confirmed by reverse transcriptase (RT)-PCR (primers and cycle conditions in Supplementary Table S3) using the S7 Fusion enzyme (Mobidiag) and a cDNA or a no-RT control template. cDNA was generated from DNAse-treated RNA. In addition, tissue-specific *Pun*-PGP-9 protein expression was examined with immunofluorescence. Freeze cracking and antibody staining were performed in a 1.5 mL tube as described elsewhere [69] with minor adaptations using 4% formaldehyde and 50% methanol for fixation followed by freeze-cracking worms three times in liquid nitrogen and thawing in water at room temperature. Following the freeze–thaw cycles, tubes were shaken at 37 °C for 1 hour. To remove the fixative, worms were centrifuged at 11,000 × *g* for 1 minute and washed with PBS-T (phosphate-buffered saline + 0.5% Triton-X100) four times, removing all liquid during the last aspiration of PBS-T. Prior to antibody staining, worms were incubated in 1 mL PBS-BSA (PBS + 1% bovine serum albumin) overnight at 4 °C under mild shaking. The next day, worms were centrifuged for 1 minute and then incubated for 24 hours at 4 °C and mild shaking in 500 µL PBS-BSA containing a monoclonal (FG4R), mouse-derived anti-FLAG IgG antibody diluted 1:200 (ab125243, A85282 antibodies.com). The following day, worms were washed again five times in PBS to remove unbound primary antibody. Once again, worms were incubated for 24 hours at 4 °C in 500 µL of PBS-BSA containing DyLight 405 conjugated polyclonal donkey anti-mouse antibodies diluted 1:300 (DyLight™ 405 AffiniPure Donkey Anti-Mouse IgG (H+L), Jackson ImmunoResearch). Following another five washes with PBS-BSA, all liquid was completely removed, and worms were transferred in ~25 µL to an untreated microscope slide. After adding a drop (~25 µL) of VECTASHIELD® mounting medium and sealing with nail polish, specimens were examined on an Eclipse Ti-U inverted research confocal microscope (Nikon, Tokyo, Japan) with a 20× and a 40× objective at excitation wavelength 405 nm to visualise antibody-specific staining (DyLight405) and 488 nm excitation to visualise pharyngeal GFP expression. Differential interference contrast (DIC) pictures were taken to visualise worm anatomy. Images were taken using VisiView 4.3.0 at 16 bit. ImageJ was used to pseudocolour and merge channels [40]. Fluorescent images at 405 nM and 488 nm excitation wavelength were visualised in the blue or green lookup table (LUT) scale provided by ImageJ.

5.4. Trashing Assay

To prepare stock solutions, IVM and MOX (Sigma-Aldrich, St. Louis, USA) were dissolved in DMSO and frozen at −20 °C. A saturated 40 mM 5-HT stock solution was prepared by

dissolving serotonin creatinine sulphate monohydrate (Sigma-Aldrich) in S-medium [67] through vigorous vortexing and was either immediately frozen at −20 °C or used directly.

Bleach-synchronised L1 [67] were grown to adult stage on NGM plates (72 h). One-day-old adults were washed with S-medium by repeatedly allowing worms to sink to the bottom of a 15 mL centrifuge tube, discarding, and refilling to remove all bacteria. The arrest of PP caused by the absence of food was confirmed visually after one hour of acclimatisation with an inverted microscope. In each individual experiment, 12 worms (only GFP-positive worms in case of transgenic strains) were transferred into 6-well plates (Sarstedt) with 2 mL S-medium final volume containing either no OP50 bacteria ($OP50^-$), OP50 bacteria at OD_{600} = 0.5 ($OP50^+$) or 4 mM 5-HT ($OP50^-$/5-HT^+ referred to as 5-HT^+ in the text for simplicity), the latter two stimulating PP, which was visually confirmed before adding the drug. Dilution series of MLs were prepared in DMSO and added to the medium resulting in a final concentration of 1% DMSO. For IVM, the final concentrations for the $OP50^-$ condition were 0.0, 10.0, 20.0, 30.0, 40.0, 50.0, and 100.0 nM IVM and for the $OP50^+$ and 5-HT^+ conditions 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, and 10 nM IVM. For MOX, final concentrations for all conditions were 0.0, 5.0, 10.0, 12.5, 15.0, 17.5, 20.0, 30, 40, 50, and 100 nM MOX. Plates were sealed with parafilm to avoid evaporation and incubated in the dark at 20 °C and 150 rpm for 18–24 hours. The next day, worms were transferred by pipetting to agar-coated Petri dishes filled with S-medium without bacteria and were allowed to acclimatise for 1 minute. Then, thrashes of individual worms were counted for one minute on a stereo-fluorescence microscope.

Caenorhabditis elegans adult worms that were incubated in S-medium without bacteria did not readily move, and even soft touch stimulus and shaking did not induce any movements. However, transfer with a pipette induced strong thrashing, hence this step was performed across all experiments and all incubation conditions to detect ML-induced paralysis. Likewise, adult worms incubated with a sufficient supply of OP50 bacteria only thrashed occasionally, but the transfer of worms by pipetting induced vigorous thrashing.

5.5. Statistical Analysis

For each concentration, strain, and condition at least 12 worms per day were tested on three separate days (total $n \geq 36$). Before $\log_{10}$ transformation of drug concentrations, vehicle controls were set to concentrations of 0.1 nM. Four-parameter non-linear logistic regression models and statistical analyses were calculated and visualised using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, California USA) constraining the bottom values to ≥0. In each graph, the negative control concentration was visualised as "0 M no drug" with a break in the x-axis. Forest plots visualising corresponding half-maximal effective concentration (EC_{50}) and 95% confidence intervals (CI) values were visualised using ggplot2 [42] in R v4.0.3 [43]. Statistical differences in EC_{50} values were calculated with the extra-sum-of-squares F test applying the Bonferroni–Holm correction for multiple testing and considering a p-value smaller than 0.05 as significant. For comparison, each *Pun*-PGP-9-expressing transgenic strain was compared to the control strain at the respective condition. Moreover, the control strain was compared to the WT at the respective condition. The EC_{50} values of the WT at the condition without food ($OP50^-$) and without food plus 5-HT (5-HT^+) were compared to the condition with food ($OP50^+$). In addition, statistical comparisons of EC_{50} values between MOX and IVM were calculated for the WT. Fold changes were calculated based on EC_{50} values. To examine the impact of the feeding stimuli and transgene expression on motility, the motility response of all worms incubated without drug (negative no drug controls) were pooled per strain and per condition (n = 72) and a Kruskal–Wallis test with a Dunn's post hoc was conducted comparing all conditions in each strain to the WT $OP50^-$ condition in GraphPad.

Supplementary Materials: The following are available online at https://www.mdpi.com/1424-8247/14/2/153/s1, Figure S1: Concentration–response curves for intestine-Pgp-9 Line 2, wildtype, and control strain; Figure S2: Concentration–response curves of *Parascaris univalens* Pgp-9-expressing transgenic, control, and wildtype strains to ivermectin and moxidectin under different conditions; Figure S3: Vector maps of expression vectors, Table S1 Ivermectin concentration-response parameters of transgenic, control and wildtype strains at different conditions; Table S2 Moxidectin concentration-response parameters of transgenic, control and wildtype strains at different conditions; Table S3: Primer

Author Contributions: A.P.G.: Conceptualisation, methodology, investigation, formal analysis, data curation and visualisation, writing—original draft preparation, review and editing, funding acquisition. J.K.: Conceptualisation, methodology, data curation and visualisation, writing—review and editing, supervision, funding acquisition. C.N.: conceptualisation, writing—review and editing, supervision. C.L.C.: conceptualisation, writing—review and editing, supervision, funding acquisition. A.H.: conceptualisation, methodology, writing—review and editing, supervision. G.v.S.-H.: conceptualisation, data curation and visualisation, writing—review and editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (grant number 111144555). Additional funding was provided by the Research Training Group 2046 "Parasite Infections: From Experimental Models to Natural Systems" also funded by the Deutsche Forschungsgemeinschaft (grant number 251133687/GRK 2046) and the Karl-Enigk Foundation grant number S0229/10019/19. This work was also supported by the German–French bilateral collaboration program Procope 2018-2019, which was funded by the Deutscher Akademischer Austausch Dienst (DAAD) (Project ID 5739054) and the Hubert Curien Partnerships program (project number 40582SM). A.G. was the grateful recipient of a travel grant from the 2017–18 Burroughs Wellcome Fund Collaborative Research Travel Award. This project was also supported through a Guest Fellowship awarded by Le Studium to G.v.S.-H. at the Institut national de la recherche agronomique (INRAE) Nouzilly, France.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Plasmids are available upon request and will be deposited on Addgene.org. The data from this study are available on request from the corresponding author.

Acknowledgements: We are grateful to Jonathan Ewbank for his guidance with *C. elegans* promotors and for providing the primer sequences for promotor amplification. We thank Jacqueline Hellinga for language proofreading the manuscript. The publication of this article was funded by Freie Universität Berlin.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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3.2.1 Supplementary Material

Supplementary files are available at: https://www.mdpi.com/1424-8247/14/2/153/s1

Pharyngeal pumping and tissue-specific transgenic P-glycoprotein expression influence macrocyclic lactone susceptibility in *Caenorhabditis elegans*

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Supplementary Files

Supplementary Figure 1 Concentration-response curves for Intestine-Pgp-9 Line 2, wildtype and control strain

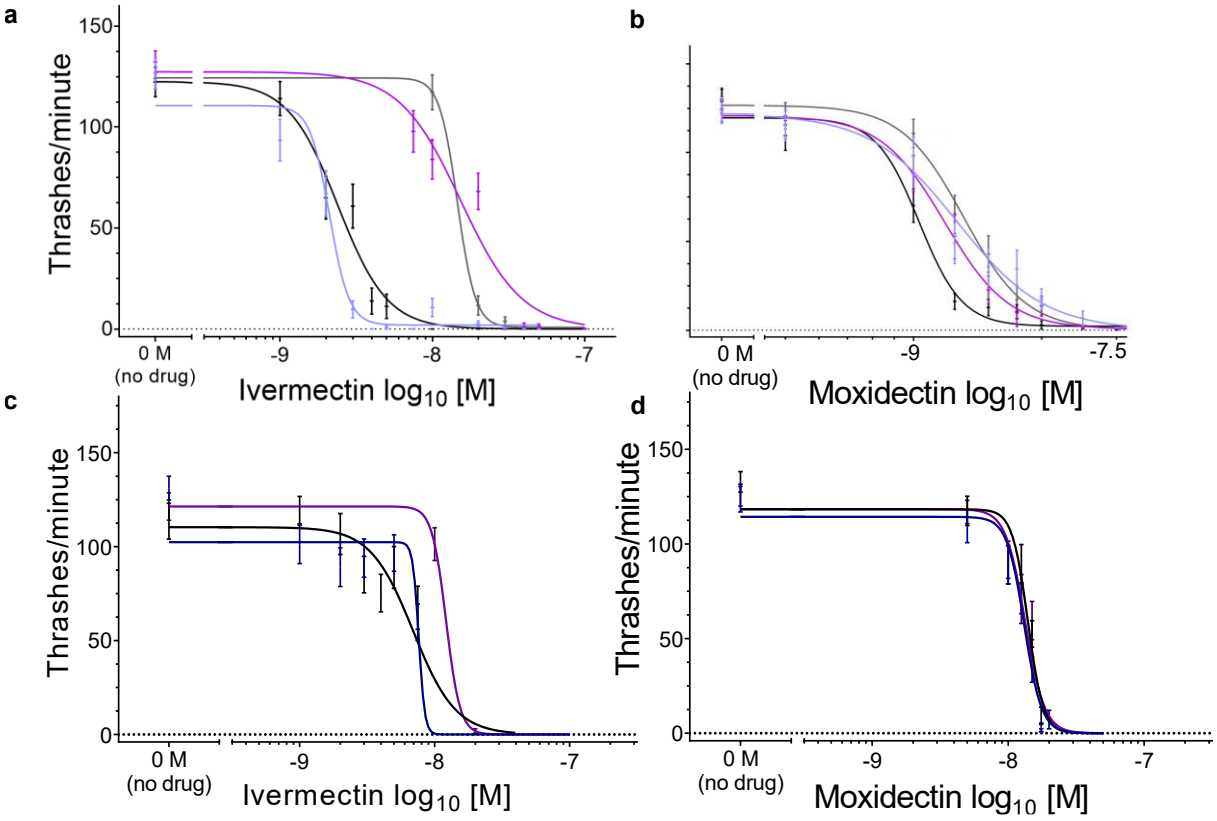
Supplementary Figure 2. All concentration-response curves of *Parascaris univalens* Pgp-9 expressing transgenic including both control and wildtype strains to ivermectin and moxidectin

Supplementary Figure 3 Vector maps of expression vectors

Supplementary Table 1 Ivermectin concentration-response parameters of transgenic, control and wildtype strains at different conditions

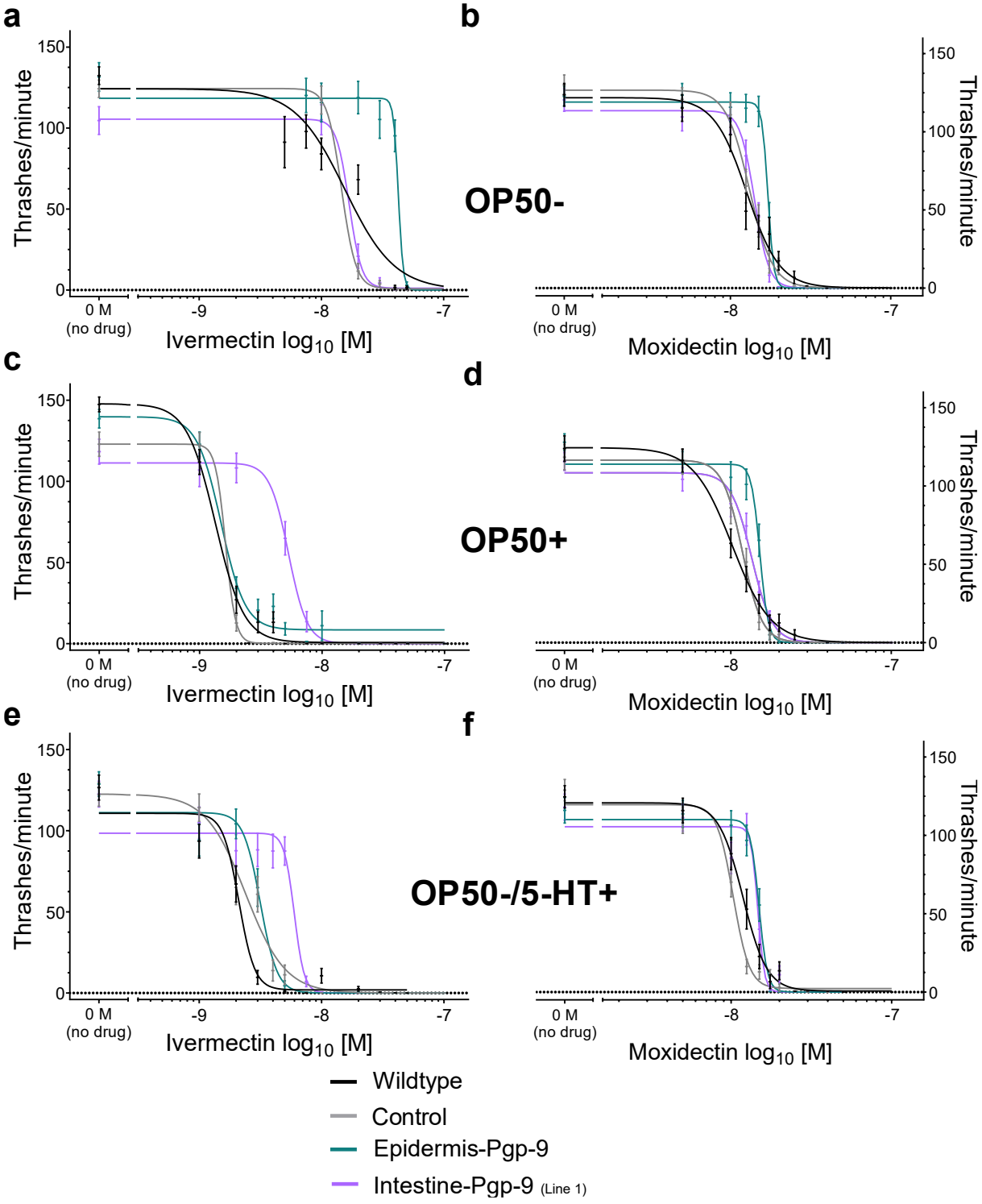
Supplementary Table 2 Moxidectin concentration-response parameters of transgenic, control and wildtype strains at different conditions

Supplementary Table 3 Primers



Supplementary Figure 1 Concentration-response curves for Intestine-Pgp-9 Line 2, wildtype and control strain

Concentration response curves were calculated and visualized in GraphPad v8.3.0 based on motility response (thrashes/minute) following ivermectin or moxidectin incubation. Concentrations were log10 transformed after setting the negative control to 0.1 nM. Concentrations response from four parameter non-linear regression models are visualized with the standard error of the mean at each concentration from 36 worms per concentration and condition. On the x-axis, the negative control was visualized as "0 M (no drug)" and separated by a break in the x-axis. Adult worms were incubated with a dilution series of ivermectin or moxidectin (final 1% DMSO concentration) for 24 hours under different conditions. **a-b** Motility response following **a** – ivermectin or **b** – moxidectin incubation in the wildtype (WT) and the control strain with pharyngeal pumping stimulation (PP) by 5-HT (black – control, blue WT) or without PP stimulation ($OP50^-$) (grey – control, purple – WT). **c-d** Motility response following **c** – ivermectin or **d** – moxidectin incubation in the intestine line 2 in the presence of PP stimulation by OP50 bacteria ($OP50^+$ - blue) and serotonin ($5\text{-}HT^+$ - black), or in the absence of a stimulus for PP ($OP50^-$ - purple). Pgp: P-glycoprotein; 5-HT is 5-hydroxytryptamine; N2Δ*Cel*Pgp-*9* is tm830 (NBRP); Transgenic strains genotypes: *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*]; *IntPgp-9Ex1* and *IntPgp-9Ex2* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*]; *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp::Cel-unc-54_3'-UTR*]

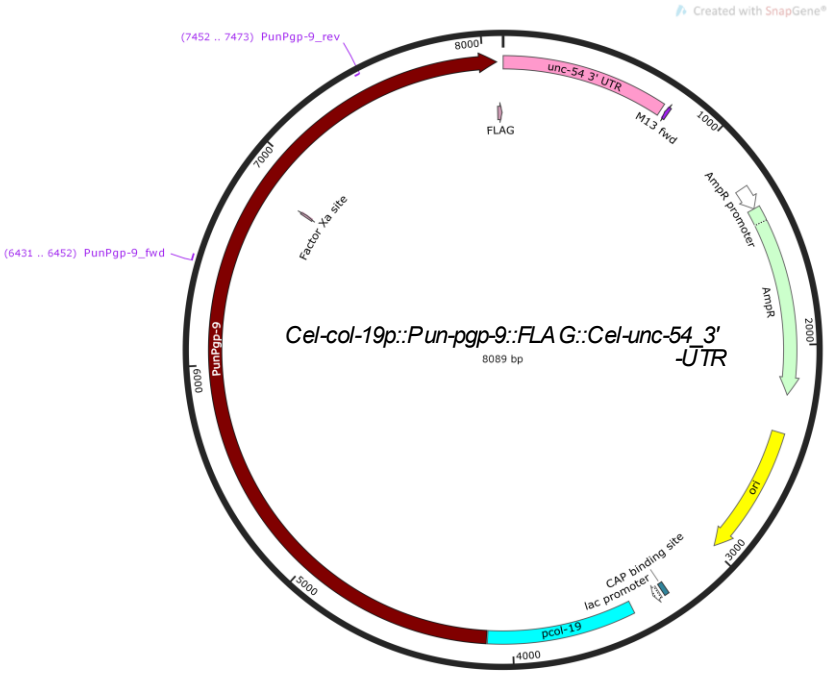


Supplementary Figure 2. Concentration-response curves of *Parascaris univalens* Pgp-9 expressing transgenic, control and wildtype strains to ivermectin and moxidectin under different conditions

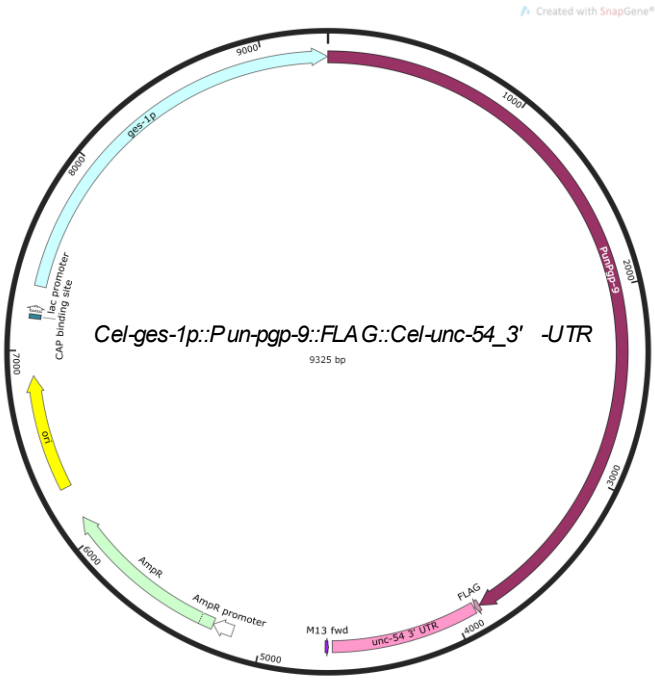
Concentration-response curves for ivermectin (a,c,e) and moxidectin (b, d, f) calculated with GraphPad v8.0.0 were visualized for 4 different strains, wildtype (WT) (black), control strain (grey), hypodermis-Pgp-9 strain (turquoise) and intestine-Pgp-9 line 1 (purple). For each strain and concentration, 36 synchronized 1-day old adult worms spread equally on three separate days were incubated for 24 hours in S-medium in the absence of OP50 bacteria ($OP50^-$) (**a**, **b**), in the presence of OP50 bacteria ($OP50^+$) (**c**, **d**) stimulating pharyngeal pumping, or in the absence of $OP50^-$ but with pharyngeal pumping stimulation by 4 mM 5-hydroxytryptamine ($OP50^-$/5-HT^+) (**e, f**). Four parameter logistic regression models were calculated and visualized along with the mean of trashes/minute (body bends) ± standard

error of the mean at each concentration. Prior to the calculation, concentrations were $\log_{10}$ transformed and the no drug negative control was set to 0.1 nM. On the x-axis, the negative control was visualized as “0 M (no drug)” and separated by a break in the axis. For both drugs a dilution series with a final DMSO concentration of 1% was used. Pgp-9: P-glycoprotein-9

a



b



Supplementary Figure 3 Vector maps of expression vectors

Vector maps of plasmids driving tissue specific PunPgp-9 expression generated with SnapGene. The backbone vector puc-19 contains elements for transformation and selection in bacteria. (a) Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR driving epidermal Pun-pgp-9 expression (b) Cel-ges-1p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR driving intestinal Pun-pgp-9 Pun-pgp-9 is Parascaris univalens P-glycoprotein-9; UTR: untranslated region;

FLAG: FLAG-tag GAC TAC AAA GAC GAT GAC or DYKDDDDK (protein sequence)

Supplementary Table 1 Ivermectin concentration-response parameters of transgenic, control and wildtype strains at different conditions

Background Strain	Strain (Genotype)[a]	*Pun*-PGP-9 expression	Condition	EC_{50}[b] (95% CI) [nM]	R^2	Fold Change[c]	p-value[d]
N2	WT	–	OP50^{-}	16.68 (11.94-19.22)	0.58	11.1 (WT OP50^{+})	0.0014
			OP50^{+}	1.50 (1.41-1.61)	0.78	–	
			5-HT^{+}	2.08 (1.97-2. 19)	0.61	1.4 (WT OP50^{+})	0.0014
N2Δ*Cel-pgp*-9	Control	–	OP50^{-}	14.60 (12.79-16.67)	0.81	0.9 (WT OP50^{-})	1
			OP50^{+}	1.51 (1.32-1.65)	0.79	1.0 (WT OP50^{+})	1
			5-HT^{+}	2.37 (2.08-2.70)	0.60	1.1 (WT 5-HT^{+})	1
	Epidermis-Pgp-9 (*EpiPgp-9Ex1)*	Epidermis	OP50^{-}	42.62 (38.20-47.54)	0.46	2.9 (Control OP50^{-})	0.0014
			OP50^{+}	1.53 (1.32-1.64)	0.69	1.0 (Control OP50^{+})	1
			5-HT^{+}	3.16 (2.93 -3.41)	0.65	1.3 (Control 5-HT^{+})	0.0014
	Intestine-Pgp-9 Line 1 (*IntPgp-9Ex1)*	Intestine	OP50^{-}	18.29 (11.76-24.13)	0.63	1.2 (Control OP50^{-})	1
			OP50^{+}	5.28 (4.81-5.84)	0.58	3.5 (Control OP50^{+})	0.0014
			5-HT^{+}	6.00 (5.14-7.04)	0.41	2.5 (Control 5-HT^{+})	0.0014

	Intestine-Pgp-9 Line 2 (*IntPgp-9Ex2)*	Intestine	OP50^{-}	12.95 (9.73-15.15)	0.78	0.9 (Control OP50^{-})	0.3102
			OP50^{+}	6.71 (4.77-9.44)	0.42	4.4 (Control OP50^{+})	0.0014
			5-HT^{+}	7.63 (6.88-8.46)	0.59	3.2 (Control 5-HT^{+})	0.0014

Concentration-response parameters correspond to Figure 2C,E, Figure 3A-D and Figure 4.
[a]N2: N2 Bristol *C. elegans* strain; N2Δ*Cel*Pgp-9: *C. elegans* strain tm830; WT: wildtype (N2 Bristol *C. elegans* strain); Transgenic strains genotypes: *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*]; *IntPgp-9Ex1* and *IntPgp-9Ex2* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*]; *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp::Cel-unc-54_3'-UTR*]
[b]EC_{50}: half maximal effective concentration
[c]Fold changes were calculated by comparing the EC_{50} of a strain and condition to the EC_{50} of a respective control which is noted in brackets
[d]p-values were calculated by comparing a pair of non-linear regression models as listed in the fold-changes column using the extra-sum-of-squares-F test, and then adjusting p-values for multiple testing with the Bonferroni-Holm method in R
PGP/*pgp*: P-glycoprotein; CI: Confidence interval

Supplementary Table 2. Moxidectin concentration-response parameters of transgenic, control and wildtype strains at different conditions

Concentration-response parameters correspond to Figure 2D, E, Figure 3A-D and Figure 4.

Background Strain	**Strain (Genotype)**[a]	***Pun*-PGP-9 expression**	**Condition**	**EC_{50}**[b] **(95% CI) [nM]**	**R^2**	**Fold Change**[c]	**p-value**[d]
N2	WT	–	OP50^{-}	12.67 (11.60-13.84)	0.59	1.2 (WT OP50^{+}) and 0.76 (IVM WT OP50^{-})	0.0024 and 0.1367
			OP50^{+}	10.19 (9.256-11.22)	0.63	6.7 (IVM WT OP50^{+})	0.0003
			5-HT^{+}	11.85 (11.12-12.63)	0.65	1.2 (WT OP50^{+}) and 7.6 (IVM 5-HT^{+})	0.0196 and 0.0003
N2Δ*Cel-pgp-9*	Control	–	OP50^{-}	13.13 (12.44-13.87)	0.72	1.0 (WT OP50^{-})	0.8892
			OP50^{+}	11.61 (11.24-12.4)	0.66	1.1 (WT OP50^{+})	0.02

			5-HT$^+$	10.31 (9.93-10.72)	0.71	0.9 (WT 5-HT$^+$)	0.0016
	Epidermis-Pgp-9 (*EpiPgp-9Ex1)*	Epidermis	OP50$^-$	17.15 (16.74-17.56)	0.69	1.3 (Control OP50$^-$)	0.0014
			OP50$^+$	15.18 (14.79-15.58)	0.69	1.3 (Control OP50$^+$)	0.0014
			5-HT$^+$	14.91 (14.48-15.35)	0.65	1.5 (Control 5-HT$^+$)	0.0014
	Intestine-Pgp-9 Line 1 (*IntPgp-9Ex1)*	Intestine	OP50$^-$	13.31 (12.57-14.09)	0.65	1.0 (Control OP50$^-$)	0.1179
			OP50$^+$	13.96 (13.28-14.67)	0.65	1.2 (Control OP50$^+$)	0.0016
			5-HT$^+$	13.14 (12.39-13.93)	0.59	1.3 (Control 5-HT$^+$)	0.0014
	Intestine-Pgp-9 Line 2 (*IntPgp-9Ex2)*	Intestine	OP50$^-$	14.13 (13.53-14.76)	0.62	1.1 (Control OP50$^-$)	0.8892
			OP50$^+$	13.49 (12.84-14.18)	0.58	1.2 (Control OP50$^+$)	0.0014
			5-HT$^+$	14.67 (14.19-15.16)	0.60	1.4 (Control 5-HT$^+$)	0.0014

[a]N2: N2 Bristol *C. elegans* strain; N2Δ*Cel*Pgp-9: *C. elegans* strain tm830; WT: wildtype (N2 Bristol *C. elegans* strain); Transgenic strains genotypes: *EpiPgp-9Ex1* [*Cel-pgp-9(-); Cel-col-19p::Pun-pgp-9::FLAG::Cel-unc-54_3'-UTR; Cel-myo-2p::gfp:: Cel-unc-54_3'UTR*]; *IntPgp-9Ex1* and *IntPgp-9Ex2* [*Cel-pgp-9(-); Cel-ges-1p::Pun-pgp-9::FLAG::Celunc-54_3'-UTR; Cel-myo-2p::gfp::Cel-unc-54_3'UTR*]; *CtrlEx1* [*Cel-pgp-9(-); Cel-myo-2p::gfp:: Cel-unc-54_3'-UTR*]
[b]EC_{50}: half maximal effective concentration
[c]Fold changes were calculated by comparing the EC_{50} of a strain and condition to the EC_{50} of a respective control which is noted in brackets
[d]p-values were calculated by comparing a pair of non-linear regression models as listed in the fold-changes column using the extra-sum-of-squares-F test, and then adjusting p-values for multiple testing with the Bonferroni-Holm method in R
PGP/*pgp*:: P-glycoprotein; CI: Confidence interval

Name	Direction	Purpose	Sequence (5'-3')	Fragment Size (bp)*	Comment
Pun-Pgp-9_fwd	Forward	RT-PCR	GATCAGATGCTCAGCAATGGTC	1043 bp	
Pun-Pgp-9_rev	Reverse	RT-PCR	ACAGTTCCATCAATTGGGTCAT		
Cel-col-19p_fwd	Forward	Promotor amplification from gDNA	catttgaaaatttgcaccaatgt	671 bp	
Cel-col-19p_rev	Reverse		catcagttcatcaacatgcg		
ges-1p_puc19_ass_fwd	Forward	Assembly	gtcgactctagaggatccccaaactccgaactatgatg	2000 bp	Overlaps puc19 and *ges-1p*
ges-1p_puc19_ass_rev	Reverse	Assembly	taatagacatctgaattcaaagataagatatgtaatag		Overlaps *ges-1p* and *Pun-pgp-9*
col-19p_puc19_ass_fwd	Forward	Assembly	gtcgactctagaggatcccccatttgaaaatttgcaccaatg	671 bp	Overlaps puc19 and pcol-19
col-19p_puc19_ass_rev	Reverse	Assembly	taatagacatcgcatgttgatgaactgatg		Overlaps *col-19*p and Pun-pgp-9
Pgp-9_pcol-19_ass_fwd	Forward	Assembly	tcaacatgcgatgtctattagatcgagtcac		Overlaps *col-19p* and Pun-pgp-9
Pgp-9_pges-1_ass_fwd	Forward	Assembly	ttgaattcagatgtctattagatcgagtcac		
Pgp-9-FLAG_3UTR_ass_rev	Reverse	Assembly	tcacttatcatcatcatccttgtaatccatgcgtgcatcaaagtc	3972 bp	Overlaps unc-54 3'UTR and Pgp-9_5'-end and integrates a FLAG-tag, combined with a primer overlapping each promotor
unc-54-5'_Pgp-9-FLAG_ass_fwd	Forward	Assembly	gattacaaggatgatgatgataagtgaaggcccatctcgcgcccg	748 bp	Overlaps unc-54 3'UTR and Pgp-9_5'-end and integrates a FLAG-tag
unc-54-5'_ass_rev	Reverse	Assembly	tgaattcgagctcggtacccccctgcaggaaacagttatgtttggtatattgggaatgtattctg		Overlaps unc-54 3'UTR and puc19

*excluding flanking region of assembly primers

Supplementary Table S3 Primers

Primers for RT-PCR and assembly of plasmid constructs. Assembly primers were generated with NEBuilder Assembly tool (New England Biolabs). Pun: Parascaris univalens. Cel: Caenorhabditis elegans. Pgp: P-glycoprotein. UTR: untranslated region; gDNA genomic DNA

3.3 Improved synergy with ivermectin of three potential novel nematode P-glycoprotein inhibitors in a *Caenorhabditis elegans* larval development assay

This article is not peer-reviewed and is only published within the scope of this dissertation.

Improved synergy with ivermectin of three potential novel nematode P-glycoprotein inhibitors in a *Caenorhabditis elegans* larval development assay

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This manuscript contains data which will later be published in a peer-reviewed publication.

Abstract

P-glycoproteins (Pgps) are considered to be contributors in macrocyclic lactone (ML) resistance mechanism in nematodes. These ATP-binding-cassette (ABC) transmembrane transporters are conserved in all eukaryotes, including mammals, and in addition to ML resistance they have been implicated with chemotherapeutic drug resistance in several human cancers. This has led to the development of large compound libraries screened for their interaction with the human Pgp. The aim of our study was to identify Pgp-is which exhibit a higher specificity to nematode Pgps than to their mammalian orthologue, so compounds with a low affinity to the human Pgp were chosen for characterization in a *Caenorhabditis elegans* larval development assay. When *C. elegans* were incubated with inhibitor and ivermectin, all substances exhibited increased ivermectin susceptibility in a concentration-dependent manner while verapamil, a known Pgp-i, did not increase ivermectin efficacy within this experimental set-up. In addition, all compounds induced a retardation of larval development at high molar concentrations (50 µM for compounds 8b, 9b and verapamil and 10 µM for compound 11c) without the addition of an ivermectin. While these results suggest that the tested compounds improve ivermectin efficacy compared to currently used Pgp-i, a more detailed characterization is needed to understand whether the observed effects are the result of inhibition of the xenobiotic detoxification machinery.

Introduction

P-glycoproteins (Pgp) are xenobiotic transporters of the ATP-binding cassette gene family subfamily B (ABCB) and are conserved in all eukaryotic phyla [1-3]. They have been implicated in resistance to chemotherapy in cancer [4, 5] and in infectious agents including the protozoan parasites *Leishmania* sp. and *Plasmodium* sp. [6], arthropod parasites [7, 8] and parasitic nematodes [9]. In nematodes, widespread anthelmintic resistance to one or multiple relevant compounds has become a global challenge to maintaining human and animal health [10-14] and Pgp have been linked to resistance against several relevant anthelmintics, most frequently to resistance against the important group of macrocyclic lactones (MLs) [9] but also against benzimidazoles [15] and monepantel [16].

While the complete elucidation of the ML resistance mechanisms has proven difficult, there is growing evidence for a contribution of Pgp within a multi-genetic context [17]. Strikingly, the association of Pgp and ML resistance has been reported in a large number of different

nematode species, e.g. sheep parasites *Haemonchus contortus [18-20]* and *Teladorsagia circumcincta* [17, 21], the canine heartworm *Dirofilaria immitis* [22, 23], and the cyathostomines [24] and the roundworm *Parascaris univalens [25]* parasitizing horses. Likewise, functional evidence for an interaction of nematode Pgp and MLs has been provided in different model systems [26-28]. Concurrently, the success of the macrocyclic lactones has resulted in the development of only very few of new anthelmintics and particularly which is a particular problem in in the veterinary sector and in equine medicine [29]. Here, the widespread emergence of macrocyclic lactone and multiple resistances severely limits safe and efficacious options to treat parasitic nematode infections. Therefore, there is an urgent need for prolonging the lifetime of established drugs, and inhibitors of Pgp could reverse resistance in populations with Pgp associated resistance. Several promising Pgp inhibitors (Pgp-i) have been reported to reverse drug resistance *in-vitro* including verapamil (VPM) [30, 31], ketoconazole, valspodar, quiercetin, pluronic P85 [32]. More recently, specifically developed so called “third generation” Pgp-i such as zosuquidar, tariquidar and crizotinib have also been shown to increase susceptibility to ivermectin (IVM), a commonly used ML, in *H. contortus in-vitro* [33, 34]. However, all current Pgp-i are unspecific and target both nematode and mammalian Pgp. In mammals deficient in Pgp (Mdr-1), treatment with ML results in neurotoxicity as a result of significantly increased ML penetrability of the blood brain barrier [35-37]. This leads to the dilemma, that unselective inhibition of Pgp targeting both nematode and mammal Pgp is similarly expected to lead to severe neurotoxicity [38]. Hence, few studies have explored the suitability of Pgp inhibition in the context of anthelmintic drug efficacy in-vivo. Only one group investigated the in-vivo administration of a known Pgp substrate, the anti-peristaltic antidiarrhea agent loperamide [39], in combination with IVM or moxidectin (MOX) and reported a considerable increase in treatment efficacy against the gastro-intestinal nematodes *Ostertagia* sp. and *Cooperia* sp. in cattle [40] as well as in lambs infected with highly IVM resistant *H. contortus* [41]. In addition, a marked increase in bioavailability potentially due to the inhibition of the intestinal elimination of IVM by Pgp [42] or another pathway was reported but importantly, no neurotoxicity.

While conserved in all eukaryotes, Pgp have diversified mostly through gene duplications to different extents in different phyla. Specifically in nematodes, the Pgp gene family has expanded to comprise a diverse repertoire of ten to fifteen different lineages [43-45] while most mammals are limited to one (e.g. humans) or two different (e.g. mice, pigs) genes [1, 46]. Noteworthy, single nucleotide polymorphisms in individual Pgp have been linked to changes in substrate specificity and affinity [47, 48]. Therefore, as single amino acid changes can alter Pgp substrate specificity it is possible that between mammal Pgp and the phylogenetically more closely related cluster of invertebrate Pgp major differences in substrate range exist and it might be possible to find Pgp-i specific to nematode Pgp. To reverse Pgp-mediated drug resistance in cancer, many new Pgp-i have been developed in the last decade [4, 49, 50]. Many of these are well characterized and some of these drugs exhibit only low or no interaction with mammalian Pgp. This large library of compounds with a low or moderate effect on mammal Pgp represents an opportunity to find compounds to reverse Pgp-mediated drug resistance in parasitic nematodes. To this end, the aim of this study was to characterize the effect of three selected potential nematode-Pgp-i utilizing a larval development assay with the model nematode *Caenorhabditis elegans*. A potential synergy of the selected compounds with MLs was examined by co-incubation of Pgp-I and ivermectin.

Materials and Methods

Biological Material

Caenorhabditis elegans strains

The *C. elegans* N2 Bristol (N2) strain was kindly provided by the Caenorhabditis elegans Center (CGC; University of Minnesota, Minneapolis, MN, USA). The strain was maintained under standard conditions on NGM plates at 20 °C [51].

Reagents

The anthelmintic ivermectin (Sigma Aldrich) was used as direct inhibitor of *C. elegans* growth. Stock solutions were prepared at 10 mM in dimethyl sulfoxide (DMSO) and stored at -20 °C until usage. The potential Pgp-i compounds (henceforth referred to as Pgp-i_c) and VPM (Table 1) were also dissolved DMSO at 10 mM and stored at -20°C until usage. Compound 11c [52] is a naphthalenyl derivative which exhibited unambiguous evidence for an interaction with the human Pgp and almost no indication of human Pgp inhibition. Substance 8d is a dimethoxy derivative which is a substrate of human Pgp, but with low to no mammal Pgp inhibitory activity [53]. Likewise, compound 9b [54], an oxazole derivative is a substrate of the human Pgp exhibiting low Pgp inhibitory activity.

Table 1 Tested potential P-glycoprotein inhibitor compounds

Compound	Molecular weight (g/mol)	Comment
11c	514.07	Ambiguous substrate of human Pgp
8d	462.034	Substrate of human Pgp
9b	490.954	Substrate of human Pgp
Verapamil	454.602	Well-characterized Pgp-inhibitor

Larval Development Inhibition Assays

Larval development inhibition assays were performed as described elsewhere [55, 56] with minor modifications. In brief, bleach synchronized first stage larvae [51] were seeded at a concentration of ca. 100 worms per well on sterile, non-coated 48 well plates (Sarstedt) in a total volume of 200 µL S-medium [51] containing IVM and/or Pgp-i_c in DMSO with a final DMSO concentration of 1%. To determine IVM susceptibility, a concentration dilution series of IVM (0 nM, 1.0 nM, 1.5 nM, 2.0 nM, 2.5 nM, 3.0 nM, 3. 5 nM, 4.0 nM, 4.5 nM, 5.0 nM, 7.5 nM and 10 nM IVM final concentration) was tested with 12 replicates equally spread on 3 independent experiments. To examine the direct impact of each Pgp-i_c on development, a dilution series of each Pgp-i_c (0.0 M, 0.1 nM, 1.0 nM, 10.0 nM, 100.0 nM, 1.0 µM, 10 µM and 50 µM Pgp-i_c) was tested with 9 replicates per concentration equally spread on 3 independent experiments. To determine the effect of each Pgp-i_c on the ivermectin detoxification machinery in developing larvae, a fixed IVM concentration (IVM+Pgp-i_c) was added additionally to each concentration of Pgp-i_c while maintaining 1% DMSO. 48-well plates were sealed with parafilm and incubated at 150 rpm and 20 °C for 52-55 hours. Following the incubation, the assay was stopped with a drop of 5% lugol's solution and the development of worms was examined. First, second and third stage larvae (L1, L2, L3) were scored as delayed developed and fourth stage larvae (L4) and adults were scored as fully developed.

Statistical Analysis

All calculations and visualizations were conducted using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, California USA) unless specified otherwise. For each well, larval development was calculated from the number of delayed developed worms as a fraction of all worms per well (0 to 1). Concentrations of IVM and Pgp-i_c were $\log_{10}$ transformed after setting the no drug concentration 0 M to 0.01 nM IVM. To determine IVM susceptibility, a four-parameter non-linear logistic regression model (model: Y=Bottom + (Top-Bottom)/(1+10^((LogEC$_{50}$-X)*HillSlope)) was calculated limiting top values to a maximum of 1 and bottom values to a minimum of 0 and visualising larval development as a concentration response curve. The effect of each Pgp-i_c on larval development in the presence or absence of a constant concentration of the known Pgp substrate and anthelmintic IVM was analysed in

R version 3.6.2 [57]. One-way analysis of variance (ANOVA) was used to determine the direct effect of a Pgp-i_c on larval development followed by a Tukey's honestly significant difference (HSD) post-hoc test to compare each condition to the no drug 0 M control and adjusting for multiple testing with the Bonferroni-Holm method. To determine the effect of each Pgp-i_c in the presence of IVM to its effect on larval development without IVM, a two-way ANOVA was conducted for each Pgp-i_c followed by a Bonferroni-Dunn's post-hoc test [58] and adjusting for multiple testing with the Bonferroni-Holm method. A corrected p-value of less than 0.05 was considered significant.

Results

First, IVM susceptibility was determined in a *C. elegans* larval development assay. An EC_{50} of 2.7 nM (2.55-2.72 nM 95 % confidence interval (CI)) was inferred from a four-parameter non-linear regression analysis with an R^2 of 0.9348. The Pgp-i_c were then tested for their effect on larval development of *C. elegans* in the absence or presence of 1 nM IVM, a concentration at which 95.7% of worms fully developed (**Fig. 1**).

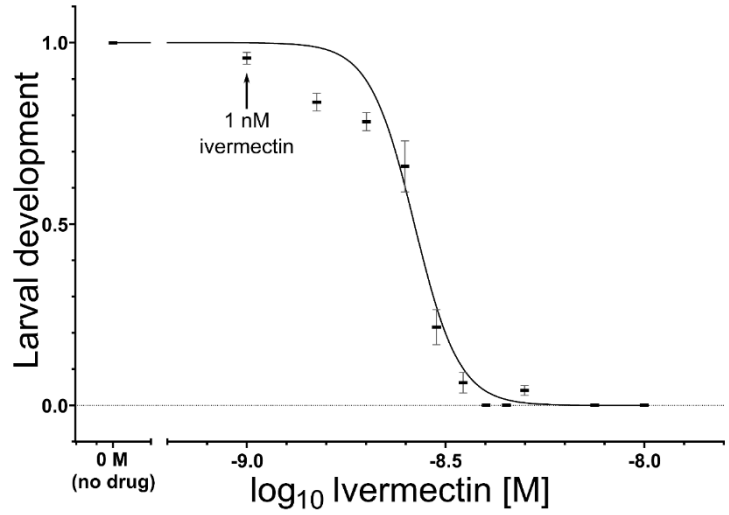

Figure 1 Ivermectin concentration dependent effect on *Caenorhabditis elegans* larval development. Relative development was calculated as the fraction of worms which fully developed (fourth stage larvae or adults). At least 100 synchronized first stage larvae of the N2 Bristol *Caenorhabditis elegans* strain were seeded in cavaties of 48 well plates in a concentration gradient of ivermectin and incubated at 20 °C for 52-55 hours and 150 rpm in the dark with a total of 12 replicates. Non-linear regression analysis was performed with GraphPad Prism v8.3.0 following a $\log_{10}$ transformation of concentrations, setting the 0 M concentration to 0.1 nM but visualizing it as "0 M (no drug)".

In the absence of IVM, all Pgp-i_c led to a developmental retardation at 50 µM and for Pgp-i_c compounds 11c and VPM also at 10 µM (all adjusted $p < 0.02$ Tukey HSD test) compared to the no-drug control. Noteworthy, for most compounds larval development was not inhibited completely but delayed significantly so that within 52-55 h larvae developed only to third stage larvae or less. In the presence of IVM and each of the tested potential Pgp-i_c (IVM+Pgp-i_c), larval development was significantly retarded ($p<0.05$, Bonferroni-Dunn's test) compared to the same concentration of Pgp-i_c but without IVM (Pgp-i_c). This statistically significant effect with IVM was observed for all new Pgp-i_c but not for VPM. In detail, in the presence of 1 nM IVM the Pgp-i_c concentration at which larval development was significantly reduced compared to the same concentration in the absence of IVM was 10 µM for compound 11c (**Fig. 2A**), 1 µM for compound 8d (**Fig. 2B**) and 50 µM for compound 9b (**Fig. 2C**) (summary in Table 2).

Table 2 Potential P-glycoprotein inhibitors - minimal compound concentrations resulting in a significant effect on larval development

	Minimal significantly[a, b] effective compound concentration (µM)	
Compound	**Compound only versus vehicle control[a]**	**Compound + 1 nM ivermectin versus compound only[b]**
11c	10	10
8d	50	1
9b	50	50
Verapamil	50	-

[a]Statistical significance ($p<0.05$) calculated using a one-way ANOVA and a Tukey's HSD post-hoc test and Holm-Bonferroni adjustment of p-values
[b]Statistical significance ($p<0.05$) calculated using a two-way ANOVA and Bonferroni-Dunn's post-hoc test and Holm-Bonferroni adjustment of p-values

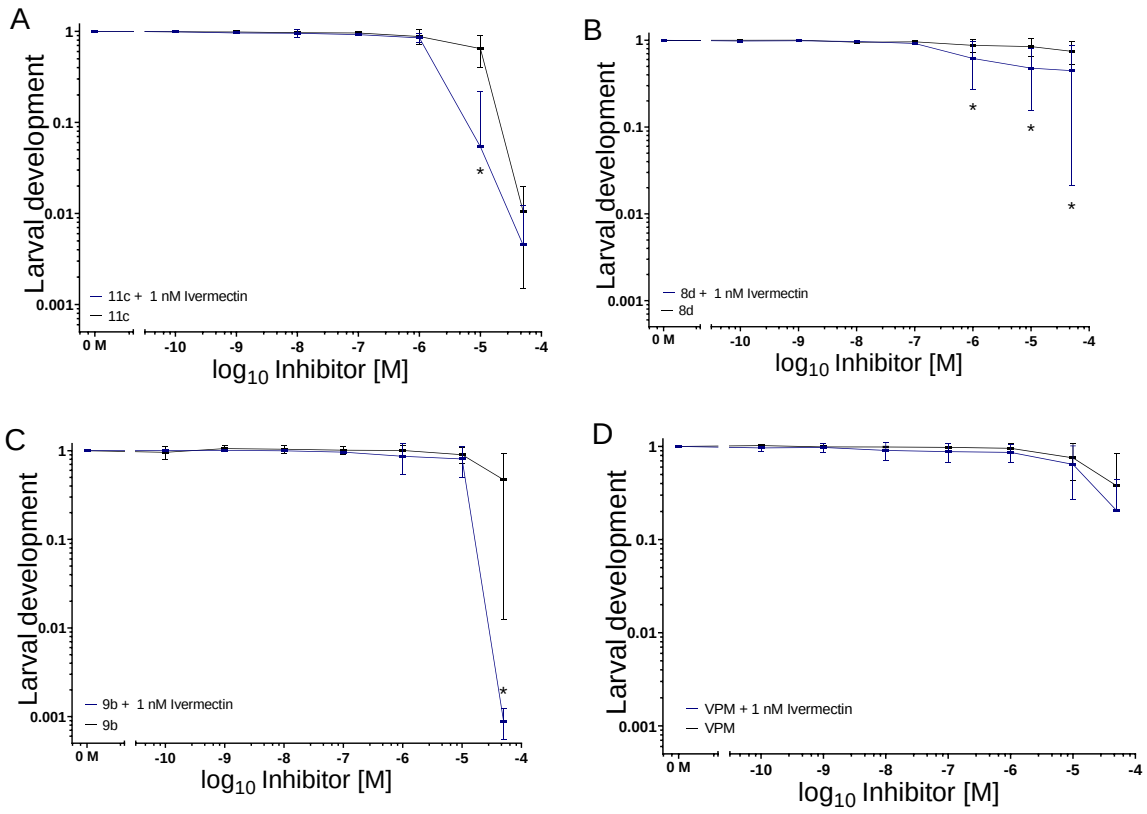

Figure 2 Effect on larval development of potential P-glycoprotein inhibitors in the presence or absence of ivermectin Larval development was calculated as the fraction of fully developed (fourth stage larvae and adults) out of all seeded worms in each well. 100 first stage larvae were incubated for 52-55 hours with different potential P-glycoprotein (Pgp) inhibitory compounds A) 11c, B) 8d, C) 9b and D) the established Pgp-i verapamil (VPM)) in the presence (blue) of absence (black) of 1 nM ivermectin, for 9 replicates spread equally on three separate days. Statistical differences (*$p<0.05$) were calculated at each concentration between the presence or absence of ivermectin using a two-way ANOVA followed by a Bonferroni-Dunn's test and Holm-Bonferroni adjustment of p-values. Due to the $\log_{10}$ transformation of the x-axis, the axis was split to visualise the negative control as 0 M. VPM: Verapamil.

Discussion

P-glycoproteins have been linked to ML resistance in many different parasitic nematodes [9, 25, 59-61], hence finding Pgp-i to reverse resistance is a promising approach to reversing resistance. In this study, three compounds originally developed to reverse drug resistance in mammalian cancer were shown to have a direct impact on *C. elegans* development as well as indirectly, using the known Pgp substrate and anthelmintic IVM, on the *C. elegans* Pgp repertoire.

Importantly, all new tested compounds induced a stronger inhibitory effect on *C. elegans* larval development in the presence of a low IVM concentration than VPM, which suggests that they might be more efficient inhibitors of nematode Pgp or xenobiotic metabolism in general. Notably, under these conditions VPM did not lead to a significantly higher inhibition of development when co-incubated with IVM compared to when incubated in the absence of IVM. However, all other tested compounds did induce retardation of development, hence showing that this approach in principle can detect subtle inhibition of the mechanism by which *C. elegans* metabolize IVM. Interestingly, all tested Pgp-i resulted in a developmental retardation when administered at 50 µM which demonstrates the burden on the whole nematode organism of high concentration of these substrates. One factor contributing to this effect could be that P-glycoproteins and potentially other metabolic components also play a role in physiological processes. For instance, *C. elegans* Pgp-2 is known to be involved in fat and cholersterol metabolism [62] which in turn is considered essential for development [63].It should be noted that the minimally significantly effective concentration does not represent a good measure for comparing the compounds for several reasons. For instance, the ranges of concentrations were chosen here for an initial screen in *C. elegans* to determine a better concentration range for a detailed analysis. This very broad, non-linear (log_{10}), range of concentrations therefore hinders a conclusion based on a quantitative comparison. However, with the scarce amount of data available, this effector size was still chosen as it at least indicates that there might be differences in the effect on the whole organism, and on the synergistic effect with ivermectin in general.

In general, the results from this approach suggest that the new tested compounds could be Pgp-i, however more detailed approaches are necessary to investigate the interaction of each compound with individual nematode Pgps. Specifically, the effect of transgenic nematode Pgp compared to mammals Pgp expression could be compared in a model system such as *Saccharomyces cerevisiae* [27] or LLC-PK1 cell line [64] or *C. elegans* overexpressing specific Pgp [28, 65] which have been used to examine the interaction of individual nematode Pgps with anthelmintics. Such approaches would shed light on the inhibitory potential of each tested compound. However, as the nematode Pgp repertoire is very large [43, 56] the advantage of the approach used in this study is that a strong effect is likely the result of a universal *C. elegans* Pgp-repertoire inhibition. In addition, it is possible that other metabolic components such as cytochrome P450 (CYPs) enzymes which are known to share a substrate range with Pgp are also inhibited by these molecules [66]. Concurrently, this broad substrate range of Pgps and CYPs foreshadow the difficulty to find inhibitors which are highly specific to the nematode xenobiotic metabolic machinery and do not affect any components of the mammal xenobiotic metabolism, which in turn, might lead to increased toxicity. The results from this study indicate that all three tested substances and structurally related compounds would be worthwhile to be investigated to determine their range of inhibited metabolic components using molecular approaches. Hence, the approach used here is well suited as a first stage screening approach. Nonetheless, the unsuitability for high or medium throughput due to the time-intensive nature of this experimental set-up restricts the applicability to a small scale and does not allow screening of large molecule libraries. More technologically advanced assays have been developed (e.g. by NemaMetrix Inc.) for *C. elegans* research which allow higher throughput and more detailed read-out of phenotype-genotype relationships, such as electrophysiological measurements [67, 68] of pharyngeal pumping as well as automated

motility analyses [69]. These approaches should be applied in future studies to allow a larger through-put of tested substances.

In conclusion, three new compounds have been characterized for their effect on *C. elegans* development. These compounds increase susceptibility to IVM compared while the well-characterized Pgp-i, VPM, did not affect IVM susceptibility under these experimental conditions. More work is needed to understand precisely which components of the xenobiotic detoxification machinery in *C. elegans* are inhibited to induce this effect.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

Deutsche Forschungsgemeinschaft Grant Number: 111144555
Deutsche Forschungsgemeinschaft Grant Number: 251133687/GRK 2046
Karl-Enigk Foundation Research Stipend Grant Number S0229/10019/19

4 DISCUSSION

The success of MLs to treat and prevent parasitic nematode infections is increasingly overshadowed by a growing number of reports of drug resistance in several nematode species (Sangster et al. 2018, Raza et al. 2019). In light of this development, there is an urgent need for a deeper understanding of the resistance mechanisms and accordingly, identification of resistance markers. However, despite extensive efforts to decipher the underlying molecular basis, the mechanisms of ML resistance remain elusive and might differ between species, population and life cycle stage (Kotze and Prichard 2016). Evidence from several studies suggests that Pgps are contributors to resistance in several nematode species including the horse roundworm *P. univalens* (Janssen et al. 2013a, Janssen et al. 2015), most likely within a multigenic context (Choi et al. 2017, Khan et al. 2020). While some studies have functionally analyzed Pgp interaction with different ML derivatives, our understanding of which Pgps and how they contribute to resistance remains fragmented.

In this PhD project, the interlinkage of three consecutive project parts has allowed considerable advancement in the understanding of the mechanism of Pgp-mediated reduced ML susceptibility in the widely ML resistant *P. univalens* and in the model nematode *C. elegans*. In addition, steps towards finding novel nematode specific Pgp-i to chemically reverse Pgp-mediated resistance have been initiated.

Briefly, the identification and comprehensive annotation of the complete Pgp repertoire in the first project part provides a basis for future studies on nematode and specifically *P. univalens* Pgps. The results of the subsequent phylogenetic and expression pattern characterization of *P. univalens* Pgps using diverse NGS resources allowed an informed choice of candidate Pgps for further functional characterization was done. In addition, the conserved strong intestinal and moderate epidermal Pgp expression in *C. elegans* and *P. univalens* led to formation of the hypothesis that Pgps play a role in the barrier function in these tissues. On this basis, functional analyses of two *P. univalens* Pgps, *Pun*Pgp-2 with moderate and *Pun*Pgp-9 with strong overall expression, were conducted using a yeast growth to characterize the interaction of specific Pgps with different anthelmintics. Subsequently, the role of Pgp expression in the intestinal and epidermal barrier against IVM and MOX was experimentally characterized by tissue-specific overexpression of *Pun*Pgp-9 in the model nematode *C. elegans*. In addition, the effect of active drug ingestion by pharyngeal pumping was demonstrated for the first time for IVM and MOX, while differences between the ML derivatives were observed. Finally, several compounds were characterized for their synergy with IVM in *C. elegans* by exploiting human Pgp (MDR-1) pre-screened compound libraries to identify novel nematode-specific Pgp-i.

7.1 Relevance of a Comprehensive Annotation of the *Parascaris univalens* P-glycoprotein Repertoire

Although Pgps have been considered candidates for ML resistance since the first report of their implication more than two decades ago (Xu et al. 1998), the diversity of Pgps in parasitic nematodes has been neglected in the absence of reliable genomic and transcriptomic reference data, hence leading to a bias in epidemiological and functional investigations towards the few experimentally validated sequences. In addition, as a result of a large research community around the model nematode *C. elegans*, the wide availability of *C. elegans* NGS resources and knowledge of gene function continue to drive the concentration of knowledge towards this model nematode. With the emergence of more affordable transcriptome and genome analyses and thus more available NGS data for an increasing number of species (Coghlan et al. 2019), the complete Pgp repertoire was described in a few relevant parasitic nematodes, i.e. *H. contortus* with 10 Pgp (Laing et al. 2013), 9 in *D. immitis* (Bourguinat et al. 2016) and 8 in *B. malayi* (Ardelli et al. 2010).

In this project for the first time, a comprehensive and reliable annotation from experimentally validated Pgp sequences has been provided for a clade 3 ascarid species, with a total of 10 Pgps in *P. univalens* including a novel ascarid-specific Pgp lineage, Pgp-18, and two gene duplications in Pgp-11 and Pgp-16 with subsequent divergence. The matching complete repertoire with orthologues for each duplicated paralog could also be identified from an *A. suum* transcriptome assembly, which allows confidence that this is the scope of the *P. univalens* Pgp repertoire. Very recently and currently only published within the scope of a dissertation, Chelladurai and Brewer also investigated the Pgp repertoire of *T. canis* (Chelladurai 2019), and found 13 Pgps based on the draft genome. Except for Pgp-11.1 and Pgp-16.2, the sequences used in the phylogenetic analysis were only *in silico* assembled predictions and only a few partial sequences were cloned and re-sequenced. The phylogenetic analysis in this PhD project also used the *T. canis* draft genome but only included complete Pgps into the analysis. Chelladurai et al. found that 10 of the genes were expressed in adults, which is most likely also the actual scope of the *T. canis* Pgp repertoire. In fact, those expressed genes also included two paralogs of Pgp-11 (already described in this project) and Pgp-16 as well as a third paralog of Pgp-16/3.2, which however clusters in a similar position in the phylogenetic tree as the novel ascarid Pgp lineage named Pgp-18. Chelladurai also report two paralogs of Pgp-9 (two completely overlapping frements of 462 and 741 aa length), but these are likely only fragments of the same gene. However, as the phylogenetic analysis suffers from poor sequence quality and coverage of most identified genes, the results of the phylogenetic analysis are not reliable and might lead to wrong conclusions of gene evolution, such as gene duplication. Along those lines, two Pgp, which the authors (supposedly incorrectly) named *Tca*Pgp-16.2 and -16.3/3.2, are too large to be Pgp, with 2130 aa and 1700 aa respectively. These are either the result of non-sense annotation and assembly or ABC-transporters not belonging to the *ABCB* gene family. Hence, while mostly in line with findings from this study, the unreliability of the findings highlights the need for validation and correct re-sequencing of NGS data prior to further analysis. Overall, the *P. univalens* and possibly ascarid Pgp repertoire in general is very similar in number to that of other parasitic nematodes, however, there are considerable differences in the Pgp lineages and their specific expression patterns. These findings highlight the differences in this gene family between different nematode species, and importantly, also to the model nematode *C. elegans*. These differences should not be neglected, particularly in light of their putative role in ML resistance. Consequentially, this study has increased the availability of NGS resources and experimental verification of annotation of the Pgps for a veterinary relevant parasitic nematode and helps counterbalance the bias in nematode biology research against *C. elegans*.

Interestingly, duplications leading to CNV of Pgps have been reported to be associated with resistance both in a candidate gene study in *T. circumcincta* (Turnbull et al. 2018) as well as in a genome-wide backcross study in *T. circumcincta* (Choi et al. 2017). Notably, the experimental set-up in this project was not tailored to identifying CNV which requires

population level genomic analyses and which in general is difficult and error-prone and requires a high quality reference genome (Choi et al. 2017). In this regard, if duplications of Pgps are driven by regular ML exposure, the recently available tools for genomic population level analyses might be able to determine Pgp CNV in *P. univalens* in the context of ML resistance on the basis of the reliable annotation provided by this project.

The phylogenetic analysis conducted in this project represents the most comprehensive phylogenetic delineation of nematode Pgps thus far. Therefore, it provides a phylogenetic reference for deciphering the role of Pgps in drug resistance and physiological processes as well as for comparative genomics. Regarding the diversity of Pgp, a major knowledge gap remains why nematodes maintain such a large repertoire of Pgps and, in line with the findings for the *P. univalens* repertoire, why new Pgp lineages continue to emerge in recent evolutionary history of nematodes. Overall, the variation in the composition of the Pgp repertoire observed in the phylogenetic analysis performed here also supports the previously suggested evolutionary models of dynamic coherent evolution of Pgps (Sheps et al. 2004b). This theory argues that the different Pgps might functionally compensate each other and thus, as a result of selection pressure exerted by the ecological environment of competitive or pathogenic species as well as by host (in case of parasites) gene loss and duplication are in balance. However, whether the differing repertoire has implications for the potential of resistance development can only be speculated without gene specific information on e.g. substrate spectrum and tissue localization/expression intensity.

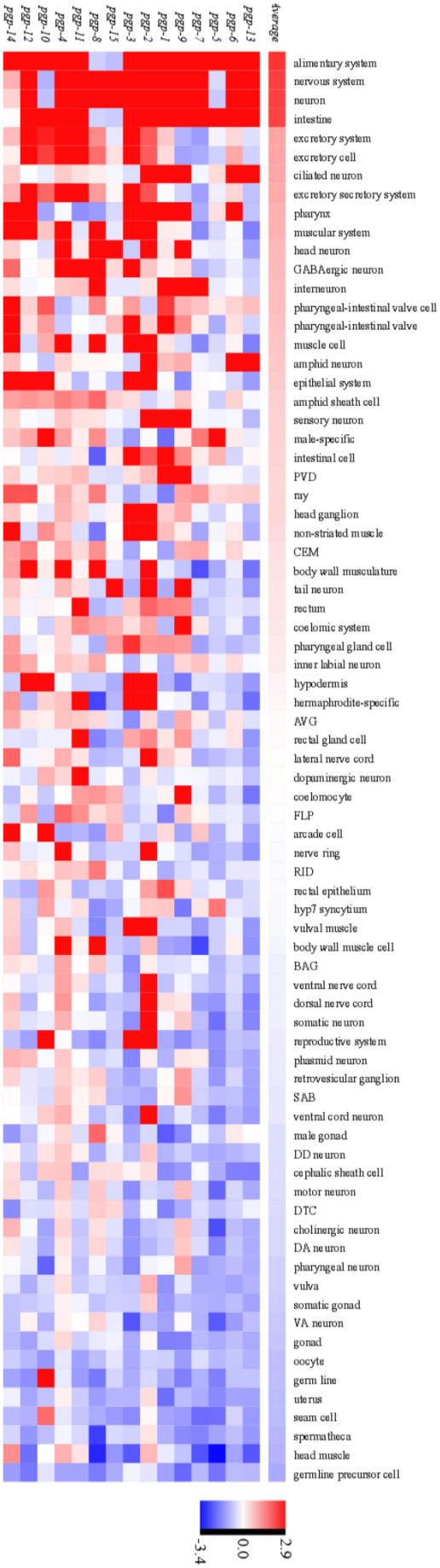
With regard to an endogenous role of Pgp, the Pgp-2 lineage forms the most separated cluster but is conserved across distant nematode species. This might suggest that the Pgp-2 lineage is rather ancient and might perform other physiological functions. In line with this assumption, *C. elegans* Pgp-2 gene has been implicated with lipid metabolism (Schroeder et al. 2007b). However, orthologues of Pgp-2 of *H. contortus* (Godoy et al. 2015b) and of *P. univalens* in the present project (Gerhard et al. 2020) were also implicated in ML transport demonstrating that orthologues of Pgp-2 can transport a diverse range of substrates.

The herein achieved considerable improvement of annotation based on complete coding sequences compared to the automatic annotation of the *P. univalens* transcriptome assembly demonstrates one of the limitations of state-of-the-art NGS analyses. Despite their incredible advantages and utility as a comprehensive resource (Doyle and Cotton 2019), their alleged superiority over conventional approaches can easily blind researchers to their limitations and technical pitfalls such as false read error rates, study bias or faulted annotation and assembly (Ioannidis 2005, Doyle and Cotton 2019, Ioannidis 2019). Nonetheless, with the increasing availability of NGS data, these resources should be used effectively but with care by the parasitological research community as they provide the most comprehensive starting point to answer many research questions, for instance by providing easy access to expression pattern analyses without additional time-consuming experiments, e.g. for quantitative PCR.

7.2 P-glycoprotein Expression Patterns in *Parascaris univalens* and *Caenorhabditis elegans*

7.2.1 The Utility of Next-Generation-Sequencing Resources to Characterize P-glycoprotein Tissue Expression Patterns

The role of Pgps in nematodes remains unclear, but tissue expression patterns may offer a starting point to form hypotheses of their function which can then be further expored by functional analyses. Here, the *P. univalens* NGS resources used in this study, i.e. the reference genome, the transcriptome assembly with tissue specific data of the corresponding raw-read transcriptome data sets and the novel raw-read transcriptome data set of worms incubated with IVM allowed a detailed characterization of tissue expression patterns of all *P. univalens* Pgps (Gerhard et al. 2020). In addition to using different independent NGS resources, the results of this comprehensive expression pattern analysis coincide with the reported tissue expression levels of two previously published *P. univalens* Pgps, *Pun*Pgp-11.1 and *Pun*Pgp-



16.1 obtained from experiments using real-time RT-PCR (Janssen et al. 2013a) and mRNA hybridization (Chelladurai and Brewer 2019), hence increasing confidence in the results. The calculated tissue expression levels for *C. elegans* Pgps presented in the first publication (Gerhard et al. 2020) are based on a single-cell data set (Cao et al. 2017) of L2 and are mostly coherent with other expression data in *C. elegans*, as shown by the pooled analysis of all available tissue-specific transcriptome data, specifically 4,342 microarray and RNA-seq experiments across 273 datasets of adult stage *C. elegans* (**Fig. 7**). This *C. elegans* tissue-ome analysis and visualization was performed using as the "Worm tissue" online tool (Kaletsky et al. 2018).

Figure 7 P-glycoprotein expression patterns in adult *C. elegans*

The figure was generated with the worm.princeton.edu tool.

(Kaletsky et al. 2018, Troyanskaya Laboratory Princeton University 2020)

In general, the strongest expression levels were observed in the intestine in both *P. univalens* and *C. elegans* (Gerhard et al. 2020), indicating that the general Pgp tissue distribution is conserved across these two species. This is also visible in **Fig. 7** for *C. elegans***,** where the strongest expression in the the alimentary system and specifically the intestine is indicated at the top of the heat map in red. This could indicate that the main function of nematode Pgps is defense against alimentary xenobiotics/toxins which was a first hint towards forming the hypotheses tested in the later project parts. In *P. univalens*, those Pgps with the highest overall expression levels, *Pun*Pgp-9, -11.1, 16.1 and 16.2, are all expressed predominantly in the intestine and secondarily, though at a considerably lower level, in the "hypodermis, cuticle, neuronal and pharynx"-tissue (German "Hautmuskelschlauch", as one sample). It should be noted, that in the first publication of this dissertation, the epidermis was addressed as the hypodermis, as this used to be the consensus in a majority of the *C. elegans* research community. This terminology was then abondened in the second publication as the result of personal communication with J. Ewbank, who advised to address it as the epidermis which is supposably the current favorable terminology or at least the trend in the *C. elegans* and nematology research community. In addition to these tissues, other Pgps (*Pun*Pgp-11.2 and -10)

with an overall very low expression level were expressed predominantly in reproductive tissues, specifically in the ovary. Possibly, this could mean that some Pgps have evolved to carry out more specialized functions in physiological processes such as lipid metabolism, which for example has been demonstrated for Pgp-2 in *C. elegans* (Schroeder et al. 2007b). The comprehensive approach visualized in **Fig. 7** indicates that in *C. elegans* most Pgps also exhibit strong expression in the nervous system. In contrast, only a few selected Pgps exhibit high expression in the excretory system and the epidermis. Assuming that xenobiotic efflux is the main function of these transporters, they could thus also play a role in barrier fortification of the epidermis and detoxification and exretion via the excretory system. Likewise, reported Pgp expression patterns vary between lineages in other parasitic nematodes but can generally be found in all tissues. For example, in *H. contortus* Pgp-2 was found to be expressed mostly in the head and in the intestine (Godoy et al. 2015b), *Hco*Pgp-9.1 in uterus of adult females (Godoy et al. 2016) and *Hco*Pgp-13 in the epidermis and other epithelial tissues as well as the pharynx and neuronal tissues (David et al. 2018). Moreover, Pgp expression can be found in the eggshell of *H. contortus* (Riou et al. 2005b).

In addition to transcriptomic data, experiments using *C. elegans* Pgp promotors driving GFP have also demonstrated a mainly intestinal expression, but with variation between individual Pgps. For example a *Celpgp-1p*::*gfp* construct drives GFP expression exclusive to the intestinal apical membrane (in L3 and L4) (Sato et al. 2007, Stutz et al. 2015) and a *Celpgp-12p*::*gfp* construct drives GFP expression exclusive to the H-shaped excretory system (Zhao et al. 2005). In contrast, the single-cell data demonstrate that at very low levels, these Pgps are also expressed in other tissues. Likewise, in *Parascaris* spp. Chelladurai et al. could also demonstrate expression of *Pun*Pgp-11.1 and *Pun*Pgp-16.1 in the H-shaped excretory system and the epidermis, just below the body wall (in addition to a pre-dominant intestinal expression (Chelladurai and Brewer 2019).

A direct comparison of expression patterns of individual Pgps in *P. univalens* and *C. elegans* shows that although the predominantly intestinal expression is conserved, the tissue expression patterns of different Pgp lineages is not conserved across these two distant relatives. In contrast, results currently only published within the scope of a dissertation report that in *T. canis* the Pgp-11.1 and Pgp-16.1 lineage are also most strongly expressed in this ascarid species (Chelladurai 2019). This suggests that the overall strongest expression of these particular Pgp lineages is conserved in ascarids. In turn, this might indicate that differentiation of nematode Pgp expression patterns is a rather slow process compared to other genetic factors driving speciation. It should be noted that Chelladurai reports expression levels in adults as fold changes over larvae rather than absolute values, hence the interpretation of the data is somewhat limited.

Expression of *Pun*Pgp-9, -11.1 and -16.2 (*Pun*Pgp-16.1 was only expressed very strongly in one of two transcriptome data sets) was consistently strongest between two independent data sets and most strongly at the intestine. This offers a potential explanation why specifically *Pun*Pgp-11.1 and *Pun*Pgp-16.1 were first described, as they had a high chance of being amplified using degenerated nested primers targeting the NBD (Janssen et al. 2013a). Interestingly, the strongly expressed Pgp-11 and Pgp-16 were duplicated in an ascarid ancestor, possibly suggesting that these genes, compared to other Pgps, might offer an advantage to ascarids in the defense against naturally occurring toxins.

Taken together, the findings from this and other studies, specifically regarding their tissue expression patterns, give rise to several hypotheses for the Pgp transmembrane transporters functional role in nematodes:

i) Barrier function against xenobiotic toxins, primarily at at tissues forming the first line of defense tissues, i.e. the apical intestinal membrane and at the epidermis-cuticle.
ii) Toxin or drug excretion via the excretory system.
iii) Protection of neuronal targets in the nervous system from toxins/drugs as a result of co-localisation in the membrane with target receptors.

iv) Transport of endogenous substrate such as lipids for homeostasis, for excretion of metabolic waste products or for transport within the organism.

Naturally, one of the first three hypotheses can also be considered as the potential mechanisms for Pgp-mediated ML resistance. The barrier hypothesis was considered the most likely mechanism for ML resistance based on the expression patterns observed and thus was explored in this project for the first time in the context of ML resistance in the second paper of this PhD thesis. In contrast, whether excretion or colocalization are relevant mechanisms of Pgp-mediated resistance remains to be investigated by a more suited experimental set-up. In principle, it is likely that Pgps can contribute by different mechanisms to reduce susceptibility in parallel. Based on the observations made of tissue expression patterns and the functional characterization of Pgp overexpression at the epidermis and the intestine, I propose to differentiate conceptual mechanisms of Pgp function in drug resistance of nematodes which are intricately linked to their tissue expression pattern: elimination by excretion (drug disposition) and barrier function (reduction of permeability of uptake barriers). Elimination by excretion will be defined here as a mechanism of detoxification which will remove the drug from the worm after it has penetrated the worm by any route. This means, that whatever the uptake route – intestine, amphids or the cuticle-epidermis - the drug will be removed from the organism by this mechanism as an active process. In this regard, this process would occur predominantly via the excretory system, but a contribution of the epidermis and intestine is also conceivable. The latter process cannot be differentiated from barrier function as nematodes simple anatomy and small size severely impose limits to their independent experimental discrimination. Barrier function will be defined here as the initial prohibition/reduction of drug uptake into the worm through a decrease of barrier permeability. For example, if the drug is taken up via the intestine, the degree of impermeability of the intestine will determine the efficacy of the barrier function.

As Pgps are functionally considered to be vacuum cleaners of substrates within the membrane (Theodoulou and Kerr 2015), it is a reasonable assumption that they can act as true impermeability modulators at the epidermal and intestinal apical membrane. At the molecular level, both conceptual functional mechanisms – elimination by excretion or barrier function – act by efflux which is the active transport of a molecule from the membrane or the intracellular space to the extracellular space. Surely, despite this conceptual distinction it is likely that both mechanisms can occur at the same time but the tissue expression pattern in a nematode species or developmental stage will impact how Pgps can contribute to resistance. Likewise, the drugs pharmacokinetics in the worm, i.e. the uptake route and compartmentalization, will determine if and how a Pgp with a specific affinity to the drug and a specific tissue expression pattern in a nematode species can contribute to reducing drug susceptibility. For MLs such pharmacokinetic parameters are unknown in nematodes. If such pharmacokinetic parameters were to be determined, the tissue expression patterns of *Parascaris* Pgps characterized here might provide further insight into the role of Pgps in drug resistance.

In addition, the type of mechanism also has other implications. For example, if enzymatic metabolization would be required for efficient drug efflux by the Pgps, then barrier function would not be of the same importance. Indeed, little is known about the interplay between xenobiotic enzymes and transporters for drug detoxification, but they can have overlapping substrate range which has been shown, for example, in humans (Kirn et al. 1999b).

7.2.2 No Inducibility of Individual *Parascaris univalens* P-glycoproteins Following Ivermectin Exposure

The inducibility of individual Pgps by ivermectin was investigated in this project based on a transcriptome data-set generated from worms incubated with ivermectin or a DMSO control which is available as the NCBI BioProject accession number PRJNA680223. The generation of this transcriptome data set was conducted prior to the start of this PhD project. In this experiment, no inducibility of Pgps by ivermectin was observed for any individual Pgp, but these results should be interpreted with care. It should be noted that generally in this transcriptome data set no explicit IVM specific response was elicited at all. While clearly this

could also be the case in an *in vivo* situation, the heavily artificial culture conditions severely limit the interpretation of these results. Recently, some of these limitations have been thoroughly explored by the Nielsen group (Scare et al. 2020). In parallel to the RNA-Seq study in this project, two other transcriptome analyses of *P. univalens* have been published investigating the response to IVM (and other drugs) of adult worms in an *in vitro* setting. Similar to the present transcriptome study, neither of the two studies could conclusively identify a drug specific response (Martin et al. 2020, Scare et al. 2020) which is in line with the findings of NGS experiments in other nematodes (Dicker et al. 2011a). However, in the Tydén group study among other xenobiotic genes including Cyt-P450 and other ABC-transporters, several Pgps (*ABCB* genes) appeared to be upregulated by 1.5 - 2.0-fold in the IVM treated worms (Martin et al. 2020). Overall, only very modest fold-changes were observed and in light of the data type, the moderate fold change and the statistical analysis, these might be false-positive findings when focusing in on candidate genes (Ioannidis 2005, Doyle and Cotton 2019, Ioannidis 2019). In contrast to the RNA-Seq data-set generated within the overall framework of the first paper (Gerhard et al. 2020), both the transcriptome from the Tydén and the Nielsen group used an even smaller number of RNA-Seq replicates. For each concentration Martin et al. generated 3 replicates (n = 3) with 3 worms per replicate while Scarce et al. generated 4 replicates (n = 4), with 2 worms per concentration. In comparison, the RNA-Seq data set presented here is based on 5 replicates (n = 5) with 2 worms per replicate but only one concentration (Gerhard et al. 2020). As a consequence of these 5 replicates, a statistical comparison of expression levels between Pgps with the Kruskal-Wallis test was possible which considerably improves the confidence of avoiding false positive finding. For example, in Fig. 2 (Gerhard et al. 2020) it becomes apparent that there is considerable variation in the constitutive expression level between individual worms which statistically increases the chances of finding a false positive in any Pgp, or worse any potential candidate gene. Based on the mean expression level, there is actually a 1.5-fold “increase” in *Pun*Pgp-16.2 in the IVM treated worms compared to the control. Hence, without sufficient replicates false positive findings in line with the prevailing hypothesis is a tempting finding in these types of analyses. Nonetheless, the genes ontology pathway analysis in Scare et al. showed that between oxibendazole and IVM different genes pathways were stimulated by the drug exposure (Scare et al. 2020). Likewise, differences between TBZ, PYR and IVM were found in Martin et al. which gives some confidence that in fact, a drug specific response was elicited (Martin et al. 2020). Overall, transcriptome analyses in *P. univalens* suggest that inducibility is most likely not a major pathway for xenobiotic metabolism to contribute to ML resistance. In accordance with findings from a back-cross analyses in *T. circumcincta* (Choi et al. 2017) and from *C. elegans* (Figueiredo et al. 2018), differential expression of Pgps (or other xenobiotic components) in ML resistant isolates rather seems to be the result of selection over several generations. This is also in line with findings in *P. univalens* (Janssen et al. 2013a). Within a transcriptomically diverse population, individuals with high constitutive expression of Pgps have a higher chance to survive treatment. Indeed, strong variability in the expression of individual Pgps was observed between individual replicates in the RNA-Seq experiment with IVM incubation (Gerhard et al. 2020, Martin et al. 2020) so while some worms would die, regular selection could drive stronger overall higher constitutive expression levels. Likewise, selection from anthelmintic treatment could shape the tissue-specific transcriptome, e.g. if worms exhibiting high intestinal or epidermal Pgp expression levels were favored. As demonstrated in the second publication of this PhD project (Gerhard et al. 2021), ML susceptibility can be reduced from Pgp overexpression at the intestine and the epidermis and the potential relevance of tissue-specific expression patterns has been overlooked in the context of anthelmintic resistance.

In contrast, several candidate gene studies in many other nematodes have reported inducibility of expression by xenobiotic components. Specifically Pgps have been reported to be upregulated upon IVM exposure in several studies e.g. in *H. contortus* (Raza et al. 2016b, Reyes-Guerrero et al. 2020) and *C. elegans* (James and Davey 2009). Concurrently, several studies report that no upregulatino was found, e.g. in *P. univalens* (Janssen et al. 2013a), T. *circumcincta* (Dicker et al. 2011a) and in another study in *H. contortus* (Mate et al. 2018).

Problematically, because of the extensive studying of Pgp, it should be considered that the number of publications reporting some sort of changes of Pgps associated with resistance is inflated as the result of publications bias towards the prevailing candidate genes. For another reason, genome-wide studies, in particular classical GWAS studies, reporting selection at loci in proximity to Pgp, need also be interpreted with care, due to the intrinsic bias of multiple testing when analyzing large genomic data sets (Ioannidis 2005). In nematodes, the strong natural genetic variation in most species surely represents a major issue for GWAS studies and the odds of finding false-positive selection signatures at any Pgp or even worse any ABC-transporter or other proposed candidate gene in general, are, unfortunately, rather high (Doyle and Cotton 2019, Ioannidis 2019, Rezansoff et al. 2019).

In summary, evidence from candidate gene studies for an upregulation of Pgps upon exposure seems to be overwhelming in some species and populations, however, transcriptome studies have thus far not provided conclusive evidence in support of these findings. Specifically, for *P. univalens* evidence from this and the other two transcriptome studies support that Pgp upregulation upon IVM exposure is not a regulatory pathway in the studied populations (Martin et al. 2020, Scare et al. 2020).

7.2.3 An Informed Choice of Candidate *P. univalens* P-glycoproteins for Functional Analyses

Orthologues of Pgp-9 have been implicated with ML resistance most often in other parasitic nematodes in candidate gene, GWAS and back-cross approaches (Dicker et al. 2011c, Issouf et al. 2014, Kaschny et al. 2015, Godoy et al. 2016, Choi et al. 2017, Turnbull et al. 2018) and it might represent one of the most efficient ML efflux transporters in the nematode repertoire. Based on two independent transcriptome analyses, *Pun*Pgp-9 was next to *Pun*Pgp-11.1 the second most strongly expressed Pgps in *P. univalens*, hence, *Pun*Pgp-9 was chosen for functional analyses in this project. In particular the strong intestinal expression was a critical lead for the development of the experimental set-up to examine the role of tissue specific Pgp expression and in this regard barrier function or detoxification.

While orthologues of Pgp-2 have also been linked to ML resistance in parasitic nematodes (Roulet and Prichard 2006, Godoy et al. 2015b), they have also been implicated with physiological processes (Schroeder et al. 2007b). Hence, it is possible that orthologues of Pgp-2 have a substrate range including both endogenous and xenobiotic substrates and might be able to perform diverse functions. Based on the analyses of two independent RNA-Seq data sets, the *Pun*Pgp-2 orthologue appears to exhibit overall low expression. In more detail, low expression at the intestine and moderate expression at the testis and ovary were found. Hence, the expression pattern of *Pun*Pgp-2 might suggest that like its *C. elegans* orthologue *Cel*Pgp-2 is involved with endogenous functions as well. Overall, *Pun*Pgp-9 exhibited higher expression in all tissues except the ovary. To analyze whether the *Pun*Pgp-2 substrate range includes relevant anthelmintics this Pgp was also chosen for analysis in a yeast growth assay.

In different species, different Pgp lineages have been implicated with resistance either by differential expression or also from functional analyses (see section 2.3.3.2 and 2.3.3.3). These observations point towards the hypothesis that MLs and specifically IVM are conserved as Pgp substrates despite functional specialization. In line with this hypothesis, each individual *C. elegans* Pgp loss-of-function strain exhibits an increased susceptibility phenotype to IVM exposure (Janssen et al. 2013d). To further test this hypothesis and to investigate whether relevant anthelmintics are substrates of two phylogenetically distant *P. univalens* Pgp, *Pun*Pgp-2 and -9, were chosen for characterization in a yeast growth assay.

As previously mentioned, one of the hypotheses that was inferred from the tissue expression patterns of Pgps in nematodes with regard to Pgp function and their role in ML resistance, is that Pgps contribute to ML resistance by reducing permeability of uptake barriers, specifically of the intestine and/or the epidermis. To test this hypothesis in general and concurrently to further characterize the function and substrate range of a promising candidate Pgp, *Pun*Pgp-9 as one of the most dominantly expressed Pgp in the intestine was chosen for further analyses

in the model nematode *C. elegans*. Notably, *Pun*Pgp-11.1 which also exhibited very strong expression in *P. univalens* was already shown to affect susceptibility to IVM in transgenic *C. elegans (Janssen et al. 2015),* hence this gene was not considered for functional analysis. In conclusion, among other factors the utilization of a diverse set of NGS resources has allowed an informed decision of candidate Pgps for further analyses.

7.3 Interaction of two *P. univalens* P-glycoproteins with Anthelmintics in a Yeast Growth Assay

The *S. cerevisiae* AD1-7 yeast growth assay has been established as a simple, cheap and largely automatically performed assay to characterize the interaction of a recombinant nematode Pgp with MLs (Kaschny et al. 2015). Kaschny et al. were able to demonstrate that recombinant *C. elongatus* Pgp-9 reduced efficacy against KCON and other directly fungicidal drugs which are known Pgp substrates. The maximum tolerated concentration of KCON (which is higher in the Pgp expressing strain than in the lacZ control) was then used for coincubation with different ML derivatives which resulted in a concentration dependent inhibition of growth in the Pgp expressing strain, but not in the lacZ expressing strain, compared to the absence of KCON. The concentration dependent inhibition of growth is considered to be the result of Pgp inhibition by the MLs. In the same manner, *Pun*Pgp-9 could be shown to interact with IVM, however, due to time constraints only this ML derivative was tested. Concurrently, no reduced susceptibility could be observed against the anthelmintic and antimycotic TBZ, as a member of the BZs. Similarly, *Pun*Pgp-2 expressed in AD1-7 *S. cerevisiae* did not reduce susceptibility to TBZ but, indirectly using KCON, appeared to interact with IVM which is also in line with findings of other Pgps from this lineage (Godoy et al. 2015b). Hence, this confirms that the interaction with MLs in the investigated Pgp lineages is conserved between species. This would suggest that these two genes should be treated with priority in candidate gene population level studies in *P. univalens*. However, with the availability of the complete *P. univalens* Pgp repertoire and access to current technology (e.g. qPCR or transcriptomics) all Pgps should be treated equally to avoid bias against these candidate genes. Furthermore, *Pun*Pgp-2 and -9 were also chosen for functional analysis based on findings from other studies. Regardless, these findings support that IVM and possibly other MLs (Kaschny et al. 2015) are conserved as substrates between phylogenetically distant Pgp. This is also in line with findings from *C. elegans* which demonstrated that all individual Pgp loss-of-function strains lead to a moderate increase in ML susceptibility (Janssen et al. 2013d). Likewise, candidate gene functional studies in different model systems support an interaction between MLs and Pgps from different Pgp lineages (Ardelli and Prichard 2013, Godoy et al. 2015b, Janssen et al. 2015, Kaschny et al. 2015, Godoy et al. 2016, Mani et al. 2016a). However, findings from Kaschny et al. showed that *Ceg*Pgp-3 expression did not result in a decreased susceptibility to KCON, but no other substrate was tested (Kaschny 2018). Hence, it is difficult to determine whether the transgene was expressed correctly in the particular strain, or whether KCON is simply not a good substrate of *Ceg*Pgp-3. Using FACS in combination with a Pgp specific antibody (C219) or an anti-V5-tag antibody, only a much smaller proportion of AD1-7*Ceg*Pgp-3 was found positive for the transgene which might explain the lack of an effect on KCON susceptibility. This lack of an effect for *Ceg*Pgp-3 was only published in the dissertation and it should be noted that as a result of publication bias, similar findings might have been concealed from the research community. This represents a general issue to functional characterization of foreign transgenes: in the absence of an effect it is very difficult to demonstrate that in fact the transgene is correctly transcribed, translated, and post-translationally modified but still does not affect the model organism's phenotype. As the highest proportion of cultured yeast cells in the Kaschny et al. study was around 20 % in the AD1-7*Ceg*Pgp-9 (Kaschny 2018) , *Pun*Pgp-2 and -9 were codon-optimized for expression in yeasts. However, due to time restrictions these lines were not characterized any further using this FACS-based analysis. In this regard, the fold increase in KCON EC_{50} between the Pgp and the lacZ transgenic AD1-7 strains was lower for AD1-7*Pun*Pgp-9 (1.86-fold) and similar for AD1-7*Pun*Pgp-2 (2.46-fold) compared to the 2.97-fold (with His-tag) and 2.20-fold (without His-tag) in AD1-7*Ceg*Pgp-9 strains.

A major advantage of the yeast growth assay is that it allows many replicates to be generated rather quickly, hence facilitating a medium-throughput analysis. Due to time restrictions only IVM was tested in this project, but in future analyses these strains as well as the strains from Kaschny et al. (2015) might be used for a more comprehensive screening, e.g. to identify nematode Pgp-is. Noteworthy, this assay has also many disadvantages, some of them are outlined in section 2.3.2.3.1 and 2.3.3.3. Generally, the main limiting factor of all currently published functional studies on nematode Pgps and ML interaction is the reliance on a secondary Pgp substrate which has a direct effect of the model system (yeast and cell culture), with Janssen et al. (2015) being the only exception(Janssen et al. 2015). Co-incubation with this substrate with a concentration series of MLs is used to deduce an interaction with MLs. In case of the yeast assay this secondary Pgp substrate is KCON (Kaschny et al. 2015) and this approach severely limits how the interaction between Pgps and ML can be interpreted. Clearly, the concentration dependent effect of each ML on the transgenic Pgp can only be quantified in relation to this secondary substrate. As Pgps have been suggested to have multiple binding sites, e.g. the H and the R site (David et al. 2016, David et al. 2018), the complex interaction of the two substrates with the Pgp is influenced by many factors for example their localization in the cell (membrane, cytoplasm), their specific affinity to the transgenic Pgp as well as to other ABC-transporters. Hence, the read-out of this assay does not allow a quantitative comparison of Pgp affinity to different ML derivatives.

Along these lines, the yeast growth assay does not appear to be precise enough to allow a gene-specific analysis of ML binding and transport characteristics between Pgps. This is due to the fact that expression levels will vary between the inserted genes and strain. For example, between two AD1-7*Pun*Pgp-2 clones (data not published in the manuscript but attached here as **Fig. S1**) the observed susceptibility differed. Likewise, between AD1-7*Ceg*Pgp-9 susceptibility to KCOn differed with or without a His-tag, with 2.97- and 2.20-fold increase in KCON EC_{50} compared to the lacZ strain (Kaschny et al. 2015). In addition, a phenotypic difference between Pgp and lacZ expression was only detected in a selected yeast strain lacking multiple (7) endogenous ABC-transporters (AD1-7) but not in another strain, JRY8012 which lacks only 3 ABC-transporters (Kaschny 2018). Similarly, as AD1-7*Ceg*Pgp-3 does not exhibit an increased KCON susceptibility, and Kaschny et al. discuss that this might also be explained by the low expression level of the transgene (Kaschny 2018). Alternatively, this could indicate that endogenous transporters might mask the potential effect of a transgenic Pgp. In the present project, two newly made lacZ control strains exhibited a higher susceptibility to KCON (EC_{50} 0.28 nM in Gerhard et al. and 0.39 nM in Kaschny et al (Kaschny et al. 2015, Gerhard et al. 2020). In addition, the assay appears to be influenced by other external factors which was indeed indicated by some ambiguous experimental outcomes in the present thesis when performed by different researchers. For example, starting the assay with too many or too few yeasts cells will either allow growth at several fold higher concentrations, or not allow the yeast to grow at all (preliminary experiments, data not shown). Likewise, KCON EC_{50} values for AD1-7*Pun*Pgp-2 (0.69 nM KCON) and AD1-7*Pun*Pgp-9 (0.52 nM KCON) were higher than in the AD1-7*Ceg*Pgp-9 strain at 1.176 nM KCON (His-tag) and 0.8696 nM KCON (no His-tag). As the EC_{50} values were lower overall, this can probably be explained by the different batches of KCON used.

In general, the findings of the present study (Gerhard et al. 2020) are in line with similar studies (Godoy et al. 2015b, Kaschny et al. 2015, Godoy et al. 2016) and suggest that both *Pun*Pgp-2 and *Pun*Pgp-9 are multidrug transporters and that their substrate range includes one of the most important and widely used MLs, IVM, but not TBZ. This has been the first functional characterization of these two *P. univalens* genes and provides evidence that functionally these genes have the capability to contribute to IVM resistance. This functional validation is necessary to provide confidence for epidemiological findings. The hypothesis that Pgps are involved in both endogenous and xenobiotic transport processes can be neither rejected nor accepted. However, the findings indicate that the substrate range of a Pgp-2 gene (i.e. *Pun*Pgp-2) includes two different xenobiotic toxins, IVM and KCON. At the same time the *C. elegans* Pgp-2 orthologue has been implicated in lipid transport (Schroeder et al. 2007b). This

might indicate that both lipophilic endogenous and exogeneous substrates fall into the Pgp-2 substrate range. As demonstrated by the differing expression patterns of Pgp lineages between species, this does not offer insights into the actual role of Pgp-2 in *P. univalens*. In a collaborative research project, the role of *Pun*Pgp-2 in cholesterol transport was also supposed to be examined, but this was abandoned as the lead researcher moved to a new position. Generally, the characterization of these two phylogenetically (and possibly functionally) rather distant Pgp, i.e. *Pun*Pgp-2 and -9, indicates that the ability to transport IVM is conserved between different Pgp. Concurrently, the findings support the prevailing assumption that there is no Pgp-mediated cross-resistance between BZs and MLs as a result of overlapping substrate range. To date, an assay to meaningfully, directly and reliably quantify and compare substrate affinity and specificity between Pgp lineages has not been developed.

7.4 The Role of Pharyngeal Pumping in the Macrocyclic Lactone Susceptibility in *Caenorhabditis elegans*

Based on observations from the preceding project parts of this PhD, several hypotheses for the role of tissue specific Pgp expression were formed (Gerhard et al. 2020). The aim of the experimental set-up was to elucidate the role of Pgps at the epidermis and the intestine against MLs while the main hypothesis was that Pgps are involved in barrier function (all hypotheses are outlined here i) - iv)). With some serendipity, considerable differences in the susceptibility of *C. elegans* WT adults to IVM became apparent in the absence or presence of PP stimulation in early pilot experiments. Subsequent experiments with MOX only led to much smaller changes in susceptibility. This finding fueled the theory that the uptake capacity might differ between the intestine and the cuticle-epidermis. Notably, uptake of anthelmintic drugs in nematodes has only been studied by a few studies over two decades ago, e.g. the uptake of levamisole or IVM in *A. suum* (Verhoeven et al. 1976) and the uptake of levamisole, TBZ and closantel in *H. contortus* (Thompson et al. 1993, Ho et al. 1994). Here, the analysis of each transgenic strains with specific intestinal or epidermal transgenic *Pun*Pgp-9 expression in the presence or absence of active drug ingestion by PP indicate that there might be differences in the tissue uptake capacities between the two ML derivatives, MOX and IVM. At least, the differences in the effect of active drug ingestion show that intestinal drug exposure differently affects the susceptibility of *C. elegans* against the two ML derivatives and the dependency of a protective effect of intestinal *Pun*Pgp-9 overexpression on the presence of active drug ingestion demonstrate that PP is necessary to expose the drug to the intestine. The differences in the physicochemical properties of the two ML derivatives might offer a good explanation for the observed differences which could lead to different tissue-specific uptake dynamics and subsequent compartmentalization.

Strikingly, PP stimulation by *E. coli* OP50 increased IVM and to a lesser degree MOX susceptibility. To control for the effects of PP as well as starvation, 5-HT was used in the absence of OP50 to stimulate PP and yielded overall similar results. Concurrently, by comparing the susceptibilities of transgenic strains with either intestinal or epidermal *Pun*Pgp-9 overexpression, it became apparent that in fact PP was directing the drug to the intestine and that this increased the overall susceptibility, probably by an increased overall uptake. The increase in susceptibility from PP is particularly strong for IVM, which suggests that intestinal IVM uptake could be more efficient than cuticular-epidermal uptake. This conclusion is also supported by the finding that in the presence of PP, despite a significant reduction of IVM susceptibility from intestinal *Pun*Pgp-9 overexpression, the EC_{50} values in all strains remained considerably lower in comparison to EC_{50} values in the absence of PP. In addition, the conclusion is supported by the observation that the IVM EC_{50} value in the epidermis-Pgp-9 strain (the strain overexpressing *Pun*Pgp-9 in the epidermis) in the absence of PP was higher than in all other combinations of drug, strain and condition. This observation also demonstrates that IVM is also taken up via the cuticle-epidermis but with a considerably lower efficiency. In line with this finding, the *C. elegans bus-8* mutant strain, which has an impaired cuticle, exhibits a significantly higher IVM susceptibility (Weeks et al. 2018b). However, Weeks et al. use PP inhibition as a read-out and incubate at much higher IVM concentrations (3 µM compared to

concentrations between1 - 50 nM in the present study) within a much shorter incubation period, making a comparison between the experiments difficult.

In contrast, a general interpretation of the findings obtained by using MOX with regard to the uptake routes was more difficult and less conspicuous as the overall shifts in susceptibility were lower (<2). Nonetheless, the best explanation for the observations made across all strains and conditions is that MOX is also taken up both via the intestine and the epidermis, with a trend towards more efficient cuticular-epidermal uptake. This interpretation is supported by the finding that the epidermis-Pgp-9 strain without PP stimulation exhibited the overall lowest EC_{50} value. At the same time, all EC_{50} values in the epidermis-Pgp-9 strain were higher than in any other combination of strain and condition. Conversely, intestinal *Pun*Pgp-9 overexpression also resulted in a significant increase in susceptibility in the presence of PP compared to WT and control strains which supports the hypothesis that MOX is also taken up via the intestine.

Little is known about the uptake routes of anthelmintics into the worm and tissue specific drug permeability. In contrast, studies investigating the genetic basis of the mode of action of anthelmintics, classically denoted as pharmacodynamics, have dove deep into the biology of nematodes with a focus on the model nematode *C. elegans* (Abongwa et al. 2017). These studies provide the framework and toolbox which were necessary to design the experimental set-up used here. With the rise of anthelmintic resistance, the interest in nematode pharmacokinetics increased as well and several studies started to investigate the worm metabolism in the worms as a pathway to resistance. These studies mostly focused on the potential role of phase I-II metabolism including Uridine 5'-diphospho-glucuronosyltransferase (UDP)-glucuronosyltransferase and Cyt-P450 enzymes (Laing et al. 2015, Ménez et al. 2016) and phase III detoxification by ABC-transporters which are also known to be involved in drug metabolism in mammals. However, investigations on the uptake routes and tissue specific barriers remained the exception. In the cattle parasitic nematode *Onchocerca ochengi* drug uptake was measured via the transcuticular and the intestinal route using [22,23-^{3}H]-labelled IVM in adults, which unfortunately, is no longer commercially available (Cross et al. 1998). The authors incubated the worms in liquid and concluded that IVM is taken up via the transcuticular route. Cross et al. were not able to determine intestinal uptake, however their control, [^{3}H]-labelled inulin, was also not taken up, hence the authors concluded that the intestine was not functional *in vitro (Cross et al. 1998)*. As Cross et al. did not stimulate pharyngeal pumping in the incubation medium, intestinal uptake was not likely to occur (Cross et al. 1998). Noteworthy, reliable stimulation of pharyngeal pumping is almost impossible for parasitic nematodes in an artificial culture medium as the underlying regulatory mechanisms are not deciphered in any parasitic nematode species. Nonetheless, the general ability of IVM to penetrate the worm via the cuticle can also be concluded from the observations in this study. The early genetic screens in *C. elegans* which were conducted to investigate the mode of action and resistance to IVM identified in addition to the GluCl also a series of genes associated with the dyf phenotype as modulators of IVM susceptibility, which lead to cuticle and amphidal defects (Dent et al. 2000). These mutants are “fluorescent DYe Filling” mutants, which have a decreased capacity to take up fluorescent dye via the amphid sensory endings (Starich et al. 1995). More recently, similar mutations were also reported in a *C. elegans* strain selected over several generations with IVM (Menez et al. 2016). Following up these findings many years later, a more detailed study screening many dyf mutant *C. elegans* strains concluded that in fact the amphidal structures are the main route of uptake for IVM (Page 2018). However, the author neglects the fact that these gene defects also massively impact cuticle formation in addition to the dyf phenotype, hence this alteration in the cuticle is also likely to influence cuticle permeability. In contrast to the experimental set-up used here, the nematodes in the Page study were kept on agar plates infused with IVM (Page 2018). Notably, these conditions are not comparable to adult gastro-intestinal parasitic nematodes because these usually dwell in the intestinal fluid. Furthermore, this approach has several pitfalls, for example IVM might not be equally spread through the agar. Nonetheless, the results presented by Page seem convincing that at least in part, the amphidal structure is a component within

the actual uptake route (Page 2018). As previously mentioned, the *bus-8* mutant cuticle defect *C. elegans* strain also exhibits an increased IVM susceptibility (Weeks et al. 2018b).

One of the most comprehensive series of studies on transcuticular uptake of drugs and other substances in nematodes was done by Geary, Ho and Thompson. Mostly, they focused on other anthelmintics and compounds than IVM but one of their studies reported that IVM was mainly transported via the cuticle although the actual data are missing (Ho et al. 1990). The experimental set-up used in these studies was to analyze the biophysics of transport of different substrates across isolated *A. suum* cuticle with and without lipid extraction. Using this method, the authors concluded that primarily size and secondarily lipophilicity influence transcuticular permeability, e.g. the cuticle is more permeable to testosterone than to the more lipophilic but considerably larger IVM molecule. However, normalization for size reveals that lipophilicity is also a major factor facilitating drug uptake (Ho et al. 1990, Ho et al. 1992, Sims et al. 1992, Ho et al. 1994, Sims et al. 1994). With a similar approach it was also demonstrated that IVM can be transported via the *A. suum* cuticle (Thompson et al. 1993). Using intact nematodes, uptake kinetics were also characterized (Thompson et al. 1993). The absence of an effect of surgical ligation of the pharynx on the uptake kinetics was interpreted as a negligible role of the intestine in the uptake of most tested substances but whether IVM was also tested with this approach is not shown in the results. Like in the Page (2018), PP was not stimulated in the worms to facilitate drug uptake (Thompson et al. 1993) but worms after surgical ligation of the pharynx were reported to fill with dye considerably slower than non-ligated worms. In these studies it could be demonstrated that levamisole, closantel and TBZ can be absorbed transcuticularly in *A. suum* (Thompson et al. 1993). Geary et al. (1993) also used IVM to successfully “chemically” ligate *H. contortus* to examine the uptake route of glucose and other substances and found glucose uptake to be independent of the intestine (Geary et al. 1993). Experiments in first stage *C. elegans* larvae exposed to alcohol to immobilize the pharynx showed that this treatment did not alter the motility paralyzing effect of IVM (Smith and Campbell 1996). In summary, findings from most studies are in line with the conclusion of the present study that transcuticular uptake of IVM is sufficient to induce paralysis even at low concentrations.

Despite the transcuticular uptake capacity discussed above, the observed strong effect of PP on IVM susceptibility indicates an even more efficient intestinal IVM uptake. The presence of this effect in *C. elegans* and other nematode species is also supported by a number of observations. In *O. volvulus* microfilariae, tolerated ML concentrations *in vitro* are 1000-fold higher than when exposed *in vivo* in the plasma (Chavasse and Davies 1990). Possibly, these differences are because the worms do not feed *in vitro* and thus only the cuticle-epidermis, which appears to be a more effective barrier to IVM than the intestine, is exposed to MLs. This is also consistent with the finding, that non-feeding larval stages are less susceptible than the feeding adult stages (Demeler 2005, Algusbi et al. 2014a). It should be noted, that the cuticle of third stage larva in several species, specifically strongylid species, can be made up of more than layers (a sheath) which likely also impacts their transcuticular permeability. In addition, another experiment from the Campbell group showed that adult *C. elegans* which were refrigerated prior to IVM incubation exhibited reduced susceptibility. In line with our findings, they discuss that the seizing of PP and the resulting absence of intestinal drug exposure which results from refrigeration is the best explanation for the observed differences (O'lone and Campbell 2001). Alternatively, they discuss whether the slowing of physiological processes might explain the differences (O'lone and Campbell 2001). In contrast, the minor affect of PP on MOX might indicate a that intestinal uptake is less efficient permeability of MOX might be an indication why this ML has retained efficacy in some IVM resistant populations (Prichard and Geary 2019).

The effective dose reaching the target tissue will be determined by the drug’s pharmacokinetics in the host as well as in the parasite. For the parasite, the life cycle stage (e.g. migrating or intestinal dwelling) as well as its feeding behaviour (e.g. sucking blood or intestinal fluid) will determine its exposure dose at different time points during treatment and the subsequent pharmakokinetic tissue distribtition. The predominantly faecal excretion of MLs by the host will

facilitate drug exposure to the cuticle-epidermis of intestinal dwelling adult parasitic nematodes following any formulation (Gokbulut et al. 2001, Laffont et al. 2002), however, the concentration in specific gastric and intestinal sections differs depending on the uptake route (Leathwick et al. 2020). In turn, these differences following drug formulation were shown to considerably influence the drug concentration reaching the parasite (Leathwick et al. 2020). In the cattle parasite *O. ostertagi*, which dwells on the surface of the abomasal mucosa and feeds by sucking on the mucosa, Leathwick et al. observed that IVM levels that in the parasite and in the mucasa, rather than the surrounding fluid, correlated well (Leathwick et al. 2020). This effect was maximized by subcutaneous drug formulation and corroborates that active drug ingestion is an important route of IVM uptake. In contrast, the IVM levels in the small-intestinal dwelling *C. oncophora* correlated well with the IVM levels in the intestinal fluid (Leathwick et al. 2020). In the the blood-sucking *H. contortus* which resides in the abomasum, the cuticle-epidermis will be exposed via the surrounding fluid following subcutaneous IVM application only during a short time window due to a low host excretion of IVM into the abomasum (Lloberas et al. 2012). The *H. contortus* intestine would be only exposed from blood sucking, but Lloberas et al. report a weak correlation with plasma levels three days after treatment, which might be attributed to the timing of the measurement. In contrast, intraruminal application leads to higher abomasal concentrations than subcutaneous formulations in sheep (Alvarez et al. 2015, Lifschitz et al. 2017). Such an intraruminal application and high abomasal drug concentrations also correlated well with IVM concentration in parasite tissue three days after exposure indicating that in this species, the uptake from the surrounding fluid could be more relevant (Lloberas et al. 2012). In a ML resistant *H. contortus* population, initial drug accumulation was not different between different ML derivatives (IVM, MOX and abamectin) but MOX concentrations dropped significantly in the following days post-treatment while not correlating with plasma or levels (Lloberas et al. 2013b). This suggests, that drug elimination also differs between the ML derivatives or that the drug's distribution into target tissues in the host differs between the drugs.

In summary, the discussed studies by the Lanusse and the Leathwick research groups nicely demonstrate how the parasite's exposure to the drug is determined by the drug's formulation, the parasite's biology and life cycle and support that intestinal drug uptake most likely increases total drug dose of the parasite. Therefore, changes at the intestinal barrier including upregulation of Pgps can be expected to have an impact on susceptibility.

In ascarids, the drug accumulation in the parasite and in relevant tissues of the host still needs to be elucidated. Due to the extensive ascarid tissue migration, it could be expect that larvae will most likely be exposed to considerably lower drug levels during this time as the migrated tissues are not sites of ML accumulation (except for the liver stage). Whether and how larvae feed during migration path is not known. In contrast, adult ascarids dwelling in the small intestine will be exposed over a prolonged period via the cuticle-epidermis and the intestine when the worm feed on the intestinal content.

In general, the differences in parasite biology will influence the exposure concentration at different barriers and thus changes at these barriers might constitute stage-specific resistance. Concurrently, these biological differences influencing the overall drug exposure might also facilitate the development of drug resistance by prolonged exposure to sublethal doses and indeed, *H. contortus*, *T. circumcincta* (sublethal concentrations in abomasum) and *P. univalens* (sublethal concentrations during migration) are among the parasites which exhibit the most widespread ML resistance. This factor should be considered in the interpretation with regard to parasite biology, drug efficacy and the potential to develop resistance.

The uptake of other relevant anthelmintics into nematodes has been analyzed in a few species as well. For example, levamisole uptake was reported to be transcuticular (Verhoeven et al. 1976) and *C. elegans* with cuticle defects were 10-times more susceptible (Lewis et al. 1980). Between BZ derivatives, drug accumulation appears to be influenced by the Log *P* (Alvarez et al. 2007). Also, Alvarez et al. conclude that transcuticular absorption is present in general but they do not rule out the intestinal route (Alvarez et al. 2007). Interestingly, to dermine uptake routes of closantel, IVM was used to to completely diminish oral uptake by inhibiting PP, but this did not affect the susceptibility to closantel (Rothwell and Sangster 1997).

To my best knowledge, the present study is the first to systematically investigate the direct effects of PP on IVM and MOX susceptibility and thereby role of the intestine as an uptake route. The evidence from these experiments supports the conclusion that both the intestine and the cuticle-epidermis represent uptake routes for IVM and MOX and this represents a reference point for future studies. Indeed, the findings demonstrate that more emphasis should be put on the uptake route and nematode pharmacokinetics, which are likely to be essential for the drug reaching the respective target tissue and general pharmacodynamics. In turn, it possible that changes in the uptake barrier can constitute a resistance pathway, thus future transcriptome studies should consider tissue specific changes. In conclusion, the novel experimental set-up employed herein for the first-time allowed insights the both intestinal and cuticular-epidermal uptake of IVM and MOX and the role of active drug ingestion to expose the intestine to the drug.

7.5 P-glycoproteins Enhance Barrier Function against Ivermectin and Moxidectin

While several studies have demonstrated an interaction of Pgps and MLs at the functional level or reported differential expression and signatures of selection in resistant compared to susceptible isolates (Janssen et al. 2015, Kaschny et al. 2015, Choi et al. 2017, Khan et al. 2020, Reyes-Guerrero et al. 2020) (see section 2.3.3.2 and 2.3.3.3 for more detail) the actual mechanism how Pgps might contribute to resistance has remained a conundrum. Based on Pgp expression patterns in *P. univalens* and other nematodes, four main hypotheses have been framed in section 7.2.1, of which three also represent possible mechanisms of resistance. In brief, these are to provide barrier function at ML uptake barriers, colocalization in neurons or excretion via the excretory system. Clearly, Pgp expression is predominant at the intestine across different nematode species (Godoy et al. 2016, Kaletsky et al. 2018, Chelladurai and Brewer 2019), but it has remained elusive whether expression in this tissue is relevant for ML resistance. Likewise, the role of epidermal expression has been unknown and to date, has not been studied. In this context, it became apparent that the routes of uptake of MLs into the worm have not been elucidated making it difficult to extrapolate the Pgp-mediated ML resistance mechanism based solely on Pgp tissue expression patterns.

Overall, the results of this thesis demonstrate that Pgps can reduce ML susceptibility in a tissue-specific manner. As this effect is dependent on active drug ingestion by PP, the most conclusive interpretation is that Pgps reduce IVM and MOX permeation through the intestine and the epidermis, but do not rule out alternative uptake routes which were suggested by other studies in *C. elegans* (Dent et al. 2000, Page 2018).

Utilization of *C. elegans* as a model nematode to analyze parasite gene function is currently the best option as few genetic manipulation tools are available in parasitic nematodes (except for *Strongyloides* spp.) and transgenesis including CRISPR/Cas9 is used widely by the *C. elegans* community. Noteworthy, knock-ins of large genes are still difficult to obtain, which is the reason why extrachromosomal arrays were generated and utilized in the present work due to time-constraints. However, a CRISPR/Cas9 gene knock-in approach was established and modified to knock-in parasite-derived Pgps in this project and will be used in further analyses by the research group. In contrast to other model systems, in the *C. elegans* model the effect of anthelmintic drugs on genotype-phenotype relationships can be directly analyzed. It should be noted that findings in *C. elegans* should be interpreted with care and should not be generalized to other nematodes without confirming evidence (Blanchard et al. 2018). Nonetheless, the closer relatedness between *C. elegans* and parasitic nematodes in contrast to model systems such as yeast or mammalian cell culture might facilitate correct post-translational modifications such as glycosylation which is important for Pgp function (Xie et al. 2010). For example, mouse Pgp has an apparent molecular weight of 140 kDa determined by westernblot due to lower levels of glycosylation in comparison to rat and human Pgp which have an apparent molecular weight of around 170 kDa (Dong et al. 1996, Dong et al. 1998). However, molecular mass was not analyzed with western blotting in this project, but the

presence of an effect of Pgps on ML susceptibility demonstrate functionality of the recombinant protein suggesting that proper glycosylation occurred and required post-translational modifications were introduced.

In general, this study provides conclusive evidence that directly links Pgp overexpression to reduced susceptibility. These findings are in line with the only other direct evidence for a contribution of Pgps to ML resistance, the *Pun*Pgp-11.1 overexpression under regulation of the native *Cel*Pgp-11 promotor in *C. elegans* (Janssen et al. 2015). In addition, the PP dependent reduction of ML susceptibility from tissue-specific *Pun*Pgp-9 overexpression support the hypothesis of barrier function of Pgps and have allowed novel insights into the functional role of Pgp. As outlined in 7.4 the intestine and the cuticle-epidermis are uptake routes for both IVM and MOX. In this regard, the reduced susceptibility to IVM and MOX from intestinal and epidermal *Pun*Pgp-9 expression compels a role in barrier function and reduced susceptibility. Most clearly, intestinal expression reduced the barrier permeability to the drug considerably when worms take up liquid with the dissolved drug from the environment by PP. Conversely, epidermal expression only affected susceptibility in the absence of PP. This suggests that Pgps can influence barrier permeability but that this effect is somewhat limited by the biophysical uptake capacities through these barriers. As a reciprocal deduction based on the results compared to the conclusion outlined in 7.4, the apparently higher intestinal permeability compared to cuticular-epidermal permeability of IVM masks the epidermal barrier enhancement of Pgp overexpression under regulation of the col-19 promotor. For MOX, the apparently higher transcuticular permeability confined the impact of intestinal *Pun*Pgp-9 overexpression on susceptibility to only a marginal fold change whereas epidermal *Pun*Pgp-9 overexpression decreased susceptibility by higher folds regardless of PP. Whether this potential of Pgps to enhance the barrier to MLs plays a role in reducing uptake of MLs in parasites remains to be elucidated.

For the intestine, the naturally strong expression of *Cel*Pgp-1 and *Cel*Pgp-3 has been implicated in barrier function to natural toxins (Lincke et al. 1993, Broeks et al. 1995). The authors postulated that the cuticle presents a mostly impermeable barrier to most toxins, thus the intestine represents a natural “porte d’entrée” for dietary and environmental toxins (Lincke et al. 1993, Broeks et al. 1995). Noteworthy, *Cel*Pgp-9 deletion did not affect the IVM or MOX susceptibility phenotype in the thrashing assays used in the present study but results in an IVM susceptibility phenotype in a development assay (Janssen et al. 2013c, Gerhard et al. 2021). However, generally the findings for *Cel*Pgp-1 and -3 are in line with the observations on the effect of transgenic *Pun*Pgp-9 overexpression in the present project which extend the functional role to the epidermis as well. Similarly, mammalian Pgps have contribute to barrier function at the blood-brain barrier to different neuronal toxins including the MLs (Schinkel et al. 1994b, Mealey et al. 2001, Van Der Sandt et al. 2001, Merola and Eubig 2012) which shows that in general the functional role of this multidrug ABC-transporter is conserved across distant phyla. In addition to barrier function, mammal Pgps have been implicated with excretion of different drugs including the MLs which are excreted via the liver and intestine in most mammals (Laffont et al. 2002, Ballent et al. 2006b, Lifschitz et al. 2006). In light of the Pgp expression in the excretory system of nematodes, excretion and drug elimination should also be considered as a potential mechanism of Pgps to reduce ML susceptibility. In parasitic nematodes, Pgps have also been implicated in the egg shell barrier to MLs (Riou et al. 2020).

Despite the col-19 and ges-1 promotors physiologically driving gene expression with other sub-cellular protein localization patterns, Pgps appeared to be transported to the outward facing epidermal membrane and the lumen-sided intestinal membrane, which is an information conserved in the aminoacid sequencec. This subcellular distribution of Pgp protein expression is in line with findings of Pgp expression in *C. elegans* where *Ce*-Pgp-1::GFP fusion constructs were shown to localize to the apical intestinal membrane (Stutz et al. 2015). Naturally, intestinal apical and outward-facing epidermal expression is a prerequisite for a barrier effect as otherwise, Pgps would increase susceptibility by transporting the substrate into the worm.

In addition, the higher lipophilicity of MOX has been argued to influence Pgp affinity (Prichard et al. 2012, Godoy et al. 2015b, Godoy et al. 2016). However, the cited values from these

publications and some other often cited publications were predicted Log *P* values e.g. using Hyperchem software or refered to experimental data prior to 2006. This experimental data will have determined the Log *P* with an outdated method which has been replaced by the slow stirring method which is recommended by the OECD 123 test guidelines (published in 2006 and recommended for all compounds with Log *P* > 4). Prior methods have underestimated log *P* for IVM, for example the Log *P* of 3.22 for IVM (Halley et al. 1989). In contrast, experimental Log P_{ow} values for IVM and MOX according to the OECD 123 test guideline are within a very close range for both derivatives, log P_{ow} 5.6 ± 0.3 (Römbke et al.) for IVM and log P_{ow} 6.49 (Health Products Regulatory Authority (Hpra) 2017) for MOX. Likewise, Log *P* values available from PubChem (only predicted) (Pubchem 2020) and manually re-calculated by XLogP3 v3.2.2 (Cheng et al. 2007) are within a very similar range, 4.1 for IVM and 4.3 for MOX. In addition, the C23 methoxime group in MOX which, in particular if split from the methyl group (e.g. at lower pH), is a strongly hydrophilic group. Hence, lipophilicity is only marginally greater for MOX but this could still influence affinity to Pgps and membrane partitioning leading to a reduced substrate access.

As a possible follow up experiment, the uptake of drugs into the worm could be quantified using the same or similar strains and fluorescent dyes such as Rhodamine 123 and Hoechst 33342 which have been established as Pgp substrates. Indeed, Rhodamine 123 is taken up by live worms (Mottram et al. 2012) and thus the incubation with this substrate would allow visualization, and possibly quantification, of the effect of Pgp overexpression on barrier permeation of a hydrophobic compound solved in the incubation medium. If available, labelled IVM, MOX or other ML derivatives could be used to directly read-out drug uptake. More generally, other ML derivatives should be screened with the approach established here to validate the barrier potential towards other MLs. In this regard, automatic motility scoring systems should be utilized as these are clearly superior to manual read-out assays. In *C. elegans*, an experiment using H^3-labelled IVM and MOX and tissue-specific Pgp expression to elucidate uptake kinetics into the worm would also allow more clear insights into barrier function of Pgps and drug uptake routes, dispersion and compartmentalization. Likewise, approaches using whole-worm MALDI-tof profiling have been established, which could be used to investigate the drugs pharmacokinetics in the worm without the need for labelled drugs (Hameed et al. 2015).

7.6 Identifying Novel Nematode Specific P-glycoprotein Inhibitors

The third project part of this thesis aimed at exploiting human Pgp-i compound libraries to identify Pgp-is which are poor inhibitors of mammal Pgps and strong inhibitors of nematode Pgp. The dataset at this stage is not complete and thus is only published within the scope of this PhD.

All three newly tested potential Pgp-i as well as VPM resulted in susceptibility to 1 nM IVM which is otherwise tolerated by the *Caenorhabditis elegans* WT strain in a larval development assay in a concentration dependent manner. Additionally, all tested substances alone led to a delay in development at high concentrations. However, for VPM no difference between the addition of IVM and absence of IVM was found suggesting that the new Pgp-i were superior regarding their effect on Pgp, or generally on the xenobiotic detoxification machinery. While many studies have linked administration of a Pgp-i in parasitic nematodes (see sections 2.3.3.2 and 2.3.3.3) to increased susceptibility, particularly in resistant strains, these might actually be a false conclusions. In fact, Cyt-P450 and Pgps have an overlapping substrate range and it is reasonable to assume that besides Pgps, other ABC-transporters are inhibited by the putative Pgp-i (Kirn et al. 1999b). The present studies thus lack the analysis of transgenic strains, for example like those which are used in the second publication. Reversal of the increased resistance phenotype from chemical transgene Pgp inhibition would better demonstrate Pgp specificity, for example by incubating the strains generated in the second manuscript with Pgp-

is. Likewise, additional investigations using the yeast growth assay would offer insight in the interaction at the molecular level.

Following regular selection with MLs, different Pgp alleles and CNV have been detected in resistant compared to susceptible or random nematode populations of *P. univalens* and *T. circumcincta* (Janssen et al. 2013a, Choi et al. 2017, Turnbull et al. 2018). Considering this adaptive evolutionary potential, regular co-administration of a Pgp-i bears the risk of the selection of resistance against Pgp-i, an evolutionary process well known from antibiotic resistance, e.g. in case of CTX-M SNPs conferring reduced susceptibility to beta-lactamase inhibitors. As an unlikely worst case scenario, a conceivable mechanism of Pgp-i resistance could be the selection of even stronger Pgp overexpression, resulting in the selection of hyper-resistant parasite populations and thereby forfeiting susceptibility of new xenobiotic drugs which fall into the Ppg substrate range in the future (De Lourdes Mottier and Prichard 2008), e.g. as suggested in case of a monepantel resistant *H. contortus* isolate (Raza et al. 2016a). Despite the potential risks, if used with care, Pgp-i are likely to extend the lifetime of MLs in ML resistant populations. Considering the effect of the tested Pgp-i on *C. elegans* even without co-administration of IVM, the synergy of a combination of compounds is considered to lead to a slower development of resistance (Cheesman et al. 2017, Yu et al. 2018b). Unfortunately, evidence (in particular from genome-wide backcross studies) has accumulated indicating that a contribution of Pgps to ML resistance might only be present in some resistant populations but this question remains unsolved. Currently, no biomarkers for ML resistance based on Pgps have been developed. For example, attempts to establish such a marker for *D. immitis* have not succeeded (Bourguinat et al. 2015, Ballesteros et al. 2018). However, with the availability of such a marker, veterinarians in the field could test for a contribution of Pgps in ML resistant populations. If positive, a specific ML formulation with inhibitors could be used to reestablish efficacy. Otherwise, the increased price of such a formulation would probably deter a regular use. Indeed, veterinary care and management at the herd level is increasingly important in veterinary practice. In summary, nematode-specific Pgp-i have immense potential to increase the lifetime of MLs.

7.7 Implications for the Field of Anthelmintic Resistance, Clinical Parasitology, and Considerations for Future Studies

With ML resistance continuously spreading (Peregrine et al. 2014, Nielsen 2016, Prichard and Geary 2019, Tinkler 2020), understanding the underlying mechanism and identifying biomarkers has become a pressing matter. This PhD project has allowed considerable advancement in understanding the mechanism of Pgp-mediated ML resistance. In fact, the first characterization of Pgp overexpression resulting in the enhanced barrier function to MLs provided the missing piece of evidence to the large number of epidemiological studies which have linked increased Pgp expression to ML resistance (Blackhall et al. 2008, Janssen et al. 2013a, Raza et al. 2016a, Whittaker et al. 2017, Saeed et al. 2019). However, whether this mechanism plays a role in parasites will need to be determined by functional reverse genetic studies in the respective resistance parasitic worm species, once these tools have been developed (Lok 2019).

As a general conclusion from the Pgp and ML resistance literature, Pgps do not seem to be relevant in all resistant populations, but rather, only in some populations and species (Williamson and Wolstenholme 2012, Choi et al. 2017, Doyle et al. 2019a, Doyle et al. 2019b, Khan et al. 2019, Khan et al. 2020) (more detail in section 2.3.3.2). Likewise, other resistance mechanisms and loci with signatures of selection differ between populations and species (Rezansoff et al. 2016, Choi et al. 2017, Luo et al. 2017, Doyle et al. 2019a, Doyle et al. 2019b, Khan et al. 2019, Laing et al. 2019, Khan et al. 2020). Despite early optimism (Prichard and Roulet 2007, Bourguinat et al. 2008), ABC-transporter associated resistance markers (e.g. SNPs or by other means) have not emerged as reliable biomarkers, possibly because of their varying extent of contribution to resistance. Concurrently, other potential markers identified from GWAS studies were reported to correlate with resistance (these are not ABC-

transporters) (Ballesteros et al. 2018). However, these genetic markers seem to be insufficiently reliable for clinical implementation. Likewise, despite extensive efforts and high quality NGS studies in *H.* contortus (Doyle et al. 2019a, Doyle and Cotton 2019, Doyle et al. 2019b, Laing et al. 2019), molecular ML resistance biomarkers have not been established and it also appears unlikely that such biomarkers will be established in equine veterinary parasitology in the near future.

In *P. univalens* and other ascarids, the availability of genomic and transcriptomic resources has considerably improved due to several recent studies (Wang et al. 2017, Bolt et al. 2018, Martin et al. 2020, Scare et al. 2020) as well as from the first publication of this PhD project. NGS resources are the basis to identify genetic markers in the future and the description of the Pgp gene family has helped in improving the annotation. For now, FECRT will remain the *in vivo* gold standard for resistance detection during epidemiological studies and clinical diagnostic. The difficulty and complexity to establish a genetic biomarker clinically had already become apparent for BZ resistance. Here, despite the identification of the beta-tubulin SNPs as the main contributors of resistance, other genetic factors (Yilmaz et al. 2019) and the complex species composition in the intestine of sheep (Avramenko et al. 2019) can influence the resistance phenotype and the corresponding genotypes. These challenges make the FECRT indispensable in a clinical setting on a single farm. Realistically for ML resistance, the apparent multi-genetic context, the findings from this project with regard to the potential of Pgps to modulate ML susceptibility as well as the general failure of identifying other causal genetic elements, loci or gene sets foreshadow the unlikeliness of finding a single universally applicable genetic marker. Only in H. contortus, the single identified QTL might lead towards the generation of a reliable marker (Doyle et al. 2018, Doyle et al. 2019b). Rather, a panel of biomarkers could offer the most reliable solution for molecular ML resistance testing, e.g. including genetic and proteomic and immunological markers. Within this panel, one or several Pgp markers could offer a path towards the cost-effective use of Pgp-i and pave the way for improved management.

With regard to other methods to detect ML resistance, shifts in the concentration-response curve between susceptible and resistant isolates vary considerably between *in vitro* assay methods used to quantify the degree of resistance, species and life cycle stage. For example *in vitro* micro-motility meter measurements resulted in shifts up to 345.6-fold for IVM and up to 13.8-fold for MOX in *Teladorsagia circumcinta* (Demeler 2005). However, in other studies using the larval migration inhibition assay, which also tests for motility, ML-resistance associated shifts in the dose-response curve in a *T. circumcinta* population were within a similar range as for the data obtained in this project (<10) and lower for MOX compared to IVM (Demeler et al. 2013a, Gerhard et al. 2021). Also, in *H. contortus* a range of resistance shifts have been reported, ranging from 1.0-108.0 for IVM and 3.87 to 32.32 for eprinomectin (Dolinská et al. 2016) in L3 using the Worminator for motility analysis. In adult *H. contortus* isolates Kirby (susceptible) and Wallangra (resistant) differences in susceptibility of 50 to 559-fold for feeding phenotypes, and 1.3 to 29-fold for motility depending on the scoring system. Generally, in populations with moderate shifts in the dose response curve, mediation by Pgps is more likely. In the data generated within the scope of this PhD project, fold-changes were only moderate, i.e. for IVM < 4.5 and for MOX < 1.6. These shifts in the concentration-response curves are lower than the observed shifts in resistant parasite populations *in vitro* tests and the concentrations used are considerably lower than effective concentrations *in vivo.* It should be noted that parasite isolates are much more genetically diverse than *C. elegans* N2 Bristol and not all individuals will carry the resistance allele(s). In total, already three *P. univalens* Pgps have been implicated functionally in ML efflux, *Pun*Pgp-2 and *Pun*Pgp-9 in this study and *Pun*Pgp-11.1 in the predecessor project (Janssen et al. 2013a, Janssen et al. 2015). This demonstrates that MLs (and explicitly IVM) are most likely conserved as substrates of different Pgps and is in line with findings from other studies (Godoy et al. 2015b, Kaschny et al. 2015, Godoy et al. 2016, Reyes-Guerrero et al. 2020).

Generally, the findings regarding the uptake routes of MLs might also have a broader applicability to parasitic nematodes of humans, animals and plants and to the field of

anthelmintic resistance. The findings regarding the role of active drug ingestion by PP propose a novel angle from which to approach anthelmintic resistance and the functional information is an important clue where to look with regard to transcriptomic changes and other changes not directly apparent from genetic material, i.e. expression changes in specific tissues masked by lack of expression changes in the majority of other tissues. Therefore, transcriptome analyses in the context of ML resistance in the future should consider a dissection of parasite tissues to uncover potential tissue specific changes. In addition, changes in barrier composition which are the result of genetic changes which are not obviously associated to be barrier associated, could lead to changes in lipid or collagen composition of the cuticle or the mucus composition of the intestine which in turn, could alter drug uptake capacities and worm pharmacokinetics. It should be taken into account that the MLs are comparatively large molecules, thus changes in the cuticle might easily result in reduced permeability, e.g. by a reduced pore size in the cuticle. In this regard, the considerably smaller size of MOX and the apparently higher cuticular-epidermal but lower intestinal permeability might be the reason why MOX has retained efficacy in IVM resistant populations (Prichard and Geary 2019). Like in the worm, MOX pharmacokinetics in the host are also different from IVM, which offers the most reasonable explanation for the observed differences (Lloberas et al. 2013b). In addition, changes in the lipid metabolism have been implicated in ML resistance in several backcross and transcriptome analyses (Laing et al. 2012, Choi et al. 2017). Possibly, lipid content in the cuticle, epidermis and the intestine influence the permeability of the lipophilic MLs.

In conclusion, Pgps are a diverse group of genes in *P. univalens* and other nematodes, they have the potential to transport MLs and to enhance the intestinal and epidermal barrier against MLs. The complexity of the underlying mechanisms of ML resistance in *P. univalens* and other nematodes remains elusive but evidence from this and other studies have consolidated Pgps as mediators of ML resistance within a multigenic context. Thus, there is an urgent need for more genomic and transcriptomic studies with a focus on tissue-specific changes, for anatomical and histological studies investigating drug uptake, dispersion and elimination in nematodes, for more functional analyses to validate identified candidates, and finally for the development of reliable panel of biomarkers. In this regard, the findings presented in this PhD project represent important advances in the understanding of Pgp-mediated ML resistance while raising new questions on the role of drug uptake via specific tissues in suscptibility.

5 SUMMARY

The Role of Nematode P-glycoproteins in the Mechanism of Macrocyclic Lactone Resistance

Control of veterinary and human parasitic nematodes relies heavily on chemotherapy with anthelmintics but drug resistance is a growing problem. Against the widely used macrocyclic lactones (MLs), drug resistance has emerged in several nematode species and this is a particular challenge in parasites of ruminants and horses. For instance, ML resistance is globally widespread in an important pathogen of foals, *Parascaris univalens*. In order to find a sustainable solution for this problematic trend, several strategies have been proposed with the aim of maintaining susceptibility or reversing resistance. These strategies focus on optimized treatment and hygiene regimens guided and complemented by reliable resistance diagnosis and improved farm management. For their implementation, a good knowledge of the underlying resistance mechanisms is considered imperative. However, the underlying mechanisms of ML resistance are mostly unknown. In the last decade, considerable efforts have helped to functionally validate several candidate genes and to better understand some aspects of the genetic basis of ML resistance at the genomic and transcriptomic level. Although uncertainty remains, the ATP-binding-cassette (ABC)-transporters and particularly members of the ABCB subfamily, the P-glycoproteins (Pgps), have been proposed as contributors to ML resistance in several nematode species. The Pgp gene family in nematodes contains several different orthologous lineages but which Pgp genes play a role in ML resistance and how, is unknown.

In a first step, the complete Pgp repertoire in the horse roundworm *P. univalens* was characterized by transcriptome-guided RT-PCR and Sanger sequencing. This allowed a considerable improvement of the annotation of a total of ten identified Pgp genes including an ascarid specific lineage and two gene duplications with subsequent divergence. Overall, this is a similarly sized repertoire compared to other nematodes, but the phylogenetic analysis revealed that the repertoire of Pgp lineages varies considerably between species. Using a diverse set of transcriptome resources, the tissue expression patterns of all *P. univalens* Pgp genes were characterized in *P. univalens* and in the model nematode *Caenorhabditis elegans*. In both species, expression of Pgps was predominant in the intestine, moderate in the epidermis or carcass tissue (in *P. univalens*, containing the epidermis, neurons, muscles and the pharynx) and low in the gonads. No indicibility of individual *P. univalens* Pgp expression was found by examining a novel transcriptome generated from adult *P. univalens* incubated with ivermectin (IVM, a commonly used ML and an avermectin) or a control. Taken together, the two examined independent *P. univalens* transcriptome data sets showed that *Pun*Pgp-9 and *Pun*Pgp-11.1 were the most strongly expressed genes followed by *Pun*Pgp-16.1 and -2. These analyses provide a basis for future studies with a focus on selected candidate Pgps.

To investigate the function of Pgps in nematodes and to examine which anthelmintics fall into the substrate range of Pgps, heterologous expression of *P. univalens* Pgps in different model organisms was employed. Two selected and phylogenetically distant *P. univalens* Pgps, *Pun*Pgp-9 and *Pun*Pgp-2, were examined for their ability to transport IVM, an ML, or thiabendazole, a benzimidazole, which is both an antimycotic and anthelmintic, by heterologous expression in an ABC-transporter deletion *Saccharomyces cerevisiae* strain.

The results of this previously established assay, indicated that IVM, but not thiabendazole, is a conserved substrate of both examined Pgps. Based on the observed strong intestinal and moderate epidermal Pgp expression observed in the first project part in *P. univalens* as well as in other studies on other nematodes, the hypothesis that nematode Pgps play a role in ML susceptibility by mediating barrier permeability was formed. However, in addition to uncertainty of the mechanisms of ML resistance, the uptake and dispersion routes of MLs in nematodes and hence the relevant drug uptake routes are unknown. To investigate the role of Pgps at two potential uptake barriers in a nematode model, transgenic *C. elegans* expressing *Pun*Pgp-9 in

the epidermis or the intestine were characterized in a motility (thrashing) assay which was modified to selectively stimulate pharyngeal pumping (PP). Strikingly, active drug ingestion by PP increased susceptibility to IVM considerably and to moxidectin (MOX, a ML belonging to the class of milbemycines) only minimally though significantly. Tissue specific expression of *Pun*Pgp-9 in the intestine reduced both MOX and IVM susceptibility when the drug was actively ingested. Expression in the epidermis slightly reduced MOX susceptibility regardless of PP but only protected against IVM when the drug was not actively ingested. These results support that nematode Pgps play a role in barrier function.

In conclusion, members of the diverse Pgp gene family are likely to be contributors to ML resistance and their protective effect against MLs appears to be brought about by reducing the intestinal and epidermal permeability. Although both tissues appear to be uptake routes, the identified direct effect of active drug ingestion on susceptibility highlights the knowledge gap between the pharmacokinetics in the host and in the parasite. This calls for specifically tailored studies on the uptake and dispersion of MLs and other anthelmintics in the worms as well as tissue-specific transcriptome analyses in the context of anthelmintic resistance. In order to reverse Pgp-mediated resistance, preliminary analyses to identify inhibitors specific to nematode Pgps were performed and first results instigate a more detailed characterization.

6 ZUSAMMENFASSUNG

Die Rolle der P-glycoproteine im Resistenzmechanismus gegen makrozyklische Lactone bei Nematoden

Die Strategie zur erfolgreichen Bekämpfung von Infektionen mit parasitischen Nematoden beruht hauptsächlich auf chemotherapeutischer Intervention mit Anthelmintika, doch die Verbreitung von Resistenzen bereitet ein zunehmendes Problem. Gegen die häufig eingesetzten makrozyklischen Lactone (MLs) bestehen Resistenzen bereits in einer Reihe von parasitischen Nematoden und insbesondere bei Parasiten von Pferden und Wiederkäuern ist diese Entwicklung eine Herausforderung. Die ML Resistenz im pathogenen Pferdespulwurm *Parascaris univalens* beispielsweise, ist global weit verbreitet. Die aktuellen Empfehlungen für eine nachhaltige Parasitenbekämpfung bauen daher auf optimierte und gezielte Behandlungs- und Desinfektionsstratgien, welche durch verbesserte Diagnosestellung und nachhaltiges Betriebsmanagement geleitet werden. Ein gutes Verständnis der zugrundeliegenden Resistenzmechanismen ist für die Umsetzung dieser Strategien unabdingbar, jedoch sind die ML Resistenzmechanismen immer noch weitgehend unbekannt. In den letzten Jahren konnten allerdings auf funktionaler, genomischer und transkriptomischer Ebene wesentliche neue Erkenntnisse gewonnen werden. Dabei zeigte sich, dass insbesondere die P-glycoproteine (Pgps) mögliche Kandidatengene für die ML Resistenz darstellen. Diese zur Subfamilie B gehörenden ATP-binding-cassette (ABC)-Transporter bilden bei Nematoden eine Genfamilie mit verschiedenen orthologen Linien, doch welche Pgps für die ML Resistenz relevant sind und wie diese Transporter zur Resistenz beitragen, ist unklar.

In der vorliegenden Arbeit wurde durch Transkriptom-geleitete RT-PCR und Sanger-Sequenzierung das gesamte Pgp-Repertoire des Pferdespulwurms *P. univalens* charakterisiert und entschlüsselt. Dabei wurde die genomische Annotation der zehn identifizierten Pgps erhebliche gegenüber der ursprünglichen automatischen Annotation verbessert. Damit ist die Anzahl von Pgp Genen ähnlich wie bei anderen Nematoden. Die phylogenetische Analyse zeigte aber auch, dass die Zusammensetzung der Pgp Gene zwischen den Nematodenspezies voneinander abweicht. Auf Basis verfügbarer Transkriptomdaten wurden die Gwebeexpressionsprofile der einzelnen Pgps in dem Modellnematoden *Caenorhabditis elegans* vergleichend untersucht. Dabei wurde eine starke Expression im Darm, eine moderate Expression in der Epidermis bzw. im Hautmuskelschlauch (in *P. univalens*) und eine niedrige Expression in den Gonaden in beiden Spezies festgestellt. Anhand eines neuen Transkriptomdatensatzes, welche durch die Inkubation adulter Würmer mit Ivermectin (IVM, ein gebräuchliches ML aus der Gruppe der Avermectine) oder einer Kontrolle generiert wurde, konnte keine Induzierbarkeit der Expression einzelner Pgps festgestellt werden. In den zwei zuvor genannten und unabhängig generierten Transkriptomdatensätzen taten sich *Pun*Pgp-9 und *Pun*Pgp-11.1 als die am stärksten exprimierten Pgps hervor, sowie in einem geringeren Ausmaß zwei *Pun*Pgp-16 Paraloge. Diese Ergebnisse bilden eine Basis für zukünftige Studien und erlauben eine gezielte Auswahl von Pgps für weitere Analysen.

Um das Substratspektrum und die Funktion nematodischer Pgps näher zu untersuchen, wurden *P. univalens* Pgps in verschiedenen Modellorganismen heterolog exprimiert. Zwei phylogenetisch entfernte *P. univalens* Pgps, *Pun*Pgp-2 und *Pun*Pgp-9, wurde durch die heterologe Expression in einem *Saccharomyces cerevisiae* Stamm mit ABC-Transporter-Deletionen untersucht. Die Ergebnisse des bereits zuvor etablierten Hefewachstumsassay deuten darauf hin, dass IVM, jedoch nicht Thiabendazole (ein Antimykotikum und Anthelmintikum aus der Gruppe der Benzimidazole), als Substrat beider untersuchter Pgps konserviert ist.

Die im ersten Projektabschnitt und auch in anderen Studien beobachtete starke Expression im Darm und moderate Expression in der Epidermis führten zu der Hypothese, dass Pgps eine Rolle in der Barrierefunktion gegenüber MLs spielen. Da die Penetrationsroute der MLs in den

Wurm nicht bekannt ist, wurde ebenfalls der Effekt aktiver oraler Ingestion durch die Stimulierung pharyngealen Pumpens (PP) untersucht. Zu diesem Zweck wurden transgene *C. elegans* mit Expression von *Pun*Pgp-9 im Darm oder in der Epidermis generiert und in einem Motilitätsassay (Thrashing Assay) untersucht, welcher für die Stimulierung von PP modifiziert wurde. Auffällig war hierbei die starke Erhöhung der Suszeptibilität durch aktive IVM Ingestion, wohingegen die Suszeptibilität gegenüber Moxidectin (MOX, ein ML in der Gruppe der Milbemycine) nur minimal erhöht wurde. Die heterologe Expression im Darm führte zu einer starken Verringerung der ML-Suszeptibilität, wenn PP stimuliert wurde, wohingegen epidermale Expression nur gegen IVM schützte, wenn PP nicht stimuliert wurde. Epidermale Expression schützte jedoch gegen MOX unabhängig von der Stimulierung von PP.

Insgesamt zeigen die Ergebnisse der vorliegenden Arbeit, dass Pgps die Suszeptibilität gegenüber MLs reduzieren können und, dass dieser Effekt auf eine Verringerung der Permeabilität der Epidermis und des Darms zurückgeführt werden kann. Der erstmalig beschriebene direkte Effekt aktiver Ingestion auf die ML Suszeptibilität verdeutlicht, wie wenig über die Aufnahme und Verteilung der Anthelmintika in Nematoden bekannt ist. Daher sollten in einem geeigneten experimentellen Rahmen die Aufnahmewege in den Wurm aber auch mögliche gewebespezifische Veränderungen von Kandidatengenen in parasitischen Nematoden näher untersucht werden. Um die Pgp-vermittelte Resistenz zu reversieren wurden zudem erste Experimente zur Identifzierung von Nematoden-spezifischen Pgp-Inhibitoren durchgeführt und die Ergebnisse bestärken die Notwendigkeit einer detaillierteren Charakterisierung.

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8 ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my thesis supervisor Prof. Dr. Georg von Samson-Himmelstjerna for trusting me with this research project, supporting my drive to conceptualize and pursue my own research ideas and for fostering my professional growth.

I would like to thank PD Dr. Jürgen Krücken for his supervision and his invaluable scientific advice, for introducing me to molecular biology and for his unconditional availability when confronted with scientific problems.

In addition, I am grateful to both of my supervisors for the numerous scientific and personal discussions, for slowing me down when my curiosity led me astray and for guiding me into the right direction.

Furthermore, I would like to extend my thanks to my thesis advisory committee, Prof. Dr. Emanuel Heitlinger for his welcoming supervision and fruitful collaboration as well as Prof. Dr Dr. Ralf Einspanier for his constructive criticism.

I would like to thank my French colleagues and friends at INRA for their hospitality and for making my research visits an amazing experience. Specifically, I want to extend my thanks to Dr. Abdallah Harmache for introducing me *C. elegans*, Dr. Claude Charvet and Dr. Cédric Neveu for providing the framework for our collaboration and Nicolas Lamassiaude for carrying out valuable experiments which (un)fortunately implied my cell-culture project's demise.

Furthermore, I would like to thank Prof. Dr. Erik Andersen for his hospitality and for his interest and recommendations made to my *C. elegans* work. I would also like to acknowledge Dr. Jonathan Ewbank who has provided critical guidance and help which have been the starting point for interesting findings and an innovative experimental set-up.

My sincere thanks go to Céline Beier who has been an excellent and reliable help in the laboratory.

Also, I would like to thank everyone part of the ZIBI and the GRK 2046 and specifically Prof. Dr. Susanne Hartmann, Dr. Juliane Kofer and Dr. Marko Janke for their support. Being a part of this community has fundamentally shaped my experience as a PhD student for the better and has taught me many valuable personal and scientific lessons. To explore the Serengeti within a research context which has truly been one of the most spectacular experiences of my life thus far and for this opportunity I would like to thank Prof. Dr. Heribert Hofer and Dr. Marion East.

I also would like to thank my colleagues at the institute for all the great memories. Particularly, I want to thank Tina, Esra, Irina and Natalie who have turned occasional frustration and tediousness into joy and laughter.

Finally, I want to extend my deepest gratitude to my family and friends, in particular Judith and my parents who provide the support necessary to overcome any obstacle.

9 FUNDING SOURCES

The project was funded from September 2016 to November 2019 by the Deutsche Forschungsgemeinschaft (DFG) (DFG grant number: 111144555).

In addition, the Karl-Enigk foundation generously provided funding for 12 months (grant number S0229/10019/19).

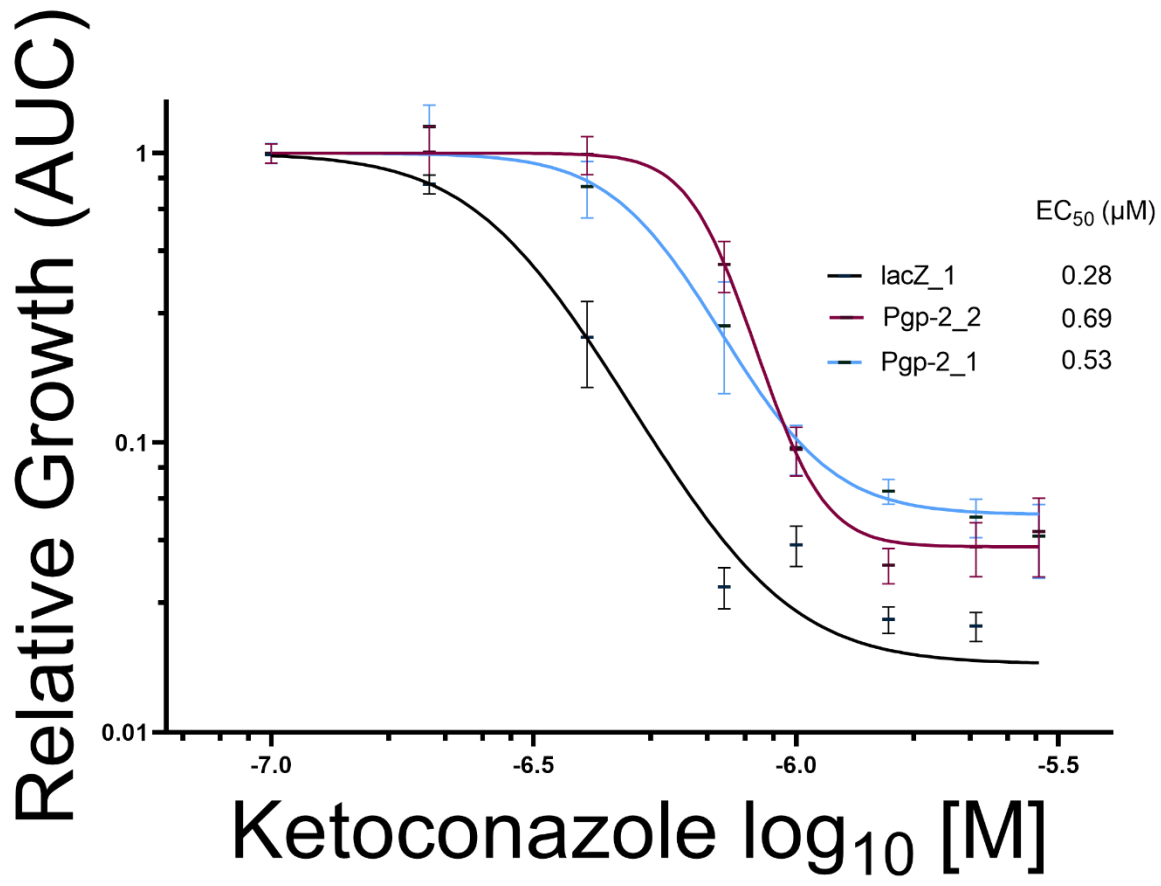
The Deutscher Akademischer Austauschdienst (DAAD) funded a two-year collobarative research exchange within the framework of Procope program. This research stay was expanded by a travel award granted by the Burroughs Wellcome Fund.

For presenting my work on international conferences the Research Training Group 2046 “Parasite Infections: From Experimental Models to Natural Systems" (DFG grant number: 251133687/GRK 2046) generously funded me on several occasions. Notably, the RTG 2046 also supported my education through funding a large number of courses including funding for a Serengeti field training course.

The Deutsche Gesellschaft für Parasitologie supported my visit to the WAAVP annual meeting, Chicago and to Prof. Erik Andersen at Northwestern University, USA.

No conflict of interest has arisen from these research fundings.

10 APPENDIX



Supplementary Fig. 1 Susceptibility to ketoconazole of two independent clones AD1-7 expressing *Pun*Pgp-2

Growth curves of *Saccharomyces cerevisiae* strain AD1-7 expressing *Pun*Pgp-2 (two independent clones _1 blue and _2 red) or a lacZ control (black). Yeast were grown on 48 well plates with 12 replicates spread equally on three independent days and were incubated for 48 hours in dilution series of ketoconazole with a final DMSO concentration of 1%. Every 10 minuztes, OD_{600} was measured. Relative growth is the normalized growth as percentage of the no drug control. Growth curves were fitted after log_{10} transformation of concentrations, setting the negative no drug control to 10^{-7} M Ketoconazole. EC_{50} values are derived from four-parameter non-linear regression models in GraphPad Prism v. 8.3.3 (GraphPad Software, San Diego, California USA, https://www.graphpad.com). Pgp: P-glycoprotein

11 LIST OF PUBLICATIONS

14.1 Peer-Reviewed Publications

Gerhard, A. P., Krücken, J., Heitlinger, E., Janssen, I., Basiaga, M., Kornaś, S., Beier, C., Nielsen, M. K., Davis, R. E., Wang, J., & von Samson-Himmelstjerna, G. (2020). **The P-glycoprotein repertoire of the equine parasitic nematode *Parascaris univalens***. *Scientific reports*, *10*(1), 13586. https://doi.org/10.1038/s41598-020-70529-6

Gerhard, A. P.; Krücken, J.; Neveu, C.; Charvet, C.L.; Harmache, A.; von Samson-Himmelstjerna, G. (2021). **Pharyngeal Pumping and Tissue-Specific Transgenic P-Glycoprotein Expression Influence Macrocyclic Lactone Susceptibility in *Caenorhabditis elegans***. *Pharmaceuticals*, *14*, 153. https://doi.org/10.3390/ph14020153

14.2 Presentations

A. P. Gerhard, J. Krücken, E. Heitlinger J. Wang, M. K. Nielsen, R. Davis, G. von Samson-Himmelstjerna (2019) **Genomic and Transcriptomic Analysis of the P-glycoprotein Gene-Family in *Parascaris* sp.,** 27th Conference of the World Association for the Advancement of veterinary Parasitology, Madison, WI, USA 2019

A. P. Gerhard, J. Krücken, E. Heitlinger J. Wang, M. K. Nielsen, R. Davis, G. von Samson-Himmelstjerna (2018) **Genomische und transkriptomische Charakterisierung der P-glycoprotein Genfamilie bei *Parascaris* sp.**, Tagung der Deutschen Veterinärmedizinischen Gesellschaft (DVG) Fachgruppe Parasitologie und parasitäre Krankheiten 2019, 17.-19.06.2019

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14.3 Poster Presentations

A. P. Gerhard, Jürgen Krücken, G. von Samson-Himmelstjerna (2019) **Relevance of P-glycoprotein mediated anthelmintic resistance mechanisms**, Center for Infection Biology and Immunology (ZIBI) Graduate School Annual Meeting, Berlin, 04.-05.04.2019

A. P. Gerhard, J. Krücken, E. Heitlinger, J. Wang, M. K. Nielsen, R. Davis, G. von Samson-Himmelstjerna (2017) **Functional analysis of the P-glycoprotein associated resistance mechanisms to macrocyclic lactones in the horse roundworm Parascaris sp.**, Center for Infection Biology and Immunology (ZIBI) Graduate School Annual Meeting, Nauen, 16.-17.03.2017

14.4 Other Publications and Presentations

A. P. Gerhard & I. Diekmann (2019) **Parasiten bei Wildtieren – moderne Technik im Serengeti National Park**, Veterinärmedizin in der Hauptstadt, Heft 34/35-2019, ISSN: 1613-4419

12 DECLARATION OF ORIGINAL AUTHORSHIP

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 11.08.2021	Alexander P. Gerhard
Ort, den (Datum)	Unterschrift